UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY.DOCKET NO./TITLE	REQUEST ID
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	68264

Acknowledgement of Change to Small Entity Status

The entity status change request below filed through Private PAIR on 07/24/2018 has been accepted.

CERTIFICATIONS:

Change of Entity Status:

X Applicant asserting small entity status. See 37 CFR 1.27.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

This portion must be completed by the signatory or signatories making the entity status change in accordance with 37 CFR 1.4(d)(4).

Signature:	/Ajay A. Jagtiani/
Name:	Ajay A. Jagtiani
Registration Number:	35205

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

110669 7590 03/13/2018

Ajay A. Jagtiani
Miles & Stockbridge
1751 Pinnacle Drive
Suite 1500
Tysons Corner, VA 22102

EXAMINER	
KEMMERER, ELIZABETH	

ART UNIT	PAPER NUMBER
1646	

NOTIFICATION DATE	DELIVERY MODE
03/13/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipdocketing@milesstockbridge.com
bgoodman@milesstockbridge.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
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In re Patent No. 7,928,057	:	
Issue Date: April 19, 2011	:	
Application No. 12/162,135	:	NOTICE
Filed: November 10, 2008	:	
Attorney Docket No. 00824.07.0001	:	

This is a notice regarding your renewed request for acceptance of a fee deficiency submission under 37 CFR 1.29(k), filed February 22, 2018.

The Office no longer investigates or rejects original or reissue applications under 37 CFR 1.56. **1098 Off. Gaz. Pat. Office 502 (January 3, 1989)**. Therefore, nothing in this Notice is intended to imply that an investigation was done.

Your fee deficiency submission is hereby **ACCEPTED**.

In accordance with the 37 CFR 1.29(k) request, status as a Micro Entity has been removed. Accordingly, all future fees paid in this patent must be paid at the small entity rate.

Inquiries related to this communication should be directed to the undersigned at (571) 272-1058.

/Angela Walker/
Angela Walker
Paralegal Specialist
Office of Petitions

Office of Petitions: Decision Count Sheet

Mailing Month 3

Application No. 12162135

For US serial numbers: enter number only, no slashes or commas. Ex: 10123456
For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: Walker, Angela

Count (1) - Palm Credit 12162135

Decision: GRANT

FINANCE WORK NEEDED
Select Check Box for YES

Decision Type: 321 - 37 CFR 1.28 TO MAKE ENTITY STATUS LARGE FR

Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED
Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

FINANCE WORK NEEDED
Select Check Box for YES

Decision Type: NONE

Notes:

Initials of Approving Official (if required)

If more than 3 decisions, attach 2nd count sheet & mark this box

Printed on: 3/8/2018

Office of Petitions: Routing Sheet

Application No. 12162135

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

- [X] **GRANTED**
- [ ] **DISMISSED**
- [ ] **DENIED**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY.DOCKET NO./TITLE	REQUEST ID
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	58610

Acknowledgement of Loss of Entitlement to Micro Entity Status

The entity status change request below filed through Private PAIR on 02/28/2018 has been accepted.

CERTIFICATIONS:

Change of Entity Status:

X Applicant asserting small entity status. See 37 CFR 1.27.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

This portion must be completed by the signatory or signatories making the entity status change in accordance with 37 CFR 1.4(d)(4).

Signature:	/Ajay A. Jagtiani/
Name:	Ajay A. Jagtiani
Registration Number:	35205

Docket No. 109025.070001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Oliver RITTER	)	Confirmation No: 8455
	)	
Application No.: 12/162,135	)	Patent No: 7,928,057
	)	
Filed: January 25, 2007	)	Issued: April 19, 2011

For: PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY, AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE

United States Patent and Trademark Office
MAIL STOP PETITION
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

RESPONSE TO NOTICE

Sir:

Applicant is in receipt of a Notice mailed February 7, 2018 in the present patent. The Notice indicates that the fee deficiency submission under 37 CPR 1.28 was not accepted because the petition was not properly signed by a person having authority to prosecute in the above-identified patent. Applicant is submitting a petition signed by the practitioner of record concurrently herewith. Accordingly, favorable consideration and granting of the petition is respectfully submitted.

The Notice further indicates that an itemization of the deficiency payment is required. It is believed that the petition filed concurrently herewith properly addresses this requirement. The deficiency in fees was paid on August 23, 2017, as evidenced by the Electronic Acknowledgement Receipt attached hereto. Accordingly, no further fees are deemed necessary.

Applicant also filed a Power of Attorney on August 23, 2017, and attach a copy of the same herewith. Although the USPTO record has been changed to show Ajay A. Jagtiani at Miles and Stockbridge PC as the Power of Attorney, it is noted that the Customer Number is incorrectly identified. The Customer Number should read 110669. Correction of the record to correctly list Customer Number 110669 is respectfully requested.

Applicants further filed a Change of Correspondence Address (Form PTO/AIA/123) on August 23, 2017 and attach a copy of the same herewith. To date the record has not yet been updated to reflect the new correspondence address and Customer Number. Correction of the record to correctly list the new correspondence address and Customer Number 110669 is respectfully requested.

Although no fees are deemed necessary, the Commissioner is hereby authorized by this paper to charge any fees which may be required, or credit any overpayment to Deposit Account 501165.

Date: February 22, 2018

Respectfully submitted,

By: /Ajay A. Jagtiani/
Ajay A. Jagtiani
Reg. No. 35,205
Attorney for Applicants

Miles & Stockbridge
1751 Pinnacle Drive
Suite 1500
Tysons Corner, VA 22102
Telephone: 703.903.9000
Facsimile: 703.610.8686

CUSTOMER NO: 110669

Docket No. 109025.070001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Oliver RITTER	)	Confirmation No: 8455
	)	
Application No.: 12/162,135	)	Patent No: 7,928,057
	)	
Filed: January 25, 2007	)	Issued: April 19, 2011

For: PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY, AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE

United States Patent and Trademark Office
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

FEE DEFICIENCY SUBMISSION AND NOTIFICATION OF CHANGE OF STATUS FROM MICRO ENTITY TO SMALL ENTITY

Sir:

Patentee inadvertently filed a certification of micro entity status on October 16, 2014. Patentee also paid the maintenance fee as micro entity in the amount of $400.00 on October 16, 2014.

Patentee respectfully submits that the certification of micro entity status was filed in error and Patentee claims small entity status. The deficiency in fees from micro entity to small entity in the amount of $400.00 was paid on August 23, 2017.

An itemization of the fee deficiency is as follows:

MICRO-ENTITY FEE	SMALL ENTITY FEE	DEFICIENCY	TOTAL FEE DUE
(Paid Erroneously)	(Actual Fee Due)		
$400	$800	-$400	$400

Patentee respectfully requests that the record be changed to reflect small entity status.

Date: February 22, 2018

Respectfully submitted,

By: /Ajay A. Jagtiani/
Ajay A. Jagtiani
Reg. No. 35,205
Attorney for Applicants

Miles & Stockbridge
1751 Pinnacle Drive
Suite 1500
Tysons Corner, VA 22102
Telephone: 703.903.9000
Facsimile: 703.610.8686

CUSTOMER NO: 110669

PTO/AIA/123 (08-12)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

CHANGE OF CORRESPONDENCE ADDRESS
Patent

Address to:
Mail Stop Post Issue
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Patent Number	7,928,057
Issue Date	April 19, 2011
Application Number	12/162,135
Filing Date	November 10, 2008
First Named Inventor	Oliver RITTER
Attorney Docket Number	109025-070001

Please change the Correspondence Address for the above-identified patent to:

☑ The address associated with Customer Number: 110669

OR

☐ **Firm or Individual Name**

Address

City | **State** | **ZIP**

Country

Telephone | **Email**

This form cannot be used to change the data associated with a Customer Number. To change the data associated with an existing Customer Number use "Request for Customer Number Data Change" (PTO/SB/124).

This form will not affect any "fee address" provided for the above-identified patent. To change a "fee address" use the "Fee Address Indication Form" (PTO/SB/47).

I am the:

☐ Patentee.

☐ If the Patentee was not the applicant for patent (37 CFR 1.42), then a Statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is enclosed or was filed on ________________. See 37 CFR 3.71.

☑ Attorney or agent of record. Registration Number 35205.

☐ Patent practitioner acting in a representative capacity whose correspondence address is the correspondence address of record. Notice has been given to the patentee or owner. Registration Number 35205.

Signature /Ajay A. Jagtiani/

Typed or Printed Name Ajay A. Jagtiani

Date August 23, 2017 | Telephone 703-903-9000

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Post Issue, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	30157480
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE
First Named Inventor/Applicant Name:	Oliver Ritter
Customer Number:	88944
Filer:	Ajay A. Jagtiani/Anna Massimo
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	00824.07.0001
Receipt Date:	23-AUG-2017
Filing Date:	10-NOV-2008
Time Stamp:	10:59:19
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$400
RAM confirmation Number	082317INTEFSW00009424501165
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	POA.pdf	131932 9c18a42a0a9515f66b0fedb63950192175fc6c3c	no	1
Warnings:					
Information:					
2	Maintenance Fee Address Change	ChangeAddressForm.pdf	247062 d7094df01c9650ead069ab69ea47f133267b0060	no	2
Warnings:					
Information:					
3	Notification of loss of entitlement to micro entity status	NotificationofChangeofStatus.pdf	40335 732600128e0ea893c1293b739f60341da5859b45	no	1
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30596 3c8dd96efbc689e46cbe92f58a8bdf65ebb2e7c1	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	449925		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 11/30/2014. OMB 0651-0051
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
12162135	July 25, 2008

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☑ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 110669

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☑ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number: 110669

OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Julius-Maximilians-Universitaet Wuerzburg

☐ Inventor or Joint Inventor (title not required below)

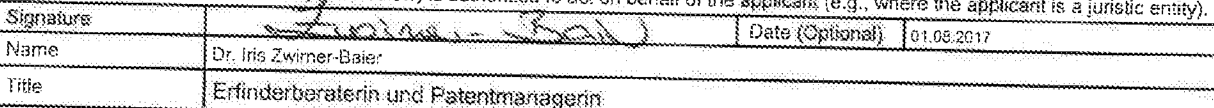
☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☑ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).



Signature		Date (Optional)	01.08.2017
Name	Dr. Iris Zwirner-Baier		
Title	Erfinderberaterin und Patentmanagerin		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	31856548
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE
First Named Inventor/Applicant Name:	Oliver Ritter
Customer Number:	110669
Filer:	Ajay A. Jagtiani/Anna Massimo
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	00824.07.0001
Receipt Date:	22-FEB-2018
Filing Date:	10-NOV-2008
Time Stamp:	11:24:57
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Petition for review by the Office of Petitions	02_22_18_ResponsetoNotice.pdf	21494 5d8b549de953e42e8a6b5acbb3fadde332a2a70d	no	2

Warnings:

Information:

2	Electronic Record Correction	02_22_18_Petition_Notification_Entity_Status.pdf	19868 119616844ff70839bcda6962d360e1f5c6928550	no	2
Warnings:					
Information:					
3	Electronic Record Correction	02_22_18_Copy_08_23_17_ChangeofAddress.pdf	282668 d45ebbb45f1aa46108e768a573da12af7c17b44b	no	2
Warnings:					
Information:					
4	Electronic Record Correction	02_22_18_Copy_08_23_17_efilingAck30157480.pdf	20758 333637a7e636a6f631f63d29256143f99149df4b	no	3
Warnings:					
Information:					
5	Electronic Record Correction	02_22_18_Copy_08_23_17_PowerAttorney.pdf	581089 70c38ea4be23794f31c63e218bfa37abc0277237	no	1
Warnings:					
Information:					
			Total Files Size (in bytes):	925877	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	REQUEST ID
12/162,135	7928057	1646	58142

PAIR Correspondence Address/Fee Address Change

The following fields have been changed to Customer Number 110669 on 02/22/2018 via Private PAIR in view of the certification copied below that authorized the change.

- Maintenance Fee Address

The address for Customer Number 110669 is:
110669
Ajay A. Jagtiani
Miles & Stockbridge
1751 Pinnacle Drive
Suite 1500
Tysons Corner, VA 22102

I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

An attorney or Agent of Record registered to practice before the Patent and Trademark Office who has been given power of attorney in this application

Signature:	/Ajay A. Jagtiani/
Name:	Ajay A. Jagtiani
Registration Number:	35205

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	REQUEST ID
12/162,135	7928057	1646	58142

PAIR Correspondence Address/Fee Address Change

The following fields have been changed to Customer Number 110669 on 02/22/2018 via Private PAIR in view of the certification copied below that authorized the change.

- Correspondence Address

The address for Customer Number 110669 is:
110669
Ajay A. Jagtiani
Miles & Stockbridge
1751 Pinnacle Drive
Suite 1500
Tysons Corner, VA 22102

I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

An attorney or Agent of Record registered to practice before the Patent and Trademark Office who has been given power of attorney in this application

Signature:	/Ajay A. Jagtiani/
Name:	Ajay A. Jagtiani
Registration Number:	35205

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

88944 7590 02/07/2018
Vedder Price P.C.
1401 I Street NW
Suite 1100
Washington, DC 20005

EXAMINER	
KEMMERER, ELIZABETH	

ART UNIT	PAPER NUMBER
1646	

NOTIFICATION DATE	DELIVERY MODE
02/07/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipdocketdc@vedderprice.com
ahughes@vedderprice.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

In re Patent No. 7,928,057 :
Issue Date: April 19, 2011 :
Application No. 12/162,135 : NOTICE
Filed: November 10, 2008 :
Attorney Docket No. 00824.07.0001 :

This is a notice regarding your request for acceptance of a fee deficiency submission under 37 CFR 1.28(c), filed August 23, 2017.

The Office no longer investigates or rejects original or reissue applications under 37 CFR 1.56. **1098 Off. Gaz. Pat. Office 502 (January 3, 1989)**. Therefore, nothing in this Notice is intended to imply that an investigation was done.

Your fee deficiency submission under 37 CFR 1.28 is hereby **NOT ACCEPTED**.

It appears that the petition was not properly signed by a person having authority to prosecute in the above-identified patent. Therefore, the request can not be accepted at this time. Petitioner's attention is directed to 37 CFR 1.33(b), which states.

> *Amendments and other papers*. Amendments and other papers, except for written assertions pursuant to § 1.27(c)(2)(iii) or (c)(2)(iv), filed in the application must be signed by:
>
> (1) A patent practitioner of record;
>
> (2) A patent practitioner not of record who acts in a representative capacity under the provisions of § 1.34; or
>
> (3) The applicant (§ 1.42). Unless otherwise specified, all papers submitted on behalf of a juristic entity must be signed by a patent practitioner.

Here, the petition appears to be signed on behalf of a juristic entity. A juristic entity (e.g., organizational assignee) must be represented by a patent practitioner even if the juristic entity is the applicant. All paper submitted on behalf of a juristic entity must be signed by a patent practitioner. A juristic entity may sign substitute statements, small entity assertions, terminal disclaimers, powers of attorney, and submissions under 37 CFR 3.73(c) for an assignee to establish ownership of patent property if the assignee is not the original applicant. See 37 CFR 1.31.

I would also like to bring to petitioner attention that petitioner only provided the calculation of the deficiency owed in the instant request. A request for acceptance of a fee deficiency submission under 37 CFR 1.28 petitioners is required to provide an itemization of the

deficiency payment. Petitioner's attention is directed to 37 CFR 1.28(c)(2) (ii) which states:

> (2) *Payment of deficiency owed*. The deficiency owed, resulting from the previous erroneous payment of small entity fees, must be paid.
>
> (ii) *Itemization of the deficiency payment*. An itemization of the total deficiency payment is required. The itemization must include the following information:
>
> (A) Each particular type of fee that was erroneously paid as a small entity, (*e.g.*, basic statutory filing fee, two-month extension of time fee) along with the current fee amount for a non-small entity;
>
> (B) The small entity fee actually paid, and when. This will permit the Office to differentiate, for example, between two one-month extension of time fees erroneously paid as a small entity but on different dates;
>
> (C) The deficiency owed amount (for each fee erroneously paid); and
>
> (D) The total deficiency payment owed, which is the sum or total of the individual deficiency owed amounts set forth in paragraph (c)(2)(ii)(C) of this section.

Applicant is given **ONE MONTH or THIRTY DAYS**, whichever is longer, from the mailing date of this decision to submit the fees required pursuant to 37 CFR 1.28(2)(i) and a replacement itemized listing of each fee erroneously paid as a small entity in compliance with 37 CFR 1.28(c)(2)(ii). **NO EXTENSION OF TIME UNDER 37 CFR 1.136 IS PERMITTED.**

Further correspondence with respect to this matter should be addressed as follows:

By Mail: Mail Stop PETITION
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

By FAX: (571) 273-8300
ATTN: Office of Petitions

By Hand: U. S. Patent and Trademark Office
Customer Service Window, Mail Stop Petitions
Randolph Building
401 Dulany Street
Alexandria, VA 22314

By Internet: EFS-Web[1]

[1] www.uspto.gov/ebc/efs_help.html (for help using EFS-Web call the Patent Electronic Business Center at (866) 217-9197)

Telephone inquiries regarding this matter should be directed to the undersigned at (571) 272-1058.

/Angela Walker/
Angela Walker
Paralegal Specialist
Office of Petitions

Office of Petitions: Decision Count Sheet

Mailing Month 2

Application No. 12162135

For US serial numbers: enter number only, no slashes or commas. Ex: 10123456
For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: Walker, Angela

Count (1) - Palm Credit 12162135

Decision: DISMISSED

FINANCE WORK NEEDED
Select Check Box for YES

Decision Type: 321 - 37 CFR 1.28 TO MAKE ENTITY STATUS LARGE FR

Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED
Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

FINANCE WORK NEEDED
Select Check Box for YES

Decision Type: NONE

Notes:

Initials of Approving Official (if required)

If more than 3 decisions, attach 2nd count sheet & mark this box

Printed on: 2/4/2018

Office of Petitions Internal Document - Ver. 5.0

Office of Petitions: Routing Sheet

Application No. 12162135

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

- [ ] **GRANTED**
- [x] **DISMISSED**
- [ ] **DENIED**

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 11/30/2014. OMB 0651-0051
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
12162135	July 25, 2008

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☑ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 110669

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☑ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number: 110669

OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Julius-Maximilians-Universitaet Wuerzburg

☐ Inventor or Joint Inventor (title not required below)

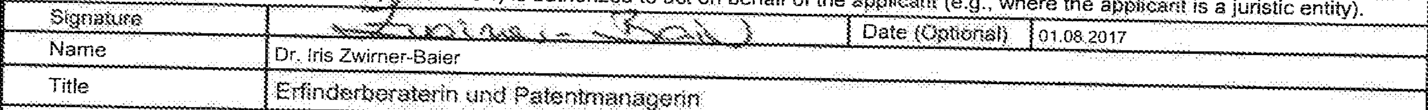
☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☑ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).



Signature		Date (Optional)	01.08.2017
Name	Dr. Iris Zwirner-Baier		
Title	Erfinderberaterin und Patentmanagerin		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Docket No. 109025.070001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Oliver RITTER	)	Confirmation No: 8455
Application No.: 12/162,135	)	Patent No: 7,928,057
Filed: January 25, 2007	)	Issued: April 19, 2011

For: PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY, AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE

United States Patent and Trademark Office
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

NOTIFICATION OF CHANGE OF STATUS FROM MICRO ENTITY TO SMALL ENTITY

Sir:

Patentee inadvertently filed a certification of micro entity status on October 16, 2014. Patentee also paid the maintenance fee as micro entity in the amount of $400.00 on October 16, 2014.

Patentee respectfully submits that the certification of micro entity status was filed in error and Patentee claims small entity status. The deficiency in fees from micro entity to small entity in the amount of $400.00 is being paid concurrently herewith.

Patentee respectfully requests that the record be changed to reflect small entity status.

Date: 4.8.2017

Respectfully submitted,

Signed: [signature]

Name of Signatory: DR. IRIS ZWIRNER-BAIER

Title of Signatory: Manager Patents and Licensing

For: Julius-Maximilians-Universitaet Weurzburg
(Patentee)

Electronic Patent Application Fee Transmittal

Application Number:	12162135
Filing Date:	10-Nov-2008
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE
First Named Inventor/Applicant Name:	Oliver Ritter
Filer:	Ajay A. Jagtiani/Anna Massimo
Attorney Docket Number:	00824.07.0001

Filed as Small Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
REQ. FOR REINSTATEMENT OF TERM REDUCED	2456	1	400	400
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**400**

Electronic Acknowledgement Receipt

EFS ID:	30157480
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE
First Named Inventor/Applicant Name:	Oliver Ritter
Customer Number:	88944
Filer:	Ajay A. Jagtiani/Anna Massimo
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	00824.07.0001
Receipt Date:	23-AUG-2017
Filing Date:	10-NOV-2008
Time Stamp:	10:59:19
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$400
RAM confirmation Number	082317INTEFSW00009424501165
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	POA.pdf	131932 9c18a42a0a9515f66b0fedb63950192175fc6c3c	no	1
Warnings:					
Information:					
2	Maintenance Fee Address Change	ChangeAddressForm.pdf	247062 d7094df01c9650ead069ab69ea47f133267b0060	no	2
Warnings:					
Information:					
3	Notification of loss of entitlement to micro entity status	NotificationofChangeofStatus.pdf	40335 732600128e0ea893c1293b739f60341da5859b45	no	1
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30596 3c8dd96efbc689e46cbe92f58a8bdf65ebb2e7c1	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	449925	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/123 (08-12)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

CHANGE OF CORRESPONDENCE ADDRESS
Patent

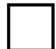
Address to:
Mail Stop Post Issue
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Patent Number	7,928,057
Issue Date	April 19, 2011
Application Number	12/162,135
Filing Date	November 10, 2008
First Named Inventor	Oliver RITTER
Attorney Docket Number	109025-070001

Please change the Correspondence Address for the above-identified patent to:

The address associated with Customer Number: 110669

OR

 **Firm *or* Individual Name**

Address

City | **State** | **ZIP**

Country

Telephone | **Email**

This form cannot be used to change the data associated with a Customer Number. To change the data associated with an existing Customer Number use "Request for Customer Number Data Change" (PTO/SB/124).

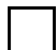
This form will not affect any "fee address" provided for the above-identified patent. To change a "fee address" use the "Fee Address Indication Form" (PTO/SB/47).

I am the:

Patentee.

If the Patentee was not the applicant for patent (37 CFR 1.42), then a Statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is enclosed or was filed on ________________. See 37 CFR 3.71.

Attorney or agent of record. Registration Number 35205.

 Patent practitioner acting in a representative capacity whose correspondence address is the correspondence address of record. Notice has been given to the patentee or owner. Registration Number 35205.

Signature /Ajay A. Jagtiani/

Typed or Printed Name Ajay A. Jagtiani

Date August 23, 2017 | Telephone 703-903-9000

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

*Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Post Issue, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	FILE WRAPPER LOCATION
12/162,135	7928057	1646	9200

Correspondence Address/Fee Address Change

The following fields have been set to Customer Number 88944 on 11/06/2012
- **Correspondence Address**
- **Maintenance Fee Address**

The address of record for Customer Number 88944 is:

88944
Vedder Price P.C.
1401 I Street NW
Suite 1100
Washington, DC 20005

PART 1 - ATTORNEY/APPLICANT COPY

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	04/19/2011	7928057	00824.07.0001	8455

22506 7590 03/30/2011

Vedder Price, PC
1401 I Street, NW
Suite 1100
Washington, DC 20005

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Oliver Ritter, Wuerzburg, GERMANY;

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

22506 7590 12/10/2010

Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

TITLE OF INVENTION: PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	03/10/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
KEMMERER, ELIZABETH	1646	514-001100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 VEDDER PRICE P.C.
2 Ajay A. Jagtiani
3 ________

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: JULIUS-MAXIMILIANS-UNIVERSITAET WUERZBURG

(B) RESIDENCE: (CITY and STATE OR COUNTRY): WUERZBURG, GERMANY

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

☒ Issue Fee
☒ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies ________

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number 22-0259 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /Ajay A. Jagtiani/ Date February 28, 2011

Typed or printed name Ajay A. Jagtiani Registration No. 35,205

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Electronic Patent Application Fee Transmittal

Application Number:	12162135
Filing Date:	10-Nov-2008
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE
First Named Inventor/Applicant Name:	Oliver Ritter
Filer:	Ajay A. Jagtiani/g mills
Attorney Docket Number:	00824.07.0001

Filed as Small Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	2501	1	755	755
Publ. Fee- early, voluntary, or normal	1504	1	300	300

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1055**

Electronic Acknowledgement Receipt

EFS ID:	9547958
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE
First Named Inventor/Applicant Name:	Oliver Ritter
Customer Number:	22506
Filer:	Ajay A. Jagtiani/g mills
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	00824.07.0001
Receipt Date:	28-FEB-2011
Filing Date:	10-NOV-2008
Time Stamp:	15:16:25
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1055
RAM confirmation Number	2233
Deposit Account	220259
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Issue Fee Payment (PTO-85B)	022811_00824070001_ASFILED_ISSUE_TRANSMITTAL.pdf	92208 f5a860bd785aaad68ff5ee45c80748bf74b726c8	no	1
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	31954 5b814113289cdff02dcb00aa0ee5ec3512c4ff03	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	124162	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Oliver RITTER	)	Confirmation No: 8455
	)	
Application No.: 12/162,135	)	Group Art Unit: 1646
	)	
Filed: November 10, 2008	)	Examiner: Elizabeth KEMMERER

For: PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY, AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE

Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS

Sir:

This is in response to the NOTICE TO FILE CORRECTED APPLICATION PAPERS dated **January 19, 2011**, the period for response to which is set to expire on **March 19, 2011**.

It is Applicants position that this Notice was incorrectly issued and that there is no need to correct the Application Papers filed on November 10, 2008.

Applicants respectfully point out that the paragraph on page 4, beginning at line 30 refers to Figure 5 (*i.e.,* Figs. 5A-5D) in total. It merely provides general information on the experiments underlying the results that have been presented in Figs. 5A-5D. Please note that only Figs. 5A-5D were originally filed in the International Application (WO 2007/085455). Therefore, Applicants submit that no further submissions or amendments to the application are required. It is respectfully requested that the Notice be withdrawn.

REMARKS

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance and favorable action is respectfully solicited.

Date: February 17, 2011

Respectfully submitted,

Vedder Price P.C.
875 15th Street, NW, Suite 725
Washington, DC 20005
Telephone: 202-312-3380
Facsimile: 202-312-3322

/Ajay A. Jagtiani/
Ajay A. Jagtiani
Attorney for Applicant(s)
Reg. No.: 35,205

CUSTOMER NO: 22506

Electronic Acknowledgement Receipt

EFS ID:	9466722
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE
First Named Inventor/Applicant Name:	Oliver Ritter
Customer Number:	22506
Filer:	Ajay A. Jagtiani/g mills
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	00824.07.0001
Receipt Date:	17-FEB-2011
Filing Date:	10-NOV-2008
Time Stamp:	13:45:55
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Post Allowance Communication - Incoming	021711_00824070001_ASFILED_Response_to_Notice_to_File_Corrected_Application_Papers.pdf	116673 8c79b14982801d2f0137193219b2fb2e845903d1	no	2

Warnings:

Information:

Total Files Size (in bytes):	116673

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

22506 7590 01/19/2011

Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

EXAMINER	
KEMMERER, ELIZABETH	

ART UNIT	PAPER NUMBER
1646	

MAIL DATE	DELIVERY MODE
01/19/2011	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Application No. : 12162135
Applicant : Ritter
Filing Date : 11/10/2008
Date Mailed : 01/19/2011

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given 2 month(s) from the mail date of this Notice within which to respond.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED. **This period for reply is NOT extendable under 37 CFR 1.136(a).**

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee,Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Carlota Erana/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 12162135

IDENTIFICATION OF SPECIFICATION/DRAWING INCONSISTENCIES

- [X] On Page 4 of the specification there is a brief description of FIG. 5, but the drawings filed 07/25/2008 do not include a drawing with that designation. Applicant must respond either by supplying the omitted drawing or by amending the specification to remove all references to that drawing.

- [ ] The drawings filed include FIG. , but the specification's brief description of the drawings does not describe a drawing with that designation. Applicant must respond either by amending the specification to add a brief description of that drawing or by correcting the drawings to remove the drawing in question.

- [ ] Drawings are present in the application and are referred to in the detailed description of the invention, but the specification does not contain a brief description of the drawings as required by 37 CFR 1.74 and 37 CFR 1.77(b)(8).

- [ ] Page of the specification refers to FIG. , but no drawing with that designation is described in the brief description of the drawings and no drawing with that designation is present in the application. Applicant must respond either by amending the specification to remove all references to that drawing, or by supplying that drawing and amending the specification to add a brief description of it.

- [ ] OTHER:

- [ ] COMMENTS:

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

22506 7590 12/10/2010

Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

EXAMINER	
KEMMERER, ELIZABETH	
ART UNIT	PAPER NUMBER
1646	

DATE MAILED: 12/10/2010

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

TITLE OF INVENTION: PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	03/10/2011

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

22506 7590 12/10/2010

Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

TITLE OF INVENTION: PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	03/10/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
KEMMERER, ELIZABETH	1646	514-001100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 ______________________

2 ______________________

3 ______________________

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies ______________________

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____________ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature ______________________________ Date ______________________________

Typed or printed name ______________________________ Registration No. ______________________________

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

22506 7590 12/10/2010

Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

EXAMINER	
KEMMERER, ELIZABETH	
ART UNIT	PAPER NUMBER
1646	

DATE MAILED: 12/10/2010

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Notice of Allowability	Application No. 12/162,135	Applicant(s) RITTER, OLIVER	
	Examiner Elizabeth C. Kemmerer, Ph.D.	Art Unit 1646	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the amendment of 26 October 2010 and the interview of 06 December 2010*.
2. ☒ The allowed claim(s) is/are *5-11 and 13-17*.
3. ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☒ All b) ☐ Some* c) ☐ None of the:
   1. ☒ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
   1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☒ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Attorney Ajay Jagtiani on 06 December 2010.

The application has been amended as follows:

5. (Currently Amended) A kit comprising the peptide of SEQ ID NO: 1,2, 5-9 or 10 ~~for diagnosing susceptibility for a myocardial and/or immunological disorder~~.

10. (Currently Amended) An antibody ~~directed against~~ that specifically binds the peptide of SEQ ID NO: 1, 2, 5-9 or 10.

11. (Currently Amended) A peptide consisting of the amino acid sequence of SEQ ID NO: 1, 2, 5-9, or 10 ~~for prophylacticly and/or therapeuticly treating a myocardial and/or immunological disorder~~.

Please cancel claim 12 without prejudice.

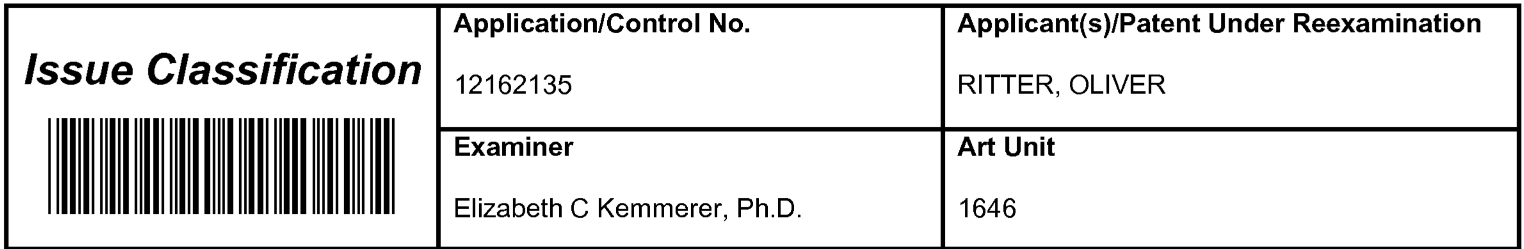
Any inquiry concerning this communication or earlier communications from the examiner should be directed to Elizabeth C. Kemmerer, Ph.D. whose telephone number is (571) 272-0874. The examiner can normally be reached on Monday through Friday, 9:00 a.m. to 5:30 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol, Ph.D. can be reached on (571) 272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/ECK/
06 December 2010

/Elizabeth C. Kemmerer/
Elizabeth C. Kemmerer, Ph.D.
Primary Examiner, Art Unit 1646



Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12162135	RITTER, OLIVER
	Examiner	Art Unit
	Elizabeth C Kemmerer, Ph.D.	1646

ORIGINAL	
CLASS	SUBCLASS
514	1.1

CROSS REFERENCE(S)

CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				
514	16.4	21.3	21.5		
530	300	324	327	387.9	
536	23.1	23.5			

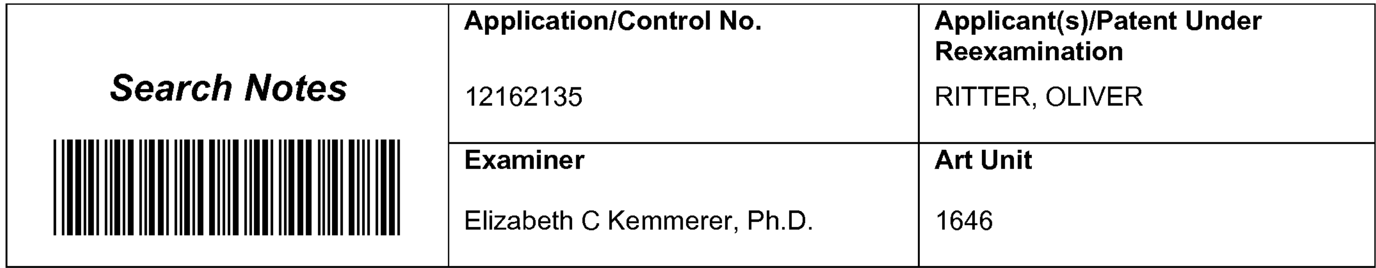
INTERNATIONAL CLASSIFICATION

CLAIMED					NON-CLAIMED
A	6	1	K	38 / 04 (2006.01.01)	
A	6	1	K	38 / 10 (2006.01.01)	
A	6	1	K	38 / 16 (2006.01.01)	
A	6	1	K	38 / 17 (2006.01.01)	
C	0	7	K	7 / 08 (2006.01.01)	
C	0	7	K	14 / 435 (2006.01.01)	
C	1	2	N	15 / 12 (2006.01.01)	
C	0	7	K	16 / 18 (2006.01.01)	

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE (Assistant Examiner)	(Date)	Total Claims Allowed: 12	
/Elizabeth C. Kemmerer/ Primary Examiner.Art Unit 1646 (Primary Examiner)	12/6/10 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure none



Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12162135	RITTER, OLIVER
	Examiner	**Art Unit**
	Elizabeth C Kemmerer, Ph.D.	1646

SEARCHED

Class	Subclass	Date	Examiner

SEARCH NOTES

Search Notes	Date	Examiner
PALM - inventor names	5/24/10	ECK
sequences searched by STIC, available in SCORE	5/24/10	ECK
EAST, DIALOG - search histories attached	5/24/10	ECK
updated	12/6/10	ECK

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner
	previous searches updated	12/6/10	ECK

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

Bib Data Sheet

CONFIRMATION NO. 8455

SERIAL NUMBER	FILING OR 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
12/162,135	11/10/2008 RULE	514	1646	00824.07.0001

APPLICANTS

Oliver Ritter, Wuerzburg, GERMANY;

** **CONTINUING DATA** **************************

This application is a 371 of PCT/EP07/00643 01/25/2007

** **FOREIGN APPLICATIONS** ********************

EUROPEAN PATENT OFFICE (EPO) 06090014.9 01/27/2006

IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** SMALL ENTITY **
** 03/17/2009

Foreign Priority claimed ☐ yes ☐ no 35 USC 119 (a-d) conditions met ☐ yes ☐ no ☐ Met after Allowance Verified and Acknowledged ____ Examiner's Signature Initials	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
	GERMANY	9	12	7

ADDRESS

22506

TITLE

PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE

FILING FEE RECEIVED	FEES: Authority has been given in Paper	
1170	No. _________ to charge/credit DEPOSIT ACCOUNT No. _________ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other ____________ ☐ Credit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Oliver RITTER	)	Confirmation No: 8455
	)	
Application No.: 12/162,135	)	Group Art Unit: 1646
	)	
Filed: November 10, 2008	)	Examiner: Elizabeth KEMMERER

For: PEPTIDE FOR INHIBITION OF CALCINEURIN

United States Patent and Trademark Office
Mail Stop Amendment
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT

Sir:

In response to the Office Action mailed **May 26, 2010**, the period for response which is set to expire on **October 26, 2010** with a **two-month** extension of time, please amend the above-captioned application, without prejudice or disclaimer as follows, as well as consider the following remarks.

Amendments to the Specification begin on page **2** of this paper.

Amendments to the Claims begin on page **3** of this paper.

Remarks/Arguments begin on page **5** of this paper.

IN THE SPECIFICATION

Please replace the title of the invention with the following title:

PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY, AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE.

IN THE CLAIMS

Please cancel Claims 1-4 without prejudice or disclaimer of the subject matter recited therein. Please amend Claims 5-12 and add Claims 13-17 to read as follows. Claims 5-17 are currently pending. This listing of claims will replace all prior versions and listings of claims in the application.

1. (Cancelled)

2. (Cancelled)

3. (Cancelled)

4. (Cancelled)

5. (Currently Amended) A kit comprising the peptide of SEQ ID NO: 1,2, 5-9 or 10 for diagnosing susceptibility for a myocardial and/or immunological disorder.

6. (Currently Amended) A therapeutic agent comprising the peptide of SEQ ID NO: 1, 2, 5-9 or 10.

7. (Currently Amended) [[A]] An ~~therapeutic~~ agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5-9 or 10.

8. (Currently Amended) A therapeutic agent comprising a peptide of at least 90% ~~preferably at least 95%, more preferred at least 98%, still more preferred 100%~~ identity of the peptide of SEQ ID NO: 1, 2, 5-9 or 10; wherein ~~he~~ the peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

9. (Previously Presented) The therapeutic agent of claim 6, further comprising a pharmaceutically acceptable carrier.

10. (Currently Amended) An antibody directed against the peptide of SEQ ID NO: 1, 2, 5-9 or 10.

11. (Currently Amended) A ~~method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:~~

~~a) providing a test sample comprising at least one cell;~~

~~b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10;~~

~~c) determining an interaction between the peptide of SEQ ID NO: 1, 2, 5-10 and cellular calcineurin; and~~

~~d) Use of using the~~ peptide of SEQ ID NO: 1, 2, 5-9 or 10 for ~~manufacturing a therapeutic agent for prophylactic~~ prophylacticly and/or ~~therapeutic~~ therapeuticly ~~treatment of~~ treating a myocardial and/or immunological disorder.

12. (Currently Amended) The ~~use method~~ peptide of claim 11, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, ~~in particular~~ hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

13. (New) A therapeutic agent comprising a peptide of at least 95 % identity of the peptide of SEQ ID NO: 1, 2, 5-9 or 10; wherein the peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

14. (New) A therapeutic agent comprising a peptide of at least 98 % identity of the peptide of SEQ ID NO: 1, 2, 5-9 or 10; wherein the peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

15. (New) A therapeutic agent comprising a peptide of 100 % identity of the peptide of SEQ ID NO: 1, 2, 5-9 or 10; wherein the peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

16. (New) The agent of claim 7, further comprising a pharmaceutically acceptable carrier.

17. (New) The therapeutic agent of claim 8, further comprising a pharmaceutically acceptable carrier.

REMARKS

A. Status of Claims

Favorable reconsideration of this application, as presently amended, is respectfully requested. Claims 5-17 are currently pending. Claims 5-8, 10 and 11 are currently amended, and Claims 13-17 are newly added. Claims 1-4 are cancelled without prejudice or disclaimer. Applicants reserve the right to file continuing or divisional applications covering the subject matter of the cancelled claims.

B. Procedural Matters

Applicant notes the Examiner's indication that the certified copy of the foreign application as required under 35 U.S.C. 119(b) has not been filed in the USPTO. However, Applicant filed a certified copy of the priority application in the USPTO on July 9, 2010. Accordingly, acknowledgment of the priority document and benefit of the foreign priority filing date is respectfully requested.

Applicant gratefully acknowledges the Examiner's acceptance of the Information Disclosure Statements filed on November 10, 2008, and the notation by the Examiner that all references listed in the accompanying SB08 forms have been considered (as none have been lined through), and made of record.

C. Response to Objections to the Claims and Specification

At page 2, the Office Action objects to the title not being sufficiently descriptive. Applicant has adopted/amended the title of the invention suggested by the examiner. Therefore, this objection should now be withdrawn. In the event that the Examiner wishes a different title, the Examiner is invited to contact Applicant's representative at 202-312-3380.

At page 3, the Office Action objected to Claim 8 for informality typographical error. Applicant has obviated this rejection by amending the word "he" to the word "the" as suggested by the Examiner. Thus, this objection is rendered moot with respect to this claim.

D. Response to Rejection under 35 U.S.C. § 112, Second Paragraph

At pages 3-5, the Office Action objects to Claims 1 through 12 for allegedly failing to particularly point out and distinctly claim the subject matter of the invention. All claims presently on file are amended to recite SEQ ID NOs: 1, 2, 5 to 9 or 10 to clarify that the sequences represent alternatives and that the recited peptide is only required to comprise one of these sequences. The amendment is supported by the description on page 21, line 18 to page 22, line 5 of the application as originally filed (WO 2007/085455 A1).

Present claim 8 has been amended to remove alternative ranges by the elimination of the language "preferably at least … ".

Present claim 11 is amended to refer to a peptide for prophylactically and/or therapeutically treating a myocardial and/or immunological disorder. This amendment is supported by claim 11 as originally filed (WO 2007/085455 A1) and page 10, lines 19 to 21 of the description as originally filed. Thus, all claims distinctly point out the claimed subject-matter and comply with 35 U.S.C. § 112, second paragraph.

Present claim 12 has been amended to remove the language "in particular."

E. Response to Rejection under 35 U.S.C. § 112, First Paragraph, for Alleged Failure to Comply with the Enablement Requirement

At pages 5-8, the Office Action rejects Claims 1 through 15 under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. Claims 1-4 are deleted from the listing of claims.

Claims 11 and 12 are amended by deleting the methods steps which were objected by the Examiner. Therefore, theses claims no longer refer to a method for diagnosing, and the objections raised under 35 U.S.C. § 112, first paragraph regarding these claims are moot.

Currently pending claim 7 is amended by deleting the word "therapeutic", as suggested by the Examiner. The claim now refers to an agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5 to 9 or 10. The amendment is supported by the disclosure of the

examples given in the description as originally filed (WO 2007/085455 A1), in particular in the paragraph dealing with the preparation and use of plasmids (page 12, lines 6 to 26).

F. Response to Rejection under 35 U.S.C. § 102

At page 9, the Office Action rejects Claim 10 under 35 U.S.C. § 102(b) for allegedly being anticipated by XP002433633 (internet article, 10 January 2005). The applicant agrees that this document discloses three antibodies (AB12233, AB3673 and AB50501) which react with calcineurin A from several species including human. However, in contrary to the Examiner's assumption, they are not suitable for binding to any of the peptides of the present application.

AB12233 was not raised against full length calcineurin A but against a peptide consisting of amino acids 264 to 283 of calcineurin A. These amino acids do not comprise any of SEQ ID NOs: 1, 2, 5 to 9 or 10 and also do not comprise the nuclear localization signal (NLS) of calcineurin A. This information can be obtained from the datasheet of the calcineurin A antibody AB12233 provided by Abcam, which is herewith submitted as enclosure 1.

The antibodies AB3673 and AB50501, although raised against full length calcineurin A protein (data sheets for AB3673 and AB50501 are submitted as enclosure 2 and 3, respectively), are unlikely to specifically bind to any of the peptides of SEQ ID NO: 1, 2, 5 to 9, or 10. The reason for this is that the NLS sequence is masked within the full length calcineurin A protein. It only gets exposed to the surface upon a change in confirmation due to the activation of calcineurin A by Ca^{2+} or proteolysis. This is evident from the application as originally filed (WO 2007/085455 A1) on page 6, lines 29 to 31 and also from Hallhuber et al, 2006 (cited in the international search report), page 632, column 2, lines 2 to 7. Additionally, the calcineurin A protein used to raise e.g. AB3673 was expressed in E. coli, as is evident from the datasheet. Since the described activation of calcineurin A is un-likely to occur in E. coli, the antibody AB3673 is unlikely to comprise any antibodies directed against the NLS domain of calcineurin A. This is further supported by the product description of AB3673 given in the datasheet which shows that this antibody only detects one single form of calcineurin A of about 62 kDa. The proteolyzed and active form of calcineurin A which is recognized by the antibody of the present

application, is shorter and cannot be detected by AB3673 as is evident from a comparison of enclosure 2 to Fig. 4 of the application as filed.

Thus, the antibodies disclosed in XP002433633 are not suitable for specifically binding the peptides of SEQ ID NO: 1, 2,5 to 9 or 10. Therefore, currently pending claim 10 is not anticipated by XP002433633 and complies with the requirements of 35 U.S.C. § 102.

CONCLUSION

In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration of this Application and the prompt allowance of all pending claims currently under consideration.

Should the Examiner feel that there are any issues outstanding after consideration of this response, the Examiner is invited to contact Ajay A. Jagtiani at (202) 312-3380 to expedite prosecution of the application.

The Commissioner is hereby authorized by this paper to charge any fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to **Deposit Account 22-0259**. **This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3) if needed.**

Respectfully submitted,

Date: October 26, 2010

/Ajay A. Jagtiani/
Ajay A. Jagtiani
Attorney for Applicant(s)
Reg. No.: 35,205

Vedder Price P.C.
875 15th Street, NW
Washington, DC 20005
Telephone: (202) 312-3320
Facsimile: (202) 312-3322
CUSTOMER NO: 22506

Enclosure 1

Data sheet for AB12233 from Abcam

Calcineurin A antibody (ab12233)

Product Name	Calcineurin A antibody
Product type	Primary antibodies
Description	Rabbit polyclonal to Calcineurin A
Immunogen	Peptide corresponding to aa 264-283 of human Calcineurin A conjugated to KLH. This sequence is identical in rat and mouse Calcineurin A.
Reacts with (species key)	Hu, Ms, Rat, Cow, Dog, Hm, Rb
Tested applications (see key)	IP, WB

Abreviews Customer reviews (see key)

Submit an Abreview

Be the first to review this antibody and earn 220 Abpoints.

Read about Abreviews

Application notes (see key)

Recommended dilutions
IP: Use at a concentration of 10 µg/ml.
WB: Use at a concentration of 2 µg/ml. Detects a band of approximately 61 kDa (predicted molecular weight: 62.5 kDa).

Not tested in other applications.
Optimal dilutions/concentrations should be determined by the end user.

Cellular localization Nuclear

Research areas
Signal Transduction >> Calcium Signaling >> Calmodulin Pathway
Signal Transduction >> Protein Phosphorylation >> Ser / Thr Phosphatases
Immunology >> Adaptive Immunity >> B Cells >> Non-CD

Relevance
Calcineurin, a major soluble calmodulin binding protein in the brain, a Ca2+/calmodulin dependent serine/threonine protein phosphatase, with a relatively narrow substrate specificity. This metalloenzyme, also known as phosphatase 2B, is a heterodimer composed of a calmodulin binding, catalytic alpha subunit 61 kDa, calcineurin A) and calcium binding beta subunit (18 kDa, calcineurin B). The Ca2+ binding subunit, calcineurin B, is immunologically conserved from yeast to mammals. The presence of 2 different calcineurin B isoforms (beta 1 and beta 2) has been reported in rat testis. The catalytic subunit of calcineurin, calcineurin A, isolated from different tissues or different organisms, exhibits some immunological heterogeneity. Further, there are at least 2 isoforms of calcineurin A in bovine brain (alpha 1 and alpha 2, 61 and 59 kDa, respectively).

Database links

Entrez Gene	286852	(Cow)	**SwissProt**	Q08209 (Human)
	5530	(Human)		
	19055	(Mouse)	**Unigene**	272458
	24674	(Rat)		

Enclosure 2

Data sheet for AB3673 from Abcam

Calcineurin A antibody (ab3673)

Product Name	Calcineurin A antibody
Product type	Primary antibodies
Description	Rabbit polyclonal to Calcineurin A
Immunogen	Full length Calcineurin A fusion expressed in E. coli.
Reacts with (species key)	Hu, Ms
Tested applications (see key)	IP, WB

Abreviews
Customer reviews
(see key)

Submit an Abreview and earn up to 220 Abpoints by reviewing this product

• WB 1 Abreview - average rating: *****

Read about Abreviews
(Read all Abreviews)

Application notes (see key)

Recommended dilutions
IP: Use at an assay dependent dilution.
WB: 1/2000 - 1/5000. Detects a band of approximately 62 kDa in brain homogenates.

Not tested in other applications.
Optimal dilutions/concentrations should be determined by the end user.

Cellular localization Nuclear

Research areas
Signal Transduction >> Calcium Signaling >> Calmodulin Pathway
Signal Transduction >> Protein Phosphorylation >> Ser / Thr Phosphatases
Immunology >> Adaptive Immunity >> B Cells >> Non-CD

Relevance
Calcineurin, a major soluble calmodulin binding protein in the brain, a Ca2+/calmodulin dependent serine/threonine protein phosphatase, with a relatively narrow substrate specificity. This metalloenzyme, also known as phosphatase 2B, is a heterodimer composed of a calmodulin binding, catalytic alpha subunit 61 kDa, calcineurin A) and calcium binding beta subunit (18 kDa, calcineurin B). The Ca2+ binding subunit, calcineurin B, is immunologically conserved from yeast to mammals. The presence of 2 different calcineurin B isoforms (beta 1 and beta 2) has been reported in rat testis. The catalytic subunit of calcineurin, calcineurin A, isolated from different tissues or different organisms, exhibits some immunological heterogeneity. Further, there are at least 2 isoforms of calcineurin A in bovine brain (alpha 1 and alpha 2, 61 and 59 kDa, respectively).

Database links

Entrez Gene	5530 (Human) 19055 (Mouse)	SwissProt	Q08209 (Human)
GeneCard	GC04M102343 (Human)	Unigene	272458 (Human)

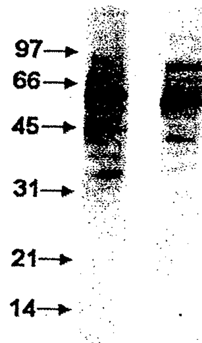
Omim	114105 (Human)
Raised in	Rabbit
Clonality	Polyclonal
Isotype	IgG
Purity	Whole antiserum
Storage buffer	Preservative: 0.01% Sodium Azide Material safety datasheet (MSDS) for this product: Sodium Azide MSDS
Form	Liquid
Concentration	Concentration not determined - why is this?
Storage instructions	Store at +4°C short term (1-2 weeks). Aliquot and store at -20°C or -80°C. Avoid repeated freeze / thaw cycles.

At Abcam, we have one centralized database to hold all of our product information, so that everything we know about this Calcineurin A antibody is on this datasheet. But please do contact us if you would like any reassurance!

See below for Calcineurin A antibody images, references, products related to ab3673 and other tools.

Calcineurin A antibody images:

- Calcineurin A antibody (ab3673)



Western Blot of wild type mouse brain homogenate with ab3673 used at 1/6000. Please note that this image was assembled from two different blots. (The numbers on the scale correspond to kD).

References for Calcineurin A antibody (ab3673)

This product has been used in:

Hoeffer CA *et al.* The Down syndrome critical region protein RCAN1 regulates long-term potentiation and memory via inhibition of phosphatase signaling. *J Neurosci* 27:13161-72 (2007). PubMed: 18045910

If you publish research using ab3673 please let us know so that we can cite the reference on this datasheet.

Customer reviews (feedback) regarding Calcineurin A antibody

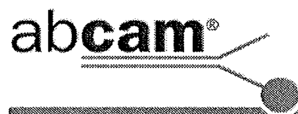
Customer FAQs regarding Calcineurin A antibody

Protocols for Calcineurin A antibody

Price and availability of products related to Calcineurin A antibody

Enclosure 3

Data sheet for AB50501 from Abcam



Calcineurin A antibody [CN-A1] (ab50501)

Product Name	Calcineurin A antibody [CN-A1]
Product type	Primary antibodies
Description	Mouse monoclonal [CN-A1] to Calcineurin A
Immunogen	Full length native protein purified from bovine brain
Reacts with (species key)	Hu, Rat, Cow
Specificity	Does not cross-react with the beta-subunit (Calcineurin B).
Tested applications (see key)	ELISA, IHC-P, WB

Abreviews Submit an Abreview

Customer reviews (see key) Be the first to review this product and earn 220 Abpoints.

Read about Abreviews

Application notes (see key)	**Recommended dilutions** ELISA: Use at an assay dependent dilution. IHC-P: Use at an assay dependent dilution. WB: 1/10000. Detects a band of approximately 61 kDa (predicted molecular weight: 59 kDa). Not yet tested in other applications. Optimal dilutions/concentrations should be determined by the end user.
Positive control (see definition)	WB: rat brain extract IHC-P: neurons in human ganglia
Cellular localization	Nuclear
Research areas	Signal Transduction >> Signaling Pathway >> Calcium Signaling >> Calmodulin Pathway Signal Transduction >> Protein Phosphorylation >> Ser / Thr Phosphatases Immunology >> Adaptive Immunity >> B Cells >> Non-CD
Relevance	Calcineurin, a major soluble calmodulin binding protein in the brain, a Ca2+/calmodulin dependent serine/threonine protein phosphatase, with a relatively narrow substrate specificity. This metalloenzyme, also known as phosphatase 2B, is a heterodimer composed of a calmodulin binding, catalytic alpha subunit 61 kDa, calcineurin A) and calcium binding beta subunit (18 kDa, calcineurin B). The Ca2+ binding subunit, calcineurin B, is immunologically conserved from yeast to mammals. The presence of 2 different calcineurin B isoforms (beta 1 and beta 2) has been reported in rat testis. The catalytic subunit of calcineurin, calcineurin A, isolated from different tissues or different organisms, exhibits some immunological heterogeneity. Further, there are at least 2 isoforms of calcineurin A in bovine brain (alpha 1 and alpha 2, 61 and 59 kDa, respectively).

Database links

Entrez Gene	286852 (Cow) 5530 (Human) 24674 (Rat)	**SwissProt**	P48452 (Cow) Q08209 (Human) P63329 (Rat)
Omim	114105 (Human)	**Unigene**	435512 (Human) 6866 (Rat)

Raised in	Mouse
Clonality	Monoclonal
Clone number	CN-A1
Isotype	IgG1
Purity	Ascites
Storage buffer	Preservative: 15mM Sodium Azide Constituents: Ascites

Material safety datasheet (MSDS) for this product:
Sodium Azide MSDS

Form	Liquid
Concentration	Batch dependent within range: 7.90 - 7.90 mg/ml Find concentration of your lot : Show
Storage instructions	Store at +4°C. Aliquot and store at -20°C or -80°C when reconstituted. Avoid repeated freeze / thaw cycles.

At Abcam, we have one centralized database to hold all of our product information, so that everything we know about this Calcineurin A antibody [CN-A1] is on this datasheet. But please do contact us if you would like any reassurance!

References for Calcineurin A antibody [CN-A1] (ab50501)

This product has been used in:

Holt M *et al.* Synaptic vesicles are constitutively active fusion machines that function independently of Ca2+. *Curr Biol* **18**:715-22 (2008). **WB.** PubMed: 18485705

If you publish research using ab50501 please let us know so that we can cite the reference on this datasheet.

Customer reviews (feedback) regarding Calcineurin A antibody [CN-A1]

Customer FAQs regarding Calcineurin A antibody [CN-A1]

Protocols for Calcineurin A antibody [CN-A1]

Price and availability of products related to Calcineurin A antibody [CN-A1]

Calcineurin A antibody [CN-A1] - related products:

Products for Immunohistochemistry (Formalin/PFA-fixed paraffin-embedded sections)

Slides

Products for Western blot

Loading controls

Lysates

Positive Controls for Calcineurin A antibody [CN-A1]

ab29474 Brain (Rat) Nuclear Lysate - normal tissue

Compatible Secondaries for Calcineurin A antibody [CN-A1]

ab2891	HRP polymer antibody
ab6563	Cy5 ® antibody
ab6726	Texas Red ® antibody
ab6728	HRP antibody
ab6729	Alkaline Phosphatase antibody
ab6785	FITC antibody
ab6788	Biotin antibody
ab7002	Phycoerythrin antibody
ab7018	Agarose antibody
ab27229	15nm Gold antibody
ab27230	10nm Gold antibody
ab27231	15nm Gold antibody
ab27233	5nm Gold antibody
ab27241	10nm Gold antibody
ab27242	20nm Gold antibody
ab27243	40nm Gold antibody
ab27244	5nm Gold antibody
ab60313	Chromeo™ 488 antibody
ab60316	Chromeo™ 546 antibody
ab60318	Chromeo™ 642 antibody
ab79091	Rat monoclonal [SB84a] Secondary Antibody to Mouse IgG2a - gamma chain

Other Calcineurin A Antibodies

See all Calcineurin A antibodies (11)...

All products are "FOR RESEARCH USE ONLY AND ARE NOT INTENDED FOR DIAGNOSTIC OR THERAPEUTIC USE"

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Doc Code: TRAN.LET

Document Description: Transmittal Letter

PTO/SB/21 (07-09)
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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	12/162,135
Filing Date	November 10, 2008
First Named Inventor	Oliver RITTER
Art Unit	1646
Examiner Name	Elizabeth KEMMERER
Attorney Docket Number	00824.07.0001
Total Number of Pages in This Submission	19

ENCLOSURES (*Check all that apply)*

- [x] Fee Transmittal Form
  - [x] Fee Attached
- [x] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [x] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) ______
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

Enclosure 1 - Data Sheet for AB12233;
Enclosure 2 - Data Sheet for AB3673; and
Enclosure 3 - Data Sheet for AB50501

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	VEDDER PRICE P.C.		
Signature	/Ajay A. Jagtiani/		
Printed name	Ajay A. Jagtiani		
Date	October 26, 2010	Reg. No.	35,205

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below:

Signature			
Typed or printed name		Date	

This collection of information is required by 37 CFR 1.5. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and1.14. This collection is estimated to 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Electronic Patent Application Fee Transmittal

Application Number:	12162135
Filing Date:	10-Nov-2008
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	Oliver Ritter
Filer:	Ajay A. Jagtiani/g mills
Attorney Docket Number:	00824.07.0001

Filed as Small Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Independent claims in excess of 3	2614	2	110	220
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	2252	1	245	245
Miscellaneous:				
		Total in USD ($)		**465**

Electronic Acknowledgement Receipt

EFS ID:	8701255
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	Oliver Ritter
Customer Number:	22506
Filer:	Ajay A. Jagtiani/g mills
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	00824.07.0001
Receipt Date:	26-OCT-2010
Filing Date:	10-NOV-2008
Time Stamp:	11:43:43
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$465
RAM confirmation Number	12036
Deposit Account	220259
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Miscellaneous Incoming Letter	102610_00824070001_ASFILED_Fee_Transmittal.pdf	116472 80d4045cac97154d841b6895204b851bb4d282ef	no	1
Warnings:					
Information:					
2	Extension of Time	102610_00824070001_ASFILED_Extension_of_Time.pdf	40028 1c2a1ca73088c9a72fd8746429f2bc63dd836c9b	no	1
Warnings:					
Information:					
3		102610_00824070001_ASFILED_NonFinal_Amendment.pdf	433685 715b38c47b58c0b142b234146f6a63c286981efa	yes	16

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Appendix to the Specification	2	2
Claims	3	4
Applicant Arguments/Remarks Made in an Amendment	5	16

Warnings:					
Information:					
4	Miscellaneous Incoming Letter	102610_00824070001_ASFILED_Transmittal_Form.pdf	45905 32560ab864f9f9f2c642e4fe74ccd76bf301d4f7	no	1
Warnings:					
Information:					
5	Fee Worksheet (PTO-875)	fee-info.pdf	32172 81515b6324d201711c09b1d57895ccbea2921cd3	no	2
Warnings:					
Information:					

Total Files Size (in bytes):	668262

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

FEE TRANSMITTAL
FY 2010

Complete if Known	
Application Serial No.	12/162,135
Filing Date	November 10, 2008
First Named Inventor	Oliver RITTER
Group No.	1646
Examiner Name	Elizabeth KEMMERER
Confirmation No.	8455

METHOD OF PAYMENT

☒ Payment Enclosed:

☐ Check ☐ Money Order ☒ Other

☒ The Commissioner is hereby authorized to credit or charge any fee indicated below for this submission to Deposit Account No. 10-0233

☐ Required Fees (copy of this sheet enclosed).

☒ Additional fee required under 37 CFR 1.16 and 1.17.

☒ Overpayment Credit.

☒ **Applicant claims small entity status**

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

Application Type	Filing	Search	Examination	Fee Paid
Utility	330	540	220	
Design	220	100	140	
Plant	220	330	170	
Reissue	330	540	650	
Provisional	220	0	0	
Small Entity Discount				
1. TOTAL				**0.00**

2. EXCESS CLAIM FEES

	Fee	Small Entity Fee ($)
Each claim over 20 or, for Reissues, each claim over 20 and more than in the original patent.	52	26
Each independent claim over 3 or, for Reissues, each independent claim more than in the original patent.	220	110

Total Claims		Extra Claims		Fee Paid ($)
13	- 20 =	0	x $52.00 =	$0.00

HP = highest number of total claims paid for, if greater than 20

Indep. Claims		Extra Claims		Fee Paid ($)
9	- 7 =	2	x $110.00 =	$220.00

HP = highest number of total claims paid for, if greater than 3

Multiple Dependent Claims	Fee($)	Small Entity fee ($)	Fee Paid ($)
	390	195	

2. TOTAL: $220.00

3. APPLICATION SIZE FEE

If the specification and drawing exceed 100 sheets of paper, the application size fee due is $270 ($135 for small entity) for each additional sheets or fraction there of. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Additional 50 or fraction thereof	Fee ($)	Fee Paid
-100=	0	/50= round up to a whole number	x	= 0.00

3. TOTAL: 0.00

CORRESPONDENCE ADDRESS

Direct all correspondence to:

Vedder Price, PC
875 15th Street, NW
Washington, DC 20005
Tel. No.: (202) 312-3320
Fax No.:(202) 312-3322
CUSTOMER 22506

FEE CALCULATION (continued)

4. ADDITIONAL FEES

Large Entity Fee($)	Small Entity Fee ($)	Fee Description	Fee Paid
130	65	Surcharge - late filing fee or oath	
50	25	Surcharge - late provisional filing fee or cover sheet	
130	130	Non-English specification	
2,520	2,520	Request for ex parte re-examination	
130	65	Extension for reply within 1st mo.	
490	245	**Extension for reply within 2nd mo.**	**$245.00**
1,110	555	Extension for reply within 3rd mo.	
1,730	865	Extension for reply within 4th mo.	
2,350	1,175	Extension for reply within 5th mo.	
540	270	Notice of Appeal	
540	270	Filing a brief in support of an appeal	
1,080	540	Request for oral hearing	
400	0	Petitions to the Director	
180	180	Submission of IDS	
810	405	Filing a submission after final rejection (37 CFR 1.129(a))	
810	405	For each additional invention to be examined (37 CFR 1.129(b))	
100	100	Certificate of Correction for applicant's error	
140	70	Submission of Terminal Disclaimer	
Other fee (Specify)			
Other fee (Specify)			
		4. TOTAL:	**245.00**

TOTAL AMOUNT SUBMITTED

$465.00

SIGNATURE BLOCK

Respectfully submitted,

Date: October 26, 2010
Reg. No.: 35,205
Tel. No.: (202) 312-3320
Fax No.: (202) 312-3322

/Ajay A. Jagtiani, Reg. No. 35,205/
Ajay A. Jagtiani
Attorney for the Applicant(s)
Vedder Price, PC
875 15th Street, NW
Washington, DC 20005

PTO/SB/22 (07-09)
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U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) **FY 2009** ***(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)***	Docket Number (Optional) 00824.07.0001
Application Number 12/162,135	Filed November 10, 2008
For Peptide for Inhibition of Calcineurin	
Art Unit 1646	Examiner Elizabeth KEMMERER

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$130	$65	$
☑ Two months (37 CFR 1.17(a)(2))	$490	$245	$ 245.00
☐ Three months (37 CFR 1.17(a)(3))	$1110	$555	$
☐ Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐ Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

☑ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 22-0259.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the ☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 35,205

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 ____________

/Ajay A. Jagtiani/	October 26, 2010
Signature	Date
Ajay A. Jagtiani	202-312-3320
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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PTO/SB/06 (07-06)
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	
12/162,135	11/10/2008	☐ To be Mailed

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
			TOTAL			TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT 10/26/2010	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 13	Minus	** 20	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 9	Minus	***7	= 2	X $110 =	220	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	**220**	OR	TOTAL ADD'L FEE	

AMENDMENT	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/Trina Steptoe/

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Europäisches Patentamt GD1	European Patent Office DG1	Office européen des brevets DG1

Bescheinigung	Certificate	Attestation
Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.	The attached documents are exact copies of the European patent application described on the following page, as originally filed.	Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécificée à la page suivante.

Patentanmeldung Nr.	**Patent application No.**	**Demande de brevet n°**

06090014.9 / EP06090014

The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP06090014

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
p.o.

R.C. van Dijk

Anmeldung Nr:
Application no.: 06090014.9
Demande no:

Anmeldetag:
Date of filing: 27.01.06
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Julius-Maximilians-Universität Würzburg
Sanderring 2
97070 Würzburg/DE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Peptide for inhibition of calcineurin

In anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen / State/Date/File no. / Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation / International Patent Classification / Classification internationale de brevets:

G01N33/50

Am Anmeldetag benannte Vertragstaaten / Contracting states designated at date of filing / Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC NL PL PT RO SE SI SK TR

EPO-BERLIN
27-01-2006

Peptide for Inhibition of Calcineurin

Field of the invention

5 The present invention relates to a method for diagnosing susceptibility for a myocardial and/or immunological disorder, a kit and a therapeutic agent comprising a peptide of SEQ ID NO: 1 or 2 and uses thereof.

Background of the invention

10 The movement of proteins over ~40 kDa into and out of the nucleus is governed by the nuclear pore complex (NPC), a multi subunit structure embedded in the nuclear envelope (Jans et al., 2000). Transcription factors and enzymes that regulate the activity of these proteins are shuttled across the nuclear envelope by proteins that recognize nuclear localization signals/sequences (NLS) and nuclear export 15 signals (NES). Positively charged NLSs are bound by importins α and/or ß – also known as karyopherins – which tether cargo to the cytosolic face of the nuclear pore complex and facilitate translocation of proteins into the nucleus. The CRM1 protein, also referred to as exportin, mediates the transfer of proteins out of the nucleus (Fornerod et al., 1997), although CRM1-independent mechanisms for nu-20 clear export exist (Kutay et al., 1997). The ability of nuclear import and export machinery to access a NLS or NES is often dictated by signalling events that lead to the exposure or masking of these regulatory sequences (Cyert, 2001). This may occur through direct modification of the target protein or via modification of an associated factor.

25

The signalling cascade of calcineurin (CnA) and the nuclear factor of activated T cells (NF-ATc) is a crucial transducer of cellular function. NF-ATc is an ubiquitous transcription factor but has particular relevance in cytokine expression and the development of pathologic myocardial hypertrophy. The formation of complexes 30 between transcription factors and DNA is crucial for the transcriptional process. Therefore, the time that transcription factors remain nuclear is a major determi-

nant for transcriptional activity. It was shown that that in addition to the transcription factor NF-ATc, the phosphatase calcineurin is also translocated to the nucleus (Burkhard et al., 2005; Frey et al., 2000; Zou et al., 2001; Shibasaki et al., 1996). Calcineurin is therefore not only responsible for dephosphorylating NF-ATc, thus enabling its nuclear import; its presence in the nucleus is also significant in ensuring the full transcriptional activity of NF-ATc (Zhu et al., 1999). The traditional understanding of calcineurin activation via sustained high Ca^{2+}-levels (Timmerman et al., 1996; Dolmetsch et al., 1997) was advanced by findings of the inventor that calcineurin is activated by proteolysis of the C-terminal auto-inhibitory domain, which leads to a constitutively nuclear translocation of calcineurin (Burkhard et al., 2005).

Calcineurin is known to suppress the immune system, and various pharmaceutical compositions are available from commercial sources. Calcineurin inhibitors belong to the group of cyclosporine, tacrolimus (Protopic®, Prograf®) and pimecrolimus. Indications are psoriasis, atopic dermatitis, rheumatism and allergies, to name a few.

US patent application No. 2003/0045679 A1 to Crawford describes compositions which are useful for inhibiting and potentiating the activity of cellular calcineurin. These compositions include peptides, peptide analogs and whole proteins. They can be used to treat calcineurin related pathologies such as cardiac, brain, immune system and developmental abnormalities.

All known calcineurin inhibitors show strong side effects like high blood pressure, renal disorders and viral and bacterial infections. The later two are due to their general immunosuppressive properties. Thus there is a still existing need for a pharmaceutical composition for the treatment of calcineurin related disorders, like myocardial disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

The solution to this problem is achieved by providing the embodiments characterized by the claims, and described further below.

5 **Summary of the invention**

The present invention is directed to a method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of (a) providing a test sample comprising at least one cell; (b) contacting the cell with a peptide of SEQ ID NO: 1 or 2; (c) determining an interaction between the peptide of SEQ ID
10 NO: 1 or 2 and cellular calcineurin; wherein a primarily nuclear localization of a complex comprising calcineurin and the peptide of SEQ ID NO: 1 or 2 indicates susceptibility for a myocardial and/or immunological disorder.

Further, the present invention is directed to a kit comprising the peptide of SEQ ID
15 NO: 1 or 2 for diagnosing a myocardial and/or immunological disorder.

The present invention also concerns a therapeutic agent comprising the peptide of SEQ ID NO: 1 or 2, a nucleic acid which encodes the peptide of SEQ ID NO: 1 or 2 or a peptide of at least 90 % identity of the peptide of SEQ ID NO: 1 or 2. The
20 peptide inhibits substantially the translocation, transport or shuffling of calcineurin and its derivates from the cytoplasm to the nucleus of a cell.

Moreover, the present invention is also directed to the use of the peptide of SEQ ID NO: 1 or 2 for manufacturing a therapeutic agent for prophylactic and/or thera-
25 peutic treatment of a myocardial and/or immunological disorder.

Brief description of the drawings

Figure 1 shows the identification of a nuclear localization signal (NLS) in calcineurin (CnA). A schematic drawing of green fluorescent protein (GFP)- and
30 FLAG-tagged CnA deletion mutants is given. The subcellular localization is indicated (c = primarily cytoplasmatic; n = primarily nuclear). NLS = nuclear localiza-

tion signal; NES = nuclear export sequence; CnB = calcineurin B binding domain; CaM = calmodulin binding domain; AID = auto-inhibitory domain; numbers correspond to CnAß amino acid sequence; EGFP and FLAG indicate tags.

5 **Figure 2** shows interactions of calcineurin (CnA) with importin ß1. Various deletion mutants were transfected into HeLa cells. Immunoprecipitation of GFP-tagged full length CnA and GFP-NLS fusion protein was performed with a GFP-antibody; the FLAG-tagged CnA 2-173 and CnA 3-143 mutants were precipitated with a FLAG-antibody. Immunodetection was performed with an importin ß1 antibody. Full
10 length calcineurin interacted with importin ß1 as demonstrated by co-immunoprecipitation whereas the truncated CnA-mutants, lacking the NLS, did not co-immunoprecipitate with importin.

Figure 3 shows the results of HeLa cells, which were transfected with the indi-
15 cated GFP-tagged CnA mutants. Immunoprecipitation of the CnA/importin complexes was performed with importin ß1 antibodies, detection with a GFP antibody. Addition of the NLS peptide (SEQ ID NO:2) abrogated calcineurin interaction with importin ß1.

20 **Figure 4** shows nuclear export signals (NES) in calcineurin (CnA). CnA lacking the NES (Δ420-434) was resistant to proteolysis by calpain. GFP-tagged full length CnA and CnA (Δ420-434) were transfected into HeLa cells. Whole cell lysates were incubated with calpain I for 30 min and the lysates run on a 5 % gel. Western blot analysis for GFP revealed proteolysis of full length CnA.

25

Figure 5 shows functional consequences of inhibition of calcineurin (CnA) nuclear import. Neonatal rat cardiomyocytes were incubated with a peptide mimicking the NLS sequence of CnA (SEQ ID NO:2) and stimulated with Ang II (100 µM). The peptide saturated CnA/importin ß1 binding capacity. Nuclear import of CnA was
30 therefore prevented. Control experiments were performed with a non-sense peptide SEQ ID NO: 3).

Figure 5A top shows that phosphatase activity of CnA was not influenced by the synthetic NLS peptide (SEQ ID NO: 2) as measured using a specific phosphor substrate of CnA.

Figure 5A bottom shows that transcriptional activity of the calcineurin/NF-ATc complex was suppressed by the inhibitory NLS peptide (SEQ ID NO: 2). Transcriptional activity was assessed with a NF-ATc luciferase reporter plasmid.

Figure 5B shows that the development of myocardial hypertrophy was also suppressed by the inhibitory NLS peptide (SEQ ID NO: 2) as demonstrated by protein synthesis **(Figure 5B top)** and cell size (magnification 600 x) **(Figure 5B bottom).**

Figure 5C shows that B-type natriuretic peptide (BNP), a molecular marker of hypertrophy, is suppressed by the use of the inhibitory NLS peptide (SEQ ID NO: 2).

Figure 5D shows the dose dependent decrease of NF-ATc transcriptional activity when treated with the NLS peptide of SEQ ID NO: 2. At higher concentrations (>1 µM, indicated by asterisk) a toxic effect is possible since values are below background levels of untreated cardiomyocytes (ctr = control).

Detailed description of the invention

The inventor has surprisingly found that a peptide which mimics the nuclear localization signal of calcineurin (SEQ ID NO: 2) successfully prevents the translocation of calcineurin from the cellular cytoplasm to the nucleus. Without intending to be bound by any theory, it is believed that the NLS peptide (SEQ ID NO: 2) binds to importin, thereby preventing complex formation between calcineurin and importin. However, complex formation between calcineurin and importin is necessary for transporting calcineurin to the nucleus, where calcineurin together with NF-ATc then act as activated transcription factor due to the constant presence of calcineurin. The NLS peptide (SEQ ID NO: 2) efficiently inhibits complex formation

and thereby blocks entry of calcineurin into the nucleus. It is further believed that that the auto-inhibitory domain (AID) not only blocks the catalytical activity of calcineurin but also masks the nuclear localization signal. Removal of the AID via a conformational change in calcineurin following Ca^{2+} activation or by proteolysis of
5 the auto-inhibitory domain leads to exposure of the nuclear localization signal and resultant nuclear translocation of calcineurin.

Myocardial disorders and immunological disorder are associated with altered transcription patterns of NF-ATc downstream targets. Therefore, the NLS peptide may
10 be used as a diagnostic tool for assessing the susceptibility for a myocardial and/or immunological disorder.

In a first aspect, the present invention is directed to a method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:
15 a) providing a test sample comprising at least one cell;
b) contacting the cell with a peptide of SEQ ID NO: 1 or 2;
c) determining an interaction between the peptide of SEQ ID NO: 1 or 2 and cellular calcineurin;

wherein a primarily nuclear localization of a complex comprising calcineurin and
20 the peptide of SEQ ID NO: 1 or 2 indicates susceptibility for a myocardial and/or immunological disorder.

As used herein the term susceptibility means predisposition or likelihood for the development of a myocardial and/or immunological disorder. The term disorder
25 means a dysfunction, a malfunction, a disease or a medical condition. The term peptide includes linear peptides, cyclic peptides and peptide analogs, derivates and salts thereof. Any modification can be employed as long as the peptide retains its ability to prevent entry of calcineurin into the nucleus of a cell. The peptides of the present invention can be incorporated in a structure comprising more
30 amino acids, and the peptides comprise preferably about 60 amino acids, more preferred about 30 to 40 amino acids, most preferred about 20 to 30 amino acids.

The test sample can be derived from an animal, in particular a mammal, preferably from a human, more preferably from a patient who is suspected to be susceptible of a myocardial and/or immunological disorder.

5

Likewise, SEQ ID NO:1 or 2 can be used for a method for diagnosing further calcineurin related disorders, like myocardial disorders, immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma,

10 bronchial asthma and allergic asthma.

In a preferred embodiment the peptide of SEQ ID NO: 1 or 2 is chemically, biologically and/or physically labeled. Examples for labeling peptides are dyes, like streptavidin-biotin labeling, fluorescent labeling, labeling by antibodies and radio-

15 active labeling.

In a further preferred embodiment the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopa-

20 thy, dilated cardiomyopathy, aortic stenosis and heart attack. The immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

In a second aspect the invention provides a kit comprising the peptide of SEQ ID

25 NO: 1 or 2 for diagnosing susceptibility for a myocardial and/or immunological disorder. Likewise, SEQ ID NO:1 or 2 can be used for a kit for diagnosing further calcineurin related disorders, like myocardial disorders, immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma,

30 bronchial asthma and allergic asthma.

In another aspect the present invention concerns a therapeutic agent comprising the peptide of SEQ ID NO: 1 or 2 and a therapeutic agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1 or 2.

5 The term nucleic acid, as used herein, includes DNA molecules, like cDNA and genomic DNA, RNA molecules, like mRNA, analogs of DNA and RNA which are generated by nucleotide analogs and derivates, fragments and homologs thereof. The nucleic acid can be single stranded or double stranded, however double stranded DNA molecules are preferred.

10

In another aspect, the present invention covers a therapeutic agent comprising a peptide of at least 90 %, preferably at least 95 %, more preferred at least 98 %, still more preferred 100 % identity of the peptide of SEQ ID NO: 1 or 2; wherein the peptide substantially inhibits the translocation of calcineurin from the cyto-
15 plasm to the nucleus of a cell. Conserved amino acid substitutions are covered by the present disclosure. The peptide of SEQ ID NO: 2 comprises three lysine amino acids (K) at positions 1, 5 and 7. When all lysine residues (K) are substituted by alanine (A) the peptides does no longer inhibit entry of calcineurin to the nucleus. However individual lysine residues may be substituted as long as the
20 peptide prevents it ability to block the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

In a preferred embodiment the therapeutic agent further comprises a pharmaceutically acceptable carrier, like fillers and excipients. Various application forms of
25 the peptides are possible. Cremes and ointments can be applied topically. In stent technology, the peptide can be used to cover a stent, which is then implanted to a patient in need thereof. Coronary stents are particularly preferred. Also intravascular pumps, in particular mini pumps can be used for delivery of the peptide to a patient.

30

In another aspect, the present invention covers the use of the peptide of SEQ ID NO: 1 or 2 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

5 In a preferred embodiment the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack. The immunological disorder is selected from the group consisting of a transplant rejection and immune suppres-
10 sion.

In yet another aspect the present invention is directed to the use of the peptide of SEQ ID NO: 1 or 2 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of calcineurin related disorders, like myocardial disorders,
15 immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

A more complete understanding of the present invention can be obtained by ref-
20 erence to the following examples, which are provided for the purpose of illustration only and are not intended to limit the scope of the invention.

Examples

A) Material and Methods

25 The following materials and methods were employed:

Cell culture

Neonatal rat cardiomyocytes of Wistar rats were isolated as described previously (Ritter et al., 2003). Cells were resuspended in minimum essential
30 medium/1 % FCS. After preplating, the cardiomyocyte containing supernatant was recovered, and cells were plated in minimum essential medium

on 6-well plates at a density of $1x10^6$ cells per well. The medium for cardiomyocytes contained 5-bromo-2'-deoxyuridine (0.1 mmol/L) to suppress fibroblast growth. Fibroblast contamination of cardiomyocyte cultures was between 4 % and 7 % as regularly determined by immunohistochemical staining for troponin T. HeLa cells were grown in DMEM/5 % FCS (Sigma). 48 hours after preparation, cells were stimulated with 10 nmol/L angiotensin II (Ang II). Cells were harvested 24 hours after stimulation. Concentrations were as follows: Ang II: 10 µM, calpeptin: 10 µM, NLS peptide and control peptide: 1 µM, respectively.

Calcineurin (CnA) activity

NF-ATc reporter plasmid comprised the Il-2 promoter followed by luciferase. Luciferase activity was determined according to the manufacturer's protocol (Promega). CnA phosphatase activity was determined using a commercial kit (CnA kit assay, Biomol) that measures effect on a specific CnA phosphosubstrate. Free PO_4 was indicated by a malachite green dye. The use of this kit has been described previously (Ritter et al., 2002).

Plasmids

Epitope-tagged derivatives of calcineurin Aβ containing amino-terminal EGFP were generated using the mammalian expression vector pEGFP-C3 (BD Bioscience Clontech). For cloning of calcineurin mutants the mammalian expression vector pCMV-Sport6 containing the directionally cloned cDNA of human calcineurin Aβ was used (Invitrogen). The following mutants have been amplified by PCR, digested with XbaI and XhoI and cloned into the XbaI and XhoI sites of the described plasmid: CnAβ(full), CnAβ 1-415, CnAβ 1-425, CnAβ 1-445, CnAβ 1-465, CnAβ 1-485 and CnAβ 171-190. For these truncated mutants different reverse oligonucleotides were designed with subsequent N-terminal binding sites. The forward primer, binding at the N-terminus of calcineurin, was identical for each plasmid apart from the CnA(171-190) mutant. For this derivative primers are bound

upstream of aa 171 and downstream of aa 190, respectively. Two internal mutants CnAβ(Δ420-434) and CnAβ(Δ420-445) were cloned into the XbaI and XhoI sites of pEGFP-C3 using a two step strategy. For deleting the short areas from aa 420 to 434 and from aa 420 to 445 two fragments have been amplified. After ligation of these two fragments the resulting whole fragment was cloned into the XbaI and XhoI sites of pEGFP-C3. FLAG-tagged calcineurin derivatives were kindly provided by the group of Ludwig Neyses (Divison of Cardiology, The University of Manchester).

Immunofluorescence and immunoprecipitation

The subcellular distribution of calcineurin was determined by immunofluorescence stainings. The primary antibodies used in fluorescence staining are described elsewhere (Burkhard et al., 2005). Secondary antibodies were Cy-3–labeled sheep anti-rabbit IgG or Cy2-conjugated mouse anti-goat IgG (Jackson Laboratories).

B) Results

The inventor's group demonstrated that posttranslational modification, specifically proteolysis of the auto-inhibitory domain (AID) of calcineurin leads to its activation and strong nuclear translocation (Burkhard et al., 2005). The calpain mediated cleavage of the c-terminal AID and the causative link to myocardial hypertrophy was demonstrated in human myocardial tissue.

In this invention the inventor demonstrates the prominent nuclear translocation of CnA in different animal models of diseased myocardium. In wild-type mice a predominantly cytosolic distribution of CnA was observed, whereas in mice that underwent aortic banding or myocardial infarction a strong nuclear localization of CnA in the hypertrophied myocardium was observed.

This is consistent with observations of nuclear import of calcineurin in cell culture models (Burkhard et al., 2005; Frey et al., 2000; Zou et al., 2001;

Zhu et al., 1999). However, the role of calcineurin within the nucleus was not been investigated before and the precise mechanism leading to nuclear import and export remains unclear.

5 **Reversible nuclear localization of calcineurin after angiotensin stimulation**

To assess whether CnA import into the nucleus is a chronic phenomenon or an acute response the inventor investigated the time course of CnA shuttling. A GFP tagged calcineurin plasmid encoding full length CnAß was
10 transfected into neonatal rat cardiomyocytes. Cells were stimulated with Ang II at 10 µM. Confocal microscopy revealed onset of nuclear translocation of calcineurin after 2h. After 4h of Ang II stimulation CnA was predominantly nuclear. After 6h maximum of intensity of the GFP-calcineurin signal was in the nucleus. Similarly, 2h after removal of Ang II from the medium
15 there was a homogenous distribution of CnA in the cytosol and the nucleus and after 4h there was perinuclear localization of CnA. 6h after removal of the stimulus calcineurin localised completely in the cytosol again. To prevent CnA from calpain mediated proteolysis, which would cause constitutive activation of CnA and therefore persistent nuclear translocation, all experi-
20 ments where performed in the presence of a membrane permeable calpain inhibitor (Burkhard et al., 2005). Calcineurin is very sensitive to pathological stimuli in cardiomyocytes and is able to induce a response within a few hours after the initial stimulation.

25 **Construction of NLS peptide mutants**

The transport of proteins into the nucleus relies on nuclear localization signal/sequences (NLS) which are bound by importins; the complex of cargo protein and importin is then transported into the nucleus. To define the regions of calcineurin that are required for nuclear import different GFP- or
30 FLAG-tagged calcineurin deletion mutants (Figure 1) were screened to assess those that entered the nucleus and those that remained cytosolic. In

general, deletion of the auto-inhibitory domain led to nuclear translocation and deletion of the region starting amino acid 173 (within the putative NLS) prevented calcineurin from entering the nucleus. The mutants 2-173 and 3-143 may have reduced catalytic phosphatase activity as the catalytic sub- 5 unit of CnA extends to amino acid 333. Therefore failure to translocate may in theory be a result of decreased phosphatase activity. Previous work however has demonstrated that catalytically inactive CnA mutants are also translocated into the nucleus (Shibasaki et al., 1996). Sequence comparisons with known NLS of other proteins enabled further delineation of the 10 putative NLS region to the sequence 171-190. Fusion of this 171-190 fragment to the GFP backbone resulted in translocation of the GFP/NLS fusion protein into the nucleus, whereas the pure GFP backbone remained cytosolic. Whilst full length CnA resides in the cytosol, it was translocated into the nucleus after Ang II stimulation due to removal of the auto-inhibitory domain 15 from the catalytic subunit and probably from the putative NLS. In contrast, deletion mutants 2-173 and 3-143 which both lacked the putative NLS remained exclusively cytosolic despite Ang II stimulation.

Interaction between calcineurin mutants and importin

20 Importin ß1 has been shown to bind the NLS of different cargo proteins (Pemberton et al., 2005). Interactions between CnA mutants and importin ß1 were assessed to determine whether the functionally defined NLS physically interacts with importin ß1. As demonstrated by co-immunoprecipitation experiments importin ß1 displayed good affinity for full 25 length calcineurin and also the deletion mutant CnA 1-415. Specifically, the interaction domain was mapped to the region 171-190 as evidenced by the ability of a fusion protein consisting of GFP and the 171-190 fragment to co-precipitate importin ß1. However, both deletion mutants 1-173 and 1-143 completely abolished the interaction between importin ß1 and CnA. These 30 data indicate that the NLS identified by functional analyses also mediate interactions between importin ß1 and calcineurin (Figure 2).

Peptide competition assay

To demonstrate further that the identified NLS in CnA is essential for the nuclear import of calcineurin, a peptide competition assay to prevent importin ß1/CnA binding was used. A peptide with the putative NLS sequence of calcineurin (AAVALLPAVLLALLAKQECKIKYSERV – SEQ ID NO: 1) was synthesised and added to the medium (the first 15 letters give N-terminal extension to increase membrane permeability, NLS sequence is underlined). However, the peptide facilitating membrane permeability is not critical, and an alternative membrane anker, harbouring two alanine residues instead of leucine residues at positions 11 and 14, respectively, can also be used (AAVALLPAVLAALAA – SEQ ID NO: 4). In control experiments a non sense peptide (AAVALLPAVLLALLAAQECAIAYSEYV – SEQ ID NO: 3) was used. Addition of the synthetic NLS peptide saturated the binding domain of importin ß1 for CnA and therefore prevented CnA binding to importin ß1. Inhibition of this interaction suppressed calcineurin nuclear import. The non-inhibitory control peptide did not interfere with the calcineurin/importin binding; accordingly, nuclear translocation of CnA was not inhibited. Also the NLS peptide abrogated the interaction of calcineurin and importin ß1 as demonstrated in co-immunoprecipitation assays (Figure 3).

Nuclear export control sequences

To identify sequences in CnA that control nuclear export serial carboxy-terminal truncation mutants with an N-terminal GFP tag of CnA were generated and examined by confocal fluorescence microscopy. Experiments were performed in the presence of a calpain inhibitor to prevent calpain induced cleavage of the auto-inhibitory domain (AID) and to ensure functional integrity of calcineurin. Cells were stimulated with Ang II for 12 h to achieve nuclear entry of CnA, subsequently the stimulus was removed to promote nuclear export. Full-length CnA (amino acids 1–524)

was re-localized exclusively to the cytosol of transfected cardiomyocytes after removal of the stimulus. An extended deletion variant (1-415) was not able to leave the nucleus any more.

5 These results demonstrated that sequences in the region c-terminal to amino acid 415 regulate nuclear export. Consistent with these findings and sequence comparisons with known NES sites, a CnA mutant lacking amino acids 420-434 remained exclusively nuclear after removal of the stimuli. Inhibition of calpain did not influence this result as the calpain cleaveage
10 site (at 424) was deleted in this mutation variant.

As different cleavage sites in the calcineurin sequence (Wu et al., 2004) have been described for calpain, the inventor assessed whether the NES sequence (423-434) really contains the calpain cleavage site. Therefore,
15 the GFP-tagged calcineurin mutant lacking the NES domain was used for further experiments. The plasmid was transfected into HeLa cells and lysates of these cells expressing this deletion variant were incubated with calpain I. Western Blot analysis was used to demonstrate that full length CnA is proteolysed by calpain whereas CnAΔ423-434 is resistant to calpain
20 mediated proteolysis (Figure 4).

Mechanism of nuclear export of calcineurin

A number of proteins that shuttle across the nuclear membrane have been identified using CRM1 as the export shuttle. Some are transcription factors
25 like NF-ATc that confer pro-hypertrophic actions. Others act within counter-regulatory pathways that repress cardiac hypertrophic growth such as the transcriptional repressor HDAC5 (McKinsey et al., 2000). To address whether CnA nuclear export is mediated by CRM1, experiments using the CRM1 specific inhibitor, leptomycin B (LMB), were performed. Agonist-
30 dependent nuclear import of full length CnA was achieved by Ang II stimulation. Calpeptin was added to prevent proteolysis of CnA. The addition of

LMB to prevent CRM 1 mediated export indeed suppressed nuclear export of CnA. Together, these findings demonstrate that LMB confines CnA to the nucleus in the cardiomyocyte by blocking its nuclear export. This supports the hypothesis that nucleo-cytoplasmic shuttling of CnA is coupled to a NES between amino acids 423-434 and is mediated by CRM1.

Nuclear accumulation of calcineurin

In vivo studies of pathological myocardial hypertrophy showed that proteolysis of the calcineurin auto-inhibitory domain at amino acid 424 results in a constitutively active calcineurin mutant lacking both the AID (aa 468-490) and the NES (423-434). To determine whether loss of the AID or disruption of the NES is responsible for strong nuclear accumulation of CnA the nuclear import and export qualities of a GFP tagged CnA mutant with a deletion of the NES was investigated. Cells were transfected with CnAΔ423-434. In this case calcineurin resided in the cytosol. Stimulation of the transfected cells with Ang II resulted in subsequent translocation of CnA into the nucleus. Based on these results the inventor concluded that the AID not only blocks the catalytical activity of CnA but also masks the NLS. Removal of the AID via a conformational change in calcineurin following Ca^{++} activation or by proteolysis of the auto-inhibitory domain leads to exposure of the NLS and resultant nuclear translocation of CnA.

Subsequent removal of the stimulating Ang II agent from the medium resulted in the CnAΔ423-434 mutant remaining nuclear, as the lack of the NES made it impossible for CRM1 to interact with CnA and to transport it back to the cytosol. The loss of the C-terminal part of CnA would therefore appear to regulate nuclear shuttling of CnA at the level of both nuclear import and export. Deprivation of the AID promotes import via importin ß1, and loss of the NES hinders nuclear export via CRM1 mediated mechanisms.

A peptide corresponding to the calcineurin NLS inhibited CnA nuclear import and maintained the overall structure of CnA. The suppression of calcineurin transport into the nucleus is important for the NF-ATc transactivational status; this peptide is therefore useful as a tool to suppress myocardial hypertrophy.

Markers for myocardial hypertrophy

Phosphatase activity, transcriptional activity, protein synthesis, cell size and makers of myocardial hypertrophy in response to the peptide related inhibition of CnA nuclear import were examined. Phosphatase activity was assessed employing a specific substrate (RII) for can (Ritter et al., 2002). Cardiomyocytes were stimulated with Ang II (10 µM), and CnA phosphatase activity was measured in the presence of the NLS peptide or a non sense control peptide. Total CnA phosphatase activity was not affected by inhibition of the access of importin ß1 to the CnA NLS (289±17 % vs. 273±11 %, n = 8, p = not significant). In contrast, transcriptional activity of the CnA/NF-ATc signalling pathway was decreased significantly by the NLS peptide (463±11 % vs. 123±8 %, n=8, $p<0.05$) (Figure 5A top). Similarly, myocardial hypertrophy, as evidenced by protein synthesis (707±21 % vs. 133±12 %, n=8, $p<0.05$), cell size (1191±91 μm^2 vs. 728±65 μm^2, n=8, $p<0.05$) (Figure 5B), and expression of brain natriuretic peptide (BNP) (163±11 % vs. 88±8 %, n=8, $p<0.05$) (Figure 5C), were suppressed by the NLS peptide. Transcriptional activity of a NF-ATc luciferase reporter plasmid was decreased when nuclear import of CnA was blocked by the NLS peptide in a dose dependent manner (Figure 5D).

These data indicated that despite full CnA phosphatase activity, CnA was unable to form effective transcriptional complexes. Activated calcineurin in itself does not appear to be sufficient to induce hypertrophy. Full transcriptional activity of CnA/NF-ATc is only possible in the presence of nuclear

calcineurin. It is thus clear that calcineurin nuclear translocation is a prerequisite to the formation of effective NF-ATc transcriptional complexes.

Conclusion

5 The inventor's data show that CRM1 not only exports NF-ATc, but also calcineurin across the nuclear envelope. To interrupt transcriptional activity of the calcineurin/NF-ATc signalling cascade CRM1 is first required to export calcineurin, so that in a second round CRM1 can access the NES of NF-ATc and subsequently proceed with its nuclear export. This mechanism is 10 prevented in myocardial hypertrophy by the proteolysis of calcineurin by calpain at amino acid 424, resulting in loss of the auto-inhibitory domain including the NES. In this scenario calcineurin remains nuclear because it is inaccessible to the export protein CRM1.

15 As import always precedes export, the inhibition of CnA nuclear import by peptide competition for the binding of the nuclear import protein importin ß1 presents a more sophisticated approach to abolishing the deleterious effects of exaggerated NF-ATc transcriptional activity. Therefore the NLS peptide is useful as a new therapeutic agent for pathologic myocardial hy- 20 pertrophy.

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Claims

1. A method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:
   a) providing a test sample comprising at least one cell;
   b) contacting the cell with a peptide of SEQ ID NO: 1 or 2;
   c) determining an interaction between the peptide of SEQ ID NO: 1 or 2 and cellular calcineurin;

   wherein a primarily nuclear localization of a complex comprising calcineurin and the peptide of SEQ ID NO: 1 or 2 indicates susceptibility for a myocardial and/or immunological disorder.

2. The method of claim 1, wherein the peptide of SEQ ID NO:1 or 2 is chemically, biologically and/or physically labeled.

3. The method of claim 1 or 2, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

4. A kit comprising the peptide of SEQ ID NO: 1 or 2 for diagnosing susceptibility for a myocardial and/or immunological disorder.

5. A therapeutic agent comprising the peptide of SEQ ID NO: 1 or 2.

6. A therapeutic agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1 or 2.

7. A therapeutic agent comprising a peptide of at least 90 %, preferably at least 95 %, more preferred at least 98 %, still more preferred 100 % identity of the peptide of SEQ ID NO: 1 or 2; wherein the peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

8. The therapeutic agent of any of the claims 5 to 7, further comprising a pharmaceutically acceptable carrier.

9. Use of the peptide of SEQ ID NO: 1 or 2 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

10. The use of claim 9, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

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Abstract

The present invention relates to a method for diagnosing susceptibility for a myocardial and/or immunological disorder disorder, a kit and a therapeutic agent comprising a peptide of SEQ ID NO: 1 or 2 and uses thereof.

Figure 1

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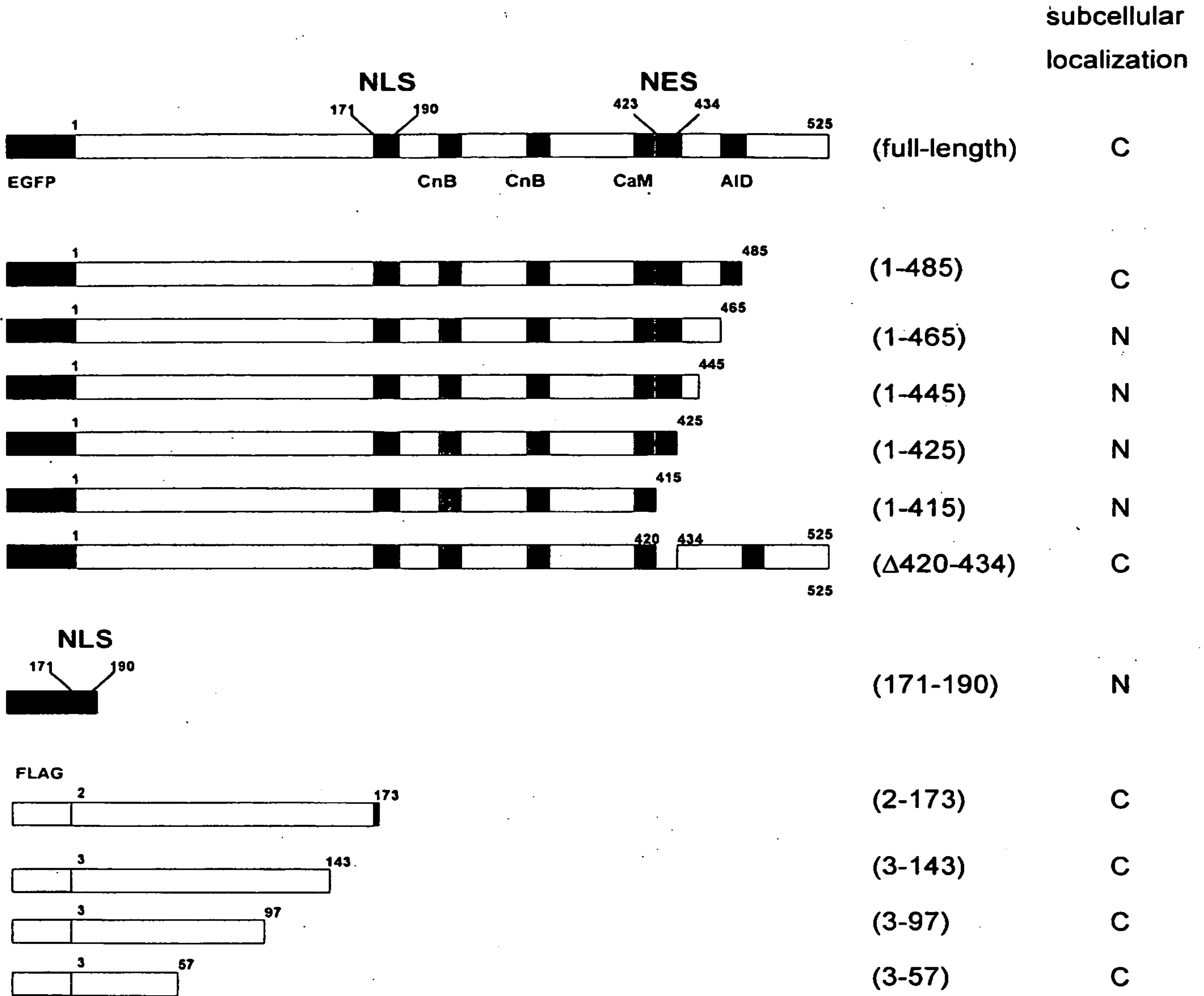


Figure 1

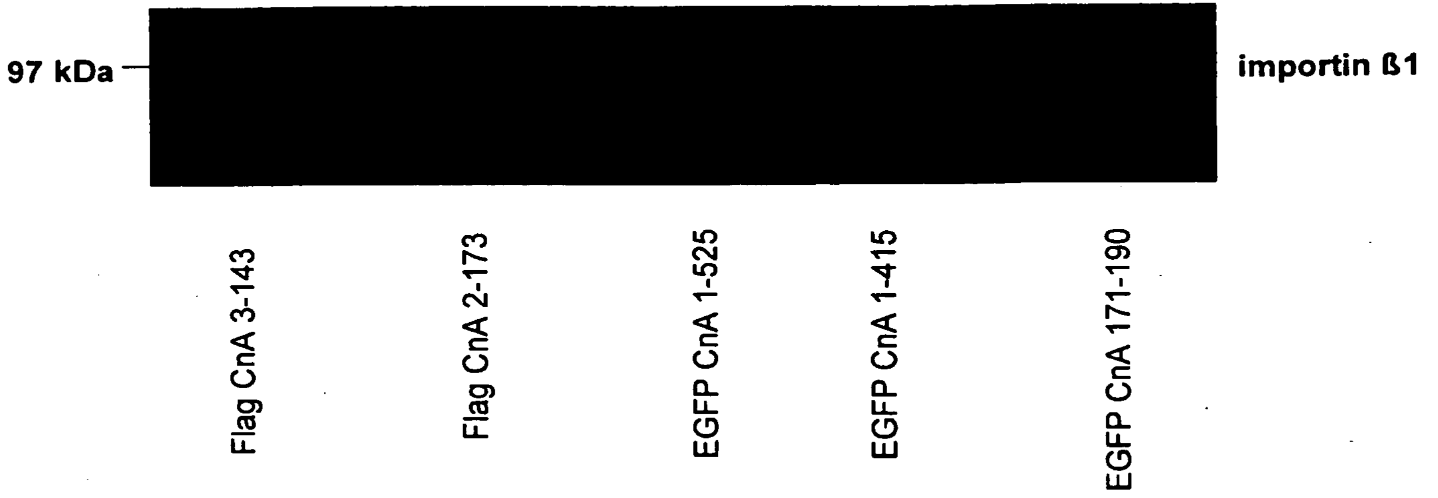
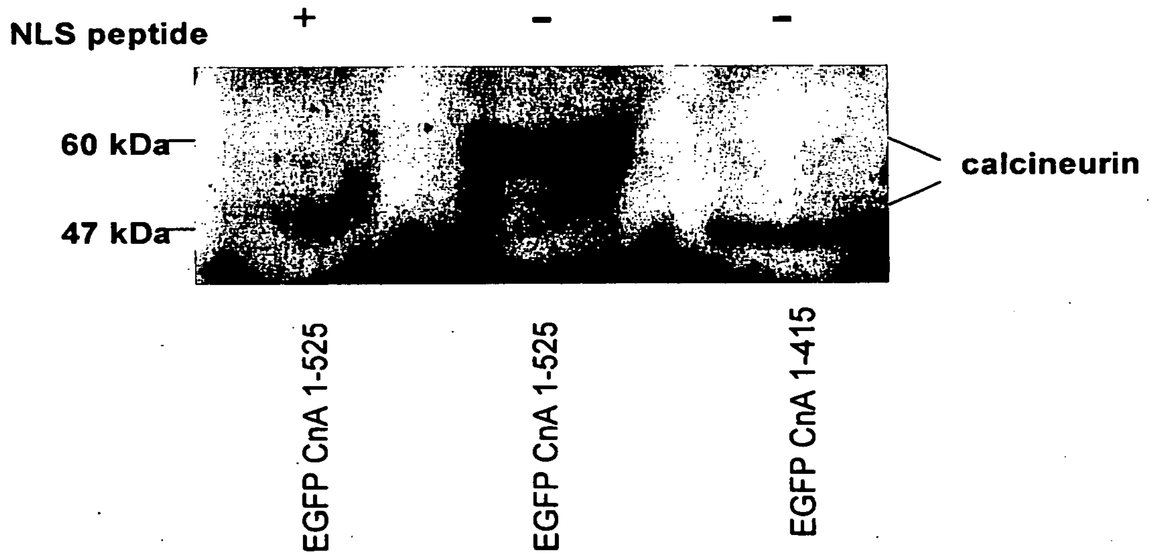


Figure 2



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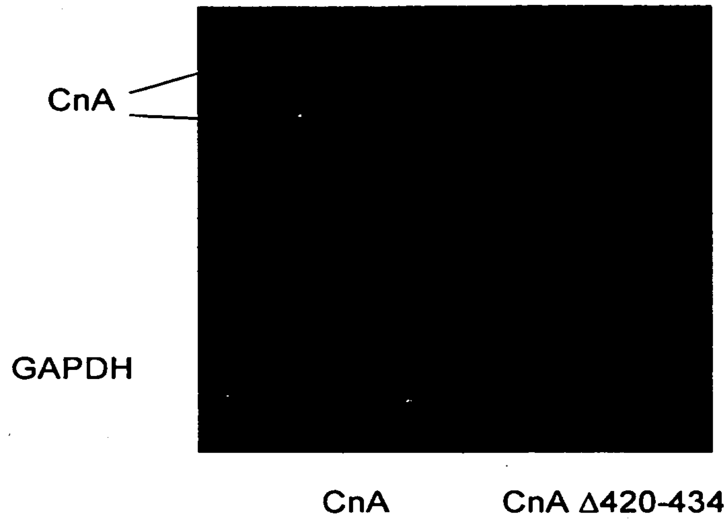

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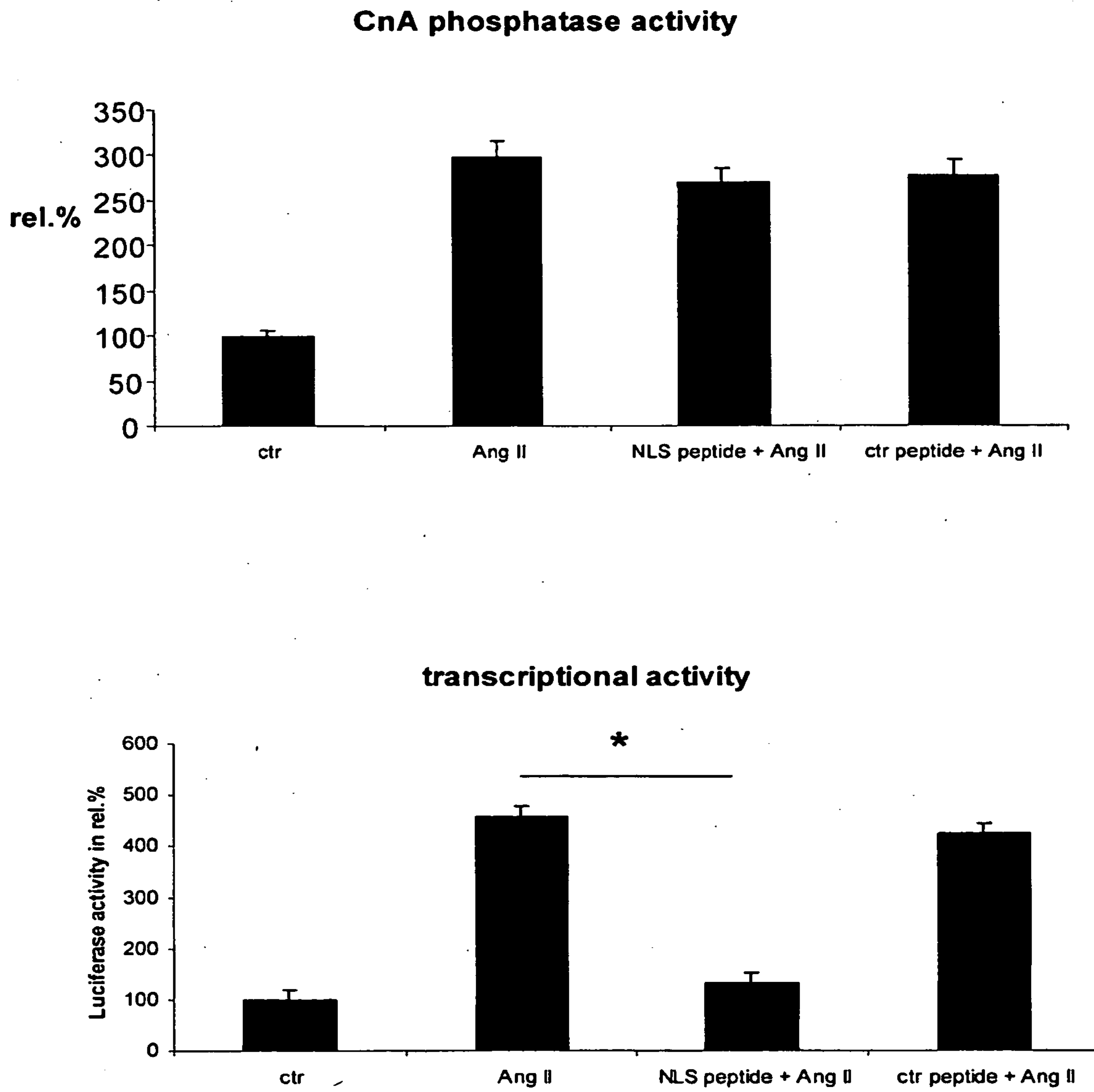

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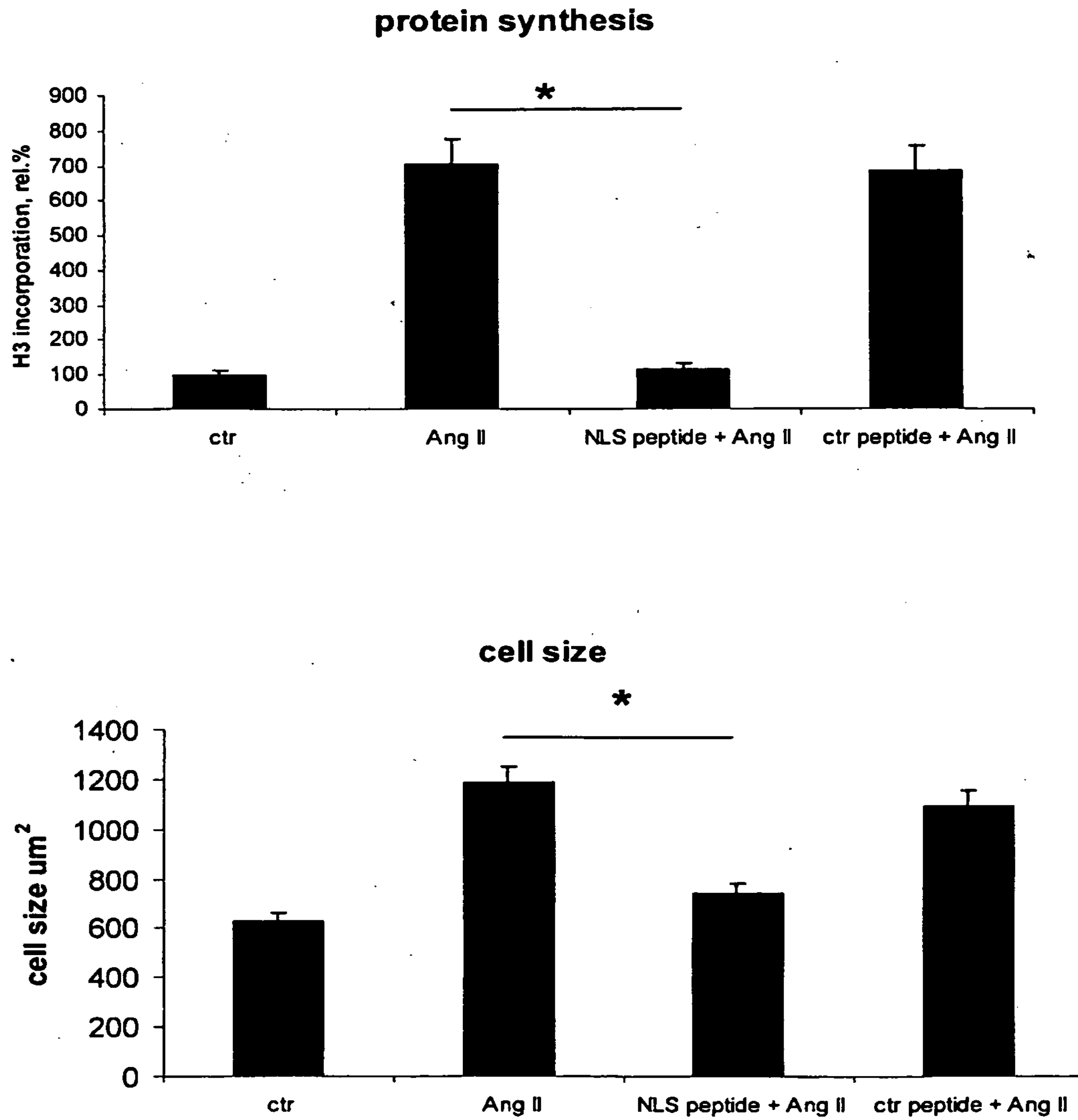

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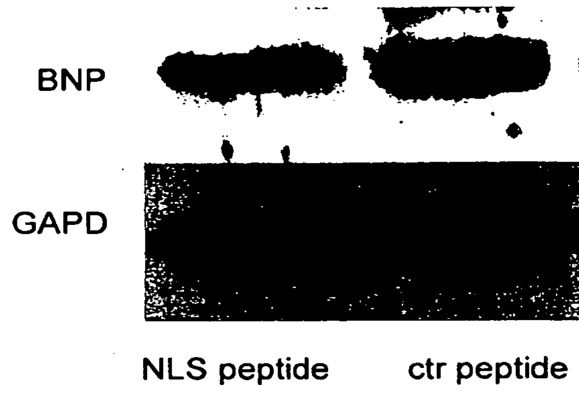

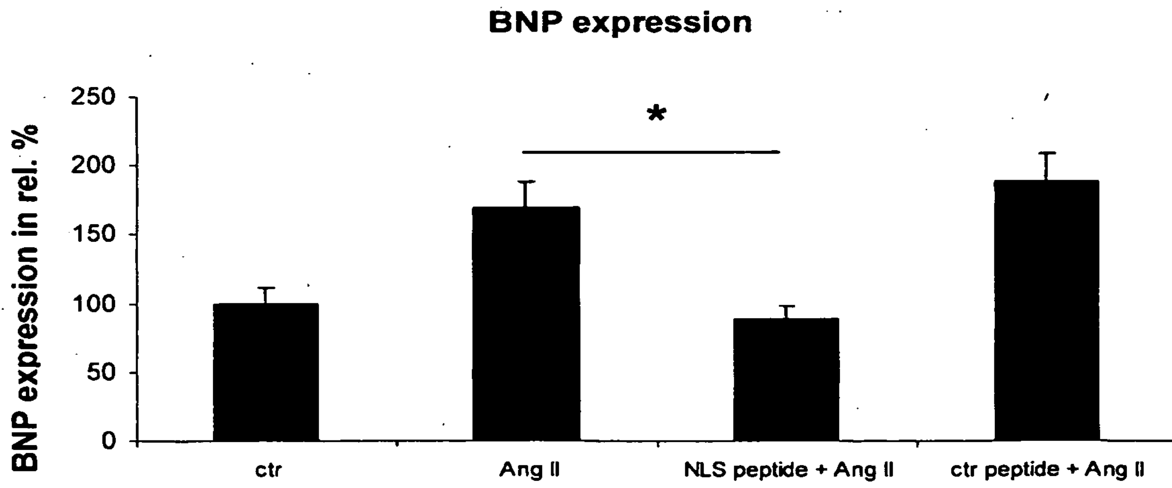

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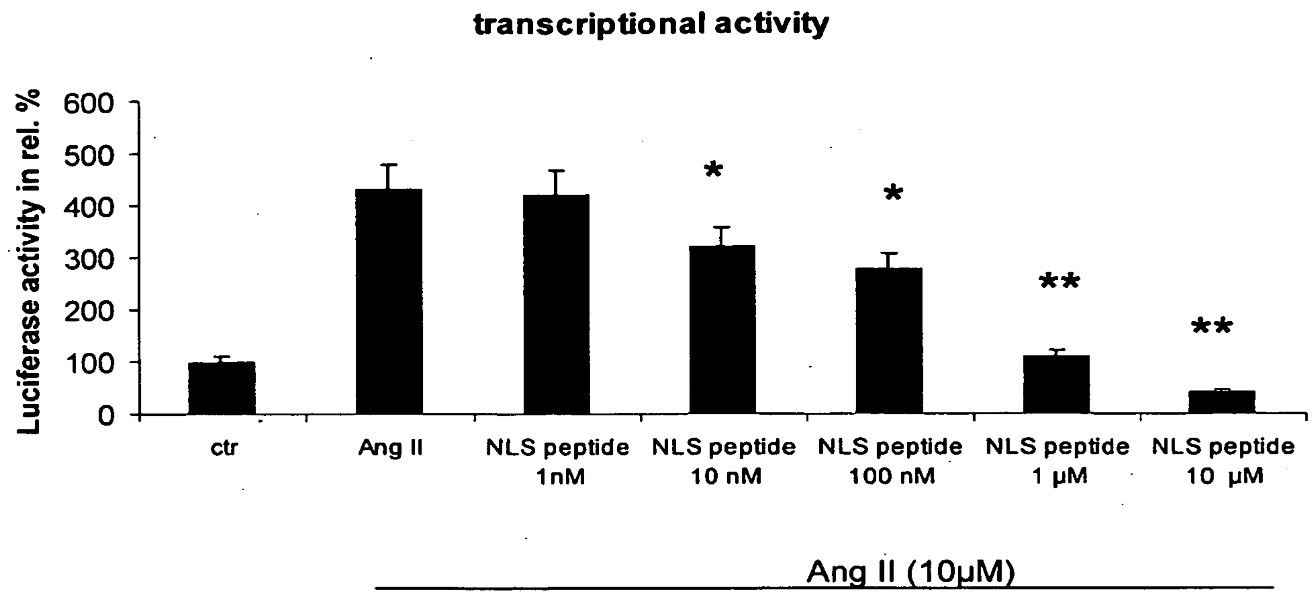

Figure 5D

SEQUENCE LISTING

EPO-BERLIN
27-01-2006

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<130> U30008

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IFW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. : 12/162,135

Applicant : OLIVER RITTER
Filed : JULY 25, 2008

Title : PEPTIDE FOR INHIBITION OF CALCINEURIN

Art Unit : 1646
Examiner : ELIZABETH KEMMERER

Atty Docket No. : 00824.07.0001

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

The below-identified communication(s) is (are) submitted in the above-captioned application or proceeding:

☒ Certified copy of European Application No. 06090014.9

Respectfully submitted,
VEDDER PRICE

Ajay A. Jagtiani
Reg. No. 35,205

VEDDER PRICE P.C.
875 15th Street, NW, Suite 725
Washington, DC 20005
(202) 312-3320 (Main)
(202) 312-3322 (Facsimile)

July 9, 2010

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

22506 7590 05/26/2010

Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

EXAMINER	
KEMMERER, ELIZABETH	

ART UNIT	PAPER NUMBER
1646	

MAIL DATE	DELIVERY MODE
05/26/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	**Application No.** 12/162,135	**Applicant(s)** RITTER, OLIVER	
	Examiner Elizabeth C. Kemmerer, Ph.D.	**Art Unit** 1646	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *31 March 2010*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-12* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-12* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *25 July 2008* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *11/10/08*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Applicant's election without traverse of Species SEQ ID NO: 1 in the reply filed on 31 March 2010 is acknowledged.

Status of Application, Amendments, And/Or Claims

The preliminary amendment of 25 July 2008 has been entered in full. Claims 1-12 are under examination.

Priority

Acknowledgment is made of applicant's claim for foreign priority based on an application filed in EPO 0609014.9 on 27 January 2006. It is noted, however, that applicant has not filed a certified copy of the foreign application as required by 35 U.S.C. 119(b). Accordingly, benefit of the foreign priority filing date is denied at this time.

Specification

The disclosure is objected to because of the following informalities: The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention to which the claims are directed.

The following title is suggested: PEPTIDE FOR INHIBITION OF CALCINEURIN, NUCLEIC ACID, ANTIBODY, AND DIAGNOSTIC AND THERAPEUTIC METHODS OF USE.

Appropriate correction is required.

The listing of references in the specification is not a proper information disclosure statement. 37 CFR 1.98(b) requires a list of all patents, publications, or other information submitted for consideration by the Office, and MPEP § 609.04(a) states, "the list may not be incorporated into the specification but must be submitted in a separate paper." Therefore, unless the references have been cited by the examiner on form PTO-892, they have not been considered.

Claim Objections

Claim 8 is objected to because of the following informalities: The word “he” in the second to last line of the claim would appear to be a typographical error wherein “the” was intended. Appropriate correction is required.

35 U.S.C. § 112, Second Paragraph

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1-12 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 1, 2, 5-8, 10, and 11 recite several sequences, but not in the alternative. Therefore, it is not clear if the recited peptide is required to have one, multiple, or all of the recited sequences. Claims 3, 4, 9, and 12 are included in this rejection since they depend from specifically mentioned claims and fail to remedy the issue.

Claims 3, 8, and 12 are further rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Regarding claims 3 and 12, the phrase "in particular" renders the claims indefinite because it is unclear whether the limitations following the phrase are part of the claimed invention. See MPEP § 2173.05(d).

Regarding claim 8, the phrases "preferably at least", “more preferred”, and “still more preferred” renders the claim indefinite because it is unclear whether the limitations following the phrases are part of the claimed invention. See MPEP § 2173.05(d).

Claim 11 is further rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The preamble of claim 11 is directed to a method of diagnosis. However, the last method step is directed to a method of manufacturing a therapeutic agent. None of the method steps relate to a method of diagnosis. Therefore, it is unclear to what sort of method the claim is directed.

35 U.S.C. § 112, First Paragraph

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-4, 11, and 12 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The claims are directed to a method of diagnosing susceptibility for a myocardial and/or immunological disorder comprising contacting a cellular test sample with a peptide of SEQ ID NO: 1, 2, 5-10, and testing for complex formation, wherein a primarily nuclear localization of the complex is indicative of susceptibility for a myocardial and/or immunological disorder. The specification discloses the peptides of SEQ ID NOS: 1, 2, and 5-10, which are based on the nuclear localization signal (NLS) of calcineurin. The peptides of SEQ ID NOS: 1 and 2 were demonstrated to block nuclear import of

calcineurin. The peptides of SEQ ID NOS: 1 and 2 were also demonstrated to reduce myocardial hypertrophy and inhibit protein synthesis in stimulated T-cells.

However, the NLS peptides of SEQ ID NOS: 1, 2, and 5-10 were not shown to bind calcineurin as required by the claims. Instead, the specification postulates that the NLS binds the protein “importin” thus competing with calcineurin for binding to importin. Indeed, since the structures of the NLS peptides are based on fragments of calcineurin, it makes sense that the NLS peptides bind other proteins that full-length calcineurin also binds. However, there is no reason to believe, nor has any evidence been provided, that the NLS peptides bind calcineurin as required by the claims.

Furthermore, while a link has been established between nuclear localization of calcineurin and myocardial hypertrophy in the specification and the prior art (e.g., Burkard et al., 2005, Circulation 111:1045-1053), such is not representative of the scope of any myocardial disorder as recited in the claims. First, it is important to appreciate the scope of the term "myocardial disorder." Such encompasses structural disorders such as heart murmurs and wall defects, as well as diverse functional disorders such as ischemia, infarction, arrhythmia, etc. The existence of a single diagnostic marker for all such diverse conditions defies sound scientific reasoning.

Furthermore, while NLS peptides were shown to inhibit protein synthesis in stimulated T-cells, such is not indicative of a diagnostic role of NLS peptides in all immunological disorders. “Immunological disorders“ is also a term that encompasses an enormous number of medical conditions, some of which have opposite symptoms. For example, immunosuppressed patients such as those with AIDS have insufficiently

active immune systems, whereas autoimmune patients such as those with rheumatoid arthritis have overactive immune systems. Again it defies scientific reasoning that one marker would be diagnostic of all such disorders.

Due to the large quantity of experimentation necessary to determine how to make a complex between calcineurin and a peptide that does not bind calcineurin and then use that interaction to diagnose a wide array of diseases, the lack of direction/guidance presented in the specification regarding the same, the absence of working examples directed to the same, the complex nature of the invention, the contradictory state of the prior art, the unpredictability of diagnosing a wide array of diseases using a single marker, and the breadth of the claims which fail to recite specific myocardial or immunological disorders, undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope.

Claim 7 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Claim 7 is directed to a therapeutic agent comprising a nucleic acid encoding the peptide of SEQ ID NOS: 1, 2, 5-10. Since the claim is directed to a *therapeutic* agent, its sole intended use is in therapy, specifically gene therapy. However, the specification does not teach the skilled artisan how to use such a therapeutic agent to treat disease.

The state of the art regarding gene therapy shows that this is still an emerging field, and very limited success has been achieved. One of the main stumbling blocks has been the achievement of a proper level of expression control. See Phillips, 2001, Journal of Pharmacy and Pharmacology 53:1169-1174 and Verma et al., 1997, Nature 389:239-242.

Given the lack of success in the prior art regarding the use of gene therapy, the specification must provide sufficient guidance for the skilled artisan to overcome past failures. However, the specification has not provided detailed guidance nor working examples directed to the approach of gene therapy using the claimed sequences to achieve therapeutic effects on any disorders.

Due to the large quantity of experimentation necessary to determine how to use the claimed agents to treat any disorder, the lack of direction/guidance presented in the specification regarding the same, the absence of working examples directed to the same, the complex nature of the invention, the contradictory state of the prior art, the unpredictability of the effect of any therapeutic agent in a complex biological system, and the breadth of the claims which fail to recite specific disorders, undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope.

Amending claim 7 to delete the word “therapeutic” would obviate the instant rejection, since compositions comprising the recited nucleic acids are useful and enabled for purposes other than gene therapy, such as nucleic acid hybridization analysis or recombinant protein production.

35 U.S.C. § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claim 10 is rejected under 35 U.S.C. 102(b) as being anticipated by XP002433633 (internet article, 10 January 2005).

XP002433633 discloses three antibody preparations that bind calcineurin A. Since calcineurin A comprises the sequence of SEQ ID NO: 2, it would be reasonable to expect that the antibodies would also inherently bind the peptides of SEQ ID NO: 1,2 , 5-10 as claimed.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Elizabeth C. Kemmerer, Ph.D. whose telephone number is (571) 272-0874. The examiner can normally be reached on Monday through Friday, 9:00 a.m. to 5:30 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol, Ph.D. can be reached on (571) 272-0835. The fax phone

number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/ECK/
24 May 2010

/Elizabeth C. Kemmerer/
Elizabeth C. Kemmerer, Ph.D.
Primary Examiner, Art Unit 1646

Notice of References Cited	Application/Control No. 12/162,135	Applicant(s)/Patent Under Reexamination RITTER, OLIVER	
	Examiner Elizabeth C. Kemmerer, Ph.D.	Art Unit 1646	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
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	J	US-			
	K	US-			
	L	US-			
	M	US-			

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	N					
	O					
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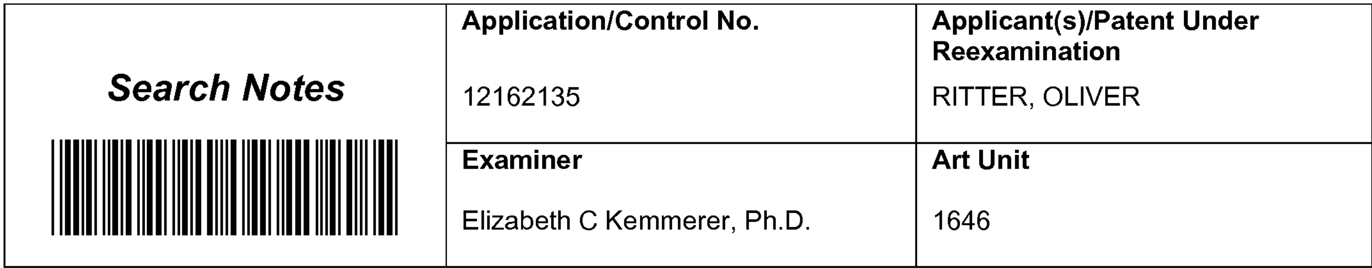
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Phillips, 2001, Journal of Pharmacy and Pharmacology 53:1169-1174
	V	Verma et al., 1997, Nature 389:239-242
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.



Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12162135	RITTER, OLIVER
	Examiner	**Art Unit**
	Elizabeth C Kemmerer, Ph.D.	1646

SEARCHED

Class	Subclass	Date	Examiner

SEARCH NOTES

Search Notes	Date	Examiner
PALM - inventor names	5/24/10	ECK
sequences searched by STIC, available in SCORE	5/24/10	ECK
EAST, DIALOG - search histories attached	5/24/10	ECK

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner

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              16   370: Science_1996-1999/Jul W3
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             109   155: MEDLINE(R)_1950-2010/May 21
               7   172: EMBASE Alert_2010/May 21
               3   357: Derwent Biotech Res.__1982-2010/May W1
              81   399: CA SEARCH(R)_1967-2010/UD=15222

   16 files have one or more items; file list includes 26 files.

? save temp; b 154,155;exs;rd
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       24may10 10:51:06 User219511 Session D819.3
           $13.76    4.454 DialUnits File411
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$13.76 Estimated cost File411
 $0.54 TELNET
$14.30 Estimated cost this search
$14.96 Estimated total session cost   4.733 DialUnits

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  File 154:MEDLINE(R) 1990-2010/May 21
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*File 154: Medline has been reloaded. Please see HELP NEWS154
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Set	Items	Description
Executing TB775878658		
	14815	CALCINEURIN
	1802031	NUCLE?
	839604	SIGNAL
	91239	CARDIOMYOPATH?
	147940	HYPERTROPH?
S1	218	CALCINEURIN AND NUCLE? AND SIGNAL AND (CARDIOMYOPATH? OR HYPERTROPH?)
S2	109	RD (unique items)

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 2/7/1   (Item 1 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

33100323   PMID: 20207814
  The %%%calcineurin%%%-myocyte enhancer factor 2c pathway mediates cardiac %%%hypertrophy%%% induced by endoplasmic reticulum stress in neonatal rat cardiomyocytes.
  Zhang Zhen-Ying; Liu Xiu-Hua; Hu Wei-Cheng; Rong Fei; Wu Xu-Dong
  Department of Pathophysiology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing, China.
  American journal of physiology. Heart and circulatory physiology (United States) May 2010, 298 (5) pH1499-509, ISSN 1522-1539--Electronic 0363-6135--Linking   Journal Code: 100901228
  Publishing Model Print-Electronic
  Document type: Journal Article; Research Support, Non-U.S. Gov't
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: MEDLINE; Completed
  Endoplasmic reticulum (ER) stress (ERS) is involved in various cardiovascular diseases. Our previous study verified that ERS took part in the development of cardiac %%%hypertrophy%%%; however, its mechanism is still unclear. This study aimed to investigate the roles of the %%%calcineurin%%% (CaN) %%%signal%%% pathway in %%%hypertrophy%%% induced by the ERS inductor thapsigargin (TG) in neonatal cardiomyocytes from Sprague-Dawley rats. Investigation of ER chaperone expression, ER staining, and calreticulin immunofluorescence were used to detect the ERS response. mRNA expression of atrial natriuretic peptide and brain natriuretic peptide, total protein synthesis rate, and cell surface area were used to

evaluate cardiac %%%hypertrophy%%% induced by TG. TG induced a significant ERS response along with %%%hypertrophy%%% in a dose- and time-dependent manner in cardiomyocytes, which was verified by treatment with tunicamycin, another ERS inducer. Furthermore, TG induced a significant elevation of the intracellular Ca(2+) level, CaN activation, and myocyte enhancer factor 2c (MEF2c) expression in a dose- and time-dependent manner in cardiomyocytes. Cyclosporine A, a CaN inhibitor, markedly suppressed MEF2c %%%nuclear%%% translocation and inhibited TG-induced %%%hypertrophy%%%. These results demonstrate that ERS induces cardiac %%%hypertrophy%%% and that the CaN-MEF2c pathway is involved in ERS-induced %%%hypertrophy%%% in cardiomyocytes.

Record Date Created: 20100505
Record Date Completed: 20100518
Date of Electronic Publication: 20100305

2/7/2 (Item 2 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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33080419 PMID: 20351294

TRPC channels are necessary mediators of pathologic cardiac %%%hypertrophy%%%.

Wu Xu; Eder Petra; Chang Baojun; Molkentin Jeffery D
Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH 45229, USA.

Proceedings of the National Academy of Sciences of the United States of America (United States) Apr 13 2010, 107 (15) p7000-5, ISSN 1091-6490 --Electronic 0027-8424--Linking Journal Code: 7505876;
Contract/Grant No.: Howard Hughes Medical Institute United States
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

Pathologic %%%hypertrophy%%% of the heart is regulated through membrane-bound receptors and intracellular signaling pathways that function, in part, by altering Ca(2+) handling and Ca(2+)-dependent signaling effectors. Transient receptor potential canonical (TRPC) channels are important mediators of Ca(2+)-dependent %%%signal%%% transduction that can sense stretch or activation of membrane-bound receptors. Here we generated cardiac-specific transgenic mice that express dominant-negative (dn) TRPC3, dnTRPC6, or dnTRPC4 toward blocking the activity of the TRPC3/6/7 or TRPC1/4/5 subfamily of channels in the heart. Remarkably, all three dn transgenic strategies attenuated the cardiac %%%hypertrophic%%% response following either neuroendocrine agonist infusion or pressure-overload stimulation. dnTRPC transgenic mice also were partially protected from loss of cardiac functional performance following long-term pressure-overload stimulation. Importantly, adult myocytes isolated from %%%hypertrophic%%% WT hearts showed a unique Ca(2+) influx activity under store-depleted conditions that was not observed in myocytes from %%%hypertrophied%%% dnTRPC3, dnTRPC6, or dnTRPC4 hearts. Moreover, dnTRPC4 inhibited the activity of the TRPC3/6/7 subfamily in the heart, suggesting that these two subfamilies function in coordinated complexes. Mechanistically, inhibition of TRPC channels in transgenic mice or in cultured neonatal myocytes significantly reduced activity in the

%%%calcineurin%%%-%%%nuclear%%% factor of activated T cells (NFAT), a known Ca(2+)-dependent %%%hypertrophy%%% -inducing pathway. Thus, TRPC channels are necessary mediators of pathologic cardiac %%%hypertrophy%%%, in part through a %%%calcineurin%%%-NFAT signaling pathway.
Record Date Created: 20100414
Record Date Completed: 20100514
Date of Electronic Publication: 20100329

2/7/3 (Item 3 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

32848786 PMID: 20177001
Reciprocal repression between microRNA-133 and %%%calcineurin%%% regulates cardiac %%%hypertrophy%%% : a novel mechanism for progressive cardiac %%%hypertrophy%%%.
Dong De-Li; Chen Chang; Huo Rong; Wang Ning; Li Zhe; Tu Yu-Jie; Hu Jun-Tao; Chu Xia; Huang Wei; Yang Bao-Feng
Department of Pharmacology, Harbin Medical University, Baojian Rd 157, Harbin 150081, Heilongjiang Province, People's Republic of China. delidong2004@yahoo.com.cn.
Hypertension (United States) Apr 2010, 55 (4) p946-52, ISSN 1524-4563--Electronic 0194-911X--Linking Journal Code: 7906255
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Cardiac %%%hypertrophy%%% involves a remodeling process of the heart in response to diverse pathological stimuli. Both %%%calcineurin%%%/ %%%nuclear%%% factor of activated T cells pathway and microRNA-133 (miR-133) have been shown to play a critical role in cardiac %%%hypertrophy%%% . It has been recognized that the expression and activity of %%%calcineurin%%% increases and miR-133 expression decreases in the %%%hypertrophic%%% heart, and inhibition of %%%calcineurin%%% or increase of miR-133 expression protects against cardiac %%%hypertrophy%%%. Here we tested the interaction between miR-133 and %%%calcineurin%%% in cardiac %%%hypertrophy%%%. Cardiac %%%hypertrophy%%% in vivo and in vitro was induced by transverse aortic constriction and phenylephrine treatment. mRNA levels were measured by using real-time PCR methods. Luciferase assays showed that transfection of miR-133 in HEK293 cells downregulated %%%calcineurin%%% expression, which was reversed by cotransfection with the miR-133-specific 2'-O-methyl antisense inhibitory oligoribonucleotides. These results were confirmed in cultured primary cardiomyocytes. miR-133 expression was downregulated, and %%%calcineurin%%% activity was enhanced in both in vivo and in vitro cardiac %%%hypertrophy%%% models. Treatment of cells and animals with cyclosporin A, an inhibitor of %%%calcineurin%%%, prevented miR-133 downregulation. Moreover, the antisense oligodeoxynucleotides against the catalytic subunits of %%%calcineurin%%% Abeta and the decoy oligodeoxynucleotides targeting %%%nuclear%%% factor of activated T cells transcription factor, a %%%calcineurin%%% downstream effector, increased miR-133 expression in cultured primary cardiomyocytes. Our data show that reciprocal repression between miR-133 and %%%calcineurin%%% regulates cardiac %%%hypertrophy%%%.
Record Date Created: 20100318
Record Date Completed: 20100409

Date of Electronic Publication: 20100222

2/7/4 (Item 4 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

32421365 PMID: 19114340
[Role of Ca2+/calmodulin-dependent %%%calcineurin%%% signaling pathway in neuropeptide Y-induced cardiac %%%hypertrophy%%% in rats]
Li Xiao-yun; Chen Min-sheng; Huang Shao-hua; Dong Qin; Li Ying-hui; Zhang Shu; Liu Zhen-xiu
Department of Cardiology, Second Affiliated Hospital of Guangzhuo Medical College, Guangzhou 510260, China. yun530@sohu.com
Nan fang yi ke da xue xue bao = Journal of Southern Medical University (China) Dec 2008, 28 (12) p2139-41, ISSN 1673-4254--Print 1673-4254--Linking Journal Code: 101266132
Publishing Model Print
Document type: English Abstract; Journal Article; Research Support, Non-U.S. Gov't
Languages: CHINESE
Main Citation Owner: NLM
Record type: MEDLINE; Completed
OBJECTIVE: To investigate the role of Ca(2+)/calmodulin-dependent %%%calcineurin%%% (CaN) signaling pathway in neuropeptide Y (NPY)-induced cardiomyocyte %%%hypertrophy%%% in rat. METHODS: Cardiomyocytes of neonatal Wistar rats were cultured in the presence of 10 and 100 nmol/L NPY, and cyclosporine A (CsA) was applied to inhibit the activity of CaN. The protein synthesis rate, c-jun mRNA expression, CaN protein expression, CaN activity and intracellular Ca(2+) concentration in the cardiomyocytes were assessed. RESULTS: Compared with the control group, (3)H-Leu incorporation and expression of c-jun mRNA in the cardiomyocytes treated with 100 nmol/L NPY increased significantly (P<0.05, P<0.001), and the effect of NPY was blocked by CsA. The activity of CaN (P<0.05), CaN expression (P<0.05), and Ca(2+) concentration in the cytoplasm (P<0.001) and %%%nuclei%%% (P<0.001) of the cells with 100 nmol/L NPY treatment also significantly increased compared with those in the control cells. CONCLUSION: NPY can induce cardiomyocyte %%%hypertrophy%%% in rats, in which process Ca(2+)/calmodulin-dependent CaN signaling pathway plays an important role.
Record Date Created: 20081230
Record Date Completed: 20100302

2/7/5 (Item 5 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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32356997 PMID: 20026769
LIM and cysteine-rich domains 1 regulates cardiac %%%hypertrophy%%% by targeting %%%calcineurin%%%/%%%nuclear%%% factor of activated T cells signaling.
Bian Zhou-Yan; Huang He; Jiang Hong; Shen Di-Fei; Yan Ling; Zhu Li-Hua; Wang Lang; Cao Feng; Liu Chen; Tang Qi-Zhu; Li Hongliang
Department of Cardiology, Renmin Hospital, Wuhan University, Wuhan, People's Republic of China.
Hypertension (United States) Feb 2010, 55 (2) p257-63, ISSN 1524-4563--Electronic 0194-911X--Linking Journal Code: 7906255

Publishing Model Print-Electronic; Comment in Hypertension. 2010 Feb;55(2):231-2 PMID 20026762
Document type: Comparative Study; Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

LIM domain proteins are important regulators in cell growth, cell fate determination, cell differentiation, and remodeling of the cell cytoskeleton. LIM and cysteine-rich domains 1 (Lmcd1) is a novel protein that contain 2 LIM domains with regular spacing in the carboxy-terminal region. However, its roles in cardiac growth remain unknown. Here, we investigated whether Lmcd1 regulates cardiac %%%hypertrophy%%% in vitro and in vivo and elucidated the underlying molecular mechanisms. We used primary cultured cardiac myocytes and cardiac-specific Lmcd1 transgenic mice. In wild-type mice subjected to the aortic banding, cardiac %%%hypertrophy%%% was evident at 8 weeks. In transgenic mice, however, cardiac %%%hypertrophy%%% was significantly greater than that in wild-type mice, as estimated by heart weight:body weight ratio, cardiomyocyte area, and echocardiographic measurements, as well as cardiac atrial natriuretic peptide and B-type natriuretic peptide mRNA and protein levels. Our results further showed that cardiac fibrosis observed in wild-type aortic banding mice was augmented in transgenic aortic banding mice. Importantly, %%%calcineurin%%% activity and %%%nuclear%%% factor of activated T cells activation level were increased more in transgenic mice than those in wild-type mice after 8-week aortic banding. In vitro experiments in cardiac myocytes further revealed that angiotensin II-induced %%%calcineurin%%% activity and %%%nuclear%%% factor of activated T cells activation were enhanced by overexpression but blunted by downregulation of Lmcd1. In conclusion, our results suggest that Lmcd1 plays a critical role in the development of cardiac %%%hypertrophy%%% via activation of %%%calcineurin%%%/%%%nuclear%%% factor of activated T cells signaling pathway.

Record Date Created: 20100121
Record Date Completed: 20100222
Date of Electronic Publication: 20091221

2/7/6 (Item 6 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

32281871 PMID: 19741299 Record Identifier: PMC2752064
Cdc42 is an antihypertrophic molecular switch in the mouse heart.
Maillet Marjorie; Lynch Jeffrey M; Sanna Bastiano; York Allen J; Zheng Yi ; Molkentin Jeffery D
Department of Pediatrics, Division of Molecular Cardiovascular Biology, University of Cincinnati, and Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA.
Journal of clinical investigation (United States) Oct 2009, 119 (10) p3079-88, ISSN 1558-8238--Electronic 0021-9738--Linking Journal Code: 7802877
Publishing Model Print-Electronic; Cites Circulation. 2008 Jan 29;117(4):545-52 PMID 18195174; Cites J Biol Chem. 2004 Apr 9;279(15):15330-8 PMID 14742426; Cites Blood. 2007 Dec 1;110(12):3853-61 PMID 17702896; Cites Proc Natl Acad Sci U S A. 2007 Mar 20;104(12):5091-6 PMID 17360364; Cites Proc Natl Acad Sci U S A. 2007 Jan 23;104(4):1248-53 PMID 17227869; Cites Genes Dev. 2006 Dec

15;20(24):3347-65 PMID 17182864; Cites Proc Natl Acad Sci U S A. 2006 Oct 31;103(44):16520-5 PMID 17050694; Cites Mol Biol Cell. 2006 Nov;17(11):4675-85 PMID 16914516; Cites Nat Rev Mol Cell Biol. 2006 Aug;7(8):589-600 PMID 16936699; Cites Nat Neurosci. 2006 Sep;9(9):1099-107 PMID 16892058; Cites Proc Natl Acad Sci U S A. 2006 May 9;103(19):7432-7 PMID 16651530; Cites Circ Res. 2006 Mar 31;98(6):730-42 PMID 16574914; Cites Circ Res. 2006 Mar 31;98(6):837-45 PMID 16514068; Cites Genes Dev. 2006 Mar 1;20(5):571-85 PMID 16510873; Cites Annu Rev Cell Dev Biol. 2005;21:247-69 PMID 16212495; Cites J Biol Chem. 2005 Sep 23;280(38):32602-8 PMID 16043490; Cites J Clin Invest. 1999 Aug;104(4):391-8 PMID 10449431; Cites Circ Res. 2004 Jan 9;94(1):110-8 PMID 14656927; Cites J Mol Cell Cardiol. 2003 Dec;35(12):1385-94 PMID 14654364; Cites Am J Physiol Heart Circ Physiol. 2004 Jan;286(1):H424-33 PMID 12969887; Cites EMBO J. 2003 Oct 1;22(19):5079-89 PMID 14517246; Cites Nat Cell Biol. 2003 Jul;5(7):633-9 PMID 12819788; Cites Biochem Biophys Res Commun. 2003 Jun 13;305(4):806-10 PMID 12767901; Cites J Clin Invest. 2003 May;111(10):1475-86 PMID 12750397; Cites Biochim Biophys Acta. 2003 Mar 17;1603(2):47-82 PMID 12618308; Cites Nature. 2002 Dec 12;420(6916):629-35 PMID 12478284; Cites Circ Res. 2002 Nov 1;91(9):776-81 PMID 12411391; Cites Mol Cell Biol. 2002 Nov;22(21):7603-13 PMID 12370307; Cites Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11363-8 PMID 12177418; Cites J Clin Invest. 2002 Jul;110(2):271-9 PMID 12122119; Cites Genes Dev. 2002 Jul 1;16(13):1587-609 PMID 12101119; Cites J Biol Chem. 2002 Jun 21;277(25):22896-901 PMID 11943770; Cites Mol Cell Biol. 2002 Apr;22(8):2799-809 PMID 11909972; Cites Proc Natl Acad Sci U S A. 2002 Mar 19;99(6):3866-71 PMID 11891332; Cites Circ Res. 2002 Mar 8;90(4):458-64 PMID 11884376; Cites Proc Natl Acad Sci U S A. 2001 Oct 9;98(21):12283-8 PMID 11593045; Cites Curr Biol. 2000 Jun 29;10(13):758-65 PMID 10898977; Cites Biochem J. 2000 Jun 1;348 Pt 2:241-55 PMID 10816416; Cites J Biol Chem. 2000 May 5;275(18):13571-9 PMID 10788473; Cites J Clin Invest. 2000 Apr;105(7):875-86 PMID 10749567; Cites J Biol Chem. 2000 Feb 4;275(5):3543-51 PMID 10652349; Cites J Cell Physiol. 2009 Jan;218(1):146-56 PMID 18780290; Cites J Mol Cell Cardiol. 2008 Apr;44(4):623-32 PMID 18339399; Cites Cell Signal. 1999 Aug;11(8):545-54 PMID 10433515; Cites EMBO J. 1997 Aug 15;16(16):4961-72 PMID 9305638; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites J Clin Invest. 1999 Jun;103(12):1627-34 PMID 10377168; Cites Science. 1997 Nov 28;278(5343):1638-41 PMID 9374467; Cites Cell. 1995 Jun 30;81(7):1147-57 PMID 7600582; Cites Cell. 1995 Jun 30;81(7):1137-46 PMID 7600581; Cites J Am Coll Cardiol. 1993 Oct;22(4 Suppl A):6A-13A PMID 8376698; Cites N Engl J Med. 1990 May 31;322(22):1561-6 PMID 2139921; Cites J Cell Biol. 1990 Jul;111(1):131-42 PMID 2195038; Cites Adv Exp Med Biol. 1983;161:249-65 PMID 6223512; Cites J Biol Chem. 2004 Jun 18;279(25):26192-200 PMID 15082723; Cites Circ Res. 2007 Nov 26;101(11):1084-95 PMID 18040025

Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM

Other Citation Owner: NLM

Record type: MEDLINE; Completed

To improve contractile function, the myocardium undergoes %%%hypertrophic%%% growth without myocyte proliferation in response to both pathologic and physiologic stimulation. Various membrane-bound receptors and intermediate %%%signal%%% transduction pathways regulate the induction of cardiac %%%hypertrophy%%%, but the cardioprotective regulatory pathways

or effectors that antagonize cardiac %%%hypertrophy%%% remain poorly understood. Here we identify the small GTPase Cdc42 as a signaling intermediate that restrained the cardiac growth response to physiologic and pathologic stimuli. Cdc42 was specifically activated in the heart after pressure overload and in cultured cardiomyocytes by multiple agonists. Mice with a heart-specific deletion of Cdc42 developed greater cardiac %%%hypertrophy%%% at 2 and 8 weeks of stimulation and transitioned more quickly into heart failure than did wild-type controls. These mice also displayed greater cardiac %%%hypertrophy%%% in response to neuroendocrine agonist infusion for 2 weeks and, more remarkably, enhanced exercise-induced %%%hypertrophy%%% and sudden death. These pathologies were associated with an inability to activate JNK following stimulation through a MEKK1/MKK4/MKK7 pathway, resulting in greater cardiac %%%nuclear%%% factor of activated T cells (NFAT) activity. Restoration of cardiac JNK signaling with an Mkk7 heart-specific transgene reversed the enhanced growth effect. These results identify what we believe to be a novel antihypertrophic and protective cardiac signaling pathway, whereby Cdc42-dependent JNK activation antagonizes %%%calcineurin%%%-NFAT activity to reduce %%%hypertrophy%%% and prevent transition to heart failure.
  Record Date Created: 20091006
  Record Date Completed: 20100209
  Date of Electronic Publication: 20090908

 2/7/7   (Item 7 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

32141313   PMID: 19996065
  Angiotensin-(1-7) prevents cardiomyocyte pathological remodeling through a nitric oxide/guanosine 3',5'-cyclic monophosphate-dependent pathway.
  Gomes Eneas R M; Lara Aline A; Almeida Pedro W M; Guimaraes Diogo; Resende Rodrigo R; Campagnole-Santos Maria J; Bader Michael; Santos Robson A S; Guatimosim Silvia
  Department of Physiology and Biophysics, Federal University of Minas Gerais, Belo Horizonte MG-CEP: 31270-901, Brazil.
  Hypertension (United States) Jan 2010, 55 (1) p153-60, ISSN 1524-4563--Electronic 0194-911X--Linking Journal Code: 7906255
  Publishing Model Print-Electronic
  Document type: Journal Article; Research Support, Non-U.S. Gov't
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: MEDLINE; Completed
  The renin-angiotensin (Ang) system plays a pivotal role in the pathogenesis of cardiovascular disease, with Ang II being the major effector of this system. Multiple lines of evidence have shown that Ang-(1-7) exerts cardioprotective effects in the heart by counterregulating Ang II actions. The questions that remain are how and where Ang-(1-7) exerts its effects. By using a combination of molecular biology, confocal microscopy, and a transgenic rat model with increased levels of circulating Ang-(1-7) (TGR[A1-7]3292), we evaluated the signaling pathways involved in Ang-(1-7) cardioprotection against Ang II-induced pathological remodeling in ventricular cardiomyocytes. Rats were infused with Ang II for 2 weeks. We found that ventricular myocytes from TGR(A1-7)3292 rats are protected from Ang II pathological remodeling characterized by Ca(2+) signaling dysfunction, %%%hypertrophic%%% fetal gene expression, glycogen synthase kinase 3beta inactivation, and %%%nuclear%%% factor of activated T-cells

%%%nuclear%%% accumulation. Moreover, cardiomyocytes from TGR(A1-7)3292 rats infused with Ang II presented increased expression levels of neuronal NO synthase. To provide a signaling pathway involved in the beneficial effects of Ang-(1-7), we treated neonatal cardiomyocytes with Ang-(1-7) and Ang II for 36 hours. Treatment of cardiomyocytes with Ang-(1-7) prevented Ang II-induced %%%hypertrophy%%% by modulating %%%calcineurin%%%/ %%%nuclear%%% factor of activated T-cell signaling cascade. Importantly, antihypertrophic effects of Ang-(1-7) on Ang II-treated cardiomyocytes were prevented by N(G)-nitro-l-arginine methyl ester and 1H-1,2,4oxadiazolo4,2-aquinoxalin-1-one, suggesting that these effects are mediated by NO/cGMP. Taken together, these data reveal a key role for NO/cGMP as a mediator of Ang-(1-7) beneficial effects in cardiac cells.
Record Date Created: 20091218
Record Date Completed: 20100121
Date of Electronic Publication: 20091207

2/7/8 (Item 8 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

31925717 PMID: 19815822 Record Identifier: NIHMS153918 Available on 11/20/10 ; PMC2783226 Available on 11/20/10
ASK1 regulates cardiomyocyte death but not %%%hypertrophy%%% in transgenic mice.
Liu Qinghang; Sargent Michelle A; York Allen J; Molkentin Jeffery D
Department of Pediatrics, Division of Molecular Cardiovascular Biology, University of Cincinnati, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229-3039, USA.
Circulation research (United States) Nov 20 2009, 105 (11) p1110-7, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103;
Contract/Grant No.: Howard Hughes Medical Institute United States
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Other Citation Owner: NLM; NLM
Record type: MEDLINE; Completed
RATIONALE: Apoptosis %%%signal%%%-regulating kinase (ASK)1 is a central upstream kinase in the greater mitogen-activated protein kinase cascade that mediates growth and death decisions in cardiac myocytes in response to diverse pathological stimuli. OBJECTIVE: However, the role that ASK1 plays in regulating the cardiac %%%hypertrophic%%% response in vivo remains controversial. METHODS AND RESULTS: Here, we generated mice with cardiac-specific and inducible overexpression of ASK1 in the heart to assess its gain-of-function effect. ASK1 transgenic mice exhibited no induction of cardiac %%%hypertrophy%%% or pathology at 3 and 12 months of age, and these mice showed an identical %%%hypertrophic%%% response to controls following 2 weeks of pressure-overload stimulation or isoproterenol infusion. Although ASK1 overexpression did not alter the cardiac %%%hypertrophic%%% response, it promoted %%%cardiomyopathy%%% and greater TUNEL following pressure-overload stimulation and myocardial infarction. Indeed, ASK1 transgenic mice showed a greater than 2-fold increase in ischemia reperfusion-induced injury to the heart compared with controls. Examination of downstream signaling showed a prominent activation of mitogen-activated protein kinase kinase 4/6 and c-Jun NH(2)-terminal

kinase (JNK)1/2 (but not p38 or extracellular %%%signal%%%-regulated kinases [ERKs]), inhibition of %%%calcineurin%%%-NFAT (%%%nuclear%%% factor of activated T cells), and induction of Bax in the hearts of ASK1 transgenic mice following 1 and 8 weeks of pressure-overload stimulation. Mechanistically, %%%cardiomyopathy%%% associated with ASK1 overexpression after 8 weeks of pressure overload was significantly reduced in the %%%calcineurin%%% Abeta-null (CnAbeta(-/-)) background. CONCLUSIONS: These results indicate that ASK1 does not directly regulate the cardiac %%%hypertrophic%%% response in vivo, but it does alter cell death and propensity to %%%cardiomyopathy%%%, in part, through a %%%calcineurin%%% -dependent mechanism.

Record Date Created: 20091120
Record Date Completed: 20091217
Date of Electronic Publication: 20091008

2/7/9 (Item 9 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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19457881 PMID: 19654000

The transcription factor MEF2C mediates cardiomyocyte %%%hypertrophy%%% induced by IGF-1 signaling.

Munoz Juan Pablo; Collao Andres; Chiong Mario; Maldonado Carola; Adasme Tatiana; Carrasco Loreto; Ocaranza Paula; Bravo Roberto; Gonzalez Leticia; Diaz-Araya Guillermo; Hidalgo Cecilia; Lavandero Sergio

Centro FONDAP Estudios Moleculares de la Celula, Universidad de Chile, Santiago, Chile.

Biochemical and biophysical research communications (United States) Oct 9 2009, 388 (1) p155-60, ISSN 1090-2104--Electronic 0006-291X--Linking Journal Code: 0372516

Publishing Model Print-Electronic
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

Myocyte enhancer factor 2C (MEF2C) plays an important role in cardiovascular development and is a key transcription factor for cardiac %%%hypertrophy%%% . Here, we describe MEF2C regulation by insulin-like growth factor-1 (IGF-1) and its role in IGF-1-induced cardiac %%%hypertrophy%%% . We found that IGF-1 addition to cultured rat cardiomyocytes activated MEF2C, as evidenced by its increased %%%nuclear%%% localization and DNA binding activity. IGF-1 stimulated MEF2 dependent-gene transcription in a time-dependent manner, as indicated by increased MEF2 promoter-driven reporter gene activity; IGF-1 also induced p38-MAPK phosphorylation, while an inhibitor of p38-MAPK decreased both effects. Additionally, inhibitors of phosphatidylinositol 3-kinase and %%%calcineurin%%% prevented IGF-1-induced MEF2 transcriptional activity. Via MEF2C-dependent signaling, IGF-1 also stimulated transcription of atrial natriuretic factor and skeletal alpha-actin but not of fos-lux reporter genes. These novel data suggest that MEF2C activation by IGF-1 mediates the pro-%%%hypertrophic%%% effects of IGF-1 on cardiac gene expression.

Record Date Created: 20090826
Record Date Completed: 20090921
Date of Electronic Publication: 20090803

2/7/10 (Item 10 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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19449982 PMID: 19592461 Record Identifier: PMC2739769 Available on 09/01/10

Regulation of soluble guanylyl cyclase-alpha1 expression in chronic hypoxia-induced pulmonary hypertension: role of NFATc3 and HuR.

de Frutos Sergio; Nitta Carlos H; Caldwell Elizabeth; Friedman Jessica; Gonzalez Bosc Laura V
Vascular Physiology Group, Department of Cell Biology and Physiology, School of Medicine, University of New Mexico, Albuquerque, New Mexico 87131, USA.

American journal of physiology. Lung cellular and molecular physiology (United States) Sep 2009, 297 (3) pL475-86, ISSN 1522-1504--Electronic 1040-0605--Linking Journal Code: 100901229
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The nitric oxide/soluble guanylyl cyclase (sGC) %%%signal%%% transduction pathway plays an important role in smooth muscle relaxation and phenotypic regulation. However, the transcriptional regulation of sGC gene expression is largely unknown. It has been shown that sGC expression increases in pulmonary arteries from chronic hypoxia-induced pulmonary hypertensive animals. Since the transcription factor NFATc3 is required for the upregulation of the smooth muscle %%%hypertrophic%%%/differentiation marker alpha-actin in pulmonary artery smooth muscle cells from chronically hypoxic mice, we hypothesized that NFATc3 is required for the regulation of sGC-alpha1 expression during chronic hypoxia. Exposure to chronic hypoxia for 2 days induced a decrease in sGC-alpha1 expression in mouse pulmonary arteries. This reduction was independent of NFATc3 but mediated by %%%nuclear%%% accumulation of the mRNA-stabilizing protein human antigen R (HuR). Consistent with our hypothesis, chronic hypoxia (21 days) upregulated pulmonary artery sGC-alpha1 expression, bringing it back to the level of the normoxic controls. This response was prevented in NFATc3 knockout and cyclosporin (%%%calcineurin%%%/NFATc inhibitor)-treated mice. Furthermore, we identified effective binding sites for NFATc in the mouse sGC-alpha1 promoter. Activation of NFATc3 increased sGC-alpha1 promoter activity in human embryonic derived kidney cells, rat aortic-derived smooth muscle cells, and human pulmonary artery smooth muscle cells. Our results suggest that NFATc3 and HuR are important regulators of sGC-alpha1 expression in pulmonary vascular smooth muscle cells during chronic hypoxia-induced pulmonary hypertension.

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19426213 PMID: 19505981 Record Identifier: PMC2746617 Available on 08/01/10

Cardiac anti-remodelling effect of aerobic training is associated with a reduction in the %%%calcineurin%%%/NFAT signalling pathway in heart failure mice.

Oliveira R S F; Ferreira J C B; Gomes E R M; Paixao N A; Rolim N P L; Medeiros A; Guatimosim S; Brum P C

School of Physical Education and Sport, University of Sao Paulo, SP, Brazil.

Journal of physiology (England) Aug 1 2009, 587 (Pt 15) p3899-910, ISSN 1469-7793--Electronic 0022-3751--Linking Journal Code: 0266262

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Cardiomyocyte %%%hypertrophy%%% occurs in response to a variety of physiological and pathological stimuli. While pathological %%%hypertrophy%%% in heart failure is usually coupled with depressed contractile function, physiological %%%hypertrophy%%% associates with increased contractility. In the present study, we explored whether 8 weeks of moderate intensity exercise training would lead to a cardiac anti-remodelling effect in an experimental model of heart failure associated with a deactivation of a pathological (%%%calcineurin%%%/NFAT, CaMKII/HDAC) or activation of a physiological (Akt-mTOR) %%%hypertrophy%%% signalling pathway. The cardiac dysfunction, exercise intolerance, left ventricle dilatation, increased heart weight and cardiomyocyte %%%hypertrophy%%% from mice lacking alpha(2A) and alpha(2C) adrenoceptors (alpha(2A)/alpha(2C)ARKO mice) were associated with sympathetic hyperactivity induced heart failure. The relative contribution of Ca(2+)-calmodulin high-affinity (%%%calcineurin%%%/NFAT) and low-affinity (CaMKII/HDAC) targets to pathological %%%hypertrophy%%% of alpha(2A)/alpha(2C)ARKO mice was verified. While %%%nuclear%%% %%%calcineurin%%% B, NFATc3 and GATA-4 translocation were significantly increased in alpha(2A)/alpha(2C)ARKO mice, no changes were observed in CaMKII/HDAC activation. As expected, cyclosporine treatment decreased %%%nuclear%%% translocation of %%%calcineurin%%% /NFAT in alpha(2A)/alpha(2C)ARKO mice, which was associated with improved ventricular function and a pronounced anti-remodelling effect. The Akt/mTOR signalling pathway was not activated in alpha(2A)/alpha(2C)ARKO mice. Exercise training improved cardiac function and exercise capacity in alpha(2A)/alpha(2C)ARKO mice and decreased heart weight and cardiomyocyte width paralleled by diminished %%%nuclear%%% NFATc3 and GATA-4 translocation as well as GATA-4 expression levels. When combined, these findings support the notion that deactivation of %%%calcineurin%%%/NFAT pathway-induced pathological %%%hypertrophy%%% is a preferential mechanism by which exercise training leads to the cardiac anti-remodelling effect in heart failure.

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miR-23a functions downstream of NFATc3 to regulate cardiac %%%hypertrophy%%%.

Lin Zhiqiang; Murtaza Iram; Wang Kun; Jiao Jianqin; Gao Jie; Li Pei-Feng
Division of Cardiovascular Research, National Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China.

Proceedings of the National Academy of Sciences of the United States of America (United States) Jul 21 2009, 106 (29) p12103-8, ISSN 1091-6490--Electronic 0027-8424--Linking Journal Code: 7505876
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Languages: ENGLISH
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Cardiac %%%hypertrophy%%% is accompanied by maladaptive cardiac remodeling, which leads to heart failure or sudden death. MicroRNAs (miRNAs) are a class of small, noncoding RNAs that mediate posttranscriptional gene silencing. Recent studies show that miRNAs are involved in the pathogenesis of %%%hypertrophy%%%, but their signaling regulations remain to be understood. Here, we report that miR-23a is a pro-%%%hypertrophic%%% miRNA, and its expression is regulated by the transcription factor, %%%nuclear%%% factor of activated T cells (NFATc3). The results showed that miR-23a expression was up-regulated upon treatment with the %%%hypertrophic%%% stimuli including isoproterenol and aldosterone. Knockdown of miR-23a could attenuate %%%hypertrophy%%% , suggesting that miR-23a is able to convey the %%%hypertrophic%%% %%%signal%%% . In exploring the molecular mechanism by which miR-23a is up-regulated, we identified that NFATc3 could directly activate miR-23a expression through the transcriptional machinery. The muscle specific ring finger protein 1, an anti-%%%hypertrophic%%% protein, was identified to be a target of miR-23a. Its translation could be suppressed by miR-23a. Our data provide a model in which the miRNA expression is regulated by the %%%hypertrophic%%% transcriptional factor.

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Effects of thapsigargin and phenylephrine on %%%calcineurin%%% and protein kinase C signaling functions in cardiac myocytes.

Prasad Anand Mohan; Inesi Giuseppe
California Pacific Medical Center Research Institute, San Francisco, CA 94107, USA.

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Neonatal rat cardiac myocytes were exposed to 10 nM thapsigargin (TG) or 20 muM phenylephrine (PE) to compare resulting alterations of Ca(2+) homeostasis. Either treatment results in resting cytosolic [Ca(2+)] rise and reduction of Ca(2+) signals in myocytes following electrical stimuli. In fact, ATP-dependent Ca(2+) transport is reduced due to catalytic inhibition of sarcoplasmic reticulum ATPase (SERCA2) by TG or reduction of SERCA2 protein expression by PE. A marked rise of %%%nuclear%%% factor of activated T cells (NFAT)-dependent expression of transfected luciferase cDNA is produced by TG or PE, which is dependent on increased NFAT dephosphorylation by activated %%%calcineurin%%% and reduced phosphorylation by inactivated glycogen synthase kinase 3beta. Expression of SERCA2 (inactivated) protein is increased following exposure to TG, whereas no %%%hypertrophy%%% is produced. On the contrary, SERCA2 expression is reduced, despite high CN activity, following protein kinase C (PKC) activation by PE (or phorbol 12-myristate 13-acetate) under conditions producing myocyte %%%hypertrophy%%%. Both effects of TG and PE are dependent on NFAT dephosphorylation by CN, as demonstrated by CN inhibition with cyclosporine (CsA). However, the %%%hypertrophy%%% program triggered by PKC activation bypasses SERCA2 transcription and expression due to competitive recruitment of NFAT and/or other transcriptional factors. A similar dependence on CN activation, but relative reduction under conditions of PKC activation, involves transcription and expression of the Na(+)/Ca(2+) exchanger-1. On the other hand, significant upregulation of transient receptor potential channel proteins is noted following PKC activation. The observed alterations of Ca(2+) homeostasis may contribute to development of contractile failure.

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Sensitivity of NFAT cycling to cytosolic calcium concentration: implications for %%%hypertrophic%%% signals in cardiac myocytes.

Cooling Michael T; Hunter Peter; Crampin Edmund J
Auckland Bioengineering Institute, The University of Auckland, New Zealand. m.cooling@auckland.ac.nz

Biophysical journal (United States) Mar 18 2009, 96 (6) p2095-104, ISSN 1542-0086--Electronic 0006-3495--Linking Journal Code: 0370626;
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Languages: ENGLISH
Main Citation Owner: NLM

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Record type: MEDLINE; Completed
The %%%nuclear%%% factor of activated T-cell (NFAT) transcription factors play an important role in many biological processes, including pathological cardiac %%%hypertrophy%%% . Stimulated by calcium signals, NFAT is translocated to the %%%nucleus%%% where it can regulate %%%hypertrophic%%% genes (excitation-transcription coupling). In excitable cells, such as myocytes, calcium is a key second messenger for multiple signaling events, including excitation-contraction coupling. Whether the calcium signals due to excitation-contraction and excitation-transcription coupling coincide or how they can be differentiated is currently unclear. Here we construct a mathematical model of NFAT cycling fitted to skeletal myocyte and baby hamster kidney cell data. The model replicates key behavior with respect to sensitivity to %%%calcineurin%%% overexpression and to calcium oscillations. Finally, we measure the sensitivity of the system to a simulated %%%hypertrophic%%% calcium %%%signal%%% , against a background excitation-contraction coupling calcium oscillation. We find that NFAT cycling is sensitive to excitation-transcription coupling even when both calcium signals are in the same cellular compartment, thus showing that separation of the signals may not be necessary in vitro.
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Record Date Completed: 20090601

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19162460 PMID: 19159628 Record Identifier: NIHMS105811 Available on 04/01/10 ; PMC2703675 Available on 04/01/10
Pressure-overload magnitude-dependence of the anti-%%%hypertrophic%%% efficacy of PDE5A inhibition.
Nagayama Takahiro; Hsu Steven; Zhang Manling; Koitabashi Norimichi; Bedja Djahida; Gabrielson Kathleen L; Takimoto Eiki; Kass David A
Division of Cardiology, Department of Medicine, Johns Hopkins Medical Institutions, Baltimore, Maryland, MD 21287, USA.
Journal of molecular and cellular cardiology (England) Apr 2009, 46 (4) p560-7, ISSN 1095-8584--Electronic 0022-2828--Linking
Journal Code: 0262322
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Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
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Record type: MEDLINE; Completed
Increased myocardial cGMP, achieved by enhancing cyclase activity or impeding cGMP hydrolysis by phosphodiesterase type-5 (PDE5A), suppresses cellular and whole organ %%%hypertrophy%%%. The efficacy of the latter also requires cyclase stimulation and may depend upon co-activation of maladaptive signaling suppressible by cGMP-stimulated kinase (cGK-1). Thus, PDE5A inhibitors could paradoxically be more effective against higher than lower magnitudes of pressure-overload stress. To test this, mice were subjected to severe or moderate trans-aortic constriction (sTAC, mTAC) for 6 wks +/-co-treatment with oral sildenafil (SIL 200 mg/kg/d). LV mass (LVM) rose 130% after 3-wks sTAC and SIL blunted this by 50%. With mTAC, LVM rose 56% at 3 wks but was unaffected by SIL, whereas a 90% increase in LVM after 6 wks was suppressed by SIL. SIL minimally altered LV function and

remodeling with mTAC until later stages that stimulated more %%%hypertrophy%%% and remodeling. SIL stimulated cGK-1 activity similarly at 3 and 6 wks of mTAC. However, pathologic stress signaling (e.g. %%%calcineurin%%% , ERK-MAPkinase) was little activated after 3-wk mTAC, unlike sTAC or later stage mTAC when activity increased and SIL suppressed it. With modest %%%hypertrophy%%% (3-wk mTAC), GSK3beta and Akt phosphorylation were unaltered but SIL enhanced it. However, with more severe %%%hypertrophy%%% (6-wk mTAC and 3-wk sTAC), both kinases were highly phosphorylated and SIL treatment reduced it. Thus, PDE5A-inhibition counters cardiac pressure-overload stress remodeling more effectively at higher than lower magnitude stress, coupled to pathologic signaling activation targetable by cGK-1 stimulation. Such regulation could impact responses of varying disease models to PDE5A inhibitors.
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19140151 PMID: 19129376
Heritable pathologic cardiac %%%hypertrophy%%% in adulthood is preceded by neonatal cardiac growth restriction.
Porrello Enzo R; Bell James R; Schertzer Jonathan D; Curl Claire L; McMullen Julie R; Mellor Kimberley M; Ritchie Rebecca H; Lynch Gordon S; Harrap Stephen B; Thomas Walter G; Delbridge Lea M D
Dept. of Physiology, The Univ. of Melbourne, Parkville, Victoria, Australia.
American journal of physiology. Regulatory, integrative and comparative physiology (United States) Mar 2009, 296 (3) pR672-80, ISSN 0363-6119 --Print 0363-6119--Linking Journal Code: 100901230
Publishing Model Print-Electronic
Document type: In Vitro; Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
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The identification of genetic factors influencing cardiac growth independently of increased load is crucial to an understanding of the molecular and cellular basis of pathological cardiac %%%hypertrophy%%%. The central aim of this investigation was to determine how pathological %%%hypertrophy%%% in the adult can be linked with disturbances in cardiomyocyte growth and viability in early neonatal development. The %%%hypertrophic%%% heart rat (HHR) model is derived from the spontaneously hypertensive rat and exhibits marked cardiac %%%hypertrophy%%%, in the absence of a pressure load at maturity. Hearts were harvested from male HHR, and control strain normal heart rats (NHR), at different stages of postnatal development [neonatal (P2), 4 wk, 6 wk, 8 wk, 12 wk, 20 wk]. Isolated neonatal cardiomyocytes were prepared to evaluate cell size, number, and binucleation. At postnatal day 2, HHR hearts were considerably smaller than control NHR (4.3 +/- 0.2 vs. 5.0 +/- 0.1 mg/g, $P < 0.05$). Cardiac growth restriction in the neonatal HHR was associated with reduced myocyte size (length and width) and an increased proportion of binucleated cardiomyocytes. Furthermore, the number of cardiomyocytes isolated from HHR neonatal hearts was significantly less (approximately 29%) than NHR. We

also observe that growth stress in the neonate is associated with accentuated PI3K and suppressed MAPK activation, although these signaling pathways are normalized in the adult heart exhibiting established %%%hypertrophy%%%. Thus, using the HHR model, we identified novel molecular and cellular mechanisms involving premature exit from the cell cycle, reduced cardiomyocyte endowment, and dysregulated trophic signaling during early development, which are implicated in the etiology of heritable cardiac %%%hypertrophy%%% in the adult.

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Interaction between TAK1-TAB1-TAB2 and RCAN1-%%%calcineurin%%% defines a signalling nodal control point.

Liu Qinghang; Busby Jennifer Caldwell; Molkentin Jeffery D

Department of Pediatrics, University of Cincinnati, Cincinnati Children's Hospital Medical Center, and the Howard Hughes Medical Institute, Cincinnati, Ohio 45229, USA.

Nature cell biology (England) Feb 2009, 11 (2) p154-61, ISSN 1476-4679--Electronic 1465-7392--Linking Journal Code: 100890575

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Publishing Model Print-Electronic; Cites EMBO J. 2000 Jul 17;19(14):3618-29 PMID 10899116; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3328-33 PMID 11248078; Cites Mol Cell. 2000 Apr;5(4):649-58 PMID 10882101; Cites Nat Med. 2000 May;6(5):556-63 PMID 10802712; Cites J Biol Chem. 2000 Mar 24;275(12):8719-25 PMID 10722714; Cites FASEB J. 2007 Oct;21(12):3023-8 PMID 17595344; Cites Trends Cell Biol. 2007 Jun;17(6):251-60 PMID 17493814; Cites J Biol Chem. 2007 Feb 9;282(6):3918-28 PMID 17158449; Cites Cell Signal. 2007 Mar;19(3):600-9 PMI D 17027227; Cites Proc Natl Acad Sci U S A. 2006 May 9;103(19):7327-32 PMID 16648267; Cites Mol Cell Biol. 2006 May;26(10):3785-97 PMID 16648474; Cites J Biol Chem. 2006 Mar 24;281(12):7717-26 PMID 16415348; Cites Cardiovasc Res. 2006 Feb 1;69(2):432-9 PMID 16360132; Cites Genes Dev. 2005 Nov 15;19(22):2668-81 PMID 16260493; Cites Nat Immunol. 2005 Nov;6(11):1087-95 PMID 16186825; Cites Proc Natl Acad Sci U S A. 2005 Sep 13;102(37):13075-80 PMID 16131541; Cites Nature. 1999 Mar 18;398(6724):252-6 PMID 10094049; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Neuron. 1999 Jul;23(3):435-47 PMID 10433257; Cites Science. 1995 Dec 22;270(5244):2008-11 PMID 8533096; Cites Science. 1996 May 24;272(5265):1179-82 PMID 8638164; Cites Mol Cell Biol. 1995 Nov;15(11):6064-74 PMID 7565759; Cites Cardiovasc Res. 2004 Aug 15;63(3):467-75 PMID 15276472; Cites Genes Dev. 2004 Jan 1;18(1):35-47 PMID 14701880; Cites EMBO J. 2003 Dec 1;22(23):6277-88 PMID 14633987; Cites Biochem Biophys Res Commun. 2003 Nov 28;311(4):1089-93 PMID 14623294; Cites Genes Dev. 2003 Sep 15;17(18):2205-32 PMID 12975316; Cites Biochem J. 2003 Sep 1;374(Pt 2):567-75 PMID 12809556; Cites Proc Natl Acad Sci U S A.

2003 Jan 21;100(2):669-74 PMID 12515860; Cites Mech Dev. 2002 Dec;119(2):239-49 PMID 12464436; Cites J Biol Chem. 2002 Aug 16;277(33):30401-7 PMID 12063245; Cites Nature. 2001 Jul 19;412(6844):346-51 PMID 11460167; Cites J Cell Sci. 2002 Jan 15;115(Pt 2):241-56 PMID 11839776; Cites Mol Cell Biol. 2001 Nov;21(21):7460-9 PMID 11585926; Cites Genes Dev. 2000 Jul 1;14(13):1595-604 PMID 10887154

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The calcium-activated protein phosphatase %%%calcineurin%%% is controlled by regulator of %%%calcineurin%%% (RCAN) in organisms ranging from yeast to mammals. Here we performed a yeast two-hybrid screen with RCAN1 as bait, identifying TAK1 binding protein 2 (TAB2) as an interacting partner. TAB2 interacted directly with RCAN1 in vitro and in vivo, recruiting TAK1, TAB1 and %%%calcineurin%%% , forming a macromolecular signalling complex. Overexpression of TAK1 and TAB1, or active TAK1(DeltaN), promoted direct phosphorylation of RCAN1 in vitro and in vivo. TAK1 phosphorylated RCAN1 at Ser 94 and Ser 136, converting RCAN1 from an inhibitor to a facilitator of %%%calcineurin%%%-NFAT signalling, and enhancing NFATc1 %%%nuclear%%% translocation, NFAT transcriptional activation and the %%%hypertrophic%%% growth of cultured cardiomyocytes. The TAK1-TAB1-TAB2 and the %%%calcineurin%%% -NFAT signalling modules did not interact in Rcan1/2- or Tab2-deficient mouse embryonic fibroblast (MEF) cultures. %%%Calcineurin%%% activation also dephosphorylated and inhibited TAK1 and TAB1, an effect that was absent in Rcan1/2 deficient MEFs. Functionally, TAK1 was indispensable for the cardiomyocyte growth response induced by pro-%%%hypertrophic%%% stimuli through %%%calcineurin%%% . These results describe a signalling relationship between two central regulatory pathways in which TAK1-TAB1-TAB2 selectively induces %%%calcineurin%%% -NFAT signalling through direct phosphorylation of RCAN1, while %%%calcineurin%%% activation diminishes TAK1 signalling by dephosphorylation of TAK1 and TAB1.

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Estrogen attenuates left ventricular and cardiomyocyte %%%hypertrophy%%% by an estrogen receptor-dependent pathway that increases %%%calcineurin%%% degradation.

Donaldson Cameron; Eder Sarah; Baker Corey; Aronovitz Mark J; Weiss Alexandra Dabreo; Hall-Porter Monica; Wang Feng; Ackerman Adam; Karas Richard H; Molkentin Jeffery D; Patten Richard D

Molecular Cardiology Research Institute, Tufts-New England Medical Center, Boston, MA 02111, USA.

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Main Citation Owner: NLM
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Left ventricular (LV) %%%hypertrophy%%% commonly develops in response to chronic hypertension and is a significant risk factor for heart failure and death. The serine-threonine phosphatase %%%calcineurin%%% (Cn)A plays a critical role in the development of pathological %%%hypertrophy%%% . Previous experimental studies in murine models show that estrogen limits pressure overload-induced %%%hypertrophy%%% ; our purpose was to explore further the mechanisms underlying this estrogen effect. Wild-type, ovariectomized female mice were treated with placebo or 17beta-estradiol (E2), followed by transverse aortic constriction (TAC), to induce pressure overload. At 2 weeks, mice underwent physiological evaluation, immediate tissue harvest, or dispersion of cardiomyocytes. E2 replacement limited TAC-induced LV and cardiomyocyte %%%hypertrophy%%% while attenuating deterioration in LV systolic function and contractility. These E2 effects were associated with reduced abundance of CnA. The primary downstream targets of CnA are the %%%nuclear%%% factor of activated T-cell (NFAT) family of transcription factors. In transgenic mice expressing a NFAT-activated promoter/luciferase reporter gene, E2 limited TAC-induced activation of NFAT. Moreover, the inhibitory effects of E2 on LV %%%hypertrophy%%% were absent in CnA knockout mice, supporting the notion that CnA is an important target of E2-mediated inhibition. In cultured rat cardiac myocytes, E2 inhibited agonist-induced %%%hypertrophy%%% while also decreasing CnA abundance and NFAT activation. Agonist stimulation also reduced CnA ubiquitination and degradation that was prevented by E2; all in vitro effects of estrogen were reversed by an estrogen receptor (ER) antagonist. These data support that E2 reduces pressure overload induced %%%hypertrophy%%% by an ER-dependent mechanism that increases CnA degradation, unveiling a novel mechanism by which E2 and ERs regulate pathological LV and cardiomyocyte growth.

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Phosphodiesterase 5 inhibition blocks pressure overload-induced cardiac %%%hypertrophy%%% independent of the %%%calcineurin%%% pathway.

Hsu Steven; Nagayama Takahiro; Koitabashi Norimichi; Zhang Manling; Zhou Liye; Bedja Djahida; Gabrielson Kathleen L; Molkentin Jeffery D; Kass David A; Takimoto Eiki
Division of Cardiology, Department of Medicine, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Ross-Building, Room 850, Baltimore, MD 21205, USA.

Cardiovascular research (England) Feb 1 2009, 81 (2) p301-9, ISSN 1755-3245--Electronic 0008-6363--Linking Journal Code: 0077427
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AIMS: Cyclic GMP (cGMP)-specific phosphodiesterase 5 (PDE5) inhibition by sildenafil (SIL) activates myocardial cGMP-dependent protein kinase G (PKG) and blunts cardiac %%%hypertrophy%%%. To date, the only documented target of PKG in myocardium is the serine-threonine phosphatase %%%calcineurin%%% (Cn), which is central to pathological cardiac %%%hypertrophy%%%. We tested whether Cn suppression is necessary in order to observe anti-%%%hypertrophic%%% effects of SIL. METHODS AND RESULTS: Mice lacking the Cn-Abeta subunit (CnAbeta(-/-)) and wild-type (WT) controls were subjected to transverse aorta constriction (TAC) with or without SIL (200 mg/kg/day, p.o.) for 3 weeks. TAC-induced elevation of Cn expression and activity in WT was absent in CnAbeta(-/-) hearts, and the latter accordingly developed less cardiac %%%hypertrophy%%% (50 vs. 100% increase in heart weight/tibia length, P < 0.03) and chamber dilation. SIL remained effective in CnAbeta(-/-) mice, increasing PKG activity similarly as in WT, suppressing %%%hypertrophy%%% and fetal gene expression, and enhancing heart function without altering afterload. TAC-stimulated calcium-calmodulin kinase II, Akt, and glycogen synthase kinase 3beta in both groups (the first rising more in CnAbeta(-/-) hearts), and SIL also suppressed these similarly. Activation of extracellular %%%signal%%% -regulated kinase observed in WT-TAC but not CnAbeta(-/-) hearts was also suppressed by SIL. CONCLUSION: PDE5A inhibition and its accompanying PKG activation blunt %%%hypertrophy%%% and improve heart function even without Cn activation. This occurs by its modulation of several alternative pathways which may result from concomitant distal targeting, or activity against a common proximal node.
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19069397   PMID: 19019835
Overexpression of prostaglandin EP3 receptors activates %%%calcineurin%%% and promotes %%%hypertrophy%%% in the murine heart.
Meyer-Kirchrath Jutta; Martin Melanie; Schooss Christina; Jacoby Christoph; Flogel Ulrich; Marzoll Andrea; Fischer Jens W; Schrader Jurgen; Schror Karsten; Hohlfeld Thomas
Institut fur Pharmakologie und Klinische Pharmakologie, Universitatsklinikum, Heinrich-Heine-Universitat, Moorenstr. 5, D-40225 Dusseldorf, Germany. meyerj@uni-duesseldorf.de
Cardiovascular research (England) Feb 1 2009, 81 (2) p310-8, ISSN 1755-3245--Electronic 0008-6363--Linking   Journal Code: 0077427
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AIMS: Prostaglandin E(2) (PGE(2)) has been shown to mediate anti-ischaemic effects and cardiomyocyte %%%hypertrophy%%% and there is evidence for an involvement of the prostaglandin EP(3)-receptor subtype. This study focuses on the EP(3)-mediated %%%hypertrophic%%% action and investigates intracellular signalling pathways of the EP(3)-receptor subtype in the murine heart. METHODS AND RESULTS: Cardiac function was analyzed in vivo by magnetic resonance imaging (MRI) in transgenic (tg) mice with cardio-specific overexpression of the EP(3) receptor in comparison with wild-type (wt) mice. Left ventricular (LV) function was determined in isolated perfused hearts subjected to 60 min of zero-flow ischaemia and 45 min of reperfusion. %%%Calcineurin%%% activity and %%%nuclear%%% activity of %%%nuclear%%% factor of activated T-cells (NFAT) were determined by a modified malachite green assay and ELISA, respectively. Extracellular matrix compounds were analyzed by RT-PCR and histology. MRI indicated a significant increase in end-diastolic and end-systolic volume in tg hearts. LV ejection fraction was severely decreased in tg hearts while the relative LV mass was significantly increased. In Langendorff perfused hearts, EP(3)-receptor overexpression resulted in a marked blunting of the ischaemia-induced increase in LV end-diastolic pressure and creatine kinase release. Analysis of EP(3)-receptor-mediated signalling revealed significantly increased %%%calcineurin%%% activity and %%%nuclear%%% activity of NFAT in tg hearts. Moreover, elevated mRNA levels of collagen types I and III as well as the collagen-binding proteoglycans biglycan and decorin were detected in tg hearts. CONCLUSION: EP(3)-receptor-mediated signalling results in a significant anti-ischaemic action and activation of the pro-%%%hypertrophic%%% %%%calcineurin%%% signalling pathway, suggesting the involvement of the EP(3) subtype in both PGE(2)-mediated cardioprotection as well as cardiac %%%hypertrophy%%%.

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Identification of FHL1 as a regulator of skeletal muscle mass: implications for human myopathy.

Cowling Belinda S; McGrath Meagan J; Nguyen Mai-Anh; Cottle Denny L; Kee Anthony J; Brown Susan; Schessl Joachim; Zou Yaqun; Joya Josephine; Bonnemann Carsten G; Hardeman Edna C; Mitchell Christina A
Department of Biochemistry and Molecular Biology, Monash University, Clayton 3800, Victoria, Australia.

Journal of cell biology (United States) Dec 15 2008, 183 (6) p1033-48, ISSN 1540-8140--Electronic 0021-9525--Linking Journal Code: 0375356
Publishing Model Print; Cites J Biol Chem. 2006 Mar 17;281(11):7666-83 PMID 16407297; Cites Cell Mol Life Sci. 2006 Feb;63(3):268-84 PMID 16389449; Cites J Cell Biol. 2006 Jan 16;172(2):233-44 PMID 16401724; Cites Nucleic Acids Res. 1999 Oct 1;27(19):e27 PMID 10481039; Cites J Biol Chem. 1999 Jul 30;274(31):21908-12 PMID 10419511; Cites Biochem J. 1999 Apr 15;339 (Pt 2):443-51 PMID 10191278; Cites Mol Cell Biol. 1999 Apr;19(4):3115-24 PMID

10082578; Cites Biochem Biophys Res Commun. 1999 Feb 16;255(2):245-50 PMID 10049693; Cites Mol Biol Cell. 1998 Oct;9(10):2905-16 PMID 9763451; Cites Genes Dev. 1998 Aug 15;12(16):2499-509 PMID 9716403; Cites Eur J Pharmacol. 2000 Mar 10;391(1-2):39-48 PMID 10720633; Cites J Biol Chem. 2000 Feb 18;275(7):4545-8 PMID 10671477; Cites Neuromuscul Disord. 1999 Dec;9(8):580-6 PMID 10619716; Cites EMBO J. 2005 Sep 7;24(17):3049-56 PMID 16079911; Cites J Appl Physiol. 2005 Apr;98(4):1396-406 PMID 15608089; Cites J Cell Physiol. 2005 Mar;202(3):787-95 PMID 15389566; Cites FASEB J. 2004 Dec;18(15):1937-9 PMID 15456738; Cites Nature. 1999 Aug 5;400(6744):581-5 PMID 10448862; Cites Nature. 1999 Aug 5;400(6744):576-81 PMID 10448861; Cites J Cell Biol. 1999 Feb 22;144(4):631-43 PMID 10037786; Cites Cell Death Differ. 2008 Aug;15(8):1221-31 PMID 18369372; Cites Physiology (Bethesda). 2008 Jun;23:160-70 PMID 18556469; Cites J Clin Invest. 2008 Mar;118(3):904-12 PMID 18274675; Cites Exp Cell Res. 2008 Mar 10;314(5):1013-29 PMID 18255059; Cites Am J Hum Genet. 2008 Jan;82(1):208-13 PMID 18179901; Cites Am J Hum Genet. 2008 Jan;82(1):88-99 PMID 18179888; Cites J Appl Physiol. 2007 Sep;103(3):1099-100 PMID 17289912; Cites Curr Biol. 2007 Aug 7;17(15):1318-25 PMID 17658256; Cites J Cell Sci. 2007 Apr 15;120(Pt 8):1423-35 PMID 17389685; Cites J Biol Chem. 2007 Mar 30;282(13):10068-78 PMID 17289669; Cites Muscle Nerve. 2007 Mar;35(3):322-6 PMID 17099882; Cites Appl Physiol Nutr Metab. 2006 Dec;31(6):782-90 PMID 17213900; Cites J Cell Sci. 2007 Jan 1;120(Pt 1):149-59 PMID 17164296; Cites J Cell Biol. 2006 Oct 9;175(1):87-97 PMID 17015617; Cites Acta Physiol (Oxf). 2006 Oct;188(2):77-89 PMID 16948795; Cites J Cell Sci. 1998 Mar;111 (Pt 6):769-79 PMID 9472005; Cites Nature. 1996 Nov 28;384(6607):349-53 PMID 8934518; Cites Biochem Biophys Res Commun. 1995 Jul 26;212(3):840-6 PMID 7626119; Cites J Neurol Sci. 1995 Jan;128(1):58-65 PMID 7722535; Cites J Biol Chem. 1993 Jan 5;268(1):719-25 PMID 7678010; Cites J Biol Chem. 1993 Jan 25;268(3):1580-5 PMID 7678408; Cites J Anat. 1992 Aug;181 (Pt 1):11-27 PMID 1294558; Cites Mol Cell Biol. 1987 Nov;7(11):4100-14 PMID 3431550; Cites Differentiation. 1990 Jun;43(3):183-91 PMID 2201580; Cites J Cell Biol. 1982 Dec;95(3):763-70 PMID 6185504; Cites Neurology. 1972 Aug;22(8):829-40 PMID 4117299; Cites Hum Mol Genet. 2004 Nov 1;13(21):2633-45 PMID 15367485; Cites Muscle Nerve. 2004 Oct;30(4):470-80 PMID 15372535; Cites Neuromuscul Disord. 2004 Sep;14(8-9):519-25 PMID 15336693; Cites Muscle Nerve. 2004 Sep;30(3):295-304 PMID 15318340; Cites J Biol Chem. 2004 Jun 18;279(25):26192-200 PMID 15082723; Cites Hum Mol Genet. 2004 Feb 15;13(4):379-88 PMID 14681302; Cites J Cell Biol. 2003 Dec 8;163(5):931-6 PMID 14662739; Cites Am J Physiol Cell Physiol. 2003 Dec;285(6):C1513-26 PMID 12917103; Cites Cell Biol Int. 2003;27(10):845-51 PMID 14499665; Cites Int Rev Cytol. 2003;225:33-89 PMID 12696590; Cites Acta Neuropathol. 2003 Mar;105(3):271-80 PMID 12557015; Cites Dev Dyn. 2003 Jan;226(1):128-38 PMID 12508234; Cites Eur J Immunol. 2002 Oct;32(10):2971-8 PMID 12355451; Cites J Cell Biol. 2002 Mar 4;156(5):771-4 PMID 11877454; Cites J Cell Biol. 2002 Feb 18;156(4):751-60 PMID 11839766; Cites Nat Cell Biol. 2001 Nov;3(11):1009-13 PMID 11715022; Cites J Cell Biol. 2001 May 28;153(5):985-98 PMID 11381084; Cites J Biol Chem. 2001 Feb 2;276(5):3524-30 PMID 11044444; Cites J Cell Biol. 2001 Apr 16;153(2):329-38 PMID 11309414; Cites Dev Biol. 2001 Apr 1;232(1):115-26 PMID 11254352; Cites Hum Mol Genet. 2001 Feb 15;10(4):317-28 PMID 11157795; Cites Acta Physiol Scand. 1999 Dec;167(4):301-5 PMID 10632630; Cites Mol Cell Biol. 2000 Sep;20(17):6600-11 PMID 10938134; Cites Mol Cell Biol Res Commun. 2000

Mar;3(3):136-40 PMID 10860860; Cites Biochem Biophys Res Commun. 2000 May 27;272(1):303-8 PMID 10872844; Cites J Cell Biol. 2000 May 1;149(3):657-66 PMID 10791979; Cites Mol Biol Cell. 2006 Apr;17(4):1570-82 PMID 16436503; Cites Ann N Y Acad Sci. 2005 Dec;1066:181-221 PMID 16533927
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Regulators of skeletal muscle mass are of interest, given the morbidity and mortality of muscle atrophy and myopathy. Four-and-a-half LIM protein 1 (FHL1) is mutated in several human myopathies, including reducing-body myopathy (RBM). The normal function of FHL1 in muscle and how it causes myopathy remains unknown. We find that FHL1 transgenic expression in mouse skeletal muscle promotes %%%hypertrophy%%% and an oxidative fiber-type switch, leading to increased whole-body strength and fatigue resistance. Additionally, FHL1 overexpression enhances myoblast fusion, resulting in %%%hypertrophic%%% myotubes in C2C12 cells, (a phenotype rescued by %%%calcineurin%%% inhibition). In FHL1-RBM C2C12 cells, there are no %%%hypertrophic%%% myotubes. FHL1 binds with the %%%calcineurin%%% -regulated transcription factor NFATc1 (%%%nuclear%%% factor of activated T cells, cytoplasmic, %%%calcineurin%%% -dependent 1), enhancing NFATc1 transcriptional activity. Mutant RBM-FHL1 forms aggregate bodies in C2C12 cells, sequestering NFATc1 and resulting in reduced NFAT %%%nuclear%%% translocation and transcriptional activity. NFATc1 also colocalizes with mutant FHL1 to reducing bodies in RBM-afflicted skeletal muscle. Therefore, via NFATc1 signaling regulation, FHL1 appears to modulate muscle mass and strength enhancement.
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Record Date Completed: 20090115

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18855154 PMID: 18776042
Activation of Na+/H+ exchanger 1 is sufficient to generate Ca2+ signals that induce cardiac %%%hypertrophy%%% and heart failure.
Nakamura Tomoe Y; Iwata Yuko; Arai Yuji; Komamura Kazuo; Wakabayashi Shigeo
Department of Molecular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan.
Circulation research (United States) Oct 10 2008, 103 (8) p891-9, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103
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Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

Activation of the sarcolemmal Na(+)/H(+) exchanger (NHE)1 is increasingly documented as a process involved in cardiac %%%hypertrophy%%% and heart failure. However, whether NHE1 activation alone is sufficient to induce such remodeling remains unknown. We generated transgenic mice that overexpress a human NHE1 with high activity in hearts. The hearts of these mice developed cardiac %%%hypertrophy%%% , contractile dysfunction, and

heart failure. In isolated transgenic myocytes, intracellular pH was elevated in Hepes buffer but not in physiological bicarbonate buffer, yet intracellular Na(+) concentrations were higher under both conditions. In addition, both diastolic and systolic Ca(2+) levels were increased as a consequence of Na(+)-induced Ca(2+) overload; this was accompanied by enhanced sarcoplasmic reticulum Ca(2+) loading via Ca(2+)/calmodulin-dependent protein kinase (CaMK)II-dependent phosphorylation of phospholamban. Negative force-frequency dependence was observed with preservation of high Ca(2+), suggesting a decrease in myofibril Ca(2+) sensitivity. Furthermore, the Ca(2+)-dependent prohypertrophic molecules %%%calcineurin%%% and CaMKII were highly activated in transgenic hearts. These effects observed in vivo and in vitro were largely prevented by the NHE1 inhibitor cariporide. Interestingly, overexpression of NHE1 in neonatal rat ventricular myocytes induced cariporide-sensitive %%%nuclear%%% translocation of NFAT (%%%nuclear%%% factor of activated T cells) and %%%nuclear%%% export of histone deacetylase 4, suggesting that increased Na(+)/H(+) exchange activity can alter %%%hypertrophy%%%-associated gene expression. However, in transgenic myocytes, contrary to exclusive translocation of histone deacetylase 4, NFAT only partially translocated to %%%nucleus%%%, possibly because of marked activation of p38, a negative regulator of NFAT signaling. We conclude that activation of NHE1 is sufficient to initiate cardiac %%%hypertrophy%%% and heart failure mainly through activation of CaMKII-histone deacetylase pathway.
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  Date of Electronic Publication: 20080905

 2/7/23   (Item 23 from file: 154)
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18826789   PMID: 18600431
  Activation of peroxisome proliferator-activated receptor gamma inhibits endothelin-1-induced cardiac %%%hypertrophy%%% via the %%%calcineurin%%% /NFAT signaling pathway.
  Bao Yingxia; Li Ruifang; Jiang Jianmin; Cai Birong; Gao Jie; Le Kang; Zhang Fangyan; Chen Shaorui; Liu Peiqing
  Laboratory of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-Sen University, 74 Zhongshan II Road, Guangzhou, 510080, People's Republic of China.
  Molecular and cellular biochemistry (Netherlands)   Oct 2008, 317 (1-2)
    p189-96, ISSN 0300-8177--Print 0300-8177--Linking Journal Code: 0364456
  Publishing Model Print-Electronic
  Document type: Journal Article; Research Support, Non-U.S. Gov't
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: MEDLINE; Completed
  Peroxisome proliferator-activated receptor gamma (PPAR-gamma) has been described as a negative regulator of cardiac %%%hypertrophy%%%. A better understanding of PPAR-gamma and cardiac %%%hypertrophy%%% may facilitate the development of novel therapeutic strategies to treat heart diseases related to cardiac %%%hypertrophy%%% by mimicking the naturally preferred mechanisms. In the present study, we investigated the interaction between PPAR-gamma and %%%calcineurin%%%/%%%nuclear%%% factor of activated T-cells (NFAT) in endothelin-1 (ET-1)-induced %%%hypertrophy%%% of neonatal rat

cardiac myocytes. The results suggest that the treatment of cultured cardiac myocytes with a PPAR-gamma ligand, rosiglitazone, inhibited the ET-1-induced increase in protein synthesis, surface area, %%%calcineurin%%% enzymatic activity, and protein expression. Both the application of rosiglitazone and overexpression of the PPAR-gamma inhibited the %%%nuclear%%% translocation of NFATc4. Moreover, co-immunoprecipitation studies showed that rosiglitazone enhanced the association between PPAR-gamma and %%%calcineurin%%% /NFAT. These results suggest that ET-1-induced cardiac %%%hypertrophy%%% is inhibited by activation of PPAR-gamma, which is at least partly due to cross-talk between PPAR-gamma and %%%calcineurin%%%/NFAT.
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18695362 PMID: 18456728
A novel signaling pathway of ADP-ribosyl cyclase activation by angiotensin II in adult rat cardiomyocytes.
Gul Rukhsana; Kim Seon-Young; Park Kwang-Hyun; Kim Byung-Ju; Kim Se-Jin; Im Mie-Jae; Kim Uh-Hyun
Dept. of Biochemistry, Chonbuk National Univ. Medical School, Keum-am dong, Jeonju, 561-182, Republic of Korea.
American journal of physiology. Heart and circulatory physiology (United States) Jul 2008, 295 (1) pH77-88, ISSN 0363-6135--Print 0363-6135--Linking Journal Code: 100901228
Publishing Model Print-Electronic; Erratum in Am J Physiol Heart Circ Physiol. 2008 Nov;295(5):H2220
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
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ADP-ribosyl cyclase (ADPR-cyclase) produces a Ca(2+)-mobilizing second messenger, cADP-ribose (cADPR), from NAD(+). In this study, we investigated the molecular basis of ADPR-cyclase activation in the ANG II signaling pathway and cellular responses in adult rat cardiomyocytes. The results showed that ANG II generated biphasic intracellular Ca(2+) concentration increases that include a rapid transient Ca(2+) elevation via inositol trisphosphate (IP(3)) receptor and sustained Ca(2+) rise via the activation of L-type Ca(2+) channel and opening of ryanodine receptor. ANG II-induced sustained Ca(2+) rise was blocked by a cADPR antagonistic analog, 8-bromo-cADPR, indicating that sustained Ca(2+) rise is mediated by cADPR. Supporting the notion, ADPR-cyclase activity and cADPR production by ANG II were increased in a time-dependent manner. Application of pharmacological inhibitors and immunological analyses revealed that cADPR formation was activated by sequential activation of Src, phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (Akt), phospholipase C (PLC)-gamma1, and IP(3)-mediated Ca(2+) %%%signal%%%. Inhibitors of these signaling molecules not only completely abolished the ANG II-induced Ca(2+) signals but also inhibited cADPR formation. Application of the cADPR antagonist and inhibitors of upstream signaling molecules of ADPR-cyclase inhibited ANG II-stimulated %%%hypertrophic%%% responses, which include %%%nuclear%%% translocation of Ca(2+)/%%%calcineurin%%%-dependent %%%nuclear%%% factor

of activated T cells 3, protein expression of transforming growth factor-beta1, and incorporation of [(3)H]leucine in cardiomyocytes. Taken together, these findings suggest that activation of ADPR-cyclase by ANG II entails a novel signaling pathway involving sequential activation of Src, PI 3-kinase/Akt, and PLC-gamma1/IP(3) and that the activation of ADPR-cyclase can lead to cardiac %%%hypertrophy%%%.
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18662199 PMID: 18577756 Record Identifier: NIHMS92837; PMC2680250
Does contractile Ca2+ control %%%calcineurin%%% -NFAT signaling and pathological %%%hypertrophy%%% in cardiac myocytes?
Houser Steven R; Molkentin Jeffery D
Department of Physiology, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140, USA. srhouser@temple.edu
Science signaling (United States) 2008, 1 (25) ppe31, ISSN 1937-9145--Electronic Journal Code: 101465400
Contract/Grant No.: P01 HL069779-06A10003; HL; NHLBI NIH HHS United States; P50 HL077101-050004; HL; NHLBI NIH HHS United States; R01 HL060562-11; HL; NHLBI NIH HHS United States; R01 HL062927-10A1; HL; NHLBI NIH HHS United States; R01 HL081104-04; HL; NHLBI NIH HHS United States; Howard Hughes Medical Institute United States
Publishing Model Electronic; Cites Circulation. 2002 Oct 29;106(18):2385-91 PMID 12403671; Cites EMBO J. 2003 Aug 1;22(15):3825-32 PMID 12881417; Cites J Physiol. 2004 Jan 15;554(Pt 2):309-20 PMID 14565991; Cites Circ Res. 2004 Jan 9;94(1):110-8 PMID 14656927; Cites Biochem Biophys Res Commun. 2004 Oct 1;322(4):1178-91 PMID 15336966; Cites Circulation. 2001 Feb 6;103(5):670-7 PMID 11156878; Cites Circulation. 2001 Jan 2;103(1):140-7 PMID 11136699; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites J Biol Chem. 2000 Jan 21;275(3):1855-63 PMID 10636885; Cites J Physiol. 2002 Oct 1;544(Pt 1):57-69 PMID 12356880; Cites Circ Res. 2002 Mar 22;90(5):578-85 PMID 11909822; Cites Cardiovasc Res. 2002 Feb 1;53(2):372-81 PMID 11827688; Cites J Cell Biol. 2001 Oct 1;155(1):27-39 PMID 11581284; Cites Chest. 2004 Dec;126(6):1926-32 PMID 15596694; Cites J Clin Invest. 2005 Mar;115(3):527-37 PMID 15765134; Cites Ann Med. 2004;36(8):584-95 PMID 15768830; Cites Ann N Y Acad Sci. 2005 Jun;1047:86-98 PMID 16093487; Cites Cell Mol Life Sci. 2006 Feb;63(3):333-42 PMID 16389460; Cites Mol Biol Cell. 2006 Apr;17(4):1570-82 PMID 16436503; Cites J Clin Invest. 2006 Mar;116(3):675-82 PMID 16511602; Cites J Mol Cell Cardiol. 2006 May;40(5):725-35 PMID 16600293; Cites Proc Natl Acad Sci U S A. 2006 May 9;103(19):7500-5 PMID 16648270; Cites J Clin Invest. 2007 Sep;117(9):2431-44 PMID 17694179; Cites Am J Physiol Cell Physiol. 2008 Mar;294(3):C715-25 PMID 18184878; Cites Subcell Biochem. 2007;45:523-37 PMI D 18193651; Cites Proc Natl Acad Sci U S A. 2008 Feb 26;105(8):2859-64 PMID 18287024; Cites J Physiol. 1988 Dec;406:115-46 PMID 2855434; Cites Nature. 1997 Apr 24;386(6627):855-8 PMID 9126747; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Nature. 1998 Apr 30;392(6679):933-6 PMI D 9582075
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In noncontractile cells, a sustained increase in total cytoplasmic Ca(2+) concentration is typically needed to activate the intracellular protein phosphatase %%%calcineurin%%% , leading to dephosphorylation of the transcription factor %%%nuclear%%% factor of activated T cells (NFAT), its %%%nuclear%%% translocation, and induction of gene expression. It remains a mystery exactly how Ca(2+)-dependent signaling pathways, such as that mediated by %%%calcineurin%%% -NFAT, are regulated in contracting cardiac myocytes given the highly specialized manner in which Ca(2+) concentration rhythmically cycles in excitation-contraction coupling. Here, we critically review evidence that supports the hypothesis that %%%calcineurin%%%-NFAT signaling is regulated by contractile Ca(2+) transients in cardiac myocytes.

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18599343 PMID: 18398669

Lipopolysaccharide induces cellular %%%hypertrophy%%% through %%%calcineurin%%%/NFAT-3 signaling pathway in H9c2 myocardiac cells.

Liu Chung-Jung; Cheng Yi-Chang; Lee Kung-Wei; Hsu Hsi-Hsien; Chu Chun-Hsien; Tsai Fuu-Jen; Tsai Chang-Hai; Chu Chia-Yih; Liu Jer-Yuh; Kuo Wei-Wen; Huang Chih-Yang
Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan.

Molecular and cellular biochemistry (Netherlands) Jun 2008, 313 (1-2) p167-78, ISSN 0300-8177--Print 0300-8177--Linking Journal Code: 0364456

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Main Citation Owner: NLM
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Evidences suggest that lipopolysaccharide (LPS) participates in the inflammatory response in the cardiovascular system; however, it is unknown if LPS is sufficient to cause the cardiac %%%hypertrophy%%%. In the present study, we treated H9c2 myocardiac cells with LPS to explore whether LPS causes cardiac %%%hypertrophy%%%, and to identify the precise molecular and cellular mechanisms behind %%%hypertrophic%%% responses. Here we show that LPS challenge induces pathological %%%hypertrophic%%% responses such as the increase in cell size, the reorganization of actin filaments, and the upregulation of %%%hypertrophy%%% markers including atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) in H9c2 cells. LPS treatment significantly promotes the activation of GATA-4 and the %%%nuclear%%% translocation of NFAT-3, which act as transcription factors mediating the development of cardiac %%%hypertrophy%%% . After administration of inhibitors including U0126 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK1/2 inhibitor), CsA (%%%calcineurin%%% inhibitor), FK506 (%%%calcineurin%%% inhibitor), and QNZ (NFkappaB

inhibitor), LPS-induced %%%hypertrophic%%% characteristic features, such as increases in cell size, actin fibers, and levels of ANP and BNP, and the %%%nuclear%%% localization of NFAT-3 are markedly inhibited only by %%%calcineurin%%% inhibitors, CsA and FK506. Collectively, these results suggest that LPS leads to myocardiac %%%hypertrophy%%% through %%%calcineurin%%% /NFAT-3 signaling pathway in H9c2 cells. Our findings further provide a link between the LPS-induced inflammatory response and the %%%calcineurin%%% /NFAT-3 signaling pathway that mediates the development of cardiac %%%hypertrophy%%%.
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Date of Electronic Publication: 20080409

2/7/27 (Item 27 from file: 154)
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18549225 PMID: 18325738
System-level investigation into the regulatory mechanism of the %%%calcineurin%%%/NFAT signaling pathway.
Shin Sung-Young; Yang Ji Min; Choo Sang-Mok; Kwon Ki-Sun; Cho Kwang-Hyun
Department of Bio and Brain Engineering and KI for the BioCentury, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea.
Cellular signalling (England) Jun 2008, 20 (6) p1117-24, ISSN 0898-6568--Print 0898-6568--Linking Journal Code: 8904683
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Calcineurn/%%%nuclear%%% factor of the activated T cell (CaN/NFAT) signaling pathway plays crucial roles in the development of cardiac %%%hypertrophy%%% , Down's syndrome, and autoimmune diseases in response to pathological stimuli. The aim of the present study is to get a system-level understanding on the regulatory mechanism of CaN/NFAT signaling pathway in consideration of the controversial roles of myocyte-enriched %%%calcineurin%%% interacting protein1 (MCIP1) for varying stress stimuli. To this end, we have developed an experimentally validated mathematical model and carried out computer simulations as well as cell-based experiments. Quantitative overexpression and knock-down experiments in C2C12 myoblasts have revealed that MCIP1 functions only as a %%%calcineurin%%% inhibitor. We have also observed a biphasic response of the NFAT activity with increasing stimuli of isoproterenol. Through extensive in silico simulations, we have discovered that the NFAT activity is primarily modulated by ERK5 and MCIP1 under mild isoproterenol stimuli whereas it is mainly modulated by atrogin1 (muscle atrophy F-box protein) under strong isoproterenol stimuli. This study shows that a system-level analysis may help understanding CaN/NFAT signaling-associated disease.
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18496086 PMID: 18375578
Cardiac cell %%%hypertrophy%%% in vitro: role of %%%calcineurin%%%/NFAT as Ca2+ %%%signal%%% integrators.
Colella Matilde; Pozzan Tullio
Department of General and Environmental Physiology, University of Bari, Bari, Italy.
Annals of the New York Academy of Sciences (United States) Mar 2008, 1123 p64-8, ISSN 0077-8923--Print 0077-8923--Linking Journal Code: 7506858
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Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Various conditions were used to investigate the importance of Ca2+ signaling in triggering %%%hypertrophy%%% in neonatal rat cardiomyocytes in vitro. An increase in cell size and sarcomere reorganization were induced not only by treatment with receptor agonists, such as angiotensin II, aldosterone, and norepinephrine, but also by a small depolarization caused by an increase in the KCl concentration of the medium. This latter treatment has no direct effects on receptor signaling. All these %%%hypertrophic%%% treatments caused a long-lasting increase in the frequency of spontaneous [Ca2+] oscillations, causing %%%nuclear%%% translocation of transfected NFAT (GFP). Cyclosporine A inhibited %%%hypertrophy%%% and NFAT translocation, but not the increased oscillation frequency. We propose here that %%%calcineurin%%% -NFAT can act as integrators of the Ca2+ %%%signal%%% and can decode alterations in the frequency even of very rapid Ca2+ oscillations.
Record Date Created: 20080331
Record Date Completed: 20080811

2/7/29 (Item 29 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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18493454 PMID: 18369159
PICOT: a multidomain scaffolding inhibitor of %%%hypertrophic%%% %%%signal%%% transduction.
Samarel Allen M
Circulation research (United States) Mar 28 2008, 102 (6) p625-7, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103
Contract/Grant No.: 2P01 HL062426; HL; NHLBI NIH HHS United States
Publishing Model Print; Comment on Circ Res. 2008 Mar 28;102(6):711-9 PMID 18258855
Document type: Comment; Editorial; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Record Date Created: 20080328
Record Date Completed: 20080410

2/7/30 (Item 30 from file: 154)
DIALOG(R)File 154:MEDLINE(R)

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18493449 PMID: 18258855
PICOT attenuates cardiac %%%hypertrophy%%% by disrupting %%%calcineurin%%%-NFAT signaling.
Jeong Dongtak; Kim Ji Myoung; Cha Hyeseon; Oh Jae Gyun; Park Jaeho; Yun Soo-Hyeon; Ju Eun-Seon; Jeon Eun-Seok; Hajjar Roger J; Park Woo Jin
Global Research Laboratory and Department of Life Science, Gwangju Institute of Science and Technology, Gwangju, Korea.
Circulation research (United States) Mar 28 2008, 102 (6) p711-9, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103
Contract/Grant No.: HL-080498-01; HL; NHLBI NIH HHS United States
Publishing Model Print-Electronic; Comment in Circ Res. 2008 Mar 28;102(6):625-7 PMID 18369159
Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
PICOT (protein kinase C-interacting cousin of thioredoxin) was previously shown to inhibit pressure overload-induced cardiac %%%hypertrophy%%%, concomitant with an increase in ventricular function and cardiomyocyte contractility. The combined analyses of glutathione S-transferase pull-down experiments and mass spectrometry enabled us to determine that PICOT directly interacts with muscle LIM protein (MLP) via its carboxyl-terminal half (PICOT-C). It was also shown that PICOT colocalizes with MLP in the Z-disc. MLP is known to play a role in anchoring %%%calcineurin%%% to the Z-disc in the sarcomere, which is critical for %%%calcineurin%%%-NFAT (%%%nuclear%%% factor of activated T cells) signaling. We, therefore, suggested that PICOT may affect %%%calcineurin%%%-NFAT signaling through its interaction with MLP. Consistent with this hypothesis, PICOT, or more specifically PICOT-C, abrogated phenylephrine-induced increases in %%%calcineurin%%% phosphatase activity, NFAT dephosphorylation/ %%%nuclear%%% translocation, and NFAT-dependent transcriptional activation in neonatal cardiomyocytes. In addition, pressure overload-induced upregulation of NFAT target genes was significantly diminished in the hearts of PICOT-overexpressing transgenic mice. PICOT interfered with MLP-%%%calcineurin%%% interactions in a dose-dependent manner. Moreover, %%%calcineurin%%% was displaced from the Z-disc, concomitant with an abrogated interaction between %%%calcineurin%%% and MLP, in the hearts of PICOT transgenic mice. Replenishment of MLP restored the %%%hypertrophic%%% responses and the increase in %%%calcineurin%%% phosphatase activity that was inhibited by PICOT in phenylephrine-treated cardiomyocytes. Finally, PICOT-C inhibited cardiac %%%hypertrophy%%% to an extent that was comparable to that of full-length PICOT. Taken together, these data suggest that PICOT inhibits cardiac %%%hypertrophy%%% largely by negatively regulating %%%calcineurin%%%-NFAT signaling via disruption of the MLP-%%%calcineurin%%% interaction.
Record Date Created: 20080328
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Date of Electronic Publication: 20080207

2/7/31 (Item 31 from file: 154)
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18441668 PMID: 18275974

T-type Ca2+ channel blockers prevent cardiac cell %%%hypertrophy%%% through an inhibition of %%%calcineurin%%%-NFAT3 activation as well as L-type Ca2+ channel blockers.

Horiba Mitsuru; Muto Takao; Ueda Norihiro; Opthof Tobias; Miwa Keiko; Hojo Mayumi; Lee Jong-Kook; Kamiya Kaichiro; Kodama Itsuo; Yasui Kenji

Department of Cardiovascular Research, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan. mhoriba@riem.nagoya-u.ac.jp

Life sciences (England) Mar 12 2008, 82 (11-12) p554-60, ISSN 0024-3205--Print 0024-3205--Linking Journal Code: 0375521

Publishing Model Print-Electronic

Document type: Journal Article; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

T-type Ca2+ channels (TCCs) are involved in cardiac cell growth and proliferation in cultured cardiomyocytes. Underlying molecular mechanisms are not well understood. In this study, we investigated the role of TCCs in %%%signal%%% transduction in cardiac %%%hypertrophy%%% compared with L-type Ca2+ channels (LCCs). Cardiomyocytes dissociated from neonatal mouse ventricles were cultured until stabilization. Cell %%%hypertrophy%%% was induced by reapplication of 1% fatal bovine serum (FBS) following a period (24 h) of FBS depletion. Cell surface area increased from 862+/-73 microm2 to 2153+/-131 microm2 by FBS stimulation in control (250+/-1.8%). T-type Ca2+ current (I(CaT)) was inhibited dose-dependently by kurtoxin (KT) and efonidipine (ED) with IC50 0.07 microM and 3.2 microM, respectively in whole-cell voltage clamp. On the other hand, 1 microM KT which inhibits I(CaT) over 90% did not effect on L-type Ca2+ current (I(CaL)). 10 microM ED had the ability of I(CaL) blockade as well as that of I(CaT) blockade. 3 microM nisoldipine (ND) suppressed I(CaL) by over 80%. The increase in cell surface area following reapplication of FBS as observed in control (250+/-1.8%) was significantly reduced in the presence of 1 microM KT (216+/-1.2%) and virtually abolished in the presence of 10 microM ED (97+/-0.8%) and 3 microM ND (80+/-1.1%). %%%Hypertrophy%%% was associated with an increase in BNP mRNA of 316+/-3.6% in control and this increase was reduced as well in the presence of 1 microM KT (254+/-1.8%) and almost abolished in the presence of 10 microM ED (116+/-1.1%) and 3 muM ND (93+/-0.8%). Immunolabeling showed that translocation of %%%nuclear%%% factor of activated T cells (NFAT3) into the %%%nucleus%%% in response to FBS stimulation was markedly inhibited by either KT or ED as well as ND. %%%Calcineurin%%% phosphatase activity was upregulated 2.2-fold by FBS, but KT, ED and ND decreased this upregulation (1.7-fold, 0.8-fold, and 0.7-fold with KT, ED and ND respectively). These results suggest that blockade of Ca2+ entry into cardiomyocytes via TCCs may block pathophysiological signaling pathways leading to %%%hypertrophy%%% as well as via LCCs. The mechanism may be the inhibition of %%%calcineurin%%% -mediated NFAT3 activation resulting in prevention of its translocation into the %%%nucleus%%%.

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Date of Electronic Publication: 20071204

2/7/32 (Item 32 from file: 154)

DIALOG(R)File 154:MEDLINE(R)

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18439758 PMID: 18287024 Record Identifier: PMC2268550

Ca2+ oscillation frequency decoding in cardiac cell %%%hypertrophy%%%: role of %%%calcineurin%%%/NFAT as Ca2+ %%%signal%%% integrators.

Colella Matilde; Grisan Francesca; Robert Valerie; Turner Jay D; Thomas Andrew P; Pozzan Tullio

Department of General and Environmental Physiology, University of Bari, 70126 Bari, Italy.

Proceedings of the National Academy of Sciences of the United States of America (United States) Feb 26 2008, 105 (8) p2859-64, ISSN 1091-6490 --Electronic 0027-8424--Linking Journal Code: 7505876

Contract/Grant No.: AA014980; AA; NIAAA NIH HHS United States

Publishing Model Print-Electronic; Cites J Physiol. 2002 Jun 1;541(Pt 2):395-409 PMID 12042347; Cites Nature. 2002 Jan 10;415(6868):206-12 PMID 11805844; Cites J Clin Invest. 2003 May;111(10):1475-86 PMID 12750397; Cites Nat Rev Mol Cell Biol. 2003 Jul;4(7):517-29 PMID 12838335; Cites J Mol Cell Cardiol. 2003 Aug;35(8):871-86 PMID 12878473; Cites EMBO J. 2003 Aug 1;22(15):3825-32 PMID 12881417; Cites J Biol Chem. 2003 Sep 26;278(39):36981-4 PMID 12881512; Cites J Physiol. 2004 Jan 15;554(Pt 2):309-20 PMID 14565991; Cites Endocrinology. 2004 Mar;145(3):1356-66 PMID 14670993; Cites Circulation. 2004 Apr 6;109(13):1580-9 PMID 15066961; Cites Nat Med. 2004 May;10(5):467-74 PMID 15122248; Cites Circulation. 2004 Jul 20;110(3):317-23 PMID 15249508; Cites Cardiovasc Res. 2004 Aug 15;63(3):467-75 PMID 15276472; Cites Cardiovasc Res. 2004 Aug 15;63(3):537-44 PMID 15276479; Cites Biochem Biophys Res Commun. 2004 Oct 1;322(4):1178-91 PMID 15336966; Cites Mol Cell Biol. 2004 Oct;24(19):8467-76 PMID 15367668; Cites J Cell Biol. 2005 Mar 14;168(6):887-97 PMID 15767461; Cites Cardiovasc Res. 2005 Aug 1;67(2):216-24 PMID 15919070; Cites J Clin Invest. 2006 Mar;116(3):675-82 PMID 16511602; Cites Biochem Soc Trans. 2006 Apr;34(Pt 2):228-31 PMID 16545082; Cites Cell Calcium. 2006 Nov-Dec;40(5-6):405-12 PMID 17030366; Cites Cardiovasc Res. 1995 Sep;30(3):419-31 PMID 7585834; Cites Circ Res. 1995 Jan;76(1):1-15 PMID 8001266; Cites Am J Physiol. 1994 Jun;266(6 Pt 2):H2443-51 PMID 8024006; Cites Science. 1993 Oct 29;262(5134):740-4 PMID 8235594; Cites Nature. 1996 Oct 31;383(6603):837-40 PMID 8893011; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Nature. 1998 Apr 30;392(6679):933-6 PMID 9582075; Cites Nature. 1998 Apr 30;392(6679):936-41 PMID 9582076; Cites Nat Med. 2000 Feb;6(2):183-8 PMID 10655107; Cites Trends Endocrinol Metab. 2000 Aug;11(6):224-6 PMID 10878752; Cites Circ Res. 2000 Oct 27;87(9):731-8 PMID 11055975; Cites Nat Med. 2000 Nov;6(11):1221-7 PMID 11062532; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3322-7 PMID 11248077; Cites EMBO J. 2001 Sep 3;20(17):4998-5007 PMID 11532963; Cites J Cell Biol. 2001 Oct 1;155(1):27-39 PMID 11581284; Cites Annu Rev Physiol. 2003;65:45-79 PMID 12524460

Document type: Comparative Study; Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM

Other Citation Owner: NLM

Record type: MEDLINE; Completed

The role of Ca(2+) signaling in triggering %%%hypertrophy%%% was investigated in neonatal rat cardiomyocytes in vitro. We show that an increase in cell size and sarcomere reorganization were elicited by receptor agonists such as Angiotensin II, aldosterone, and norepinephrine and by a small rise in medium KCl concentration, a treatment devoid of direct effects on receptor functions. All these treatments increased the

frequency of spontaneous [Ca(2+)] transients, caused %%%nuclear%%% translocation of transfected NFAT(GFP), and increased the expression of a NFAT-sensitive reporter gene. There was no increase in Ca(2+) spark frequency in the whole cell or in the perinuclear region under these conditions. %%%Hypertrophy%%% and NFAT translocation but not the increased frequency of [Ca(2+)] transients were inhibited by the %%%calcineurin%%% inhibitor cyclosporine A. %%%Hypertrophy%%% by the different stimuli was insensitive to inhibition of myofilament contraction. We concluded that %%%calcineurin%%%-NFAT can act as integrators of the contractile Ca(2+) %%%signal%%%, and that they can decode alterations in the frequency even of rapid Ca(2+) oscillations.
Record Date Created: 20080228
Record Date Completed: 20080429
Date of Electronic Publication: 20080219

2/7/33 (Item 33 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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18260458 PMID: 18059617
Ca2+/calmodulin-based signalling in the regulation of the muscle fibre phenotype and its therapeutic potential via modulation of utrophin A and myostatin expression.
Michel Robin N; Chin Eva R; Chakkalakal Joe V; Eibl Joe K; Jasmin Bernard J
Department of Chemistry and Biochemistry,Concordia University, The Richard J. Renaud Science Complex, Montreal, QC H4B 1R6, Canada. rmichel@alcor.concordia.ca
Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme (Canada) Oct 2007, 32 (5) p921-9, ISSN 1715-5312--Print Journal Code: 101264333
Publishing Model Print; Erratum in Appl Physiol Nutr Metab. 2007 Dec;32(6):1274
Document type: Journal Article; Research Support, Non-U.S. Gov't; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Ca2+ signalling plays an important role in excitation-contraction coupling and the resultant force output of skeletal muscle. It is also known to play a crucial role in modulating both short- and long-term muscle cellular phenotypic adaptations associated with these events. Ca2+ signalling via the Ca2+/calmodulin (CaM)-dependent phosphatase %%%calcineurin%%% (CnA) and via Ca2+/CaM-dependent kinases, such as CaMKI and CaMKII, is known to regulate %%%hypertrophic%%% growth in response to overload, to direct slow versus fast fibre gene expression, and to contribute to mitochondrial biogenesis. The CnA- and CaMK-dependent regulation of the downstream transcription factors %%%nuclear%%% factor of activated T cells (NFAT) and myocyte-specific enhancer factor 2 are known to activate muscle-specific genes associated with a slower, more oxidative fibre phenotype. We have also recently shown the expression of utrophin A, a cytoskeletal protein that accumulates at the neuromuscular junction and plays a role in maturation of the postsynaptic apparatus, to be regulated by CnA-NFAT and Ca2+/CaM signalling. This regulation is fibre-type specific and potentiated by interactions with the transcriptional regulators and coactivators GA binding protein (also known as %%%nuclear%%% respiratory factor 2) and peroxisome proliferator-activated receptor-gamma coactivator

1 alpha. Another downstream target of CnA signalling may be myostatin, a transforming growth factor-beta family member that is a negative regulator of muscle growth. While the list of the downstream targets of CnA/NFAT- and Ca2+/CaM-dependent signalling is emerging, the precise interaction of these pathways with the Ca2+-independent pathways p38 mitogen-activated protein kinase, extracellular %%%signal%%% -regulated kinases 1 and 2, phosphoinositide-3 kinase, and protein kinase B (Akt/PKB) must also be considered when deciphering fibre responses and plasticity to altered contractile load. (43 Refs.)
Record Date Created: 20071206
Record Date Completed: 20080125

2/7/34 (Item 34 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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18245749 PMID: 18034994
Activation of the %%%calcineurin%%%/NFAT signalling cascade starts early in human %%%hypertrophic%%% myocardium.
Diedrichs H; Hagemeister J; Chi M; Boelck B; Muller-Ehmsen J; Schneider C A
Laboratory of Muscle Research and Molecular Cardiology, Department of Internal Medicine, University of Cologne, Cologne, Germany. Holger.Diedrichs@uni-koeln.de
Journal of international medical research (England) Nov-Dec 2007, 35 (6) p803-18, ISSN 0300-0605--Print 0300-0605--Linking Journal Code: 0346411
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Cardiac %%%hypertrophy%%% is an independent risk factor for heart failure. Recent studies on gene regulation of proteins have involved intracellular Ca2+ homeostasis. The Ca2+-sensitive phosphatase, %%%calcineurin%%%, is one potential regulator of the %%%hypertrophic%%% response, so we aimed to investigate the %%%calcineurin%%%-dependent %%%signal%%% pathway at different stages of %%%hypertrophy%%% in human myocardium. We found the %%%calcineurin%%% pathway to be significantly activated in %%%hypertrophic%%% compared with non-%%%hypertrophic%%% myocardium as demonstrated by increased %%%calcineurin%%% activity and expression of %%%calcineurin%%% A-beta and B, and GATA-4, and a shift of phosphorylated cytoplasmic NFAT-3 into the %%%nucleus%%% as dephosphorylated %%%nuclear%%% NFAT-3. There was a tendency for these changes to be more pronounced in the decompensated compared with the compensated %%%hypertrophic%%% myocardium. The present study provides evidence for significant activation of the Ca2+-triggered %%%calcineurin%%% pathway in %%%hypertrophic%%% humans. Already present in compensated %%%hypertrophy%%% it showed a tendency to a further increase following transition to decompensated %%%hypertrophy%%%.
Record Date Created: 20071123
Record Date Completed: 20080226

2/7/35 (Item 35 from file: 154)
DIALOG(R)File 154:MEDLINE(R)

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17932237   PMID: 17450511
  Signaling pathways mediating cardiac myocyte gene expression in physiological and stress responses.
  Clerk Angela; Cullingford Timothy E; Fuller Stephen J; Giraldo Alejandro; Markou Thomais; Pikkarainen Sampsa; Sugden Peter H
  NHLI Division, Faculty of Medicine, Imperial College London, London, UK. a.clerk@imperial.ac.uk
  Journal of cellular physiology (United States) Aug 2007, 212 (2) p311-22, ISSN 0021-9541--Print 0021-9541--Linking Journal Code: 0050222
  Publishing Model Print
  Document type: Journal Article; Research Support, Non-U.S. Gov't; Review
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: MEDLINE; Completed
  The contractile cells in the heart (the cardiac myocytes) are terminally differentiated. In response to pathophysiological stresses, cardiac myocytes undergo %%%hypertrophic%%% growth or apoptosis, responses associated with the development of cardiac pathologies. There has been much effort expended in gaining an understanding of the stimuli which promote these responses, and in identifying the intracellular signaling pathways which are activated and potentially involved. These signaling pathways presumably modulate gene and protein expression to elicit the end-stage response. For the regulation of gene expression, the %%%signal%%% may traverse the cytoplasm to modulate %%%nuclear%%%-localized transcription factors as occurs with the mitogen-activated protein kinase or protein kinase B/Akt cascades. Alternatively, the %%%signal%%% may promote translocation of transcription factors from the cytoplasm to the %%%nucleus%%% as is seen with the %%%calcineurin%%%/NFAT and JAK/STAT systems. We present an overview of the principal signaling pathways implicated in the regulation of gene expression in cardiac myocyte pathophysiology, and summarize the current understanding of these pathways, the transcription factors they regulate and the changes in gene expression associated with the development of cardiac pathologies. Finally, we discuss how intracellular signaling and gene expression may be integrated to elicit the overall change in cellular phenotype. (93 Refs.)
  Record Date Created: 20070604
  Record Date Completed: 20070816

 2/7/36   (Item 36 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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17874495   PMID: 17415416   Record Identifier: PMC1838928
  Modulation of adverse cardiac remodeling by STARS, a mediator of MEF2 signaling and SRF activity.
  Kuwahara Koichiro; Teg Pipes Gordon C; McAnally John; Richardson James A; Hill Joseph A; Bassel-Duby Rhonda; Olson Eric N
  Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA.
  Journal of clinical investigation (United States) May 2007, 117 (5) p1324-34, ISSN 0021-9738--Print 0021-9738--Linking Journal Code: 7802877
  Publishing Model Print-Electronic; Cites Cell. 2001 Feb 23;104(4):557-67 PMID 11239412; Cites Annu Rev Cell Dev Biol.

1998;14:167-96 PMID 9891782; Cites Cell. 2001 Jun 29;105(7):851-62 PMID 11439182; Cites Trends Biochem Sci. 2002 Jan;27(1):40-7 PMID 11796223; Cites J Biol Chem. 2002 Jul 5;277(27):24453-9 PMID 11983702; Cites Cell. 2002 Jul 26;110(2):153-62 PMID 12150924; Cites Nat Med. 2002 Nov;8(11):1303-9 PMID 12379849; Cites Proc Natl Acad Sci U S A. 2002 Nov 12;99(23):14855-60 PMID 12397177; Cites Cell. 2002 Dec 27;111(7):943-55 PMID 12507422; Cites Annu Rev Physiol. 2003;65:45-79 PMID 12524460; Cites Cell. 2003 May 2;113(3):329-42 PMID 12732141; Cites Proc Natl Acad Sci U S A. 2003 Jun 10;100(12):7129-34 PMID 12756293; Cites J Mol Cell Cardiol. 2003 Jun;35(6):577-93 PMID 12788374; Cites Proc Natl Acad Sci U S A. 2003 Aug 5;100(16):9366-70 PMID 12867591; Cites Circulation. 2003 Jul 29;108(4):407-13 PMID 12874181; Cites Mol Cell Biol. 2003 Sep;23(18):6597-608 PMID 12944485; Cites J Cell Biol. 1992 Dec;119(6):1649-56 PMID 1334962; Cites Genes Dev. 2006 Jun 15;20(12):1545-56 PMID 16778073; Cites Mol Cell Biol. 1989 Nov;9(11):5022-33 PMID 2601707; Cites Circ Res. 1995 Dec;77(6):1060-9 PMID 7586217; Cites Mol Cell Biol. 1995 Apr;15(4):1870-8 PMID 7891680; Cites EMBO J. 2000 May 2;19(9):1963-73 PMID 10790363; Cites J Clin Invest. 2000 May;105(10):1395-406 PMID 10811847; Cites Circulation. 2000 Jun 20;101(24):2863-9 PMID 10859294; Cites Mol Cell Biol. 2001 Feb;21(4):1036-44 PMID 11158291; Cites Mol Cell Biol. 2001 Mar;21(6):2085-97 PMID 11238943; Cites Mol Cell Biol. 2004 Feb;24(3):1081-95 PMID 14729955; Cites Mol Cell Biol. 2004 Jun;24(12):5281-9 PMID 15169892; Cites Nat Med. 2004 Dec;10(12):1336-43 PMID 15543153; Cites Proc Natl Acad Sci U S A. 2004 Dec 7;101(49):17132-7 PMID 15569937; Cites Mol Cell. 2004 Dec 22;16(6):867-80 PMID 15610731; Cites Mol Cell Biol. 2005 Apr;25(8):3173-81 PMID 15798203; Cites Science. 2005 Jun 10;308(5728):1599-603 PMID 15802564; Cites J Mol Cell Cardiol. 2005 Sep;39(3):479-89 PMID 15950986; Cites Genes Dev. 2005 Sep 1;19(17):2066-77 PMID 16140986; Cites Circulation. 2005 Nov 8;112(19):2930-9 PMID 16260633; Cites Heart Fail Rev. 2005 Sep;10(3):211-23 PMID 16416044; Cites J Biol Chem. 2006 Apr 7;281(14):9152-62 PMID 16469744; Cites Circ Res. 2006 Apr 28;98(8):1089-97 PMID 16556869; Cites Mol Cell Biol. 1994 Jan;14(1):686-99 PMID 8264638; Cites Science. 1997 May 30;276(5317):1404-7 PMID 9162005; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Am J Physiol Heart Circ Physiol. 2001 Apr;280(4):H1782-92 PMID 11247792

Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM

Other Citation Owner: NLM

Record type: MEDLINE; Completed

Cytoskeletal proteins have been implicated in the pathogenesis of %%%cardiomyopathy%%% , but how the cytoskeleton influences the transcriptional alterations associated with adverse cardiac remodeling remains unclear. Striated muscle activator of Rho signaling (STARS) is a muscle-specific actin-binding protein localized to the Z disc that activates serum response factor-dependent (SRF-dependent) transcription by inducing %%%nuclear%%% translocation of the myocardin-related SRF coactivators MRTF-A and -B. We show that STARS expression is upregulated in mouse models of cardiac %%%hypertrophy%%% and in failing human hearts. A conserved region of the STARS promoter containing an essential binding site for myocyte enhancer factor-2 (MEF2), a stress-responsive transcriptional activator, mediates cardiac expression of STARS, which in turn activates SRF target genes. Forced overexpression of STARS in the heart sensitizes the heart to pressure overload and %%%calcineurin%%% signaling, resulting

in exaggerated deterioration in cardiac function in response to these %%%hypertrophic%%% stimuli. These findings suggest that STARS modulates the responsiveness of the heart to stress signaling by functioning as a cytoskeletal intermediary between MEF2 and SRF.

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Record Date Completed: 20070613
Date of Electronic Publication: 20070405

2/7/37   (Item 37 from file: 154)
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17862956   PMID: 17463165
Zinc inhibits doxorubicin-activated %%%calcineurin%%% %%%signal%%% transduction pathway in H9c2 embryonic rat cardiac cells.
Merten Kevyn E; Jiang Youchun; Kang Y James
Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40202, USA.
Experimental biology and medicine (Maywood, N.J.) (United States) May 2007, 232 (5) p682-9, ISSN 1535-3702--Print 1535-3699--Linking
Journal Code: 100973463
Contract/Grant No.: ES011564; ES; NIEHS NIH HHS United States; HL59225; HL; NHLBI NIH HHS United States; HL63760; HL; NHLBI NIH HHS United States
Publishing Model Print
Document type: Journal Article; Research Support, N.I.H., Extramural
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Elevation of the zinc-binding protein metallothionein (MT) in the heart inhibits doxorubicin (DOX)-induced myocardial apoptosis and heart %%%hypertrophy%%%. Zinc release from MT in response to oxidative stress has been suggested as a mechanism of action of MT protection from DOX toxicity, and %%%calcineurin%%% is involved in the signaling pathways leading to myocardial apoptosis and heart %%%hypertrophy%%%. The present study was undertaken to determine if zinc can modulate the DOX-activated %%%calcineurin%%% signaling pathway. H9c2 cells were treated with 1 muM DOX, and zinc release was monitored by a zinc ion-specific fluorophore, zinquin ethyl ester. Additionally, DOX-activated %%%calcineurin%%% signaling was detected by a %%%calcineurin%%%-dependent %%%nuclear%%% factor of activated T-cell reporter. DOX treatment induced an increase in intracellular labile zinc and activated %%%calcineurin%%% signaling. Pretreatment of H9c2 cells with a zinc-specific, membrane-permeable chelating agent, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), inhibited the increase in intracellular labile zinc and increased the DOX-activated %%%calcineurin%%% signaling. Pretreatment of H9c2 cells with exogenously added zinc attenuated the DOX-activated %%%calcineurin%%% signaling in a dose-dependent manner. However, neither TPEN nor addition of exogenous zinc affected DOX-induced cellular %%%hypertrophy%%% or DOX-induced decrease in cell viability. Additionally, inhibition of DOX-induced %%%calcineurin%%% signaling with the %%%calcineurin%%% inhibitors cyclosporine A or tacrolimus (FK506) failed to restrict the DOX-induced decrease in cell viability. These results indicate that zinc suppresses DOX-induced %%%calcineurin%%% signaling in H9c2 cells; however, %%%calcineurin%%% signaling is not involved in the DOX-induced decrease in cell viability in H9c2 cells. (It had been shown previously that %%%calcineurin%%% is also not necessary for DOX-induced H9c2 cell

%%%hypertrophy.)%%%.
Record Date Created: 20070427
Record Date Completed: 20070611

2/7/38 (Item 38 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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17796692 PMID: 17266062
Long-term activation of adenosine monophosphate-activated protein kinase attenuates pressure-overload-induced cardiac %%%hypertrophy%%%.
Li Hong-Liang; Yin Ran; Chen Dandan; Liu Dan; Wang Dong; Yang Qinglin; Dong Yu-Gang
National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College Beijing, 100005, PR China.
Journal of cellular biochemistry (United States) Apr 1 2007, 100 (5) p1086-99, ISSN 0730-2312--Print 0730-2312--Linking Journal Code: 8205768
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Recent in vitro studies suggest that adenosine monophosphate (AMP)-activated protein kinase (AMPK) exerts inhibitory effects on cardiac %%%hypertrophy%%% . However, it is unclear whether long-term activation of AMPK will affect cardiac %%%hypertrophy%%% in vivo. In these reports, we investigate the in vivo effects of long-term AMPK activation on cardiac %%%hypertrophy%%% and the related molecular mechanisms. To examine the effects of AMPK activation in the development of pressure overload-induced cardiac %%%hypertrophy%%% , we administered 5-aminoimidazole 1 carboxamide ribonucleoside (AICAR, 0.5 mg/g body wt), a specific activator of AMPK, to rats with transaortic constriction (TAC) for 7 weeks. We found that long-term AMPK activation attenuated cardiac %%%hypertrophy%%% , and improved cardiac function in rats subjected to TAC. Furthermore, long-term AMPK activation attenuated protein synthesis, diminished %%%calcineurin%%%-%%%nuclear%%% factor of activated T cells (NFAT) and %%%nuclear%%% factor kappaB (NF-kappaB) signaling in pressure overload-induced %%%hypertrophic%%% hearts. Our in vitro experiments further proved that activation of AMPK by infection of AdAMPK blocked cardiac %%%hypertrophy%%% and NFAT, NF-kappaB, and MAPK %%%signal%%% pathways. The present study demonstrates for the first time that pharmacological activation of AMPK inhibits cardiac %%%hypertrophy%%% in through blocking signaling transduction pathways that are involved in cardiac growth. It presents a potential therapy strategy to inhibit pathological cardiac %%%hypertrophy%%% by increasing the activity of AMPK. c 2007 Wiley-Liss, Inc.
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17579277 PMID: 17099778 Record Identifier: PMC1635163
TRPC6 fulfills a %%%calcineurin%%% signaling circuit during pathologic cardiac remodeling.
Kuwahara Koichiro; Wang Yanggan; McAnally John; Richardson James A; Bassel-Duby Rhonda; Hill Joseph A; Olson Eric N
Department of Molecular Biology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390-9148, USA.
Journal of clinical investigation (United States) Dec 2006, 116 (12) p3114-26, ISSN 0021-9738--Print 0021-9738--Linking Journal Code: 7802877
Publishing Model Print-Electronic; Cites Proc Natl Acad Sci U S A. 2002 Jan 22;99(2):907-12 PMID 11782539; Cites J Biol Chem. 2002 Mar 22;277(12):10251-5 PMID 11786544; Cites Am J Physiol Cell Physiol. 2002 Feb;282(2):C347-59 PMID 11788346; Cites J Biol Chem. 2002 Apr 19;277(16):14266-73 PMID 11827959; Cites Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4586-91 PMID 11904392; Cites Physiol Rev. 2002 Apr;82(2):429-72 PMI D 11917094; Cites Cell Calcium. 2002 Feb;31(2):89-96 PMID 11969249; Cites Cell. 2002 Apr;109 Suppl:S67-79 PMID 11983154; Cites J Biol Chem. 2003 Oct 3;278(40):39014-9 PMID 12857742; Cites Mol Cell Biol. 2003 Aug;23(15):5143-64 PMID 12861002; Cites J Biol Chem. 1999 Apr 30;274(18):12811-8 PMID 10212267; Cites Am J Physiol Heart Circ Physiol. 2000 Feb;278(2):H412-9 PMID 10666070; Cites Circ Res. 2000 Mar 3;86(4):386-90 PMID 10700442; Cites J Biol Chem. 2000 Mar 24;275(12):8719-25 PMID 10722714; Cites J Clin Invest. 2000 Apr;105(7):875-86 PMID 10749567; Cites J Mol Med. 2000;78(1):14-25 PMID 10759026; Cites Circulation. 2000 Jun 20;101(24):2863-9 PMID 10859294; Cites J Immunol. 2000 Jul 1;165(1):297-305 PMID 10861065; Cites Genes Dev. 2003 Aug 15;17(16):1937-56 PMID 12893779; Cites Circ Res. 2004 Jan 9;94(1):110-8 PMID 14656927; Cites Circ Res. 2004 Feb 20;94(3):e18-26 PMID 14739160; Cites J Card Fail. 2003 Dec;9(6):469-74 PMID 14966788; Cites Lancet. 2004 Jun 5;363(9424):1881-91 PMID 15183628; Cites Proc Natl Acad Sci U S A. 2004 Jun 15;101(24):8969-74 PMID 15184684; Cites Proc Natl Acad Sci U S A. 2004 Jun 22;101(25):9387-92 PMID 15199180; Cites Am J Respir Crit Care Med. 2004 Nov 15;170(10):1101-7 PMID 15317671; Cites Biochem Biophys Res Commun. 2004 Oct 1;322(4):1178-91 PMID 15336966; Cites Nat Med. 2004 Dec;10(12):1336-43 PMID 15543153; Cites J Am Coll Cardiol. 2004 Dec 21;44(12):2390-7 PMID 15607403; Cites Annu Rev Physiol. 2005;67:69-98 PMID 15709953; Cites Sci STKE. 2005 Feb 22;2005(272):re3 PMID 15728426; Cites J Clin Invest. 2005 Mar;115(3):527-37 PMID 15765134; Cites Circulation. 2005 May 10;111(18):2339-46 PMID 15867177; Cites Mol Cell Biol. 2005 Aug;25(15):6629-38 PMID 16024798; Cites Mol Cell Biol. 2005 Aug;25(16):6980-9 PMID 16055711; Cites Annu Rev Genomics Hum Genet. 2005;6:185-216 PMID 16124859; Cites J Biol Chem. 2005 Dec 2;280(48):39786-94 PMID 16204251; Cites J Physiol. 2006 Apr 15;572(Pt 2):359-77 PMID 16439426; Cites J Clin Invest. 2006 Mar;116(3):675-82 PMID 16511602; Cites Nature. 2006 May 11;441(7090):179-85 PMID 16582901; Cites Circulation. 2006 Apr 18;113(15):1849-56 PMID 16618832; Cites Nat Cell Biol. 2006 Jul;8(7):771-3 PMID 16733527; Cites J Biol Chem. 2006 Jul 28;281(30):20661-5 PMID 16766533; Cites J Biol Chem. 2006 Aug 25;281(34):24979-90 PMID 16807233; Cites FASEB J. 2006 Aug;20(10):1660-70 P MID 16873889; Cites Nat Cell Biol. 2006 Sep;8(9):1003-10 PMID 16906149; Cites Nature. 2006 Sep 14;443(7108):230-3 PMID 16921383; Cites Nature. 2006 Sep 14;443(7108):226-9 PMID 16921385; Cites Biochem Biophys Res Commun. 1997 Oct 9;239(1):279-83 PMID 9345310; Cites J Clin Invest. 1997 Nov 1;100(9):2362-70 PMID 9410916; Cites J Clin Invest. 1998 Jan 1;101(1):51-61 PMID 9421465; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites J Mol Cell Cardiol. 1998 Nov;30(11):2269-80 PMID 9925364;

Cites Nature. 1999 Jan 21;397(6716):259-63 PMID 9930701; Cites Mol Cell Biol. 2000 Sep;20(17):6600-11 PMID 10938134; Cites J Mol Cell Cardiol. 2000 Sep;32(9):1595-607 PMID 10966823; Cites Microsc Res Tech. 2000 Sep 15;50(6):522-31 PMID 10998641; Cites N Engl J Med. 2000 Dec 7;343(23):1688-96 PMID 11106718; Cites Circ Res. 2000 Dec 8;87(12):E61-8 PMID 11110780; Cites Circ Res. 2001 Feb 16;88(3):325-32 PMID 11179201; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3322-7 PMID 11248077; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3328-33 PMID 11248078; Cites Proc Natl Acad Sci U S A. 2002 May 28;99(11):7461-6 PMID 12032305; Cites Mol Cell Biol. 2002 Nov;22(21):7603-13 PMID 12370307; Cites Am J Physiol Cell Physiol. 2002 Dec;283(6):C1761-75 PMID 12388056; Cites Mol Med. 2002 Nov;8(11):750-60 PMID 12520092; Cites Annu Rev Physiol. 2003;65:45-79 PMID 12524460; Cites Am J Physiol Cell Physiol. 2003 Feb;284(2):C316-30 PMID 12529250; Cites Brain Res Mol Brain Res. 2002 Dec 30;109(1-2):95-104 PMID 12531519; Cites J Mol Cell Cardiol. 2003 May;35(5):461-71 PMID 12738228; Cites Nat Immunol. 2001 Apr;2(4):316-24 PMID 11276202; Cites Proc Natl Acad Sci U S A. 2001 Nov 6;98(23):13108-13 PMID 11606756

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The heart responds to injury and chronic pressure overload by pathologic growth and remodeling, which frequently result in heart failure and sudden death. Calcium-dependent signaling pathways promote cardiac growth and associated changes in gene expression in response to stress. The calcium/calmodulin-dependent phosphatase %%%calcineurin%%%, which signals to %%%nuclear%%% factor of activated T cells (NFAT) transcription factors, serves as a transducer of calcium signals and is sufficient and necessary for pathologic cardiac %%%hypertrophy%%% and remodeling. Transient receptor potential (TRP) proteins regulate cation entry into cells in response to a variety of signals, and in skeletal muscle, expression of TRP cation channel, subfamily C, member 3 (TRPC3) is increased in response to neurostimulation and %%%calcineurin%%% signaling. Here we show that TRPC6 was upregulated in mouse hearts in response to activated %%%calcineurin%%% and pressure overload, as well as in failing human hearts. Two conserved NFAT consensus sites in the promoter of the TRPC6 gene conferred responsiveness to cardiac stress. Cardiac-specific overexpression of TRPC6 in transgenic mice resulted in heightened sensitivity to stress, a propensity for lethal cardiac growth and heart failure, and an increase in NFAT-dependent expression of beta-myosin heavy chain, a sensitive marker for pathologic %%%hypertrophy%%% . These findings implicate TRPC6 as a positive regulator of %%%calcineurin%%%-NFAT signaling and a key component of a calcium-dependent regulatory loop that drives pathologic cardiac remodeling.

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17519179 PMID: 16950785

Canonical transient receptor potential channels promote cardiomyocyte %%%hypertrophy%%% through activation of %%%calcineurin%%% signaling.
Bush Erik W; Hood David B; Papst Philip J; Chapo Joseph A; Minobe Wayne; Bristow Michael R; Olson Eric N; McKinsey Timothy A
Myogen, Inc., Westminster, Colorado 80021, USA. erik.bush@myogen.com
Journal of biological chemistry (United States) Nov 3 2006, 281 (44) p33487-96, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
The calcium/calmodulin-dependent phosphatase %%%calcineurin%%% plays a central role in the control of cardiomyocyte %%%hypertrophy%%% in response to pathological stimuli. Although %%%calcineurin%%% is present at high levels in normal heart, its activity appears to be unaffected by calcium during the course of a cardiac cycle. The mechanism(s) whereby %%%calcineurin%%% is selectively activated by calcium under pathological conditions has remained unclear. Here, we demonstrate that diverse signals for cardiac %%%hypertrophy%%% stimulate expression of canonical transient receptor potential (TRPC) channels. TRPC consists of a family of seven membrane-spanning nonselective cation channels that have been implicated in the nonvoltage-gated influx of calcium in response to G protein-coupled receptor signaling, receptor tyrosine kinase signaling, and depletion of internal calcium stores. TRPC3 expression is up-regulated in multiple rodent models of pathological cardiac %%%hypertrophy%%%, whereas TRPC5 expression is induced in failing human heart. We demonstrate that TRPC promotes cardiomyocyte %%%hypertrophy%%% through activation of %%%calcineurin%%% and its downstream effector, the %%%nuclear%%% factor of activated T cells transcription factor. These results define a novel role for TRPC channels in the control of cardiac growth, and suggest that a TRPC-derived pool of calcium contributes to selective activation of %%%calcineurin%%% in diseased heart.
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Date of Electronic Publication: 20060901

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17506287 PMID: 17046757
Switching feedback mechanisms realize the dual role of MCIP in the regulation of %%%calcineurin%%% activity.
Shin Sung-Young; Choo Sang-Mok; Kim Dongsan; Baek Song Joon; Wolkenhauer Olaf; Cho Kwang-Hyun
School of Electrical Engineering, University of Ulsan, Ulsan 680-749, Republic of Korea.
FEBS letters (Netherlands) Oct 30 2006, 580 (25) p5965-73, ISSN 0014-5793--Print 0014-5793--Linking Journal Code: 0155157
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Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
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Record type: MEDLINE; Completed
%%%Calcineurin%%% (CaN) assists T-cell activation, growth and differentiation of skeletal and cardiac myocytes, memory, and apoptosis. It also activates transcription of the %%%nuclear%%% factor of activated T-cells (NFAT) family including %%%hypertrophic%%% target genes. It has been reported that the modulatory %%%calcineurin%%%-interacting protein (MCIP) inhibits the CaN activity and thereby reduces the %%%hypertrophic%%% response. However, it has been shown that MCIP facilitates or permits the %%%hypertrophic%%% response under some stress conditions such as isoproterenol infusion or pressure overload by transverse aortic constriction. As there is no direct experimental evidence that can explain these paradoxical phenomena, there has been a controversy concerning the functional role of MCIP in developing the %%%hypertrophic%%% response. It is therefore crucial to establish a hypothesis that can clearly explain these phenomena. Towards this end, we propose in this paper a hypothesis that is based on available experimental evidence as well as mathematical modeling and computer simulations. We hypothesize that there is a threshold in the %%%nuclear%%% NFAT concentration above which MCIP is switched on. Below this threshold, the inhibition of active CaN by MCIP is negligible, while the activated protein kinase increases the dissociation rate of the CaN/MCIP complex. This leads to an augmentation of active CaN. This mechanism realizes the positive effect (i.e., removing any negative feedback) of MCIP in the %%%hypertrophic%%% response. On the other hand, the over-expression of active CaN increases %%%nuclear%%% NFAT to values above the threshold, while CaN is inhibited through binding of MCIP (expressed by the %%%nuclear%%% NFAT). This mechanism realizes the introduction of a negative feedback mechanism. To unravel this switching feedback mechanism, we have developed a mathematical model for which computer simulations are in agreement with the existing experimental data. The simulations demonstrate how the apparently paradoxical behavior can emerge as a result of cellular conditions.
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Date of Electronic Publication: 20061005

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17442819 PMID: 16931796
Inhibition of %%%nuclear%%% import of %%%calcineurin%%% prevents myocardial %%%hypertrophy%%%.
Hallhuber Matthias; Burkard Natalie; Wu Rongxue; Buch Mamta H; Engelhardt Stefan; Hein Lutz; Neyses Ludwig; Schuh Kai; Ritter Oliver
Department of Medicine I, DFG-Research Center for Experimental Biomedicine, University of Wuerzburg, Germany.
Circulation research (United States) Sep 15 2006, 99 (6) p626-35, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
The time that transcription factors remain %%%nuclear%%% is a major determinant for transcriptional activity. It has recently been demonstrated that the phosphatase %%%calcineurin%%% is translocated to the %%%nucleus%%%

with the transcription factor %%%nuclear%%% factor of activated T cells (NF-AT). This study identifies a %%%nuclear%%% localization sequence (NLS) and a %%%nuclear%%% export %%%signal%%% (NES) in the sequence of %%%calcineurin%%%. Furthermore we identified the %%%nuclear%%% cargo protein importinbeta(1) to be responsible for %%%nuclear%%% translocation of %%%calcineurin%%%. Inhibition of the %%%calcineurin%%% /importin interaction by a competitive peptide (KQECKIKYSERV), which mimicked the %%%calcineurin%%% NLS, prevented %%%nuclear%%% entry of %%%calcineurin%%%. A noninhibitory control peptide did not interfere with the %%%calcineurin%%% /importin binding. Using this approach, we were able to prevent the development of myocardial %%%hypertrophy%%%. In angiotensin II-stimulated cardiomyocytes, [(3)H]-leucine incorporation (159%+/-9 versus 111%+/-11; P<0.01) and cell size were suppressed significantly by the NLS peptide compared with a control peptide. The NLS peptide inhibited %%%calcineurin%%% /NF-AT transcriptional activity (227%+/-11 versus 133%+/-8; P<0.01), whereas %%%calcineurin%%% phosphatase activity was unaffected (298%+/-9 versus 270%+/-11; P=NS). We conclude that %%%calcineurin%%% is not only capable of dephosphorylating NF-AT, thus enabling its %%%nuclear%%% import, but the presence of %%%calcineurin%%% in the %%%nucleus%%% is also important for full NF-AT transcriptional activity.
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Date of Electronic Publication: 20060824

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17379895 PMID: 16828070
%%%Calcineurin%%% regulates NFAT-dependent iNOS expression and protection of cardiomyocytes: co-operation with Src tyrosine kinase.
Obasanjo-Blackshire Kofo; Mesquita Rui; Jabr Rita I; Molkentin Jeffery D; Hart Stephen L; Marber Michael S; Xia Yang; Heads Richard J
Cardiovascular Division, King's College London School of Medicine, Department of Cardiology, The Rayne Institute, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, UK.
Cardiovascular research (Netherlands) Sep 1 2006, 71 (4) p672-83, ISSN 0008-6363--Print 0008-6363--Linking Journal Code: 0077427
Publishing Model Print-Electronic; Comment in Cardiovasc Res. 2006 Sep 1;71(4):612-4 PMID 16876148
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
OBJECTIVE: To determine the role of %%%calcineurin%%% and Src tyrosine kinase in the regulation of inducible nitric oxide synthase (iNOS) expression and protection in cardiomyocytes. METHODS: iNOS expression was studied in isolated neonatal rat ventricular myocyte cultures in response to bacterial lipopolysaccharide (LPS) or following transfection with constitutively active %%%calcineurin%%% or Src and in hearts isolated from wild-type or calcineruin Abeta knockout mice. Cell injury in response to simulated ischemia-reperfusion was studied following overexpression of active %%%calcineurin%%%. Regulation of the iNOS gene promoter by %%%calcineurin%%% was studied using promoter-luciferase reporter and chromatin immunoprecipitation assays. RESULTS: Overexpression of

constitutively active Src co-operated with [Ca2+]c elevation to induce iNOS expression, and LPS-induced iNOS expression was abrogated by pharmacological inhibition of %%%calcineurin%%% or tyrosine kinase. LPS also induced tyrosine kinase-dependent but %%%calcineurin%%%-independent phosphorylation of Src Tyr418. LPS induced myocardial iNOS expression in wild-type but not %%%calcineurin%%% Abeta knockout mice. Overexpression of constitutively active calcinuerin in isolated cardiomyocytes caused dephosphorylation and %%%nuclear%%% accumulation of the c1 isoform of %%%nuclear%%% factor of activated T-cells (NFATc1), induced strong iNOS expression, and induced NOS-dependent protection against simulated ischemia-reperfusion prior to cardiomyocyte %%%hypertrophy%%% . Co-transfection of a mouse iNOS promoter-luciferase reporter in combination with active %%%calcineurin%%% and wild-type or dominant negative Src confirmed that constitutive activation of %%%calcineurin%%% was sufficient for transactivation. Chromatin immunoprecipitation confirmed %%%calcineurin%%% -dependent in vivo binding of NFATc1 to consensus sites within the iNOS promoter. CONCLUSIONS: These results support a cardioprotective role for %%%calcineurin%%% mediated by NFAT-dependent induction of iNOS expression and co-operativity between %%%calcineurin%%% and Src.

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17352905 PMID: 16873889 Record Identifier: NIHMS92847; PMC2693319
%%%Calcineurin%%%-dependent %%%cardiomyopathy%%% is activated by TRPC in the adult mouse heart.
Nakayama Hiroyuki; Wilkin Benjamin J; Bodi Ilona; Molkentin Jeffery D
Department of Pediatrics, University of Cincinnati, Children's Hospital Medical Center, Cincinnati, Ohio, USA.
FASEB journal - official publication of the Federation of American Societies for Experimental Biology (United States) Aug 2006, 20 (10) p1660-70, ISSN 1530-6860--Electronic 0892-6638--Linking Journal Code: 8804484
Contract/Grant No.: P01 HL069779-06A10003; HL; NHLBI NIH HHS United States; P50 HL077101-050004; HL; NHLBI NIH HHS United States; R01 HL060562-11; HL; NHLBI NIH HHS United States; R01 HL062927-10A1; HL; NHLBI NIH HHS United States; R01 HL081104-04; HL; NHLBI NIH HHS United States; Howard Hughes Medical Institute United States
Publishing Model Print; Cites Cell Physiol Biochem. 1999;9(4-5):270-83 PM ID 10575202; Cites J Biol Chem. 2003 Oct 10;278(41):40088-96 PMID 12874277; Cites Cell. 2002 Apr;109 Suppl:S67-79 PMID 11983154; Cites Cell Calcium. 2002 Feb;31(2):89-96 PMID 11969249; Cites Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4586-91 PMID 11904392; Cites J Cell Biol. 2002 Mar 4;156(5):771-4 PMID 11877454; Cites J Biol Chem. 2002 Apr 19;277(16):14266-73 PMID 11827959; Cites Cardiovasc Res. 2002 Feb 1;53(2):372-81 PMID 11827688; Cites J Cell Biol. 2001 Oct 1;155(1):27-39 PMID 11581284; Cites Curr Opin Genet Dev. 2001 Oct;11(5):505-12 PMID 11532391; Cites Nat Immunol. 2001 Apr;2(4):316-24 PMI D 11276202; Cites Circ Res. 2000 Dec 8;87(12):1087-94 PMID 11110764; Cites Nat Immunol. 2000 Nov;1(5):402-12 PMID 11062500; Cites J Biol Chem. 2000 Dec 15;275(50):39055-60 PMID 10984475; Cites J Biol Chem. 2000 May

5;275(18):13571-9 PMID 10788473; Cites Genes Dev. 2003 Sep 15;17(18):2205-32 PMID 12975316; Cites Am J Physiol Heart Circ Physiol. 2004 Mar;286(3):H1124-32 PMID 14630640; Cites Nature. 2003 Dec 4;426(6966):517-24 PMID 14654832; Cites Circ Res. 1999 Feb 5;84(2):210-9 PMID 9933253; Cites J Biol Chem. 1998 May 29;273(22):13367-70 PMID 9593662; Cites Nature. 1998 Apr 30;392(6679):933-6 PMID 9582075; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Biochem Biophys Res Commun. 1997 Oct 9;239(1):279-83 PMID 9345310; Cites Nature. 1997 Apr 24;386(6627):855-8 PMID 9126747; Cites Nature. 1996 Oct 31;383(6603):837-40 PMID 8893011; Cites Science. 1985 Nov 29;230(4729):1040-3 PMID 3933112; Cites Physiol Rev. 2005 Apr;85(2):757-810 PMID 15788710; Cites J Cell Biol. 2005 Mar 14;168(6):887-97 PMID 15767461; Cites Proc Natl Acad Sci U S A. 2005 Feb 22;102(8):3099-104 PMID 15710901; Cites Proc Natl Acad Sci U S A. 2004 Jun 22;101(25):9387-92 PMID 15199180; Cites J Biol Chem. 2004 Apr 9;279(15):15524-30 PMID 14749328; Cites Circ Res. 2004 Jan 9;94(1):110-8 PMID 14656927

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The manner in which Ca2+-sensitive signaling proteins are activated in contracting cardiomyocytes is an intriguing theoretical problem given that the cytoplasm is continually bathed with systolic Ca2+ concentrations that should maximally activate most Ca2+-sensitive signaling kinases and phosphatases. Store-operated Ca2+ entry, partially attributed to transient receptor potential (TRP) proteins, can mediate activation of the Ca2+-sensitive phosphatase %%%calcineurin%%% in nonexcitable cells. Here we investigated the gain-of-function phenotype associated with TRPC3 expression in the mouse heart using transgenesis to examine the potential role of store-operated Ca2+ entry in regulating cardiac %%%calcineurin%%% activation and ensuing %%%hypertrophy%%%/myopathy. Adult myocytes isolated from TRPC3 transgenic mice showed abundant store-operated Ca2+ entry that was inhibited with SKF96365 but not verapamil or KB-R7943. Associated with this induction in store-operated Ca2+ entry, TRPC3 transgenic mice showed increased %%%calcineurin%%%-%%%nuclear%%% factor of activated T cells (NFAT) activation in vivo, %%%cardiomyopathy%%%, and increased %%%hypertrophy%%% after neuroendocrine agonist or pressure overload stimulation. The %%%cardiomyopathic%%% phenotype and increased %%%hypertrophy%%% after pressure overload stimulation were blocked by targeted disruption of the %%%calcineurin%%% Abeta gene. Thus, enhanced store-operated Ca2+ entry in the heart can regulate %%%calcineurin%%%-NFAT signaling in vivo, which could secondarily impact the %%%hypertrophic%%% response and %%%cardiomyopathy%%%.

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17345594 PMID: 16847152
MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in %%%calcineurin%%%-induced heart failure.

van Oort Ralph J; van Rooij Eva; Bourajjaj Meriem; Schimmel Joost; Jansen Maurits A; van der Nagel Roel; Doevendans Pieter A; Schneider Michael D; van Echteld Cees J A; De Windt Leon J
Hubrecht Laboratory, and Interuniversity Cardiology Institute Netherlands, Royal Netherlands Academy of Sciences, Utrecht, Netherlands.
Circulation (United States) Jul 25 2006, 114 (4) p298-308, ISSN 1524-4539--Electronic 0009-7322--Linking Journal Code: 0147763
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Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
BACKGROUND: %%%Hypertrophic%%% growth, a risk factor for mortality in heart disease, is driven by reprogramming of cardiac gene expression. Although the transcription factor myocyte enhancer factor-2 (MEF2) is a common end point for several %%%hypertrophic%%% pathways, its precise cardiac gene targets and function in cardiac remodeling remain to be elucidated. METHODS AND RESULTS: We report the existence of synergistic interactions between the %%%nuclear%%% factor of activated T cells and MEF2 transcription factors triggered by %%%calcineurin%%% signaling. To circumvent the embryonic lethality and mitochondrial deficiency associated with germ-line null mutations for MEF2C and MEF2A respectively, we used conditional transgenesis to express a dominant-negative form of MEF2 in the murine postnatal heart and combined this with magnetic resonance imaging to assess MEF2 transcriptional function in Ca2+/%%%calcineurin%%%-induced cardiac remodeling. Surprisingly, end-diastolic and end-systolic ventricular dimensions and contractility were normalized in the presence of severely %%%hypertrophied%%% left ventricular walls on MEF2 inhibition in %%%calcineurin%%% transgenic mice. In line, we generated lines of transgenic mice expressing MEF2A in the heart, which displayed primarily chamber dilation. Microarray profiling indicated that MEF2 promotes a gene profile functioning primarily to or at the %%%nucleus%%%, cytoskeletal and microtubular networks, and mitochondria. CONCLUSIONS: These findings assign a novel function to MEF2 transcription factors in the postnatal heart, where they activate a genetic program that minimally affects cardiac growth yet promotes chamber dilation, mechanical dysfunction, and dilated %%%cardiomyopathy%%%.
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17283587 PMID: 16460834
The mAKAP signaling complex: integration of cAMP, calcium, and MAP kinase signaling pathways.
Dodge-Kafka Kimberly L; Kapiloff Michael S
Pat and Jim Calhoun Center for Cardiology, University of Connecticut Health Center, 263 Farmington Ave, Farmington, CT 06030, USA. dodge@uchc.edu
European journal of cell biology (Germany) Jul 2006, 85 (7) p593-602 , ISSN 0171-9335--Print 0171-9335--Linking Journal Code: 7906240
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Following its production by adenylyl cyclases, the second messenger cAMP is in involved in pleiotrophic %%%signal%%% transduction. The effectors of cAMP include the cAMP-dependent protein kinase (PKA), the guanine %%%nucleotide%%% exchange factor Epac (exchange protein activated by cAMP), and cAMP-dependent ion channels. In turn, cAMP signaling is attenuated by phosphodiesterase-catalyzed degradation. The association of cAMP effectors and the enzymes that regulate cAMP concentration into signaling complexes helps to explain the differential signaling initiated by members of the G(s)-protein coupled receptor family. The %%%signal%%% transduction complex formed by the scaffold protein mAKAP (muscle A kinase-anchoring protein) at the %%%nuclear%%% envelope of both striated myocytes and neurons contains three cAMP-binding proteins, PKA, Epac1, and the phosphodiesterase PDE4D3. In addition, the mAKAP complex also contains components of the ERK5 MAP kinase signaling pathway, the calcium release channel ryanodine receptor and the phosphatases PP2A as well as %%%calcineurin%%%. Analysis of the mAKAP complex illustrates how a macromolecular complex can serve as a node in the intracellular signaling network of cardiac myocytes to integrate multiple cAMP signals with those of calcium and MAP kinases to regulate the %%%hypertrophic%%% actions of several hormones. (32 Refs.)

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Record Date Completed: 20060914
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2/7/47 (Item 47 from file: 154)
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17214058 PMID: 16648267 Record Identifier: PMC1464340

Modulatory %%%calcineurin%%%-interacting proteins 1 and 2 function as %%%calcineurin%%% facilitators in vivo.

Sanna Bastiano; Brandt Eric B; Kaiser Robert A; Pfluger Paul; Witt Sandy A; Kimball Thomas R; van Rooij Eva; De Windt Leon J; Rothenberg Marc E; Tschop Matthias H; Benoit Stephen C; Molkentin Jeffery D
Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati, 3333 Burnet Avenue, Cincinnati, OH 45229, USA.

Proceedings of the National Academy of Sciences of the United States of America (United States) May 9 2006, 103 (19) p7327-32, ISSN 0027-8424 --Print 0027-8424--Linking Journal Code: 7505876

Publishing Model Print-Electronic; Cites Neuron. 1999 Jul;23(3):435-47 PMID 10433257; Cites Cell. 1998 Jan 9;92(1):39-49 PMID 9489698; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites J Biol Chem. 2000 Feb 18;275(7):4545-8 PMID 10671477; Cites J Biol Chem. 2000 Mar 24;275(12):8719-25 PMID 10722714; Cites Genomics. 2000 Mar 15;64(3):252-63 PMID 10756093; Cites Circulation. 2000 May 23;101(20):2431-7 PMID 10821822; Cites Circulation. 2000 Jun 20;101(24):2863-9 PMID 10859294; Cites Hum Mol Genet. 2000 Jul 1;9(11):1681-90 PMID 10861295; Cites Genes Dev. 2000 Jul 1;14(13):1595-604 PMID 10887154; Cites EMBO J. 2000 Jul 17;19(14):3618-29 PMID 10899116; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3328-33 PMID 11248078; Cites Cell. 2001 Nov 30;107(5):617-29 PMID 11733061; Cites J Biol Chem. 2002 Mar 22;277(12):10251-5 PMID 11786544; Cites Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4586-91 PMID 11904392;

Cites FASEB J. 2002 Jun;16(8):814-24 PMID 12039863; Cites Proc Natl Acad Sci U S A. 2002 Jul 9;99(14):9398-403 PMID 12091710; Cites Arch Biochem Biophys. 2002 Aug 1;404(1):71-9 PMID 12127071; Cites Proc Natl Acad Sci U S A. 2003 Jan 21;100(2):669-74 PMID 12515860; Cites J Mol Biol. 2003 Apr 18;328(1):147-56 PMID 12684004; Cites Mol Cell Biol. 2003 Jun;23(12):4331-43 PMID 12773574; Cites Biochem J. 2003 Sep 1;374(Pt 2):567-75 PMID 12809556; Cites Proc Natl Acad Sci U S A. 2003 Jul 22;100(15):8987-92 PMID 12851457; Cites Nat Immunol. 2003 Sep;4(9):874-81 PMID 12925851; Cites Genes Dev. 2003 Sep 15;17(18):2205-32 PMID 12975316; Cites Circ Res. 2004 Jan 9;94(1):91-9 PMID 14615291; Cites Biochem Biophys Res Commun. 2003 Nov 28;311(4):1089-93 PMID 14623294; Cites Biochem Biophys Res Commun. 2003 Nov 28;311(4):1195-208 PMID 14623305; Cites Circ Res. 2004 Jan 9;94(1):110-8 PMID 14656927; Cites Genes Dev. 2004 Jan 1;18(1):35-47 PMID 14701880; Cites J Biol Chem. 2004 Apr 9;279(15):15524-30 PMID 14749328; Cites Blood. 2004 Jul 1;104(1):149-58 PMID 15016650; Cites Immunity. 2004 Mar;20(3):255-66 PMID 15030770; Cites J Biol Chem. 2004 Jun 18;279(25):26192-200 PMID 15082723; Cites Endocrinology. 2004 Oct;145(10):4645-52 PMID 15231700; Cites J Vasc Res. 2004 Jul-Aug;41(4):334-44 PMID 15263820; Cites J Mol Biol. 2004 Oct 1;342(5):1659-74 PMID 15364589; Cites J Biol Chem. 2004 Nov 26;279(48):50537-54 PMID 15448146; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites J Biol Chem. 1998 May 29;273(22):13367-70 PMID 9593662; Cites Genes Dev. 1998 Aug 1;12(15):2424-33 PMID 9694806; Cites Genes Dev. 1998 Aug 15;12(16):2499-509 PMID 9716403; Cites Arch Biochem Biophys. 2005 Jul 1;439(1):121-8 PMID 15935327; Cites Proc Natl Acad Sci U S A. 2005 Sep 13;102(37):13075-80 PMID 16131541; Cites Eukaryot Cell. 2005 Sep;4(9):1526-38 PMID 16151246; Cites Science. 1995 Jan 6;267(5194):108-11 PMID 7528941; Cites Mol Cell Biol. 1995 Nov;15(11):6064-74 PMID 7565759; Cites Science. 1996 Mar 15;271(5255):1589-92 PMID 8599116; Cites Mol Cell Biol. 1999 Oct;19(10):7245-54 PMID 10490659

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Languages: ENGLISH

Main Citation Owner: NLM

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Record type: MEDLINE; Completed

The calcium-activated phosphatase %%%calcineurin%%% is regulated by a binding cofactor known as modulatory %%%calcineurin%%%-interacting protein (MCIP) in yeast up through mammals. The physiologic function of MCIP remains an area of ongoing investigation, because both positive and negative %%%calcineurin%%% regulatory effects have been reported. Here we disrupted the mcip1 and mcip2 genes in the mouse and provide multiple lines of evidence that endogenous MCIP functions as a %%%calcineurin%%% facilitator in vivo. Mouse embryonic fibroblasts deficient in both mcip1/2 showed impaired activation of %%%nuclear%%% factor of activated T cells (NFAT), suggesting that MCIP is required for efficient %%%calcineurin%%% -NFAT coupling. Mice deficient in mcip1/2 showed a dramatic impairment in cardiac %%%hypertrophy%%% induced by pressure overload, neuroendocrine stimulation, or exercise, similar to mice lacking %%%calcineurin%%% Abeta. Moreover, simultaneous deletion of %%%calcineurin%%% Abeta in the mcip1/2-null background did not rescue impaired %%%hypertrophic%%% growth after pressure overload. Slow/oxidative fiber-type switching in skeletal muscle after exercise stimulation was also impaired in mcip1/2 mice, similar to %%%calcineurin%%% Abeta-null mice. Moreover, CD4(+) T cells from mcip1/2-null mice showed enhanced apoptosis that was further increased by loss of %%%calcineurin%%% Abeta. Finally, mcip1/2-null mice displayed a

neurologic phenotype that was similar to %%%calcineurin%%% Abeta-null mice, such as increased locomotor activity and impaired working memory. Thus, a loss-of-function analysis suggests that MCIPs serve either a permissive or facilitative function for %%%calcineurin%%%-NFAT signaling in vivo.
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Record Date Completed: 20060719
Date of Electronic Publication: 20060428

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17196302 PMID: 16648474 Record Identifier: PMC1489013
Direct interaction and reciprocal regulation between ASK1 and %%%calcineurin%%%-NFAT control cardiomyocyte death and growth.
Liu Qinghang; Wilkins Benjamin J; Lee Yong J; Ichijo Hidenori; Molkentin Jeffery D
Department of Pediatrics, University of Cincinnati, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave., MLC7020, Cincinnati Ohio 45229-3039, USA.
Molecular and cellular biology (United States) May 2006, 26 (10) p3785-97, ISSN 0270-7306--Print 0270-7306--Linking Journal Code: 8109087
Publishing Model Print; Cites Proc Natl Acad Sci U S A. 2001 Jul 3;98(14):7783-8 PMID 11427728; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Mol Cell Biol. 2001 Nov;21(21):7460-9 PMID 11585926; Cites EMBO J. 2001 Nov 1;20(21):6028-36 PMID 11689443; Cites Circulation. 2002 Jan 29;105(4):509-15 PMID 11815436; Cites J Cell Physiol. 2002 Apr;191(1):95-104 PMID 11920685; Cites Science. 1998 Sep 18;281(5384):1860-3 PMID 9743501; Cites J Biol Chem. 1998 Oct 23;273(43):28392-8 PMID 9774466; Cites Physiol Rev. 1999 Jan;79(1):143-80 PMID 9922370; Cites Cell. 2002 Apr;109 Suppl:S67-79 PMID 11983154; Cites J Biol Chem. 2002 Jul 5;277(27):24390-8 PMID 11994297; Cites J Physiol. 2002 May 15;541(Pt 1):1-8 PMID 12015416; Cites J Biol Chem. 2002 Nov 29;277(48):46566-75 PMID 12244106; Cites Nat Cell Biol. 2003 Jan;5(1):28-37 PMID 12510191; Cites Cell Struct Funct. 2003 Feb;28(1):23-9 PMID 12655147; Cites Circ Res. 2003 Apr 18;92(7):725-31 PMID 12663489; Cites Biochem J. 2003 Aug 1;373(Pt 3):845-53 PMID 12723971; Cites J Clin Invest. 2003 May;111(10):1475-86 PMID 12750397; Cites J Biol Chem. 1999 Mar 19;274(12):8208-16 PMID 10075725; Cites Cell. 1999 Mar 5;96(5):611-4 PMID 10089876; Cites Science. 1999 Apr 9;284(5412):339-43 PMI D 10195903; Cites Curr Opin Cell Biol. 1999 Apr;11(2):211-8 PMID 10209154; Cites Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8511-5 PMID 10411906; Cites Proc Natl Acad Sci U S A. 1999 Oct 12;96(21):12016-20 PMID 10518568; Cites Mol Cell Biol. 2000 Jan;20(1):196-204 PMID 10594022; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites Circ Res. 2000 Feb 18;86(3):255-63 PMID 10679475; Cites J Biol Chem. 2000 Mar 3;275(9):6417-20 PMID 10692444; Cites J Biol Chem. 2000 May 5;275(18):13571-9 PMID 10788473; Cites J Biol Chem. 2000 Aug 25;275(34):26576-81 PMID 10849426; Cites Prostate. 2000 Jun 1;43(4):303-17 PMID 10861750; Cites J Biol Chem. 2000 Nov 3;275(44):34528-33 PMID 10931827; Cites J Physiol. 2000 Nov 15;529 Pt 1:11-21 PMID 11080247; Cites Mol Cell Biol. 2001 Feb;21(3):893-901 PMID 11154276; Cites EMBO Rep. 2001 Mar;2(3):222-8 PMID 11266364; Cites Mol Cell Biol. 2001 Jul;21(14):4818-28 PMID 11416155; Cites Circ Res. 2001 Jun 22;88(12):1239-46 PMID 11420299; Cites Am J Pathol. 2003

Jul;163(1):243-51 PMID 12819028; Cites Genes Dev. 2003 Sep 15;17(18):2205-32 PMID 12975316; Cites EMBO J. 2003 Oct 1;22(19):5079-89 PMID 14517246; Cites Circ Res. 2003 Oct 31;93(9):874-83 PMID 14551246; Cites Circ Res. 2004 Jan 9;94(1):91-9 PMID 14615291; Cites Circ Res. 2004 Jan 9;94(1):110-8 PMID 14656927; Cites Proc Natl Acad Sci U S A. 2003 Dec 23;100(26):15883-8 PMID 14665690; Cites J Biol Chem. 2004 Mar 12;279(11):10442-9 PMID 14688258; Cites Exp Cell Res. 2004 Apr 1;294(2):581-91 PMID 15023544; Cites J Biol Chem. 2004 Jun 18;279(25):26192-200 PMID 15082723; Cites Oncogene. 2004 Jun 24;23(29):5099-104 PMID 15094778; Cites Circulation. 2004 Jul 20;110(3):317-23 PMID 15249508; Cites Cardiovasc Res. 2004 Aug 15;63(3):467-75 PMID 15276472; Cites J Clin Invest. 2004 Oct;114(7):937-43 PMID 15467832; Cites Mol Cell Biol. 2005 Feb;25(3):865-78 PMID 15657416; Cites Biochem Biophys Res Commun. 2005 Jul 29;333(2):562-7 PMID 15953587; Cites J Immunol. 1995 Jun 15;154(12):6346-54 PMID 7539018; Cites J Cell Biol. 1995 Nov;131(3):735-43 PMID 7593193; Cites FEBS Lett. 1996 Oct 7;394(3):321-4 PMID 8830666; Cites Nature. 1996 Oct 3;383(6599):434-7 PMID 8837775; Cites Science. 1997 Jan 3;275(5296):90-4 PMID 8974401; Cites Biochem Biophys Res Commun. 1997 Oct 29;239(3):905-10 PMID 9367868; Cites J Neurochem. 1998 Feb;70(2):677-87 PMID 9453562; Cites EMBO J. 1998 May 1;17(9):2596-606 PMID 9564042; Cites J Cell Biol. 2001 Oct 1;155(1):27-39 PMID 11581284

Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM

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Record type: MEDLINE; Completed

The calcium-calmodulin-activated protein phosphatase %%%calcineurin%%% functions as a key mediator of diverse biologic processes, including differentiation, apoptosis, growth, and adaptive responses, in part through dephosphorylation and activation of %%%nuclear%%% factor of activated T-cell (NFAT) transcription factors. Apoptosis %%%signal%%%-regulating kinase 1 (ASK1) is an upstream component of the mitogen-activated protein kinases that serves as a pivotal regulator of cytokine-, oxidative-, and stress-induced cell death. Here, we performed a yeast two-hybrid screen with %%%calcineurin%%% B as bait, which identified ASK1 as a direct physical interacting partner. The C-terminal 218 amino acids of ASK1 were sufficient to mediate interaction with %%%calcineurin%%% B in yeast, as well as in mammalian cell lysates. Importantly, endogenous calcium binding B subunit (CnB) protein interacted with endogenous ASK1 protein in cardiomyocytes at baseline, suggesting that the interaction observed in yeast was of potential biologic relevance. Indeed, %%%calcineurin%%% directly dephosphorylated ASK1 at serine 967 using purified proteins or mammalian cell lysates. Dephosphorylation of ASK1 serine 967 by %%%calcineurin%%% promoted its disassociation from 14-3-3 proteins, resulting in ASK1 activation. %%%Calcineurin%%% and ASK1 cooperatively enhanced cardiomyocyte apoptosis, while expression of a dominant negative ASK1 blocked %%%calcineurin%%% -induced apoptosis. Mouse embryonic fibroblasts deficient in ask1 were also partially resistant to %%%calcineurin%%%- or ionomycin-induced apoptosis. Finally, ASK1 negatively regulated %%%calcineurin%%% -NFAT signaling indirectly through c-Jun NH2-terminal kinase (JNK)- and p38-mediated phosphorylation of NFAT, which blocked %%%calcineurin%%%- and agonist-dependent %%%hypertrophic%%% growth of cardiomyocytes. Thus, ASK1 and %%%calcineurin%%% -NFAT constitute a feedback regulatory circuit in which %%%calcineurin%%% positively regulates

ASK1 through direct dephosphorylation, while ASK1 negatively regulates %%%calcineurin%%% -NFAT signaling through p38- and JNK-mediated NFAT phosphorylation.
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  Record Date Completed: 20060609

 2/7/49   (Item 49 from file: 154)
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17163955   PMID: 16611217
  A cell-permeable NFAT inhibitor peptide prevents pressure-overload cardiac %%%hypertrophy%%%.
  Kuriyama Mitsuhito; Matsushita Masayuki; Tateishi Atsushi; Moriwaki Akiyoshi; Tomizawa Kazuhito; Ishino Kozo; Sano Shunji; Matsui Hideki
  Department of Physiology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan.
  Chemical biology & drug design (England) Mar 2006, 67 (3) p238-43, ISSN 1747-0277--Print Journal Code: 101262549
  Publishing Model Print
  Document type: Journal Article; Research Support, Non-U.S. Gov't
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: MEDLINE; Completed
  The activation of the %%%calcineurin%%%-%%%nuclear%%% factor of activated T cells cascade during the development of pressure-overload cardiac %%%hypertrophy%%% has been previously reported in a number of studies. In addition, numerous pharmacological studies involving %%%calcineurin%%% inhibitors such as FK506 and cyclosporine A have now demonstrated that these agents can prevent such %%%hypertrophic%%% responses in the heart. However, little is known regarding the roles of the %%%calcineurin%%% downstream effector--%%%nuclear%%% factor of activated T cells. Our present study has further examined the roles of %%%nuclear%%% factor of activated T cells in pressure-overload cardiac %%%hypertrophy%%% by employing a recently developed cell-permeable %%%nuclear%%% factor of activated T cells inhibitor peptide. Rat hearts were subjected to pressure overload attributable by 4 weeks of aortic banding, and then treated with this cell-permeable %%%nuclear%%% factor of activated T cells inhibitor peptide and a control peptide. Treatment with the inhibitor was found to significantly decrease the heart weight/body weight ratio, the size of cardiac myocytes, and the serum brain natriuretic peptide and atrial natriuretic peptide levels. These results suggest that %%%nuclear%%% factor of activated T cells functions in a key role in the development of cardiac %%%hypertrophy%%% during pressure overload. Inhibition of %%%nuclear%%% factor of activated T cells by a specific inhibitor peptide is a suitable method for characterization of the molecular mechanisms underlying cardiac %%%hypertrophy%%% as well as in the search for new promising therapies for disease.
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  Record Date Completed: 20070309

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17137227 PMID: 16369686

Role of %%%calcineurin%%% in Porphyromonas gingivalis-induced myocardial cell %%%hypertrophy%%% and apoptosis.

Lee Shin-Da; Kuo Wei-Wen; Lin Ding-Yu; Chen Ter-Hsin; Kuo Wu-Hsien; Hsu Hsi-Hsien; Chen Jian-Zhi; Liu Jer-Yuh; Yeh Yu-Lan; Huang Chih-Yang

School of Physical Therapy, Chung-Shan Medical University, Taichung, Taiwan.

Journal of biomedical science (Netherlands) Mar 2006, 13 (2) p251-60 , ISSN 1021-7770--Print 1021-7770--Linking Journal Code: 9421567

Publishing Model Print-Electronic

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

BACKGROUND AND OBJECTIVE: Periodontal pathogen Porphyromonas gingivalis (P. gingivalis) increased cardiomyocyte %%%hypertrophy%%% and apoptosis whereas Actinobaeillus actinomycetemcomitans and Prevotella intermedia had no effects. The purpose of this study is to clarify the role of %%%calcineurin%%% signaling pathway in P. gingivalis-induced H9c2 myocardial cell %%%hypertrophy%%% and apoptosis. METHODS: DNA fragmentation, %%%nuclear%%% condensation, cellular morphology, %%%calcineurin%%% protein, Bcl2-associated death promoter (Bad) and %%%nuclear%%% factor of activated T cell (NFAT)-3 protein products in cultured H9c2 myocardial cell were measured by agarose gel electrophoresis, DAPI, immunofluorescence, and Western blotting following P. gingivalis and/or pre-administration of CsA (%%%calcineurin%%% inhibitors cyclosporin A). RESULTS: P. gingivalis not only increased %%%calcineurin%%% protein, NFAT-3 protein products and cellular %%%hypertrophy%%%, but also increased DNA fragmentation, %%%nuclear%%% condensation and Bad protein products in H9c2 cells. The increased cellular sizes, DNA fragmentation, %%%nuclear%%% condensation, and Bad of H9c2 cells treated with P. gingivalis were all significantly reduced after pre-administration of CsA. CONCLUSION: Our findings suggest that the activity of %%%calcineurin%%% %%%signal%%% pathway may be initiated by P. gingivalis and further lead to cell %%%hypertrophy%%% and death in culture H9c2 myocardial cells.

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17122959 PMID: 16415348

Protein kinase-mediated regulation of %%%calcineurin%%% through the phosphorylation of modulatory %%%calcineurin%%%-interacting protein 1.

Abbasi Shahrzad; Lee Jiing-Dwan; Su Bing; Chen Xiang; Alcon Joseph L; Yang JianHua; Kellems Rodney E; Xia Yang

Department of Biochemistry and Molecular Biology, The University of Texas-Houston Medical School, Houston, Texas 77030, USA.

Journal of biological chemistry (United States) Mar 24 2006, 281 (12) p7717-26, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R

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Document type: Journal Article; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM
Record type: MEDLINE; Completed

%%%Calcineurin%%% is a serine/threonine protein phosphatase that plays a critical role in many physiologic processes such as T-cell activation, skeletal myocyte differentiation, and cardiac %%%hypertrophy%%% . We previously showed that active MEKK3 is capable of stimulating %%%calcineurin%%%/%%%nuclear%%% factor of activated T-cells (NFAT) signaling in cardiac myocytes through phosphorylation of modulatory %%%calcineurin%%% -interacting protein 1 (MCIP1). However, the protein kinases that function downstream of MEKK3 to mediate MCIP1 phosphorylation and the mechanism of MCIP1-mediated %%%calcineurin%%% regulation have not been defined. Here, we show that MEK5 and big MAP kinase 1 (BMK1) function downstream of MEKK3 in a signaling cascade that induces %%%calcineurin%%% activity through phosphorylation of MCIP1. Genetic studies showed that BMK1-deficient mouse lung fibroblasts failed to mediate MCIP1 phosphorylation and activate %%%calcineurin%%% /NFAT in response to angiotensin II, a potent NFAT activator. Conversely, restoring BMK1 to the deficient cells restored angiotensin II-mediated %%%calcineurin%%%/NFAT activation. Thus, using BMK1-deficient mouse lung fibroblast cells, we provided the genetic evidence that BMK1 is required for angiotensin II-mediated %%%calcineurin%%% /NFAT activation through MICP1 phosphorylation. Finally, we discovered that phosphorylated MCIP1 dissociates from %%%calcineurin%%% and binds with 14-3-3, thereby relieving its inhibitory effect on %%%calcineurin%%% activity. In summary, our findings reveal a previously unrecognized essential regulatory role of mitogen-activated protein kinase signaling in %%%calcineurin%%% activation through the reversible phosphorylation of a %%%calcineurin%%%-interacting protein, MCIP1.

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Date of Electronic Publication: 20060116

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17056470 PMID: 16389460
Activation of the %%%calcineurin%%% signaling pathway induces atrial %%%hypertrophy%%% during atrial fibrillation.

Bukowska A; Lendeckel U; Hirte D; Wolke C; Striggow F; Rohnert P; Huth C; Klein H U; Goette A
Institute of Experimental Internal Medicine, University Hospital Magdeburg, Leipziger Strasse 44, 39120 Magdeburg, Germany.

Cellular and molecular life sciences - CMLS (Switzerland) Feb 2006, 63 (3) p333-42, ISSN 1420-682X--Print 1420-682X--Linking Journal Code: 9705402
Publishing Model Print
Document type: In Vitro; Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

Atrial tachyarrhythmia (AF) alters intracellular calcium homeostasis and induces cellular %%%hypertrophy%%% of atrial myocytes. The impact of the calcium-dependent %%%calcineurin%%% pathway on the development of AF-induced atrial %%%hypertrophy%%% has not yet been analyzed. In this

study, atrial tissue samples from patients with sinus rhythm and chronic persistent atrial fibrillation (CAF) were used to determine changes in expression and activity of %%%calcineurin%%% A (CnA), and its relation to CnA-regulated transcription factors NFATc1-4, and %%%hypertrophic%%% markers ANP, troponin I, and beta-MHC. CnA phosphatase activity and CnAbeta protein contents were significantly upregulated in patients with CAF. %%%Calcineurin%%% activation led to dephosphorylation, redistribution, and subsequent accumulation of NFATc3 in %%%nuclei%%% during CAF, and expression of %%%hypertrophic%%% genes was increased. CAF-dependent changes were reproduced by ex vivo pacing (2-4 Hz) of human atrial tissue slices. FK506 abolished the %%%hypertrophic%%% response induced by electrical-field stimulation. Atrial tachyarrhythmia causes atrial %%%hypertrophy%%% by activation of the CnA %%%signal%%% pathway, which thereby contributes to structural remodeling of human atria.

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Record Date Completed: 20060316

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DIALOG(R)File 154:MEDLINE(R)

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16965628 PMID: 16496058

L-type calcium channel blocker suppresses %%%calcineurin%%% %%%signal%%% pathway and development of right ventricular %%%hypertrophy%%%.

Liu Chen-Zhou; Tan Jian-Xin; Wang You; Huang Yu-Ge; Huang Di-Lan

Department of Pediatrics, Affiliated Hospital of Guangdong Medical College, China.

Journal of the Formosan Medical Association = Taiwan yi zhi (China) Nov 2005, 104 (11) p798-803, ISSN 0929-6646--Print 0929-6646--Linking

Journal Code: 9214933

Publishing Model Print

Document type: Journal Article; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

BACKGROUND AND PURPOSE: Many studies have shown that L-type calcium channel blockers can prevent and treat right ventricular %%%hypertrophy%%% (RVH). In order to identify the mechanism, we investigated the role of the %%%calcineurin%%% %%%signal%%% pathway in the progression of RVH induced by chronic hypoxia and the effects of an L-type calcium channel blocker on the pathway. METHODS: Rats were allocated to 1 of 3 groups (n=10 for each): chronic hypoxia group, amlodipine treatment group (30 mg/kg/day, administered via gavage); and control group. Rats in the amlodipine treatment group and the chronic hypoxia group were exposed to normobaric chronic hypoxia (9.5%-10.5% oxygen). We investigated the changes of right ventricle (RV) to left ventricle (LV) and interventricular septum (S) weight ratio [RV/(LV+S)], RV to body weight (BW) ratio (RV/BW), %%%calcineurin%%% A beta (CnAbeta) mRNA levels, cardiac myosin heavy chain beta (beta-MHC) mRNA levels and protein expression of CnAbeta, %%%nuclear%%% factor 3 of activated T cell (NFAT3), and beta-MHC. RESULTS: After 21 days, RV/(LV+S) and RV/BW were significantly higher in the chronic hypoxia group than in the control group and the amlodipine group (p<0.01). The expression of CnAbeta mRNA and protein, NFAT3 protein, beta-MHC mRNA and protein in RV of the chronic hypoxia group was higher than that of the control group and the amlodipine treatment group (p<0.01). CONCLUSIONS: The %%%calcineurin%%% %%%signal%%% pathway plays a critical role in the

progression of RVH induced by chronic hypoxia. L-type calcium channel blockade suppresses the development of RVH by inhibiting this pathway.
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Record Date Completed: 20060330

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DIALOG(R)File 154:MEDLINE(R)
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16916976 PMID: 16269655
cAMP-binding protein Epac induces cardiomyocyte %%%hypertrophy%%%.
Morel Eric; Marcantoni Andrea; Gastineau Monique; Birkedal Rikke; Rochais Francesca; Garnier Anne; Lompre Anne-Marie; Vandecasteele Gregoire; Lezoualc'h Frank
Cardiologie Cellulaire et Moleculaire, Inserm U-446, IFR-75, Faculte de Pharmacie, Universite Paris XI, 5 Rue JB Clement, 92296 Chatenay Malabry, France.
Circulation research (United States) Dec 9 2005, 97 (12) p1296-304, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
cAMP is one of the most important second messenger in the heart. The discovery of Epac as a guanine exchange factor (GEF), which is directly activated by cAMP, raises the question of the role of this protein in cardiac cells. Here we show that Epac activation leads to morphological changes and induces expression of cardiac %%%hypertrophic%%% markers. This process is associated with a Ca2+-dependent activation of the small GTPase, Rac. In addition, we found that Epac activates a prohypertrophic signaling pathway, which involves the Ca2+ sensitive phosphatase, %%%calcineurin%%%, and its primary downstream effector, NFAT. Rac is involved in Epac-induced NFAT dependent cardiomyocyte %%%hypertrophy%%%. Blockade of either %%%calcineurin%%% or Rac activity blunts the %%%hypertrophic%%% response elicited by Epac indicating these signaling molecules coordinately regulate cardiac gene expression and cellular growth. Our results thus open new insights into the signaling pathways by which cAMP may mediate its biological effects and identify Epac as a new positive regulator of cardiac growth.
Record Date Created: 20051212
Record Date Completed: 20051228
Date of Electronic Publication: 20051103

2/7/55 (Item 55 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

16853118 PMID: 16126726
The essential role of MEKK3 signaling in angiotensin II-induced %%%calcineurin%%%/%%%nuclear%%% factor of activated T-cells activation.
Abbasi Shahrzad; Su Bing; Kellems Rodney E; Yang Jianhua; Xia Yang
Department of Biochemistry and Molecular Biology, University of Texas at Houston Medical School, Houston, Texas 77030, USA.
Journal of biological chemistry (United States) Nov 4 2005, 280 (44)

p36737-46, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
%%%Calcineurin%%% is a serine/threonine protein phosphatase that plays a critical role in many physiologic processes, such as T-cell activation, apoptosis, skeletal myocyte differentiation, and cardiac %%%hypertrophy%%%. We determined that active MEKK3 was capable of activating %%%calcineurin%%% /%%%nuclear%%% factor of activated T-cells (NFAT) signaling in cardiac myocytes and reprogramming cardiac gene expression. In contrast, small interference RNA directed against MEKK3 and a dominant negative form of MEKK3 caused the reduction of NFAT activation in response to angiotensin II in cardiac myocytes. Genetic studies showed that MEKK3-deficient mouse embryo fibroblasts failed to activate %%%calcineurin%%%/NFAT in response to angiotensin II, a potent NFAT activator. Conversely, restoring MEKK3 to the MEKK3-deficient cells restored angiotensin II-mediated %%%calcineurin%%% /NFAT activation. We determined that angiotensin II induced MEKK3 phosphorylation. Thus, MEKK3 functions downstream of the AT1 receptor and is essential for %%%calcineurin%%%/NFAT activation. Finally, we determined that MEKK3-mediated activation of %%%calcineurin%%% /NFAT signaling was associated with the phosphorylation of modulatory %%%calcineurin%%% -interacting protein 1 at Ser(108) and Ser(112). Taken together, our studies reveal a previously unrecognized novel essential regulatory role of MEKK3 signaling in %%%calcineurin%%%/NFAT activation.
Record Date Created: 20051031
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Date of Electronic Publication: 20050826

2/7/56 (Item 56 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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16827427 PMID: 16185906
The molecular regulation of exercised-induced muscle fibre %%%hypertrophy%%% in the common carp: expression of MyoD, PCNA and components of the %%%calcineurin%%%-signalling pathway.
Martin C I; Johnston I A
Gatty Marine Laboratory, School of Biology, University of St Andrews, St Andrews, Scotland, KY16 8LB, UK.
Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology (England) Nov 2005, 142 (3) p324-34, ISSN 1096-4959--Print 1096-4959--Linking Journal Code: 9516061
Publishing Model Print-Electronic
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Growth was investigated over 16 d in juvenile common carp (Cyprinus carpio L.) held in either static water (tank rested, TR16) or exercised in a flume at 2.5-3.2 body lengths s-1 for 18 h a day (exercised, E16). Relative to the start of the experiment (TR0), the TR16 group showed a 31% increase in body mass (specific growth rate, 1.57% d-1), whereas there was no net change in the E16 group. There was, however, a significant

exercise-induced %%%hypertrophy%%% of slow muscle fibres with average fibre cross-sectional area (FCSA) increasing by 35% in the E16 group, compared with 11% in the TR16 group. In contrast, FCSA of fast muscle fibres increased by 34% in the TR16 group compared to just 18% in the E16 group. The relative concentrations and subcellular localisation of proteins hypothesised to play a role in the regulation of muscle growth were measured. MyoD concentration was similar in the TR0, TR16 and E16 groups in both slow and fast muscle. However, there was a small (5%-10%) but statistically significant increase in %%%nuclear%%% localisation of MyoD in those groups showing a significant increase in FCSA over the time course of the experiment. PCNA concentration was 31% and 12% higher in the TR16 than in either the TR0 or E16 groups for slow and fast muscle, respectively. Exercise resulted in a approximately 10% increase in %%%nuclear%%% factor of T-cells (NFAT2) concentration in slow muscle but no change in NFAT2 localisation. %%%Calcineurin%%% B concentration was similar in tank rested and exercised groups. The results do not support a major role for the %%%calcineurin%%%-signalling pathway in the regulation of muscle %%%hypertrophy%%% in the common carp.
Record Date Created: 20051017
Record Date Completed: 20060105
Date of Electronic Publication: 20050926

2/7/57 (Item 57 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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16716513 PMID: 16093487
Calcium signaling in cardiac ventricular myocytes.
Bers Donald M; Guo Tao
Department of Physiology, Loyola University Chicago, 2160 S. First Ave, Maywood, IL 60153, USA. dbers@lumc.edu
Annals of the New York Academy of Sciences (United States) Jun 2005, 1047 p86-98, ISSN 0077-8923--Print 0077-8923--Linking Journal Code: 7506858
Contract/Grant No.: HL-30077; HL; NHLBI NIH HHS United States; HL-64724; HL; NHLBI NIH HHS United States
Publishing Model Print
Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Calcium (Ca) is a multifunctional regulator of diverse cellular functions. In cardiac muscle Ca is a direct central mediator of electrical activation, ion channel gating, and excitation-contraction (E-C) coupling that all occur on the millisecond time scale. The key amplification step in E-C coupling is under tight control of very local [Ca]. Ca also directly activates signaling via kinases and phosphatases (e.g., Ca-calmodulin-dependent protein kinase [CaMKII] and %%%calcineurin%%%) that occur over a longer time scale (seconds to minutes), and the co-localization of these Ca-dependent modulators to their targets and to Ca is also critical in distinct signaling pathways. Finally, Ca-dependent signaling is also involved in long-term (minutes to hours/days) alterations in gene expression (or excitation-transcription coupling). These pathways are involved in %%%hypertrophy%%% and heart failure, and they can alter the

expression of some of the key Ca regulatory proteins involved in E-C coupling and their regulation by kinases and phosphatases. There may again be physical microenvironments involved in this %%%nuclear%%% transcription, such that they sense a discrete Ca %%%signal%%% that is distinct from that involved in E-C coupling. In this way cells can use Ca signaling in multiple ways that function in spatially and temporally distinct manners. (90 Refs.)
Record Date Created: 20050811
Record Date Completed: 20051028

2/7/58 (Item 58 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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16660385 PMID: 15899894 Record Identifier: NIHMS3724; PMC1249515
Estrogen inhibits cardiomyocyte %%%hypertrophy%%% in vitro. Antagonism of %%%calcineurin%%%-related %%%hypertrophy%%% through induction of MCIP1.
Pedram Ali; Razandi Mahnaz; Aitkenhead Mark; Levin Ellis R
Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, California 90822, USA.
Journal of biological chemistry (United States) Jul 15 2005, 280 (28) p26339-48, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R
Contract/Grant No.: CA-100366; CA; NCI NIH HHS United States; HL-59890; HL; NHLBI NIH HHS United States; R01 CA100366-01A2; CA; NCI NIH HHS United States
Publishing Model Print-Electronic; Cites Mol Cell Biol. 2005 Feb;25(3):865-78 PMID 15657416; Cites Hypertension. 2003 Jan;41(1):99-107 PMID 12511537; Cites Am J Physiol. 1996 Apr;270(4 Pt 2):H1342-9 PMID 8967374 ; Cites Biochim Biophys Acta. 1997 Apr 24;1356(2):221-8 PMID 9150279; Cites J Mol Cell Cardiol. 1997 Sep;29(9):2545-59 PMID 9299377; Cites Am J Cardiol. 1999 Apr 1;83(7):1132-4, A9 PMID 10190535; Cites N Engl J Med. 1999 Apr 22;340(16):1279-80 PMID 10210713; Cites J Cardiovasc Pharmacol. 1999 Dec;34(6):870-8 PMID 10598132; Cites Cardiovasc Res. 1999 Aug 15;43(3):666-74 PMID 10690338; Cites Biochem J. 2000 Apr 1;347 Pt 1:275-84 PMID 10727428; Cites Hum Mol Genet. 2000 Jul 1;9(11):1681-90 PMID 10861295; Cites J Biol Chem. 2000 Dec 1;275(48):37895-901 PMID 10984495; Cites Nature. 2000 Sep 28;407(6803):538-41 PMID 11029009; Cites J Hypertens. 2001 Feb;19(2):269-78 PMID 11212970; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3328-33 PMID 11248078; Cites Circulation. 2001 Sep 18;104(12):1419-23 PMID 11560859; Cites Proc Natl Acad Sci U S A. 2001 Sep 25;98(20):11765-70 PMID 11562484; Cites Circulation. 2001 Nov 6;104(19):2300-4 PMID 11696469; Cites Mol Endocrinol. 2002 Jan;16(1):100-15 PMID 11773442; Cites Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4586-91 PMID 11904392; Cites Nature. 2002 Mar 21;416(6878):334-8 PMID 11907581; Cites Card Electrophysiol Rev. 2002 Feb;6(1-2):18-25 PMID 11984010; Cites Circulation. 2002 May 14;105(19):2242-3 PMID 12010903; Cites Hypertens Res. 2002 Mar;25(2):153-9 PMID 12047028; Cites J Biol Chem. 2002 Aug 16;277(33):30401-7 PMID 12063245; Cites Proc Natl Acad Sci U S A. 2003 Jan 21;100(2):669-74 PMID 12515860; Cites Can J Cardiol. 2002 Dec;18(12):1331-9 PMID 12518185; Cites J Biol Chem. 2003 Sep 26;278(39):36981-4 PMID 12881512; Cites J Mol Cell Cardiol. 2003 Sep;35(9):1121-33 PMID 12967635; Cites Hypertension. 2003 Dec;42(6):1177-82 PMID 14581297; Cites Circulation. 2004 Jan 20;109(2):269-76 PMID 14718400; Cites J Pharmacol Exp Ther. 2004 Jul;310(1):43-51 PMID 15007106; Cites Proc Natl Acad Sci U S A. 2004 Mar 23;101(12):4130-5 PMID 15024130;

Cites Science. 2004 Apr 9;304(5668):292-6 PMID 15073377; Cites Science. 2004 May 28;304(5675):1269-73 PMID 15166356; Cites Mol Endocrinol. 2004 Dec;18(12):2854-65 PMID 15231873; Cites Proc Natl Acad Sci U S A. 2004 Sep 28;101(39):14234-9 PMID 15375213; Cites Mol Cell Biol. 2002 Nov;22(21):7603-13 PMID 12370307; Cites J Biol Chem. 2002 Dec 27;277(52):50768-75 PMID 12372818; Cites Circ Res. 2002 Nov 1;91(9):821-9 PMID 12411397; Cites J Biol Chem. 2005 Jun 3;280(22):21594-9 PMID 15793309

Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, U.S. Gov't, Non-P.H.S.; Research Support, U.S. Gov't, P.H.S.

Languages: ENGLISH

Main Citation Owner: NLM

Other Citation Owner: NLM; NLM

Record type: MEDLINE; Completed

Evidence from in vivo studies suggests that some inputs to cardiac %%%hypertrophy%%% are opposed by the actions of estrogen. However, the mechanisms of E2 action in this respect are mainly unknown. An important pathway that is utilized by multiple %%%hypertrophic%%% stimuli involves the activation of the tyrosine phosphatase, %%%calcineurin%%% (PP2B). Here we show that 17beta-estradiol (E2) significantly prevents angiotensin II (AngII)- or endothelin-1 (ET-1)-induced new protein synthesis, skeletal muscle actin expression, and increased surface area in cultured rat cardiomyocytes. ET-1 stimulated %%%calcineurin%%% phosphatase activity, resulting in new protein synthesis, and both were prevented by E2. E2 induced the MCIP1 gene, an inhibitor of %%%calcineurin%%% activity, via phosphatidylinositol 3-kinase, transcriptional, and mRNA stability mechanisms. Small interfering RNA for MCIP1 significantly reversed both the E2 restraint of protein synthesis and the inhibition of AngII-induced %%%calcineurin%%% activity. AngII-induced the translocation of the %%%hypertrophic%%% transcription factor, NF-AT, to the %%%nucleus%%% of the cardiomyocyte and stimulated NF-AT transcriptional activity. Both were prevented by E2. AngII also stimulated the activation of ERK and protein kinase C, contributing to cardiac %%%hypertrophy%%%. E2 inhibited these pathways, related to the stimulation of atrial natriuretic peptide production and secretion. Thus, restraint of %%%calcineurin%%% and kinase signaling to the %%%hypertrophic%%% program underlie these important effects of E2.

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Record Date Completed: 20050912

Date of Electronic Publication: 20050516

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16593026 PMID: 15793309

Local atrial natriuretic peptide signaling prevents hypertensive cardiac %%%hypertrophy%%% in endothelial nitric-oxide synthase-deficient mice.

Bubikat Alexander; De Windt Leon J; Zetsche Bernd; Fabritz Larissa; Sickler Heidrun; Eckardt Dominik; Godecke Axel; Baba Hideo A; Kuhn Michaela

Department of Pharmacology and Toxicology, Universitatsklinikum Munster, D-48129 Munster, Germany.

Journal of biological chemistry (United States) Jun 3 2005, 280 (22) p21594-9, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R

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Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

The crucial functions of atrial natriuretic peptide (ANP) and endothelial nitric oxide/NO in the regulation of arterial blood pressure have been emphasized by the hypertensive phenotype of mice with systemic inactivation of either the guanylyl cyclase-A receptor for ANP (GC-A-/-) or endothelial nitric-oxide synthase (eNOS-/-). Intriguingly, similar levels of arterial hypertension are accompanied by marked cardiac %%%hypertrophy%%% in GC-A-/-, but not in eNOS-/-, mice, suggesting that changes in local pathways regulating cardiac growth accelerate cardiac %%%hypertrophy%%% in the former and protect the heart of the latter. Our recent observations in mice with conditional, cardiomyocyte-restricted GC-A deletion demonstrated that ANP locally inhibits cardiomyocyte growth. Abolition of these local, protective effects may enhance the cardiac %%%hypertrophic%%% response of GC-A-/- mice to persistent increases in hemodynamic load. Notably, eNOS-/- mice exhibit markedly increased cardiac ANP levels, suggesting that increased activation of cardiac GC-A can prevent hypertensive heart disease. To test this hypothesis, we generated mice with systemic inactivation of eNOS and cardiomyocyte-restricted deletion of GC-A by crossing eNOS-/- and cardiomyocyte-restricted GC-A-deficient mice. Cardiac deletion of GC-A did not affect arterial hypertension but significantly exacerbated cardiac %%%hypertrophy%%% and fibrosis in eNOS-/- mice. This was accompanied by marked cardiac activation of both the mitogen-activated protein kinase (MAPK) ERK 1/2 and the phosphatase %%%calcineurin%%%. Our observations suggest that local ANP/GC-A/cyclic GMP signaling counter-regulates MAPK/ERK- and %%%calcineurin%%%/%%%nuclear%%% factor of activated T cells-dependent pathways of cardiac myocyte growth in hypertensive eNOS-/- mice.

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Record Date Completed: 20050712
Date of Electronic Publication: 20050326

2/7/60 (Item 60 from file: 154)
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16586588 PMID: 15914652

The role of myostatin and the %%%calcineurin%%%-signalling pathway in regulating muscle mass in response to exercise training in the rainbow trout Oncorhynchus mykiss Walbaum.

Martin C I; Johnston I A
Gatty Marine Laboratory, School of Biology, University of St Andrews, St Andrews, Scotland KY16 8LB, UK.

Journal of experimental biology (England) Jun 2005, 208 (Pt 11) p2083-90, ISSN 0022-0949--Print 0022-0949--Linking Journal Code: 0243705
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

Rainbow trout Oncorhynchus mykiss Walbaum were exercised at 0.8 and 1.6 body lengths s(-1) for 18 h a day over a 30 day period. Exercise resulted

in a 24-30% increase in the average cross-sectional area of fast muscle fibres relative to tank-rested controls. The concentrations of growth factors and transcription factors hypothesised to play a role in regulating exercise-induced muscle fibre %%%hypertrophy%%% were measured. Exercise training resulted in a minor increase in %%%calcineurin%%% localisation in the %%%nucleus%%%. However, %%%nuclear%%% factor of T-cells 2 (NFAT2) %%%nuclear%%% localisation did not follow a pattern that was consistent with NFAT2-mediated transcriptional activity and changes in %%%calcineurin%%% signaling. The active peptide of myostatin, a negative regulator of muscle growth in mammals, was downregulated in exercise groups relative to tank-rested controls, but only by 6-7%. It was concluded that myostatin and %%%calcineurin%%% signaling do not play a major role in regulating exercise-induced muscle %%%hypertrophy%%% in trout.
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  Record Date Completed: 20050809

2/7/61 (Item 61 from file: 154)
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16465451 PMID: 15765135 Record Identifier: PMC1052006
  Toward transcriptional therapies for the failing heart: chemical screens to modulate genes.
  McKinsey Timothy A; Olson Eric N
  Myogen Inc., Westminster, Colorado 80021, USA. timothy.mckinsey@myogen.com
  Journal of clinical investigation (United States) Mar 2005, 115 (3) p538-46, ISSN 0021-9738--Print 0021-9738--Linking Journal Code: 7802877
  Publishing Model Print; Cites Mol Cell Biol. 2003 May;23(10):3593-606 PMID 12724418; Cites J Biol Chem. 2003 Aug 1;278(31):28930-7 PMID 12761226; Cites Circulation. 2003 Jul 1;107(25):3223-9 PMID 12810613; Cites J Mol Cell Cardiol. 2003 Aug;35(8):887-9 PMID 12878474; Cites J Biol Chem. 2003 Sep 26;278(39):36981-4 PMID 12881512; Cites Genes Dev. 2003 Aug 15;17(16):1937-56 PMID 12893779; Cites J Clin Invest. 2003 Sep;112(6):863-71 PMID 12975471; Cites Science. 2003 Nov 28;302(5650):1578-81 PMID 14645853; Cites JAMA. 2004 Nov 17;292(19):2343-9 PMID 15547161; Cites JAMA. 2004 Nov 17;292(19):2350-6 PMID 15547162; Cites JAMA. 2004 Nov 17;292(19):2396-8 PMID 15547168; Cites Mol Cell Biol. 2004 Dec;24(24):10636-49 PMID 15572669; Cites FEBS Lett. 1996 Aug 26;392(2):77-80 PMID 8772178; Cites J Clin Invest. 1997 Nov 1;100(9):2362-70 PMID 9410916; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Circ Res. 1998 Aug 24;83(4):345-52 PMID 9721691; Cites Cell. 1999 Sep 3;98(5):555-8 PMID 10490095; Cites Circ Res. 2000 Mar 3;86(4):386-90 PMID 10700442; Cites J Biol Chem. 2000 May 12;275(19):14466-75 PMID 10799529; Cites Nat Rev Mol Cell Biol. 2003 Dec;4(12):938-47 PMID 14685172; Cites Circ Res. 2004 Feb 20;94(3):e18-26 PMID 14739160; Cites Proc Natl Acad Sci U S A. 2004 Mar 2;101(9):2870-5 PMID 14976250; Cites Circulation. 2004 Mar 16;109(10):1196-205 PMID 15023894; Cites Trends Genet. 2004 Apr;20(4):206-13 PMID 15041175; Cites Circulation. 2004 Apr 6;109(13):1580-9 PMID 15066961; Cites Proc Natl Acad Sci U S A. 2004 Jun 29;101(26):9578-83 PMID 15210966; Cites Curr Pharm Des. 2004;10(19):2289-98 PMID 15279609; Cites EMBO J. 2004 Sep 1;23(17):3559-69 PMID 15297879; Cites Mol Cell Biol. 2004 Oct;24(19):8374-85 PMID 15367659; Cites Mol Cell Biol. 2004 Oct;24(19):8467-76 PMID 15367668; Cites Am J Physiol Heart Circ

Physiol. 2005 Mar;288(3):H1477-90 PMID 15388503; Cites Circ Res. 2004 Nov 26;95(11):1091-9 PMID 15514163; Cites J Biol Chem. 2000 Jul 21;275(29):22563-7 PMID 10825153; Cites Proc Natl Acad Sci U S A. 2000 Jul 5;97(14):7835-40 PMID 10869435; Cites Mol Cell. 2000 Mar;5(3):589-95 PMID 10882143; Cites EMBO J. 2000 Aug 15;19(16):4323-31 PMID 10944115; Cites J Cell Biol. 2000 Oct 2;151(1):117-30 PMID 11018058; Cites Circulation. 2000 Nov 14;102(20 Suppl 4):IV14-23 PMID 11080127; Cites Nature. 2000 Nov 2;408(6808):106-11 PMID 11081517; Cites Circ Res. 2000 Dec 8;87(12):E61-8 PMID 11110780; Cites Proc Natl Acad Sci U S A. 2000 Dec 19;97(26):14400-5 PMID 11114197; Cites Trends Cardiovasc Med. 2000 Jan;10(1):19-22 PMID 11150724; Cites Annu Rev Physiol. 2001;63:391-426 PMID 11181961; Cites Am Heart J. 2001 Mar;141(3):334-41 PMID 11231428; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):2947-9 PMID 11248009; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3328-33 PMID 11248078; Cites Circulation. 2001 Jun 19;103(24):2973-9 PMID 11413089; Cites Mol Cell Biol. 2001 Sep;21(17):5992-6005 PMID 11486037; Cites Mol Cell Biol. 2001 Sep;21(18):6312-21 PMID 11509672; Cites Proc Natl Acad Sci U S A. 2002 Jan 22;99(2):907-12 PMID 11782539; Cites J Biol Chem. 2002 Mar 22;277(12):10251-5 PMID 11786544; Cites Trends Biochem Sci. 2002 Jan;27(1):40-7 PMID 11796223; Cites Nature. 2002 Jan 10;415(6868):206-12 PM ID 11805844; Cites Circulation. 2002 Jan 29;105(4):446-51 PMID 11815426; Cites Trends Cell Biol. 2002 Apr;12(4):193-200 PMID 11978539; Cites N Engl J Med. 2002 May 2;346(18):1357-65 PMID 11986409; Cites Circ Res. 2002 Jun 14;90(11):1150-2 PMID 12065316; Cites Cell. 2002 Aug 23;110(4):479-88 PMID 12202037; Cites Nat Med. 2002 Nov;8(11):1310-7 PMID 12368904; Cites Nat Med. 2002 Nov;8(11):1303-9 PMID 12379849; Cites Proc Natl Acad Sci U S A. 2003 Jan 21;100(2):669-74 PMID 12515860; Cites Mol Med. 2002 Nov;8(11):750-60 PMID 12520092; Cites Annu Rev Physiol. 2003;65:45-79 PMID 12524460; Cites Proc Natl Acad Sci U S A. 2003 Feb 18;100(4):1711-6 PMID 12578979; Cites J Biol Chem. 2003 May 23;278(21):19134-40 PMID 12624111; Cites Circulation. 2003 Mar 11;107(9):1234-6 PMID 12628939; Cites Curr Opin Cell Biol. 2003 Apr;15(2):172-83 PMID 12648673; Cites Circulation. 2003 Mar 25;107(11):1456-9 PMID 12654597; Cites J Am Coll Cardiol. 2003 Apr 2;41(7):1096-106 PMID 12679207; Cites Nature. 2003 Apr 17;422(6933):730-4 P MID 12700764; Cites Trends Genet. 2003 May;19(5):263-8 PMID 12711218; Cites Trends Genet. 2003 May;19(5):286-93 PMID 12711221

Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.; Review

Languages: ENGLISH

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In response to acute and chronic stresses, the heart frequently undergoes a remodeling process that is accompanied by myocyte %%%hypertrophy%%%, impaired contractility, and pump failure, often culminating in sudden death. The existence of redundant signaling pathways that trigger heart failure poses challenges for therapeutic intervention. Cardiac remodeling is associated with the activation of a pathological gene program that weakens cardiac performance. Thus, targeting the disease process at the level of gene expression represents a potentially powerful therapeutic approach. In this review, we describe strategies for normalizing gene expression in the failing heart with small molecules that control %%%signal%%% transduction pathways directed at transcription factors and associated chromatin-modifying enzymes. (72 Refs.)

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2/7/62 (Item 62 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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16411128 PMID: 15665834
Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac %%%hypertrophy%%%.
Takimoto Eiki; Champion Hunter C; Li Manxiang; Belardi Diego; Ren Shuxun; Rodriguez E Rene; Bedja Djahida; Gabrielson Kathleen L; Wang Yibin; Kass David A
Division of Cardiology, Department of Medicine, Johns Hopkins Medical Institutions, Ross 835, 720 Rutland Avenue, Baltimore, Maryland 21205, USA.
Nature medicine (United States) Feb 2005, 11 (2) p214-22, ISSN 1078-8956--Print 1078-8956--Linking Journal Code: 9502015
Contract/Grant No.: AG18324; AG; NIA NIH HHS United States; HL-47511; HL; NHLBI NIH HHS United States; P01-HL59408; HL; NHLBI NIH HHS United States
Publishing Model Print-Electronic; Comment in Nat Med. 2005 Feb;11(2):115-6 PMID 15692588
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Sustained cardiac pressure overload induces %%%hypertrophy%%% and pathological remodeling, frequently leading to heart failure. Genetically engineered hyperstimulation of guanosine 3',5'-cyclic monophosphate (cGMP) synthesis counters this response. Here, we show that blocking the intrinsic catabolism of cGMP with an oral phosphodiesterase-5A (PDE5A) inhibitor (sildenafil) suppresses chamber and myocyte %%%hypertrophy%%%, and improves in vivo heart function in mice exposed to chronic pressure overload induced by transverse aortic constriction. Sildenafil also reverses pre-established %%%hypertrophy%%% induced by pressure load while restoring chamber function to normal. cGMP catabolism by PDE5A increases in pressure-loaded hearts, leading to activation of cGMP-dependent protein kinase with inhibition of PDE5A. PDE5A inhibition deactivates multiple %%%hypertrophy%%% signaling pathways triggered by pressure load (the %%%calcineurin%%% /NFAT, phosphoinositide-3 kinase (PI3K)/Akt, and ERK1/2 signaling pathways). But it does not suppress %%%hypertrophy%%% induced by overexpression of %%%calcineurin%%% in vitro or Akt in vivo, suggesting upstream targeting of these pathways. PDE5A inhibition may provide a new treatment strategy for cardiac %%%hypertrophy%%% and remodeling.
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16389705 PMID: 15657416 Record Identifier: PMC544001
Direct and indirect interactions between %%%calcineurin%%%-NFAT and MEK1-extracellular %%%signal%%% -regulated kinase 1/2 signaling pathways regulate cardiac gene expression and cellular growth.
Sanna Bastiano; Bueno Orlando F; Dai Yan-Shan; Wilkins Benjamin J; Molkentin Jeffery D

Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave., MLC7020, Cincinnati, OH 45229-3039, USA.
Molecular and cellular biology (United States) Feb 2005, 25 (3) p865-78, ISSN 0270-7306--Print 0270-7306--Linking Journal Code: 8109087
Publishing Model Print; Cites Hum Mol Genet. 2000 Jul 1;9(11):1681-90 PMID 10861295; Cites Biochem Biophys Res Commun. 1991 Jan 15;174(1):393-8 PMID 1846543; Cites J Biol Chem. 2000 Dec 1;275(48):37895-901 PMID 10984495; Cites J Cell Biol. 2000 Oct 2;151(1):117-30 PMID 11018058; Cites J Biol Chem. 2001 Feb 2;276(5):3524-30 PMID 11044444; Cites EMBO J. 2000 Dec 1;19(23):6341-50 PMID 11101507; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3322-7 PMID 11248077; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3328-33 PMID 11248078; Cites Circulation. 2001 Jul 3;104(1):97-101 PMID 11435345; Cites Circulation. 2001 Jul 3;104(1):102-8 PMID 11435346; Cites J Biol Chem. 2001 Sep 14;276(37):34983-9 PMID 11435416; Cites J Cell Biol. 2001 Oct 1;155(1):27-39 PMID 11581284; Cites Mol Cell Biol. 2001 Nov;21(21):7460-9 PMID 11585926; Cites Proc Natl Acad Sci U S A. 2001 Oct 9;98(21):12283-8 PMID 11593045; Cites Proc Natl Acad Sci U S A. 2002 Jan 22;99(2):907-12 PMID 11782539; Cites Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4586-91 PMID 11904392; Cites Mol Cell Biol. 2002 Jun;22(11):3892-904 PMID 11997522; Cites J Biol Chem. 1991 Apr 25;266(12):7759-68 PMID 1850419; Cites Mol Cell Biol. 1991 Dec;11(12):6286-95 PMID 1944289; Cites Science. 1995 Jan 6;267(5194):108-11 PMID 7528941; Cites Histochem J. 1995 Apr;27(4):291-9 PMID 7635761; Cites Immunity. 1995 May;2(5):473-83 PMID 7749982; Cites Circ Res. 1996 Jun;78(6):954-61 PMID 8635245; Cites EMBO J. 1996 Aug 1;15(15):3923-33 PMID 8670897; Cites Science. 1997 Mar 28;275(5308):1930-4 PMID 9072970; Cites Nature. 1997 Apr 24;386(6627):855-8 PMID 9126747; Cites Science. 1997 Nov 28;278(5343):1638-41 PMID 9374467; Cites Mol Immunol. 1997 Jun;34(8-9):663-9 PMID 9393969; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites J Biol Chem. 1998 May 29;273(22):13367-70 PMID 9593662; Cites Cell. 1998 May 29;93(5):851-61 PMID 9630228; Cites Immunity. 1998 Jun;8(6):703-11 PMID 9655484; Cites J Biol Chem. 1998 Jul 17;273(29):18325-31 PMID 9660798; Cites Circ Res. 1998 Aug 24;83(4):345-52 PMID 9721691; Cites Physiol Rev. 1999 Jan;79(1):143-80 PMID 9922370; Cites Cell. 1999 Mar 5;96(5):611-4 PMID 10089876; Cites Science. 1999 Sep 24;285(5436):2129-33 PMID 10497131; Cites J Biol Chem. 2000 Jan 21;275(3):1855-63 PMID 10636885; Cites J Biol Chem. 2000 Feb 4;275(5):3543-51 PMID 10652349; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites Circ Res. 2000 Feb 18;86(3):255-63 PMID 10679475; Cites Immunity. 2000 Feb;12(2):151-60 PMID 10714681; Cites J Biol Chem. 2000 Mar 24;275(12):8719-25 PMID 10722714; Cites J Biol Chem. 2000 May 5;275(18):13571-9 PMID 10788473; Cites J Biol Chem. 2000 May 5;275(18):13872-8 PMID 10788511; Cites J Biol Chem. 2002 Jul 26;277(30):27073-80 PMID 12021260; Cites J Biol Chem. 2002 Dec 13;277(50):48664-76 PMID 12351631; Cites Circ Res. 2002 Oct 4;91(7):640-7 PMID 12364393; Cites Mol Cell Biol. 2002 Nov;22(21):7603-13 PMID 12370307; Cites Circ Res. 2002 Nov 1;91(9):776-81 PMID 12411391; Cites FASEB J. 2003 Apr;17(6):749-51 PMID 12594183; Cites J Clin Invest. 2003 May;111(10):1475-86 PMID 12750397; Cites Mol Cell Biol. 2003 Oct;23(19):7030-43 PMID 12972619; Cites Genes Dev. 2003 Sep 15;17(18):2205-32 PMID 12975316; Cites Proc Natl Acad Sci U S A. 1992 Jan 15;89(2):529-33 PMID 1309945; Cites EMBO J. 2003 Oct 1;22(19):5079-89 PMID 14517246; Cites Circ Res. 2004 Jan 9;94(1):110-8 PMID

14656927; Cites Mol Cell Biol. 2004 Feb;24(3):1081-95 PMID 14729955; Cites Immunity. 2004 Mar;20(3):255-66 PMID 15030770; Cites J Biol Chem. 2004 Jun 18;279(25):26192-200 PMID 15082723; Cites EMBO J. 1992 Mar;11(3):1045-54 PMID 1547771; Cites J Mol Cell Cardiol. 2000 Jun;32(6):947-60 PMID 10888249

Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.

Languages: ENGLISH

Main Citation Owner: NLM

Other Citation Owner: NLM

Record type: MEDLINE; Completed

MEK1, a member of the mitogen-activated protein kinase (MAPK) cascade that directly activates extracellular %%%signal%%%-regulated kinase (ERK), induces cardiac %%%hypertrophy%%% in transgenic mice. %%%Calcineurin%%% is a calcium-regulated protein phosphatase that also functions as a positive regulator of cardiac %%%hypertrophic%%% growth through a direct mechanism involving activation of %%%nuclear%%% factor of activated T-cell (NFAT) transcription factors. Here we determined that %%%calcineurin%%%-NFAT and MEK1-ERK1/2 signaling pathways are interdependent in cardiomyocytes, where they directly coregulate the %%%hypertrophic%%% growth response. For example, genetic deletion of the %%%calcineurin%%% Abeta gene reduced the %%%hypertrophic%%% response elicited by an activated MEK1 transgene in the heart, while inhibition of %%%calcineurin%%% or NFAT in cultured neonatal cardiomyocytes also blunted the %%%hypertrophic%%% response driven by activated MEK1. Conversely, targeted inhibition of MEK1-ERK1/2 signaling in cultured cardiomyocytes attenuated the %%%hypertrophic%%% growth response directed by activated %%%calcineurin%%%. However, targeted inhibition of MEK1-ERK1/2 signaling did not directly affect %%%calcineurin%%% -NFAT activation, nor was MEK1-ERK1/2 activation altered by targeted inhibition of %%%calcineurin%%% -NFAT. Mechanistically, we show that MEK1-ERK1/2 signaling augments NFAT transcriptional activity independent of %%%calcineurin%%%, independent of changes in NFAT %%%nuclear%%% localization, and independent of alterations in NFAT transactivation potential. In contrast, MEK1-ERK1/2 signaling enhances NFAT-dependent gene expression through an indirect mechanism involving induction of cardiac AP-1 activity, which functions as a necessary NFAT-interacting partner. As a second mechanism, MEK1-ERK1/2 and %%%calcineurin%%%-NFAT proteins form a complex in cardiac myocytes, resulting in direct phosphorylation of NFATc3 within its C terminus. MEK1-ERK1/2-mediated phosphorylation of NFATc3 directly augmented its DNA binding activity, while inhibition of MEK1-ERK1/2 signaling reduced NFATc3 DNA binding activity. Collectively, these results indicate that %%%calcineurin%%%-NFAT and MEK1-ERK1/2 pathways constitute a codependent signaling module in cardiomyocytes that coordinately regulates the growth response through two distinct mechanisms.

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2/7/64 (Item 64 from file: 154)

DIALOG(R)File 154:MEDLINE(R)

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16374315 PMID: 15389566

%%%Hypertrophy%%% and transcriptional regulation induced in myogenic cell line L6-C5 by an increase of extracellular calcium.

De Arcangelis V; Coletti D; Canato M; Molinaro M; Adamo S; Reggiani C; Naro F

Dipartimento di Istologia ed Embriologia Medica, Universita di Roma La Sapienza, Italy.
Journal of cellular physiology (United States) Mar 2005, 202 (3) p787-95, ISSN 0021-9541--Print 0021-9541--Linking Journal Code: 0050222
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Calcium plays a pivotal role in the establishment of the differentiated phenotype in myogenic cells but the involved molecular mechanisms are still matter of debate. Here we studied the effects of exposing L6-C5 myogenic cells to high extracellular Ca2+ concentration ([Ca2+]o), which induces an increase of intracellular calcium ([Ca2+]i) without involving Ca2+ release from the intracellular stores but exclusively due to plasma membrane influx (Naro et al., 2003). Exposure of L6-C5 cells to [Ca2+]o up to 20 mM for 30 min, before shifting them into a differentiative medium, induced the appearance of multinucleated, myosin-positive myotubes, much larger than in control cells with an increased protein/DNA ratio. These large myotubes showed %%%nuclear%%% accumulation of the %%%hypertrophy%%% marker GATA-2. The %%%hypertrophic%%% growth of these cells was blocked by cyclosporin A (CsA), FK506, or overexpression of a %%%calcineurin%%%-dominant negative protein, suggesting the involvement in this process of the Ca2+ responsive phosphatase %%%calcineurin%%% . Furthermore, transient exposure of L6-C5 cells to high [Ca2+]o increased the expression of luciferase reporter driven by myoglobin (Mb) and beta-MHC promoters but not IIB-MHC and MCK promoters. Luciferase transcription driven by CK promoter was, instead, enhanced by mobilizing Ca2+ from the intracellular stores. These data indicate that a transient increase of [Ca2+]i due to plasma-membrane influx is sufficient to induce a %%%hypertrophic%%% phenotype and an increased expression of slow-fiber genes but not fast-fiber genes. 2004 Wiley-Liss, Inc.
Record Date Created: 20050103
Record Date Completed: 20050407

2/7/65 (Item 65 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

16361687 PMID: 15582708
Neuropeptide Y induces cardiomyocyte %%%hypertrophy%%% via %%%calcineurin%%% signaling in rats.
Chen Minsheng; Li Xiaoyun; Dong Qi; Li Yinghui; Liang Wenbin
Department of Cardiology, The Second Affiliated Hospital of Guangzhou Medical College, Chang Gang East Road No. 250, Guangzhou 510260, PR China. dongqistar@hotmail.com
Regulatory peptides (Netherlands) Feb 15 2005, 125 (1-3) p9-15, ISSN 0167-0115--Print 0167-0115--Linking Journal Code: 8100479
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Neuropeptide Y (NPY) has been shown to participate in cardiac %%%hypertrophy%%% . However, the mechanisms by which NPY induces cardiomyocyte %%%hypertrophy%%% are poorly understood. This study tested

the hypothesis that NPY induces cardiomyocyte %%%hypertrophy%%% through Ca2+/CaM-dependent %%%calcineurin%%% (CaN) pathway in cultured neonatal rat cardiomyocytes. After 24-h treatment, NPY (100 nM) significantly increased 3H-leucine incorporation and c-Jun mRNA expression, concomitant with augment of CaN activity and protein level in cardiomyocytes compared to those cells without NPY treatment. The enhancement of 3H-leucine incorporation and c-Jun mRNA expression in cardiomyocytes treated with NPY were markedly inhibited by cyclosporine A (CsA), a selective inhibitor of CaN. We also investigated the effect of NPY on intracellular Ca2+ level in cardiomyocytes. There were no obvious changes in intracellular Ca2+ level of cytoplasm and %%%nucleus%%% in cardiomyocytes treated with NPY (100 nM) for 10 min. However, NPY significantly increased intracellular Ca2+ level of cytoplasm and %%%nucleus%%% in cardiomyocytes after 24-h treatment. The result suggested that NPY could induce %%%hypertrophy%%% of cardiomyocytes via Ca2+/CaM-dependent CaN %%%signal%%% pathway. The enhancement of [Ca2+]i caused by NPY may activate CaN %%%signal%%% pathways to mediate cardiac %%%hypertrophy%%%.

Record Date Created: 20041207

Record Date Completed: 20050524

2/7/66 (Item 66 from file: 154)

DIALOG(R)File 154:MEDLINE(R)

(c) format only 2010 Dialog. All rts. reserv.

16082835 PMID: 15294044

The role of calcium and calcium/calmodulin-dependent kinases in skeletal muscle plasticity and mitochondrial biogenesis.

Chin Eva R

Department of Cardiovascular and Metabolic Diseases, Pfizer Global Research & Development, Eastern Point Rd., MS8220-3120, Groton, CT 06340, USA. eva r chin@groton.pfizer.com

Proceedings of the Nutrition Society (England) May 2004, 63 (2) p279-86, ISSN 0029-6651--Print 0029-6651--Linking Journal Code: 7505881

Publishing Model Print

Document type: Journal Article; Review

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Intracellular Ca(2+) plays an important role in skeletal muscle excitation-contraction coupling and also in excitation-transcription coupling. Activity-dependent alterations in muscle gene expression as a result of increased load (i.e. resistance or endurance training) or decreased activity (i.e. immobilization or injury) are tightly linked to the level of muscle excitation. Differential expression of genes in slow- and fast-twitch fibres is also dependent on fibre activation. Both these biological phenomena are, therefore, tightly linked to the amplitude and duration of the Ca(2+) transient, a %%%signal%%% decoded downstream by Ca(2+)-dependent transcriptional pathways. Evidence is mounting that the %%%calcineurin%%%-%%%nuclear%%% factor of activated T-cells pathway and the Ca(2+)/calmodulin-dependent kinases (CaMK) II and IV play important roles in regulating oxidative enzyme expression, mitochondrial biogenesis and expression of fibre-type specific myofibrillar proteins. CaMKII is known to decode frequency-dependent information and is activated during %%%hypertrophic%%% growth and endurance adaptations. Thus, it was hypothesized that CaMKII, and possibly CaMKIV, are down regulated during muscle atrophy and levels of expression of CaMKII alpha, -II beta, -II

gamma and -IV were assessed in skeletal muscles from young, aged and denervated rats. The results indicate that CaMKII gamma, but not CaMKIIalpha or -beta, is up regulated in aged and denervated soleus muscle and that CaMKIV is absent in skeletal but not cardiac muscle. Whether CaMKII gamma up-regulation is part of the pathology of wasting or a result of some adaptational response to atrophy is not known. Future studies will be important in determining whether insights from the adaptational response of muscle to increased loads will provide pharmacological approaches for increasing muscle strength or endurance to counter muscle wasting. (36 Refs.)

Record Date Created: 20040805

Record Date Completed: 20050408

2/7/67 (Item 67 from file: 154)

DIALOG(R)File 154:MEDLINE(R)

(c) format only 2010 Dialog. All rts. reserv.

16066013 PMID: 15276480

Heme oxygenase-1 inhibition of MAP kinases, %%%calcineurin%%%/NFAT signaling, and %%%hypertrophy%%% in cardiac myocytes.

Tongers Jorn; Fiedler Beate; Konig Danny; Kempf Tibor; Klein Gunnar; Heineke Jorg; Kraft Theresia; Gambaryan Stepan; Lohmann Suzanne M; Drexler Helmut; Wollert Kai C

Department of Cardiology and Angiology, Hannover Medical School, 30625 Hannover, Germany.

Cardiovascular research (Netherlands) Aug 15 2004, 63 (3) p545-52, ISSN 0008-6363--Print 0008-6363--Linking Journal Code: 0077427

Publishing Model Print

Document type: Journal Article; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

OBJECTIVE: Heme oxygenases (HO) are the rate-limiting enzymes in heme degradation, catalyzing the breakdown of heme to equimolar quantities of biliverdin (BV), carbon monoxide (CO), and ferrous iron. The inducible HO isoform, HO-1, confers protection against ischemia/reperfusion (I/R)-injury in the heart. We hypothesized that HO-1 and its catalytic by-products constitute an antihypertrophic signaling module in cardiac myocytes. METHODS AND RESULTS: The G protein-coupled receptor (GPCR) agonist endothelin-1 (ET-1) (30 nmol/l) stimulated a robust %%%hypertrophic%%% response in cardiac myocytes isolated from 1- to 3-day-old Sprague-Dawley rats, with increases in cell surface area (planimetry), sarcomere assembly (confocal laser scanning microscopy), and prepro-atrial natriuretic peptide (ANP) mRNA expression. Adenoviral overexpression of HO-1, but not beta-galactosidase, significantly inhibited ET-1 induced cardiac myocyte %%%hypertrophy%%%. The antihypertrophic effects of HO-1 were mimicked by BV (10 micromol/l) and the CO-releasing molecule [Ru(CO)3Cl2]2 (10 micromol/l), strongly suggesting a critical involvement of BV and CO in the antihypertrophic effects of HO-1. Both BV and CO suppressed extracellular %%%signal%%% -regulated kinases (ERK1/ERK2) and p38 mitogen-activated protein kinase (MAPK) activation by ET-1 stimulation. Moreover, BV and CO inhibited the prohypertrophic %%%calcineurin%%% /NFAT pathway. This inhibition occurred upstream from %%%calcineurin%%% because BV and CO inhibited NFAT activation in response to ET-1 stimulation but not in response to adenoviral expression of a constitutively active %%%calcineurin%%% mutant. Upstream-inhibition of the %%%calcineurin%%%/NFAT

pathway by CO occurred independent from cGMP and cGMP-dependent protein kinase type I (PKG I). CONCLUSIONS: Heme oxygenase-1 and its catalytic by-products, BV and CO, constitute a novel antihypertrophic signaling pathway in cardiac myocytes. Biliverdin and CO inhibition of MAPKs and %%%calcineurin%%% /NFAT signaling provides a mechanistic framework how heme degradation products may promote their antihypertrophic effects. Copryright 2004 European Society of Cardiology
Record Date Created: 20040727
Record Date Completed: 20041007

2/7/68 (Item 68 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

16066012 PMID: 15276479
Contractile arrest reveals calcium-dependent stimulation of SERCA2a mRNA expression in cultured ventricular cardiomyocytes.
Vlasblom Ronald; Muller Alice; Musters Rene J P; Zuidwijk Marian J; Van Hardeveld Cornelis; Paulus Walter J; Simonides Warner S
Laboratory for Physiology, Institute for Cardiovascular Research (ICaR-VU), VU University Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.
Cardiovascular research (Netherlands) Aug 15 2004, 63 (3) p537-44, ISSN 0008-6363--Print 0008-6363--Linking Journal Code: 0077427
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
OBJECTIVE: Downregulation of sarco-endoplasmic reticulum calcium ATPase 2a (SERCA2a) expression is a critical marker of pathological myocardial %%%hypertrophy%%% . The effects of calcium-dependent signaling and of contractile activity on the regulation of myocardial SERCA2a expression remain unclear. The present study dissociates effects of calcium-dependent signaling through %%%calcineurin%%% (CN) and calmodulin dependent protein kinase-II (CAMK-II), from effects of contractile activity in spontaneously contracting rat neonatal ventricular cardiomyocytes (NVCM) using 2,3-butanedione monoxime (BDM), which arrests contractions but maintains calcium fluxes. METHODS: SERCA2a mRNA expression was analysed using Northern hybridisation in spontaneously contracting NVCM (control) and in NVCM treated with either BDM, L-type Ca2+-channel blocker (verapamil), CN-blocker (cyclosporin A; CsA), CAMK-II blocker (KN-93), or combinations thereof. Transient transfection of the CN-dependent transcription factor %%%nuclear%%% factor of activated T-lymphocytes (NFATc), coupled to GFP, was used to detect NFAT %%%nuclear%%% translocation. The effects of CN/CAMK-II-dependent signaling were further dissected into effects of the transcription factors NFATc4 and myocyte enhancer factor 2c (MEF2c) on the activity of various SERCA2a promoter fragments using transient transfection assays. RESULTS: Treatment with BDM induced a 2.5-fold rise in SERCA2a mRNA, which was abolished by addition of verapamil and was reduced by addition of CsA (-40%) and KN-93 (-20%). NFAT %%%nuclear%%% translocation was similar in control and BDM-treated NVCM. SERCA2a promoter activity was stimulated by NFATc4 and MEF2c, but only when both factors were co-transfected. CONCLUSION: Following contractile arrest with BDM, upregulation of SERCA2a mRNA expression by CN/CAMK-II signaling becomes evident. This upregulation is likely the result of synergistic stimulation

of SERCA2a promoter activity by NFATc4 and MEF2c. Contractile activity opposes this upregulation through distinct and independent pathways.
Copryright 2004 European Society of Cardiology
Record Date Created: 20040727
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2/7/69 (Item 69 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

16066005 PMID: 15276472
%%%Calcineurin%%%-NFAT signaling regulates the cardiac %%%hypertrophic%%% response in coordination with the MAPKs.
Molkentin Jeffery D
Division of Molecular Cardiovascular Biology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA. jeff.molkentin@cchmc.org
Cardiovascular research (Netherlands) Aug 15 2004, 63 (3) p467-75,
ISSN 0008-6363--Print 0008-6363--Linking Journal Code: 0077427
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Prolonged cardiac %%%hypertrophy%%% of pathologic etiology is associated with arrhythmia, sudden death, decompensation, and dilated %%%cardiomyopathy%%% . In an attempt to understand the mechanisms that underlie the %%%hypertrophic%%% response, extensive investigation has centered on a characterization of the molecular pathways that initiate or maintain the pathologic growth of individual cardiac myocytes. While a large number of %%%signal%%% transduction cascades have been identified as critical regulators of cardiac %%%hypertrophy%%% , here the scientific evidence implicating the protein phosphatase %%%calcineurin%%% (PP2B) and the mitogen-activated protein kinases (MAPK) as co-regulators of reactive %%%hypertrophy%%% will be discussed. Gain- and loss-of-function studies in genetically altered mice and in cultured cardiomyocytes have demonstrated the necessity and sufficiency of %%%calcineurin%%% to regulate pathologic cardiac %%%hypertrophy%%%. However, using similar approaches, the %%%hypertrophic%%% regulatory role attributed to various branches of the MAPK signaling pathway has been less conclusive, although a loose consensus suggests that the c-Jun N-terminal kinases (JNK) and p38 kinases function as mediators of dilated %%%cardiomyopathy%%%, while extracellular %%%signal%%%-regulated kinases (ERKs) function as regulators of %%%hypertrophy%%%. More recently, the actions of %%%calcineurin%%% and MAPK signaling pathways have been shown to be co-dependent such that unitary activation of %%%calcineurin%%% in myocytes leads to up-regulation in ERK and JNK signaling, but down-regulation in p38 signaling. Conversely, unitary activation of JNK or p38 in cardiac myocytes leads to down-regulation of %%%calcineurin%%% effectiveness by directly antagonizing %%%nuclear%%% factor of activated T cells (NFAT) %%%nuclear%%% occupancy. Thus, an emerging paradigm suggests that %%%calcineurin%%%-NFAT and MAPK signaling pathways are inter-dependent and together orchestrate the cardiac %%%hypertrophic%%% response. Copryright 2004 European Society of Cardiology (91 Refs.)
Record Date Created: 20040727

Record Date Completed: 20041007

2/7/70 (Item 70 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

16066003 PMID: 15276470
Interference of antihypertrophic molecules and signaling pathways with the Ca2+-%%%calcineurin%%%-NFAT cascade in cardiac myocytes.
Fiedler Beate; Wollert Kai C
Department of Cardiology and Angiology, Hannover Medical School, Carl-Neuberg Str. 1, 30625, Hannover, Germany.
Cardiovascular research (Netherlands) Aug 15 2004, 63 (3) p450-7, ISSN 0008-6363--Print 0008-6363--Linking Journal Code: 0077427
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Cardiac %%%hypertrophy%%% occurs in a number of disease states associated with chronic increases in cardiac work load. Although cardiac %%%hypertrophy%%% may initially represent an adaptive response of the myocardium, ultimately, it often progresses to ventricular dilatation and heart failure. Much investigation has focused on the signaling pathways controlling cardiac %%%hypertrophy%%% at the level of the single cardiac myocyte. One prohypertrophic pathway that has received much attention involves the ubiquitously expressed Ca2+/calmodulin-activated phosphatase %%%calcineurin%%%. Upon activation by Ca2+, %%%calcineurin%%% dephosphorylates %%%nuclear%%% factor of activated T cell (NFAT) transcription factors, leading to their %%%nuclear%%% translocation. As common in complex biological systems, cardiac %%%hypertrophy%%% is controlled simultaneously by stimulatory (prohypertrophic) and counter-regulatory (antihypertrophic) pathways. Given the potent prohypertrophic effects of the Ca2+-%%%calcineurin%%% -NFAT pathway in cardiac myocytes, it is not surprising that the activity of this pathway is tightly controlled at multiple levels. Inhibitory mechanisms upstream (nitric oxide (NO), cGMP, cGMP-dependent protein kinase type I (PKG I), heme oxygenase-1 (HO-1), biliverdin, carbon monoxide (CO)) and downstream from %%%calcineurin%%% (glycogen synthase kinase-3 (GSK3), c-Jun N-terminal kinases (JNKs), p38 mitogen-activated protein kinase (MAPKs)) have been described. Moreover, several inhibitors directly target %%%calcineurin%%% enzymatic activity (cyclosporine A (CsA), tacrolimus (FK506), %%%calcineurin%%%-binding protein-1 (Cabin-1)/%%%calcineurin%%%-inhibitory protein (Cain), A-kinase-anchoring protein-79 (AKAP79), %%%calcineurin%%% B homology protein (CHP), MCIPs, VIVIT). Considering the dominant role of the %%%calcineurin%%% pathway in cardiac %%%hypertrophy%%% and failure, %%%calcineurin%%% -inhibitory strategies may lead to the identification of novel therapeutic approaches for patients with cardiac disease. Copryright 2004 European Society of Cardiology (79 Refs.)
Record Date Created: 20040727
Record Date Completed: 20041007

2/7/71 (Item 71 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

16000195 PMID: 15201149
Mechanotransduction in cardiac myocytes.
Lammerding Jan; Kamm Roger D; Lee Richard T
Biological Engineering Division, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.
Annals of the New York Academy of Sciences (United States) May 2004, 1015 p53-70, ISSN 0077-8923--Print 0077-8923--Linking Journal Code: 7506858
Publishing Model Print
Document type: Journal Article; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Cardiac myocytes react to diverse mechanical demands with a multitude of transient and long-term responses to normalize the cellular mechanical environment. Several stretch-activated signaling pathways have been identified, most prominently guanine %%%nucleotide%%% binding proteins (G-proteins), mitogen-activated protein kinases (MAPK), Janus-associated kinase/%%%signal%%% transducers and activators of transcription (JAK/STAT), protein kinase C (PKC), %%%calcineurin%%% , intracellular calcium regulation, and several autocrine and paracrine factors. Multiple levels of crosstalk exist between pathways. The cellular response to changes in the mechanical environment can lead to cardiac myocyte %%%hypertrophy%%%, cellular growth that can be accompanied by pathological myocyte dysfunction, and tissue fibrosis. Several candidates for the primary mechanosensor in cardiac myocytes have been identified, ranging from stretch-activated ion channels in the membrane to yet-unknown mechanosensitive mechanisms in the %%%nucleus%%% . New and refined experimental techniques will exploit advances in molecular biology and biological imaging to study mechanotransduction in isolated cells and genetically engineered mice to explore the function of individual proteins. (101 Refs.)
Record Date Created: 20040617
Record Date Completed: 20040728

2/7/72 (Item 72 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

15994559 PMID: 15082723
Genetic loss of %%%calcineurin%%% blocks mechanical overload-induced skeletal muscle fiber type switching but not %%%hypertrophy%%%.
Parsons Stephanie A; Millay Douglas P; Wilkins Benjamin J; Bueno Orlando F; Tsika Gretchen L; Neilson Joel R; Liberatore Christine M; Yutzey Katherine E; Crabtree Gerald R; Tsika Richard W; Molkentin Jeffery D
Department of Pediatrics, University of Cincinnati, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229-3039, USA.
Journal of biological chemistry (United States) Jun 18 2004, 279 (25) p26192-200, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R
Contract/Grant No.: 5T32 HL07382; HL; NHLBI NIH HHS United States
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Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.

Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

The serine/threonine phosphatase %%%calcineurin%%% is an important regulator of calcium-activated intracellular responses in eukaryotic cells. In higher eukaryotes, calcium/calmodulin-mediated activation of %%%calcineurin%%% facilitates direct dephosphorylation and %%%nuclear%%% translocation of the transcription factor %%%nuclear%%% factor of activated T-cells (NFAT). Recently, controversy has surrounded the role of %%%calcineurin%%% in mediating skeletal muscle cell %%%hypertrophy%%%. Here we examined the ability of %%%calcineurin%%%-deficient mice to undergo skeletal muscle %%%hypertrophic%%% growth following mechanical overload (MOV) stimulation or insulin-like growth factor-1 (IGF-1) stimulation. Two distinct models of %%%calcineurin%%% deficiency were employed: %%%calcineurin%%% Abeta gene-targeted mice, which show a approximately 50% reduction in total %%%calcineurin%%%, and %%%calcineurin%%% B1-LoxP-targeted mice crossed with a myosin light chain 1f cre knock-in allele, which show a greater than 80% loss of total %%%calcineurin%%% only in skeletal muscle. %%%Calcineurin%%% Abeta-/- and %%%calcineurin%%% B1-LoxP(fl/fl)-MLC-cre mice show essentially no defects in muscle growth in response to IGF-1 treatment or MOV stimulation, although %%%calcineurin%%% Abeta-/- mice show a basal defect in total fiber number in the plantaris and a mild secondary reduction in growth, consistent with a developmental defect in myogenesis. Both groups of gene-targeted mice show normal increases in Akt activation following MOV or IGF-1 stimulation. However, overload-mediated fiber-type switching was dramatically impaired in %%%calcineurin%%% B1-LoxP(fl/fl)-MLC-cre mice. NFAT-luciferase reporter transgenic mice failed to show a correlation between IGF-1- or MOV-induced %%%hypertrophy%%% and %%%calcineurin%%%-NFAT-dependent signaling in vivo. We conclude that %%%calcineurin%%% expression is important during myogenesis and fiber-type switching, but not for muscle growth in response to %%%hypertrophic%%% stimuli.

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15886337 PMID: 14742441

Involvement of %%%calcineurin%%% in transforming growth factor-beta-mediated regulation of extracellular matrix accumulation.

Gooch Jennifer L; Gorin Yves; Zhang Bin-Xian; Abboud Hanna E
Department of Medicine, Division of Nephrology, University of Texas Health Science Center, San Antonio, Texas 78229-3900, USA. Gooch@uthscsa.edu

Journal of biological chemistry (United States) Apr 9 2004, 279 (15) p15561-70, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R
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Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.
Languages: ENGLISH
Main Citation Owner: NLM

Record type: MEDLINE; Completed

%%%Calcineurin%%% is a calcium-dependent, serine/threonine phosphatase that functions as a signaling intermediate. In this study, we investigated the role of %%%calcineurin%%% in transforming growth factor-beta (TGF-beta)-mediated cellular effects and examined the signaling pathway involved in activation of %%%calcineurin%%%. %%%Calcineurin%%% is activated by TGF-beta in a time- and dose-dependent manner. Consistent with increased phosphatase activity, the %%%calcineurin%%% substrate, NFATc1, is dephosphorylated and transported to the %%%nucleus%%%. Inhibition of %%%calcineurin%%% prior to the addition of TGF-beta revealed that %%%calcineurin%%% is required for TGF-beta-mediated accumulation of extracellular matrix (ECM) proteins but not cell %%%hypertrophy%%% . Conversely, overexpression of constitutively active %%%calcineurin%%% was sufficient to induce ECM protein expression. The mechanism of %%%calcineurin%%% activation by TGF-beta was found to be induction of a low, sustained increase of intracellular calcium. Chelation of extracellular calcium blocked both TGF-beta-mediated calcium influx and %%%calcineurin%%% activity. Finally, calcium entry was found to be dependent upon generation of reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide. Accordingly, inhibition of ROS generation also blocked TGF-beta-mediated %%%calcineurin%%% phosphatase activity and decreased ECM accumulation. In conclusion, this study describes a new pathway for TGF-beta-mediated regulation of ECM via generation of ROS, calcium influx, and activation of %%%calcineurin%%%.

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Date of Electronic Publication: 20040123

2/7/74 (Item 74 from file: 154)
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15844120 PMID: 15012912

Increased regulatory activity of the %%%calcineurin%%%/NFAT pathway in human heart failure.

Diedrichs Holger; Chi Mei; Boelck Birgit; Mehlhorn Uwe; Schwinger Robert H G
Laboratory of Muscle Research and Molecular Cardiology, University of Cologne, Joseph-Stelzmann-Str. 9, 50924 Cologne, Germany.

European journal of heart failure - journal of the Working Group on Heart Failure of the European Society of Cardiology (Netherlands) Jan 2004, 6 (1) p3-9, ISSN 1388-9842--Print 1388-9842--Linking Journal Code: 100887595
Publishing Model Print; Erratum in Eur J Heart Fail. 2004 Oct;6(6):823 Note Mehlhorm, Uwe [corrected to Mehlhorn, Uwe]
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

BACKGROUND: Cardiac %%%hypertrophy%%% may initiate progression to a compromised cardiac function. While the clinical consequences of %%%hypertrophy%%% are well understood, only little is known about the underlying molecular pathways. As reported from animal experiments, the Ca(2+)-calmodulin activated phosphatase %%%calcineurin%%% and its downstream transcriptional effector NFAT have been implicated as transducers of the %%%hypertrophic%%% response. METHODS AND RESULTS: To

study whether the %%%calcineurin%%% pathway is activated in human heart failure, we investigated samples of human left ventricular myocardium from patients with dilated (idiopathic) %%%cardiomyopathy%%% (DCM, NYHA IV, n=8) in comparison with non-failing controls (NF, n=8). We not only analyzed the pathway by measuring the %%%calcineurin%%% activity, but also by determination of the protein expression of the %%%calcineurin%%% B subunit and additional key markers of the %%%calcineurin%%% signaling cascade (NFAT-3, GATA-4). %%%Calcineurin%%% enzymatic activity was increased by 80% in human dilated %%%cardiomyopathy%%% compared with non-failing human hearts (135.424+/-11.69 and 83.484+/-1.81 nmol Pi/min per microl). This was in line with increased protein expression of %%%calcineurin%%% B in DCM (71.18+9.11 vs. 46.41+/-11.23 densitometric units (DU)/microg protein). In order to verify the activated %%%calcineurin%%% pathway as described in animal models, we compared the protein expression of NFAT-3 in homogenates within %%%nuclear%%% extracts. In %%%nuclear%%% extracts the protein level of NFAT-3 was increased in dilated %%%cardiomyopathy%%% compared with non-failing myocardium (104.01+/-8.85 vs. 71.47+/-8.79 DU/microg protein). In contrast, in homogenates the expression of NFAT-3 was higher in the non-failing tissue indicating subcellular redistribution (19.56+/-3.36 vs. 25.84+/-3.16 DU/microg protein). The protein expression of GATA-4 was increased in DCM (43.14+/-2.89 vs. 29.87+/-2.17 DU/microg protein). CONCLUSIONS: In human heart failure (DCM) the %%%calcineurin%%% signaling pathway is activated not only by an increased activity of %%%calcineurin%%% and expression of GATA-4, but also by the shift from dephosphorylated NFAT-3 to the %%%nucleus%%% indicating subcellular redistribution and regulatory activation.

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15790799 PMID: 14749501

Cardiomyocyte calcium and calcium/calmodulin-dependent protein kinase II: friends or foes?

Zhang Tong; Miyamoto Shigeki; Brown Joan Heller
Department of Pharmacology, University of California, San Diego, La Jolla, California 92093, USA.

Recent progress in hormone research (United States) 2004, 59 p141-68 , ISSN 0079-9963--Print 0079-9963--Linking Journal Code: 0404471
Contract/Grant No.: HL-28143; HL; NHLBI NIH HHS United States; HL-46345; HL; NHLBI NIH HHS United States
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Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
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Calcium (Ca(2+)) is a critical second messenger in cell signaling. Elevated intracellular Ca(2+) can activate numerous Ca(2+)-regulated enzymes. These enzymes have different subcellular localizations and may respond to distinct modes of Ca(2+) mobilization. In cardiac muscle, Ca(2+) plays a central role in regulating contractility, gene expression, %%%hypertrophy%%%, and apoptosis. Many cellular responses to Ca(2+) signals are mediated by Ca(2+)/calmodulin-dependent enzymes, among which is the

Ca(2+)/calmodulin-dependent protein kinase II (CaMKII). Putative substrates for CaMKII include proteins involved in regulating Ca(2+) storage and release, transcription factors, and ion channels. The major isoform of CaMKII in the heart is CaMKIIdelta. Two cardiac splice variants, CaMKIIdelta(B) and delta(C), differ in whether they contain a %%%nuclear%%% localization sequence. Our laboratory has examined the hypothesis that the %%%nuclear%%% delta(B) and the cytoplasmic delta(C) isoforms respond to different Ca(2+) stimuli and have distinct effects on %%%hypertrophic%%% cardiac growth and Ca(2+) handling. We have shown that pressure overload-induced %%%hypertrophy%%% differentially affects the %%%nuclear%%% delta(B) and the cytoplasmic delta(C) isoforms of CaMKII. Additionally, using isolated myocytes and transgenic mouse models, we demonstrated that the %%%nuclear%%% CaMKIIdelta(B) isoform plays a key role in cardiac gene expression associated with cardiac %%%hypertrophy%%% . The cytoplasmic CaMKIIdelta(C) isoform phosphorylates substrates involved in Ca(2+) handling. Dysregulation of intracellular Ca(2+) and resulting changes in excitation-contraction coupling characterize heart failure and can be induced by in vivo overexpression of CaMKIIdelta(C) and phosphorylation of its substrates. The differential location of CaMKII isoforms and their relative activation by physiological vs. pathological stimuli may provide a paradigm for exploring and elucidating how Ca(2+)/CaMKII pathways can serve as both friends and foes in the heart. (133 Refs.)
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Record Date Completed: 20040429

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DIALOG(R)File 154:MEDLINE(R)
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15778030 PMID: 14729474
%%%Calcineurin%%% signaling and NFAT activation in cardiovascular and skeletal muscle development.
Schulz Robert A; Yutzey Katherine E
Department of Biochemistry and Molecular Biology, Graduate Program in Genes and Development, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. raschulz@mdanderson.org
Developmental biology (United States) Feb 1 2004, 266 (1) p1-16, ISSN 0012-1606--Print 0012-1606--Linking Journal Code: 0372762
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
%%%Calcineurin%%% signaling has been implicated in a broad spectrum of developmental processes in a variety of organ systems. %%%Calcineurin%%% is a calmodulin-dependent, calcium-activated protein phosphatase composed of catalytic and regulatory subunits. The serine/threonine-specific phosphatase functions within a %%%signal%%% transduction pathway that regulates gene expression and biological responses in many developmentally important cell types. %%%Calcineurin%%% signaling was first defined in T lymphocytes as a regulator of %%%nuclear%%% factor of activated T cells (NFAT) transcription factor %%%nuclear%%% translocation and activation. Recent studies have demonstrated the vital nature of calcium/ %%%calcineurin%%% /NFAT signaling in cardiovascular and skeletal muscle development in vertebrates. Inhibition, mutation, or forced expression of

%%%calcineurin%%% pathway genes result in defects or alterations in cardiomyocyte maturation, heart valve formation, vascular development, skeletal muscle differentiation and fiber-type switching, and cardiac and skeletal muscle %%%hypertrophy%%%. Conserved %%%calcineurin%%% genes are found in invertebrates such as Drosophila and Caenorhabditis elegans, and genetic studies have demonstrated specific myogenic functions for the phosphatase in their development. The ability to investigate %%%calcineurin%%% signaling pathways in vertebrates and model genetic organisms provides a great potential to more fully comprehend the functions of %%%calcineurin%%% and its interacting genes in heart, blood vessel, and muscle development. (129 Refs.)
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Record Date Completed: 20040330

2/7/77 (Item 77 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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15771729 PMID: 14656927
%%%Calcineurin%%% /NFAT coupling participates in pathological, but not physiological, cardiac %%%hypertrophy%%%.
Wilkins Benjamin J; Dai Yan-Shan; Bueno Orlando F; Parsons Stephanie A; Xu Jian; Plank David M; Jones Fred; Kimball Thomas R; Molkentin Jeffery D
Division of Molecular Cardiovascular Biology, Department of Pediatrics, Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, Ohio 45229-3039, USA.
Circulation research (United States) Jan 9 2004, 94 (1) p110-8,
ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103
Contract/Grant No.: 5T32 HL07382; HL; NHLBI NIH HHS United States
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Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
%%%Calcineurin%%% (PP2B) is a calcium/calmodulin-activated, serine-threonine phosphatase that transmits signals to the %%%nucleus%%% through the dephosphorylation and translocation of %%%nuclear%%% factor of activated T cell (NFAT) transcription factors. Whereas %%%calcineurin%%% -NFAT signaling has been implicated in regulating the %%%hypertrophic%%% growth of the myocardium, considerable controversy persists as to its role in maintaining versus initiating %%%hypertrophy%%% , its role in pathological versus physiological %%%hypertrophy%%%, and its role in heart failure. To address these issues, NFAT-luciferase reporter transgenic mice were generated and characterized. These mice showed robust and %%%calcineurin%%% -specific activation in the heart that was inhibited with cyclosporin A. In the adult heart, NFAT-luciferase activity was upregulated in a delayed, but sustained manner throughout eight weeks of pathological cardiac %%%hypertrophy%%% induced by pressure-overload, or more dramatically following myocardial infarction-induced heart failure. In contrast, physiological %%%hypertrophy%%% as produced in two separate models of exercise training failed to show significant %%%calcineurin%%% -NFAT coupling in the heart at multiple time points, despite measurable increases in heart to body weight ratios. Moreover, stimulation of %%%hypertrophy%%% with growth hormone-insulin-like growth factor-1 (GH-IGF-1) failed to activate %%%calcineurin%%%-NFAT signaling in the heart

or in culture, despite %%%hypertrophy%%%, activation of Akt, and activation of p70 S6K. %%%Calcineurin%%% Abeta gene-targeted mice also showed a normal %%%hypertrophic%%% response after GH-IGF-1 infusion. Lastly, exercise- or GH-IGF-1-induced cardiac growth failed to show induction of %%%hypertrophic%%% marker gene expression compared with pressure-overloaded animals. Although a direct cause-and-effect relationship between NFAT-luciferase activity and pathological %%%hypertrophy%%% was not proven here, our results support the hypothesis that separable signaling pathways regulate pathological versus physiological %%%hypertrophic%%% growth of the myocardium, with %%%calcineurin%%% -NFAT potentially serving a regulatory role that is more specialized for maladaptive %%%hypertrophy%%% and heart failure.
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Date of Electronic Publication: 20031201

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15662155 PMID: 14645134
Regulation of gene expression by cyclic GMP.
Pilz Renate B; Casteel Darren E
Department of Medicine and Cancer Center, University of California at San Diego, 9500 Gilman Dr, La Jolla, Calif 92093-0652, USA. rpilz@ucsd.edu
Circulation research (United States) Nov 28 2003, 93 (11) p1034-46, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103
Contract/Grant No.: R01-GM55586; GM; NIGMS NIH HHS United States
Publishing Model Print
Document type: Journal Article; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Cyclic GMP, produced in response to nitric oxide and natriuretic peptides, is a key regulator of vascular smooth muscle cell contractility, growth, and differentiation, and is implicated in opposing the pathophysiology of hypertension, cardiac %%%hypertrophy%%% , atherosclerosis, and vascular injury/restenosis. cGMP regulates gene expression both positively and negatively at transcriptional as well as at posttranscriptional levels. cGMP-regulated transcription factors include the cAMP-response element binding protein CREB, the serum response factor SRF, and the %%%nuclear%%% factor of activated T cells NF/AT. cGMP can regulate CREB directly, through phosphorylation by cGMP-dependent protein kinase, or indirectly, through activation of mitogen-activated protein kinase pathways; regulation of SRF and NF/AT by cGMP is indirect, through modulation of RhoA and %%%calcineurin%%% signaling, respectively. Downregulation of the RNA-binding protein HuR by cGMP leads to destabilization of guanylate cyclase mRNA, but this posttranscriptional mechanism may affect many more cGMP-regulated genes. In this review, we discuss the role of cGMP-regulated gene expression in (patho)physiological processes most relevant to the cardiovascular system, such as regulation of vascular tone, cardiac %%%hypertrophy%%%, phenotypic modulation of vascular smooth muscle cells, and regulation of cell proliferation and apoptosis. (144 Refs.)
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Record Date Completed: 20031211

2/7/79 (Item 79 from file: 154)
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15643106 PMID: 14623297
%%%Calcineurin%%% anchoring and cell signaling.
Dodge Kimberly L; Scott John D
Howard Hughes Medical Institute, Vollum Institute, Oregon Health and Sciences University, Portland, OR, USA.
Biochemical and biophysical research communications (United States) Nov 28 2003, 311 (4) p1111-5, ISSN 0006-291X--Print 0006-291X--Linking Journal Code: 0372516
Publishing Model Print
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
The targeting of phosphatase PP2B or %%%calcineurin%%% toward certain substrates synchronizes a variety of physiological processes. This review emphasizes how the targeting of %%%calcineurin%%% through interaction with various anchoring proteins facilitates phosphatase regulation of T-cell activation, neuronal excitability and cardiac %%%hypertrophy%%%. (37 Refs.)
Record Date Created: 20031118
Record Date Completed: 20040209

2/7/80 (Item 80 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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15566991 PMID: 14517246 Record Identifier: PMC204458
c-Jun N-terminal kinases (JNK) antagonize cardiac growth through cross-talk with calcineurin-NFAT signaling.
Liang Qiangrong; Bueno Orlando F; Wilkins Benjamin J; Kuan Chia-Yi; Xia Ying; Molkentin Jeffery D
Department of Pediatrics, University of Cincinnati, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA.
EMBO journal (England) Oct 1 2003, 22 (19) p5079-89, ISSN 0261-4189 --Print 0261-4189--Linking Journal Code: 8208664
Publishing Model Print; Cites Cell. 2001 Jun 29;105(7):863-75 PMID 11439183; Cites Circulation. 2000 Jul 25;102(4):470-9 PMID 10908222; Cites J Biol Chem. 2000 Dec 22;275(51):40120-7 PMID 10969079; Cites Cell. 2000 Oct 13;103(2):239-52 PMID 11057897; Cites Biochem J. 2000 Nov 15;352 Pt 1:145-54 PMID 11062067; Cites Circulation. 2001 Mar 13;103(10):1453-8 PMID 11245652; Cites Methods Enzymol. 2001;332:319-36 PMID 11305107; Cites EMBO J. 2001 Jun 1;20(11):2757-67 PMID 11387209; Cites Proc Natl Acad Sci U S A. 2001 Oct 9;98(21):12283-8 PMID 11593045; Cites Proc Natl Acad Sci U S A. 2002 Jan 22;99(2):907-12 PMID 11782539; Cites Curr Opin Genet Dev. 2002 Feb;12(1):14-21 PMID 11790549; Cites J Biol Chem. 2002 Apr 19;277(16):14266-73 PMID 11827959; Cites Proc Natl Acad Sci U S A. 2002 Mar 19;99(6):3866-71 PMID 11891332; Cites Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4586-91 PMID 11904392; Cites Cell. 2002 Apr;109 Suppl:S67-79 PMID 11983154; Cites Mol Cell Biol. 2002

Jun;22(11):3892-904 PMID 11997522; Cites J Physiol. 2002 May 15;541(Pt 1):1-8 PMID 12015416; Cites Mol Cell. 2002 May;9(5):945-56 PMID 12049732; Cites Mol Cell Biol. 2002 Jul;22(13):4929-42 PMID 12052897; Cites Proc Natl Acad Sci U S A. 2002 Jul 9;99(14):9398-403 PMID 12091710; Cites J Clin Invest. 2002 Jul;110(2):271-9 PMID 12122119; Cites Circ Res. 2002 Oct 4;91(7):640-7 PMID 12364393; Cites Mol Cell Biol. 2002 Nov;22(21):7603-13 PMID 12370307; Cites J Clin Invest. 2003 May;111(10):1475-86 PMID 12750397; Cites Biochem J. 1977 Dec 15;168(3):599-601 PMID 147077; Cites Nucleic Acids Res. 1989 Aug 11;17(15):6419 PMID 2771659; Cites Science. 1995 Jan 20;267(5196):389-93 PMID 7824938; Cites EMBO J. 1996 Jun 3;15(11):2760-70 PMID 8654373; Cites J Biol Chem. 1996 Oct 4;271(40):24313-6 PMID 8798679; Cites J Biol Chem. 1996 Nov 1;271(44):27205-8 PMID 8910287; Cites J Biol Chem. 1997 May 30;272(22):14057-61 PMID 9162028; Cites Nature. 1997 Oct 23;389(6653):865-70 PMID 9349820; Cites Science. 1997 Nov 28;278(5343):1638-41 PMID 9374467; Cites J Biol Chem. 1998 Mar 6;273(10):5423-6 PMID 9488659; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites J Clin Invest. 1998 Oct 1;102(7):1311-20 PMID 9769323; Cites Cell. 1999 Mar 5;96(5):611-4 PMID 10089876; Cites Curr Opin Cell Biol. 1999 Apr;11(2):211-8 PMID 10209154; Cites Neuron. 1999 Apr;22(4):667-76 PMID 10230788; Cites J Clin Invest. 1999 Aug;104(4):391-8 PMID 10449431; Cites Mol Cell Biol. 1999 Nov;19(11):7539-48 PMID 10523642; Cites Cell Death Differ. 1999 Oct;6(10):987-91 PMID 10556976; Cites Mech Dev. 1999 Dec;89(1-2):115-24 PMID 10559486; Cites J Biol Chem. 2000 Feb 4;275(5):3543-51 PMID 10652349; Cites J Biol Chem. 2000 May 5;275(18):13571-9 PMID 10788473; Cites J Biol Chem. 2000 May 5;275(18):13872-8 PMID 10788511; Cites Mol Cell Biol. 2001 Nov;21(21):7460-9 PMID 11585926

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Languages: ENGLISH
Main Citation Owner: NLM
Other Citation Owner: NLM
Record type: MEDLINE; Completed

The c-Jun N-terminal kinase (JNK) branch of the mitogen-activated protein kinase (MAPK) signaling pathway regulates cellular differentiation, stress responsiveness and apoptosis in multicellular eukaryotic organisms. Here we investigated the functional importance of JNK signaling in regulating differentiated cellular growth in the post-mitotic myocardium. JNK1/2 gene-targeted mice and transgenic mice expressing dominant negative JNK1/2 were determined to have enhanced myocardial growth following stress stimulation or with normal aging. A mechanism underlying this effect was suggested by the observation that JNK directly regulated nuclear factor of activated T-cell (NFAT) activation in culture and in transgenic mice containing an NFAT-dependent luciferase reporter. Moreover, calcineurin Abeta gene targeting abrogated the pro-growth effects associated with JNK inhibition in the heart, while expression of an MKK7-JNK1 fusion protein in the heart partially reduced calcineurin-mediated cardiac hypertrophy. Collectively, these results indicate that JNK signaling antagonizes the differentiated growth response of the myocardium through direct cross-talk with the calcineurin-NFAT pathway. These results also suggest that myocardial JNK activation is primarily dedicated to modulating %%%calcineurin%%% -NFAT signaling in the regulation of differentiated heart growth.

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15361579   PMID: 12750397   Record Identifier: PMC155046
Targeted inhibition of p38 MAPK promotes %%%hypertrophic%%% %%%cardiomyopathy%%% through upregulation of %%%calcineurin%%% -NFAT signaling.
Braz Julian C; Bueno Orlando F; Liang Qiangrong; Wilkins Benjamin J; Dai Yan-Shan; Parsons Stephanie; Braunwart Joseph; Glascock Betty J; Klevitsky Raisa; Kimball Thomas F; Hewett Timothy E; Molkentin Jeffery D
Department of Pediatrics, University of Cincinnati, Children's Hospital Medical Center, Cincinnati, Ohio, USA.
Journal of clinical investigation (United States) May 2003, 111 (10) p1475-86, ISSN 0021-9738--Print 0021-9738--Linking Journal Code: 7802877
Contract/Grant No.: 5T32 HL07382; HL; NHLBI NIH HHS United States; HL10336; HL; NHLBI NIH HHS United States
Publishing Model Print; Cites Curr Opin Cell Biol. 1999 Apr;11(2):211-8 PMID 10209154; Cites J Clin Invest. 1998 Oct 1;102(7):1311-20 PMID 9769323; Cites Hypertension. 2000 Jan;35(1 Pt 2):188-92 PMID 10642296; Cites J Biol Chem. 2000 Feb 4;275(5):3543-51 PMID 10652349; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites Cardiovasc Res. 2000 Jan 1;45(1):82-91 PMID 10728317; Cites J Biol Chem. 2000 May 5;275(18):13571-9 PMID 10788473; Cites J Biol Chem. 2000 May 5;275(18):13872-8 PMID 10788511; Cites Nat Med. 2000 May;6(5):482-3 PMID 10802676; Cites Nat Med. 2000 May;6(5):556-63 PMID 10802712; Cites Circulation. 2000 May 23;101(20):2431-7 PMID 10821822; Cites Circulation. 2000 Jul 25;102(4):470-9 PMID 10908222; Cites Mol Cell. 2000 Jul;6(1):109-16 PMID 10949032; Cites Proc Natl Acad Sci U S A. 2000 Sep 12;97(19):10454-9 PMID 10973481; Cites Circ Res. 2001 Jan 19;88(1):88-96 PMID 11139479; Cites Annu Rev Physiol. 2001;63:391-426 PMID 11181961; Cites Proc Natl Acad Sci U S A. 2001 Jun 5;98(12):6668-73 PMID 11381115; Cites J Mol Cell Cardiol. 2001 Aug;33(8):1527-40 PMID 11448140; Cites Nature. 2001 Jul 19;412(6844):346-51 PMID 11460167; Cites J Cell Biol. 2001 Oct 1;155(1):27-39 PMID 11581284; Cites Proc Natl Acad Sci U S A. 2001 Oct 9;98(21):12283-8 PMID 11593045; Cites Proc Natl Acad Sci U S A. 2002 Jan 22;99(2):907-12 PMID 11782539; Cites J Biol Chem. 2002 Apr 19;277(16):14266-73 PMID 11827959; Cites Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4586-91 PMID 11904392; Cites J Mol Cell Cardiol. 2002 Apr;34(4):413-26 PMID 11991731; Cites Mol Cell Biol. 2002 Jun;22(11):3892-904 PMID 11997522; Cites Proc Natl Acad Sci U S A. 2002 Jul 9;99(14):9398-403 PMID 12091710; Cites Mol Cell Biol. 2002 Nov;22(21):7603-13 PMID 12370307; Cites N Engl J Med. 1990 May 31;322(22):1561-6 PMID 2139921; Cites J Am Coll Cardiol. 1993 Jul;22(1):6-7 PMID 8509564; Cites Mol Cell Biol. 1996 Mar;16(3):1247-55 PMID 8622669; Cites J Clin Invest. 1996 Oct 15;98(8):1906-17 PMID 8878443; Cites J Biol Chem. 1996 Nov 22;271(47):29876-81 PMID 8939929; Cites Circ Res. 1997 May;80(5):655-64 PMI D 9130446; Cites Biochem Biophys Res Commun. 1997 Jun 27;235(3):533-8 PMID 9207191; Cites J Cell Biol. 1997 Oct 6;139(1):115-27 PMID 9314533; Cites J Biol Chem. 1997 Nov 28;272(48):30122-8 PMID 9374491; Cites J Biol Chem. 1998 Jan 16;273(3):1741-8 PMID 9430721; Cites J Biol Chem. 1998 Jan 23;273(4):2161-8 PMID 9442057; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Mol Cell Biol. 1998 Jun;18(6):3518-26 PMID 9584192; Cites J Cell Biol. 1998 Jul 27;142(2):523-35 PMID 9679149; Cites J Clin Invest. 1999 Dec;104(11):1603-12 PMID 10587524

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Languages: ENGLISH
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The MAPKs are important transducers of growth and stress stimuli in virtually all eukaryotic cell types. In the mammalian heart, MAPK signaling pathways have been hypothesized to regulate myocyte growth in response to developmental signals or physiologic and pathologic stimuli. Here we generated cardiac-specific transgenic mice expressing dominant-negative mutants of p38alpha, MKK3, or MKK6. Remarkably, attenuation of cardiac p38 activity produced a progressive growth response and myopathy in the heart that correlated with the degree of enzymatic inhibition. Moreover, dominant-negative p38alpha, MKK3, and MKK6 transgenic mice each showed enhanced cardiac %%%hypertrophy%%% following aortic banding, Ang II infusion, isoproterenol infusion, or phenylephrine infusion for 14 days. A mechanism underlying this enhanced-growth profile was suggested by the observation that dominant-negative p38alpha directly augmented %%%nuclear%%% factor of activated T cells (NFAT) transcriptional activity and its %%%nuclear%%% translocation. In vivo, NFAT-dependent luciferase reporter transgenic mice showed enhanced activation in the presence of the dominant-negative p38alpha transgene before and after the onset of cardiac %%%hypertrophy%%%. More significantly, genetic disruption of the %%%calcineurin%%% Abeta gene rescued %%%hypertrophic%%% %%%cardiomyopathy%%%%%
%%%% and depressed functional capacity observed in p38-inhibited mice.%%%
%%%Collectively, these observations indicate that reduced p38 signaling in the%%%
%%%heart promotes myocyte growth through a mechanism involving enhanced%%%
%%%%%%calcineurin%%%-NFAT signaling.%%%
%%% Record Date Created: 20030516%%%
%%% Record Date Completed: 20030604%%%
%%%%%%

2/7/82 (Item 82 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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15314874 PMID: 12695656
Cyclins that don't cycle--cyclin T/cyclin-dependent kinase-9 determines cardiac muscle cell size.
Sano Motoaki; Schneider Michael D
Center for Cardiovascular Development, Department of Medicine, Baylor College of Medicine, Houston, Texas 77030, USA.
Cell cycle (Georgetown, Tex.) (United States) Mar-Apr 2003, 2 (2) p99-104, ISSN 1538-4101--Print 1551-4005--Linking Journal Code: 101137841
Publishing Model Print
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
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A subset of cyclin-dependent protein kinases--Cdk7, Cdk8, and Cdk9--participates directly, in complex ways, with the fundamental machinery for gene transcription, as elements of general transcription

factors whose substrate is the C-terminal domain (CTD) of RNA polymerase II. Here, we review recent data implicating the CTD kinase Cdk9 as a critical determinant of cardiac %%%hypertrophy%%%, in vitro and in vivo. Diverse trophic signals that increase cardiac mass all activated Cdk9 (work load, the small G-protein Gaq, and the calcium-dependent phosphatase %%%calcineurin%%% in mouse myocardium; endothelin-1, a %%%hypertrophic%%% agonist, in cultured cardiomyocytes). Little or no change occurred in levels of the kinase or its activator, cyclin T. Instead, in all four %%%hypertrophic%%% models, Cdk9 activation involves the dissociation of 7SK small %%%nuclear%%% RNA (snRNA), an endogenous inhibitor. In culture, dominant-negative Cdk9 blocked ET-1-induced %%%hypertrophy%%%, whereas an anti-sense "knockdown" of 7SK snRNA provoked spontaneous cell growth. In trans-genie mice, concordant with these results, activation of Cdk9 activity via cardiac-specific overexpression of cyclin Tl suffices to provoke %%%hypertrophy%%%. Together, these findings implicate Cdk9 activity as a pivotal regulator of pathophysiological heart growth. Because %%%hypertrophy%%% , in turn, is a cardinal risk factor for developing cardiac pump failure, these results support the logic of examining Cdk9 as a potential drug target in heart disease. (63 Refs.)
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2/7/83 (Item 83 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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15172732 PMID: 12858558
Calcium-dependent gene regulation in myocyte %%%hypertrophy%%% and remodeling.
Williams R S; Rosenberg P
Departments of Medicine and Pharmacology, Duke University Medical Center, Durham, North Carolina 27701, USA.
Cold Spring Harbor symposia on quantitative biology (United States) 2002, 67 p339-44, ISSN 0091-7451--Print 0091-7451--Linking
Journal Code: 1256107
Contract/Grant No.: R01-AR-40849; AR; NIAMS NIH HHS United States
Publishing Model Print
Document type: Journal Article; Research Support, U.S. Gov't, P.H.S.; Review
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Main Citation Owner: NLM
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(23 Refs.)
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2/7/84 (Item 84 from file: 154)
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15095523 PMID: 12226086
Requirement of %%%nuclear%%% factor of activated T-cells in %%%calcineurin%%%-mediated cardiomyocyte %%%hypertrophy%%%.
van Rooij Eva; Doevendans Pieter A; de Theije Chiel C; Babiker Fawzi A; Molkentin Jeffery D; de Windt Leon J

Department of Cardiology, Cardiovascular Research Institute Maastricht, University Hospital, P. Debyelaan 25, the Netherlands.
Journal of biological chemistry (United States) Dec 13 2002, 277 (50) p48617-26, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R
Contract/Grant No.: HL07382; HL; NHLBI NIH HHS United States; HL60562; HL ; NHLBI NIH HHS United States
Publishing Model Print-Electronic
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
The calcium-activated phosphatase %%%calcineurin%%% has been implicated as a critical intracellular %%%signal%%% transducer of cardiomyocyte %%%hypertrophy%%%. Although previous data suggested the %%%nuclear%%% factor of activated T-cells (NFAT) as its sole transcriptional effector, the absolute requirement of NFAT as a mediator of %%%calcineurin%%% signaling has not been examined in the heart. We therefore investigated the expression and activation profile of NFAT genes in the heart. Four members (NFATc1-c4) are expressed in cardiomyocytes, elicit %%%nuclear%%% translocation upon %%%calcineurin%%% activation, and are able to drive transactivation of cardiac promoter luciferase constructs. To define the necessary function of NFAT factors as %%%hypertrophic%%% transducers, a dominant negative NFAT construct was created, encompassing part of the N-terminal region of NFATc4 containing a conserved %%%calcineurin%%% -binding motif. Cotransfection of this construct dose-dependently abrogated promoter activation, irrespective of the NFAT isoform used, whereas a control construct with the %%%calcineurin%%% -binding motif mutated displayed no such effects. Adenoviral gene transfer of dominant negative NFAT rendered cardiomyocytes resistant toward all aspects of %%%calcineurin%%% or agonist-induced cardiomyocyte %%%hypertrophy%%% , whereas adenoviral gene transfer of the control construct had no discernable effect on these parameters. These results indicate that multiple NFAT isoforms are expressed in cardiomyocytes where they function as necessary transducers of %%%calcineurin%%% in facilitating cardiomyocyte %%%hypertrophy%%%.
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15084935 PMID: 12457775
%%%Calcineurin%%% signaling and neural control of skeletal muscle fiber type and size.
Schiaffino Stefano; Serrano AntonioL
Dept of Biomedical Sciences, CNR Institute of Neurosciences, University of Padova, Viale G. Colombo 3, Italy. stefano.schiaffino@unipd.it
Trends in pharmacological sciences (England) Dec 2002, 23 (12) p569-75, ISSN 0165-6147--Print 0165-6147--Linking Journal Code: 7906158
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Review
Languages: ENGLISH

Main Citation Owner: NLM
Record type: MEDLINE; Completed
Nerve activity controls muscle contractile function and muscle gene expression. Although excitation-contraction coupling is well characterized, excitation-transcription coupling is still poorly understood. Pharmacological and genetic approaches have been used to dissect the signaling pathways that mediate the effect of nerve activity on muscle fiber type and size. In particular, the role of %%%calcineurin%%% has recently been the subject of intensive investigation and debate. The identification of the transduction pathways involved in neuromuscular signaling has implications for the development of new therapeutic strategies to prevent muscle wasting and loss of muscle power resulting from aging, disuse and neuromuscular disorders. (60 Refs.)
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Record Date Completed: 20030211

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DIALOG(R)File 154:MEDLINE(R)
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14937909 PMID: 12177418 Record Identifier: PMC123262
Inhibition of %%%calcineurin%%%-NFAT %%%hypertrophy%%% signaling by cGMP-dependent protein kinase type I in cardiac myocytes.
Fiedler Beate; Lohmann Suzanne M; Smolenski Albert; Linnemuller Stephan; Pieske Burkert; Schroder Frank; Molkentin Jeffery D; Drexler Helmut; Wollert Kai C
Department of Cardiology and Angiology, Hannover Medical School, 30625 Hannover, Germany.
Proceedings of the National Academy of Sciences of the United States of America (United States) Aug 20 2002, 99 (17) p11363-8, ISSN 0027-8424 --Print 0027-8424--Linking Journal Code: 7505876
Publishing Model Print-Electronic; Cites Circulation. 1999 Aug 31;100(9):999-1008 PMID 10468532; Cites Mol Cell Biol. 1998 Dec;18(12):6983-94 PMID 9819386; Cites Nature. 1999 Oct 14;401(6754):703-8 PMID 10537109; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites Circ Res. 2000 Feb 18;86(3):255-63 PMID 10679475; Cites Nature. 2000 Mar 9;404(6774):197-201 P MID 10724174; Cites J Cell Sci. 2000 May;113 (Pt 10):1671-6 PMID 10769198; Cites J Biol Chem. 2000 Jul 14;275(28):21722-9 PMID 10783386; Cites J Biol Chem. 2000 May 5;275(18):13571-9 PMID 10788473; Cites EMBO J. 2000 May 2;19(9):2046-55 PMID 10790371; Cites J Biol Chem. 2000 May 19;275(20):15239-45 PMID 10809760; Cites J Clin Invest. 2000 May;105(10):1395-406 PMID 10811847; Cites J Biol Chem. 2000 Aug 18;275(33):25723-32 PMID 10851246; Cites J Cell Biol. 2000 Oct 2;151(1):117-30 PMID 11018058; Cites J Biol Chem. 2001 Feb 2;276(5):3524-30 PMID 11044444; Cites Nat Med. 2000 Nov;6(11):1221-7 PMID 11062532; Cites Circ Res. 2000 Nov 10;87(10):937-45 PMID 11073891; Cites Cardiovasc Res. 2000 Dec;48(3):367-74 PMID 11090832; Cites J Biol Chem. 2001 Jan 26;276(4):2313-6 PMID 11096121; Cites Circ Res. 2001 Jan 19;88(1):88-96 PMID 11139479; Cites Annu Rev Physiol. 2001;63:391-426 PMID 11181961; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3328-33 PMID 11248078; Cites J Biol Chem. 2001 May 11;276(19):15913-9 PMID 11279073; Cites Cardiovasc Res. 2001 Jun;50(3):486-94 PMID 11376624; Cites Circulation. 2001 Sep 11;104(11):1286-91 PMID 11551881; Cites Circulation. 2001 Oct 2;104(14):1670-5 PMID 11581147; Cites J Biol Chem. 2002 Feb 22;277(8):6214-22 PMID 11739394; Cites Hypertension. 2002

Jan;39(1):87-92 PMID 11799084; Cites Proc Natl Acad Sci U S A. 1991 Feb 15;88(4):1197-201 PMID 1705030; Cites J Biol Chem. 1991 Aug 25;266(24):15910-6 PMID 1714900; Cites Circ Res. 1995 Oct;77(4):803-12 PMID 7554127; Cites J Biol Chem. 1994 Oct 21;269(42):26227-33 PMID 7929338; Cites Hypertension. 1996 Jan;27(1):14-8 PMID 8591877; Cites Am J Physiol. 1997 Dec;273(6 Pt 2):H2659-63 PMID 9435601; Cites J Clin Invest. 1998 Feb 15;101(4):812-8 PMID 9466976; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites J Mol Cell Cardiol. 1998 Oct;30(10):1955-65 PMID 9799650; Cites N Engl J Med. 1999 Oct 21;341(17):1276-83 PMID 10528039

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Languages: ENGLISH
Main Citation Owner: NLM
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Recent investigation has focused on identifying signaling pathways that inhibit cardiac %%%hypertrophy%%%, a major risk factor for cardiovascular morbidity and mortality. In this context, nitric oxide (NO), signaling via cGMP and cGMP-dependent protein kinase type I (PKG I), has been recognized as a negative regulator of cardiac myocyte (CM) %%%hypertrophy%%%. However, the underlying mechanisms are poorly understood. Here, we show that PKG I inhibits CM %%%hypertrophy%%% by targeting the %%%calcineurin%%%-NFAT signaling pathway. %%%Calcineurin%%% , a Ca2+-dependent phosphatase, promotes %%%hypertrophy%%% in part by activating NFAT transcription factors which induce expression of %%%hypertrophic%%% genes, including brain natriuretic peptide (BNP). Activation of PKG I by NO/cGMP in CM suppressed NFAT transcriptional activity, BNP induction, and cell enlargement in response to alpha(1)-adrenoreceptor stimulation but not in response to adenoviral expression of a Ca2+-independent, constitutively active %%%calcineurin%%% mutant, thus demonstrating NO-cGMP-PKG I inhibition of %%%calcineurin%%%-NFAT signaling upstream of %%%calcineurin%%% . PKG I suppressed single L-type Ca2+-channel open probability, [Ca2+]i transient amplitude, and, most importantly, L-type Ca2+-channel current-induced NFAT activation, indicating that PKG I targets Ca2+-dependent steps upstream of %%%calcineurin%%% . Adenoviral expression of PKG I enhanced NO/cGMP inhibitory effects upstream of %%%calcineurin%%%, confirming that PKG I mediates NO/cGMP inhibition of %%%calcineurin%%% -NFAT signaling. In CM overexpressing PKG I, NO/cGMP also suppressed BNP induction and cell enlargement but not NFAT activation elicited by constitutively active %%%calcineurin%%% , which is consistent with additional, NFAT-independent inhibitory effect(s) of PKG I downstream of %%%calcineurin%%%. Inhibition of %%%calcineurin%%% -NFAT signaling by PKG I provides a framework for understanding how NO inhibits cardiac myocyte %%%hypertrophy%%%.

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14793891 PMID: 12010908

%%%Calcineurin%%% in human heart %%%hypertrophy%%%.

Ritter Oliver; Hack Susanne; Schuh Kai; Rothlein Nicola; Perrot Andreas; Osterziel Karl J; Schulte Hagen D; Neyses Ludwig
Department of Medicine, University of Wuerzburg, Germany.
Circulation (United States) May 14 2002, 105 (19) p2265-9, ISSN

1524-4539--Electronic 0009-7322--Linking Journal Code: 0147763
  Publishing Model Print; Comment in Circulation. 2002 May 14;105(19):2242-3 PMID 12010903
  Document type: Journal Article
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: MEDLINE; Completed
  BACKGROUND: In animal models, increased signaling through the %%%calcineurin%%% pathway has been shown to be sufficient for the development of cardiac %%%hypertrophy%%%. %%%Calcineurin%%% activity has been reported to be elevated in the myocardium of patients with congestive heart failure. In contrast, few data are available about %%%calcineurin%%% activity in patients with pressure overload or %%%cardiomyopathic%%% %%%hypertrophy%%% who are not in cardiac failure. METHODS AND RESULTS: We investigated %%%calcineurin%%% activity and protein expression in 2 different forms of cardiac %%%hypertrophy%%%: %%%hypertrophic%%% obstructive %%%cardiomyopathy%%% (HOCM) and aortic stenosis (AS). We found that the C-terminus of %%%calcineurin%%% A protein containing the autoinhibitory domain was less abundant in myocardial %%%hypertrophy%%% than in normal heart, which suggests the possibility of proteolysis. No new splice variants could be detected by reverse-transcription polymerase chain reaction. This resulted in a significant elevation of %%%calcineurin%%% enzymatic activity in HOCM and AS compared with 6 normal hearts. Increased %%%calcineurin%%% phosphatase activity caused increased migration of NF-AT2 (%%%nuclear%%% factor of activated T cells 2) in SDS-PAGE compatible with pronounced NF-AT dephosphorylation in %%%hypertrophied%%% myocardial tissue. CONCLUSIONS: %%%Hypertrophy%%% in HOCM and AS without heart failure is characterized by a significant increase in %%%calcineurin%%% activity. This might occur by (partial) proteolysis of the %%%calcineurin%%% A C-terminus containing the autoinhibitory domain. Increased %%%calcineurin%%% activity has functional relevance, as shown by altered NF-AT phosphorylation state. Although %%%hypertrophy%%% in AS and HOCM may be initiated by different upstream triggers (internal versus external fiber overload), in both cases, there is activation of %%%calcineurin%%%, which suggests an involvement of this pathway in the pathogenesis of human cardiac %%%hypertrophy%%%.
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  Record Date Completed: 20020524

 2/7/88   (Item 88 from file: 154)
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14782539   PMID: 11997253
  Maintenance of muscle mass is not dependent on the %%%calcineurin%%%-NFAT pathway.
  Dupont-Versteegden Esther E; Knox Micheal; Gurley Cathy M; Houle John D; Peterson Charlotte A
  Department of Geriatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205, USA. dupontesthere@uams.edu
  American journal of physiology. Cell physiology (United States) Jun 2002, 282 (6) pC1387-95, ISSN 0363-6143--Print 0363-6143--Linking
Journal Code: 100901225
  Contract/Grant No.: NS-40008; NS; NINDS NIH HHS United States
  Publishing Model Print
  Document type: Journal Article; Research Support, Non-U.S. Gov't;

Research Support, U.S. Gov't, P.H.S.
Languages: ENGLISH
Main Citation Owner: NLM
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In this study, the role of the %%%calcineurin%%% pathway in skeletal muscle atrophy and atrophy-reducing interventions was investigated in rat soleus muscles. Because %%%calcineurin%%% has been suggested to be involved in skeletal and cardiac muscle %%%hypertrophy%%%, we hypothesized that blocking %%%calcineurin%%% activity would eliminate beneficial effects of interventions that maintain muscle mass in the face of atrophy-inducing stimuli. Hindlimb suspension and spinal cord transection were used to induce atrophy, and intermittent reloading and exercise were used to reduce atrophy. Cyclosporin (CsA, 25 mg x kg(-1) x day(-1)) was administered to block %%%calcineurin%%% activity. Soleus muscles were studied 14 days after the onset of atrophy. CsA administration did not inhibit the beneficial effects of the two muscle-maintaining interventions, nor did it change muscle mass in control or atrophied muscles, suggesting that %%%calcineurin%%% does not play a role in regulating muscle size during atrophy. However, %%%calcineurin%%% abundance was increased in atrophied soleus muscles, and this was associated with %%%nuclear%%% localization of NFATc1 (a %%%nuclear%%% factor of activated T cells). Therefore, results suggest that %%%calcineurin%%% may be playing opposing roles during skeletal muscle atrophy and under muscle mass-maintaining conditions.

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14656878 PMID: 11782539 Record Identifier: PMC117404

Activated glycogen synthase-3 beta suppresses cardiac %%%hypertrophy%%% in vivo.

Antos Christopher L; McKinsey Timothy A; Frey Norbert; Kutschke William; McAnally John; Shelton John M; Richardson James A; Hill Joseph A; Olson Eric N
Department of Molecular Biology, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75390-9148, USA.

Proceedings of the National Academy of Sciences of the United States of America (United States) Jan 22 2002, 99 (2) p907-12, ISSN 0027-8424--Print 0027-8424--Linking Journal Code: 7505876
Publishing Model Print-Electronic; Cites EMBO J. 1997 Apr 15;16(8):1888-900 PMID 9155015; Cites Genes Dev. 1997 Apr 15;11(8):957-72 PMID 9136925; Cites J Clin Invest. 1998 Feb 15;101(4):812-8 PMID 9466976; Cites J Biol Chem. 1998 Mar 6;273(10):5423-6 PMID 9488659; Cites Cell. 1998 Mar 6;92(5):687-96 PMID 9506523; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites Mol Cell Biol. 1998 Jun;18(6):3518-26 PMID 9584192; Cites Circ Res. 1998 Aug 24;83(4):345-52 PMID 9721691; Cites Genes Dev. 1998 Nov 15;12(22):3499-511 PMID 9832503; Cites Mol Cell Biol. 2000 Jul;20(14):5227-34 PMID 10866678; Cites Cell. 2000 Jun 23;101(7):689-92 PMID 10892739; Cites N Engl J Med. 2000 Jul 27;343(4):246-53 PMID 10911006; Cites J Cell Biol. 2000 Oct 2;151(1):117-30 PMID 11018058; Cites Circ Res. 2000 Oct 27;87(9):731-8 PMID 11055975; Cites Nat Med. 2000 Nov;6(11):1221-7 PMID 11062532; Cites Circulation. 2001 Feb 6;103(5):670-7 PMID 11156878; Cites Proc Natl Acad Sci U S A. 2001 Feb

27;98(5):2703-6 PMID 11226303; Cites Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):2947-9 PMID 11248009; Cites Dev Biol. 2001 Jul 15;235(2):303-13 PMID 11437438; Cites Cell. 2001 Jun 15;105(6):721-32 PMID 11440715; Cites Annu Rev Genomics Hum Genet. 2000;1:179-223 PMID 11701629; Cites Cell. 1991 Feb 8;64(3):573-84 PMID 1846781; Cites J Biol Chem. 1991 May 15;266(14):9180-5 PMID 2026617; Cites N Engl J Med. 1990 May 31;322(22):1561-6 PMID 2139921; Cites Proc Natl Acad Sci U S A. 1986 Nov;83(21):8348-50 PMID 3022291; Cites Science. 1988 Jul 8;241(4862):202-5 PMID 3260404; Cites J Biol Chem. 1995 Aug 25;270(34):19898-907 PMID 7650004; Cites Immunity. 1995 May;2(5):461-72 PMID 7749981; Cites Biochem J. 1994 Nov 1;303 (Pt 3):701-4 PMID 7980435; Cites Science. 1993 Oct 29;262(5134):750-4 PMID 8235597; Cites Nature. 1995 Dec 21-28;378(6559):785-9 PMID 8524413; Cites Cell. 1999 Mar 5;96(5):611-4 PMID 10089876; Cites J Biol Chem. 1999 Aug 27;274(35):24858-64 PMID 10455158; Cites Cell. 1999 Sep 3;98(5):555-8 PMID 10490095; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites J Biol Chem. 2000 Feb 18;275(7):4693-8 PMID 10671499; Cites Circ Res. 2000 Feb 18;86(3):255-63 PMID 10679475; Cites J Biol Chem. 2000 Mar 24;275(12):8719-25 PMID 10722714; Cites J Biol Chem. 2000 May 12;275(19):14466-75 PMID 10799529; Cites J Clin Invest. 2000 May;105(10):1339-42 PMID 10811840; Cites EMBO J. 2000 Jun 1;19(11):2537-48 PMID 10835352; Cites Circulation. 2000 Jun 20;101(24):2863-9 PMID 10859294; Cites Circ Res. 1997 Feb;80(2):228-41 PMID 9012745; Cites Science. 1997 Mar 28;275(5308):1930-4 PMID 9072970; Cites Science. 1997 Nov 28;278(5343):1638-41 PMID 9374467

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The adult myocardium responds to a variety of pathologic stimuli by %%%hypertrophic%%% growth that frequently progresses to heart failure. The calcium/calmodulin-dependent protein phosphatase %%%calcineurin%%% is a potent transducer of %%%hypertrophic%%% stimuli. %%%Calcineurin%%% dephosphorylates members of the %%%nuclear%%% factor of activated T cell (NFAT) family of transcription factors, which results in their translocation to the %%%nucleus%%% and activation of calcium-dependent genes. Glycogen synthase kinase-3 (GSK-3) phosphorylates NFAT proteins and antagonizes the actions of %%%calcineurin%%% by stimulating NFAT %%%nuclear%%% export. To determine whether activated GSK-3 can act as an antagonist of %%%hypertrophic%%% signaling in the adult heart in vivo, we generated transgenic mice that express a constitutively active form of GSK-3 beta under control of a cardiac-specific promoter. These mice were physiologically normal under nonstressed conditions, but their ability to mount a %%%hypertrophic%%% response to %%%calcineurin%%% activation was severely impaired. Similarly, cardiac-specific expression of activated GSK-3 beta diminished %%%hypertrophy%%% in response to chronic beta-adrenergic stimulation and pressure overload. These findings reveal a role for GSK-3 beta as an inhibitor of %%%hypertrophic%%% signaling in the intact myocardium and suggest that elevation of cardiac GSK-3 beta activity may provide clinical benefit in the treatment of pathologic %%%hypertrophy%%% and heart failure.

Record Date Created: 20020123

Record Date Completed: 20020429

Date of Electronic Publication: 20020108

2/7/90 (Item 90 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14579134 PMID: 11817656
Signalling pathways in cardiac myocyte %%%hypertrophy%%%.
Sugden P H
National Heart and Lung Institute Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, London, UK. p.sugden@ic.ac.uk
Annals of medicine (England) Dec 2001, 33 (9) p611-22, ISSN 0785-3890--Print 0785-3890--Linking Journal Code: 8906388
Publishing Model Print
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
In response to a requirement for increased contractile power in vivo, mammalian cardiac myocytes adapt through a %%%hypertrophic%%% response (cell enlargement in the absence of cell division). This response can be simulated by exposing isolated myocytes in primary culture to alpha-adrenergic agonists or the vasoactive peptide, endothelin-1. The signalling pathways responsible for %%%hypertrophic%%% growth have been actively studied, and it is likely that reversible protein phosphorylation and dephosphorylation are involved. Three signalling pathways show particular potential as regulators of the response, ie protein kinase C (PKC), mitogen-activated protein kinase (MAPK) cascades, and %%%calcineurin%%% . These species are thought to regulate the rate and specificity of gene transcription ultimately through modifying the transactivating activity of %%%nuclear%%% transcription factors. There are three pertinent MAPK cascades, the extracellular %%%signal%%%-regulated kinase (ERK) cascade, the c-Jun N-terminal kinase (JNK or SAPK1) cascade, and the p38-MAPK (SAPK2-5) cascade. PKC participates in the activation of the ERK cascade but does not contribute significantly to the activation of the two remaining cascades. %%%Calcineurin%%% (or protein phosphatase 2B) is activated by increases in [Ca2+i] through the [Ca2+]-sensing protein, calmodulin. In this review, I discuss the evidence for and against the involvement of these signalling proteins in the induction of myocyte %%%hypertrophy%%% and emphasize that the ERK cascade should perhaps feature more widely in the collective consciousness. (136 Refs.)
Record Date Created: 20020130
Record Date Completed: 20020510

2/7/91 (Item 91 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14513360 PMID: 11715023
Akt/mTOR pathway is a crucial regulator of skeletal muscle %%%hypertrophy%%% and can prevent muscle atrophy in vivo.
Bodine S C; Stitt T N; Gonzalez M; Kline W O; Stover G L; Bauerlein R; Zlotchenko E; Scrimgeour A; Lawrence J C; Glass D J; Yancopoulos G D
Regeneron Pharmaceuticals, Inc. 777 Old Saw Mill River Road, Tarrytown, New York 10591-6707, USA. sue.bodine@regeneron.com
Nature cell biology (England) Nov 2001, 3 (11) p1014-9, ISSN

1465-7392--Print 1465-7392--Linking   Journal Code: 100890575
Publishing Model Print; Comment in Nat Cell Biol. 2002 Mar;4(3):E46; author reply E46-7 PMID 11875443
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Skeletal muscles adapt to changes in their workload by regulating fibre size by unknown mechanisms. The roles of two signalling pathways implicated in muscle %%%hypertrophy%%% on the basis of findings in vitro, Akt/mTOR (mammalian target of rapamycin) and %%%calcineurin%%%/NFAT (%%%nuclear%%% factor of activated T cells), were investigated in several models of skeletal muscle %%%hypertrophy%%% and atrophy in vivo. The Akt/mTOR pathway was upregulated during %%%hypertrophy%%% and downregulated during muscle atrophy. Furthermore, rapamycin, a selective blocker of mTOR, blocked %%%hypertrophy%%% in all models tested, without causing atrophy in control muscles. In contrast, the %%%calcineurin%%% pathway was not activated during %%%hypertrophy%%% in vivo, and inhibitors of %%%calcineurin%%%, cyclosporin A and FK506 did not blunt %%%hypertrophy%%%. Finally, genetic activation of the Akt/mTOR pathway was sufficient to cause %%%hypertrophy%%% and prevent atrophy in vivo, whereas genetic blockade of this pathway blocked %%%hypertrophy%%% in vivo. We conclude that the activation of the Akt/mTOR pathway and its downstream targets, p70S6K and PHAS-1/4E-BP1, is requisitely involved in regulating skeletal muscle fibre size, and that activation of the Akt/mTOR pathway can oppose muscle atrophy induced by disuse.
Record Date Created: 20011120
Record Date Completed: 20011219

2/7/92   (Item 92 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14513359   PMID: 11715022
Mediation of IGF-1-induced skeletal myotube %%%hypertrophy%%% by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways.
Rommel C; Bodine S C; Clarke B A; Rossman R; Nunez L; Stitt T N; Yancopoulos G D; Glass D J
Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707, USA.
Nature cell biology (England) Nov 2001, 3 (11) p1009-13, ISSN 1465-7392--Print 1465-7392--Linking   Journal Code: 100890575
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Skeletal muscle is composed of multinucleated fibres, formed after the differentiation and fusion of myoblast precursors. Skeletal muscle atrophy and %%%hypertrophy%%% refer to changes in the diameter of these pre-existing muscle fibres. The prevention of atrophy would provide an obvious clinical benefit; insulin-like growth factor 1 (IGF-1) is a promising anti-atrophy agent because of its ability to promote %%%hypertrophy%%%. However, the signalling pathways by which IGF-1 promotes %%%hypertrophy%%% remain unclear, with roles suggested for both the %%%calcineurin%%%/NFAT (%%%nuclear%%% factor of activated T cells) pathway

and the PtdIns-3-OH kinase (PI(3)K)/Akt pathway. Here we employ a battery of approaches to examine these pathways during the %%%hypertrophic%%% response of cultured myotubes to IGF-1. We report that Akt promotes %%%hypertrophy%%% by activating downstream signalling pathways previously implicated in activating protein synthesis: the pathways downstream of mammalian target of rapamycin (mTOR) and the pathway activated by phosphorylating and thereby inhibiting glycogen synthase kinase 3 (GSK3). In contrast, in addition to demonstrating that %%%calcineurin%%% does not mediate IGF-1-induced %%%hypertrophy%%% , we show that IGF-1 unexpectedly acts via Akt to antagonize %%%calcineurin%%% signalling during myotube %%%hypertrophy%%%.
Record Date Created: 20011120
Record Date Completed: 20011219

2/7/93 (Item 93 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14327099 PMID: 11435346
Isoproterenol activates extracellular %%%signal%%% -regulated protein kinases in cardiomyocytes through %%%calcineurin%%%.
Zou Y; Yao A; Zhu W; Kudoh S; Hiroi Y; Shimoyama M; Uozumi H; Kohmoto O; Takahashi T; Shibasaki F; Nagai R; Yazaki Y; Komuro I
Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba.
Circulation (United States) Jul 3 2001, 104 (1) p102-8, ISSN 1524-4539--Electronic 0009-7322--Linking Journal Code: 0147763
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
BACKGROUND: Extracellular %%%signal%%%-regulated kinases (ERKs) and %%%calcineurin%%% have been reported to play important roles in the development of cardiac %%%hypertrophy%%% . We examined here the relation between %%%calcineurin%%% and ERKs in cardiomyocytes. METHODS AND RESULTS: Isoproterenol activated ERKs in cultured cardiomyocytes of neonatal rats, and the activation was abolished by chelation of extracellular Ca(2+) with EGTA, blockade of L-type Ca(2+) channels with nifedipine, or depletion of intracellular Ca(2+) stores with thapsigargin. Isoproterenol-induced activation of ERKs was also significantly suppressed by %%%calcineurin%%% inhibitors in cultured cardiomyocytes as well as in the hearts of mice. Isoproterenol failed to activate ERKs in either the cultured cardiomyocytes or the hearts of mice that overexpress the dominant negative mutant of %%%calcineurin%%% . Isoproterenol elevated intracellular Ca(2+) levels at both systolic and diastolic phases and dose-dependently activated %%%calcineurin%%%. Inhibition of %%%calcineurin%%% also attenuated isoproterenol-stimulated phosphorylation of Src, Shc, and Raf-1 kinase. The immunocytochemistry revealed that %%%calcineurin%%% was localized in the Z band, and isoproterenol induced translocation of %%%calcineurin%%% and ERKs into the %%%nucleus%%%. CONCLUSIONS: %%%Calcineurin%%%, which is activated by marked elevation of intracellular Ca(2+) levels by the Ca(2+)-induced Ca(2+) release mechanism, regulates isoproterenol-induced activation of ERKs in cardiomyocytes.
Record Date Created: 20010703
Record Date Completed: 20010802

2/7/94   (Item 94 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14270923   PMID: 11044444
Ras regulates NFAT3 activity in cardiac myocytes.
Ichida M; Finkel T
Laboratory of Molecular Biology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892, USA.
Journal of biological chemistry (United States)   Feb 2 2001, 276 (5) p3524-30, ISSN 0021-9258--Print 0021-9258--Linking Journal Code: 2985121R
Publishing Model Print-Electronic
Document type: In Vitro; Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Multiple distinct %%%signal%%% transduction pathways have been implicated in the development of cardiac myocyte %%%hypertrophy%%%. These %%%hypertrophic%%% pathways include those regulated by the Ras superfamily of small GTPases and a separate %%%calcineurin%%%-regulated pathway that culminates in the activation of the transcription factor NFAT3. In this report, we demonstrate a functional interaction between Ras-regulated and %%%calcineurin%%%-regulated pathways. In particular, expression in neonatal myocytes of a constitutively active form of Ras (V12ras), but not activating mutants of Rac1, RhoA, or Cdc42, results in an increase in NFAT activity. Similarly, expression of an activated Ras, but not other small GTPases, results in the %%%nuclear%%% translocation of an NFAT3 fusion protein. Expression of a dominant negative ras gene product blocks phenylephrine-stimulated NFAT transcriptional activity and the ligand-stimulated NFAT3 %%%nuclear%%% localization. Ras proteins appear to function upstream of %%%calcineurin%%%, because cyclosporin A blocks the ability of V12ras to stimulate NFAT-dependent transcription and %%%nuclear%%% localization. Similarly, expression of a dominant negative ras gene inhibits phenylephrine-stimulated %%%calcineurin%%% activity. Pharmacological inhibition of MEK1 or expression of a dominant negative form of c-Raf or ERK2 inhibits phenylephrine-stimulated NFAT3 activation. Conversely, NFAT activity was stimulated by expression of constitutively active forms of c-Raf or MEK1. Taken together, these results imply that, in cardiac myocytes, a Ras-regulated pathway involving stimulation of mitogen-activated protein kinase regulates NFAT3 activity.
Record Date Created: 20010523
Record Date Completed: 20010621
Date of Electronic Publication: 20001023

2/7/95   (Item 95 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14205262   PMID: 11289309
Molecular genetic analysis of the %%%calcineurin%%% signaling pathways.
Sugiura R; Sio S O; Shuntoh H; Kuno T
Department of Pharmacology, Kobe University School of Medicine, Japan.
Cellular and molecular life sciences - CMLS (Switzerland)   Feb 2001, 58

(2) p278-88, ISSN 1420-682X--Print 1420-682X--Linking Journal Code: 9705402
Publishing Model Print
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
%%%Calcineurin%%% is a Ca2+- and calmodulin-regulated protein phosphatase that is important in Ca2+-mediated %%%signal%%% transduction. Recent application of the powerful techniques of molecular genetics has demonstrated that %%%calcineurin%%% is involved in the regulation of critical biological processes such as T cell activation, muscle %%%hypertrophy%%% , memory development, glucan synthesis, ion homeostasis, and cell cycle control. Notably, specific transcription factors have been shown to play a key role in regulating these functions, and their %%%calcineurin%%%-mediated dephosphorylation and %%%nuclear%%% translocation appear to be a central event in the %%%signal%%% transduction pathways. This review focuses on recent progress in these areas and discusses the evidence for cross-talk between %%%calcineurin%%% and other signaling pathways. (76 Refs.)
Record Date Created: 20010405
Record Date Completed: 20010419

2/7/96 (Item 96 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14177439 PMID: 11248077 Record Identifier: PMC30652
Targeted inhibition of %%%calcineurin%%% attenuates cardiac %%%hypertrophy%%% in vivo.
De Windt L J; Lim H W; Bueno O F; Liang Q; Delling U; Braz J C; Glascock B J; Kimball T F; del Monte F; Hajjar R J; Molkentin J D
Divisions of Molecular Cardiovascular Biology and Cardiology, Department of Pediatrics, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, USA.
Proceedings of the National Academy of Sciences of the United States of America (United States) Mar 13 2001, 98 (6) p3322-7, ISSN 0027-8424--Print 0027-8424--Linking Journal Code: 7505876
Contract/Grant No.: HL50361; HL; NHLBI NIH HHS United States; HL52318; HL ; NHLBI NIH HHS United States; HL57623; HL; NHLBI NIH HHS United States; HL62927; HL; NHLBI NIH HHS United States; HL69562; HL; NHLBI NIH HHS United States
Publishing Model Print; Cites Circ Res. 1999 Apr 2;84(6):623-32 PMID 10189350; Cites Circ Res. 1999 Apr 2;84(6):722-8 PMID 10189360; Cites Circ Res. 1999 Apr 2;84(6):729-34 PMID 10189361; Cites Circ Res. 1999 Apr 2;84(6):735-40 PMID 10189362; Cites J Clin Invest. 1999 Jul;104(1):21-9 PMID 10393695; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites J Clin Invest. 1999 Aug;104(4):391-8 PMID 10449431; Cites N Engl J Med. 1999 Oct 21;341(17):1276-83 PMID 10528039; Cites Curr Biol. 1999 Oct 21;9(20):1203-6 PMID 10531040; Cites Science. 1999 Oct 22;286(5440):790-3 PMID 10531067; Cites Circulation. 1999 Dec 14;100(24):2449-54 PMID 10595959; Cites Proc Natl Acad Sci U S A. 2000 Jan 18;97(2):793-8 PMID 10639159; Cites Hypertension. 2000 Jan;35(1 Pt 2):360-6 PMID 10642325; Cites Circ Res. 2000 Feb 18;86(3):255-63 PMID 10679475; Cites Circulation. 2000 Feb 22;101(7):790-6 PMID 10683354; Cites J Biol Chem. 2000 Mar

24;275(12):8719-25 PMID 10722714; Cites J Mol Cell Cardiol. 2000 Apr;32(4):697-709 PMID 10756124; Cites Circulation. 2000 May 9;101(18):2134-7 PMID 10801751; Cites J Clin Invest. 2000 May;105(10):1395-406 PMID 10811847; Cites Circulation. 2000 May 23;101(20):2431-7 PMID 10821822; Cites Circulation. 2000 Jun 20;101(24):2863-9 PMID 10859294; Cites Hum Mol Genet. 2000 Jul 1;9(11):1681-90 PMID 10861295; Cites J Biol Chem. 2000 Dec 29;275(52):40867-73 PMID 11016940; Cites Circulation. 2000 Oct 17;102(16):1996-2004 PMID 11034951; Cites Circulation. 2000 Oct 31;102(18):2269-75 PMID 11056104; Cites Biochem J. 1977 Dec 15;168(3):599-601 PMID 147077; Cites N Engl J Med. 1990 May 31;322(22):1561-6 PMID 2139921; Cites Science. 1995 Jan 6;267(5194):108-11 PMID 7528941; Cites J Am Coll Cardiol. 1993 Jul;22(1):6-7 PMID 8509564; Cites Curr Opin Cardiol. 1997 Jul;12(4):375-81 PMID 9263649; Cites FEBS Lett. 1997 Aug 18;413(2):354-8 PMID 9280312; Cites Proc Natl Acad Sci U S A. 1998 Apr 28;95(9):5251-6 PMID 9560262; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites J Biol Chem. 1998 May 29;273(22):13367-70 PMID 9593662; Cites Immunity. 1998 Jun;8(6):703-11 PMID 9655484; Cites J Biol Chem. 1998 Jul 17;273(29):18325-31 PMID 9660798; Cites Science. 1998 Sep 11;281(5383):1690-3 PMID 9733519; Cites Nat Med. 1998 Oct;4(10):1092-3 PMID 9771723; Cites Proc Natl Acad Sci U S A. 1998 Nov 10;95(23):13893-8 PMID 9811897; Cites Transplant Proc. 1999 Feb-Mar;31(1-2):804-5 PMID 10083347; Cites Cell. 1999 Mar 5;96(5):611-4 PMID 10089876; Comment in Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):2947-9 PMID 11248009

Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.

Languages: ENGLISH

Main Citation Owner: NLM

Other Citation Owner: NLM

Record type: MEDLINE; Completed

The Ca(2+)-calmodulin-activated Ser/Thr protein phosphatase %%%calcineurin%%% and the downstream transcriptional effectors of %%%calcineurin%%%, %%%nuclear%%% factor of activated T cells, have been implicated in the %%%hypertrophic%%% response of the myocardium. Recently, the %%%calcineurin%%% inhibitory agents cyclosporine A and FK506 have been extensively used to evaluate the importance of this signaling pathway in rodent models of cardiac %%%hypertrophy%%% . However, pharmacologic approaches have rendered equivocal results necessitating more specific or genetic-based inhibitory strategies. In this regard, we have generated Tg mice expressing the %%%calcineurin%%% inhibitory domains of Cain/Cabin-1 and A-kinase anchoring protein 79 specifically in the heart. DeltaCain and DeltaA-kinase-anchoring protein Tg mice demonstrated reduced cardiac %%%calcineurin%%% activity and reduced %%%hypertrophy%%% in response to catecholamine infusion or pressure overload. In a second approach, adenoviral-mediated gene transfer of DeltaCain was performed in the adult rat myocardium to evaluate the effectiveness of an acute intervention and any potential species dependency. DeltaCain adenoviral gene transfer inhibited cardiac %%%calcineurin%%% activity and reduced %%%hypertrophy%%% in response to pressure overload without reducing aortic pressure. These results provide genetic evidence implicating %%%calcineurin%%% as an important mediator of the cardiac %%%hypertrophic%%% response in vivo.

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Record Date Completed: 20011204

2/7/97 (Item 97 from file: 154)

DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14087050 PMID: 11701629
From the sarcomere to the %%%nucleus%%%: role of genetics and signaling in structural heart disease.
Nicol R L; Frey N; Olson E N
Department of Molecular Biology, University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines Blvd., Dallas, Texas 75390-9148, USA.
Annual review of genomics and human genetics (United States) 2000, 1 p179-223, ISSN 1527-8204--Print 1527-8204--Linking Journal Code: 100911346
Publishing Model Print
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
The identification of genetic mutations underlying familial structural heart disease has provided exciting new insights into how alterations in structural components of the cardiomyocyte lead to different forms of %%%cardiomyopathy%%% . Specifically, mutations in components of the sarcomere are frequently associated with %%%hypertrophic%%% %%%cardiomyopathy%%% , whereas mutations in cytoskeletal proteins lead to dilated %%%cardiomyopathy%%% . In addition, extrinsic stresses such as hypertension and valvular disease can produce myocardial remodeling that is very similar to that observed in genetic %%%cardiomyopathy%%% . For myocardial remodeling to occur, changes in gene expression must occur; therefore, changes in contractile function or wall stress must be communicated to the %%%nucleus%%% via %%%signal%%% transduction pathways. The identity of these signaling pathways has become a key question in molecular biology. Numerous signaling molecules have been implicated in the development of %%%hypertrophy%%% and failure, including the beta-adrenergic receptor, G alpha(q) and downstream effectors, mitogen-activated protein kinase pathways, and the Ca(2+)-regulated phosphatase, %%%calcineurin%%%. In the past it has been difficult to discern which signaling molecules actually contributed to disease progression in vivo; however, the development of numerous transgenic and knockout mouse models of %%%cardiomyopathy%%% is now allowing the direct testing of stimulatory and inhibitory molecules in the mouse heart. From this work it has been possible to identify signaling molecules and pathways that are required for different aspects of disease progression in vivo. In particular, a number of signaling pathways have now been identified that may be key regulators of changes in myocardial structure and function in response to mutations in structural components of the cardiomyocyte. Myocardial structure and %%%signal%%% transduction are now merging into a common field of research that will lead to a more complete understanding of the molecular mechanisms that underly heart disease. (278 Refs.)
Record Date Created: 20011109
Record Date Completed: 20020208

2/7/98 (Item 98 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

13974674 PMID: 11073891
Calmodulin kinases II and IV and %%%calcineurin%%% are involved in

leukemia inhibitory factor-induced cardiac %%%hypertrophy%%% in rats.
Kato T; Sano M; Miyoshi S; Sato T; Hakuno D; Ishida H; Kinoshita-Nakazawa H; Fukuda K; Ogawa S
Cardiopulmonary Division, Department of Internal Medicine, Keio University, Tokyo, Japan.
Circulation research (UNITED STATES) Nov 10 2000, 87 (10) p937-45, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
We recently reported that leukemia inhibitory factor (LIF) enhances Ca(2+)](i) through an increase in L-type Ca(2+) current (I(Ca,L)) in adult cardiomyocytes. The aim of this study was to investigate whether LIF activates Ca(2+)-dependent signaling molecules, such as %%%calcineurin%%% and calmodulin kinases II and IV (CaMKII and CaMKIV), and, if so, whether these Ca(2+)-mediated signaling events contribute to LIF-mediated cardiac %%%hypertrophy%%% . We first confirmed that LIF increased I(Ca,L) and [Ca(2+)](i) in primary cultured rat neonatal cardiomyocytes. %%%Calcineurin%%%, CaMKII, and CaMKIV activities increased at 2 minutes and peaked by 1.6-, 2.2-, and 2.2-fold, respectively, at 15 minutes. Nicardipine or verapamil fully inhibited these activities. Autophosphorylation of CaMKII was also observed to parallel the timing of CaMKII activity, and this phosphorylation was blocked by nicardipine, verapamil, or EGTA. LIF treatment led to a 3-fold increase in %%%nuclear%%% factor of activated T cell-luciferase activity. To confirm that inositol triphosphate (IP(3))-induced Ca(2+) release from sarcoplasmic reticulum was not involved in this process, IP(3) content and phosphorylation of phospholipase Cgamma were investigated. LIF did not increase IP(3) content or phosphorylate phospholipase Cgamma. KN62 (an inhibitor of CaMKII and CaMKIV) attenuated c-fos, brain natriuretic peptide, alpha-skeletal actin, and atrial natriuretic peptide expression. KN62 suppressed the LIF-induced increase in [(3)H]phenylalanine uptake and cell size. Cyclosporin A and FK506 slightly attenuated brain natriuretic peptide but did not affect c-fos or atrial natriuretic peptide expression. Cyclosporin A significantly reduced the LIF-induced increase in [(3)H]phenylalanine uptake. These findings indicated that LIF activated CaMKII, CaMKIV, and %%%calcineurin%%% through an increase in I:(Ca,L) and [Ca(2+)](i) and that CaMKII, CaMKIV, and %%%calcineurin%%% are critically involved in LIF-induced cardiac %%%hypertrophy%%%.
Record Date Created: 20001117
Record Date Completed: 20001130

2/7/99 (Item 99 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

13958127 PMID: 11055975
%%%Calcineurin%%% and beyond: cardiac %%%hypertrophic%%% signaling.
Molkentin J D
Department of Pediatrics, University of Cincinnati, Division of Molecular Cardiovascular Biology, Children's Hospital Medical Center, Cincinnati, Ohio, USA. molkj0@chmcc.org
Circulation research (UNITED STATES) Oct 27 2000, 87 (9) p731-8, ISSN 1524-4571--Electronic 0009-7330--Linking Journal Code: 0047103

Contract/Grant No.: HL-62927; HL; NHLBI NIH HHS United States; HL52318; HL; NHLBI NIH HHS United States; HL69562; HL; NHLBI NIH HHS United States
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
In response to increased ventricular wall tension or neurohumoral stimuli, the myocardium undergoes an adaptive %%%hypertrophy%%% response that temporarily augments pump function. Although initially beneficial, sustained cardiac %%%hypertrophy%%% can lead to decompensation and %%%cardiomyopathy%%% . Recent studies have focused on characterizing the molecular mechanisms that underlie cardiac %%%hypertrophy%%%. An increasing number of %%%signal%%% transduction pathways have been identified as important regulators of the %%%hypertrophic%%% response, including the low-molecular weight GTPases (Ras, RhoA, and Rac), mitogen-activated protein kinases, protein kinase C, and %%%calcineurin%%%. This review will discuss an emerging body of evidence that implicates the calcium-calmodulin-activated protein phosphatase %%%calcineurin%%% as a physiological regulator of the cardiac %%%hypertrophic%%% response. Although the sufficiency of %%%calcineurin%%% to promote cardiomyocyte %%%hypertrophy%%% in vivo and in vitro is established, its overall necessity as a %%%hypertrophic%%% mediator is currently an area of ongoing debate. The use of the %%%calcineurin%%%-inhibitory agents cyclosporine A and FK506 have suggested a necessary role for %%%calcineurin%%% in many, but not all, animal models of %%%hypertrophy%%% or %%%cardiomyopathy%%%. The evidence implicating a role for %%%calcineurin%%% signaling in the heart will be weighed against a growing body of literature suggesting necessary roles for a diverse array of intracellular signaling pathways, highlighting the multifactorial nature of the %%%hypertrophic%%% program.
(84 Refs.)
Record Date Created: 20001103
Record Date Completed: 20001207

2/7/100 (Item 100 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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13860072 PMID: 10938134 Record Identifier: PMC86143
A %%%calcineurin%%% -NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression.
Delling U; Tureckova J; Lim H W; De Windt L J; Rotwein P; Molkentin J D
Department of Pediatrics, University of Cincinnati, and Division of Molecular Cardiovascular Biology, Children's Hospital Medical Center, Cincinnati, Ohio 45229-3039, USA.
Molecular and cellular biology (UNITED STATES) Sep 2000, 20 (17) p6600-11, ISSN 0270-7306--Print 0270-7306--Linking Journal Code: 8109087
Contract/Grant No.: DK-42748; DK; NIDDK NIH HHS United States; HL-62927; HL; NHLBI NIH HHS United States; HL-69562; HL; NHLBI NIH HHS United States
Publishing Model Print; Cites Proc Natl Acad Sci U S A. 1999 Mar 16;96(6):3281-6 PMID 10077675; Cites J Cell Physiol. 1996 Nov;169(2):227-34 PMID 8908189; Cites Cell. 1999 Mar 5;96(5):611-4 PMID 10089876; Cites Biochem J. 1999 Apr 15;339 (Pt 2):443-51 PMID 10191278; Cites Proc Natl Acad Sci U S A. 1999 Jun 22;96(13):7214-9 PMID 10377394;

Cites J Biol Chem. 1999 Jul 30;274(31):21908-12 PMID 10419511; Cites J Cell Sci. 1999 Sep;112 (Pt 17):2895-901 PMID 10444384; Cites Nature. 1999 Aug 5;400(6744):576-81 PMID 10448861; Cites Nature. 1999 Aug 5;400(6744):581-5 PMID 10448862; Cites J Biol Chem. 1999 Dec 31;274(53):37821-6 PMID 10608845; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites J Biol Chem. 2000 Feb 18;275(7):4545-8 PMID 10671477; Cites J Biol Chem. 1992 Dec 15;267(35):25129-34 PMID 1334082; Cites Cell. 1992 Dec 24;71(7):1181-94 PMID 1335366; Cites J Gen Physiol. 1991 Sep;98(3):615-35 PMID 1761971; Cites Proc Natl Acad Sci U S A. 1988 Aug;85(15):5728-32 PMID 2456580; Cites J Biol Chem. 1989 Aug 15;264(23):13810-7 PMID 2474537; Cites Am J Physiol. 1987 Aug;253(2 Pt 1):C296-300 PMID 2956887; Cites J Biol Chem. 1986 Jan 5;261(1):376-80 PMID 3941082; Cites Cell. 1983 Apr;32(4):1171-80 PMID 6839359; Cites J Biol Chem. 1993 Oct 25;268(30):22305-12 PMID 7693664; Cites Mol Cell Biol. 1995 May;15(5):2697-706 PMID 7739550; Cites Immunity. 1995 May;2(5):461-72 PMID 7749981; Cites Mol Cell Biol. 1995 Jul;15(7):3823-9 PMID 7791789; Cites Science. 1995 Feb 17;267(5200):1022-4 PMID 7863328; Cites Nature. 1994 Jun 9;369(6480):497-502 PMID 8202141; Cites Endocrinology. 1993 Aug;133(2):474-81 PMID 8393762; Cites Cell. 1995 Dec 29;83(7):1125-36 PMID 8548800; Cites J Biol Chem. 1996 Apr 12;271(15):9009-13 PMID 8621548; Cites J Cell Biol. 1996 May;133(3):683-93 PMID 8636241; Cites Mol Cell Biol. 1997 Feb;17(2):1010-26 PMID 9001254; Cites Cell Struct Funct. 1997 Feb;22(1):37-43 PMID 9113388; Cites Nature. 1997 Apr 24;386(6627):855-8 PMID 9126747; Cites Annu Rev Immunol. 1997;15:707-47 PMID 9143705; Cites FEBS Lett. 1997 Aug 18;413(2):354-8 PMID 9280312; Cites Muscle Nerve. 1997 Nov;20(11):1404-13 PMID 9342157; Cites Circ Res. 1998 Jan 9-23;82(1):94-105 PMID 9440708; Cites Mol Endocrinol. 1998 Jan;12(1):66-77 PMID 9440811; Cites Cell. 1998 Apr 17;93(2):215-28 PMID 9568714; Cites J Appl Physiol. 1998 Jun;84(6):1967-75 PMID 9609791; Cites J Biol Chem. 1998 Jul 10;273(28):17696-701 PMID 9651367; Cites Immunity. 1998 Jun;8(6):703-11 PMID 9655484; Cites J Biol Chem. 1998 Jul 17;273(29):18325-31 PMID 9660798; Cites Cell Tissue Res. 1996 Aug;285(2):297-303 PMID 8766166; Cites Curr Opin Genet Dev. 1996 Aug;6(4):445-53 PMID 8791524; Cites Genes Dev. 1996 Oct 1;10(19):2478-90 PMID 8843199; Cites Genes Dev. 1998 Aug 15;12(16):2499-509 PMID 9716403; Cites Mol Biol Cell. 1998 Oct;9(10):2905-16 PMID 9763451; Cites Proc Natl Acad Sci U S A. 1998 Oct 13;95(21):12358-63 PMID 9770491; Cites Proc Natl Acad Sci U S A. 1998 Nov 24;95(24):14179-83 PMID 9826674; Cites Proc Natl Acad Sci U S A. 1998 Dec 22;95(26):15603-7 PMID 9861016; Cites Biofactors. 1998;8(3-4):273-81 PMID 9914829; Cites J Biol Chem. 1999 Feb 19;274(8):5193-200 PMID 9988769; Cites Mol Cell Biol. 1996 Nov;16(11):6065-74 PMID 8887636; Cites J Virol. 1996 Nov;70(11):7498-509 PMID 8892868; Cites Am J Physiol. 1996 Oct;271(4 Pt 1):C1409-14 PMID 8897848; Cites J Biol Chem. 1996 Oct 25;271(43):26981-8 PMID 8900184; Cites Mol Cell Biol. 1999 Apr;19(4):3115-24 PMID 10082578

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Languages: ENGLISH

Main Citation Owner: NLM

Other Citation Owner: NLM

Record type: MEDLINE; Completed

The differentiation and maturation of skeletal muscle cells into functional fibers is coordinated largely by inductive signals which act through discrete intracellular %%%signal%%% transduction pathways. Recently, the calcium-activated phosphatase %%%calcineurin%%% (PP2B) and

the family of transcription factors known as NFAT have been implicated in the regulation of myocyte %%%hypertrophy%%% and fiber type specificity. Here we present an analysis of the intracellular mechanisms which underlie myocyte differentiation and fiber type specificity due to an insulinlike growth factor 1 (IGF-1)-%%%calcineurin%%%-NFAT %%%signal%%% transduction pathway. We demonstrate that %%%calcineurin%%% enzymatic activity is transiently increased during the initiation of myogenic differentiation in cultured C2C12 cells and that this increase is associated with NFATc3 %%%nuclear%%% translocation. Adenovirus-mediated gene transfer of an activated %%%calcineurin%%% protein (AdCnA) potentiates C2C12 and Sol8 myocyte differentiation, while adenovirus-mediated gene transfer of noncompetitive %%%calcineurin%%%-inhibitory peptides (cain or DeltaAKAP79) attenuates differentiation. AdCnA infection was also sufficient to rescue myocyte differentiation in an IGF-depleted myoblast cell line. Using 10T1/2 cells, we demonstrate that MyoD-directed myogenesis is dramatically enhanced by either %%%calcineurin%%% or NFATc3 cotransfection, while a %%%calcineurin%%% inhibitory peptide (cain) blocks differentiation. Enhanced myogenic differentiation directed by %%%calcineurin%%%, but not NFATc3, preferentially specifies slow myosin heavy-chain expression, while enhanced differentiation through mitogen-activated protein kinase kinase 6 (MKK6) promotes fast myosin heavy-chain expression. These data indicate that a signaling pathway involving IGF-%%%calcineurin%%%-NFATc3 enhances myogenic differentiation whereas %%%calcineurin%%% acts through other factors to promote the slow fiber type program.
Record Date Created: 20000921
Record Date Completed: 20000921

2/7/101 (Item 101 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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13849410 PMID: 10924357
Tetracycline-inducible CaM kinase II silences %%%hypertrophy%%%-sensitive gene expression in rat neonate cardiomyocytes.
Valencia T G; Roberts L D; Zeng H; Grant S R
Laboratory of Cardiac and Vascular Molecular Genetics, University of North Texas Health Science Center at Fort Worth, Fort Worth, Texas, 76107, USA.
Biochemical and biophysical research communications (UNITED STATES) Aug 11 2000, 274 (3) p803-10, ISSN 0006-291X--Print 0006-291X--Linking
Journal Code: 0372516
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Recent work from this laboratory both in rat primary cardiomyocytes and in ventricular tissue of transgenic mouse models of induced %%%hypertrophy%%% has identified two Ca(2+)/calmodulin-dependent %%%nuclear%%% signaling cascades. The first involves the phosphatase %%%calcineurin%%% (CaN). The second is the CaM kinase kinase cascade which involves CaM kinase I and CaM kinase IV. Each of these signaling cascades strongly up-regulate transcription of %%%hypertrophy%%%-sensitive genes in the rat ventricular cardiomyocyte. We have documented that over-expression of an active form of CaM kinase II silenced transcriptional induction of %%%hypertrophy%%% -sensitive genes. The purpose of this study was to

generate an inducible CaM kinase II expression system and correlate its expression with the silencing of %%%hypertrophic%%%-sensitive reporters. A truncated form of CaM KII, CaM KII (1-290) was subcloned downstream and proximal to a promoter under transcriptional control (induction) of the tetracycline-regulated transcription factor, tet-TransActivator (tTA). %%%Hypertrophy%%%-sensitive reporter activity in primary cardiomyocytes was silenced when tet-inducible CaM KII was co-expressed with plasmids harboring active forms of CaN, CaM KI or CaM KIV. For instance, induced CaM KII expression silenced CaN, CaM kinase I, or CaM kinase IV driven ANF reporter activity 4.9-, 2.9-, and 6.9-fold below their maximal values, respectively. Myocyte exposure to doxycycline (DOX) blocked tTA-driven CaM KII expression and restored CaN/CaM KI or CaN/CaM KIV driven reporter activation. This study demonstrates, for the first time, that active CaM KII silences Ca(2+)-sensitive %%%nuclear%%% signaling cascades for transcriptional up-regulation of cardiomyocyte %%%hypertrophy%%%. Copyright 2000 Academic Press.
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Record Date Completed: 20000915

2/7/102 (Item 102 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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13803457 PMID: 10869527
Mechanical stress-induced cardiac %%%hypertrophy%%%: mechanisms and %%%signal%%% transduction pathways.
Ruwhof C; van der Laarse A
Department of Cardiology, Leiden University Medical Center, P.O. Box 9600, 2300 RC, Leiden, The Netherlands.
Cardiovascular research (NETHERLANDS) Jul 2000, 47 (1) p23-37, ISSN 0008-6363--Print 0008-6363--Linking Journal Code: 0077427
Publishing Model Print
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Cardiac %%%hypertrophy%%% is a well known response to increased hemodynamic load. Mechanical stress is considered to be the trigger inducing a growth response in the overloaded myocardium. Furthermore, mechanical stress induces the release of growth-promoting factors, such as angiotensin II, endothelin-1, and transforming growth factor-beta, which provide a second line of growth induction. In this review, we will focus on the primary effects of mechanical stress: how mechanical stress may be sensed, and which %%%signal%%% transduction pathways may couple mechanical stress to modulation of gene expression, and to increased protein synthesis. Mechanical stress may be coupled to intracellular signals that are responsible for the %%%hypertrophic%%% response via integrins and the cytoskeleton or via sarcolemmal proteins, such as phospholipases, ion channels and ion exchangers. The %%%signal%%% transduction pathways that may be involved belong to two groups: (1) the mitogen-activated protein kinases (MAPK) pathway; and (2) the janus kinase/%%%signal%%% transducers and activators of transcription (JAK/STAT) pathway. The MAPK pathway can be subdivided into the extracellular-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), and the 38-kDa MAPK (p38 MAPK) pathway. Alternatively, the stress %%%signal%%% may be directly submitted to the %%%nucleus%%% via the cytoskeleton without the involvement of %%%signal%%%

transduction pathways. Finally, by promoting an increase in intracellular Ca2+ concentration stretch may stimulate the calcium/calmodulin-dependent phosphatase %%%calcineurin%%%, a novel %%%hypertrophic%%% signalling pathway. (208 Refs.)
Record Date Created: 20000929
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2/7/103 (Item 103 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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13780180 PMID: 10842305
Remodeling muscles with %%%calcineurin%%%.
Olson E N; Williams R S
Department of Molecular Biology, University of Texas, Southwestern Medical Center at Dallas, Texas. eolson@hamon.swmed.edu
BioEssays - news and reviews in molecular, cellular and developmental biology (ENGLAND) Jun 2000, 22 (6) p510-9, ISSN 0265-9247--Print 0265-9247--Linking Journal Code: 8510851
Publishing Model Print; Erratum in Bioessays 2000 Nov;22(11):1049
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Ca(2+) signaling plays a central role in %%%hypertrophic%%% growth of cardiac and skeletal muscle in response to mechanical load and a variety of signals. However, the mechanisms whereby alterations in Ca(2+) in the cytoplasm activate the %%%hypertrophic%%% response and result in longterm changes in muscle gene expression are unclear. The Ca(2+), calmodulin-dependent protein phosphatase %%%calcineurin%%% has been proposed to control cardiac and skeletal muscle %%%hypertrophy%%% by acting as a Ca(2+) sensor that couples prolonged changes in Ca(2+) levels to reprogramming of muscle gene expression. %%%Calcineurin%%% also controls the contractile and metabolic properties of skeletal muscle by activating the slow muscle fiber-specific gene program, which is dependent on Ca(2+) signaling. Transcription factors of the NFAT and MEF2 families serve as endpoints for the signaling pathways whereby %%%calcineurin%%% controls muscle %%%hypertrophy%%% and fiber-type. We consider these findings in the context of a model for Ca(2+)-regulated gene expression in muscle cells and discuss potential implications of these findings for pharmacologic modification of cardiac and skeletal muscle function. BioEssays 22:510-519, 2000. Copyright 2000 John Wiley & Sons, Inc. (79 Refs.)
Record Date Created: 20000929
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2/7/104 (Item 104 from file: 154)
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13756209 PMID: 10811847 Record Identifier: PMC315462
CaM kinase signaling induces cardiac %%%hypertrophy%%% and activates the MEF2 transcription factor in vivo.
Passier R; Zeng H; Frey N; Naya F J; Nicol R L; McKinsey T A; Overbeek P; Richardson J A; Grant S R; Olson E N
Department of Molecular Biology, The University of Texas Southwestern

Medical Center at Dallas, Dallas, Texas 75235-9148, USA.
Journal of clinical investigation (UNITED STATES) May 2000, 105 (10) p1395-406, ISSN 0021-9738--Print 0021-9738--Linking Journal Code: 7802877
Publishing Model Print;
Cites J Biol Chem. 1991 Aug 25;266(24):15910-6 PMID 1714900; Cites J Biol Chem. 1992 Jun 15;267(17):11665-8 PMID 1376309; Cites J Biol Chem. 1991 May 15;266(14):9180-5 PMID 2026617; Cites Experientia. 1990 Jan 15;46(1):81-4 PMID 2153573; Cites Mol Cell Biol. 1989 Nov;9(11):5022-33 PMID 2601707; Cites Fed Proc. 1986 Jun;45(7):2106-10 PMID 3011520; Cites J Biol Chem. 1986 Oct 5;261(28):13333-41 PMID 3759968; Cites J Biol Chem. 1994 Apr 1;269(13):9466-72 PMID 7511588; Cites Circ Res. 1995 May;76(5):907-14 PMID 7729009; Cites Annu Rev Physiol. 1995;57:417-45 PMID 7778873; Cites Am J Physiol. 1994 Dec;267(6 Pt 2):H2193-203 PMID 7810719; Cites J Biol Chem. 1995 Jan 6;270(1):410-7 PMID 7814403; Cites Genes Dev. 1994 Nov 1;8(21):2527-39 PMID 7958915; Cites Mol Cell Biol. 1994 Sep;14(9):6107-16 PMID 8065343; Cites J Biol Chem. 1994 Jun 17;269(24):16754-60 PMID 8206998; Cites Cell. 1993 Dec 3;75(5):977-84 PMID 8252633; Cites Circ Res. 1999 Apr 2;84(6):623-32 PMID 10189350; Cites Genes Dev. 1992 Sep;6(9):1783-98 PMID 1516833; Cites J Biol Chem. 1991 Jun 15;266(17):11144-52 PMID 1645727; Cites Hypertension. 1990 Jan;15(1):20-8 P MID 1688546; Cites J Biol Chem. 1993 Dec 15;268(35):26171-8 PMID 8253736; Cites Development. 1993 Aug;118(4):1095-106 PMID 8269842; Cites Endocrinolo gy. 1993 Jul;133(1):376-88 PMID 8319584; Cites Circ Res. 1993 Sep;73(3):424-38 PMID 8348687; Cites Mol Cell Biol. 1993 Jan;13(1):600-12 PMID 8417355; Cites Endocrinology. 1996 Mar;137(3):1108-17 PMID 8603581; Cites J Biol Chem. 1996 May 3;271(18):10827-33 PMID 8631897; Cites J Exp Med. 1996 Jul 1;184(1):101-12 PMID 8691123; Cites J Biol Chem. 1996 Aug 30;271(35):21542-8 PMID 8702940; Cites Annu Rev Physiol. 1997;59:551-71 PMID 9074777; Cites Nature. 1997 Apr 24;386(6627):855-8 PMID 9126747; Cites Science. 1997 May 30;276(5317):1404-7 PMID 9162005; Cites Proc Natl Acad Sci U S A. 1997 Jul 8;94(14):7543-8 PMID 9207128; Cites J Biol Chem. 1997 Dec 5;272(49):31203-8 PMID 9388275; Cites Circulation. 1997 Dec 2;96(11):3943-53 PMID 9403619; Cites Circulation. 1998 Apr 21;97(15):1433-5 PMID 9576421; Cites Cardiovasc Res. 1998 Feb;37(2):381-404 PMID 9614495; Cites J Clin Invest. 1998 Jun 1;101(11):2415-26 PMID 9616213; Cites Science. 1998 Sep 11;281(5383):1690-3 PMID 9733519; Cites Development. 1998 Nov;125(22):4565-74 PMID 9778514; Cites Proc Natl Acad Sci U S A. 1998 Nov 10;95(23):13893-8 PMID 9811897; Cites J Biol Chem. 1998 Nov 27;273(48):31880-9 PMID 9822657; Cites Annu Rev Cell Dev Biol. 1998;14:167-96 PMID 9891782; Cites J Immunol. 1999 Feb 15;162(4):2057-63 PMID 9973478; Cites Circ Res. 1999 Apr 2;84(6):713-21 PMID 10189359; Cites Circ Res. 1999 Apr 2;84(6):722-8 PMID 10189360; Cites Circ Res. 1999 Apr 2;84(6):729-34 PMID 10189361; Cites Circ Res. 1999 Apr 2;84(6):735-40 PMID 10189362; Cites Development. 1999 May;126(10):2045-52 PMID 10207130; Cites Hypertens Res. 1999 Mar;22(1):1-9 PMID 10221344; Cites Curr Opin Genet Dev. 1999 Jun;9(3):267-74 PMID 10377279; Cites Cardiovasc Res. 1999 Apr;42(1):254-61 PMID 10435018; Cites Cell. 1999 Sep 3;98(5):555-8 PMID 10490095; Cites J Biol Chem. 1999 Oct 22;274(43):31102-7 PMID 10521511; Cites Curr Biol. 1999 Oct 21;9(20):1203-6 PMID 10531040; Cites Circulation. 1999 Dec 14;100(24):2449-54 PMID 10595959; Cites Curr Opin Cell Biol. 1999 Dec;11(6):683-8 PMID 10600704; Cites J Biol Chem. 2000 Jan 7;275(1):197-209 PMID 10617605; Cites Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1196-201 PMID 10655507; Cites Proc Natl Acad Sci U S A. 2000 Apr 11;97(8):4070-5 PMID 10737771; Cites J Biol Chem. 1992 Jan 15;267(2):1198-203 PMID 1309765; Cites J Biol Chem. 1992 Mar

15;267(8):5211-6 PMID 1371996; Comment on J Clin Invest. 2000 May;105(10):1339-42 PMID 10811840
Document type: Comment; Journal Article; Research Support, Non-U.S. Gov't ; Research Support, U.S. Gov't, P.H.S.
Languages: ENGLISH
Main Citation Owner: NLM
Other Citation Owner: NLM
Record type: MEDLINE; Completed
%%%Hypertrophic%%% growth is an adaptive response of the heart to diverse pathological stimuli and is characterized by cardiomyocyte enlargement, sarcomere assembly, and activation of a fetal program of cardiac gene expression. A variety of Ca(2+)-dependent %%%signal%%% transduction pathways have been implicated in cardiac %%%hypertrophy%%%, but whether these pathways are independent or interdependent and whether there is specificity among them are unclear. Previously, we showed that activation of the Ca(2+)/calmodulin-dependent protein phosphatase %%%calcineurin%%% or its target transcription factor NFAT3 was sufficient to evoke myocardial %%%hypertrophy%%% in vivo. Here, we show that activated Ca(2+)/calmodulin-dependent protein kinases-I and -IV (CaMKI and CaMKIV) also induce %%%hypertrophic%%% responses in cardiomyocytes in vitro and that CaMKIV overexpressing mice develop cardiac %%%hypertrophy%%% with increased left ventricular end-diastolic diameter and decreased fractional shortening. Crossing this transgenic line with mice expressing a constitutively activated form of NFAT3 revealed synergy between these signaling pathways. We further show that CaMKIV activates the transcription factor MEF2 through a posttranslational mechanism in the %%%hypertrophic%%% heart in vivo. Activated %%%calcineurin%%% is a less efficient activator of MEF2-dependent transcription, suggesting that the %%%calcineurin%%%/NFAT and CaMK/MEF2 pathways act in parallel. These findings identify MEF2 as a downstream target for CaMK signaling in the %%%hypertrophic%%% heart and suggest that the CaMK and %%%calcineurin%%% pathways preferentially target different transcription factors to induce cardiac %%%hypertrophy%%%.
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2/7/105 (Item 105 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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13539551 PMID: 10448862
IGF-1 induces skeletal myocyte %%%hypertrophy%%% through %%%calcineurin%%% in association with GATA-2 and NF-ATc1.
Musaro A; McCullagh K J; Naya F J; Olson E N; Rosenthal N Rosenthal N Harvard Med Sch, Boston, MA
Cardiovascular Research Center, Massachusetts General Hospital-East, Charlestown 02129, USA.
Nature (ENGLAND) Aug 5 1999, 400 (6744) p581-5, ISSN 0028-0836--Print 0028-0836--Linking Journal Code: 0410462
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, Non-P.H.S.; Research Support, U.S. Gov't, P.H.S.
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Localized synthesis of insulin-like growth factors (IGFs) has been

broadly implicated in skeletal muscle growth, %%%hypertrophy%%% and regeneration. Virally delivered IGF-1 genes induce local skeletal muscle %%%hypertrophy%%% and attenuate age-related skeletal muscle atrophy, restoring and improving muscle mass and strength in mice. Here we show that the molecular pathways underlying the %%%hypertrophic%%% action of IGF-1 in skeletal muscle are similar to those responsible for cardiac %%%hypertrophy%%% . Transfected IGF-1 gene expression in postmitotic skeletal myocytes activates %%%calcineurin%%%-mediated calcium signalling by inducing %%%calcineurin%%% transcripts and %%%nuclear%%% localization of %%%calcineurin%%% protein. Expression of activated %%%calcineurin%%% mimics the effects of IGF-1, whereas expression of a dominant-negative %%%calcineurin%%% mutant or addition of cyclosporin, a %%%calcineurin%%% inhibitor, represses myocyte differentiation and %%%hypertrophy%%%. Either IGF-1 or activated %%%calcineurin%%% induces expression of the transcription factor GATA-2, which accumulates in a subset of myocyte %%%nuclei%%%, where it associates with %%%calcineurin%%% and a specific dephosphorylated isoform of the transcription factor NF-ATc1. Thus, IGF-1 induces %%%calcineurin%%% -mediated signalling and activation of GATA-2, a marker of skeletal muscle %%%hypertrophy%%%, which cooperates with selected NF-ATc isoforms to activate gene expression programs.

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Record Date Completed: 19990902

2/7/106 (Item 106 from file: 154)

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13539550 PMID: 10448861

Skeletal muscle %%%hypertrophy%%% is mediated by a Ca2+-dependent %%%calcineurin%%% signalling pathway.

Semsarian C; Wu M J; Ju Y K; Marciniec T; Yeoh T; Allen D G; Harvey R P; Graham R M

Victor Chang Cardiac Research Institute, St Vincent's Hospital, New South Wales, Australia.

Nature (ENGLAND) Aug 5 1999, 400 (6744) p576-81, ISSN 0028-0836--Print 0028-0836--Linking Journal Code: 0410462

Publishing Model Print

Document type: Journal Article; Research Support, Non-U.S. Gov't

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Skeletal muscle %%%hypertrophy%%% and regeneration are important adaptive responses to both physical activity and pathological stimuli. Failure to maintain these processes underlies the loss of skeletal muscle mass and strength that occurs with ageing and in myopathies. Here we show that stable expression of a gene encoding insulin-like growth factor 1 (IGF-1) in C2C12 skeletal muscle cells, or treatment of these cells with recombinant IGF-1 or with insulin and dexamethasone, results in %%%hypertrophy%%% of differentiated myotubes and a switch to glycolytic metabolism. Treatment with IGF-1 or insulin and dexamethasone mobilizes intracellular calcium, activates the Ca2+/calmodulin-dependent phosphatase %%%calcineurin%%%, and induces the %%%nuclear%%% translocation of the transcription factor NF-ATc1. %%%Hypertrophy%%% is suppressed by the %%%calcineurin%%% inhibitors cyclosporin A or FK506, but not by inhibitors of the MAP-kinase or phosphatidylinositol-3-OH kinase pathways. Injecting rat latissimus dorsi muscle with a plasmid encoding IGF-1 also activates

%%%calcineurin%%% , mobilizes satellite cells and causes a switch to glycolytic metabolism. We propose that growth-factor-induced skeletal-muscle %%%hypertrophy%%% and changes in myofibre phenotype are mediated by calcium mobilization and are critically regulated by the %%%calcineurin%%%/NF-ATc1 signalling pathway.
Record Date Created: 19990902
Record Date Completed: 19990902

2/7/107 (Item 107 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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13530710 PMID: 10440234
Signaling pathways mediating the response to %%%hypertrophic%%% stress in the heart.
Force T; Hajjar R; Del Monte F; Rosenzweig A; Choukroun G
Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, Boston 02129, USA.
Gene expression (UNITED STATES) 1999, 7 (4-6) p337-48, ISSN 1052-2166--Print 1052-2166--Linking Journal Code: 9200651
Contract/Grant No.: HL50361; HL; NHLBI NIH HHS United States; HL54202; HL ; NHLBI NIH HHS United States; HL57623; HL; NHLBI NIH HHS United States; +
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Cardiac %%%hypertrophy%%% is an increase in the mass of the heart. It is a major risk factor for the development of myocardial infarction and congestive heart failure, diseases that afflict millions of patients worldwide. %%%Hypertrophy%%% can be caused by intrinsic defects of the proteins of the contractile apparatus of the heart, or by extrinsic stimuli such as hypertension. In this review, we will focus on the cytosolic %%%signal%%% transduction pathways that mediate the %%%hypertrophic%%% response to extrinsic stimuli. Although a large number of signaling molecules have been implicated in the %%%hypertrophic%%% response, we will review data that, we believe, suggest there may be only a few molecules that serve as signaling funnels through which many %%%hypertrophic%%% signals must pass on their way to the %%%nucleus%%%. These include the stress response protein kinases (the stress-activated protein kinases or SAPKs, and, possibly, the p38 kinases) and %%%calcineurin%%% . These molecules have as their primary targets transcription factors, many of which have been implicated in the complex yet stereotypic genetic response to %%%hypertrophic%%% stress. In most cases, it is not possible at present to complete the link from %%%hypertrophic%%% stimulus through a specific signaling molecule and a specific transcription factor to the induction of a specific gene that initiates a particular biologic response. We will attempt to identify some of the most important areas where major questions remain in the hopes of stimulating further research into this major cause of death and disability. (74 Refs.)
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Record Date Completed: 19991020

2/7/108 (Item 108 from file: 154)

DIALOG(R)File 154:MEDLINE(R)
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13475943 PMID: 10377279
Cardiac %%%hypertrophy%%%: sorting out the circuitry.
McKinsey T A; Olson E N
Department of Molecular Biology and Oncology University of Texas Southwestern Medical Center at Dallas, 75235-9148, USA.
Current opinion in genetics & development (ENGLAND) Jun 1999, 9 (3) p267-74, ISSN 0959-437X--Print 0959-437X--Linking Journal Code: 9111375
Publishing Model Print
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Cardiac %%%hypertrophy%%% is an adaptive response of the heart to a variety of intrinsic and extrinsic stimuli. The %%%hypertrophic%%% response, during which cardiomyocytes increase in size without undergoing cell division, initially serves to compensate for decreased cardiac output; however, prolonged %%%hypertrophy%%% can become detrimental, resulting in dilated %%%cardiomyopathy%%% and heart failure. Cardiac %%%hypertrophy%%% requires coupling of intracellular %%%signal%%% transduction systems with transcription factors that activate and maintain the %%%hypertrophic%%% program. Over the past year, signaling pathways involving G proteins, mitogen-activated protein kinases and calcium-responsive phosphatases have emerged as critical regulators of cardiac %%%hypertrophy%%%. (54 Refs.)
Record Date Created: 20000316
Record Date Completed: 20000316

2/7/109 (Item 109 from file: 154)
DIALOG(R)File 154:MEDLINE(R)
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12816985 PMID: 9568714
A %%%calcineurin%%%-dependent transcriptional pathway for cardiac %%%hypertrophy%%%.
Molkentin J D; Lu J R; Antos C L; Markham B; Richardson J; Robbins J; Grant S R; Olson E N
Department of Molecular Biology and Oncology, The University of Texas Southwestern Medical Center at Dallas, 75225-9148, USA.
Cell (UNITED STATES) Apr 17 1998, 93 (2) p215-28, ISSN 0092-8674--Print 0092-8674--Linking Journal Code: 0413066
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
In response to numerous pathologic stimuli, the myocardium undergoes a %%%hypertrophic%%% response characterized by increased myocardial cell size and activation of fetal cardiac genes. We show that cardiac %%%hypertrophy%%% is induced by the calcium-dependent phosphatase %%%calcineurin%%% , which dephosphorylates the transcription factor NF-AT3, enabling it to translocate to the %%%nucleus%%%. NF-AT3 interacts with the cardiac zinc finger transcription factor GATA4, resulting in synergistic activation of cardiac transcription. Transgenic mice that express activated

forms of %%%calcineurin%%% or NF-AT3 in the heart develop cardiac %%%hypertrophy%%% and heart failure that mimic human heart disease. Pharmacologic inhibition of %%%calcineurin%%% activity blocks %%%hypertrophy%%% in vivo and in vitro. These results define a novel %%%hypertrophic%%% signaling pathway and suggest pharmacologic approaches to prevent cardiac %%%hypertrophy%%% and heart failure.

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  Record Date Completed: 19980521
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              $30.52  109 Type(s) in Format  7
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            $4.03    1.089 DialUnits File155
     $4.03  Estimated cost File155
            OneSearch, 2 files,  3.371 DialUnits FileOS
     $0.54  TELNET
    $43.53  Estimated cost this search
    $58.49  Estimated total session cost   8.103 DialUnits
Logoff: level 05.29.00 D  10:52:44
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	5	calcineurin and ritter.in.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2010/05/24 11:45
L2	382	calcineurin same signal same nucle$	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2010/05/24 11:46
L3	117	calcineurin same (cardiomyopathy or hypertrophic)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/05/24 11:47

5/ 24/ 10 11:47:52 AM
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BIB DATA SHEET

CONFIRMATION NO. 8455

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
12/162,135	11/10/2008 **RULE**	435	1646	00824.07.0001

APPLICANTS
Oliver Ritter, Wuerzburg, GERMANY;

** **CONTINUING DATA** *************************
This application is a 371 of PCT/EP07/00643 01/25/2007

** **FOREIGN APPLICATIONS** *************************
EUROPEAN PATENT OFFICE (EPO) 06090014.9 01/27/2006

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03/17/2009

Foreign Priority claimed ☑ Yes ☐ No 35 USC 119(a-d) conditions met ☐ Yes ☑ No Verified and Acknowledged /ELIZABETH KEMMERER/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		GERMANY	9	12	7

ADDRESS

Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005
UNITED STATES

TITLE

PEPTIDE FOR INHIBITION OF CALCINEURIN

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950	No.____________ to charge/credit DEPOSIT ACCOUNT No.____________ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other ________________ ☐ Credit

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Sheet	1	of	3

Complete If Known	
Applicant Number	12/162,135
Filing Date	July 25, 2008
First Named Inventor	Oliver RITTER
Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	ROTE-0007-UT1

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US- 2003/0045679 A1	03-06-03	Crawford	
		US- 2003/0060399 A1	06-27-03	Brophy et al.	
		US-			
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Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		WO 00/28011	05-18-00	President & Fellows of Harvard		
		WO 02/50097 A2	06-27-02	Allergene Ltd.		

Examiner's Signature	/Elizabeth C. Kemmerer/	Date Considered	05/24/2010

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Applicant Number	12/162,135
Filing Date	July 25, 2008
First Named Inventor	Oliver RITTER
Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	ROTE-0007-UT1

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		Anonymous, Internet Article, Calcineurin A Antibodies, January, 2005	
		Aramburu, J., et al., “Affinity-Driven Peptide Selection of an NFAT Inhibitor More Selective than Cyclosporin A,” Science, Vol. 285, pages 2129-2133, September, 1999	
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		Wu, H., et al., “Critical Role of Calpain-Mediated Cleavage of Calcineurin in Excitotoxic Neurodegeneration,” J. of Biology Chemistry, Vol. 279, pages 4929-4940, 2004	

Examiner's Signature	/Elizabeth C. Kemmerer/	Date Considered	05/24/2010

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Complete If Known	
Applicant Number	12/162,135
Filing Date	July 25, 2008
First Named Inventor	Oliver RITTER
Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	ROTE-0007-UT1

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		Zhu, J., et al., "NF-AT Activation Requires Suppression of Crm1-Dependent Export by Calcineurin," Nature, Vol. 398, pages 256-260, 1999	
		Zou, Y., et al., "Isopreterenol Activates Extracellular Signal-Regulated Protein Kinases in Cardiomyocytes through Calcineurin," Circulation, Vol. 104, pages 102-108, 2001	
		.	

Examiner's Signature	/Elizabeth C. Kemmerer/	Date Considered	05/24/2010

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Oliver RITTER	)	Confirmation No: 8455
	)	
Application No.: 12/162,135	)	Group Art Unit: 1646
	)	
Filed: November 10, 2008	)	Examiner: Elizabeth KEMMERER

For: PEPTIDE FOR INHIBITION OF CALCINEURIN

United States Patent and Trademark Office
Mail Stop Amendment (Restriction)
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

RESPONSE TO RESTRICTION REQUIREMENT

Sir:

In response to the Restriction Requirement mailed **January 15, 2010**, the period for response which is extended to **April 15, 2010** by filing a petition for a two-month extension of time, has been reviewed and the comments of the U.S. Patent and Trademark Office have been considered. Applicants hereby elect **Species SEQ ID NO 1**, without traverse. Species **SEQ ID NO 1** is covered by all pending claims.

REMARKS

The Examiner has required a restriction between the following species:

SEQ ID NO 1
SEQ ID NO 2
SEQ ID NO 5
SEQ ID NO 6
SEQ ID NO 7
SEQ ID NO 8
SEQ ID NO 9
SEQ ID NO 10

Applicants elect the claims of **Species SEQ ID NO 1**, without traverse, *i.e.* **claims 1-12**, for prosecution in the instant application. Applicants reserve the right to file a divisional application for the subject matter covered by the remaining groups. The inventor for the invention of the elected claims is the same as the inventor of record in the application.

CONCLUSION

In view of the foregoing, it is respectfully submitted that this application is now in condition for allowance and favorable action is respectfully solicited.

The Commissioner is hereby authorized by this paper to charge any fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to **Deposit Account 22-0259**.

Respectfully submitted,

Date: March 31, 2010

/Ajay A. Jagtiani, Reg. No. 35,205/
Ajay A. Jagtiani
Attorney for Applicant(s)
Reg. No.: 35,205

Vedder Price P.C.
875 15th Street, NW
Washington, DC 20005
Telephone: (202) 312-3320
Facsimile: (202) 312-3322
CUSTOMER NO: 22506

Electronic Patent Application Fee Transmittal

Application Number:	12162135
Filing Date:	10-Nov-2008
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	Oliver Ritter
Filer:	Ajay A. Jagtiani/g mills
Attorney Docket Number:	00824.07.0001

Filed as Small Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 2 months with $0 paid	2252	1	245	245

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**245**

Electronic Acknowledgement Receipt

EFS ID:	7321695
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	Oliver Ritter
Customer Number:	22506
Filer:	Ajay A. Jagtiani/g mills
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	00824.07.0001
Receipt Date:	31-MAR-2010
Filing Date:	10-NOV-2008
Time Stamp:	08:16:47
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$245
RAM confirmation Number	6881
Deposit Account	220259
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Miscellaneous Incoming Letter	033110_00824070001_ASFILED_TRANSMITTAL.pdf	43825 2d0dcef8da6cb4d2cdcdb3474d55f2a6e579e546	no	1
Warnings:					
Information:					
2	Extension of Time	033110_00824070001_ASFILED_EXTENSION_OF_TIME.pdf	40899 0a37606addf812b7581543499263098c07c04a42	no	1
Warnings:					
Information:					
3	Response to Election / Restriction Filed	033110_00824070001_ASFILED_RESPONSE.pdf	30733 cc1711874d9aabeea4147c4cc7abbee02724195e	no	2
Warnings:					
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4	Fee Worksheet (PTO-875)	fee-info.pdf	30312 75202ec5db57a91647a91a7c032baad76bb7e993	no	2
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TRANSMITTAL FORM

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Application Number	12/162,135
Filing Date	November 10, 2008
First Named Inventor	Oliver RITTER
Art Unit	1646
Examiner Name	Elizabeth KEMMERER
Attorney Docket Number	00824.07.0001
Total Number of Pages in This Submission	4

ENCLOSURES (*Check all that apply*)

- ☐ Fee Transmittal Form
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- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☑ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
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- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
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- ☐ Status Letter
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  Response to Restriction Requirement

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	VEDDER PRICE P.C.		
Signature	/Ajay A. Jagtiani/		
Printed name	Ajay A. Jagtiani		
Date	March 31, 2010	Reg. No.	35,205

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Application Number 12/162,135	Filed November 10, 2008
For PEPTIDE FOR INHIBITION OF CALCINEURIN	
Art Unit 1646	Examiner Elizabeth KEMMERER

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$130	$65	$
☑ Two months (37 CFR 1.17(a)(2))	$490	$245	$ 245.00
☐ Three months (37 CFR 1.17(a)(3))	$1110	$555	$
☐ Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐ Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

☑ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☑ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 22-2059.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 35,205

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 ____________

/Ajay A. Jagtiani/	March 31, 2010
Signature	Date
Ajay A. Jagtiani	202-312-3320
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001	8455

22506 7590 01/15/2010

Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

EXAMINER	
KEMMERER, ELIZABETH	

ART UNIT	PAPER NUMBER
1646	

MAIL DATE	DELIVERY MODE
01/15/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)
	12/162,135	RITTER, OLIVER
	Examiner	Art Unit
	Elizabeth C. Kemmerer, Ph.D.	1646

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *1* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *25 July 2008*.

2a)☐ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-12* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *1-12* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

The species are as follows:

SEQ ID NO: 1

SEQ ID NO: 2

SEQ ID NO: 5

SEQ ID NO: 6

SEQ ID NO: 7

SEQ ID NO: 8

SEQ ID NO: 9

SEQ ID NO: 10

Applicant is required, in reply to this action, to elect a single species to which the claims shall be restricted if no generic claim is finally held to be allowable. The reply must also identify the claims readable on the elected species, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered non-responsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 CFR 1.141. If claims

are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each sequence defines a separate contribution over the prior art, requiring a separate search of literature and sequence databases.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species or invention to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention or species may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Elizabeth C. Kemmerer, Ph.D. whose telephone number is (571) 272-0874. The examiner can normally be reached on Monday through Friday, 9:00 a.m. to 5:30 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol, Ph.D. can be reached on (571) 272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/ECK/
16 December 2009

/Elizabeth C. Kemmerer/
Elizabeth C. Kemmerer, Ph.D.
Primary Examiner, Art Unit 1646

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
12/162,135	11/10/2008	Oliver Ritter	00824.07.0001

CONFIRMATION NO. 8455

POA ACCEPTANCE LETTER

OC000000039474641

22506
Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

Date Mailed: 01/05/2010

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 02/26/2009.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/blgray/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
12/162,135	11/10/2008	Oliver Ritter	ROTE-0007-UT1

CONFIRMATION NO. 8455

PUBLICATION NOTICE

OC000000036761834

22506
Vedder Price, PC
875 15th Street, NW
Suite 725
Washington, DC 20005

Title:PEPTIDE FOR INHIBITION OF CALCINEURIN

Publication No.US-2009-0170765-A1
Publication Date:07/02/2009

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
12/162,135	Oliver Ritter	ROTE-0007-UT1

22506
JAGTIANI + GUTTAG
10363-A DEMOCRACY LANE
FAIRFAX, VA 22030

INTERNATIONAL APPLICATION NO.	
PCT/EP07/00643	
I.A. FILING DATE	PRIORITY DATE
01/25/2007	01/27/2006

CONFIRMATION NO. 8455
371 ACCEPTANCE LETTER

OC000000035049234

Date Mailed: 03/20/2009

NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office in its capacity as a Designated / Elected Office (37 CFR 1.495), has determined that the above identified international application has met the requirements of 35 U.S.C. 371, and is ACCEPTED for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above and the relevant dates are:

11/10/2008	11/10/2008
DATE OF RECEIPT OF 35 U.S.C. 371(c)(1), (c)(2) and (c)(4) REQUIREMENTS	DATE OF COMPLETION OF ALL 35 U.S.C. 371 REQUIREMENTS

A Filing Receipt (PTO-103X) will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE " FILING DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1), (c)(2) and (c)(4) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN ABOVE.** *The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363).* Once the Filing Receipt has been received, send all correspondence to the Group Art Unit designated thereon.

The following items have been received:

- Indication of Small Entity Status
- Copy of the International Application filed on 07/25/2008
- Copy of the International Search Report filed on 07/25/2008
- Preliminary Amendments filed on 07/25/2008
- Information Disclosure Statements filed on 11/10/2008
- Biochemical Sequence Diskette filed on 11/10/2008
- Oath or Declaration filed on 11/10/2008
- Biochemical Sequence Listing filed on 11/10/2008
- U.S. Basic National Fees filed on 07/25/2008
- Assignment filed on 07/25/2008
- Power of Attorney filed on 02/26/2009

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

JOHN L ANDERSON

Telephone: (703) 308-9140 EXT 211

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
12/162,135	11/10/2008	1656	950	ROTE-0007-UT1	12	7

CONFIRMATION NO. 8455

22506
JAGTIANI + GUTTAG
10363-A DEMOCRACY LANE
FAIRFAX, VA 22030

FILING RECEIPT

Date Mailed: 03/20/2009

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Oliver Ritter, Wuerzburg, GERMANY;

Assignment For Published Patent Application

Julius-Maximillans-Universitaet Wuerzburg, Wuerzburg, GERMANY

Power of Attorney: The patent practitioners associated with Customer Number 22506

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/EP07/00643 01/25/2007

Foreign Applications

EUROPEAN PATENT OFFICE (EPO) 06090014.9 01/27/2006

If Required, Foreign Filing License Granted: 03/17/2009

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 12/162,135**

Projected Publication Date: 07/02/2009

Non-Publication Request: No

Early Publication Request: No

**** SMALL ENTITY ****

Title

PEPTIDE FOR INHIBITION OF CALCINEURIN

Preliminary Class

435

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as

set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

DO/EO WORKSHEET

U.S. Appl. No. 12/162135 International Appl. No. EP2007/000643

Application filed by: ☐ 20 months ☐ 30 months

WIPO PUBLICATION INFORMATION:

Publication No.: WO2007/085455 A1 Publication Language: ☑ English ☐ Japanese ☐ German ☐ French ☐ Other: ______ Screening Done by: [signature]

Publication Date: Aug 2, 2007 Not Published: ☐ U.S. only designated ☐ EP request

INTERNATIONAL APPLICATION PAPERS IN THE APPLICATION FILE:

- ☑ International Application *(RECORD COPY)*
- ☐ Article 19 Amendments
- ☐ PCT/IB/331
- ☐ PCT/IPEA/409 IPER (PCT/IPEA/416 on front)
- ☐ Annexes to 409
- ☐ Priority Document (s) No. ______
- ☐ International Appl. on Double Sided Paper *(COPIES MADE)*
- ☐ Request form PCT/RO/101
- ☑ PCT/ISA/210 - Search Report
- ☐ Search Report References
- ☐ Other: ______

RECEIPTS FROM THE APPLICANT *(other than checked above)*:

- ☑ Basic National Fee *(or authorization to charge)*
- ☑ Description
- ☑ Claims
- ☑ Words in the Drawing Figure(s) - (# of dwgs. 9)
- ☐ Article 19 Amendments
  - ☐ english transl. of annexes NOT present
  - ☐ entered ☐ not entered:
    - ☐ not a page for page substitution
    - ☐ other: ______
- ☐ Annexes to 409
  - ☐ english transl. of annexes NOT present
  - ☐ entered ☐ not entered:
    - ☐ not a page for page substitution
    - ☐ other: ______
- ☑ Preliminary Amendment(s) Filed on: 1. July 25, 2008 2. ______ 3. ______
- ☑ Information Disclosure Statement(s) Filed on: 1. Nov 10, 2008 2. ______ 3. ______
- ☑ Assignment Document
- ☑ Power of Attorney/ Change of Address — signed by agent
- ☐ Substitute Specification Filed on: 1. ______ 2. ______
- ☑ Small Entity
- ☑ Oath/ Declaration (executed)
  - ☐ surcharge was paid at the time of filing
- ☑ DNA Diskette ☑ Sequence Listing 11/10/08
- ☐ Other: 1. ______ 2. ______

NOTES: ☑ I.A. used as Specification ☐ Other:

35 U.S.C. 371 - Receipt of Request (PTO-1390)	July 25, 2008
Date Acceptable Oath/Declaration Received	Nov 10, 2008
Date of Completion of requirements under 35 U.S.C. 371	Nov 10, 2008
102(e) Date	Nov 10, 2008
Date of Completion of DO/EO 903 - Notification of Acceptance	Mar 17, 2009
Date of Completion of DO/EO 905 - Notification of Missing Requirements	
Date of Completion of DO/EO 906 - Notification of Missing 102(e) Requirements	
Date of Completion of DO/EO 907 - Notification of Acceptance for 102(e) Date	
Date of Completion of DO/EO 909 - Notification of Abandonment	
Date of Completion of DO/EO 911 - Application Accepted Under 35 U.S.C. 111	
Date of Completion of DO/EO 916 - Notification of Defective Response	
Date of Completion of DO/EO 910 - Notification to Comply w/ Seq. Requirements	

Docket No: ROTE-0007-UT1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: OLIVER RITTER	)	Confirmation No: 8455
	)	
Application No.: 12/162,135	)	Group Art Unit: UNASSIGNED
	)	
Filed: JULY 25, 2008	)	Examiner: UNASSIGNED

For: PEPTIDE FOR INHIBITION OF CALCINEURIN

United States Patent and Trademark Office
Customer Service Window, Mail Stop Amendment
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

POWER OF ATTORNEY

Sir:

Please recognize Steven B. Kelber, Registration No. 30,073, as an attorney of record in the above-referenced patent application. Also please update the correspondence records at the United States Patent and Trademark Office for this application to correspond with Customer Number 22506. For the convenience of the Office, attached to this filing is a copy of the Decision on Petition for Reinstatement for Steven B. Kelber, executed by Harry I. Moatz, Director of Enrollment and Discipline on February 3, 2009.

The Office is invited to contact the undersigned if there are any outstanding issues after consideration to expedite entry of this Power of Attorney and updating of the correspondence address to Customer Number 22506.

Respectfully submitted,

/Ajay A. Jagtiani/
Ajay A. Jagtiani
Attorney for Applicant
Reg. No.: 35,205

Customer No. 22506

Date: February 26, 2009

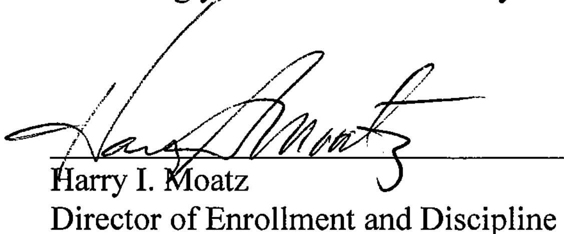
Patent Administrator
Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030
Telephone: (703) 591-2664
Facsimile: (703) 591-5907
CUSTOMER NO: 22506

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
OFFICE OF ENROLLMENT AND DISCIPLINE

In re:

Steven B. Kelber	:	DECISION ON PETITION
Jagtiani & Guttag	:	FOR REINSTATEMENT
10363-A Democracy Lane	:	
Fairfax, VA 22030	:	

Consideration has been given to the Petition for Reinstatement of Steven B. Kelber dated December 23, 2008. Mr. Kelber's attorney has filed on Mr. Kelber's behalf an Affidavit of Compliance and a Supplemental Affidavit of Compliance pursuant to 37 CFR § 11.58(b)(2) with documentation in support of the Petition. Mr. Kelber's attorney also sent to OED a letter dated January 30, 2009, responding to OED's communication dated December 31, 2008. After careful review of the Petition, supporting affidavits, documentation, and letter dated January 30, 2009, the Director of Enrollment and Discipline has determined that reinstatement of Mr. Kelber to practice before the United States Patent and Trademark Office is appropriate. Accordingly, the Petition is hereby GRANTED.



Harry I. Moatz
Director of Enrollment and Discipline

Date

Electronic Acknowledgement Receipt

EFS ID:	4865025
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	OLIVER RITTER
Customer Number:	22506
Filer:	Ajay A. Jagtiani/SUSAN MAHON
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	ROTE-0007-UT1
Receipt Date:	26-FEB-2009
Filing Date:	
Time Stamp:	15:07:03
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	2-26-09-ROTE-0007-UT1-POAAddingSteveback.pdf	69209 a5743685ecfd6f311c8079d44e9c1916d0a5886d	no	1

Warnings:

Information:

2	Miscellaneous Incoming Letter	1-DecisiononPetitionforReinstatement.pdf	33442 29cbae9a8f67cbbc0337126d85965a65054e83b7	no	1

Warnings:

Information:

Total Files Size (in bytes):	102651

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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Sequence Listing was accepted.

If you need help call the Patent Electronic Business Center at (866) 217-9197 (toll free).

Reviewer: Anne Corrigan

Timestamp: [year=2008; month=12; day=3; hr=9; min=37; sec=30; ms=836;]

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Validated By CRFValidator v 1.0.3

Application No: 12162135 Version No: 1.0

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Finished: 2008-11-10 11:31:12.932
Elapsed: 0 hr(s) 0 min(s) 2 sec(s) 110 ms
Total Warnings: 10
Total Errors: 0
No. of SeqIDs Defined: 10
Actual SeqID Count: 10

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PTO/SB/83 (04-08)
Approved for use through 12/31/2008. OMB 0651-0035
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

REQUEST FOR WITHDRAWAL AS ATTORNEY OR AGENT AND CHANGE OF CORRESPONDENCE ADDRESS

Application Number	12/162,135
Filing Date	JULY 25, 2008
First Named Inventor	RITTER, OLIVER
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	ROTE-0007-UT1

To: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Please withdraw me as attorney or agent for the above identified patent application, and

☐ all the practitioners of record;

☐ the practitioners (with registration numbers) of record listed on the attached paper(s); or

☐ the practitioners of record associated with Customer Number: ____________

NOTE: The immediately preceding box should only be marked when the practitioners were appointed using the listed Customer Number.

The reason(s) for this request are those described in 37 CFR :

☐ 10.40(b)(1)	☐ 10.40(b)(2)	☐ 10.40(b)(3)	☐ 10.40(b)(4)
☐ 10.40(c)(1)(i)	☐ 10.40(c)(1)(ii)	☐ 10.40(c)(1)(iii)	☐ 10.40(c)(1)(iv)
☐ 10.40(c)(1)(v)	☐ 10.40(c)(1)(vi)	☐ 10.40(c)(2)	☐ 10.40(c)(3)
☐ 10.40(c)(4)	☐ 10.40(c)(5)	☑ 10.40(c)(6) Please explain below:	

The Petitioner has been suspended from practice before the United States Patent and Trademark Office for sixty (60) days pursuant to the provisions of 37 C.F.R. Section 1.158.

Certifications

Check each box below that is factually correct. WARNING: If a box is left unchecked, the request will likely not be approved.

1. ☑ I/We have given reasonable notice to the client, prior to the expiration of the response period, that the practitioner(s) intend to withdraw from employment.

2. ☑ I/We have delivered to the client or a duly authorized representative of the client all papers and property (including funds) to which the client is entitled.

3. ☑ I/We have notified the client of any responses that may be due and the time frame within which the client must respond.

Please provide an explanation, if necessary:

Petitioner has been suspended from practice before the United States Patent and Trademark Office for a period of sixty (60) days effective October 24, 2008. Proceeding No: D2006-13.

This collection of information is required by 37 CFR 1.36. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

REQUEST FOR WITHDRAWAL AS ATTORNEY OR AGENT AND CHANGE OF CORRESPONDENCE ADDRESS

Complete the following section only when the correspondence address will change. *Changes of address will only be accepted to an inventor or an assignee that has properly made itself of record pursuant to 37 CFR 3.71.*

Change the correspondence address and direct all future correspondence to:

A. ☐ The address of the inventor or assignee associated with Customer Number: ____________

OR

B. ☐ Inventor or Assignee name			
Address			
City	State	Zip	Country
Telephone		Email	

I am authorized to sign on behalf of myself and all withdrawing practitioners.

Signature	/Steven B. Kelber/		
Name	Steven B. Kelber	Registration No.	30,073
Address	Jagtiani + Guttag, LLP, 10363-A Democracy Lane		
City Fairfax	State Virginia	Zip 22030	Country United States
Date	November 10, 2008	Telephone No.	703-591-2664

NOTE: ***Withdrawal is effective when approved rather than when received.***

This collection of information is required by 37 CFR 1.36. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	4257891
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	OLIVER RITTER
Customer Number:	22506
Filer:	Steven B. Kelber/SUSAN MAHON
Filer Authorized By:	Steven B. Kelber
Attorney Docket Number:	ROTE-0007-UT1
Receipt Date:	10-NOV-2008
Filing Date:	
Time Stamp:	11:13:36
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	11-10-08-ROTE-0007-UT1-SBK-Withdrawl-at-PTO.pdf	296611 cbcdbabc8da4f0cb65522800daa2119d14eca761	no	3

Warnings:

Information:

Total Files Size (in bytes):	296611

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No. ROTE-0007-UT1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: RITTER, Oliver	)	Confirmation No: 8455
Application No.: 12/162,135	)	Group Art Unit: Unassigned
Filed: July 25, 2008	)	Examiner: Unassigned

For: PEPTIDE FOR INHIBITION OF CALCINEURIN

United States Patent and Trademark Office
Customer Service Window, Mail Stop Amendment
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

Sir:

This is in response to the Notification of Missing Requirements dated October 3, 2008, the period for response to which is set to expire on December 3, 2008.

The attached executed Declaration is submitted in compliance with 37 CFR 1.497(a) and (b).

The attached Statement and computer readable copies of the Sequence Listing are submitted in compliance with C.F.R. 1.821-1.825.

Although it is believed that no fees are currently due, the Commissioner is hereby authorized to charge any additional fees which may be required under 37 CFR §§ 1.16(e) or credit any overpayment to **Deposit Account Number 10-0233.**

Respectfully submitted,

/Ajay A. Jagtiani/Reg. No. 35,205
Ajay A. Jagtiani
Attorney for Applicant(s)
Reg. No.: 35,205

Customer No. 22506

Date: November 10, 2008

Patent Administrator
Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030
Telephone: (703) 591-2664
Facsimile: (703) 591-5907
CUSTOMER NO: 22506

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION	Attorney Docket No.	ROTE-0007-UT1
	First Named Inventor	Oliver RITTER
	COMPLETE IF KNOWN	
	Application Serial Number	12/162,135
☐ Declaration Submitted with Initial Filing ☒ Declaration Submitted after Initial Filing (surcharge 37 CFR 1.16(e) required)	Filing Date	July 25, 2008
	Group Art Unit	Unassigned
	Examiner Name	Unassigned

As a below named inventor, I hereby declare that:

My (our) residence, mailing address, and citizenship are as stated below next to my name. I (we) believe I (we) am (are) the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PEPTIDE FOR INHIBITION OF CALCINEURIN

the specification of which:

☐ is attached hereto;

OR

☒ was filed on (07/25/2008) as United States Application Serial Number or PCT International Application Number 12/162,135 and was amended on (MM/DD/YYYY) ______ *(if applicable).*

I (we) hereby state that I (we) have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above. I (we) acknowledge the duty to disclose to the Patent Office all information known by me to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information known by me which became available between the filing date of the prior application and the national or Patent Cooperation Treaty (PCT) or international filing date of the continuation-in-part application.

For Assigned or Obligated to be Assigned Inventions: By executing this Power of Attorney, (I am)/(we are) allowing Jagtiani + Guttag, LLLP (the Firm) to prosecute the above-referenced patent application and to prosecute any matters that arise before the Patent Office during the enforceable life of the above-identified application, any patent that issues from this application and any future applications and/or patents, in any country, that claim priority to the above-identified application/patent. Although the Power states that (I am)/(we are) appointing the Firm with "power of attorney" for the sole purpose of prosecuting the matters arising before the Patent Office in connection with the patent and patent application, that appointment is understood solely in light of my (our) nominal interest in the application/ patent. I/we understand that, despite the use of the phrase "power of attorney," the executed power of attorney does not create any attorney-client relationship between me/us and the Firm, or between the Firm and any other person. The assignee in law or in fact will remain the Firm's sole client with respect to the application and/or patent. Accordingly, the firm understands, and my/our signature on the Power confirms, that the Firm has no attorney-client relationship with Assignor/inventor, or with me/us personally.

I (we) hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f),172 or 365(b) of any foreign application(s) for patent, or inventor's certificate(s), or 365(a) of any Patent Cooperation Treaty (PCT) or international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, or inventor's certificate(s), or of any PCT or international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	**Country**	**Foreign Filing Date (MM/DD/YYYY)**	**Priority Not Claimed**	**Certified Copy Attached? YES**	**NO**
06090014.9	EP	01/27/2006	☐	☐	☒
PCT/EP2007/000643	WIPO	01/25/2007	☐	☐	☒
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet attached hereto.

I (we) hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Serial Number(s)	**Filing Date (MM/DD/YYYY)**	
		☐ Additional provisional application serial numbers are listed on a supplemental priority data sheet attached hereto.

DECLARATION – Utility or Design Patent Application

I (we) hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c), of any Patent Cooperation Treaty or international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or Patent Cooperation Treaty or International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or Patent Cooperation Treaty or international filing date of this application.

U.S. Parent Application or PCT Parent Serial Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

☐ Additional U.S. or Patent Cooperation Treaty or international application numbers are listed on a supplemental priority data sheet attached hereto.

As a named inventor, I (we) hereby appoint(s) the practitioners associated with:

Customer Number: 22506

as attorney(s) and/or agent(s) to prosecute and transact all business in the U.S. Patent and Trademark Office in connection therewith. Customer Number 22506 is assigned to Jagtiani + Guttag, LLLP, 10363-A Democracy Lane, Fairfax, VA 22030.

As a named inventor, I (we) hereby grant additional Power of Attorney to the attorney(s) and/or agent(s) named below to file and prosecute **foreign national applications** in any and all countries of the world, regional patent applications under the European Patent Convention and/or international applications under the Patent Cooperation Treaty based upon the patent application identified above, including power to meet all designated office requirements for designated states.

Name	Reg. No.	Name	Reg. No.
Ajay A. Jagtiani	Reg. No.: 35,205	David J. Lanzotti	Reg. No. 60,765
Mark J. Guttag	Reg. No.: 33,057		
Steven B. Kelber	Reg. No.: 30,073		
Eric W. Guttag	Reg. No.: 28,853		
Kimberly O. Snead	Reg. No. 45,199		

☐ Additional registered practitioners named on supplemental Registered Practitioner Information sheet attached hereto.

Authorization To Permit Access to Application by Participating Offices

☑ If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), and any other intellectual property offices in which a foreign application claiming priority to the above-identified application is filed access to the above-identified patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JP,or other intellectual property office in which a foreign application claiming priority to the above-identified application is filed to have access to the application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the application-as-filed with respect to: 1) the above-identified application, 2) any foreign application to which the above-identified application claims priority under 35 USC 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the above-identified US application, and 3) any U.S. application from which benefit is sought in the above-identified application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing the Authorization to Permit Access to Application by Participating Offices.

Direct all correspondence to:

Patent Administrator
Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030
Tel. No.: (703) 591-2664
Fax No.: (703) 591-5907

CUSTOMER NO 22506

WARNING

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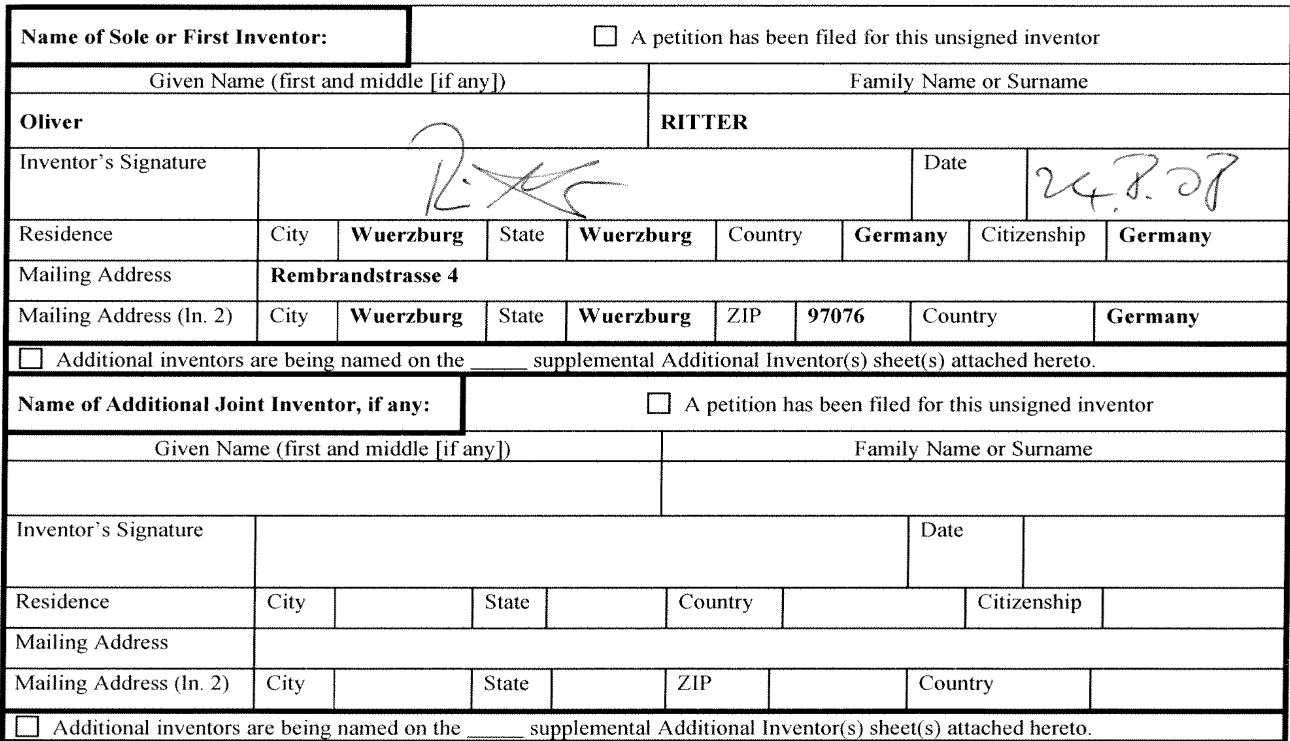
I (we) hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Oliver	**RITTER**

Inventor's Signature	[signature]			Date	24.8.08		
Residence	City	**Wuerzburg**	State	**Wuerzburg**	Country	**Germany**	Citizenship	**Germany**
Mailing Address	**Rembrandstrasse 4**							
Mailing Address (ln. 2)	City	**Wuerzburg**	State	**Wuerzburg**	ZIP	**97076**	Country	**Germany**

☐ Additional inventors are being named on the _____ supplemental Additional Inventor(s) sheet(s) attached hereto.

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname

Inventor's Signature				Date				
Residence	City		State		Country		Citizenship	
Mailing Address								
Mailing Address (ln. 2)	City		State		ZIP		Country	

☐ Additional inventors are being named on the _____ supplemental Additional Inventor(s) sheet(s) attached hereto.

Docket No. ROTE-0007-UT1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: RITTER, Oliver) Confirmation No: 8455

Application No.: 12/162,135) Group Art Unit: Unassigned

Filed: July 25, 2008) Examiner: Unassigned

For: PEPTIDE FOR INHIBITION OF CALCINEURIN

United States Patent and Trademark Office
Mail Stop Amendment
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

STATEMENT UNDER 37 C.F.R. 1.821-1.825

Sir:

I, Ajay A. Jagtiani, declare as follows:

1. That the contents of this paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. 1.821(c) and (e), respectively, are the same in compliance with 1.821 (f).

2. That the submission herein, filed in accordance with 37 C.F.R. 1.821 (g), does not include new matter.

Respectfully submitted,

Date: November 10, 2008

/Ajay A. Jagtiani/Reg. No. 35,205
Ajay A. Jagtiani
Attorney for Applicant(s)
Reg. No.: 35,205

Customer No. 22506

Patent Administrator
Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030
Telephone: (703) 591-2664
Facsimile: (703) 591-5907
CUSTOMER NO: 22506

Electronic Acknowledgement Receipt

EFS ID:	4258033
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	OLIVER RITTER
Customer Number:	22506
Filer:	Ajay A. Jagtiani/g mills
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	ROTE-0007-UT1
Receipt Date:	10-NOV-2008
Filing Date:	
Time Stamp:	11:30:20
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	111008_ROTE0007UT1_AsFiled_Transmittal.pdf	108640 2423c1b2cce6a2cc3bc8b1d3166b73018c4e1fc3	no	1

Warnings:

Information:

2	Applicant Response to Pre-Exam Formalities Notice	111008_ROTE0007UT1_AsFiled_Response_to_Missing_Requirements.pdf	96066 0e2ac8374640d64fe8195fa5a64cee9df05d8313	no	1
Warnings:					
Information:					
3	Oath or Declaration filed	111008_ROTE0007UT1_Executed_Declaration.pdf	328860 80f6e1715bc0abae3bd18a3e941d680c0fe1b1ed	no	3
Warnings:					
Information:					
4	Miscellaneous Incoming Letter	111008_ROTE0007UT1_AsFiled_Sequence_Listing_Statement.pdf	90193 cd3a12669e4600c5c2e82cb8d797bb26ffa65faf	no	1
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Information:					
5	Sequence Listing (Text File)	111008_ROTE0007UT1SequenceListing.txt	3688	no	0
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

TRANSMITTAL FORM

Application Serial Number	12/162,135
Filing Date	July 25, 2008
First Named Inventor	RITTER, Oliver
Group Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket No.	ROTE-0007-UT1
Patent No.	Not applicable
Issue Date	Not applicable

ENCLOSURES ***(check all that apply)***

☐ Fee Transmittal Form
- ☐ Check Attached
- ☐ Copy of Fee Transmittal Form

☐ Amendment/Response
- ☐ Preliminary
- ☐ After Final
- ☐ Affidavits/declaration(s)
- ☐ Letter to Official Draftsperson including Drawings [Total Sheets _____]

☐ Petition for Extension of Time (1/2/3 months)

☒ Information Disclosure Statement
- ☒ Form PTO-1449
- ☒ Copies of IDS Citations

☐ Certified Copy of Priority Document(s)

☒ Sequence Listing submission
- ☐ Paper Copy/CD
- ☒ Computer Readable Copy
- ☒ Statement verifying identity of above

☐ Copy of Notice to File Missing Parts of Application (PTO-1553)

☐ Formal Drawing(s)

☐ Request For Continued Examination (RCE) Transmittal

☐ Power of Attorney (Revocation of Prior Powers)

☐ Terminal Disclaimer

☒ Executed Declaration and Power of Attorney for Utility or Design Patent Application (**3** pages)

☐ Small Entity Statement

☐ CD(s) for large table or computer program

☐ Amendment After Allowance

☐ Request for Certificate of Correction
- ☐ Certificate of Correction (in duplicate)

☐ Notice of Appeal to Board of Patent Appeals and Interferences

☐ Appeal Brief (in triplicate)

☐ Status Inquiry

☐ Return Receipt Postcard

☐ Certificate of Facsimile Transmission under 37 C.F.R. 1.8

☒ Additional Enclosure(s) *(please identify below)*

Response to Notification of Missing Requirements

CORRESPONDENCE ADDRESS

Direct all correspondence to:

Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030
Tel. No.: (703) 591-2664
Fax No.: (703) 591-5907
CUSTOMER NO: 22506

SIGNATURE BLOCK

Respectfully submitted,

Date: November 10, 2008
Reg. No.: 35,205
Tel. No.: (703) 591-8664
Fax No.: (703) 591-5907

/Ajay A. Jagtiani, Reg. No. 35,205/
Ajay A. Jagtiani
Attorney for the Applicants
Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030

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Application Number: 12162135 Document Date: 11/10/2008

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- Sequence Listing

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

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Form Revision Date: February 8, 2006

Attorney Docket No.: ROTE-0007-UT1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	RITTER, Oliver	Confirmation No.: 8455
Appl. No.:	12/162,135	Examiner: Unassigned
Filing Date:	July 25, 2008	Art Unit: Unassigned
Title:	PEPTIDE FOR INHIBITION OF CALCINEURIN	

INFORMATION DISCLOSURE STATEMENT
UNDER 37 CFR §1.56 and 37 CFR §1.97

Commissioner for Patents
Customer Service Window, Mail Stop Amendment
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

Listed on the attached Forms PTO/SB/08a and PTO/SB/08b are documents known to applicants in order to comply with applicants' duty of disclosure pursuant to 37 CFR §1.56. Submitted herewith for the Examiner's convenience are copies of the listed foreign document(s) and non-patent literature document(s). These materials are being submitted to comply with the provisions of 37 CFR §1.97-§1.98.

The citation of any document herewith, is not intended as an admission that such document constitutes prior art against the claims of the present application or is considered to be material to patentability as defined in 37 CFR §1.56(b). Applicants do not waive any rights to take any action which would be appropriate to antedate or otherwise remove as a competent reference any document which is determined to be a *prima facie* prior art reference against the claims of the present application.

TIMING/FEE

This Information Disclosure Statement is being submitted before the first office action on the merits. Therefore, no fee is believed due in connection with this paper. However, if any fee is due, the Commissioner is hereby authorized to charge any deficiency or credit any overpayment in connection with this fee to Deposit Account No. 10-0233.

CONCLUSION

Applicants respectfully request that the listed document be considered by the Examiner and be made of record in the present application and that an initialed copy of Forms PTO/SB/08a and PTO/SB08b be returned in accordance with M.P.E.P. §609.

Respectfully submitted,

Date: November 10, 2008

By Ajay A. Jagtiani, Reg. No. 35,205/

Ajay A. Jagtiani
Attorney for Applicant(s)
Registration No. 35,205
Customer No. 22506

Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030
Telephone: (703) 591-2664
Facsimile: (703) 591-5907
CUSTOMER NO: 22506

Substitute for Form PTO-1449

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 3

Complete If Known	
Applicant Number	12/162,135
Filing Date	July 25, 2008
First Named Inventor	Oliver RITTER
Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	ROTE-0007-UT1

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US- 2003/0045679 A1	03-06-03	Crawford	
		US- 2003/0060399 A1	06-27-03	Brophy et al.	
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		WO 00/28011	05-18-00	President & Fellows of Harvard		
		WO 02/50097 A2	06-27-02	Allergene Ltd.		

Examiner's Signature		Date Considered	

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PTO/SB/08b (08-08)
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Substitute for Form PTO-1449

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Complete If Known	
Applicant Number	12/162,135
Filing Date	July 25, 2008
First Named Inventor	Oliver RITTER
Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	ROTE-0007-UT1

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		Anonymous, Internet Article, Calcineurin A Antibodies, January, 2005	
		Aramburu, J., et al., “Affinity-Driven Peptide Selection of an NFAT Inhibitor More Selective than Cyclosporin A,” Science, Vol. 285, pages 2129-2133, September, 1999	
		Burkard, N., et al., “Targeted Proteolysis Sustains Calcineurin Activation,” Circulation, Vol. 111, pages 1045-1053, 2005	
		Cyert, M.S., “Regulation of Nuclear Localization during Signaling,” J. of Biology Chemistry, Vol. 276, No. 24, pages 20805-20808, June, 2001	
		Dolmetsch, R., et al., “Differential Activation of Transcription Factors Induced by CA^{2+} Response Amplitude and Duration,” Nature, Vol. 386, pages 855-858, April, 1997	
		Fornerod, M., et al., “CRM1 is an Export Receptor for Leucine-Rich Nuclear Export Signals,” Cell, Vol. 90, pages 1051-1060, September, 1997	
		Frey, N., et al., Calsarcins, a Novel Family of Sarcomeric Calcineurin-Binding Proteins,” Proc. Natl. Acad. Sci., pages 14632-14637, December, 2000	
		Gasiorowski, J., et al., “Mechanisms of Nuclear Transport and Interventions,” Advanced Drug Delivery Reviews, Vol. 55, pages 703-716, 2003	
		Hallhuber, M., et al., “Inhibition of Nuclear Import of Calcineurin Prevents Myocardial Hypertrophy, Circulation Research, Vol. 99, pages 626-635, 2006	
		Hallhuber, M., et al., “New Approach to Prevent Myocardial Hypertrophy,” Future Cardiology, Vol. 3, pages 91-98, 2007	
		Hogan, P.G., et al., “Modification by Nuclear Export?” Nature, Vol. 398, pages 200-201, 1999	
		Jans, D., et al. “Nuclear Targeting Signal Recognition: A Key Control Point in Nuclear Transport?” BioEssays, Vol. 22, pages 532-544, 2000	
		Kutay, U., et al., “Export of Importin α from the Nucleus is Mediated by a Specific Nuclear Transport Factor,” Cell, Vol. 90, pages 1061-1071, September, 1997	
		McKinsey, T., et al., “Signal-Dependent Nuclear Export of a Histone Deacetylase Regulates Muscle Differentiation, Nature, Vol. 408, pages 106-111, November, 2000	
		Pemberton, L., et al., “Mechanisms of Receptor-Mediated Nuclear Import and Nuclear Export,” Traffic, Vol. 6, pages 187-198, 2005	
		Ritter, O., et al., “Calcineurin in Human Heart Hypertrophy,” Circulation, Vol. 105, pages 2265-2269, 2002	
		Ritter, O., et al., “AT_2-Receptor Activation Regulates Myocardial eNOS Expression Via the Calcineurin-NF-AT Pathway,” FASEB Journal, Vol., 17, pages 283-285, 2003	
		Shibasaki, F., et al., “Role of Kinases and the Phosphatase Calcineurin in the Nuclear Shuttling of Transcription Factor NF-AT4,” Nature, Vol. 382, pages 370-373, July, 1996	
		Timmerman, L., et al., “Rapid Shuttling of NF-AT in Discrimination of CA^{2+} Signals and Immunosuppression,” Nature, Vol., 383, pages 837-840, 1996	
		Wilkins, B., et al., “Calcineurin/NFAT Coupling Participates in Pathological, but not Physiological, Cardiac Hypertrophy,” Circulation Research Vol. 94, pages 110-118, 2004	
		Wu, H., et al., “Critical Role of Calpain-Mediated Cleavage of Calcineurin in Excitotoxic Neurodegeneration,” J. of Biology Chemistry, Vol. 279, pages 4929-4940, 2004	

Examiner's Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.
1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached.

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
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Substitute for Form PTO-1449

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	3	of	3

Complete If Known	
Applicant Number	12/162,135
Filing Date	July 25, 2008
First Named Inventor	Oliver RITTER
Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	ROTE-0007-UT1

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		Zhu, J., et al., “NF-AT Activation Requires Suppression of Crm1-Dependent Export by Calcineurin,” Nature, Vol. 398, pages 256-260, 1999	
		Zou, Y., et al., “Isopreterenol Activates Extracellular Signal-Regulated Protein Kinases in Cardiomyocytes through Calcineurin,” Circulation, Vol. 104, pages 102-108, 2001	
		.	

Examiner's Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.
1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached.

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(51) International Patent Classification 7 : C12N 15/00	A2	(11) International Publication Number: WO 00/28011 (43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/US99/25766

(22) International Filing Date: 5 November 1999 (05.11.99)

(30) Priority Data:
60/107,473 6 November 1998 (06.11.98) US

(71) Applicant *(for all designated States except US)*: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 124 Mount Auburn Street, Cambridge, MA 02138-5701 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CLEMONS, Paul, A. [US/US]; 23 Elm Street #109, Somerville, 02143 (US). GLADSTONE, Brian, G. [GB/US]; 50 Harbor Point Boulevard #208, Dorchester, MA 02125 (US). SETH, Abhinav [US/US]; 300 Engle Street, Tenafly, NJ 07670 (US). SCHREIBER, Stuart, L. [US/US]; 434 Marlborough Street, Boston, MA 02115 (US).

(74) Agent: HAUSDORFF, Sharon, F.; Ariad Pharmaceuticals, Inc., 26 Landsdowne Street, Cambridge, MA 02139-4234 (US).

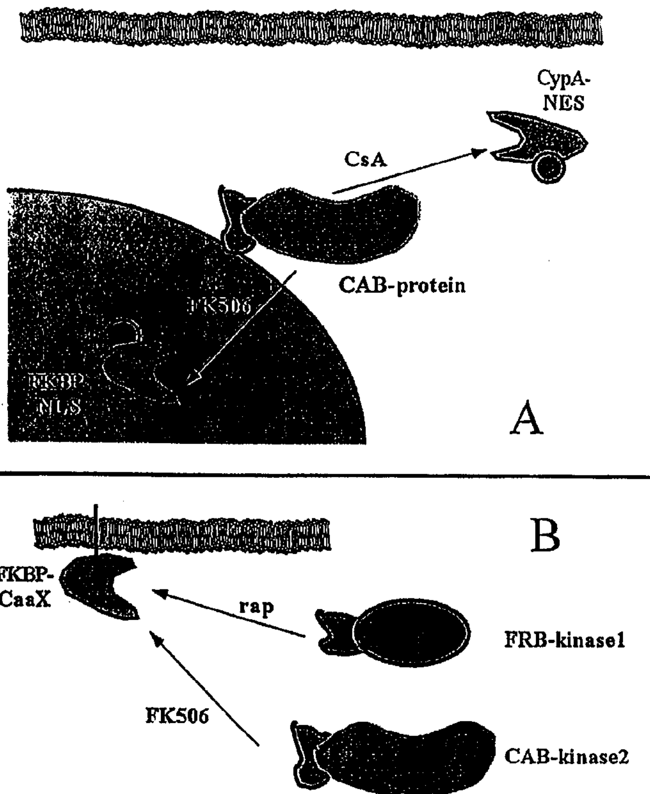
(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: FK506-BASED REGULATION OF BIOLOGICAL EVENTS

(57) Abstract

Materials and methods are disclosed for regulation of biological events such as target gene transcription and growth, proliferation or differentiation of engineered cells.



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FK506-Based Regulation Of Biological Events

This work was supported in part by grant number GM-52067 from the National
5 Institute of General Medical Sciences. Accordingly, the US government has certain rights in this invention.

Background of the Invention

FK506 is a natural product which binds to an FK506-binding protein, FKBP,
10 with high affinity to form an FK506:FKBP complex. Reported Kd values for that interaction are as low as 400 pM. The FK506:FKBP complex binds with high affinity to the protein phosphatase calcineurin to form a tripartite, [FKBP:FK506]:[calcineurin], complex. Calcineurin is a heterodimer of a catalytic subunit (calcineurin A) and a regulatory subunit (calcineurin B.) In this tripartite
15 complex FK506 acts as a dimerizer or adapter to join FKBP to calcineurin.

Numerous naturally occurring FK506 binding proteins (FKBPs) are known. See e.g. Kay, 1996, Biochem. J. **314**:361-385 (review). FKBP proteins have been used for their ligand-binding properties in biological switches based on ligand-mediated multimerization of immunophilin-based recombinant proteins as disclosed e.g. in
20 Spencer et al, 1993, Science **262**:1019-1024 and in WO 94/18317.

Cyclosporin A is another macrocyclic natural product of interest. It binds to the protein cyclophilin to form a complex which also binds to calcineurin to form an immunosuppressive complex. Cyclosporin thus also acts as a dimerizer. While the potent immunosuppressive activity of FK506 and cyclosporin would limit their utility
25 as a dimerizer, especially in animals, this invention harnesses their dimerizing potential (and that of related compounds) while avoiding their profound, inherent limitations.

Summary of the Invention

30 This invention concerns new configurations for biological switches and provides new methods and materials for regulating biological events, particularly in animal cells. Those biological events include, for example, gene transcription, activation of an intracellular signal transduction pathway (leading, for example, to gene expression, cell proliferation or apoptotic cell death), gene knock-out, blockade of
35 expression of a gene, and inhibition of the function of a gene product. The invention relies upon two types of fusion proteins which when complexed through mutual

binding to a common ligand, are capable of actuating, directly or indirectly, the desired event.

This invention encompasses recombinant DNA constructs encoding those fusion proteins; DNA vectors containing one or more of those constructs; the fusion proteins encoded by the foregoing constructs; cells, especially animal cells, transduced with (i.e., containing and capable of expressing) one or more of the DNA constructs described herein; small molecules (bivalent or multivalent multimerizing agents) which bind to and are capable of inducing multimerization of the fusion protein molecules; and methods for preparing and using the foregoing.

More specifically, this invention provides methods and materials for making and using genetically engineered cells which are responsive to the presence of an FKBP/CAB ligand or a cyclophilin/CAB ligand. The invention relies upon the introduction into cells of recombinant DNAs encoding a set of fusion proteins which are capable of forming a complex with each other in the presence of ligand. Contacting such genetically engineered cells with a ligand results in complex formation between the fusion proteins and initiation of a biological response. One of the fusion proteins contains one or more copies of a calcineurin A/calcineurin B domain (CAB) and at least one heterologous protein domain. The second fusion protein contains one or more copies of a domain derived from an FKBP protein which is capable of binding to an FKBP/CAB ligand and forming a complex with a CAB-containing protein. The second fusion protein may alternatively contain one or more copies of a cyclophilin domain which is capable of binding cyclosporin or other cyclophilin/CAB ligand and forming a complex with a CAB-containing protein. The second fusion protein also contains at least one heterologous domain which may be the same or different from a heterologous domain of the first fusion protein. CAB and FKBP domains for use in fusion proteins of this invention may be selected from naturally occurring proteins and may be variously modified, as is discussed in detail below. While CAB, FKBP and heterologous domains derived from various species may be used, human peptide sequences or variants thereof are preferred for human gene therapy applications. Operationally, the CAB and FKBP domains serve as receptor (or "ligand-binding") domains and direct the complex formation between the fusion proteins under the mediation of ligand molecules. The nature of the biological response triggered by ligand-mediated complexes is determined by the heterologous domains of the fusion proteins. The heterologous domains are therefore also referred to as "action" domains.

Various heterologous protein domains may be used in these fusion proteins, including, among others, DNA binding domains, transcription regulatory domains and

cellular signaling domains. In one aspect of the invention, the two fusion proteins (one of which contains at least one CAB domain, the other contains at least one FKBP or cyclophilin domain) each contain at least one different heterologous domain, i.e., a heterologous domain not contained in the other fusion protein. For example, in certain embodiments, one of the fusion proteins contains at least one DNA binding domain and the other fusion protein contains at least one transcription activation domain. Ligand-mediated association of the fusion proteins represents the formation of a transcription factor complex and leads to initiation of transcription of a target gene linked to a DNA sequence recognized by (i.e., capable of binding with) a DNA-binding domain on one of the fusion proteins. In other embodiments, one of the fusion proteins contains at least one domain capable of directing the fusion protein to a particular cellular location such as the cell membrane, nucleus, etc. Localization domains which target the cell membrane include domains such as a myristoylation site or a transmembrane region of a receptor protein or other membrane-spanning protein. The other fusion protein contains a signalling domain capable, upon membrane localization and/or clustering, of activating a cellular signal transduction pathway. Examples of signalling domains include an intracellular domain of a growth factor or cytokine receptor, an apoptosis triggering domain such as the intracellular domain of FAS or TNF-R1, and domains derived from other intracellular signalling proteins such as SOS, Raf, lck, ZAP-70, caspases, etc. A number of illustrative signalling proteins are disclosed in WO 94/18317 (see e.g. pages 23 - 26). In still other embodiments, each of the fusion proteins contains at least one CAB domain and at least one FKBP domain and/or a cyclophilin domain, as well as one or more heterologous domains. Such fusion proteins are capable of homodimerization in the presence of ligand. In general, domains containing peptide sequence endogenous to the host cell are preferred. Thus, for human gene therapy applications, domains of human origin are of particular interest.

Recombinant DNA molecules encoding the fusion proteins are also provided, as are vectors capable of directing their expression, particularly in eukaryotic cells, of which yeast and animal cells are of particular interest. In view of the constituent components of the fusion proteins, the recombinant DNA molecules which encode them are capable of selectively hybridizing (a) to a DNA molecule encoding a given fusion protein's ligand-binding domain (CAB domain, FKBP domain or cyclophilin domain) or a protein containing such a domain and (b) to a DNA molecule encoding the heterologous domain or a protein from which the heterologous protein domain was

derived. DNAs are also encompassed which would be capable of so hybridizing but for the degeneracy of the genetic code.

Using DNA sequences encoding the fusion proteins of this invention and vectors capable of directing their expression in eukaryotic cells, one may genetically engineer cells for a number of important uses. To do so, one first provides an expression vector or DNA construct for directing the expression in a eukaryotic (preferably animal) cell of the desired fusion protein and then introduces the recombinant DNA into the cells in a manner permitting DNA uptake and expression of the introduced DNA in at least a portion of the cells. One may use any of the various methods and materials for introducing DNA into cells for heterologous gene expression, a variety of which are well known and/or commercially available.

One object of this invention is thus to provide an animal cell containing recombinant DNAs encoding two fusion proteins as described herein. One of the fusion proteins is capable of binding to ligand and contains at least one FKBP or cyclophilin domain and at least one domain that is heterologous thereto. The second fusion protein contains at least one CAB domain and at least one domain heterologous thereto and is capable of forming a tripartite complex with the first fusion protein and one or more molecules of ligand. In some embodiments one or more of the heterologous domains present on one of the fusion proteins are also present on the other fusion protein, i.e., the two fusion proteins have one or more common heterologous domains. In other embodiments, each fusion protein contains one or more different heterologous domains.

A specific object of this invention is to provide animal cells engineered such that contacting the cells with ligand leads to transcription of a target gene. Such cells contain, in addition to recombinant DNAs encoding the two fusion proteins, a target gene construct which comprises a target gene operably linked to a DNA sequence which is responsive to the presence of a complex of the fusion proteins with the ligand. In certain embodiments the cells are responsive to contact with a ligand which binds to the FKBP fusion protein and CAB fusion protein with a detectable preference over binding to endogenous FKBP or CAB-containing proteins of the host cell. Similarly, ligands which bind cyclophilin-containing fusion proteins with greater affinity than their binding to endogenous cyclophilin may be desirable.

Another specific object of this invention is to provide animal cells engineered such that contacting the cells with the ligand stimulates cell growth, differentiation or proliferation. In such cells, at least one of the heterologous domains on at least one of the fusion proteins is a domain such as the intracellular domain of a receptor for a

hormone which mediates cell growth, differentiation or proliferation. Cell growth, differentiation and/or proliferation follow clustering of the receptor intracellular signalling domains. Such clustering occurs in nature following hormone binding, and in engineered cells of this invention following contact with ligand.

5 Cells of human origin are preferred for human gene therapy applications, although cell types of various origins (human or other species) may be used, and may, if desired, be encapsulated within a biocompatible material for use in human subjects.

Another object of the invention is to provide materials and methods for producing the foregoing engineered cells. This object is met by providing recombinant 10 DNAs encoding the fusion proteins, together with any ancillary recombinant DNAs such as a target gene construct, and introducing the recombinant DNAs into the host cells under conditions permitting DNA uptake by cells. Such transduction may be effected ex vivo, using host cells maintained in culture. Cells that are engineered in culture may subsequently be introduced into a host organism, e.g. in ex vivo gene 15 therapy applications. Doing so thus constitutes a method for providing a host organism, preferably a human or non-human mammal, which is responsive (as described herein) to the presence of ligand. Alternatively transduction may be effected in vivo, using host cells present in a human or non-human host organism. In such cases, the DNA molecules are introduced directly into the host organism under 20 conditions permitting uptake of the DNA by one or more of the host organism's cells. This approach thus constitutes an alternative method for providing a host organism, preferably a human or non-human mammal, which is responsive (as described herein) to the presence of ligand. Various materials and methods for the introduction of DNA into cells in culture or in whole organisms are known in the art and may be adapted 25 for use in practicing this invention.

Other objects are achieved using the engineered cells described herein. For instance, a method is provided for multimerizing the fusion proteins of this invention by contacting cells engineered as described herein with an effective amount of ligand, permitting the ligand to form a complex with the fusion proteins. In embodiments in 30 which multimerization of the fusion proteins triggers transcription of a target gene, this constitutes a method for activating the expression of the target gene. In embodiments in which the fusion proteins contain one or more signalling domains, this constitutes a method for activating a cellular signal transduction pathway. These methods may be carried out in cell culture or in whole organisms, including human patients. In the 35 former case, the ligand is added to the culture medium. In the latter case, the ligand (which may be in the form of a pharmaceutical or veterinary composition) is

administered to the whole organism, e.g., orally, parenterally, etc. Preferably, the dose or ligand administered to an animal is below the dosage level that would cause undue immunosuppression in the recipient.

A further object of this invention is to provide kits for use in the genetic engineering of cells or human or non-human animals as described herein. One such kit contains recombinant DNA constructs encoding a pair of fusion proteins of this invention. The recombinant DNA constructs will generally be in the form of eukaryotic expression vectors suitable for introduction into animal cells and capable of directing the expression of the fusion proteins therein. The kit may also contain a sample of ligand capable of forming a complex with the encoded fusion proteins. The kit may further contain a multimerization antagonist such as rapamycin or some other compound capable of binding to one of the fusion proteins but incapable of forming a complex with both. In certain embodiments, the recombinant DNA constructs encoding the fusion proteins will contain a cloning site in place of DNA encoding one or more of the heterologous domains, thus permitting the practitioner to introduce DNA encoding a heterologous domain of choice. In some embodiments the kit may also contain a target gene construct containing a target gene or cloning site linked to a DNA sequence responsive to the presence of the complexed fusion proteins, as described in more detail elsewhere.

Brief Description of the Figures

Figure 1: Constructs used in the CAB dimerization system.

Figure 2: Reaction scheme for synthesis of E/Z C40-phenyl-FK506.

Figure 3: Styrene analogs to be used in cross-metathesis reaction with FK506.

Figure 4: Effect of varying the number of CAB domains on secreted alkaline phosphatase activity.

Figure 5: Secreted alkaline phosphatase activity induced by full length CABs and mini CABs.

Figure 6: Schematic depictions of three-construct systems involving the CAB dimerization domain. **A** Recruitment of a CAB fusion protein to either FKBP in the

context of a nuclear localization signal (NLS) or cyclophilin A (CypA) in the context of a nuclear export signal (NES). **B.** Recruitment of kinases fused to either the CAB domain or the FRB domain to FKBP localized to the plasma membrane by virtue of a CaaX isoprenylation signal.

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Figure 7: Effect of C40 derivatization on calcineurin-dependent reporter gene activity. Results represent the average of at least three independent experiments per derivative. Abbreviations: Nap, naphthyl; Ph, phenyl; PhOPh, phenoylphenyl; TMS, trimethylsilyl; FPh, fluorophenyl; Iph, iodophenyl.

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Figure 8: Effect of high-level fCAB expression on NFAT-SEAP reporter gene activity. SEAP assays were carried out as described above except that aliquots of transfected cells were grown up separately for analysis by Western blotting. **A.** Western blot using antibodies (3F10, Gibco) directed against the HA epitope tag., and showing the

15 expression of fCAB protein as a function of the concentration of transfected DNA. **B.** SEAP assay results showing the high constitutive reporter activity in the presence of overexpressed fCAB. Data shown is for 4000ng of transfected fCAB DNA.

Figure 9: Tuning of phosphatase assay as a function of fCAB construct DNA

20 concentration. SEAP assays were carried out as described above using the indicated amount of fCAB construct DNA. Data represent the average of two independent experiments.

Figure 10: Development of FK506-mediated transcription assay using CAB-p65 fusion

25 proteins. SEAP assays were carried out as described using the optimized concentrations of each DNA species and the indicated dose of FK506 or FK506 derivative. **A.** SEAP assay results showing the activation of transcription by FK506 and two derivatives. Data represent the average of three independent experiments. **B.** SEAP assay results showing the behavior of the dose response with many

30 closely-spaced doses over a narrow concentration range. Data represent the results of three independent experiments.

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Detailed Description of the Invention

Definitions

The definitions of terms as they are used herein will be helpful for a full understanding of the present disclosure.

"Activate" as applied to the expression or transcription of a gene denotes a directly or indirectly observable increase in the production of a gene product, e.g., an RNA or polypeptide encoded by the gene.

"Capable of selectively hybridizing" as that phrase is used herein means that two DNA molecules are susceptible to hybridization with one another, despite the presence of other DNA molecules, under hybridization conditions which can be chosen or readily determined empirically by the practitioner of ordinary skill in this art. Such treatments include conditions of high stringency such as washing extensively with buffers containing 0.2 to 6 x SSC, and/or containing 0.1% to 1% SDS, at temperatures ranging from room temperature to 65-75°C. See for example F.M. Ausubel et al., Eds, Short Protocols in Molecular Biology, Units 6.3 and 6.4 (John Wiley and Sons, New York, 3d Edition, 1995).

"Cells", **"host cells"** or **"recombinant host cells"** refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

"Cell line" refers to a population of cells capable of continuous or prolonged growth and division in vitro. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

A **"cloning site"**, also sometimes referred to as a "multiple cloning site"or a "polylinker" is a region within a vector which contains multiple sites for restriction enzyme cleavage, thus rendering the vector suitable for cloning of exogenous genes.

A **"coding sequence"** or a sequence which "encodes" a particular polypeptide or RNA, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of an appropriate expression control sequence. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a

translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from procaryotic or eukaryotic mRNA, genomic DNA sequences from procaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

5 **"Composite"**, **"fusion"**, and **"recombinant"** denote a material such as a nucleic acid, nucleic acid sequence or polypeptide which contains at least two constituent portions which are mutually heterologous in the sense that they are not otherwise found directly (covalently) linked in nature, e.g. are not found in the same continuous polypeptide or gene in nature, at least not in the same order or orientation 10 or with the same spacing present in the composite, fusion or recombinant product. Such materials contain components derived from at least two different proteins or genes or from at least two non-adjacent portions of the same protein or gene. In general, "composite" refers to portions of different proteins or nucleic acids which are joined together to form a single functional unit, while "fusion" generally refers to two 15 or more functional units which are linked together. "Recombinant" is generally used in the context of nucleic acids or nucleic acid sequences.

The term **"conjoint"**, with respect to administration of two or more viruses, refers to the simultaneous, sequential or separate dosing of the individual virus provided that some overlap occurs in the simultaneous presence of the viruses in one 20 or more cells of the animal.

A **"construct"**, e.g., a "nucleic acid construct" or "DNA construct" refers to a nucleic acid or nucleic acid sequence.

"Derived from" denotes a peptide or nucleotide sequence selected from within a given sequence. A peptide or nucleotide sequence derived from a named sequence 25 may further contain a small number of modifications relative to the parent sequence, in most cases representing deletion, replacement or insertion of less than about 15%, preferably less than about 10%, and in many cases less than about 5%, of amino acid residues or bases present in the parent sequence. In the case of DNAs, one DNA molecule is also considered to be derived from another if the two are capable of 30 selectively hybridizing to one another. Polypeptides or polypeptide sequences are also considered to be derived from a reference polypeptide or polypeptide sequence if any DNAs encoding the two polypeptides or sequences are capable of selectively hybridizing to one another. Typically, a derived peptide sequence will differ from a parent sequence by the replacement of up to 5 amino acids, in many cases up to 3 35 amino acids, and very often by 0 or 1 amino acids. A derived nucleic acid sequence will differ from a parent sequence by the replacement of up to 15 bases, in many cases

up to 9 bases, and very often by 0 - 3 bases. In some cases the amino acid(s) or base(s) is/are added or deleted rather than replaced.

"Dimerization", **"oligomerization"** and **"multimerization"** are used interchangeably herein and refer to the association or clustering of two or more protein molecules, mediated by the binding of a drug to at least one of the proteins. In preferred embodiments, the multimerization is mediated by the binding of two or more such protein molecules to a common divalent or multivalent drug. The formation of a complex comprising two or more protein molecules, each of which containing one or more FKBP domains, together with one or more molecules of an FKBP ligand which is at least divalent (e.g. FK1012 or AP1510) is an example of such association or clustering. In cases where at least one of the proteins contains more than one drug binding domain, e.g., where at least one of the proteins contains three FKBP domains, the presence of a divalent drug leads to the clustering of more than two protein molecules. Embodiments in which the drug is more than divalent (e.g. trivalent) in its ability to bind to proteins bearing drug binding domains also can result in clustering of more than two protein molecules. The formation of a tripartite complex comprising a protein containing at least one CAB domain, a protein containing at least one FKBP domain and a ligand molecule is another example of such protein clustering. In certain embodiments of this invention, fusion proteins contain multiple CAB and/or FKBP domains. Complexes of such proteins may contain more than one molecule of ligand or other dimerizing agent and more than one copy of one or more of the constituent proteins. Again, such multimeric complexes are still referred to herein as tripartite complexes to indicate the presence of the three types of constituent molecules, even if one or more are represented by multiple copies. The formation of complexes containing at least one divalent drug and at least two protein molecules, each of which contains at least one drug binding domain, may be referred to as "oligomerization" or "multimerization", or simply as "dimerization", "clustering" or association".

"Dimerizer" denotes a compound which brings together two or more proteins in a multimeric complex.

"Divalent", as that term is applied to ligands in this document, denotes a ligand which is capable of complexing with at least two protein molecules which contain ligand binding domains, to form a three (or greater number)-component complex.

"Domain" refers to a portion of a protein or polypeptide. In the art, the term "domain" may refer to a portion of a protein having a discrete secondary structure. However, as will be apparent from the context used herein, the term "domain" as used

in this document does not necessariy connote a given secondary structure. Rather, a peptide sequence is referred to herein as a "domain" simply to denote a polypeptide sequence from a defined source, or having or conferring an intended or observed activity. Domains can be derived from naturally occurring proteins or may comprise
5 non-naturally-occurring sequence.

"DNA recognition sequence" means a DNA sequence which is capable of binding to one or more DNA-binding domains, e.g., of a transcription factor or an engineered polypeptide.

"Endogenous" refers to molecules which are naturally occurring in a cell, i.e.
10 prior to the genetic engineering or infection of the cell.

"Exogenous" refers to molecules which are not naturally present in the cell, and which have been, e.g., introduced by transfection or transduction of the cell (or the parent cell thereof).

FKBPs (FK506 binding proteins) are the cytosolic receptors for macrolides
15 such as FK506, FK520 and rapamycin and are highly conserved across species lines. For the purpose of this disclosure, FKBPs are proteins or protein domains which are capable of binding to an FKBP/CAB ligand and further forming a tripartite complex with calcineurin or a CAB-containing protein. An FKBP domain may also be referred to as a "FK506 binding domain". Information concerning the nucleotide sequences,
20 cloning, and other aspects of various FKBP species is already known in the art, permitting the synthesis or cloning of DNA encoding the desired FKBP peptide sequence, e.g., using well known methods and PCR primers based on published sequences. See e.g. Staendart et al, 1990, Nature 346, 671-674 (human FKBP12); Kay, 1996, Biochem. J. 314, 361-385 (review). Homologous FKBP proteins in other
25 mammalian species, in yeast, and in other organsims are also known in the art and may be used in the fusion proteins disclosed herein. See e.g. Kay, 1996, Biochem. J. 314, 361-385 (review). The size of FKBP domains for use in this invention are usually 90-100 amino acids, although this varies, depending on which FKBP protein is employed. An FKBP domain of a fusion protein of this invention will be capable of
30 binding to an FKBP/CAB ligand and participating in a tripartite complex with calcineurin or a CAB-containing protein (as may be determined by any means, direct or indirect, for detecting such binding). The peptide sequence of an FKBP domain of an FKBP fusion protein of this invention comprises (a) a naturally occurring FKBP peptide sequence, preferably derived from the human FKBP12 protein (exemplified
35 below) or a peptide sequence derived from another human FKBP, from a murine or other mammalian FKBP, or from some other animal, yeast or fungal FKBP; (b) a

variant of a naturally occurring FKBP sequence in which up to about ten (preferably 1-5, more preferably 1-3, and in some embodiments just one) amino acids of the naturally-occurring peptide sequence have been deleted, inserted, or replaced with substitute amino acids; or (c) a peptide sequence encoded by a DNA sequence capable of selectively hybridizing to a DNA molecule encoding a naturally occurring FKBP or by a DNA sequence which would be capable, but for the degeneracy of the genetic code, of selectively hybridizing to a DNA molecule encoding a naturally occurring FKBP.

An **"FKBP/CAB ligand"** is a compound, e.g. FK506 or an analog, homolog, derivative or mimetic of any of the foregoing, which binds to an FKBP protein to form a complex which binds to calcineurin or a CAB protein. Similarly, a "cyclophilin/CAB ligand" is a compound, e.g. cyclosporin or an analog, homolog, derivative or mimetic of any of the foregoing, which binds to a cyclophilin protein to form a complex which binds to calcineurin or a CAB protein.

"Gene" refers to a nucleic acid molecule or sequence comprising an open reading frame and including at least one exon and (optionally) an intron sequence. The term "intron" refers to a DNA sequence present in a given gene which is not translated into protein and is generally found between exons.

"Genetically engineered cells" denotes cells which have been modified by the introduction of recombinant or heterologous nucleic acids (e.g. one or more DNA constructs or their RNA counterparts) and further includes the progeny of such cells which retain part or all of such genetic modification.

"Heterologous" as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally joined together, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, in the case of a cell transduced with a nucleic acid construct which is not normally present in the cell, the cell and the construct would be considered mutually heterologous for purposes of this invention. Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used herein.

"Interact" as used herein is meant to include detectable interactions between molecules, such as can be detected using, for example, a yeast or mammalian two hybrid assay or by immunoprecipitation. The term interact is also meant to include "binding" interactions between molecules. Interactions may be, for example, protein-protein, protein-nucleic acid, protein-small molecule or small molecule-nucleic acid in nature.

"Ligand" refers to any molecule which is capable of interacting with a corresponding protein or protein domain. A ligand can be naturally occurring, or the ligand can be partially or wholly synthetic. The term "modified ligand" refers to a ligand which has been modified such that it does not significantly interact with the naturally occurring receptor of the ligand in its non modified form. As used herein, ligand refers to an FKBP/CAB ligand.

"Minimal promoter" refers to the minimal expression control sequence that is necessary for initiating transcription of a selected DNA sequence to which it is operably linked.

The terms **"promoter"** and **"expression control sequence"** further encompass "tissue specific" promoters and expression control sequences, i.e., promoters and expression control sequences which effect expression of the selected DNA sequence preferentially in specific cells (e.g., cells of a specific tissue). Gene expression occurs preferentially in a specific cell if expression in this cell type is significantly higher than expression in other cell types. The terms "promoter" and " expression control sequence" also encompass so-called "leaky" promoters and " expression control sequences", which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well. These terms also encompass non-tissue specific promoters and expression control sequences which are active in most cell types. Furthermore, a promoter or expression control sequence can be constitutive i.e. one which is active basally or inducible, i.e., a promoter or expression control sequence which is active primarily in response to a stimulus. A stimulus can be, e.g., a molecule, such as a hormone, a cytokine, a heavy metal, phorbol esters, cyclic AMP (cAMP), or retinoic acid.

"Nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, derivatives, variants and analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides. For simplicity, the term DNA is often used herein to refer to any nucleic acid.

A **"nucleic acid binding domain"** refers to a polypeptide which interacts, or binds, with a higher affinity to a nucleic acid having a specific nucleotide sequence relative to a nucleic acid having a nucleotide sequence which is essentially unrelated to the specific nucleotide sequence. In a preferred embodiment, a nucleic acid binding 5 domain is a "DNA binding domain".

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, an expression control sequence operably linked to a coding sequence permits expression of the coding sequence. The control sequence need not be contiguous with 10 the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Protein", **"polypeptide"** and **"peptide"** are used interchangeably herein when 15 referring to a gene product, e.g., as may be encoded by a coding sequence.

A **"recombinant virus"** is a complete virus particle in which the packaged nucleic acid contains a heterologous portion.

"Subunit", when referring to the subunit of an activation domain, refers to a portion of the transcription activation domain.

20 A **"target gene"** is a nucleic acid of interest, the expression of which is modulated according to the methods of the invention. The target gene can be endogenous or exogenous and can integrate into a cell's genome, or remain episomal. The target gene can encode a protein or be a non coding nucleic acid, e.g, a nucleic acid which is transcribed into an antisense RNA or a ribozyme.

25 A **"therapeutically effective dose"** of ligand denotes a treatment- or prophylaxis-effective dose, e.g., a dose which yields detectable target gene transcription or cell growth, proliferation, differentiation, death, etc. in the genetically engineered cell, or a dose which is predicted to be treatment- or prophylaxis-effective by extrapolation from data obtained in animal or cell culture models. A 30 therapeutically effective dose is ususally preferred for the treatment of a human or non-human mammal.

"Transcription control element" denotes a regulatory DNA sequence, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. The term "enhancer" is 35 intended to include regulatory elements capable of increasing, stimulating, or enhancing transcription from a promoter. Such transcription regulatory components

can be present upstream of a coding region, or in certain cases (e.g. enhancers), in other locations as well, such as in introns, exons, coding regions, and 3' flanking sequences.

"Transcription factor" refers to any protein or modified form thereof that is involved in the initiation of transcription but which is not itself a part of the
5 polymerase. Transcription factors are proteins or modified forms thereof, which interact preferentially with specific nucleic acid sequences, i.e., regulatory elements. Some transcription factors are active when they are in the form of a monomer. Alternatively, other transcription factors are active in the form of oligomers consisting of two or more identical proteins or different proteins (heterodimer). The factors have
10 different actions during the transcription initiation: they may interact with other factors, with the RNA polymerase, with the entire complex, with activators, or with DNA. Transcription factors usually contain one or more transcription regulatory domains.

"Transcription regulatory domain" refers to any domain which regulates
15 transcription, and includes both activation and repression domains. The term " transcription activation domain" denotes a domain in a transcription factor which positively regulates (increases) the rate of gene transcription. The term " transcription repression domain" denotes a domain in a transcription factor which negatively regulates (inhibits or decreases) the rate of gene transcription.

20 **"Transfection"** means the introduction of a naked nucleic acid molecule into a recipient cell. **"Infection"** refers to the process wherein a virus enters the cell in a manner whereby the genetic material of the virus can be expressed in the cell. A "productive infection" refers to the process wherein a virus enters the cell, is replicated, and then released from the cell (sometimes referred to as a "lytic" infection).
25 **"Transduction"** encompasses the introduction of nucleic acid into cells by any means.

"Transgene" refers to a nucleic acid sequence which has been introduced into a cell. Daughter cells deriving from a cell in which a transgene has been introduced are also said to contain the transgene (unless it has been deleted). A transgene can encode, e.g., a polypeptide, partly or entirely heterologous to the animal or cell into
30 which it is introduced, or comprises or is derived from an endogenous gene of the animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the recipient's genome in such a way as to alter that genome. (e.g., it is inserted at a location which differs from that of the natural gene). Alternatively, a transgene can also be present in an episome. A transgene can include one or more
35 expression control sequences and any other nucleic acid, (e.g. intron), that may be necessary or desirable for optimal expression of a selected coding sequence.

"**Transient transfection**" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, e.g., where episomal DNA is transcribed into mRNA and translated into protein. A cell has been "stably transfected" with a nucleic acid construct when the nucleic acid construct has been integrated into the genome of that cell.

By "**virus**" is meant a complete virus, such as a wild-type (wt) virus particle comprising a nucleic acid genome associated with a capsid protein coat, or a recombinant virus particle as described above. For example, an adenovirus is a complete virus particle, comprising an Ad nucleic acid genome associated with an Ad capsid protein coat.

"**Wild-type**" means naturally occurring in a normal cell.

This invention involves methods and materials for multimerizing fusion proteins in genetically engineered cells using a novel dimerization-based biological switch. The design and implementation of various dimerization-based biological switches has been reported, inter alia, in Spencer et al and in various international patent applications cited herein. Other accounts of successful application of this general approach have also been reported. Fusion proteins containing a domain from human FRAP fused to an effector domain have been disclosed in Rivera et al, 1996, Nature Medicine 2, 1028-1032 and in WO 96/41865 (Clackson et al) and WO 95/33052 (Berlin et al). As noted previously, the fusion proteins are designed such that association of the effector domains, through ligand-mediated "dimerization" or "multimerization" of the fusion proteins which contain them, triggers a desired biological event such as transcription of a desired gene, cell death, cell proliferation, etc. For example, clustering of fusion proteins containing an action domain derived from the intracellular portion of the T cell receptor CD3 zeta domain triggers transcription of a gene under the transcription control of the IL-2 promoter or promoter elements derived therefrom. In other embodiments, the action domain comprises a domain derived from the intracellular portion of a protein such as FAS or the TNF-alpha receptor (TNFalpha-R1), which are capable, upon oligomerization, of triggering apoptosis of the cell. In still other embodiments, the action domains comprise a DNA-binding domain such as GAL4 or ZFHD1 and a transcription activation domain such as VP16 or p65, paired such that oligomerization of the fusion

proteins represents assembly of a transcription factor complex which triggers transcription of a gene linked to a DNA sequence recognized by (capable of specific binding interaction with) the DNA binding domain.

Fusion proteins containing one or more ligand-binding domains and one or more action domains, e.g. for activation of transcription of a target gene, triggering cell death or other signal transduction pathway, cellular localization, cell proliferation etc., are disclosed in WO 94/18317, PCT/US94/08008, Spencer et al, supra and Blau et al. (PNAS 1997 94:3076). The design and use of such fusion proteins for ligand-mediated gene-knock out and for ligand-mediated blockade of gene expression or inhibition of gene product function are disclosed in PCT/US95/10591. Novel DNA binding domains and DNA sequences to which they bind which are useful in embodiments involving regulated transcription of a target gene are disclosed, e.g., in Pomeranz et al, 1995, Science 267:93-96. Those references provide substantial information, guidance and examples relating to the design, construction and use of DNA constructs encoding analogous fusion proteins, target gene constructs, and other aspects which may also be useful to the practitioner of the subject invention.

By appropriate choice of fusion proteins, this invention permits one to activate the transcription of a desired gene; actuate cell growth, proliferation, differentiation or apoptosis, or trigger other biological events in engineered cells in an FK506-dependent manner analogous to the systems described in the patent documents and other references cited above. The engineered cells, preferably animal cells, may be growing or maintained in culture or may be present within whole organisms, as in the case of human gene therapy, transgenic animals, and other such applications. An FKBP/CAB ligand is administered to the cell culture or to the organism containing the engineered cells, as the case may be, in an amount effective to multimerize the FKBP fusion proteins and CAB fusion proteins (as may be observed indirectly by monitoring target gene transcription, apoptosis or other biological process so triggered). In the case of administration to whole organisms, the ligand may be administered in a composition containing the ligand and one or more acceptable veterinary or pharmaceutical diluents and/or excipients.

A compound which binds to one of the fusion proteins but does not form tripartite complexes with both fusion proteins may be used as a multimerization antagonist. As such it may be administered to the engineered cells, or to organisms containing them (preferably in a composition as described above in the case of administration to whole animals), in an amount effective for blocking or reversing the effect of the ligand, i.e. for preventing, inhibiting or disrupting multimerization of the

fusion proteins. For instance, rapamycin, a rapalog, or any of the many synthetic FKBP ligands which do not form tripartite complexes with FKBP and CAB may be used as an antagonist.

One important aspect of this invention provides materials and methods for ligand-dependent, direct activation of transcription of a desired gene. In one such embodiment, a set of two or more different fusion proteins, and corresponding DNA constructs capable of directing their expression, is provided. One such fusion protein contains as its action domain(s) one or more transcription activation domains. The other fusion protein contains as its action domain(s) one or more DNA-binding domains. The selected ligand is capable of binding to both fusion proteins to form a dimeric or multimeric complex thus containing at least one DNA binding domain and at least one transcription activation domain. Formation of such complexes leads to activation of transcription of a target gene linked to, and under the transcription control of, a DNA sequence to which the DNA-binding domain is capable of binding, as can be observed by monitoring directly or indirectly the presence or concentration of the target gene product.

Preferably the DNA binding domain, and a fusion protein containing it, binds to its recognized DNA sequence with sufficient selectivity so that binding to the selected DNA sequence can be observed (directly or indirectly) despite the presence of other, often numerous other, DNA sequences. Preferably, binding of the fusion protein comprising the DNA-binding domain to the selected DNA sequence is at least two, more preferably three and even more preferably more than four orders of magnitude greater than binding to any one alternative DNA sequence, as measured by in vitro binding studies or by measuring relative rates or levels of transcription of genes associated with the selected DNA sequence as compared with any alternative DNA sequences.

Cells which have been genetically engineered to contain such a set of constructs, together with any desired accessory constructs, may be used in applications involving ligand-mediated, regulated actuation of the desired biological event, be it regulated transcription of a desired gene, regulated triggering of a signal transduction pathway such as the triggering of apoptosis, or another event. Cells engineered for regulatable expression of a target gene, for instance, can be used for regulated production of a desired protein (or other gene product) encoded by the target gene. Such cells may be grown in culture by conventional means. Addition of ligand to the culture medium containing the cells leads to expression of the target gene by the cells and production of the protein encoded by that gene. Expression of the

gene and production of the protein can be turned off by withholding further ligand from the medium, by removing residual ligand from the medium, or by adding to the medium a multimerization antagonist reagent.

Engineered cells of this invention can also be produced and/or used in vivo, to
5 modify whole organisms, preferably animals, especially humans, e.g. such that the cells produce a desired protein or other result within the animal containing them. Such uses include gene therapy applications.

Embodiments involving regulatable actuation of apoptosis provide engineered cells susceptible to FK506-inducible cell death. Such engineered cells can be eliminated
10 from a cell culture or host organism after they have served their intended purposed (e.g. production of a desired protein or other product), if they have or develop unwanted properties, or if they are no longer useful, safe or desired. Elimination is effected by adding ligand to the medium or administering it to the host organism. In such cases, the action domains of the fusion proteins are protein domains such as the
15 intracellular domains of FAS or TNF-R1, downstream components of their signaling pathways or other protein domains which upon oligomerization trigger apoptosis.

Said differently, this invention provides a method for achieving any of those objectives, e.g. activation of transcription of a target gene (typically a heterologous gene for a therapeutic protein), cell growth or proliferation, cell death or some other
20 selected biological event, in an animal, preferably a human patient, in need thereof and containing engineered cells of this invention. That method involves administering to the animal a pharmaceutical composition containing the ligand by a route of administration and in an amount effective to cause multimerization of the fusion proteins in at least a portion of the engineered cells. Multimerization may be detected
25 indirectly by detecting the occurrence of target gene expression, cell growth, proliferation or death, or other objective for which the fusion proteins were designed and the cells genetically engineered.

This invention further encompasses a pharmaceutical composition comprising a multimerization antagonist of this invention in admixture with a pharmaceutically
30 acceptable carrier and optionally with one or more pharmaceutically acceptable excipients for inhibiting or otherwise reducing, in whole or part, the extent of multimerization of fusion proteins in engineered cells of this invention in a subject, and thus for de-activating the transcription of a target gene, for example, or turning off another biological result of this invention. Thus, the use of the multimerizing ligands
35 and of the multimerization antagonist reagents to prepare pharmaceutical

compositions and achieve their pharmacologic results is encompassed by this invention.

Also disclosed is a method for providing a host organism, preferably an animal, typically a non-human mammal or a human subject, responsive to a ligand of this invention. The method involves introducing into the organism cells which have been engineered in accordance with this invention, i.e. containing one or more nucleic acid constructs encoding the fusion proteins, and so forth. The engineered cells may be encapsulated using any of a variety of materials and methods before being introduced into the host organism. Alternatively, one can introduce the nucleic acid constructs of this invention into a host organism, e.g. a mammal, under conditions permitting incorporation thereof into one or more cells of the host mammal, e.g. using viral vectors, introduction of DNA by injection or via catheter, etc.

Also provided are kits for producing cells responsive to a ligand of this invention. One such kit contains one or more nucleic acid constructs encoding and capable of directing the expression of fusion proteins which, upon ligand-mediated oligomerization, trigger the desired biological response. The kit may contain a quantity of a ligand capable of multimerizing the fusion protein molecules encoded by the construct(s) of the kit, and may contain in addition a quantity of a multimerization antagonist. The kit may further contain a nucleic acid construct encoding a target gene (or cloning site) linked to a cognate DNA sequence which is recognized by the dimerized fusion proteins permitting transcription of a gene linked to that cognate DNA sequence in the presence of multimerized fusion protein molecules. The constructs may be associated with one or more selection markers for convenient selection of transfectants, as well as other conventional vector elements useful for replication in prokaryotes, for expression in eukaryotes, and the like. The selection markers may be the same or different for each different construct, permitting the selection of cells which contain each such construct(s).

The accessory construct for introducing into cells a target gene in association with a cognate DNA sequence may contain a cloning site in place of a target gene. A kit containing such a construct permits the engineering of cells for regulatable expression of a gene to be provided by the practitioner.

Other kits of this invention may contain one or two (or more) nucleic acid constructs for fusion proteins in which one or more contain a cloning site in place of the transcription activator or DNA binding protein, permitting the user to insert whichever such domain s/he wishes. Such a kit may optionally include other elements

as described above, e.g. a nucleic acid construct for a target gene with or without a cognate DNA sequence for a pre-selected DNA binding domain.

Any of the kits may also contain positive control cells which were stably transformed with constructs of this invention such that they express a reporter gene (for CAT, beta-galactosidase or any conveniently detectable gene product) in response to exposure of the cells to the ligand. Reagents for detecting and/or quantifying the expression of the reporter gene may also be provided.

For further information and guidance on the design, construction and use of such systems or components thereof which may be adapted for use in practising the subject invention, reference to the following publications is suggested: Spencer et al, 1993, supra; Rivera et al, 1996, supra; Spencer et al, 1996, Current Biology 6, 839-847; Luo et al, 1996, Nature, 383, 181-185; Ho et al, 1996, Nature 382, 822-826; Belshaw et al, 1996, Proc. Natl. Acad. Sci. USA 93, 4604-4607; Spencer, 1996, TIG 12(5), 181-187; Spencer et al, 1995, Proc., Natl. Acad. Sci. USA 92, 9805-9809; Holsinger et al, 1995, Proc. Natl. Acad. Sci. USA 92, 9810-9814; Pruschy et al, 1994, Chemistry & Biology 1(3),163-172; and published international patent applications WO 94/18317, WO 95/02684, WO 95/33052, WO 96/20951 and WO 96/41865.

FKBP domains and fusion proteins

The FKBP fusion protein comprises at least one FKBP domain containing all or part of the peptide sequence of an FKBP domain and at least one heterologous action domain. This fusion protein must be capable of binding to ligand, preferably with a Kd value below about 100 nM, more preferably below about 10 nM and even more preferably below about 1 nM, as measured by direct binding measurement (e.g. fluorescence quenching), competition binding measurement (e.g. versus FK506), inhibition of FKBP enzyme activity (rotamase), or other assay methodology. Typically the fusion protein will contain one or more protein domains comprising peptide sequence selected from that of a naturally occurring FKBP protein such as human FKBP12, e.g. as described in International Patent Application PCT/US94/01617. That peptide sequence may be modified to adjust the binding specificity, usually with replacement, insertion or deletion of 10 or fewer, preferably 5 or fewer, in some cases 1-3, and often 1, amino acid residue. Such modifications are elected in certain embodiments to yield one or both of the following binding profiles: (a) binding of a ligand to the modified FKBP domain, or fusion protein containing it, preferably at least one, and more preferably at least two, and even more preferably three or four or

more, orders of magnitude better (by any measure) than to FKBP12 or the FKBP endogenous to the host cells to be engineered; and (b) binding of the FKBP:ligand complex to the CAB fusion protein, preferably at least one, and more preferably at least two, and even more preferably at least three, orders of magnitude better (by any
5 measure) than to the calcineurin endogenous to the host cell to be engineered.

The FKBP fusion protein also contains at least one heterologous action domain, i.e., a protein domain containing non-FKBP peptide sequence. The action domain may be a DNA-binding domain, transcription activation domain, transcription repression domain, cellular localization domain, intracellular signal transduction domain, etc.,
10 e.g. as described elsewhere herein or in PCT/US94/01617 or the other cited references. Generally speaking, the action domain is capable of directing the fusion protein to a selected cellular location or of initiating a biological effect upon association or aggregation with another action domain, for instance, upon multimerization of proteins containing the same or different action domains.

15 A recombinant nucleic acid encoding such a fusion protein will be capable of selectively hybridizing to a DNA encoding the parent FKBP protein, e.g. human FKBP12, or would be capable of such hybridization but for the degeneracy of the genetic code. Since these fusion proteins contain an action domain derived from another protein, e.g. Gal4, ZFHD1, p65, VP16, FAS, CD3 zeta chain, etc., the
20 recombinant DNA encoding the fusion protein will also be capable of selectively hybridizing to a DNA encoding that other protein, or would be capable of such hybridization but for the degeneracy of the genetic code.

FKBP fusion proteins of this invention, as well as CAB fusion proteins discussed in further detail below, may contain one or more copies of one or more
25 different ligand binding domains and one or more copies of one or more action domains. The ligand binding domain(s) (i.e., FKBP and CAB domains) may be N-terminal, C-terminal, or interspersed with respect to the action domain(s). Embodiments involving multiple copies of a ligand binding domain usually have 2 , 3 or 4 such copies. For example, an FKBP fusion protein may contain 2, 3 or 4 FKBP
30 domains. The various domains of the FKBP fusion proteins (and of the CAB fusion proteins discussed below) are optionally separated by linking peptide regions which may be derived from one of the adjacent domains or may be heterologous.

Illustrative examples of FKBP fusion proteins useful in the practice of this invention include the FKBP fusion proteins disclosed in PCT/US94/01617 (Stanford
35 & Harvard), PCT/US94/08008 (Stanford & Harvard), Spencer et al (supra), PCT/US95/10591 (ARIAD), PCT/US95/06722 (Mitotix, Inc.) and other references

cited herein; the FKBP fusion proteins disclosed in the examples which follow; variants of any of the foregoing FKBP fusion proteins which contain up to 10 (preferably 1-5) amino acid insertions, deletions or substitutions in one or more of the FKBP domains and which are still capable of binding to a ligand; variants of any of
5 the foregoing FKBP fusion proteins which contain one or more copies of an FKBP domain which is encoded by a DNA sequence capable of selectively hybridizing to a DNA sequence encoding a naturally occurring FKBP domain and which are still capable of binding to a ligand; variants of any of the foregoing in which one or more heterologous action domains are deleted, replaced or supplemented with a different
10 heterologous action domain; variants of any of the foregoing FKBP fusion proteins which are capable of binding to an FKBP/CAB ligand and which contain an FKBP domain derived from a non-human source; and variants of any of the foregoing FKBP fusion proteins which contain one or more amino acid residues corresponding to Tyr26, Phe36, Asp37, Arg42, Phe46, Phe48, Glu54, Val55, or Phe99 of human
15 FKBP12 in which one or more of those amino acid residues is replaced by a different amino acid, the variant being capable of binding to a ligand.

For instance, in a number of cases the FKBP fusion proteins comprise multiple copies of an FKBP domain containing amino acids 1-107 of human FKBP12, separated by the 2-amino acid linker Thr-Arg encoded by ACTAGA, the ligation
20 product of DNAs digested respectively with the restriction endonucleases SpeI and XbaI. The following table provides illustrative subsets of mutant FKBP domains based on the foregoing FKBP12 sequence:

Illustrative Mutant FKBPs

25

F36A	Y26V	F46A	W59A
F36V	Y26S	F48H	H87W
F36M	D37A	F48L	H87R
F36S	I90A	F48A	F36V/F99A
F99A	I91A	E54A	F36V/F99G
F99G	F46H	E54K	F36M/F99A
Y26A	F46L	V55A	F36M/F99G

note: Entries identify the native amino acid by single letter code and sequence position, followed by the replacement amino acid in the mutant. Thus, F36V designates a human FKBP12 sequence in which phenylalanine at position 36 is replaced by valine. F36V/F99A indicates a double mutation in which phenylalanine
5 at positions 36 and 99 are replaced by valine and alanine, respectively.

Cyclophilin domains and fusion proteins

Cyclophilin fusion proteins may contain all or part of murine cyclophilin C (e.g. residues 36-212; Freidman et al., Cell 664(1991)799-806) or human cyclophilin
10 **C (Genbank Accession number S71018; Schneider et al., Biochemistry 33 (27), 8218-8224 (1994)). These fusion proteins contain a heterologous action domain and form complexes with a cyclophilin/CAB ligand, such as cyclosporin, as described above for FKBP fusion proteins. Cyclophilin domains may also be** modified to adjust the binding specificity, usually with replacement, insertion or
15 deletion of 10 or fewer, preferably 5 or fewer, in some cases 1-3, and often 1, amino acid residue, again as described in detail above for FKBP domains. In general, any description of FKBP domains and fusion proteins and their use in this invention is applicable to cyclophilin domains as well. Cyclophilin domains and their use in chimeric proteins are described in US 5,830,462, in particular example 4c, the full
20 contents of which are incorporated herein by reference.

CAB domains and fusion proteins

The structure of FKBP-FK506 complexed to calcineurin phosphatase (Griffith
25 et al., Cell, 82:507-522, 1995) has been reported. Calcineurin A (residues 12-394) was shown to be effective as a dimerization domain using a three hybrid system in yeast using three FKBPs fused to Gal4 and residues 12-394 of murine calcineurin A fused C-terminally to the Gal4 activation domain (Ho, 1996 Nature. 382:822-826). Addition of FK506 activated transcription of a reporter gene in these cells. This system is not
30 optimal, however, because it requires a complex to form between endogenous calcineurin B and the calcineurin A fusion protein. In addition, the calcineurin fusion protein might interfere with the calcineurin signaling pathway.

The present invention provides a "minimal" calcineurin domain, termed a CAB, which is a smaller, more manipulatable domain that can be used in a general
35 way to control dimerization of proteins for the purposes of regulating biological processes. A CAB domain of this invention must be able to form a complex with

FKBP in the presence of an FKBP/CAB ligand. In other embodiments, the CAB domain can form a tripartite complex with a cyclophilin domain and cyclosporin. Thus, one of the most compelling potential features of the CAB as a dimerization domain is its ability to dock with two different partners under the control of two
5 different ligands. With this system, a single dimerization domain (i.e. the CAB) can interact with two other protein partners simultaneously or competitively, depending on the particular drug added. The same is also true for FKBP which is now capable of being recruited to either FRAP in the presence of rapamycin or a rapalog or to CAB in the presence of an FKBP/CAB ligand.

10 Since the CAB should bind both the FK506:FKBP complex and the cyclosporin:cyclophilin complex, one can engineer cells or animals in which a fusion of the CAB to a heterologous action domain is present in the same cell as two other fusion proteins, one containing FKBP and one containing cyclophilin. Addition of FK506 to these cells results in the formation of an FKBP/FK506/CAB complex, while
15 addition of cyclosporin to the same cell would result in a cyclophilin/cyclosporin/CAB complex. An example of this situation is illustrated in Figure 6A.

Furthermore, since FKBP is required to mediate the interactions between both FK506 or rapamycin and their cellular targets, one can engineer cells or animals in
20 which a fusion of the CAB to a heterologous action domain is present in the same cell as a fusion protein containing an FRB domain. FRB domains are described in detail in WO 95/33052 and in WO 96/41865. In this embodiment, addition of FK506 to the cell induces the formation of an FKBP/FK506/CAB complex, while addition of rapamycin should result in the formation of an FKBP/rapamycin/FRB complex. This
25 allows for complex order of addition experiments in cell culture or for more complex control over signal transduction processes that require localization or protein association for function. An example of this situation is illustrated in Figure 6B.

CAB domains of this invention are composite ligand binding domains, comprising a portion of calcineurin A and a portion of calcineurin B, such that the
30 resulting composite ligand binding domain contains the surface of calcineurin phosphatase that contacts the FKBP-FK506 complex. The region of calcineurin that has been truncated contains the autoregulatory and calmodulin domains, which are not involved in FKBP binding. The portion of calcineurin A used in the examples includes residues 12 to 394 or residues 12 to 370 of human calcineurin A in the full
35 length CABS, however, equivalent regions of the calcineurin gene from other species could also be used. For example, one may desire to use the sequence of the mouse or

rat genes for the construction of transgenic animals. The N or C terminus of the calcineurin A portion could also be shortened or extended, if desired. The 12-394 CABs contain an active phosphatase domain, while the 12-370 CABs have an H151A mutation in calcineurin A that abolishes the phosphatase activity.

5 The calcineurin B portion of the CABs described in the examples contains residues 2 or 3 to 170 of human calcineurin B. The N-terminal methionine (residue 1) or methionine and glycine (residue 1 and residue 2) can be removed to prevent processing or myristoylation of calcineurin B at its N-terminus and to remove an alternative start site for translation. The calcineurin B portion of the composite 10 domain may also be shortened, if desired. Literature references and Genbank accession numbers for various calcineurin genes are given in the table below:

	Calcineurin A		**Calcineurin B**	
Species	**Genbank #**	**Reference**	**Genbank #**	**Reference**
Human	M29550	Guerini and Klee, PNAS USA 86, 9183-9187 (1989).	M30773	Guerini et al., DNA 8(9), 675-682 (1989).
Rat	D90035	Ito et al., BBRC 163(3), 1492-1497 (1989).	D14568	Chang et al., BBA 1217(2), 174-180 (1994).
Bovine	U33868	Griffith et al., Cell, 82:507-522, (1995)	X71666	Nargang et al., DNA seq 4(5), 313-318 (1994).
Mouse	M81483	Guerini and Klee, PNAS USA 86, 9183-9187 (1989).	S43864	Ueki et al., BBRC 187(1), 537-543 (1992)

The distance between residue 370 of calcineurin A and the N-terminus of 15 calcineurin B is 17.5 Angstroms, as determined from the crystal structure. Thus, when the CAB domain is constructed in such a way that the calcineurin A portion is N-

terminal to the calcineurin B portion, only a small linker region is necessary to connect the two. In fact, since the structure indicates that calcineurin A maintains a rigid structure only as far as residue 370, the C-terminus of calcineurin A can be linked directly to the N-terminus of calcineurin B, thereby using residues 370-394 of calcineurin A as the linker region. Hence, a preferred embodiment of the invention comprises a CAB domain composed of residues 12-394 of calcineurin A fused N-terminally to residues 3-170 of calcineurin B.

The CAB fusion protein comprises at least one CAB domain and at least one heterologous action domain, i.e., a protein domain containing non-CAB peptide sequence. The action domain may be a DNA-binding domain, transcription activation domain, transcription repression domain, cellular localization domain, intracellular signal transduction domain, etc., e.g. as described elsewhere herein or in PCT/US94/01617 or the other cited references. Generally speaking, the action domain is capable of directing the fusion protein to a selected cellular location or of initiating a biological effect upon association or aggregation with another action domain, for instance, upon multimerization of proteins containing the same or different action domains. As described in the examples, both CAB-VP16 fusion proteins and CAB-GAL4 fusion proteins are functional in SEAP reporter assays. As in the case of FKBP fusion proteins, the CAB domains should be able to hybridize with either calcineurin A or calcineurin B or portions thereof, or would be able to but for the degeneracy of the genetic code.

A shorter version of the CAB domain, termed the mini CAB, comprises residues 340-394 of calcineurin A fused N-terminally to residues 3-170 of calcineurin B. This construct eliminates the phosphatase domain of calcineurin and provides a less bulky protein for use in the dimerization system. Example 8 shows that the mini CAB can work as well as the full length CABs in reporter assays. As described above for FKBPs, CAB fusion proteins of this invention may contain one or more copies of the ligand binding domain and one or more copies of one or more action domains. The CAB domain may be N-terminal, C-terminal, or interspersed with respect to the action domain(s). Embodiments involving multiple copies of a ligand binding domain usually have 2 , 3 or 4 such copies. For example, a CAB fusion protein may contain 2, 3, or 4 CAB domains, although currently, the preferred embodiment for regulated transcription contains 2 CAB domains.

Mixed fusion proteins

A third type of fusion protein comprises one or more FKBP domains, one or more heterologous action domains, and one or more CAB domains as described for the CAB fusion proteins.

5 Mixed fusion protein molecules are capable of forming homodimeric or homomultimeric protein complexes in the presence of an FKBP/CAB ligand to which they bind. Embodiments involving mixed fusion proteins have the advantage of requiring the introduction into cells of a single recombinant nucleic acid construct in place of two recombinant nucleic acid constructs otherwise required to direct the 10 expression of both an FKBP fusion protein and a CAB fusion protein.

A recombinant DNA encoding a mixed fusion protein will be capable of selectively hybridizing to a DNA encoding an FKBP protein, a DNA encoding calcineurin A or B, and a heterologous DNA sequence encoding the protein from which one or more effector domains is derived (e.g. Gal4, VP16, Fas, CD3 zeta chain, etc.), 15 or would be capable of such hybridization but for the degeneracy of the genetic code.

Heterologous domains

As mentioned above, the heterologous action domains of the fusion proteins are protein domains which, upon mutual association of the fusion proteins bearing 20 them, are capable of triggering (or inhibiting) events such as DNA-binding and/or transcription of a target gene; actuating cell growth, differentiation, proliferation or apoptosis; directing proteins to a particular celllular location; or actuating other biological events.

Embodiments involving regulatable gene transcription involve the use of target 25 gene constructs which comprise a target gene (which encodes a polypeptide, antisense RNA, ribozyme, etc. of interest) under the transcription control of a DNA element responsive to the association or multimerization of the heterologous domains of the 1st and 2d fusion proteins.

In embodiments of the invention involving direct activation of transcription, the 30 heterologous domains of the 1st and 2nd fusion proteins comprise a DNA binding domain such as Gal4 or a fusion DNA binding domain such as ZFHD1, and a transcription activation domain such as those derived from VP16 or p65, respectively. The multimerization of a fusion protein containing such a transcription activation domain to a fusion protein containing a DNA binding domain targets the transcription 35 factor to the expression control sequence to which the DNA binding domain binds, and thus activates the transcription of a target gene linked to that expression control

sequence. Foregoing the transcription activation domain or substituting a repressor domain (see PCT/US94/01617) in place of a transcription activation domain provides an analogous fusion protein useful for inhibiting transcription of a target gene. Composite DNA binding domains and DNA sequences to which they bind are
5 disclosed in Pomerantz et al, 1995, supra, the contents of which are incorporated herein by reference. Such composite DNA binding domains may be used as DNA binding domains in the practice of this invention, together with a target gene construct containing the cognate DNA sequences to which the composite DBD binds.

In embodiments involving indirect activation of transcription, the heterologous
10 domains of the fusion proteins are action domains of signaling proteins which upon aggregation or multimerization trigger the activation of transcription under the control of a responsive promoter. For example, the signaling domain may be the intracellular domain of the zeta subunit of the T cell receptor, which, upon aggregation, triggers transcription of a gene linked to the IL-2 promoter or a derivative thereof (e.g. iterated
15 NF-AT binding sites). Alternatively, the signaling domain may be a cell surface receptor such as the erythropoietin receptor, which can initiate cell proliferation upon multimerization.

In another aspect of the invention, the heterologous domains are protein domains which upon mutual association are capable of triggering cell death.
20 Examples of such domains are the intracellular domains of the Fas antigen or of the TNF R1. Fusion proteins containing a Fas domain can be designed and prepared by analogy to the disclosure of PCT/US94/01617.

Engineered receptor domains

25 As noted previously, the FKBP and CAB domains may contain peptide sequence selected from the peptide sequences of naturally occurring FKBP and CAB domains. Naturally occurring sequences include those of human FKBP12 and portions of human calcineurin A or calcineurin B. Alternatively, the peptide sequences may be derived from such naturally occurring peptide sequences but contain generally up to
30 10, and preferably 1-5, mutations in one or both such peptide sequences. As disclosed in greater detail elswhere herein, such mutations can confer a number of important features. For instance, an FKBP domain may be modified such that it is capable of binding an improved ligand preferentially, i.e. at least one, preferably two, and even more preferably three or four or more orders of magnitude more effectively, with
35 respect to ligand binding by the unmodified FKBP domain. A CAB domain may be modified such that it is capable of binding a (modified or unmodified) FKBP:ligand

complex preferentially, i.e. at least one, preferably two, and even more preferably three orders of magnitude more effectively, with respect to the unmodified CAB domain. FKBP and CAB domains may be modified such that they are capable of forming a tripartite complex with an improved ligand, preferentially, i.e. at least one,
5 preferably two, and even more preferably three orders of magnitude more effectively, with respect to unmodified FKBP and CAB domains.

Methods for identifying FKBP mutations that confer enhanced ability to bind derivatives of FK506 containing various substituents ("bumps") were disclosed in US 5,830,462. In this approach, molecular modelling is used to identify candidate amino
10 acid substitutions in the FKBP domain that would accommodate the ligand substituent(s), and site-directed mutagenesis may then be used to engineer the protein mutations so identified. The mutants are expressed by standard methods and their binding affinity for the ligands measured, for example by inhibition of rotamase activity, or by competition for binding with a molecule such as FK506, if the mutant
15 retains appropriate activity/affinity.

A similar strategy can be used to identify bump-hole pairs for the CAB domain. An exemplary FKBP/CAB ligand is C40-phenyl-FK506. This compound appears to be at least 30-fold less immunosuppressive than FK506 as measured by its dose-dependent suppression of PMA/ionomycin-induced activation of an NFAT-
20 SEAP reporter in transient transfection experiments.

Without being bound by a particular theory, the inability of C40-phenyl-FK506 to inhibit NFAT-SEAP activity can be understood as a failure of the FKBP12/C40-phenyl-FK506 complex to bind endogenous calcineurin. The inhibition of NFAT-SEAP activity by FK506 is known to require binding of the FKBP12/FK506 complex to
25 calcineurin, which results in a loss of calcineurin phosphatase activity (J. Liu, et al. Cell,1991; 66: 807-815. J. Liu et al., Biochemistry, 1992; 31: 3896-3901). Indeed, the crystal structure of FKBP12/FK506 with the calcineurin heterodimer reveals that the terminal C39-C40 olefin of FK506 protrudes into the protein-protein interface between calcineurin A and calcineurin B (J.P. Griffith, et al Cell, 1995; 82(3): 507-522.)
30 Grafting a model of the phenyl group of C40-phenyl-FK506 onto this structure allows the identification of several residues of calcineurin (W352, S353, and F356 from calcineurin A; L116, M119, and V120 from calcineurin B) whose side chains might be responsible for the steric interference which abrogates binding. Each of the residues in question is part of the CAB minimal binding domain structure. Thus, these residues
35 may be mutated randomly or rationally to obtain a "holed" CAB which

accommodates bumped FK506. These mutations may be inserted randomly or in a directed fashion, as described above for FKBP.

An alternative to iterative engineering and testing of single or multiple mutants is to co-randomize structurally-identified residues that are or would be in contact with or near one or more ligand or FK506 substituents. A collection of polypeptides containing FKBP or CAB domains randomized at the identified positions (such as are noted in the foregoing paragraph) is prepared e.g. using conventional synthetic or genetic methods. Such a collection represents a set of receptor domains containing replacement amino acids at one or more of such positions. The collection is screened and variants are selected which possess the desired ligand binding properties. In general, randomizing several residues simultaneously is expected to yield compensating mutants of higher affinity and specificity for a given bumped ligand as it maximizes the likelihood of beneficial cooperative interactions between sidechains. Techniques for preparing libraries randomized at discrete positions are known and include primer-directed mutagenesis using degenerate oligonucleotides, PCR with degenerate oligonucleotides, and cassette mutagenesis with degenerate oligonucleotides (see for example Lowman, H.B, and Wells, J.A. Methods: Comp. Methods Enzymol. 1991. 3, 205-216; Dennis, M.S. and Lazarus, R.A. 1994. J. Biol. Chem. 269, 22129-22136; and references therein).

We further contemplate that in many cases, randomization of only the few residues in or near direct contact with a given position in FK506 may not completely explore all the possible variations in FKBP or CAB conformation that could optimally accommodate a ligand substituent (bump). Thus the construction is also envisaged of unbiased libraries containing random substitutions that are not based on structural considerations, to identify subtle mutations or combinations thereof that confer preferential binding to bumped ligands. Several suitable mutagenesis schemes have been described, including alanine-scanning mutagenesis (Cunningham and Wells (1989) Science 244, 1081-1085), PCR misincorporation mutagenesis (see eg. Cadwell and Joyce,1992, PCR Meth. Applic. 2, 28-33), and 'DNA shuffling' (Stemmer, 1994, Nature 370, 389-391 and Crameri et al, 1996, Nature Medicine 2, 100-103). These techniques produce libraries of random mutants, or sets of single mutants, that are then searched by screening or selection approaches.

In many cases, an effective strategy to identify the best mutants for preferential binding of a given bump is a combination of structure-based and unbiased approaches. See Clackson and Wells, 1994, Trends Biotechnology 12, 173-184 (review). For example we contemplate the construction of libraries in which key

contact residues are randomized by PCR with degenerate oligonucleotides, but with amplification performed using error-promoting conditions to introduce further mutations at random sites. A further example is the combination of component DNA fragments from structure-based and unbiased random libraries using DNA shuffling.

5 Screening of libraries for desirable mutations may be performed by use of a yeast 2-hybrid system (Fields and Song (1989) Nature 340, 245-246). For example, a CAB-VP16 fusion may be introduced into one vector, and a library of randomized FKBP sequences cloned into a separate GAL4 fusion vector. Yeast co-transformants are treated with ligand, and those harboring complementary FKBP mutants are 10 identified by for example beta-galactosidase or luciferase production (a screen), or survival on plates lacking an essential nutrient (a selection), as appropriate for the vectors used. The requirement for bumped FK506 to bridge the FKBP-CAB interaction is a useful screen to eliminate false positives.

A further strategy for isolating modified ligand-binding domains from libraries 15 of FKBP (or cyclophilin or CAB) mutants utilizes a genetic selection for functional dimer formation described by Hu et. al. (Hu, J.C., et al. 1990. Science. 250:1400-1403; for review see Hu, J.C. 1995. Structure. 3:431-433). This strategy utilizes the fact that the bacteriophage lambda repressor cI binds to DNA as a homodimer and that binding of such homodimers to operator DNA prevents transcription of phage genes 20 involved in the lytic pathway of the phage life cycle. Thus, bacterial cells expressing functional lambda repressor are immune to lysis by superinfecting phage lambda. Repressor protein comprises an amino terminal DNA binding domain (amino acids 1-92), joined by a 40 amino acid flexible linker to a carboxy terminal dimerization domain. The isolated N-terminal domain binds to DNA with low affinity due to 25 inefficient dimer formation. High affinity DNA binding can be restored with heterologous dimerization domains such as the GCN4 "leucine zipper". Hu et al have described a system in which phage immunity is used as a genetic selection to isolate GCN4 leucine zipper mutants capable of mediating lambda repressor dimer formation from a large population of sequences (Hu et. al., 1990).

30 For example, to use the lambda repressor system to identify FRAP mutants complementary to bumped ligands, lambda repressor-FRAP libraries bearing mutant FRAP sequences are transformed into E. coli cells expressing wildtype lambda repressor-FKBP protein. Plasmids expressing FRAP mutants are isolated from those colonies that survive lysis on bacterial plates containing high titres of lambda phage 35 and "bumped" FK506 compounds. Alternatively, to isolate FKBP mutants, the above strategy is repeated with lambda repressor-FKBP libraries bearing mutant FKBP

sequences transformed into E. coli cells expressing wildtype lambda repressor-FRAP protein.

A further alternative is to clone the randomized FKBP sequences into a vector for phage display, allowing in vitro selection of the variants that bind best to the
5 ligand. Affinity selection in vitro may be performed in a number of ways. For example, ligand is mixed with the library phage pool in solution in the presence of CAB tagged with an affinity handle (for example a hexa-histidine tag, or GST), and the resultant complexes are captured on the appropriate affinity matrix to enrich for phage displaying FKBP harboring complementary mutations. Techniques for phage display
10 have been described, and other in vitro selection selection systems can also be contemplated (for example display on lambda phage, display on plasmids, display on baculovirus). Furthermore, selection and screening strategies can also be used to improve other properties of benefit in the application of this invention, such as enhanced stability in vivo. For a review see Clackson, T. & Wells, J.A. 1994. Trends
15 Biotechnol. 12, 173-184.

Additionally, in optimizing the receptor domains of this invention, it should be appreciated that immunogenicity of a polypeptide sequence is thought to require the binding of peptides by MHC proteins and the recognition of the presented peptides as foreign by endogenous T-cell receptors. It may be preferable, at least in human gene
20 therapy applications, to tailor a given foreign peptide sequence, including junction peptide sequences, to minimize the probability of its being immunologically presented in humans. For example, peptide binding to human MHC class I molecules has strict requirements for certain residues at key 'anchor' positions in the bound peptide: eg. HLA-A2 requires leucine, methionine or isoleucine at position 2 and leucine or valine
25 at the C-terminus (for review see Stern and Wiley (1994) Structure 2, 145-251). Thus in engineering proteins in the practice of this invention, this periodicity of these residues is preferably avoided, especially in human gene therapy applications. The foregoing applies to all protein engineering aspects of the invention, including without limitation the engineering of point mutations into receptor domains, and to the choice
30 or design of boundaries between the various protein domains.

Ligands

Modified FKBP ligands for use with engineered FKBP domains have been extensively described in the scientific literature and in published patent applications,
35 including WO 94/18317 and US 5,830,462. A number of modified FKBP/CAB ligands have been identified and synthesized, including C40-phenyl-FK506, C40-p-

phenoxyphenyl-FK506, C40-p-biphenyl-FK506, C40-beta-naphthyl-FK506, C40-m-fluorophenyl-FK506, C40-p-iodophenyl-FK506, and "C41"-trimethylsilyl-FK506. The synthesis of FKBP/CAB ligands is described in Example 9.

Each of these compounds is made using the appropriate styrene derivative, except for "C41"-trimethylsilyl-FK506 which uses allyltrimethylsilane, coupled to FK506's terminal olefin using the Grubbs catalyst to facilitate the olefin metathesis chemistry. As shown in figure 3, any styrene derivative may be added at the C40 position of FK506 using the methods described in Example 9. By such means one may prepare a wide variety of aryl- and heteroaryl-substituted C40 FK506 derivatives which can be used in the practice of this invention.

Modified cyclosporin A derivatives have also been prepared and are described in detail in US 5,830,462, especially example 22, and in WO 98/08956, example 1, the full contents of which are incorporated herein by reference.

Other components, design features and applications

The fusion proteins may contain as a heterologous domain a cellular localization domain such as a membrane retention domain. See e.g. PCT/US94/01617, especially pages 26-27. Briefly, a membrane retention domain can be isolated from any convenient membrane-bound protein, whether endogenous to the host cell or not. The membrane retention domain may be a transmembrane retention domain, i.e., an amino acid sequence which extends across the membrane as in the case of cell surface proteins, incluing many receptors. The transmembrane peptide sequence may be extended to span part or all of an extracellular and/or intracellular domain as well. Alternatively, the membrane retention domain may be a lipid membrane retention domain such as a myristoylation or palmitoylation site which permits association with the lipids of the cell surface membrane. Lipid membrane retention domains will usually be added at the 5' end of the coding sequence for N-terminal binding to the membrane and, proximal to the 3' end for C-terminal binding. Peptide sequences involving post-translational processing to provide for lipid membrane binding are described by Carr, et al., PNAS USA (1988) 79, 6128; Aitken, et al., FEBS Lett. (1982) 150, 314; Henderson, et al., PNAS USA (1983) 80, 319; Schulz, et al., Virology (1984), 123, 2131; Dellman, et al., Nature (1985) 314, 374; and reviewed in Ann. Rev. of Biochem. (1988) 57, 69. An amino acid sequence of interest includes the sequence M-G-S-S-K-S-K-P-K-D-P-S-Q-R. Various DNA sequences can be used to encode such sequences in the various fusion proteins of this invention. Other localization domains include organelle-targeting domains and sequences such as

-K-D-E-L and -H-D-E-L which target proteins bearing them to the endoplasmic reticulum, as well as nuclear localization sequences which are particularly useful for fusion proteins designed for (direct) transcription regulation. Various cellular localization sequences and signals are well known in the art.

5 Other fusion proteins may contain a bundling domain as a heterologous domain. These domains, such as the lac repressor tetramerization domain constitutively oligomerize proteins containing such domains. Bundling domains may be used to deliver additional copies of activation domains or DNA binding domains to a given promoter.

10 Further details which may be used in the practice of the subject invention relating to the design, assembly and use of constructs encoding fusion proteins containing various action domains including cytoplasmic signal initiation domains such as the CD3 zeta chain, nuclear transcription factor domains including among others VP16 and GAL4, domains capable of triggering apoptosis including the Fas
15 cytoplasmic domain and others are disclosed in PCT/US94/01617 and PCT/US95/10591. The latter international application further discloses additional features particularly applicable to the creation of genetically engineered animals which may be used as disease models in biopharmaceutical research. Those features include the use of tissue specific regulatory elements in the constructs for expression of the
20 fusion proteins and the application of regulated transcription to the expression of Cre recombinase as the target gene leading to the elimination of a gene of interest flanked by loxP sequences. Alternatively, flp and its cognate recognition sequences may be used instead of Cre and lox. Those features may be adapted to the subject invention.

In various cases, especially in embodiments involving whole animals containing
25 cells engineered in accordance with this invention, it will often be preferred, and in some cases required, that the various domains of the fusion proteins be derived from proteins of the same species as the host cell. Thus, for genetic engineering of human cells, it is often preferred that the heterologous domains (as well as the FKBP and CAB domains) be of human origin, rather than of bacterial, yeast or other non-human
30 source.

We also note that epitope tags may also be incorporated into fusion proteins of this invention to permit convenient detection.

Tissue-specific or cell-type specific expression

35 It will be preferred in certain embodiments, that the fusion proteins be expressed in a cell-specific or tissue-specific manner. Such specificity of expression

may be achieved by operably linking one ore more of the DNA sequences encoding the fusion protein(s) to a cell-type specific transcription regulatory sequence (e.g. promoter/enhancer). Numerous cell-type specific transcription regulatory sequences are known. Others may be obtained from genes which are expressed in a cell-specific
5 manner. See e.g. PCT/US95/10591, especially pp. 36-37.

For example, constructs for expressing the fusion proteins may contain regulatory sequences derived from known genes for specific expression in selected tissues.

Representative examples are tabulated below:

10

Tissue	Gene	Reference
lens	g2-crystallin	Breitman, M.L., Clapoff, S., Rossant, J., Tsui, L.C., Golde, L.M., Maxwell, I.H., Bernstin, A. (1987) Genetic Ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice. Science 238: 1563-1565
	aA-crystallin	Landel, C.P., Zhao, J., Bok, D., Evans, G.A. (1988) Lens-specific expression of a recombinant ricin induces developmental defects in the eyes of transgenic mice. Genes Dev. 2: 1168-1178
		Kaur, S., key, B., Stock, J., McNeish, J.D., Akeson, R., Potter, S.S. (1989) Targeted ablation of alpha-crystallin-synthesizing cells produces lens-deficient eyes in transgenic mice. Development 105: 613-619
pituitary - somatrophic cells	Growth hormone	Behringer, R.R., Mathews, L.S., Palmiter, R.D., Brinster, R.L. (1988) Dwarf mice produced by genetic ablation of growth hormone-expressing cells. Genes Dev. 2: 453-461

pancreas	Insulin-Elastase - acinar cell specific	Ornitz, D.M., Palmiter, R.D., Hammer, R.E., Brinster, R.L., Swift, G.H., MacDonald, R.J. (1985) Specific expression of an elastase-human growth fusion in pancreatic acinar cells of transgeneic mice. Nature 131: 600-603 Palmiter, R.D., Behringer, R.R., Quaife, C.J., Maxwell, F., Maxwell, I.H., Brinster, R.L. (1987) Cell lineage ablation in transgeneic mice by cell-specific expression of a toxin gene. Cell 50: 435-443
T cells	lck promoter	Chaffin, K.E., Beals, C.R., Wilkie, T.M., Forbush, K.A., Simon, M.I., Perlmutter, R.M. (1990) EMBO Journal 9: 3821-3829
B cells	Immunoglobulin kappa light chain	Borelli, E., Heyman, R., Hsi, M., Evans, R.M. (1988) Targeting of an inducible toxic phenotype in animal cells. Proc. Natl. Acad. Sci. USA 85: 7572-7576 Heyman, R.A., Borrelli, E., Lesley, J., Anderson, D., Richmond, D.D., Baird, S.M., Hyman, R., Evans, R.M. (1989) Thymidine kinase obliteration: creation of transgenic mice with controlled immunodeficiencies. Proc. Natl. Acad. Sci. USA 86: 2698-2702
Schwann cells	P_0 promoter	Messing, A., Behringer, R.R., Hammang, J.P. Palmiter, RD, Brinster, RL, Lemke, G. ,P0 promoter directs espression of reporter and toxin genes to Schwann cells of transgenic mice. Neuron 8: 507-520 1992
	Myelin basic protein	Miskimins, R. Knapp, L., Dewey,MJ, Zhang, X. Cell and tissue-specific expression of a heterologous gene under control of the myelin basic protein gene promoter in trangenic mice. Brain Res Dev Brain Res 1992 Vol 65: 217-21

spermatids	protamine	Breitman, M.L., Rombola, H., Maxwell, I.H., Klintworth, G.K., Bernstein, A. (1990) Genetic ablation in transgenic mice with attenuated diphtheria toxin A gene. Mol. Cell. Biol. 10: 474-479
lung	Lung surfacant gene	Ornitz, D.M., Palmiter, R.D., Hammer, R.E., Brinster, R.L., Swift, G.H., MacDonald, R.J. (1985) Specific expression of an elastase-human growth fusion in pancreatic acinar cells of transgeneic mice. Nature 131: 600-603
adipocyte P2		Ross, S.R, Braves, RA, Spiegelman, BM Targeted expression of a toxin gene to adipose tissue: transgenic mice resistant to obesity Genes and Dev 7: 1318-24 1993
muscle	myosin light chain	Lee, KJ, Ross, RS, Rockman, HA, Harris, AN, O'Brien, TX, van-Bilsen, M., Shubeita, HE, Kandolf, R., Brem, G., Prices et alJ. BIol. Chem. 1992 Aug 5, 267: 15875-85
	Alpha actin	Muscat, GE., Perry, S. , Prentice, H. Kedes, L. The human skeletal alpha-actin gene is regulated by a muscle-specific enhancer that binds three nuclear factors. Gene Expression 2, 111-26, 1992
neurons	neurofilament proteins	Reeben, M. Halmekyto, M. Alhonen, L. Sinervirta, R. Saarma, M. Janne,J. Tissue-specific expression of rat light neurofilament promoter-driven reporter gene in transgenic mice. BBRC 1993: 192: 465-70
liver	tyrosine aminotransferase, albumin, apolipoproteins	

5

Target genes

As used herein, the term "target gene" refers to a gene, whose transcription is stimulated according to the method of the invention. In one preferred embodiment, the gene is integrated in the chromosomal DNA of a cell. Alternatively, the gene is episomal. A cell comprising a target gene is referred to herein as a "target cell".

In a preferred embodiment of the invention, the target gene is an endogenous gene. As used herein, the term "endogenous gene" refers to a gene which is naturally present in a cell, in its natural environment, i.e., not a gene which has been introduced into the cell by genetic engineering. The endogenous gene can be any gene having a promoter that is recognized by at least one transcription factor. In a preferred embodiment, the promoter or any regulatory element thereof, of the endogenous gene ("endogenous promoter" and "endogenous regulatory element", respectively), is recognized by a known, preferably cloned, DNA binding protein, whether it is a transcription activator or repressor. Alternatively, if no DNA binding protein is known to interact with a target promoter, it is possible to clone such a factor using techniques well known in the art without undue experimentation, such as screening of expression libraries with at least a portion of the target promoter. Furthermore, the affinity of binding of a DNA binding domain to a target sequence can be improved according to methods known in the art. Such methods comprise, e.g., introducing mutations into the DNA binding domain and screening for mutants having increased DNA binding affinity.

In another embodiment of the invention, the target gene is an endogenous gene, which contains an exogenous target sequence. The exogenous target sequence can be inserted into the endogenous promoter or substitute at least a portion of the endogenous promoter. In preferred embodiments, the exogenous promoter or regulatory element introduced into the endogenous target promoter is recognized by a DNA binding protein, capable of binding with high affinity and specificity to a target sequence. In a preferred embodiment, the DNA binding protein is human. However, the DNA binding protein can be from any other species. For example, the DNA binding protein can be from the yeast GAL4 protein.

In yet another embodiment, the target gene is an exogenous gene. In a preferred embodiment, the exogenous gene is integrated into the chromosomal DNA of a cell. The exogenous gene can be inserted into the chromosomal DNA, or the exogenous gene can substitute for at least a portion of an endogenous gene. The target gene can be present in a single copy or in multiple copies. In view of the experimental results described herein, it is not necessary that the target gene be present in more than one

copy. However, if even higher levels of protein encoded by the target gene is desired, multiple copies of the gene can be used.

In one embodiment, the taget gene construct enables transcription of a target gene to be regulated by a transcription factor in accordance with this invention comprises a DNA molecule which includes a synthetic transcription unit typically consisting of: (1) one copy or multiple copies of a DNA sequence recognized with high-affinity by the DNA binding domain of a fusion protein which includes a transcription activator, or of a protein which recruits the transcription activator; (2) a promoter sequence consisting minimally of a TATA box and initiator sequence but optionally including other transcription factor binding sites; (3) a coding sequence for a desired gene product, including sequences that promote the initiation and termination of translation, if appropriate; (4) an optional sequence consisting of a splice donor, splice acceptor, and intervening intron DNA; and (5) a sequence directing cleavage and polyadenylation of the resulting RNA transcript.

A wide variety of genes can be employed as the target gene, including genes that encode a therapeutic protein, antisense sequence or ribozyme of interest. The target gene can be any sequence of interest which provides a desired phenotype. It can encode a surface membrane protein, a secreted protein, a cytoplasmic protein, or there can be a plurality of target genes encoding different products. The target gene may be an antisense sequence which can modulate a particular pathway by inhibiting a transcription regulation protein or turn on a particular pathway by inhibiting the translation of an inhibitor of the pathway. The target gene can encode a ribozyme which may modulate a particular pathway by interfering, at the RNA level, with the expression of a relevant transcription regulator or with the expression of an inhibitor of a particular pathway. The proteins which are expressed, singly or in combination, can involve homing, cytotoxicity, proliferation, immune response, inflammatory response, clotting or dissolving of clots, hormonal regulation, etc. The proteins expressed may be naturally-occurring proteins, mutants of naturally-occurring proteins, unique sequences, or combinations thereof.

Various secreted products include hormones, such as insulin, human growth hormone, glucagon, pituitary releasing factor, ACTH, melanotropin, relaxin, etc.; growth factors, such as EGF, IGF-1, TGF–α or – β, PDGF, G-CSF, M-CSF, GM-CSF, FGF, erythropoietin, thrombopoietin, megakaryocytic stimulating and growth factors, etc.; interleukins, such as IL-1 to -13; TNF-α and - β, etc.; and enzymes and other factors, such as tissue plasminogen activator, members of the complement cascade, perforins, superoxide dismutase, coagulation factors, antithrombin-III, Factor VIIIc,

Factor VIIIvW, Factor IX, a-antitrypsin, protein C, protein S, endorphins, dynorphin, bone morphogenetic protein, etc.

The gene can encode a naturally-occurring surface membrane protein or a protein made so by introduction of an appropriate signal peptide and transmembrane sequence. Various such proteins include homing receptors, e.g. L-selectin (Mel-14), blood-related proteins, particularly having a kringle structure, e.g. Factor VIIIc, Factor VIIIvW, hematopoietic cell markers, e.g. CD3, CD4, CD8, B-cell receptor, TCR subunits α, β, γ, δ, CD10, CD19, CD28, CD33, CD38, CD41, etc., receptors, such as the interleukin receptors IL-2R, IL-4R, etc., channel proteins for influx or efflux of ions, e.g. Ca+2, K+, Na+, Cl- and the like; CFTR, tyrosine activation motif, zap-70, etc.

Proteins may be modified for transport to a vesicle for exocytosis. By adding the sequence from a protein which is directed to vesicles, where the sequence is modified proximal to one or the other terminus, or situated in an analogous position to the protein source, the modified protein will be directed to the Golgi apparatus for packaging in a vesicle. This process in conjunction with the presence of the fusion proteins for exocytosis allows for rapid transfer of the proteins to the extracellular medium and a relatively high localized concentration.

Also, intracellular proteins can be of interest, such as proteins in metabolic pathways, regulatory proteins, steroid receptors, transcription factors, etc., depending upon the nature of the host cell. Some of the proteins indicated above can also serve as intracellular proteins.

By way of further illustration, in T-cells, one may wish to introduce genes encoding one or both chains of a T-cell receptor. For B-cells, one could provide the heavy and light chains for an immunoglobulin for secretion. For cutaneous cells, e.g. keratinocytes, particularly stem cell keratinocytes, one could provide for protection against infection, by secreting -, or -interferon, antichemotactic factors, proteases specific for bacterial cell wall proteins, etc.

In addition to providing for expression of a gene having therapeutic value, there will be many situations where one may wish to direct a cell to a particular site. The site can include anatomical sites, such as lymph nodes, mucosal tissue, skin, synovium, lung or other internal organs or functional sites, such as clots, injured sites, sites of surgical manipulation, inflammation, infection, etc. By providing for expression of surface membrane proteins which will direct the host cell to the particular site by providing for binding at the host target site to a naturally-occurring epitope, localized concentrations of a secreted product can be achieved. Proteins of interest include homing receptors, e.g. L-selectin, GMP140, CLAM-1, etc., or

addressins, e.g. ELAM-1, PNAd, LNAd, etc., clot binding proteins, or cell surface proteins that respond to localized gradients of chemotactic factors. There are numerous situations where one would wish to direct cells to a particular site, where release of a therapeutic product could be of great value.

5 For use in gene therapy, the target gene can encode any gene product that is beneficial to a subject. The gene product can be a secreted protein, a membraneous protein, or a cytoplasmic protein. Preferred secreted proteins include growth factors, differentiation factors, cytokines, interleukins, tPA, and erythropoietin. Preferred membraneous proteins include receptors, e.g, growth factor or cytokine receptors or 10 proteins mediating apoptosis, e.g., Fas receptor. Other candidate therapeutic genes are disclosed in PCT/US93/01617.

In yet another embodiment, a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcription regulatory sequences of an endogenous gene, can be used to introduce recognition elements for a 15 DNA binding activity of one of the subject engineered proteins. A vareity of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

20 **Design and assembly of the DNA constructs**

Constructs may be designed in accordance with the principles, illustrative examples and materials and methods disclosed in the patent documents and scientific literature cited herein, each of which is incorporated herein by reference, with modifications and further exemplification as described herein. Components of the 25 constructs can be prepared in conventional ways, where the coding sequences and regulatory regions may be isolated, as appropriate, ligated, cloned in an appropriate cloning host, analyzed by restriction or sequencing, or other convenient means. Particularly, using PCR, individual fragments including all or portions of a functional unit may be isolated, where one or more mutations may be introduced using "primer 30 repair", ligation, in vitro mutagenesis, etc. as appropriate. In the case of DNA constructs encoding fusion proteins, DNA sequences encoding individual domains and sub-domains are joined such that they constitute a single open reading frame encoding a fusion protein capable of being translated in cells or cell lysates into a single polypeptide harboring all component domains. The DNA construct encoding the 35 fusion protein may then be placed into a vector that directs the expression of the protein in the appropriate cell type(s). For biochemical analysis of the encoded fusion

protein, it may be desirable to construct plasmids that direct the expression of the protein in bacteria or in reticulocyte-lysate systems. For use in the production of proteins in mammalian cells, the protein-encoding sequence is introduced into an expression vector that directs expression in these cells. Expression vectors suitable for such uses are well known in the art. Various sorts of such vectors are commercially available.

Constructs encoding the fusion proteins and target genes of this invention can be introduced into the cells as one or more DNA molecules or constructs, in many cases in association with one or more markers to allow for selection of host cells which contain the construct(s). The construct(s) once completed and demonstrated to have the appropriate sequences may then be introduced into a host cell by any convenient means. The constructs may be incorporated into vectors capable of episomal replication (e.g. BPV or EBV vectors) or into vectors designed for integration into the host cells' chromosomes. The constructs may be integrated and packaged into non-replicating, defective viral genomes like Adenovirus, Adeno-associated virus (AAV), or Herpes simplex virus (HSV) or others, including retroviral vectors, for infection or transduction into cells. Viral delivery systems are discussed in greater detail below. Alternatively, the construct may be introduced by protoplast fusion, electro-poration, biolistics, calcium phosphate transfection, lipofection, microinjection of DNA or the like. The host cells will in some cases be grown and expanded in culture before introduction of the construct(s), followed by the appropriate treatment for introduction of the construct(s) and integration of the construct(s). The cells will then be expanded and screened by virtue of a marker present in the constructs. Various markers which may be used successfully include hprt, neomycin resistance, thymidine kinase, hygromycin resistance, etc., and various cell-surface markers such as Tac, CD8, CD3, Thy1 and the NGF receptor.

In some instances, one may have a target site for homologous recombination, where it is desired that a construct be integrated at a particular locus. For example, one can delete and/or replace an endogenous gene (at the same locus or elsewhere) with a recombinant target construct of this invention. For homologous recombination, one may generally use either Ω or O-vectors. See, for example, Thomas and Capecchi, Cell (1987) 51, 503-512; Mansour, et al., Nature (1988) 336, 348-352; and Joyner, et al., Nature (1989) 338, 153-156.

The constructs may be introduced as a single DNA molecule encoding all of the genes, or different DNA molecules having one or more genes. The constructs may be introduced simultaneously or consecutively, each with the same or different markers.

Vectors containing useful elements such as bacterial or yeast origins of replication, selectable and/or amplifiable markers, promoter/enhancer elements for expression in procaryotes or eucaryotes, and mammalian expression control elements, etc. which may be used to prepare stocks of construct DNAs and for carrying out 5 transfections are well known in the art, and many are commercially available.

Delivery of Nucleic Acid: Ex vivo and in vivo

Any means for the introduction of heterologous nucleic acids into host cells, especially eucaryotic cells, an in particular animal cells, preferably human or non- 10 human mammalian cells, may be adapted to the practice of this invention. For the purpose of this discussion, the various nucleic acid constructs described herein may together be referred to as the transgene. Ex vivo approaches for delivery of DNA include calcium phosphate precipitation, electroporation, lipofection and infection via viral vectors. Two general in vivo gene therapy approaches include (a) the delivery of 15 "naked", lipid-complexed or liposome-formulated or otherwise formulated DNA and (b) the delivery of the heterologous nucleic acids via viral vectors. In the former approach, prior to formulation of DNA, e.g. with lipid, a plasmid containing a transgene bearing the desired DNA constructs may first be experimentally optimized for expression (e.g., inclusion of an intron in the 5' untranslated region and elimination 20 of unnecessary sequences (Felgner, et al., Ann NY Acad Sci 126-139, 1995). Formulation of DNA, e.g. with various lipid or liposome materials, may then be effected using known methods and materials and delivered to the recipient mammal.

While various viral vectors may be used in the practice of this invention, retroviral, AAV, and adenovirus-based approaches are of particular interest. See, for 25 example, Dubensky et al. (1984) Proc. Natl. Acad. Sci. USA 81, 7529-7533; Kaneda et al., (1989) Science 243,375-378; Hiebert et al. (1989) Proc. Natl. Acad. Sci. USA 86, 3594-3598; Hatzoglu et al. (1990) J. Biol. Chem. 265, 17285-17293 and Ferry, et al. (1991) Proc. Natl. Acad. Sci. USA 88, 8377-8381. When using viral vectors, the recombinant nucleic acids may be delivered in a single virus, or may be divided into 30 two or more viruses. For example, the fusion protein constructs could be delivered by one virus, while the target gene could be on a second virus. The target gene virus may further comprise an additional transcription regulatory domain construct. The following additional guidance on the choice and use of viral vectors may be helpful to the practitioner.

35

Viral Vectors:

Adenoviral vectors

A viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. Knowledge of the genetic organization of adenovirus, a 36 kB, linear and double-stranded DNA virus, allows substitution of a large piece of adenoviral DNA with foreign sequences up to 8 kB. In contrast to retrovirus, the infection of adenoviral DNA into host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in the human.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. Both ends of the viral genome contain 100-200 base pair (bp) inverted terminal repeats (ITR), at the 3' and 5' terminal regions of the adenovirus genome which are cis elements necessary for viral DNA replication and packaging. See, e.g., Gingeras et al. (1982) J. Biol. Chem.257:13475-13491. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan (1990) Radiotherap. Oncol. 19:197). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al., (1988) BioTechniques 6:616; Rosenfeld et al., (1991) Science 252:431-434; and Rosenfeld et al., (1992) Cell 68:143-155). Suitable adenoviruses derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled

in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al., (1992) cited *supra*), endothelial cells (Lemarchand et al., (1992) PNAS USA 89:6482-6486), hepatocytes (Herz and Gerard, (1993) PNAS USA 90:2812-2816) and muscle cells (Quantin et al., (1992) PNAS USA 89:2581-2584).

Adenoviruses have also been used in vaccine development (Grunhaus and Horwitz (1992) Siminar in Virology 3:237; Graham and Prevec (1992) Biotechnology 20:363). Experiments in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld et al. (1991) ; Rosenfeld et al. (1992) Cell 68:143), muscle injection (Ragot et al. (1993) Nature 361:647), peripheral intravenous injection (Herz and Gerard (1993) Proc. Natl. Acad. Sci. U.S.A. 90:2812), and stereotactic inoculation into the brain (Le Gal La Salle et al. (1993) Science 254:988).

Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and in vivo. This group of viruses can be obtained in high titers, e.g., 10^9 - 10^{11} plaque-forming unit (PFU)/ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus are episomal, and therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch et al., 1963; Top et al., 1971), demonstrating their safety and therapeutic potential as in vivo gene transfer vectors. Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., *supra*; Haj-Ahmand and Graham (1986) J. Virol. 57:267).

The invention provides recombinant adenoviruses, pAdΔ, which have been deleted of adenovirus cis-elements necessary for replication and virion encapsidation and which contains a target gene and/or one or more genes encoding fusion proteins of the invention. Productive viral infection with pAdD requires a helper adenovirus, which alone or with a packaging cell line, supplies sufficient gene sequences necessary for a productive viral infection. Preferred helper viruses are altered in one or more native gene sequences which direct efficient packaging, to thereby produce a helper virus whose packaging function or ability to replicate is "crippled" or disabled. Such recombinant adenoviruses are further described in published PCT application No. PCT/US95/14017 having publication No. WO 96/13597 by Wilson et al.

Most replication-defective adenoviruses currently in use and favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but may retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al., (1979) Cell 16:683; Berkner et al., *supra;* and Graham et al., in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Accordingly, a preferred adenovirus of the invention is an adenovirus in which the E1 and E3 genes have been deleted or mutated, and in which the E1 gene has been replaced with a gene encoding a fusion protein and/or reporter gene of the invention. An E1/E3 deleted adenovirus allows for an insert of up to 8 kb. A preferred viral backbone is dl327 which is deleted in E1a, E1b, and E3. Deletion within adenovirus genes other than E1 and E3 region genes may also be useful to further reduce viral genome size, allowing thereby the insertion of larger genes of interest. In addition, since replication and viral protein expression in these viruses is reduced or eliminated *in vivo,* the immune response of the infected host to the virus and viral protein is also reduced. By decreasing the host immune response, the persistence of expression of the inserted gene is increased. Such vectors are further described in published PCT application No. PCT/US94/06338 having publication No. WO 94/28938 by Wilson and Engelhardt.

Other than the requirement that the adenovirus be replication defective, or at least conditionally defective, the nature of the adenovirus is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus. As stated above, the typical virus according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the nucleic acid of interest at the position from which the E1 coding sequences have been removed. However, the position of insertion of the nucleic acid of interest in a region within the adenovirus sequences is not critical to the present invention. For example, the nucleic acid of interest may also be inserted in lieu of the deleted E3 region in E3 replacement viruses as described previously by Karlsson et. al. (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Various adenoviruses have been shown to be of use in the transfer of genes to mammals, including humans. Replication-deficient adenovirus has been used to express marker proteins and CFTR in the pulmonary epithelium. Because of their ability to efficiently infect dividing cells, their tropism for the lung, and the relative ease of generation of high titer stocks, adenoviruses have been the subject of much research in the last few years, and various viruses have been used to deliver genes to the lungs of human subjects (Zabner et al., Cell 75:207-216, 1993; Crystal, et al., Nat Genet. 8:42-51, 1994; Boucher, et al., Hum Gene Ther 5:615-639, 1994). The first generation E1a deleted adenovirus has been improved upon with a second generation that includes a temperature-sensitive E2a viral protein, designed to express less viral protein and thereby make the virally infected cell less of a target for the immune system (Goldman et al., Human Gene Therapy 6:839-851,1995). More recently, a viral vector deleted of all viral open reading frames has been reported (Fisher et al., Virology 217:11-22, 1996). Moreover, it has been shown that expression of viral IL-10 inhibits the immune response to adenoviral antigen (Qin et al., Human Gene Therapy 8:1365-1374, 1997).

Adenoviruses can also be cell type specific, i.e., infect only restricted types of cells and/or express a transgene only in restricted types of cells. For example, the viruses comprise a gene under the transcription control of a transcription initiation region specifically regulated by target host cells, as described e.g., in U.S. Patent No. 5,698,443, by Henderson and Schuur, issued December 16, 1997. Thus, replication competent adenoviruses can be restricted to certain cells by, e.g., inserting a cell specific response element to regulate a synthesis of a protein necessary for replication, e.g., E1A or E1B.

Techniques for introducing viruses into a variety of cells including hepatocytes, pancreatic and biliary eptithelial cells, endothelial cells and described in the following published PCT applications: PCT/US96/03041 (WO 96/26286) by Wilson et al. (hepatocytes); PCT/US91/09700 (WO 92/12242) by Mulligan and Wilson (hepatocytes); and PCT/US89/00422 (WO 89/07136) by Wilson and Mulligan (hepatocytes); PCT/US94/05187 (WO 94/26915) by J.M. Wilson (pancreatic cells); and PCT/US91/08127 (WO 92/07573) by Rafield et al. and PCT/US88/04383 (WO 89/05345) by Wilson and Mulligan (endothelial cells).

DNA sequences of a number of adenovirus types are available from Genbank. For example, human adenovirus type 5 has GenBank Accession No.M73260. The adenovirus DNA sequences may be obtained from any of the 42 human adenovirus types currently identified. Various adenovirus strains are available from the American

Type Culture Collection, Rockville, Maryland, or by request from a number of commercial and academic sources. A transgene as described herein may be incorporated into any adenovirus and delivery protocol, by the same methods (restriction digest, linker ligation or filling in of ends, and ligation) used to insert the
5 CFTR or other genes into the vectors.

In another embodiment, the invention provides a virus, e.g, adenovirus, which is a recombinant replication defective virus comprising the DNA of, or corresponding to, at least a portion of the genome of said virus, capable of infecting a mammalian cell, and a first expression sequence comprising a gene of interest operably linked to an
10 expression control sequence, flanked on each side by the cis-acting terminal repeat sequence of a transposon, said expression sequence flanked by the DNA of the virus. The recombinant virus is capable of infecting a mammalian cell and capable of expressing the gene of interest and tansferring it to the chromatin of said cell in vivo or in vitro in the presence of a transposase. The virus can further comprise a gene
15 encoding a suitable trans-acting transposase operably linked to an expression control sequence. Such a recombinant virus is capable of infecting a mammalian cell and capable of expressing the selected gene and transferring it to the chromatin of the infected cell in vivo or in vitro, when in the presence of a transposase. These viruses are further described in published PCT application No. WO 97/15679 by Kelley and
20 Wilson.

The viruses of the invention can be administered to a host animal in such a manner that a potential immune reaction to the viruses is reduced. This can be achieved, e.g., by administering together with the virus a selected immune modulator, which substantially reduces the occurrence of neutralizing antibody responses directed
25 against the virus encoded antigens and/or cytolytic T cell elimination of the viral protein containing cell. The immune modulator can be administered simultaneously or prior to administration of the viruses. The immune modulator can be, e.g., selected from the group consisting of a cytokine, an agent capable of depleting or inhibiting CD4+ T cells, and anti-T cell antibody, an agent capable of bloking the interaction
30 between CD40 ligand on a T cell and CD40 on a B cell, an agent capable of bloking the interaction between the CD28 or CTLA4 ligand on a T cell and B7 on a B cell, and cyclphosphamide. This technique is further described in published PCT application No. WO 96/26285 by Wilson et al.

A preferred helper cell line is 293 (ATCC Accession No. CRL1573). This
35 helper cell line, also termed a "packaging cell line" was developed by Frank Graham (Graham et al. (1987) J. Gen. Virol. 36:59-72 and Graham (1977) J.General Virology

68:937-940) and provides E1A and E1B in trans. However, helper cell lines may also be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells.

Adenovirus producer cell lines can include one or more of the adenoviral genes E1, E2a, and E4 DNA sequence, for packaging adenovirus in which one or more of these genes have been mutated or deleted are described, e.g., in WO 96/18418 by Kadan et al.; WO 95/346671 by Kovesdi et al.; WO94/28152 by Imler et al.; WO 95/02697 by Perrocaudet et al., WO96/14061 by Wang et al.

AAV

Yet another viral system useful for delivery of the subject fusion genes is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review, see Muzyczka et al., Curr. Topics in Micro. and Immunol. (1992) 158:97-129).

AAV has not been associated with the cause of any disease. AAV is not a transforming or oncogenic virus. AAV integration into chromosomes of human cell lines does not cause any significant alteration in the growth properties or morphological characteristics of the cells. These properties of AAV also recommend it as a potentially useful human gene therapy vector.

AAV is also one of the few viruses that may integrate its DNA into non-dividing cells, e.g., pulmonary epithelial cells, and exhibits a high frequency of stable integration (see for example Flotte et al., (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et al., (1989) J. Virol. 63:3822-3828; and McLaughlin et al., (1989) J. Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., (1985) Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., (1984) PNAS USA 81:6466-6470; Tratschin et al., (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al., (1988) Mol. Endocrinol. 2:32-39; Tratschin et al., (1984) J. Virol. 51:611-619; and Flotte et al., (1993) J. Biol. Chem. 268:3781-3790).

The AAV-based expression vector to be used typically includes the 145 nucleotide AAV inverted terminal repeats (ITRs) flanking a restriction site that can be used for subcloning of the transgene, either directly using the restriction site available, or by excision of the transgene with restriction enzymes followed by blunting of the 5 ends, ligation of appropriate DNA linkers, restriction digestion, and ligation into the site between the ITRs. By "adeno-associated virus inverted terminal repeats" or "AAV ITRs" is meant the palindromic regions which comprise the terminal repeats at the 3' and 5' ends of the AAV genome. The AAV ITR regions provide sequences for packaging the AAV provirus (i.e., the AAV genome) into the AAV viral capsid. The 10 ITR regions also form secondary structures which act as self-primers for AAV replication. Samulski et al. (J. Virol. 63:3822, 1989), for example, describes AAV ITR sequences and structures. The nucleotide sequences of AAV ITR regions are known. See, e.g., Kotin, R. M. (1994) Human Gene Therapy 5:793-801; Berns, K. I. "Parvoviridae and their Replication" in Fundamental Virology, 2nd Edition, (B. N. 15 Fields and D. M. Knipe, eds.) for the AAV-2 sequence. As used herein, an "AAV ITR" need not have the wild-type nucleotide sequence depicted, but may be altered, e.g., by the insertion, deletion or substitution of nucleotides. Additionally, the AAV ITR may be derived from any of several AAV serotypes, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAVX7, etc. Furthermore, 5' and 3' ITRs which 20 flank a selected nucleotide sequence in an AAV vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, i.e., to allow for excision and rescue of the sequence of interest from a host cell genome or vector, and to allow integration of the heterologous sequence into the recipient cell genome when the rep gene is present in the cell (either on the same or 25 on a different vector).

The capacity of AAV vectors is about 4.4 kb. The following proteins have been expressed using various AAV-based vectors, and a variety of promoter/enhancers: neomycin phosphotransferase, chloramphenicol acetyl transferase, Fanconi's anemia gene, cystic fibrosis transmembrane conductance 30 regulator, and granulocyte macrophage colony-stimulating factor (Kotin, R.M., Human Gene Therapy 5:793-801, 1994, Table I). A transgene incorporating the various DNA constructs of this invention can similarly be included in an AAV-based vector. As an alternative to inclusion of a constitutive promoter such as CMV to drive expression of the recombinant DNA encoding the fusion protein(s), e.g. fusion proteins comprising 35 an activation domain or DNA-binding domain, an AAV promoter can be used (ITR itself or AAV p5 (Flotte, et al. J. Biol.Chem. 268:3781-3790, 1993)).

Such a vector can be packaged into AAV virions by reported methods. For example, a human cell line such as 293 can be co-transfected with the AAV-based expression vector and another plasmid containing open reading frames encoding AAV rep and cap (which are obligatory for replication and packaging of the recombinant viral construct) under the control of endogenous AAV promoters or a heterologous promoter. In the absence of helper virus, the rep proteins Rep68 and Rep78 prevent accumulation of the replicative form, but upon superinfection with adenovirus or herpes virus, these proteins permit replication from the ITRs (present only in the construct containing the transgene) and expression of the viral capsid proteins. This system results in packaging of the transgene DNA into AAV virions (Carter, B.J., Current Opinion in Biotechnology 3:533-539, 1992; Kotin, R.M, Human Gene Therapy 5:793-801, 1994)). Typically, three days after transfection, recombinant AAV is harvested from the cells along with adenoviurs and the contaminating adenovirus is then inactivated by heat treatment.

Methods to improve the titer of AAV can also be used to express the transgene in an AAV virion. Such strategies include, but are not limited to: stable expression of the ITR-flanked transgene in a cell line followed by transfection with a second plasmid to direct viral packaging; use of a cell line that expresses AAV proteins inducibly, such as temperature-sensitive inducible expression or pharmacologically inducible expression. Alternatively, a cell can be infected with a first AAV including a 5' ITR, a 3' ITR flanking a heterologous gene, and a second AAV vector which includes an inducible origin of replication, e.g., SV40 origin of replication, which is capable of being induced by an agent, such as the SV40 T antigen and which includes DNA sequences encoding the AAV rep and cap proteins. Upon induction by an agent, the second AAV vector may replicate to a high copy number, and thereby increased numbers of infectious AAV particles may be generated (see, e.g, U.S. Patent No. 5,693,531 by Chiorini et al., issued December 2, 1997. In yet another method for producing large amounts of recombinant AAV, a fusion plasmid is used which incorporate the Epstein Barr Nuclear Antigen (EBNA) gene , the latent origin of replication of Epstein Barr virus (oriP) and an AAV genome. These plasmids are maintained as a multicopy extra-chromosomal elements in cells, such as in 293 cells. Upon addition of wild-type helper functions, these cells will produce high amounts of recombinant AAV (U.S. Patent 5,691,176 by Lebkowski et al., issued Nov. 25, 1997). In another system, an AAV packaging plasmid is provided that allows expression of the rep gene, wherein the p5 promoter, which normally controls rep expression, is replaced with a heterologous promoter (U.S. Patent 5,658,776, by Flotte et al., issued Aug. 19, 1997).

Additionally, one may increase the efficiency of AAV transduction by treating the cells with an agent that facilitates the conversion of the single stranded form to the double stranded form, as described in Wilson et al., WO96/39530.

AAV stocks can be produced as described in Hermonat and Muzyczka (1984) PNAS 81:6466, modified by using the pAAV/Ad described by Samulski et al. (1989) J. Virol. 63:3822. Concentration and purification of the virus can be achieved by reported methods such as banding in cesium chloride gradients, as was used for the initial report of AAV expression in vivo (Flotte, et al. J.Biol. Chem. 268:3781-3790, 1993) or chromatographic purification, as described in O'Riordan et al., WO97/08298.

Methods for *in vitro* packaging AAV vectors are also available and have the advantage that there is no size limitation of the DNA packaged into the particles (see, U.S. Patent No. 5,688,676, by Zhou et al., issued Nov. 18, 1997). This procedure involves the preparation of cell free packaging extracts.

For additional detailed guidance on AAV technology which may be useful in the practice of the subject invention, including methods and materials for the incorporation of a transgene, the propagation and purification of the recombinant AAV containing the transgene, and its use in transfecting cells and mammals, see e.g. Carter et al, US Patent No. 4,797,368 (10 Jan 1989); Muzyczka et al, US Patent No. 5,139,941 (18 Aug 1992); Lebkowski et al, US Patent No. 5,173,414 (22 Dec 1992); Srivastava, US Patent No. 5,252,479 (12 Oct 1993); Lebkowski et al, US Patent No. 5,354,678 (11 Oct 1994); Shenk et al, US Patent No. 5,436,146(25 July 1995); Chatterjee et al, US Patent No. 5,454,935 (12 Dec 1995), Carter et al WO 93/24641 (published 9 Dec 1993), and Natsoulis, U.S. Patent No. 5,622,856 (April 22, 1997). Further information regarding AAVs and the adenovirus or herpes helper functions required can be found in the following articles.Berns and Bohensky (1987), "Adeno-Associated Viruses: An Update", Advanced in Virus Research, Academic Press, 33:243-306. The genome of AAV is described in Laughlin et al. (1983) "Cloning of infectious adeno-associated virus genomes in bacterial plasmids", Gene, 23: 65-73. Expression of AAV is described in Beaton et al. (1989) "Expression from the Adeno-associated virus p5 and p19 promoters is negatively regulated in trans by the rep protein", J. Virol., 63:4450-4454. Construction of rAAV is described in a number of publications: Tratschin et al. (1984) "Adeno-associated virus vector for high frequency integration, expression and rescue of genes in mammalian cells", Mol. Cell. Biol., 4:2072-2081; Hermonat and Muzyczka (1984) "Use of adeno-associated virus as a mammalian DNA cloning vector: Transduction of neomycin resistance into

mammalian tissue culture cells", Proc. Natl. Acad. Sci. USA, 81:6466-6470; McLaughlin et al. (1988) "Adeno-associated virus general transduction vectors: Analysis of Proviral Structures", J. Virol., 62:1963-1973; and Samulski et al. (1989) "Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression", J. Virol., 63:3822-3828. Cell lines that can be infected by rAAV are those described in Lebkowski et al. (1988) "Adeno-associated virus: a vector system for efficient introduction and integration of DNA into a variety of mammalian cell types", Mol. Cell. Biol., 8:3988-3996. "Producer" or "packaging" cell lines used in manufacturing recombinant retroviruses are described in Dougherty et al. (1989) J. Virol., 63:3209-3212; and Markowitz et al. (1988) J. Virol., 62:1120-1124.

Hybrid Adenovirus-AAV

Hybrid Adenovirus-AAV is represented by an adenovirus capsid containing a nucleic acid comprising a portion of an adenovirus, and 5' and 3' ITR sequences from an AAV which flank a selected transgene under the control of a promoter. See e.g. Wilson et al, International Patent Application Publication No. WO 96/13598. This hybrid virus is characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome in the presence of the rep gene. This virus is capable of infecting virtually all cell types (conferred by its adenovirus sequences) and stable long term transgene integration into the host cell genome (conferred by its AAV sequences).

The adenovirus nucleic acid sequences employed in the this vector can range from a minimum sequence amount, which requires the use of a helper virus to produce the hybrid virus particle, to only selected deletions of adenovirus genes, which deleted gene products can be supplied in the hybrid viral process by a packaging cell. For example, a hybrid virus can comprise the 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication). The left terminal sequence (5') sequence of the Ad5 genome that can be used spans bp 1 to about 360 of the conventional adenovirus genome (also referred to as map units 0-1) and includes the 5' ITR and the packaging/enhancer domain. The 3' adenovirus sequences of the hybrid virus include the right terminal 3' ITR sequence which is about 580 nucleotides (about bp 35,353- end of the adenovirus, referred to as about map units 98.4-100.

The AAV sequences useful in the hybrid vector are viral sequences from which the rep and cap polypeptide encoding sequences are deleted and are usually the cis acting 5' and 3' ITR sequences. Thus, the AAV ITR sequences are flanked by the selected adenovirus sequences and the AAV ITR sequences themselves flank a selected

transgene. The preparation of the hybrid vector is further described in detail in published PCT application entitled "Hybrid Adenovirus-AAV Virus and Method of Use Thereof", WO 96/13598 by Wilson et al.

For additional detailed guidance on adenovirus and hybrid adenovirus-AAV
5 technology which may be useful in the practice of the subject invention, including methods and materials for the incorporation of a transgene, the propagation and purification of recombinant virus containing the transgene, and its use in transfecting cells and mammals, see also Wilson et al, WO 94/28938, WO 96/13597 and WO 96/26285, and references cited therein.

10

Retroviruses

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin (1990) Retroviridae and their Replication" In Fields,
15 Knipe ed. Virology. New York: Raven Press). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsial proteins, polymerase enzyme, and envelope components,
20 respectively. A sequence found upstream from the gag gene, termed psi , functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin (1990), *supra*).

25 In order to construct a retroviral vector, a nucleic acid of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and psi components is constructed (Mann et al. (1983) Cell 33:153). When a recombinant plasmid containing a human cDNA,
30 together with the retroviral LTR and psi sequences is introduced into this cell line (by calcium phosphate precipitation for example), the psi sequence allows the RNA transcript of the recombinant plasmid to be packaged into viruses, which are then secreted into the culture media (Nicolas and Rubenstein (1988) "Retroviral Vectors", In: Rodriguez and Denhardt ed. Vectors: A Survey of Molecular Cloning Vectors and
35 their Uses. Stoneham:Butterworth; Temin, (1986) "Retrovirus Vectors for Gene Transfer: Efficient Integration into and Exprssion of Exogenous DNA in Vertebrate

Cell Genome", In: Kucherlapati ed. Gene Transfer. New York: Plenum Press; Mann et al., 1983, *supra*). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviruses are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind et al. (1975) Virology 67:242).

A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) Blood 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by nucleic acid encoding a fusion protein of the present invention, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al., (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. A preferred retroviral vector is a pSR MSVtkNeo (Muller et al. (1991) Mol. Cell Biol. 11:1785 and pSR MSV(XbaI) (Sawyers et al. (1995) J. Exp. Med. 181:307) and derivatives thereof. For example, the unique BamHI sites in both of these vectors can be removed by digesting the vectors with BamHI, filling in with Klenow and religating to produce pSMTN2 and pSMTX2, respectively, as described in PCT/US96/09948 by Clackson et al. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Crip, Cre, 2 and Am.

Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or in vivo (see for example Eglitis et al., (1985) Science 230:1395-1398; Danos and Mulligan, (1988) PNAS USA 85:6460-6464; Wilson et al., (1988) PNAS USA 85:3014-3018; Armentano et al., (1990) PNAS USA 87:6141-6145; Huber et al., (1991) PNAS USA 88:8039-8043; Ferry et al., (1991) PNAS USA 88:8377-8381; Chowdhury et al., (1991) Science 254:1802-1805; van Beusechem et al., (1992) PNAS USA 89:7640-7644; Kay et al.,

(1992) Human Gene Therapy 3:641-647; Dai et al., (1992) PNAS USA 89:10892-10895; Hwu et al., (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

5 Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the virus (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviruses include:
10 coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al., (1989) PNAS USA 86:9079-9083; Julan et al., (1992) J. Gen Virol 73:3251-3255; and Goud et al., (1983) Virology 163:251-254); or coupling cell surface ligands to the viral env proteins (Neda et al., (1991) J. Biol. Chem. 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose
15 to convert the env protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

20 **Other Viral Systems**

Other viral systems that may have application in gene therapy have been derived from herpes virus, e.g., Herpes Simplex Virus (U.S. Patent No. 5,631,236 by Woo et al., issued May 20, 1997), vaccinia virus (Ridgeway (1988) Ridgeway, "Mammalian expression vectors," In: Rodriguez R L, Denhardt D T, ed. Vectors: A
25 survey of molecular cloning vectors and their uses. Stoneham: Butterworth,; Baichwal and Sugden (1986) "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press; Coupar et al. (1988) Gene, 68:1-10), and several RNA viruses. Preferred viruses include an alphavirus, a poxivirus, an arena virus, a
30 vaccinia virus, a polio virus, and the like. In particular, herpes virus may provide a unique strategy for persistence of the recombinant gene in cells of the central nervous system and ocular tissue (Pepose et al., (1994) Invest Ophthalmol Vis Sci 35:2662-2666). They offer several attractive features for various mammalian cells (Friedmann (1989) Science, 244:1275-1281 ; Ridgeway, 1988, *supra*; Baichwal and Sugden, 1986,
35 *supra*; Coupar et al., 1988; Horwich et al.(1990) J.Virol., 64:642-650).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich et al., 1990, *supra*). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang et al. recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang et al. (1991) Hepatology, 14:124A).

Administration of Viral Vectors

Generally the viral particles are transferred to a biologically compatible solution or pharmaceutically acceptable delivery vehicle, such as sterile saline, or other aqueous or non-aqueous isotonic sterile injection solutions or suspensions, numerous examples of which are well known in the art, including Ringer's, phosphate buffered saline, or other similar vehicles. Delivery of the recombinant viral vector can be carried out via any of several routes of administration, including intramuscular injection, intravenous administration, subcutaneous injection, intrahepatic administration, catheterization (including cardiac catheterization), intracranial injection, nebulization/inhalation or by instillation via bronchoscopy.

Preferably, the DNA or recombinant virus is administered in sufficient amounts to transfect cells within the recipient's target cells, including without limitation, muscle cells, liver cells, various airway epithelial cells and smooth muscle cells, neurons, cardiac muscle cells, etc. and provide sufficient levels of transgene expression to provide for observable ligand-responsive secretion of a target protein, preferably at a level providing therapeutic benefit without undue adverse effects.

Optimal dosages of DNA or virus depends on a variety of factors, as discussed previously, and may thus vary somewhat from patient to patient. Again, therapeutically effective doses of viruses are considered to be in the range of about 20 to about 50 ml of saline solution containing concentrations of from about 1×10^7 to about 1×10^{10} pfu of virus/ml, e.g. from 1×10^8 to 1×10^9 pfu of virus/ml.

Host Cells

This invention is particularly useful for the engineering of animal cells and in applications involving the use of such engineered animal cells. The animal cells may be insect, worm or mammalian cells. While various mammalian cells may be used, including, by way of example, equine, bovine, ovine, canine, feline, murine, and non-human primate cells, human cells are of particular interest. Among the various species, various types of cells may be used, such as hematopoietic, neural, glial, mesenchymal, cutaneous, mucosal, stromal, muscle (including smooth muscle cells), spleen, reticulo-endothelial, epithelial, endothelial, hepatic, kidney, gastrointestinal, pulmonary, fibroblast, and other cell types. Of particular interest are hematopoietic cells, which may include any of the nucleated cells which may be involved with the erythroid, lymphoid or myelomonocytic lineages, as well as myoblasts and fibroblasts. Also of interest are stem and progenitor cells, such as hematopoietic, neural, stromal, muscle, hepatic, pulmonary, gastrointestinal and mesenchymal stem cells

The cells may be autologous cells, syngeneic cells, allogeneic cells and even in some cases, xenogeneic cells with respect to an intended host organism. The cells may be modified by changing the major histocompatibility complex ("MHC") profile, by inactivating ß2-microglobulin to prevent the formation of functional Class I MHC molecules, inactivation of Class II molecules, providing for expression of one or more MHC molecules, enhancing or inactivating cytotoxic capabilities by enhancing or inhibiting the expression of genes associated with the cytotoxic activity, or the like.

In some instances specific clones or oligoclonal cells may be of interest, where the cells have a particular specificity, such as T cells and B cells having a specific antigen specificity or homing target site specificity.

Introduction of Constructs into Animals

Cells which have been modified ex vivo with the DNA constructs may be grown in culture under selective conditions and cells which are selected as having the desired construct(s) may then be expanded and further analyzed, using, for example, the polymerase chain reaction for determining the presence of the construct in the host cells and/or assays for the production of the desired gene product(s). Once modified host cells have been identified, they may then be used as planned, e.g. grown in culture or introduced into a host organism.

Depending upon the nature of the cells, the cells may be introduced into a host organism, e.g. a mammal, in a wide variety of ways. Hematopoietic cells may be

administered by injection into the vascular system, there being usually at least about 10^4 cells and generally not more than about 10^{10} cells. The number of cells which are employed will depend upon a number of circumstances, the purpose for the introduction, the lifetime of the cells, the protocol to be used, for example, the number
5 of administrations, the ability of the cells to multiply, the stability of the therapeutic agent, the physiologic need for the therapeutic agent, and the like. Generally, for myoblasts or fibroblasts for example, the number of cells will be at least about 104 and not more than about 109 and may be applied as a dispersion, generally being injected at or near the site of interest. The cells will usually be in a physiologically-
10 acceptable medium.

Cells engineered in accordance with this invention may also be encapsulated, e.g. using conventional biocompatible materials and methods, prior to implantation into the host organism or patient for the production of a therapeutic protein. See e.g. Hguyen et al, Tissue Implant Systems and Methods for Sustaining viable High Cell
15 Densities within a Host, US Patent No. 5,314,471 (Baxter International, Inc.); Uludag and Sefton, 1993, J Biomed. Mater. Res. 27(10):1213-24 (HepG2 cells/hydroxyethyl methacrylate-methyl methacrylate membranes); Chang et al, 1993, Hum Gene Ther 4(4):433-40 (mouse Ltk- cells expressing hGH/immunoprotective perm-selective alginate microcapsules; Reddy et al, 1993, J Infect Dis 168(4):1082-3 (alginate); Tai
20 and Sun, 1993, FASEB J 7(11):1061-9 (mouse fibroblasts expressing hGH/alginate-poly-L-lysine-alginate membrane); Ao et al, 1995, Transplanataion Proc. 27(6):3349, 3350 (alginate); Rajotte et al, 1995, Transplantation Proc. 27(6):3389 (alginate); Lakey et al, 1995, Transplantation Proc. 27(6):3266 (alginate); Korbutt et al, 1995, Transplantation Proc. 27(6):3212 (alginate); Dorian et al, US Patent No. 5,429,821
25 (alginate); Emerich et al, 1993, Exp Neurol 122(1):37-47 (polymer-encapsulated PC12 cells); Sagen et al, 1993, J Neurosci 13(6):2415-23 (bovine chromaffin cells encapsulated in semipermeable polymer membrane and implanted into rat spinal subarachnoid space); Aebischer et al, 1994, Exp Neurol 126(2):151-8 (polymer-encapsulated rat PC12 cells implanted into monkeys; see also Aebischer, WO
30 92/19595); Savelkoul et al, 1994, J Immunol Methods 170(2):185-96 (encapsulated hybridomas producing antibodies; encapsulated transfected cell lines expressing various cytokines); Winn et al, 1994, PNAS USA 91(6):2324-8 (engineered BHK cells expressing human nerve growth factor encapsulated in an immunoisolation polymeric device and transplanted into rats); Emerich et al, 1994, Prog Neuropsychopharmacol
35 Biol Psychiatry 18(5):935-46 (polymer-encapsulated PC12 cells implanted into rats); Kordower et al, 1994, PNAS USA 91(23):10898-902 (polymer-encapsulated

engineered BHK cells expressing hNGF implanted into monkeys) and Butler et al WO 95/04521 (encapsulated device). The cells may then be introduced in encapsulated form into an animal host, preferably a mammal and more preferably a human subject in need thereof. Preferably the encapsulating material is semipermeable, permitting release into the host of secreted proteins produced by the encapsulated cells. In many embodiments the semipermeable encapsulation renders the encapsulated cells immunologically isolated from the host organism in which the encapsulated cells are introduced. In those embodiments the cells to be encapsulated may express one or more fusion proteins containing component domains derived from proteins of the host species and/or from viral proteins or proteins from species other than the host species. For example in such cases the fusion proteins may contain elements derived from GAL4 and VP16. The cells may be derived from one or more individuals other than the recipient and may be derived from a species other than that of the recipient organism or patient.

Instead of ex vivo modification of the cells, in many situations one may wish to modify cells in vivo. For this purpose, various techniques have been developed for modification of target tissue and cells in vivo. A number of viral vectors have been developed, such as adenovirus, adeno-associated virus, and retroviruses, as discussed above, which allow for transfection and, in some cases, integration of the virus into the host. See, for example, Dubensky et al. (1984) Proc. Natl. Acad. Sci. USA 81, 7529-7533; Kaneda et al., (1989) Science 243,375-378; Hiebert et al. (1989) Proc. Natl. Acad. Sci. USA 86, 3594-3598; Hatzoglu et al. (1990) J. Biol. Chem. 265, 17285-17293 and Ferry, et al. (1991) Proc. Natl. Acad. Sci. USA 88, 8377-8381. The vector may be administered by injection, e.g. intravascularly or intramuscularly, inhalation, or other parenteral mode. Non-viral delivery methods such as administration of the DNA via complexes with liposomes or by injection, catheter or biolistics may also be used.

In accordance with in vivo genetic modification, the manner of the modification will depend on the nature of the tissue, the efficiency of cellular modification required, the number of opportunities to modify the particular cells, the accessibility of the tissue to the DNA composition to be introduced, and the like. By employing an attenuated or modified retrovirus carrying a target transcription initiation region, if desired, one can activate the virus using one of the subject transcription factor constructs, so that the virus may be produced and transfect adjacent cells.

The DNA introduction need not result in integration in every case. In some situations, transient maintenance of the DNA introduced may be sufficient. In this way, one could have a short term effect, where cells could be introduced into the host

and then turned on after a predetermined time, for example, after the cells have been able to home to a particular site.

Binding properties, Assays

5 FK506 is known to bind to the human protein, FKBP12 and to form a tripartite complex with the serine/threonine phosphatase, calcineurin. FK506 analogs may be characterized and compared to FK506 with respect to their ability to bind to human FKBP12 and/or to form tripartite complexes with fusion proteins containing human FKBP12 and CABs. See, for example, WO 96/41865 (Clackson et al). That 10 application discloses various materials and methods which can be used to quantify the ability of a compound to bind to human FKBP12 or to form a tripartite complex with (*i.e.*, "heterodimerize") proteins comprising human FKBP12 and the FRB domain of human FRAP, respectively. Such assays include fluorescence polarization assays to measure binding. Also included are cell based transcription assays in which the ability 15 of a compound to form the tripartite complex is measured indirectly by correlation with the observed level of reporter gene product produced by engineered mammalian cells in the presence of the compound. Corresponding cell-based assays may also be conducted in engineered yeast cells. See *e.g.* WO 95/33052 (Berlin et al).

It will often be preferred that the ligands of this invention be physiologically 20 acceptable (i.e., lack undue toxicity toward the cell or organism with which it is to be used), can be taken orally by animals (i.e., is orally active in applications in whole animals, including gene therapy), and/or can cross cellular and other membranes, as necessary for a particular application.

In addition, preferred ligands are those which bind preferentially to mutant 25 immunophilins (by way of non-limiting example, a human FKBP in which Phe36 is replaced with a different amino acid, preferably an amino acid with a less bulky R group such as valine or alanine) over native or naturally-ocurring immunophilins. For example, such compounds may bind preferentially to mutant FKBPs at least an order of magnitude better than they bind to human FKBP12, and in some cases may bind to 30 mutant FKBPs greater than 2 or even 3 or more orders of magnitude better than they do to human FKBP12, as determined by any scientifically valid or art-accepted assay methodology.

Binding affinities of various ligands of this invention with respect to human FKBP12, variants thereof or other immunophilin proteins may be determined by 35 adaptation of known methods used in the case of FKBP. For instance, the practitioner may measure the ability of a compound of this invention to compete with the binding

of a known ligand to the protein of interest. See e.g. Sierkierka et al, 1989, Nature 341, 755-757 (test compound competes with binding of labeled FK506 derivative to FKBP).

One set of preferred ligands of this invention which binds, to human FKBP12, to a mutant thereof as discussed above, or to a fusion protein containing such FKBP domains, with a Kd value below about 200 nM, more preferably below about 50 nM , even more preferably below about 10 nM, and even more preferably below about 1 nM, as measured by direct binding measurement (e.g. fluorescence quenching), competition binding measurement (e.g. versus FK506), inhibition of FKBP enzyme activity (rotamase), or other assay methodology. In one subset of such compounds, the FKBP domain is one in which phenylalanine at position 36 has been replaced with an amino acid having a less bulky side chain, e.g. alanine, valine, methionine or serine.

A Competitive Binding FP Assay is described in detail in the examples which follow. That assay permits the in vitro measurement of an IC50 value for a given compound which reflects its ability to bind to an FKBP protein in competition with a labeled FKBP ligand, such as, for example, FK506.

One preferred class of compounds of this invention are those ligands which have an IC50 value in the Competitive Binding FP Assay better than 1000 nM, preferably better than 300 nM, more preferably better than 100 nM, and even more preferably better than 10 nM with respect to a given FKBP domain and ligand pair, e.g. human FKBP12 or a variant thereof with up to 10, preferably up to 5 amino acid replacements, with a flouresceinated FK506 standard. In one subset of that class, the FKBP domain has one of the abovementioned modifications at position 36.

The ability of the ligands to multimerize fusion proteins may be measured in cell-based assays by measuring the occurrence of an event triggered by such multimerization. For instance, one may use cells containing and capable of expressing DNA encoding a first fusion protein comprising one or more FKBP- domains and one or more action domains as well as DNA encoding a second fusion protein containing an CAB domain and one or more action domains capable, upon multimerization, of actuating a biological response. We prefer to use cells which further contain a reporter gene under the transcription control of a regulatory element (i.e., promoter) which is responsive to the multimerization of the fusion proteins. The design and preparation of illustrative components and their use in so engineered cells is described in WO96/41865 and the other international patent applications referred to in this and the foregoing section. The cells are grown or maintained in culture. A ligand is added to the culture medium and after a suitable incubation period (to permit gene expression and secretion, e.g. several hours or overnight) the presence of the reporter

gene product is measured. Positive results, i.e., multimerization, correlates with transcription of the reporter gene as observed by the appearance of the reporter gene product. The reporter gene product may be a conveniently detectable protein (e.g. by ELISA) or may catalyze the production of a conveniently detectable product (e.g. colored). Materials and methods for producing appropriate cell lines for conducting such assays are disclosed in the international patent applications cited above in this section. Typically used target genes include by way of example SEAP, hGH, beta-galactosidase, Green Fluorescent Protein and luciferase, for which convenient assays are commercially available.

Another preferred class of compounds of this invention are those which are capable of inducing a detectable signal in a 2-hybrid transcription assay based on fusion proteins containing an FKBP domain. Preferably, the FKBP domain is an FKBP domain other than wild-type human FKBP12.

Another assay for measuring the ability of the ligands to multimerize fusion proteins, like the FKBP-based transcription assay, is a cell-based assay which measures the occurrence of an event triggered by such multimerization. In this case, one uses cells which constitutively express a detectable product. The cells also contain and are capable of expressing DNAs encoding fusion proteins comprising one or more immunophilin-derived ligand binding domains and one or more action domains, such as the intracellular domain of FAS, capable, upon multimerization, of triggering cell death. The design and preparation of illustrative components and their use in so engineering cells is described in WO95/02684. See also WO96/41865. The cells are maintained or cultured in a culture medium permitting cell growth or continued viability. The cells or medium are assayed for the presence of the constitutive cellular product, and a baseline level of reporter is thus established. One may use cells engineered for constitutive production of hGH or any other conveniently detectable product to serve as the reporter. The compound to be tested is addded to the medium, the cells are incubated, and the cell lysate or medium is tested for the presence of reporter at one or more time points. Decrease in reporter production indicates cell death, an indirect measure of multimerization of the fusion proteins.

Another preferred class of compounds of this invention are those which are capable of inducing a detectable signal in such an FKBP/CAB-based apoptosis assay. Preferably, the FKBP domain is an FKBP domain other than wild-type human FKBP12. In some cases, the FKBP domain is modified, as discussed above. Also preferably, the CAB domain is a CAB domain other than wild-type CAB.

Conducting such assays permits the practitioner to select ligands possessing the desired IC50 values and/or binding preference for a mutant FKBP over wild-type human FKBP12. The Competitive Binding FP Assay permits one to select monomers or ligands which possess the desired IC50 values and/or binding preference for a mutant FKBP or wild-type FKBP relative to a control, such as FK506.

Applications

The ligands and ligand binding domains can be used as described in WO94/18317, WO95/02684, WO96/20951, WO95/41865, e.g. to regulatably activate the transcription of a desired gene, delete a target gene, actuate apoptosis, or trigger other biological events in engineered cells growing in culture or in whole organisms, including in gene therapy applications. The following are non-limiting examples of applications of the subject invention.

1. **Regulated gene therapy.** In many instances, the ability to switch a therapeutic gene on and off at will or the ability to titrate expression with precision are important for therapeutic efficacy. This invention is particularly well suited for achieving regulated expression of a therapeutic target gene in the context of human gene therapy. One example uses a pair of fusion proteins of this invention (one containing at least one CAB domain, the other containing at least one FKBP domain), a ligand capable of dimerizing the fusion proteins, and a target gene construct to be expressed. One of the fusion proteins comprises a DNA-binding domain, preferably a composite DNA-binding domain as described in Pomerantz et al, supra, as the heterologous action domain. The second fusion protein comprises a transcription activating domain as the heterologous action domain. The improved ligand is capable of binding to both fusion proteins and thus of effectively cross-linking the fusion proteins. DNA molecules encoding and capable of directing the expression of these fusion proteins are introduced into the cells to be engineered. Also introduced into the cells is a target gene linked to a DNA sequence to which the DNA-binding domain is capable of binding. Contacting the engineered cells or their progeny with the improved ligand (by administering it to the animal or patient) leads to assembly of the transcription factor complex and hence to expression of the target gene. The design and use of similar components is disclosed in PCT/US93/01617 and in WO 96/41865 (Clackson et al). In practice, the level of target gene expression should be a function of the number or concentration of fusion transcription factor complexes,

which should in turn be a function of the concentration of the improved ligand. Dose (of improved ligand)-responsive gene expression is typically observed.

The improved ligand may be administered to the patient as desired to activate transcription of the target gene. Depending upon the binding affinity of the improved ligand, the response desired, the manner of administration, the biological half-life of the ligand and/or target gene mRNA, the number of engineered cells present, various protocols may be employed. The improved ligand may be administered by various routes, including parenterally or orally. The number of administrations will depend upon the factors described above. The improved ligand may be taken orally as a pill, powder, or dispersion; bucally; sublingually; injected intravascularly, intraperitoneally, intramuscularly, subcutaneously; by inhalation, or the like. The improved ligand (and monomeric antagonist compound) may be formulated using conventional methods and materials well known in the art for the various routes of administration. The precise dose and particular method of administration will depend upon the above factors and be determined by the attending physician or human or animal healthcare provider. For the most part, the manner of administration will be determined empirically.

In the event that transcription activation by the improved ligand is to be reversed or terminated, a monomeric compound which can compete with the improved ligand may be administered. Thus, in the case of an adverse reaction or the desire to terminate the therapeutic effect, an antagonist to the dimerizing agent can be administered in any convenient way, particularly intravascularly, if a rapid reversal is desired. Alternatively, one may provide for the presence of an inactivation domain (or transcription silencer) with a ligand binding domain. In another approach, cells may be eliminated through apoptosis via signalling through Fas or TNF receptor as described elsewhere. See International Patent Applications PCT/US94/01617 and PCT/US94/08008.

The particular dosage of the improved ligand for any application may be determined in accordance with the procedures used for therapeutic dosage monitoring, where maintenance of a particular level of expression is desired over an extended period of times, for example, greater than about two weeks, or where there is repetitive therapy, with individual or repeated doses of improved ligand over short periods of time, with extended intervals, for example, two weeks or more. A dose of the improved ligand within a predetermined range would be given and monitored for response, so as to obtain a time-expression level relationship, as well as observing therapeutic response. Depending on the levels observed during the time period and

the therapeutic response, one could provide a larger or smaller dose the next time, following the response. This process would be iteratively repeated until one obtained a dosage within the therapeutic range. Where the improved ligand is chronically administered, once the maintenance dosage of the improved ligand is determined, one could then do assays at extended intervals to be assured that the cellular system is providing the appropriate response and level of the expression product.

It should be appreciated that the system is subject to many variables, such as the cellular response to the improved ligand, the efficiency of expression and, as appropriate, the level of secretion, the activity of the expression product, the particular need of the patient, which may vary with time and circumstances, the rate of loss of the cellular activity as a result of loss of cells or expression activity of individual cells, and the like.

2. Production of recombinant proteins and viruses. Production of recombinant therapeutic proteins for commercial and investigational purposes is often achieved through the use of mammalian cell lines engineered to express the protein at high level. The use of mammalian cells, rather than bacteria or yeast, is indicated where the proper function of the protein requires post-translational modifications not generally performed by heterologous cells. Examples of proteins produced commercially this way include erythropoietin, tissue plasminogen activator, clotting factors such as Factor VIII:c, antibodies, etc. The cost of producing proteins in this fashion is directly related to the level of expression achieved in the engineered cells. A second limitation on the production of such proteins is toxicity to the host cell: Protein expression may prevent cells from growing to high density, sharply reducing production levels. Therefore, the ability to tightly control protein expression, as described for regulated gene therapy, permits cells to be grown to high density in the absence of protein production. Only after an optimum cell density is reached, is expression of the gene activated and the protein product subsequently harvested.

A similar problem is encountered in the construction and use of "packaging lines" for the production of recombinant viruses for commercial (e.g., gene therapy) and experimental use. These cell lines are engineered to produce viral proteins required for the assembly of infectious viral particles harboring defective recombinant genomes. Viral vectors that are dependent on such packaging lines include retrovirus, adenovirus, and adeno-associated virus. In the latter case, the titer of the virus stock obtained from a packaging line is directly related to the level of production of the viral rep and core proteins. But these proteins are highly toxic to the host cells. Therefore, it

has proven difficult to generate high-titer recombinant AAV viruses. This invention provides a solution to this problem, by allowing the construction of packaging lines in which the rep and core genes are placed under the control of regulatable transcription factors of the design described here. The packaging cell line can be grown to high density, infected with helper virus, and transfected with the recombinant viral genome. Then, expression of the viral proteins encoded by the packaging cells is induced by the addition of dimerizing agent to allow the production of virus at high titer.

3. Biological research. This invention is applicable to a wide range of biological experiments in which precise control over a target gene is desired. These include: (1) expression of a protein or RNA of interest for biochemical purification; (2) regulated expression of a protein or RNA of interest in tissue culture cells (or in vivo, via engineered cells) for the purposes of evaluating its biological function; (3) regulated expression of a protein or RNA of interest in transgenic animals for the purposes of evaluating its biological function; (4) regulating the expression of a gene encoding another regulatory protein, ribozyme or antisense molecule that acts on an endogenous gene for the purposes of evaluating the biological function of that gene. Transgenic animal models and other applications in which the components of this invention may be adapted include those disclosed in PCT/US95/10591.

This invention further provides kits useful for the foregoing applications. Such kits contain DNA constructs encoding and capable of directing the expression of fusion proteins of this invention (and may contain additional domains as discussed above) and, in embodiments involving regulated gene transcription, a target gene construct containing a target gene linked to one or more transcriptioal control elements which are activated by the multimerization of the fusion proteins. Alternatively, the target gene construct may contain a cloning site for insertion of a desired target gene by the practitioner. Such kits may also contain a sample of a dimerizing agent capable of dimerizing the two recombinant proteins and activating transcription of the target gene.

Formulations, dosage and administration

By virtue of its capacity to promote protein-protein interactions, a ligand of this invention may be used in pharmaceutical compositions and methods for promoting formation of complexes of fusion proteins of this invention in a human or non-human mammal containing genetically engineered cells of this invention.

The preferred method of such treatment or prevention is by administering to the mammal an effective amount of the compound to promote measurable formation of such complexes in the engineered cells, or preferably, to promote measurable actuation of the desired biological event triggered by such complexation, e.g.
5 transcription of a target gene, apoptosis of engineered cells, etc.

Therapeutic/Prophylactic Administration & Pharmaceutical Compositions

The ligands can exist in free form or, where appropriate, in salt form. Pharmaceutically acceptable salts of many types of compounds and their preparation
10 are well-known to those of skill in the art. The pharmaceutically acceptable salts of compounds of this invention include the conventional non-toxic salts or the quaternary ammonium salts of such compounds which are formed, for example, from inorganic or organic acids of bases.

The compounds of the invention may form hydrates or solvates. It is known to
15 those of skill in the art that charged compounds form hydrated species when lyophilized with water, or form solvated species when concentrated in a solution with an appropriate organic solvent.

This invention also relates to pharmaceutical compositions comprising a therapeutically (or prophylactically) effective amount of the compound, and one or
20 more pharmaceutically acceptable carriers and/or other excipients. Carriers include e.g. saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof, and are discussed in greater detail below. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule,
25 sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc*. Formulation may involve mixing, granulating and compressing or
30 dissolving the ingredients as appropriate to the desired preparation.

The pharmaceutical carrier employed may be, for example, either a solid or liquid.

Illustrative solid carrier include lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid and the like. A solid carrier can
35 include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or

tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions ,and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins.

Illustrative liquid carriers include syrup, peanut oil, olive oil, water, etc. Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant. Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by, for example, intramuscular, intraperitoneal or subcutaneous injection. Sterile solutions can also be administered intravenously. The compound can also be administered orally either in liquid or solid composition form.

The carrier or excipient may include time delay material well known to the art, such as glyceryl monostearate or glyceryl distearate along or with a wax, ethylcellulose, hydroxypropylmethylcellulose, methylmethacrylate and the like. When formulated for oral administration, 0.01% Tween 80 in PHOSAL PG-50 (phospholipid concentrate with 1,2-propylene glycol, A. Nattermann & Cie. GmbH) has been recognized as providing an acceptable oral formulation for other compounds, and may be adapted to formulations for various compounds of this invention.

A wide variety of pharmaceutical forms can be employed. If a solid carrier is used, the preparation can be tableted, placed in a hard gelatin capsule in powder or pellet form or in the form of a troche or lozenge. The amount of solid carrier will vary widely but preferably will be from about 25 mg to about 1 g. If a liquid carrier is used, the preparation will be in the form of a syrup, emulsion, soft gelatin capsule, sterile injectable solution or suspension in an ampule or vial or nonaqueous liquid suspension.

To obtain a stable water soluble dosage form, a pharmaceutically acceptable salt of the multimerizer may be dissolved in an aqueous solution of an organic or inorganic acid, such as a 0.3M solution of succinic acid or citric acid. Alternatively, acidic derivatives can be dissolved in suitable basic solutions. If a soluble salt form is not available, the compound is dissolved in a suitable cosolvent or combinations thereof. Examples of such suitable cosolvents include, but are not limited to, alcohol, propylene glycol, polyethylene glycol 300, polysorbate 80, glycerin, polyoxyethylated fatty acids, fatty alcohols or glycerin hydroxy fatty acids esters and the like in concentrations ranging from 0-60% of the total volume.

Various delivery systems are known and can be used to administer the multimerizer, or the various formulations thereof, including tablets, capsules, injectable solutions, encapsulation in liposomes, microparticles, microcapsules, *etc.* Methods of introduction include but are not limited to dermal, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, pulmonary, epidural, ocular and (as is usually preferred) oral routes. The compound may be administered by any convenient or otherwise appropriate route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents. Administration can be systemic or local. For treatment or prophylaxis of nasal, bronchial or pulmonary conditions, preferred routes of administration are oral, nasal or via a bronchial aerosol or nebulizer.

In certain embodiments, it may be desirable to administer the compound locally to an area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, by injection, by means of a catheter, by means of a suppository, or by means of a skin patch or implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In a specific embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous

administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the side of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

Administration to an individual of an effective amount of the compound can also be accomplished topically by administering the compound(s) directly to the affected area of the skin of the individual. For this purpose, the compound is administered or applied in a composition including a pharmacologically acceptable topical carrier, such as a gel, an ointment, a lotion, or a cream, which includes, without limitation, such carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oils.

Other topical carriers include liquid petroleum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylene monolaurate (5%) in water, or sodium lauryl sulfate (5%) in water. Other materials such as anti-oxidants, humectants, viscosity stabilizers, and similar agents may be added as necessary. Percutaneous penetration enhancers such as Azone may also be included.

In addition, in certain instances, it is expected that the compound may be disposed within devices placed upon, in, or under the skin. Such devices include patches, implants, and injections which release the compound into the skin, by either passive or active release mechanisms.

Materials and methods for producing the various formulations are well known in the art and may be adapted for practicing the subject invention. See *e.g.* US Patent Nos. 5,182,293 and 4,837,311 (tablets, capsules and other oral formulations as well as intravenous formulations) and European Patent Application Publication Nos. 0 649 659 (published April 26, 1995; illustrative formulation for IV administration) and 0 648 494 (published April 19, 1995; illustrative formulation for oral administration).

The effective dose of the compound will typically be in the range of about 0.01 to about 50 mg/kgs, preferably about 0.1 to about 10 mg/kg of mammalian body weight, administered in single or multiple doses. Generally, the compound may be

administered to patients in need of such treatment in a daily dose range of about 1 to about 2000 mg per patient.

The amount of compound which will be effective in the treatment or prevention of a particular disorder or condition will depend in part on the characteristics of the fusion proteins to be multimerized, the characteristics and location of the genetically engineered cells, and on the nature of the disorder or condition, which can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. The precise dosage level should be determined by the attending physician or other health care provider and will depend upon well known factors, including route of administration, and the age, body weight, sex and general health of the individual; the nature, severity and clinical stage of the disease; the use (or not) of concomitant therapies; and the nature and extent of genetic engineering of cells in the patient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers containing one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. The notice or package insert may contain instructions for use of an improved ligand of this invention, consistent with the disclosure herein.

Examples

Example 1: Construction of human CAB fusion proteins

5 All constructs were made in the pcDNA3/m.jy2 vector which was derived from the pcDNA3 vector by mutation of backbone SalI sites and insertion of a new polylinker region. (B. Stockwell, J. Yang). The plasmid contains an ampicillin resistance gene which allows for selection in E. coli and a cytomegalovirus promoter which allows for high plasmid gene expression. The pcDNA3/m.jy2 plasmid was
10 chosen instead of PBJ5, a plasmid used in many of the other dimerization systems, because its restriction digest profile eased the cloning of the calcineurin constructs.

Polymerase Chain Reaction

Primers were designed for the 3′ and 5′ ends of all calcineurin constructs used
15 in this project. The primers integrated specific restriction sites into the ends of each gene to facilitate ligation of the gene into the pcDNA3/m.jy2 vector. The primers were synthesized on a Perkin Elmer Applied Biosystems 394 DNA/RNA Synthesizer Machine and purified using a Perkin Elmer Purification Protocol.

PCR Reactions were carried out on an M5 Research Mini-Cycler. The general
20 PCR protocol included incubation at 95ßC for 5 minutes. 30 seconds were then allowed for annealing at 55ßC. This was followed by extension for one minute at 72ßC and then a one minute incubation at 95ßC. The annealing, extension and incubation steps were repeated 25-30 times. The resulting reaction mixture was stored at 4ßC until further work up. PCR products were purified using a Qiagen PCR
25 Purification Kit and the protocol therein.

Oligos for construction of calcineurin A fragments:

CNA sequence is in bold

30

ï hCNA 5' PCR Oligo Start at residue 12

___Junk___ _Xho1_

5' cggg ccc ccc ctc gag **tct acg acc gac agg gtg gtg aaa gc** 3'

35

Note: g->t is a silent mutation that destroys the Sal1 site.

ï hCNA 5' PCR Oligo Start at residue 340

5

Junk Xho1

5' atat aaa tcg ctc gag **cca tac tgg ctt cca aat ttc atg g** 3'

10

ï hCNA 5' PCR Oligo Start at Residue 350

Junk Xho1

5' atat aaa tcg ctc gag **ttt act tgg tcc ctt cca ttt gtt ggg g** 3'

15

ï hCNA 3' PCR Oligo End at Residue 370

20 Junk See Note ApaI Junk SalI

5' cca gta ggg tct aga tct ggg ccc acg ata taa gtc gac **gtt gag gac**

att tac cag c 3'

25 Note: tct aga tct = overlapping XbaI and BglII sites.

ï hCNA 3' PCR Oligo End at Residue 394

30

See Note STP FLAG Peptide SalI

5' ttaa tct aga tct tca ctt gtc atc gtc atc ttt ata gtc gac **ctc**

ttt ccg ggc tgc agc tg 3'

35

Note: tct aga tct = overlapping XbaI and BglII sites.

Oligos Designed for Human calcineurin B:

(**Bold** portion is CNB sequence)

5

ï hCNB 5' PCR Oligo Start at residue 2

10 Junk XhoI

5' atat aaa tcg ctc gag **gga aat gag gca agt tat cct ttg g** 3'

15 ï hCNB 5' PCR Oligo Start at residue 3

Junk XhoI

5' atat aaa tcg ctc gag **aat gag gca agt tat cct ttg g** 3'

20

ï hCNB 3' PCR Oligo with 3' FLAG peptide and Stop

See Note ApaI STP FLAG Peptide

25 5' ttaa tct aga tct ggg ccc tca ctt gtc atc gtc atc ttt ata

SalI

gtc gac **cac atc tac cac cat c** 3'

30 Note: tct aga tct = overlapping XbaI and BglII sites.

Oligos Designed for Constructing CNA-CNB Linkers:

(**Bold** portion is CNA sequence)

35

ï 3' PCR Primer for hCNA to Generate 24 Amino Acid Template Linker (to residue 370)

5 Junk ApaI See Note Linker

5' cga ttt atat ggg ccc tct aga tct aga acc aga acc aga acc aga

Linker

acc aga acc aga acc aga acc aga acc aga acc acc aga acc aga acc

10

acc **gtt gag gac att tac cag c** 3'

Note: tct aga tct = overlapping XbaI and BglII sites.

15

ï 3' PCR Primer for Randomizing the Length of the CNA-CNB Linker (Register 1 oligo)

20

Junk See Note ApaI Junk SalI

5' g aat cgc aaa tct aga tct ggg ccc gtc atc ttt ata gtc gac acc

aga acc aga acc 3'

25

Note: tct aga tct = overlapping XbaI and BglII sites.

30 ï 3' PCR Primer for Randomizing the Length of the CNA-CNB Linker (Register 2 oligo)

Junk See Note ApaI Junk SalI

5' g aat cgc aaa tct aga tct ggg ccc gtc atc ttt ata gtc gac aga

35

acc aga acc aga 3'

Note: tct aga tct = overlapping XbaI and BglII sites.

PCR Conditions (for all CNA and CNB fragments):

Step	Temperature (∞C)	Time (sec)
1.	95	180
2.	37	30
3.	72	60
4.	95	60
5.	37	30
6.	72	60
7.	Goto Step 4 9 times	
8.	95	60
9.	55	30
10.	72	60
11.	Goto Step 8 25 times	
12.	72	300
13.	4	

PCR Conditions (for randomization of Linker Length):

Step	Temperature (∞C)	Time (sec)
1.	95	180
2.	37	30
3.	72	60
4.	95	60
5.	45	30
6.	72	60
7.	Goto Step 4 9 times	
8.	95	60
9.	55	30
10.	72	60
11.	Goto step 8 25 times	

12.	72	300
13	4	

5 **Example 2: Method of Linking Calcineurin A to Calcineurin B**

The method of linking calcineurin A to calcineurin B in the CABS falls into two categories. The first method used fragments of the gene encoding calcineurin A (residues 12-370, 12-394, 340-370, 340-394, 350-370, or 350-394) generated by PCR to contain a 5' Xho1 restriction site directly before the codons for residues 12, 340, or 350
10 and a 3' Sal1 restriction site directly following the codons for residues 370 or 394. The fragments of the gene encoding calcineurin B (residues 2-170 or 3-170) were also generated by PCR to contain a 5' Xho1 restriction site directly before the codons for residues 2 or 3 and a 3' Sal1 restriction site directly following the codon for residue 170. Ligation of the two fragments were performed such that the fragment of
15 calcineurin A is 5' to calcineurin B and the site of ligation is the result of a Sal1/Xho1 fusion (gtcgag). This results in two additional codons encoding a Valine and a Glutamate between the calcineurin A portion of the CABS and the calcineurin B portion of these CABS.

The second method of linking the CABS was to generate a pool of calcineurin A
20 fragments with linkers, ranging in size from 6 to 24 amino acids, attached directly after residue 370. These fragments were PCR'ed such that they also contained a 5' Xho1 restriction site directly before the codons for residues 12, 340, 350 and a 3' Sal1 restriction site directly following the codons encoding the flexible linker. The calcineurin B fragments were the same as those described in the previous paragraph.
25 The ligation was performed such that the fragment of calcineurin A is 5' to calcineurin B and the site of ligation is the result of a Sal1/Xho1 fusion (gtcgag). This results in two additional codons encoding a Valine and a Glutamate between the calcineurin A-linker portion of the CABS and the calcineurin B portion of these CABS.

Generation of the variously lengthed flexible linkers on calcineurin A was
30 accomplished through a two step PCR procedure developed for this purpose. The following bases were added after the codon for residue 370 of calcineurin A by PCR:

CNA residue 370-

35 GGTGGTTCTGGTTCTGGTGGTTCTGGTTCTGGTTCTGGTTCTGG
TTCTGGTTCTGGTTCTGGTTCTGGTTCT

This encodes for the following flexible longest length linker:

GGSGSGGSGSGSGSGSGSGSGSGS

PCR was then performed on the above template with two primers that contained the following complimentary sequence:

Primer 1:

5' GTC GAC AGA ACC AGA ACC AGA 3'

Primer 2:

5' GTC GAC ACC AGA ACC AGA ACC 3'

and a Sal 1 restriction site (gtc gac). Upon PCR with both primers that can anneal in many registers of the template calcineurin A, fragments of calcineurin A containing from 7 to 24 amino acids of the flexible linker were generated. Interestingly, all of the fragments contained the amino acids GGSGS followed by the appropriate number of single alternating Glycines and Serines. The predicted PCR products should have two GGSGS repeats, but we recovered only one in all of the clones. Moreover, this strategy also provided fragments that had longer linkers than what we had predicted because the second PCR step allows the linker to grow.

Example 3: Construction of FKBP and CAB fusion proteins

All CNA constructs were derived from human calcineurin A (a isoform). All CNB constructs were derived from human calcineurin B. CNB_M contains an N-terminal methionine, while CNB_{MG} contains both a methionine and a glycine residue. Residues 1-147 from GAL4 are used for the GAL4 DNA binding domain (GE). Residues 413-490 from VP16 are used as the VP16 transactivation domain (VE). Each VE construct contains an N-terminal nuclear localization sequence (NLS) from SV40 large T antigen, while all GE constructs contain an NLS within the GAL4 sequence. All GE constructs contain a FLAG epitope tag and all VE constructs contain a FLU epitope tag. All FKBP constructs contain the entirety of the human coding sequence. The FRB construct contains residues 2025-2114 of the human FRAP protein. The UAS-SEAP reporter gene contains 5 upstream Gal4-binding sites (UAS sites) and a minimal interleukin 2 basic promoter and encodes SEcreted Alkaline Phosphatase, a heat-stable alkaline phosphatase.

The same basic digestion, ligation and transformation procedure was used to obtain each construct. Calcineurin A fragments were put into the pcDNA3/m.jy2 vector by digesting the PCR product with XhoI and XbaI and ligating into vector that had been cut with the same enzymes. CNB was ligated into the vector using XhoI and

ApaI restriction sites. FKBP3-VE and FKBP3-GE were ligated from PBJ5 into the pcDNA3/m.jy2 vector using the SacII and EcoRI enzymes. These basic parent vectors allowed for the generation of all future constructs since calcineurin A and calcineurin B fragments could be liberated by digestion with XhoI and SalI or XhoI and ApaI while VE could be liberated with SalI and ApaI. In addition, GE, CNA or CNB containing vectors could be created by digestion with SalI and ApaI. Since XhoI and SalI generate complementary sticky ends, all the calcineurin constructs used in this project could be created from different combinations of the digests described above and from digests of successive generations of constructs.

After vector and insert DNAs were digested with the appropriate restriction enzymes, the products were then run on either a 1% or 2% agarose gel (1% was used for all products greater than 300 basepairs). The desired DNA bands were then isolated using a Qiagen Gel Purification Kit and the protocol therein. Vector and insert bands were ligated from 4 hours to overnight at 16ßC in an 11ml volume with 1 ml T4 DNA ligase and 1 ml NEB ligation buffer and a 2:1 (or greater) ratio of insert to vector. The ligation reaction was then transformed into subcloning efficiency DH5a bacterial cells obtained from GIBCO BRL according to the protocol supplied with the cells. For certain inefficient ligations, such as SalI/ApaI ligations, maximum efficiency DH5a cells were used. Positive transformants were selected for using ampicillin. A few positive colonies from each ligation were chosen for further analysis. The Wizard Mini-Prep Kit and protocol therein were used to isolate plasmid DNA from these positive colonies. The DNA was subjected to restriction digest analysis to screen for false positive clones. DNA from true positive colonies was prepared using a Wizard Midi-Prep Kit and protocol therein. The prepared DNA was quantitated using the relation:

[double stranded DNA] = (50 mg/ml)*(A260)

where A260 equals the absorbance of the DNA sample at 260 nm wavelength light.

Example 4: Reporter- based Assay for Activation of Transcription:

The transcription assay that we utilize has been described in WO94/18317 and in Rivera et al., 1996 supra. The general assay is as follows.

10 million T-antigen transformed Jurkat T-cells growing in log phase at 37 degrees with 5% CO2 in RPMI medium supplemented with 10% FBS, Penicillin and Streptomycin, and Glutamate are centrifuged for 5 minutes at 1000xg. This pellet is washed with RPMI without phenol-red and re-pelleted by centrifugation at 1000xg for 5 minutes. 200 microliters of phenol-free RPMI are used to resuspend each 10 million

cells. 200 microliters of these cells are then incubated for 10 minutes at room temperature with 1 microgram of the UAS-SEAP reporter, 1 microgram of the DNA binding domain containing construct, and 5 micrograms of the activation domain containing construct. Electroporation is performed at 250 mV, 129 Ohms on a BTX electroporator. The cells are allowed to recover at room temperature for another 10 minutes. They are then resuspended in 10 mLs of RPMI w/o phenol red supplemented with 10% FBS, penicillin, streptomycin, and Glutamate and returned to the 37 degree incubator with 5% CO2 for 24 hrs.

After recovery, the cells are pelleted by centrifugation at 1000xg for 5 minutes and resuspended in 5 mLs of RPMI w/o phenol red supplemented with 10% FBS, penecillin, streptomycin, and Glutamate. 100 microliters of this cell suspension is plated on a 96 well plate. Appropriate dilutions of the dimerizer from an ethanolic solution (2 micromolar to 0.2 nanomolar) are made in the same media that the cells were resuspended in such that the concentration of ethanol is less than 1%. 100 microliters of these dilutions in media are added to the wells containing cells in the 96 well plate. The cells are again placed in the 37% incubator with 5% CO2 for 24-48 hrs.

After incubation with the dimerizer the 96 well plates containing cells are wrapped in Saran Wrap and heated to 65-75 degrees C for two hours. After heat treatment, the plates are placed at room temperature until cool. 100 microliters of each of the wells was transferred to a new plate containing 100 microliters of the following solution. For each 96 well plate prepare 11mLs of 2M diethanolamine pH 10 (with CO2) with 132 microliters of a solution of 1 mL 2M diethanolamine pH 10 (with CO2) containing 25.6 milligrams of 4-MethylumbuliferylPhosphate (MUP). These plates are then returned to 37 degrees for 1-24 hours and are read on a microplate reader using a standard FITC filter set. The maximal reading should not exceed 1-2000 as this indicates that the substrate is nearly used up.

Example 5: Activation of Transcription using CAB fusion proteins

The CABS have been extensively tested in a three-hybrid-like transcription assay as described in Spencer et al, 1993, Science 262:1019-1024, in WO 94/18317, in Rivera et al.,1996, Nature Medicine 2, 1028-1032 and in WO 96/41865 (Clackson et al). As described above, multimeric CABS, single full length or mini CABS or portions of the CABS have been fused C-terminally to the Gal4-DNA binding domain and placed between an N-terminal NLS and a C-terminal VP16 activation domain. Three copies of FKBP act as the other dimerization domain and are fused to either the C-

terminus of Gal4 or between an N terminal NLS and the VP16 activation domain. The reporter contains the upstream activating sequence of GAL4 (12 tandem copies) followed by the secreted alkaline phosphatase gene (SEAP). FK506 titration was used to elicit dimerizer dependent transcription activation of SEAP which was
5 detected on a fluorescence plate reader using methylumbyleriferyl-phosphate (MUP) as a substrate. The vector used for expression is a version of pCDNA3 from Pharmacia Biotech that has had its Xho1 and Sal1 restriction sites destroyed and a new polylinker inserted for ease of cloning. The cells used in these experiments are T-antigen transformed Jurkat T-cells.

10 The summary of our data in this transcription assay is as follows. The full length CABS (residues 12-394 of calcineurin A fused to residue 2 or to residue 3 of Calcinuerin B) are functional when fused to VP16 or to GAL4. They have an EC50 of ~ 1 nanoMolar.

We identified residues 340-370 of calcineurin A as being sufficient to form a
15 complex with calcineurin B; residues 350-370 fail to complex with calcineurin B. This is also reflected in the fusion proteins using the mini-CABS, which all function to varying degrees with respect to total level of activation but behave almost identically with respect to EC50.

The linker length does seem to be able to modulate the amount of activity elicited
20 by the CABS, but not the EC50s of the CABS, in the following way. The smallest linkers (7 or 8 amino acids) and the longest linkers (16-18 amino acids) tested appear to be the best with a total activation of around 8-10 fold and are comparable to the direct fusion between residues 340-394 of calcineurin A and residues 2 or 3-170 of calcineurin B.

25 The two versions of calcineurin B differ slightly in two respects. Calcineurin B from residues 3-370 seems to give a higher overall activation by about 10 % but also seem to have a slightly higher background association with "free" calcineurin B and calcineurin A. Calcineurin B from residues 2-370 has a slightly lower overall activation but seems to interact less with free calcineurin A or "free" calcineurin B. It
30 should be noted that this interaction with "free" calcineurin A or calcineurin B is extremely minimal. The average EC50 for activation for all of these constructs is between 1 and 3 nanoMolar.

The multimers of minicabs (340-394 of CNA and 3-170 of CNB) have been tested as fusions to VP16. 3, and 4 tandem CABS function identically to the best single
35 CABS mentioned above when recruited to Gal4 fused to 3 FKBPs. 2 tandem CABS

function twice as well with regard to total activation (~20 fold) and have an EC50 that is shifted to between 0.1 and 0.3 nanoMolar with FK506.

Example 6: Creation and Testing of miniCAB Proteins

5 Our analysis suggested that the N-terminal region of the CNA domain of CAB could be removed and the resulting protein could still be expected to bind FK506-FKBP. Four such minimal CAB proteins were created by PCR and ligation. Two of the miniCABs contained CNA residues 340-394 fused to either CNB_{MG} or CNB_M (340miniCABs). The other two miniCABs contained CNA residues 350-394
10 fused to either CNB_{MG} or CNB_M (350miniCABs). Each of these miniCABs was also fused to the VP16 transactivation domain.

Both of the 340miniCAB-VE constructs were able to stimulate SEAP activity when cotransfected with $FKBP_3$-GE in an FK506-dependent manner under standard SEAP assay conditions. Consistent with the relative activity of CNB_{MG} and CNB_M,
15 340miniCAB$_{MG}$-VE was able to induce higher SEAP activity than 340miniCAB$_M$-VE. In contrast, neither of the 350miniCABs were able to stimulate SEAP activity in similar experiments. These results indicate that residues 340-394 of CNA represent a minimal domain of CNA that is able to form functional interactions with both CNB and FKBP-FK506 at the resolution detected by the SEAP assay.
20 The 340miniCAB$_{MG}$-VE construct also stimulates higher SEAP activity (6.5 0.6 fold) than the corresponding full length CAB_{MG}-VE (4.0 0.4 fold). The finding that the miniCABs produce higher SEAP activation than the full CABs might be attributable to the smaller miniCAB proteins being more stable or more efficiently translated or folded than the larger full length CAB proteins. The EC_{50} of the
25 miniCABs is about 3 nM, an order of magnitude greater than for full length CAB_{MG}-VE. This result is understandable given the fact that the full length CAB-VE is able to make more binding contacts with FK506-FKBP than the 340miniCABs

As with the full length CABs, assays were done to study whether the miniCABs were working through an inter- or intra- molecular mechanism. Like the
30 full length CABs, the 340miniCAB-VEs showed only slight interaction with CNA-GE. In addition, both 340miniCAB-VE's showed only slight interaction with CNB-GE. This is in contrast to full CAB_{MG}-VE which shows a high level of interaction with CNB-GE. Perhaps the less bulky CNA domain of the 340miniCAB-VE is less efficient in finding intermolecular binding partners than the

larger CNA domain of the full CAB. These are promising results for the use of miniCABs as dimerization domains since they imply that both miniCAB-VE proteins, unlike full CAB_{MG}-VE, do not participate in unwanted intermolecular interactions which could lower the efficiency of the miniCAB proteins or cause them
5 to interfere with the functioning of other proteins. Since the miniCABs do not seem to participate significantly in intermolecular interactions, this implies that the CNA and CNB domains of these proteins work together, and not independently in an intramolecular fashion, to form an FK506-FKBP binding site.

The results obtained imply that the 340miniCAB-VEs function as efficient
10 dimerization domains in an intramolecular fashion, as designed. In addition, the 340miniCAB-VEs lack the catalytic domain of the full length CABs and, at 24 kDa, are approximately 35 kDa smaller.

Improving the miniCAB System by Placing Multiple Dimerization Domains in
15 *Series*

In previous dimerization systems, it had been observed that multiple dimerization domains fused to both the GAL4 DNA binding domain and the VP16 transactivation domain could increase the maximal amount of SEAP activity induced by the CID. For example, it was found that three FKBP molecules fused in
20 tandem led to higher SEAP activity than did one, two or four FKBP molecules in series (Belshaw, PJ. et al. "Controlling Protein Association and Subcellular Localization with a Synthetic Ligand that Induces Heterodimerization of Proteins". PNAS. 1996. 93:4604-4607.) This is why $FKBP_3$ constructs have been used for all the experiments above. The optimal number of dimerization domains in tandem may be
25 different for each domain. Multiple dimerization domains in series in a protein would increase the number of ligand binding sites on that protein. This would increase the effective affinity of a ligand for that protein. Too many dimerization domains in series would add excess bulk to the fusion protein and cause it to have unfavorable steric interactions with its environment. The balance between these
30 factors determines the ideal number of dimerization domains to put in series for a particular construct.

In an effort to improve the miniCAB dimerization system, constructs with one, two, three or four 340miniCABs in series fused to VE were created through ligations of existing constructs (called $(340miniCAB)_n$-VE where n = 1,2,3,4). There was a 2
35 residue linker between each 340miniCAB subunit due to the restriction profile of the miniCAB constructs. The multiple 340miniCAB constructs were only made with

CNB_{MG} containing miniCABs since these gave the best signal in the dimerization assay.

Figure 4 shows that two 340miniCAB domains in series give rise to the highest SEAP activity (9.9 0.4 fold) in the presence of FK506 when cotransfected with
5 $FKBP_3$-GE. One and three 340miniCABs in series result in similar levels of SEAP activity while four miniCABs give a very low signal. The EC_{50} of two and three miniCABs in series is similar with a value of approximately 1.3 nM and is lower than that for one miniCAB. Like 340miniCAB$_{MG}$-VE, (340miniCAB$_{MG}$)$_2$-VE shows little intermolecular association with CNA-GE or CNB_{MG}-GE, thereby implying that the
10 activity of this construct is due to its CNA and CNB domains working together in an intramolecular fashion.

Currently, the (340miniCAB$_{MG}$)$_2$ domain represents the most optimized CAB dimerization reagent.

15 <u>Example 7: Implementing CAB Mediated Dimerization in Contexts Other than CAB-VE</u>

In order to show that CAB truly represents a useful new dimerization domain, it must function in the context of activation domains other than VP16. A simple test of CAB versatility would be to create a new transcription activation system in which
20 CAB-GE could be shown to heterodimerize with FKBP-VE. This is the same as the original CAB-VE system except that the activation domains to which CAB and FKBP are fused have been swapped. To implement the new system, six CAB-GE constructs were made. Two of these were full length CABs with either CNB_{MG} or CNB_M. The other four were 340miniCABs and 350miniCABs with either form of
25 CNB. In order to test these proteins, an FKBP-VE fusion protein was needed. As with $FKBP_3$-GE, $FKBP_3$-VE was already available in a PBJ5 vector and was easily transferred to the pcDNA3/m.jy2 vector used for all constructs in this project.

We found that the CAB-GE's work similarly to the CAB-VE constructs. However, the full length CAB_{MG}-GE protein only enhance SEAP activity 2.0 fold
30 whereas the full length CAB_{MG}-VE protein could enhance it by approximately 4 fold. Both full CAB-GE and CAB-VE are able to stimulate SEAP activity when heterodimerized to $FKBP_3$-GE, demonstrating the versatility of the full CAB domain. In addition, they do not stimulate activity in the absence of FK506. This result implies that the presence of an intact phosphatase active site on the full length CABs

is not responsible for enhanced SEAP activity, a concern raised initially by the constitutive association experiments between CNA and CNB described above.

The 340miniCAB-GE constructs enhance SEAP activity to the same level as the 340miniCAB-VE constructs. No difference was seen between 340miniCAB$_{MG}$-GE
5 and 340miniCAB$_{M}$-GE. Like 340miniCAB-VE, 340miniCAB$_{MG}$-GE does not show significant constitutive activity with CNA or CNB and thus, seems to work through an intramolecular mechanism as opposed to an intermolecular mechanism.

Example 8: Probing the Difference between full length CAB-GE and
10 **miniCAB-GE**

Further experiments were done to explore why the full length CAB$_{MG}$-GE construct was not working as well as its 340miniCAB$_{MG}$-GE counterpart (a trend also observed with the VE CAB proteins). The normal SEAP assay protocol calls for transfecting a 1:5 ratio of GE construct DNA to VE construct DNA. This ratio has
15 been empirically determined to give the best results for the FK1012 and rapamycin dimerization systems. Presumably, the ratio of transfected DNA affects the ratio of translated GE and VE containing proteins. A series of SEAP assays were performed with full CAB$_{MG}$-GE in which the ratio of transfected GE DNA to VE DNA was changed from 1:5 (normal) to 1:1 to 5:1 while keeping the total amount of transfected
20 DNA constant. As the amount of full CAB$_{MG}$-GE DNA was increased the enhancement of SEAP activity increased as well.

A similar series of experiments was performed for 340miniCAB$_{MG}$-GE, but changes in the GE DNA to VE DNA transfection ratio did not affect maximum induced SEAP activity as significantly as for the full CAB$_{MG}$-GE construct.
25 Interestingly, in the 5:1 GE DNA to VE DNA experiment, the maximum amount of SEAP activity induced by full length CAB$_{MG}$-GE was equal to that produced by 340miniCAB$_{MG}$-GE. Even though protein expression levels were never directly examined, these results suggest that greater expression of full CAB$_{MG}$-GE, assuming more transfected DNA leads to higher levels of expression, increases its
30 performance as a dimerization domain up to the level achieved by 340miniCAB$_{MG}$-GE. This finding supports the previously presented suggestion that miniCABs perform better than full CABs because their smaller size makes them more stable or more efficiently translated or folded than the larger full length CAB proteins.

Example 9: Synthesis of C40 bumped FK506

Extraction/Purification of FK506

Powder from PrografTM brand pharmaceutical tacrolimus capsules was suspended in water and FK506 was extracted using 3/7 volume of EtOAc and collected by rotary evaporation. Crude FK506 extract was purified by size exclusion chromatography using a Japan Analytical Industries LC- 908 recycling preparative HPLC equipped with tandem J14 and J11 9C13 columns (herafter referred to as "Sizing JAI"). The identity of this material was confirmed by TLC, FAB-MS, and proton NMR.

Synthesis of C40-phenyl-FK506

12mg of purified FK506 was sealed with a stir bar in a flame-dried 20ml roundbottom flask and the flask purged with argon. 12ml of CH2Cl2, freshly distilled over CaH2, was injected and stirred briefly to solubilize the FK506. 0.25mL of styrene (150 eq.) was injected, along with 2.2ml CHCl2 containing 3.6mg (0.3 eq) of the Grubbs catalyst, bis-tricyclohexylphosphine benzylidine ruthenium chloride. The final reaction concentration of FK506 was thus about 1 mM. This reaction mixture was stirred under argon for 24h at room temperature. Products and unreacted SM were collected by rotary evaporation, solubilized in ethyl acetate, and filtered through Celite. At this point, fresh solvent, styrene, and catalyst were added in the same proportions andthe reaction repeated for an additional 24h.

Purification of C40-phenyl-FK506

Following rotary evaporation and filtration as described above, the product/SM mixture was resuspended in CH2Cl2 and separated by flash chromatography using a stepwise gradient of a) CH2Cl2, b) 18:1:1 CH2Cl2: iPrOH:PhH, c) 12:1:1 CH2Cl2:iPrOH:PhH, and d) 9:2:2 CH2Cl2:iPrOH:PhH. Column fractions were analyzed by TLC and compared with standards to facilitate pooling into three major fractions which were analyzed by FAB-MS. Fraction I contained styrene and stilbene, fraction II contained FK506 and C40-phenyl-FK506, and fraction III contained FK1012 (the FK506 self-metathesis product). The components of fraction II were separated by two rounds of HPLC. First, the mixture was separated by affinity

HPLC using a Japan Analytical Industries LC-908 recycling preparative

HPLC equipped with two tandem JAIgel GS310 columns (hereafter referred to as "Affinity JAI"). Crude C40-phenyl-FK506 as well as crude recovered FK506 were obtained in this fashion. Second, each crude fraction from the Affinity JAI was purified to homogeneity by Sizing JAI as described above. The identity of each
5 purified compound was confirmed by FAB-MS and proton-NMR analysis.

Synthesis/Purification of other C40-derivatized FK506s

In each case, the synthetic route to preparing these derivatives is essentially that described above, using the appropriate terminal olefin compound (usually a
10 substituted styrene) instead of styrene itself. For example, p-phenoxystyrene was used in the synthesis of C40-p-phenyoxy-phenyl-FK506, and m-fluorostyrene was using in the synthesis of C40- m-fluorophenyl-FK506. The principal means of following reaction progress was FAB-MS, with emphasis on the appearance of a parent ion peak which corresponds to the desired product. During purification,
15 FAB-MS was also used to quickly identify the components of JAI fractions. In this case, however, emphasis was placed upon the absence of the parent ion peak corresponding to FK506. This focus is necessary since FK506 is competitive with each product in biological applications, so its absolute absence was considered more important than was maximizing product yields. In some cases, the sequence of JAI
20 purification steps were reversed (i.e. Sizing JAI first, then Affinity JAI), or more such steps were added, in pursuit of this goal.

Example 10: Construction of CAB/p65 fusion proteins

CAB/p65 fusion proteins were prepared in the pBJ5.2X vector, which contains
25 an SV40 origin of replication for high-copy number in mammalian cells transformed with the large tumor antigen. This version of pBJ5 does not contain a selection cassette for mammalian cells and contains the ampicillin-resistance cassette for selection in bacterial cells. This plasmid contains an SRa promoter for high-level expression in mammalian cells. The polylinker sequence of this plasmid consists of
30 the following elements (HA is the hemagglutinin epitope tag):

NotI/SacII—Kozak—ATG—XhoI—SalI—HA—SpeI—TAA

Primers containing restriction sites were prepared in order to amplify two different
35 fragments of calcineurin A, corresponding the the full-length CAB (fCAB) and the

minimal CAB (mCAB). These PCR fragments were subcloned directionally into the polylinker to generate the following constructs:

ATG—XhoI— CnA (12-394) —SalI—SpeI—TAA—EcoRI
5 **ATG—XhoI— CnA (340-394) —SalI—SpeI—TAA—EcoRI**

Primers containing restriction sites were prepared in order to amplify the fragment of calcineurin B corresponding to residues 3-170 of this protein. These PCR fragments were subcloned directionally into the constructs already containing calcineurin A
10 fragments, producing the following CAB constructs (S\X represents a fusion of complementart SalI and XhoI restriction sites which can no longer be cut by either enzyme):

ATG—XhoI—CnA (12-394)—(S\X)—Cn B (3-170)—SalI—HA—SpeI—TAA
15 **ATG—XhoI—CnA (340-394) —(S\X)—Cn B (3-170)—SalI—HA—SpeI—TAA**

To study the ability of the CABs to mediate transcriptional activation in the context of FKBP:FK506, a (XhoI/SpeI) fragment containing the transcriptional activation domain of the p65 subunit of NF-kB was inserted into (SalI/SpeI) digested mCAB
20 constructs. This fusion results in another (SalI/XhoI) fusion which cannot be cut by either enzyme. A similar strategy is possible to generate multimers of the CAB domain, greatly facilitating the production of these reagents. Since all of the restriction enzymes within the coding region are 6-base cutters, they preserve the reading frame for protein synthesis. The mature CAB should have the following
25 amino acid sequence:

NH_2-Met-Leu-Glu-(CnA frag)-Val-Glu-(CnB frag)-Val-Asp-Thr-Ser-COOH

New mCAB-p65 constructs were verified by sequence analysis.

30

Example 11: Demonstration of FK506-derivatives as "bumps" by calcineurin inhibition assay.

To demonstrate that the FK506 derivatives are less effective at binding
35 calcineurin than is FK506 itself, we performed reporter gene assays that are sensitive to the action of FK506 on calcineurin. In particular, we transfected human TAg

Jurkat T cells with a reporter construct, NFAT-SEAP, which consists of 12 copies of the NFAT response element fused upstream of the gene for secreted alkaline phosphatase (SEAP). Following transfection, the cells were exposed to phorbol myristate acetate (PMA) and ionomycin (IO), which together are able to activate
5 NFAT-SEAP reporter activity. FK506, by inhibiting calcineurin, antagonizes the effect of PMA+IO in a dose-dependent manner. Specifically, ten million Jurkat cells per condition were transfected with 500ng of NFAT-SEAP reporter construct by electroporation (40ms pulse) and aliquoted at 0.3×10^6/well into a 96-well microtiter plate. PMA (50ng/mL) and IO (1μM) were added to all cells, and serial dilutions of
10 FK506, FK506 derivative, or vehicle, were added. Cells were incubated 36 hours in a tissue culture incubator, then heat-inactivated for 2 hours at 65°C. Following heating, 100μL of cell supernatants were transferred to a replica plate containing 100μL of assay buffer (120μM 4-methylumbelliferyl phosphate in 2M diethanolamine bicarbonate, pH 10). Assays were incubated 8 hours before reading on a Fluoroskan plate reader. The
15 results of these experiments are shown in Figure 7.

We assumed that the reduced ability of the FK506 derivatives (as judged by their higher IC_{50} in this assay) to antagonize reporter gene activity is due to an impairment in the ability of the FKBP:ligand complex to bind calcineurin. Other possibilities for the reduced activity of derivatives in this assay are 1) compound
20 insolubility in tissue culture media, 2) inability of compound to penetrate the cell membrane, or 3) inability of compound to bind to FKBP. To rule out these other possibilities, we tested two of the derivatives for their ability to suppress a rapamycin-dependent effect in mink lung cells (Paul A Clemons and Brent Stockwell, unpublished observations). As both the phenyl and phenoxyphenyl derivatives were
25 able to suppress the effects of rapamycin in this context, we believe that each is soluble, cell-permeant, and able to bind FKBP.

Example 12: Effect of overexpression of full-length CABs (fCABs) on reporter gene activity.

30 In contrast to the assay described in the previous section, we wanted to test the ability of the bumped compounds to inhibit the phosphatase activity of the full length CABs (fCABs) upon transient transfection of these constructs. Initial experiments involved the co-transfection of 500-5000ng of fCAB construct DNA along with 500ng of NFAT-SEAP reporter gene. Figure 8 shows the results of these
35 experiments, in which a high level of fCAB overexpression overcame the ability of

FK506 or its derivatives to inhibit reporter gene activity. Also shown is a Western blot corresponding to each concentration of construct DNA transfected.

To tune the phosphatase assay in the context of transiently transfected fCABs, we performed SEAP assays as described above while varying the
5 concentration of fCAB construct DNA between 3.2ng and 320ng. The results of these experiments are shown in Figure 9.

Example 13: Optimization of CAB transcription system based on mCAB-p65 constructs.

10 In order to screen for mutants CABs which can restore binding to FK506 derivatives, we prepared fusions of the mCAB module to the N-terminus of the activation domain of p65, as described above. Our rationale for the use of this activation domain was that the original transcription construct, with the mCAB fused to VP16, gave low signal amplitudes in the transcription assay. We hoped
15 that changing activation domains would improve this signal and give a stable EC_{50} for transcriptional activation. Both of these are required for effective screening of pools of mutants using the transcription assay.

Briefly, we transfected human TAg Jurkat T cells with a a reporter construct (GRE-SEAP) containing 12 copies of the Gal4 response element upstream of the gene
20 for SEAP, along with Gal4-$(FKBP)_3$ and the mCAB-p65 fusion. FK506, a derivative, or vehicle were added to cells in a 96-well plate as with the NFAT-SEAP assay. Initial trials gave very promising results, so we undertook to optimize the transcription system by systematic variation of DNA concentrations for each of the three constructs. In three successive experiments, we found the following optimal
25 DNA concentrations for these transfections:

GRE-SEAP reporter gene	500ng
Gal4-$(FKBP)_3$ DB fusion	1500ng
mCAB-p65 AD fusion	500ng

30

Following optimization of DNA concentrations, we tested FK506 and two derivatives in the transcription assay. The results of this experiment are shown in Figure 10A. The amplitudes of activation in these experiments are sufficient to warrant an attempt to screen pools of CAB mutants for compensatory binding.
35 To address the stability of the dose response in these cells, we carried out a finer titration of FK506 and phenyl-FK506. These data are shown in Figure 10B,

which illustrates this property and indicates that the transcription system comprises a viable assay for screening pools of CAB mutants for compensatory binding.

Example 14: Mutagenesis strategies for CAB "hole" discovery.

5 As described above, we envision the use of the improved transcription system to screen pools of mutants for compensatory binding to FK506 derivatives. Put simply, this involves transfection of mixtures of DNA species corresponding to mCAB-p65 mutants, then looking for leftward shifts in the dose-response curves depicted in Figure 10.

10 To mutagenize the mCAB-p65 constructs, we prepared degenerate, internal primer pairs at each of the amino acid positions to be mutagenized. These primers are fully degenerate at the codon corresponding to their respective amino acid position (the positions are the same six positions outlined in the original document). Using each primer and the corresponding (antiparallel) primer outside the coding 15 region (in the vector arms), two degenerate, overlapping PCR products were generated. In a secondary PCR reaction, these two fragments were used as template and amplified by overlap extension using the two external primers. These final products were digested and placed back into the pBJ5.2X host vector. It is these libraries that will be screened in the transcription assay for compensatory mutants.

20 Another strategy for identifying potential compensatory mutants is one involving a yeast three-hybrid system. In such a system, yeast are transformed with a DNA-binding fusion to one dimerization domain and an activation domain fusion to the other dimerization domain, along with an appropriately responsive reporter which drives expression of a nutritional marker gene. Yeast transformants are 25 screened for the recovery of growth at a concentration of FK506 or derivative which does not permit growth of yeast harboring the "wild-type" mCAB-p65 construct. The advantage of such a system is that many more clones can be screened simultaneously, allowing multiple amino acid positions in the mCAB to be randomized simultaneously. Use of such three-hybrid systems for screening 30 mutagenized libraries of FKBP and FRB domains are described in USSN 5,830,462 and WO 96/41865.

The system used to screen CAB libraries is based on the MatchMaker LexA Two-Hybrid System from Clonetech (Catalog # K1609-1). It utilizes two vectors, pLex-A and pB42AD, that contain a DNA binding domain from Lex-A and an 35 activation domain (B42), respectively. In addition, the yeast strain EGY48 contains an integrated LEU2 reporter under the control of the $LexA_{op(X6)}$-Leu2 operator

enabling one to directly screen for LexA induced reporter activation on LEU2 dropout plates.

The normal polylinker from pB42AD was removed and replaced with the polylinker described below. I have fused single copies of FKBP and the CABS C-terminal to both domains.

LexA-miniCAB-E (E = Flag epitope tag)
B42AD-FKBP-E (E = Flag epitope tag)

Using this system, ~5 million different mutant mCABS can be screened simultaneously on a single petri dish using very small amounts of compounds. Some or all of the residues that will putatively be in contact with the allyl sidechain, or a substituent on the allyl sidechain, of FK506 can be mutated individually or simultaneously by PCR with the oligonucleotides indicated below. These libraries are then ligated into the Lex-A-miniCAB-E construct and electroporated into DH5-alpha e-coli electrocompetent cells from Gibco BRL. The DNA is amplified in DH5-alphas, purified, and transformed into the yeast strain EGY48 that are expressing B42AD-FKBP-E. The library will be further amplified in yeast by growth on -HIS-TRP plates with Glucose as the sugar source (for selection of both the LexA-mCAB and B42AD-FKBP constructs) then plated onto -HIS-TRP-LEU Galactose/Raffinose plates containing 100nM concentrations of each of the bump compounds. Colonies that grow will be checked by plating onto -HIS-TRP-LEU Galactose/Raffinose plates in the absence of compounds to distinguish between mutants that allow constitutive assocation (ie. False positives) from those that facilitate drug induced association. The colonies that do not grow on the previous control will then be re-plated onto -HIS-TRP-LEU +Galactose/Raffinose with 100 nM of each of the individual bump compounds to be sure that the compounds induce growth. Those that grow will then be compared by plating them onto -HIS-TRP-LEU +Galactose/Raffinose plates with 3 fold dilutions of the bump compounds. The clones that allow the lowest concentration of drug to induce growth will be further analyzed. The DNA from the best postive clones will be rescued or PCR'd to give template for sequencing of the positive hits. These mutants will then be tested in one or more of the assays (ie. transcription assay with mCABS) that we have developed in cell culture for verification of the improvement of affinity of the miniCABS for the bump compound in the context of FKBP. The shuttling of these mutant miniCABS

into these other systems can be accomplished by a simple ligation since they all contain a 5′ Xho1 site and a 3′ Sal 1 site and a 3′ Apa1 site.

Preparation of the Constructs:

5

A. pB42AD constructs.

The pB42AD vector was digested with EcoR1 and Xho1, gel purified and ligated to the following polylinker to give pB42AD-PL3. All of the constructs made with
10 PB42AD are in this pB42AD-PL3 vector.

Polylinker Oligos for pB42AD:

The following two oligonucleotides were phosporylated with polynucleotide
15 kinase, annealed, and ligated into pB42AD that had been digested with EcoR1 and Xho 1 to give a new polylinker with the following restriction sites in order.

5′ Xho1-Spacer-Sal1-Nco1-BstEII-BspEI-AflII-ApaI-EcoR1 3′

20

5′ tcg acg aat tcg ggc ccc tta agt ccg gag gtc acc cat ggg tcg acg tcg gtc gta gac tcg aga 3′

5′ aat ttc tcg agt cta cga ccg acg tcg acc cat ggg tga cct ccg gac tta agg ggc ccg
25 aat tcg 3′

The miniCABS and FKBP from previously reported constructs were then digested with Xho1 and EcoR1 and ligated directly into pB42AD-PL.

30

B. pLexA constructs.

The following oligos were used with standard PCR conditions to generate an FKBP fragment with a 5′ EcoR1 and a 3′ BamHI. pLexA and this fragment were
35 digested with EcoR1 and BamH1, gel purified, and ligated.

FKBP oligos:

5 1. FKBP Y2H^{ex} 5′ (5′ oligo for FKBP with EcoR1 and Xho1 restriction sites

EcoR1 Xho1 Met
5′ c ggg ccc ccc gaa ttc ctc gag atg ggc gtg cag gtg gag ac 3′

10 2. FKBP Y2H^{sb} 3′ (3′ oligo for FKBP with 3′Sal 1 and BamH1 restriction sites. No stop codon)

BamH1 Sal1 E
5′ ggg tct gga tcc gtg gac ttc cag ttt tag aag ctc g 3′

15

Construction of the miniCABS for pLexA:

mCABS were PCR'd with standard PCR conditions off of my original miniCAB template with the followin oligos and digested with NcoI and BamHI. This
20 gel purified fragment was ligated to gel purified pLexA digested with BamHI and NcoI.

3. mCAB Y2H^{ex} 5′ (5′ oligo for mCAB starting at residue 340 of CNA with 5′ BamH1 and Xho1 sites._

25

BamH1 Xho1 Pro340
5′ a tat aaa tcg gga tc cgt ctc gag cca tac tgg ctt cca aat ttc atg g 3′

4. mCAB Y2H^{sfann} 3′ (3′ oligo for mCAB with 3′ Sal1, Flag, Stop, Apa1, Not1, and
30 Nco1)

Nco1 Not1 Apa1 stp ----------Flag----------- Sal1
5′ tct ttaa cca tgg cgg ccg c ggg ccc tca ctt gtc atc gtc atc ttt ata gtc gac cac atc tac cac cat c 3′

35

C. Mutagenic Oligos for mCABs. The following are the oligonucleotides used to generate the libraries of mutant mCABS. They were constructed with the codon NN(G/C) (N= G, A, T, or C) at each of the following positions that we mutagenized. The numbering scheme from each of the independent proteins in the mCAB is retained.

For the CNB portion an overlap mutagenesis strategy was employed. An N-terminal fragment without mutations was PCR'd from my original miniCAB template. This has a region that will anneal with a C-terminal fragment generated by PCR with the indicated oligonucleotides. These two fragments were mixed and subjected to PCR with standard conditions to generate a fragment with an N-terminal Pflm1 site and a C-terminal Apa1 site. Since this reaction generated a visible fragment of the proper size it was then further amplified with oligo 4. above (mCAB Y2H^{sfann} 3') and the Pflm1 oligo below to generate a significant amount of product. This was then digested with Pflm1 and Nco1 and ligated into pLexA-mCAB-E that had been digested with Pflm1 and Nco1 and gel purified. The CNA portion of the mCABS was mutagenized with the oligos under 2. below by PCR to generate a small fragment of the mCAB with a 3' Pflm1 site and a 5'Xho site. This was ligated into pLexA-mCABE digested with Pflm1 and Xho1. To generate the 5 position library this can be ligated into pLexA-mCABE library with the 3 positions mutagenized.

1. Overlap extension mutagenesis of the CNB portion of the mCABS.

5' oligo for generation of the N-terminal portion of CNB for overlap.

Pflm1

5' ct tgg tcc ctt cca ttt gtt ggg gaa aaa gtg act gag 3'

3' oligo for generation of the N-terminal portion of CNB for overlap.

5' ggg aac aat ctg aaa gat aca cag tta cag c 3'

5' mutagenic oligo for generation of C-terminal portion of CNB for overlap.

Val119 Met118 Leu115

5′ gctg taa ctg tgt atc ttt cag att gtt ccc (g/c)NN (g/c)NN cat ctt (g/c)NN tac ctg gaa gag ttc ccc 3′

3′ mutagenic oligo for generation of C-terminal portion of CNB for overlap.

XbaI BglII ApaI Stp ---------FLAG--------- SalI 170

5′ ttaa tct aga tct ggg ccc tca ctt gtc atc gtc atc ttt ata gtc gac cac atc tac cac cat c 3′

2. Mutagenic oligos for the CNA portion of mCABS.

5′ Mutagenic oligo with 5′ Xho I site.

XhoI 340

5′ atat aaa tcg ctc gag cca tac tgg ctt cca aat ttc atg g 3′

3′ Mutagenic oligo

PflM1 353 352

5′ ctc agt cac ttt ttc ccc aac aaa tgg aag (g/c)NN (g/c)NN agt aaa aac atc cat g 3′

Example 15: Use of the CAB domain in complex with multiple binding partners

In order to engineer a cell containing both FKBP/FK506/CAB complexes as well as cyclophilin/cyclosporin/CAB complexes, one would first test the ability of the CAB domain to form cyclophilin/cyclosporin/CAB complexes by replacing FKBP directly with cyclophilin in the transcription assay described in example 4. Thus, the cell would be transfected with a first fusion protein containing cyclophilin and a DNA binding domain such as GAL4, and a second fusion protein containing a CAB domain fused to a transcription activation domain such as p65. Reporter gene expression would be detected following addition of cyclosporin to the cells. Such experiments have been described in WO 98/08956, especially in Example 3. Once formation of cyclophilin/cyclosporin/CAB complexes is demonstrated, one would

then show that cells containing both cyclophilin and FKBP fusion proteins could form the appropriate complexes upon addition of the correct ligand.

A proof of principle experiment for each of the modes of CAB function illustrated in figure5 would involve fluorescent proteins. To demonstrate the recruitment of either FRB or CAB to the same (FKBP) dock, we would make fusions of each of these modules to a different fluorescent protein, such as BFP and GFP, then cotransfect these constructs with a nuclear (or membrane-localized) FKBP construct. Treatment with rapamycin, FK506, or both should result in translocation of either or both of the fluorescence signals to the nucleus (or membrane). To demonstrate the docking of CAB with either FKBP or cyclophilin, we would make fusions of each of these immunophilins to a different localization signal sequence, such as those for nuclear or membrane localization, then cotransfect these constructs with CAB-GFP. Treatment with FK506, cyclosporin, or both, should result in translocation of green fluorescence signal to the appropriate location. These experiments would ideally be backed up immunoprecipitation studies. For example, one could use an antibody to calcineurin or the CAB domain to immunoprecipitate the CAB domain in the presence of FK506 or cyclosporin. One would then run the immunoprecipitates on SDS-page, perform a Western transfer and detect the complex formation by blotting with antibodies to either FKBP or cyclophilin.

A use for this system has been envisioned in the conditional regulation of the protein kinases Akt and PDK1. These kinases are regulated by their proximity to each other and to the membrane in a complex way. Using the first mode of localization discussed above, but replacing each of BFP and GFP with one of these kinases, we could perform order of addition experiments with rapamycin and FK506 in which we monitor treated cells for readouts known to be downstream of one or the other of these kinases. Because these kinases impinge, directly or indirectly, on pathways involving the
cellular targets of both rapamycin and FK506, one would preferably have "bump-hole" solutions for each of these immunophilin-ligand complexes in order to carry out these experiments.

The full disclosure of each of the patent documents and scientific papers cited herein is hereby incorporated by reference. Those documents serve to illustrate the state of the art in various aspects of this invention. Numerous modifications and variations of the present invention should be apparent to one of
5 skill in the art. Such modifications and variations, including design choices in selecting a heterologous action domain, improved ligand, fusion protein design, DNA formulation, viral vector or other DNA delivery means, manner and route of transgene administration, etc. are intended to be encompassed by the scope of the invention and of the appended claims.

Claims:

1. A recombinant nucleic acid encoding a CAB domain, comprising a portion of calcineurin A and a portion of calcineurin B, wherein the CAB domain forms a tripartite complex with an FKBP/CAB ligand and an FKBP domain.

2. The recombinant nucleic acid of claim 1 wherein the calcineurin A portion of the CAB domain comprises a peptide sequence selected from any of the following peptide sequences: residues 12-394 of human calcineurin A, residues 12-370 of human calcineurin A or residues 340-394 of human calcineurin A.

3. The recombinant nucleic acid of claim 1 wherein the calcineurin B portion of the CAB domain comprises residues 3-170 of human calcineurin B.

4. The recombinant nucleic acid of claim 1, 2 or 3 comprising a nucleic acid sequence encoding a calcineurin A and/or calcineurin B peptide sequence which differs from a naturally occurring calcineurin peptide sequence by up to ten amino acid substitutions, deletions or insertions.

5. A recombinant nucleic acid encoding a fusion protein comprising at least one CAB domain of claim 1 and at least one additional domain that is heterologous thereto.

6. The recombinant nucleic acid of claim 5 wherein the heterologous domain is selected from the group comprising a DNA binding domain, a transcription regulatory domain, a cellular localizing domain and a signaling domain.

7. The recombinant nucleic acid of claim 6 wherein the heterologous domain is or is derived from a lexA, GAL4 or composite DNA binding domain.

8. The recombinant nucleic acid of claim 6 wherein the heterologous domain is or is derived from a p65, VP16 or AP domain.

9. The recombinant nucleic acid of claim 6 wherein the heterologous domain is or is derived from a KRAB domain or a ssn-6/TUP-1 domain.

10. The recombinant nucleic acid of claim 6 wherein the heterologous domain is or is derived from an intracellular domain of a cell surface receptor.

5 11. A recombinant nucleic acid encoding a fusion protein containing one or more CAB domains which form a tripartite complex with an FKBP domain-containing protein and a non-naturally occurring FKBP/CAB ligand preferentially over FK506.

12. A nucleic acid composition, comprising a first recombinant nucleic acid of
10 any of claims 5-11 and a second recombinant nucleic acid encoding a fusion protein comprising at least one FKBP domain and at least one additional domain that is heterologous thereto.

13. A nucleic acid composition of claim 12 wherein the second nucleic acid
15 encodes a fusion protein containing a heterologous domain that is the same or different from the heterologous domain on the first fusion protein.

14. The nucleic acid composition of claim 13 wherein the first fusion protein comprises a CAB domain and a transcription activation domain and the second
20 fusion protein comprises an FKBP domain and a DNA binding domain.

15. The nucleic acid composition of claim 13 wherein the first fusion protein comprises a CAB domain and a DNA binding domain and the second fusion protein comprises an FKBP domain and a transcription activation domain.

25

16. A nucleic acid composition of claim 12 wherein the first and second fusion proteins form a ligand dependent complex in the presence of ligand, and wherein the complex initiates a detectable biological signal.

30 17. The nucleic acid composition of claim 16 wherein the biological signal is selected from the group comprising transcription, cell proliferation, cell differentiation, apoptosis.

18. The nucleic acid composition of claim 12 wherein the composition further
35 comprises a target gene construct.

19. A fusion protein encoded by the recombinant nucleic acid of any of claims 5-11.

20. A vector comprising a recombinant nucleic acid of any of claims 1-3 or 5-11.

21. A vector comprising a recombinant nucleic acid of claim 4.

22. A vector comprising a nucleic acid composition of claim 12.

23. The vector of claim 20 wherein the vector is a viral vector.

24. The vector of claim 22 wherein the vector is a viral vector.

25. The vector of claim 23 or 24 wherein the viral vector is selected from the group consisting of adenovirus, AAV, herpesvirus, retrovirus, hybrid adenovirus/AAV, poxvirus, lentivirus.

26. A host cell comprising a recombinant nucleic acid of any of claims 1-3 or 5-11.

27. A host cell comprising a nucleic acid composition of claim 12.

28. A host cell cell of claim 26 which is of human origin.

29. A host cell cell of claim 27 which is of human origin.

30. A host cell of claim 26 which is encapsulated within a biocompatible material.

31. A host cell of claim 27 which is encapsulated within a biocompatible material.

32. A non-human animal containing host cells of claim 26.

33. A non-human animal containing host cells of claim 27.

34. A method for producing genetically engineered host cells comprising introducing into the cells a recombinant nucleic acid of any of claims 1-3 or 5-11 under conditions permitting DNA uptake by cells.

5 35. A method for producing genetically engineered host cells comprising introducing into the cells the nucleic acid compositions of any of claims 12-18 under conditions permitting DNA uptake by cells.

36. The method of claim 34 wherein the nucleic acids are introduced ex vivo.

10

37. The method of claim 35 wherein the nucleic acids are introduced ex vivo.

38. The method of claim 34 wherein the cells are present within an organism.

15 39. The method of claim 35 wherein the cells are present within an organism.

40. A method for multimerizing fusion proteins in the cell of claim 27 which comprises contacting the cells with an effective amount of a ligand under conditions permitting it to form a complex with the fusion proteins.

20

41. A method for initiating a detectable biological signal in cells of claim 27 which comprises contacting the cells with a ligand capable of forming a complex with the fusion proteins, in an effective amount permitting gene expression.

25 42. A method of claim 40 or 41 wherein the cells are grown in a culture medium and the contacting is effected by adding the ligand to the culture medium.

43. A method of claim 40 or 41 wherein the cells are present within a host organism and the contacting is effected by administering the ligand to the host
30 organism.

44. A method of claim 43 wherein the host organism is a mammal and the ligand is administered by oral, bucal, sublingual, transdermal, subcutaneous, intramuscular, intravenous, intra-joint or inhalation administration in an appropriate vehicle
35 therefor.

45. A method for providing animal cells responsive to a ligand which comprises introducing into host animal cells a nucleic acid composition of any of claims 12-18.

46. A kit which comprises the nucleic acid composition of any of claims 12-18 with an appropriate package insert.

47. A kit of claim 46 which further comprises a ligand capable of forming a complex with the fusion proteins.

48. A kit of claim 47 which further comprises a multimerization antagonist.

49. A kit of claim 46 in which one or more of the DNA constructs contains a cloning site in place of a heterologous domain.

50. A kit of claim 46 in which the target gene construct contains a cloning site in place of a target gene.

Figure 1

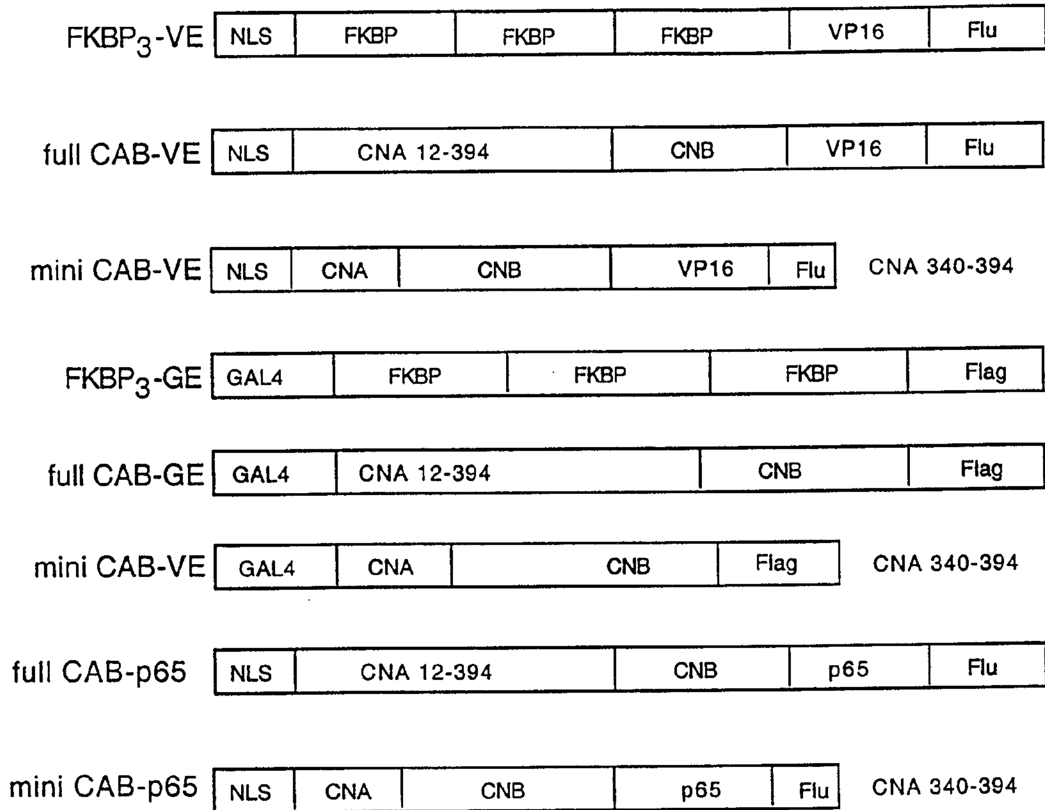

Figure 2

Initial reaction scheme for direct synthesis of *(E/Z)*-C40-phenyl-FK506.

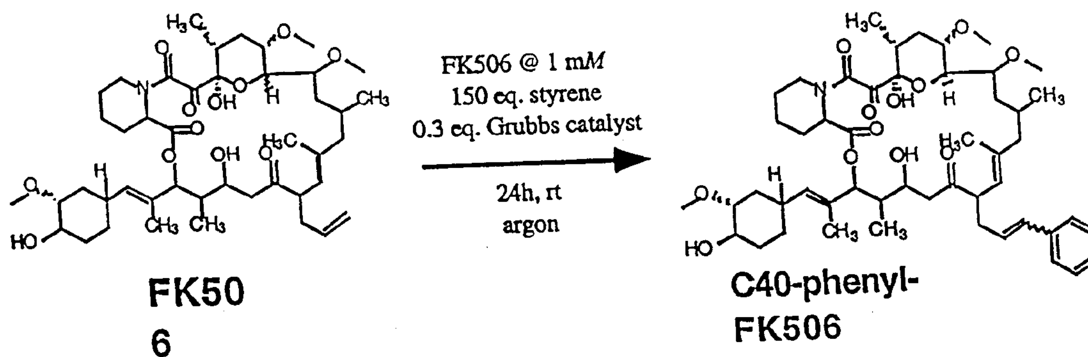

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Figure 3

Styrene and its analogs to be used in cross-metathesis reaction with FK506.

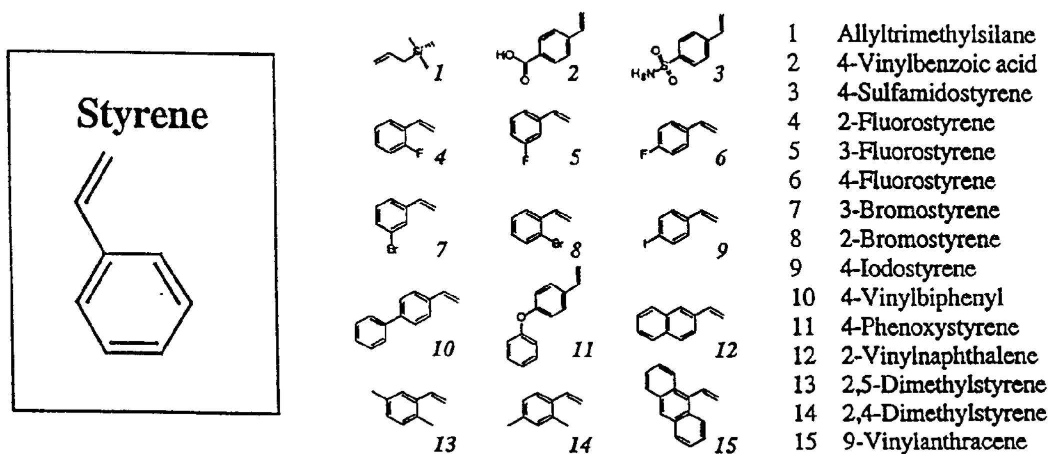

Figure 4

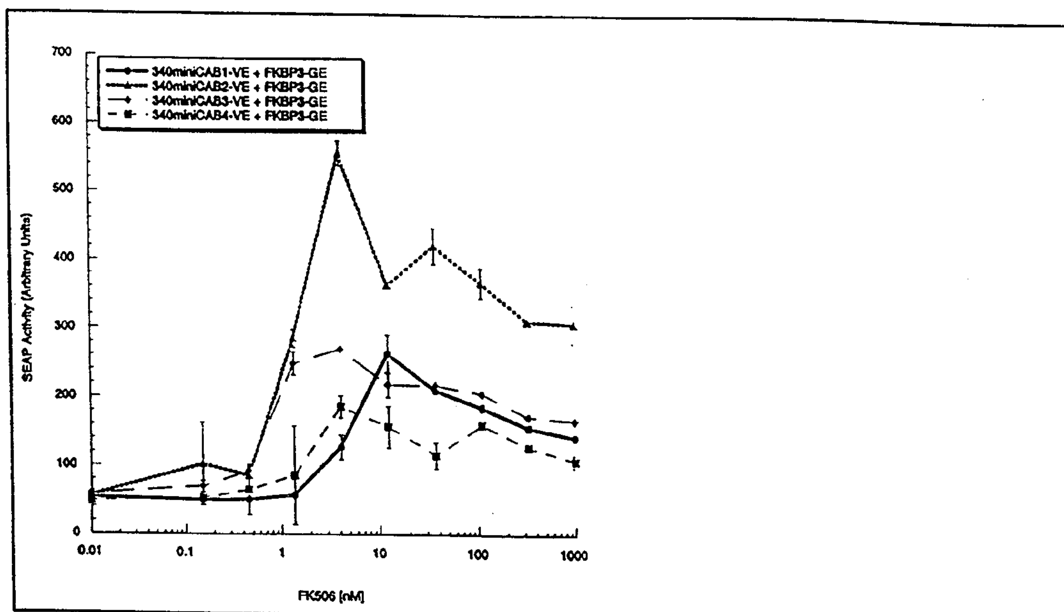

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Figure 5

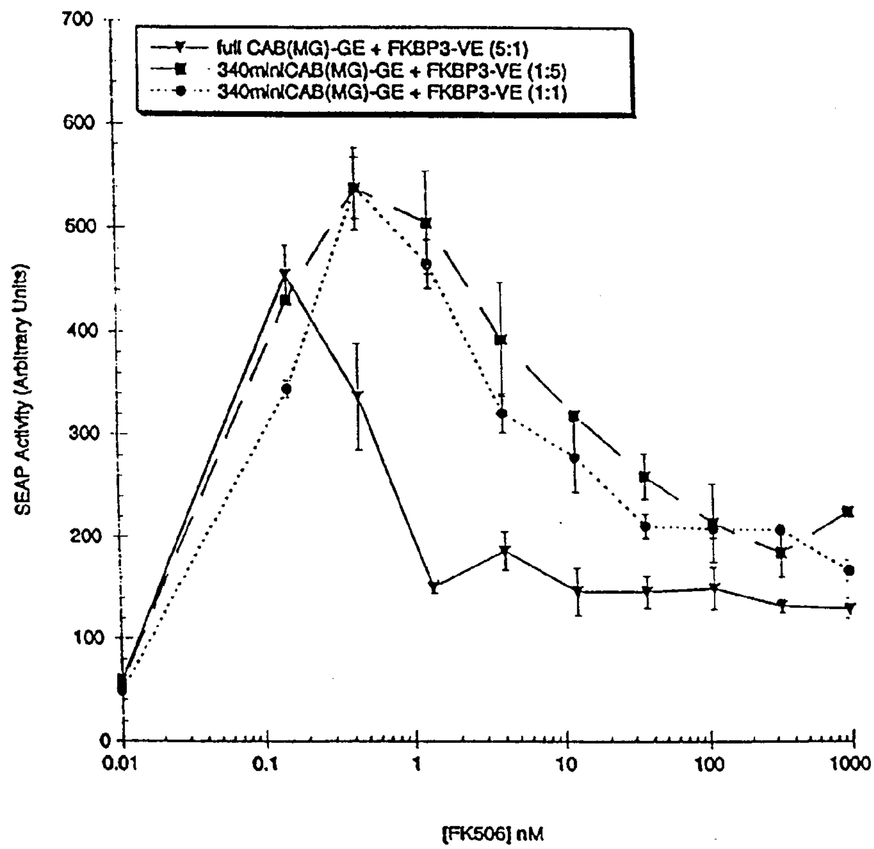

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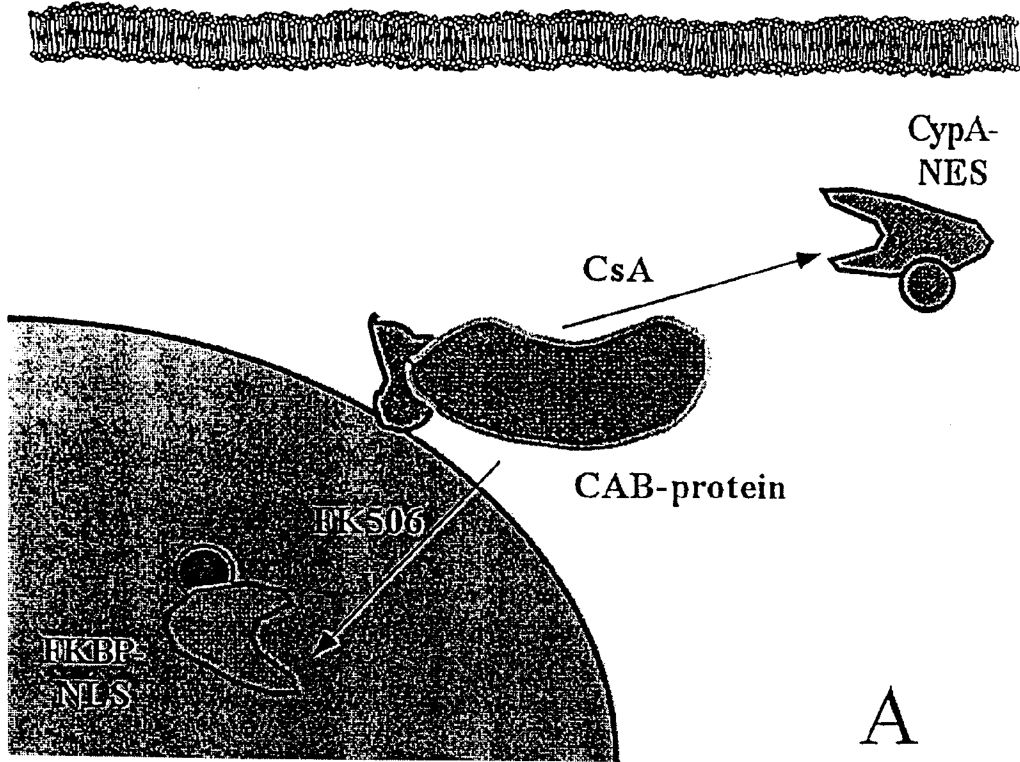
Figure 6



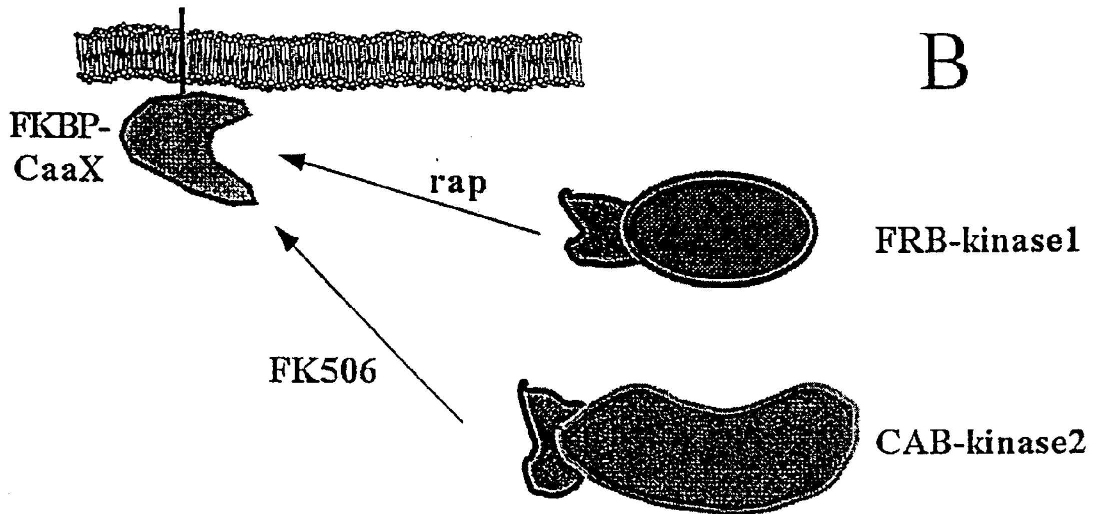


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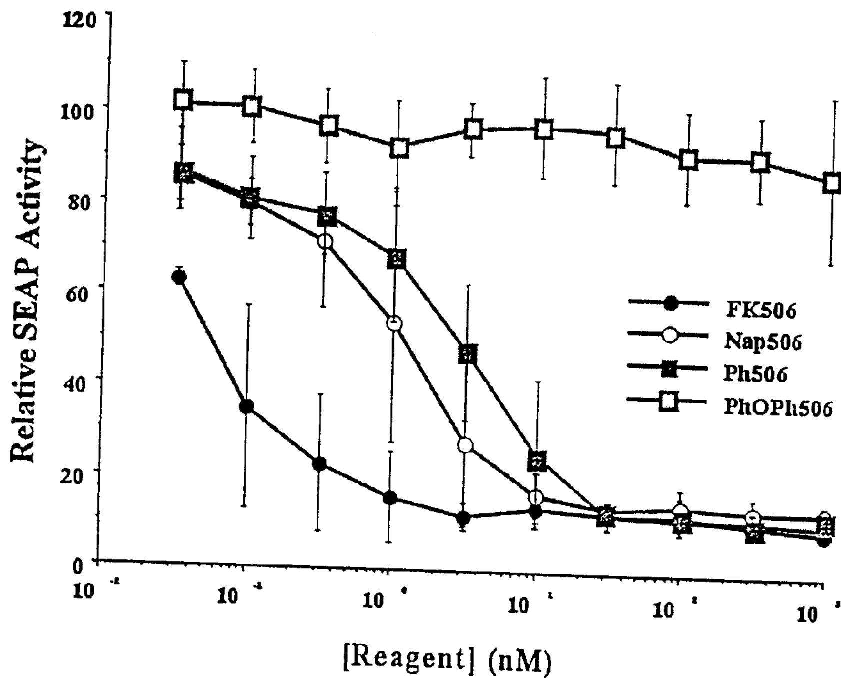
Figure 7



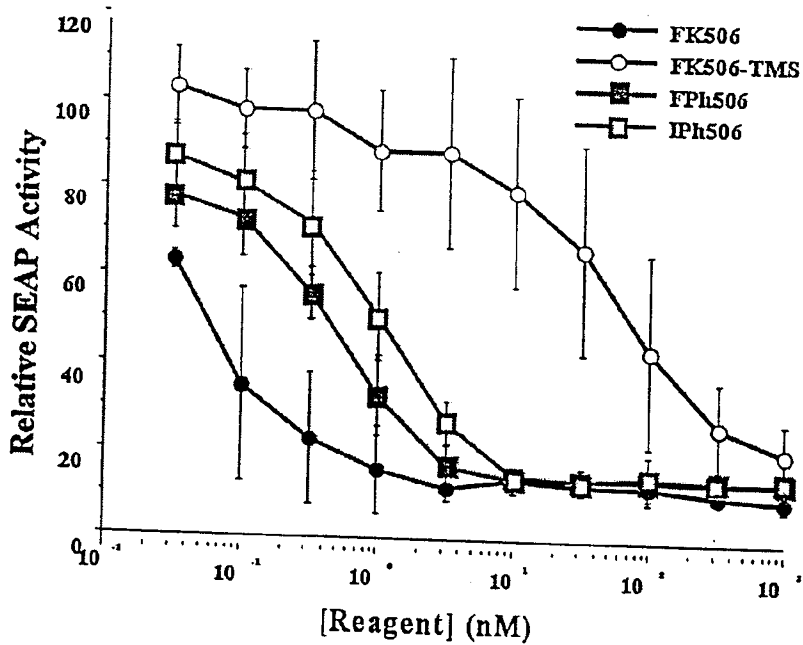
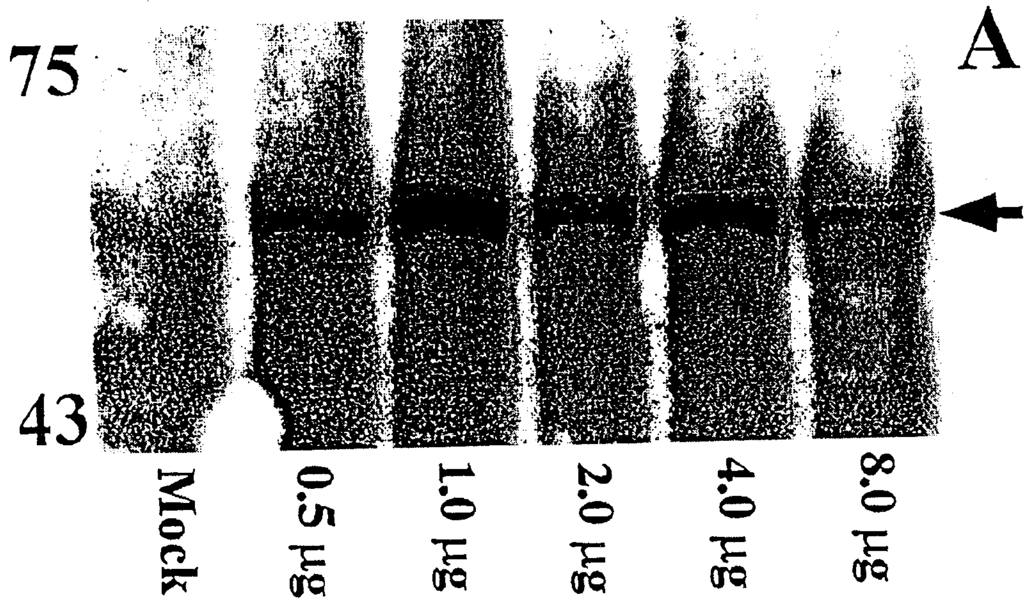


Figure 8



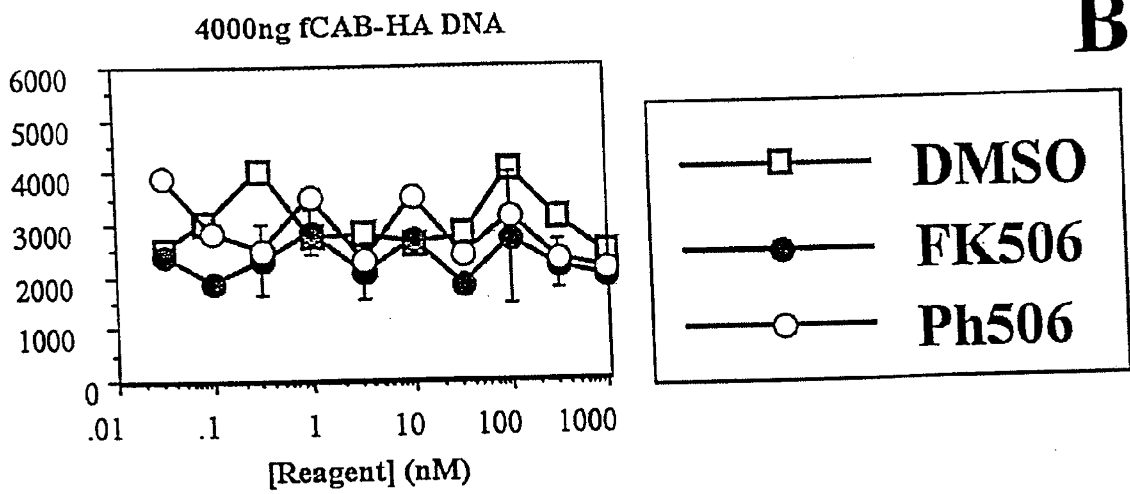


Figure 9

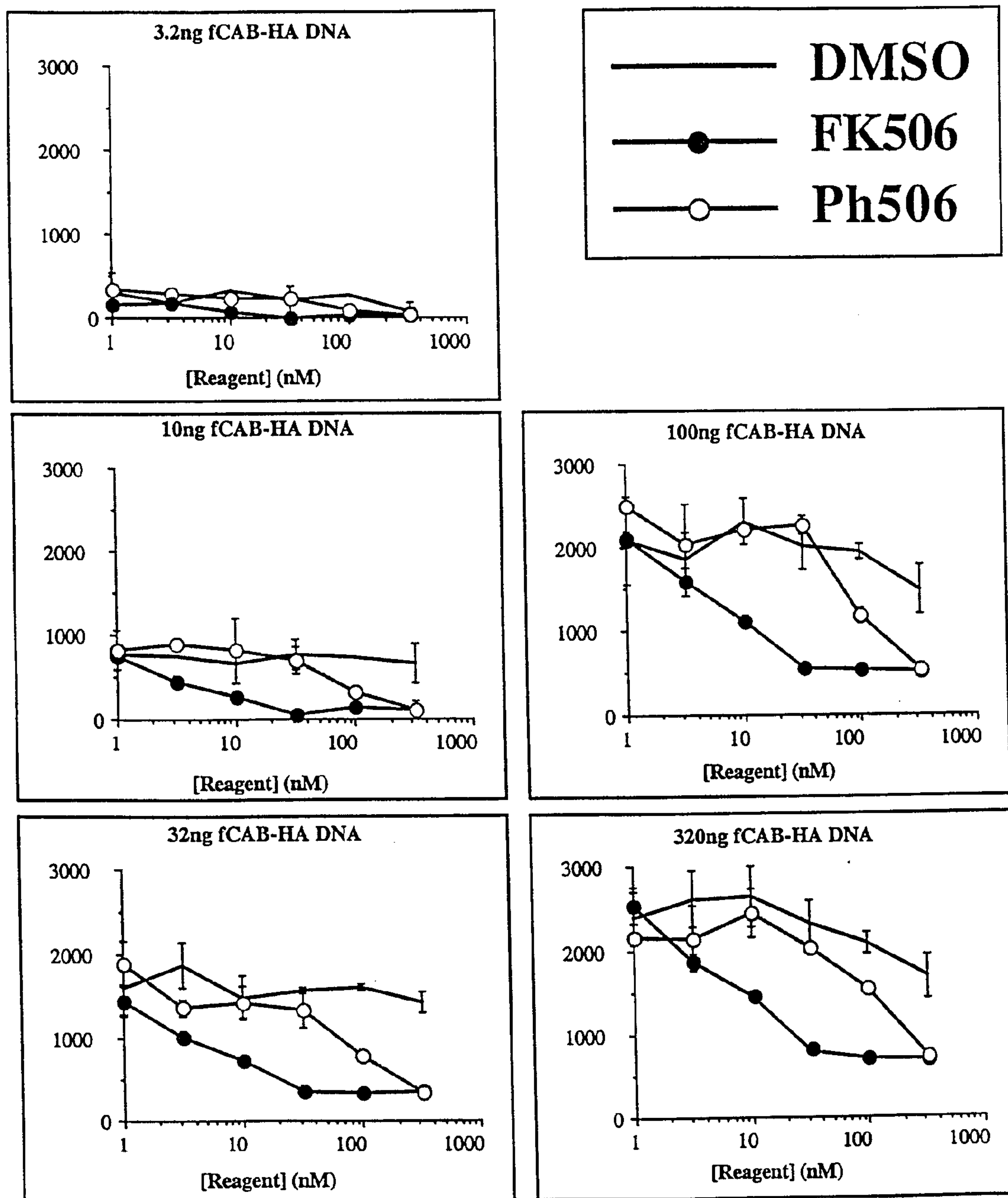


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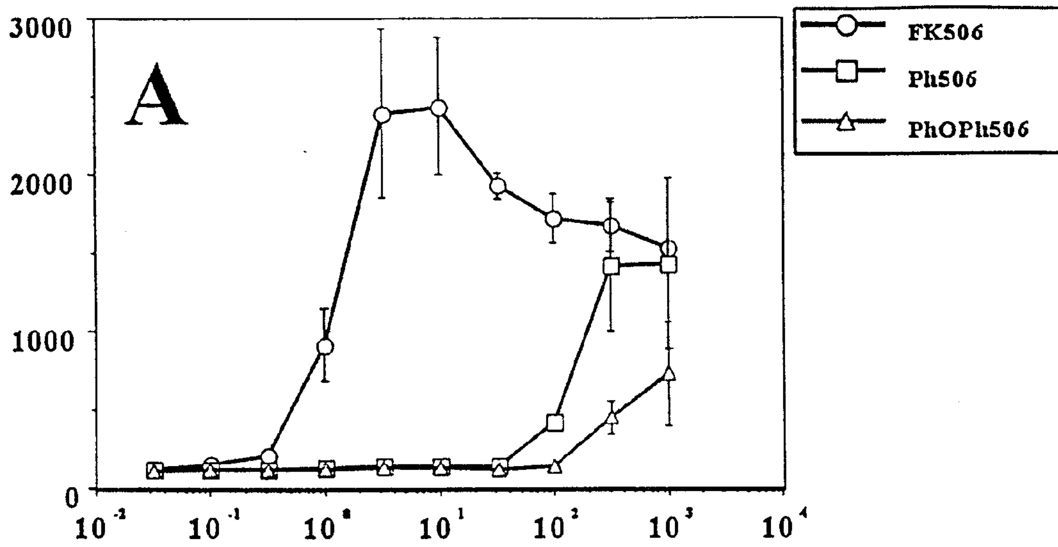
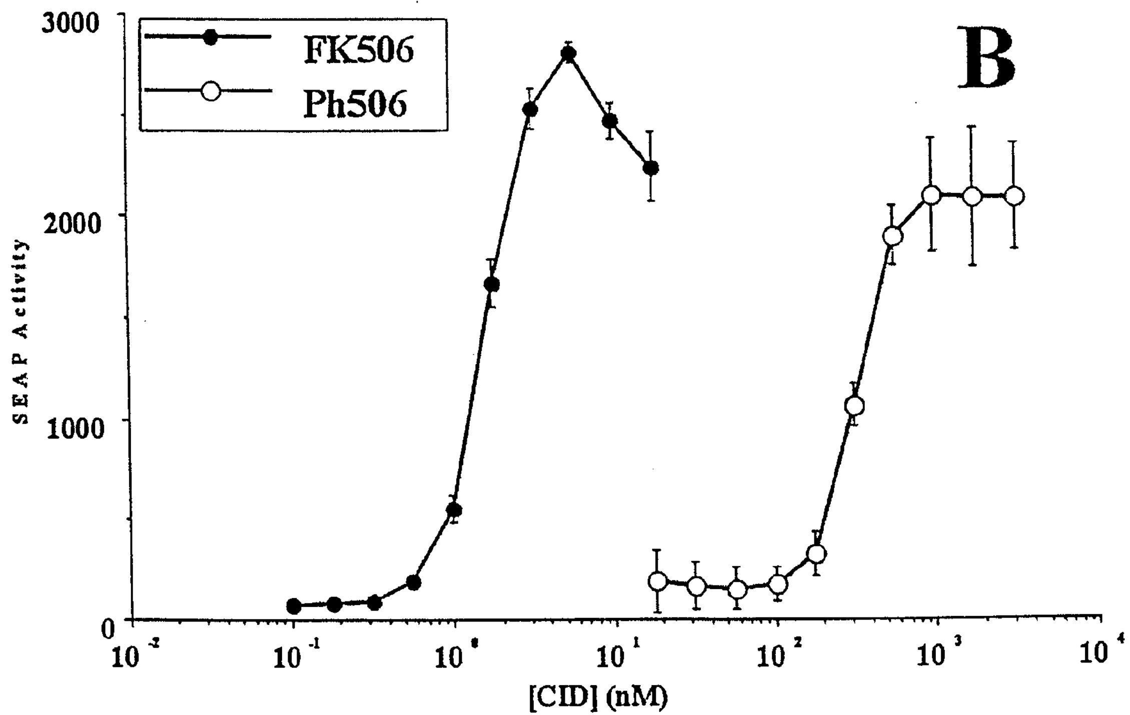
Figure 10





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(71) Applicant *(for all designated States except US)*: **ALLERGENE LTD.** [IL/IL]; 2a Katzir Street, Tel Hashomer, 52656 Ramat Gan (IL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **EISENBERG, Ronit** [IL/IL]; 6 Lotus Street, 74047 Ness-Ziona (IL). **RAZ, Tamar** [IL/IL]; 72/12 He-Beiyar Street, 48056 Rosh Haayin (IL).

(74) Agent: **WEBB, Cynthia**; Webb & Associates, P.O. Box 2189, 76121 Rehovot (IL).

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WO 02/50097 A2

(54) Title: ANTI-ALLERGIC COMPLEX MOLECULES

(57) Abstract: The present invention discloses novel anti-allergic complex molecules, and in particular, peptidic or peptidomimetic molecules, comprising a first part which is competent for cell penetration and a second part which is able to reduce or abolish mast cell degranulation, in particular to reduce or abolish allergy mediators, including histamine secretion from mast cells and protein kinase activation, wherein the first part is connected to the second part via a linker or a direct bond that creates a conformational constraint by forming a bend or turn.

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ANTI-ALLERGIC COMPLEX MOLECULES

FIELD OF THE INVENTION

The present invention discloses novel anti-allergic complex molecules, and in
5 particular, peptidic or peptidomimetic molecules, comprising a first part which is competent for cell penetration and a second part which is able to reduce or abolish mast cell degranulation, in particular to reduce or abolish allergy mediators such as histamine secretion from mast cells, wherein the first part is connected to the second part via a linker or a direct bond that creates a conformational constraint by forming a bend or turn.

10

BACKGROUND OF THE INVENTION

Allergic diseases, including nasal allergy, asthma, urticaria and angioedema, are among the most common diseases encountered by physicians in their clinical practice. Allergy refers to certain diseases in which a wide spectrum of biologically active
15 substances, released from activated mast cells, cause tissue inflammation and organ dysfunction. In essence, any allergic reaction may lead to tissue damage in one or more target organs (see for example Lichtenstein, 1993).

On the cellular level, mast cells are significant mediators of the allergic reaction and are packed with 500 to 1000 granules in which the mediators of the inflammatory
20 reactions are stored. These include vasoactive mediators such as histamine, chemotactic mediators and proteolytic enzymes. In addition, following the activation of mast cells, a number of mediators are generated *de novo* and released. These include arachidonic acid metabolites such as leukotrienes and prostaglandins and a number of multifunctional cytokines. Mast cell derived factors also recruit and activate additional inflammatory
25 cells, such as eosinophils, neutrophils and mononuclear cells. Therefore, mast cell derived

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mediators possess all the requisite properties to induce the symptoms of itching, swelling, coughing and choking that are associated with an allergic reaction (Bienenstock et al., 1987). These mediators are released in response to processes which occur through a number of different pathways within mast cells. Thus, therapeutic treatments for allergy
5 and related inflammatory conditions must intervene at some point in the allergenic pathway in order to be effective.

Current therapies against allergy include H_1 and H_2 blockers, which block the biological activities of histamine. Examples include chlorpheniramine, azatidine, ketotifen, loratidine and others. However, anti-histamines cannot counteract the
10 inflammatory reactions effected by the additional mediators released alongside histamine. Therefore, anti-histamines cannot provide a reliable protection against allergy.

A better allergy treatment would block the secretory process by preventing mast cell degranulation. Drugs which are currently available for this purpose include hydrocortisone and disodium cromoglycate. However, disodium cromoglycate cannot
15 inhibit all types of histamine secretion, and is not always completely effective. Steroids, on the other hand, are effective for blocking mast cell degranulation, but have many unacceptable side effects. Therefore, therapeutic agents which could prevent mast cell degranulation without significant side effects, and could thus prevent or significantly reduce the occurrence of clinical symptoms associated with allergy, such as neurogenic
20 inflammation (see below for details), would be very useful for the treatment of allergy and related conditions.

Mast cell degranulation is a complex process involving at least two different pathways. Mast cells secrete their granular contents in a process of regulated exocytosis (degranulation) by two major pathways, the IgE (immunoglobulin E) dependent pathway
25 and the IgE independent pathway. The IgE dependent pathway is invoked in response to

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an immunological trigger, brought about by aggregation of the high affinity receptors ($F_{c\epsilon}RI$) for IgE, which are present on the cell surface of mast cells. This response involves crosslinking of cell bound IgE antibodies by the corresponding antigens (allergens).

The IgE-independent or peptidergic pathway is invoked in response to a number of polycationic compounds, collectively known as the basic secretagogues of mast cells. These compounds include the synthetic compound 48/80, naturally occurring polyamines and positively charged peptides, such as the neurotransmitter substance P (Ennis et al., 1980; Sagi-Eisenberg 1993; Chahdi et al., 1998).

The ability of substance P to induce mast cell degranulation, together with the observed presence of mast cells clustered around nerve endings which contain substance P, implicate mast cells as the mediators of substance-P induced neurogenic inflammation (Foreman 1987a,b; Pearce et al., 1989). It is well established that in the skin and elsewhere neurogenic inflammation, through the release of neurotransmitters such as substance P, is a contributor to a variety of diseases such as acute urticaria, psychogenic asthma, interstitial cystitis, bowel diseases, migraines, multiple sclerosis and more (Reviewed by Theoharides 1996). In addition, this IgE independent pathway of degranulation can also be evoked by snake, bee and wasp venoms, bacterial toxins and certain drugs such as opiates.

Although the signal transduction pathways by which mast cell degranulation is activated are not yet fully resolved, a number of cellular events have been shown to occur after stimulation of the mast cells. These include activation of phospholipases such as PLC, PLD and PLA2, elevation of cytosolic Ca^{2+} and activation of serine and tyrosine kinases (reviewed by Sagi-Eisenberg, R. "Signal Transmission Pathways in Mast Cell Exocytosis". In: The Handbook of Immunopharmacology. Academic Press, UK. pp. 71-88, 1993).

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Within these processes, however, the involvement of GTP-binding proteins (G-proteins) is well established. For example, the introduction of nonhydrolyzable analogues of GTP, such as GTP-γ-S, into ATP^{-4} permeabilized mast cells, stimulates PLC activity and degranulation.

5 From these and other observations, the involvement of at least two different G-proteins, one involved in PLC and Ca^{2+} activation (G_P) and one directly regulating exocytosis (G_E), has been suggested (Gomperts 1990; Gomperts et al., 1991; reviewed by Sagi-Eisenberg 1993). Indeed, it was subsequently demonstrated that basic secretagogues induce histamine secretion by interacting directly with G_E, a pertussis toxin-sensitive 10 heterotrimeric G protein, in a receptor-independent manner (Aridor et al., 1990; Aridor & Sagi-Eisenberg 1990). This G-protein was subsequently identified as Gi_3, which appears to mediate the peptidergic pathway leading to exocytosis in mast cells. In particular, a synthetic peptide which corresponds to the C terminal sequence of $G\alpha i_3$ (KNNLKECGLY) was able to inhibit histamine release when introduced, into 15 permeabilized mast cells (Aridor et al., 1993).

However, the cell membrane is generally impermeable to most peptides. Therefore, the use of a peptide as a therapeutic agent, directed against an intracellular target, requires a special mechanism to enable the peptide to overcome the membrane permeability barrier.

20 One possible approach is based on the fusion of the selected peptide with a specific hydrophobic sequence, comprising the "h" region of a signal peptide sequence. Examples of such hydrophobic regions are the signal sequence of the Kaposi fibroblast growth factor (AAVALLPAVLLALLAP; Liu et al., 1995; Rojas et al., 1997) and the signal sequence within human integrin β_3 (VTVLALGALAGVGVG; Liu et al., 1996; 25 Review by Hawiger 1997).

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Specific importation of biologically active molecules into cells by linking an importation-competent signal peptide to the molecule of interest was disclosed in U.S. Patent No. 5,807,746, although only *in vitro* studies were described, such that the signal peptide was not shown to function *in vivo*. The signal peptide causes the entire complex
5 to be imported into the cell, where theoretically the biologically active molecule could then have its effect. Although such direct importation could serve to introduce the therapeutic compound into the cell, , the efficacy of the complex may be limited, such that the biologically active molecule may have little or no effect. The variables which may affect the efficacy of the biologically active molecule include the effect of linking the
10 molecule to the signal peptide, which may result in an inactive hybrid molecule; unpredictable effects of the entire complex within the cell; and even the inability of the entire complex to be imported into the cell, despite the presence of the signal peptide.

In addition, identifying a suitable biologically active molecule for treatment of allergy may also be difficult. For example, linking a non-peptide molecule, such as a
15 known secretion-blocking compound, to a signal peptide is both difficult and may result in an unstable molecule. A peptide could be used as the secretion-blocking compound, but then such a peptide must be carefully selected and tested. Finally, the entire complex would require testing, particularly *in vivo*, since the ability to penetrate a cell in tissue culture does not necessarily predict the efficacy of the complex in a human or animal
20 subject. U.S. Patent No. 5,807,746 therefore suffers from the drawback that only *in vitro* data is disclosed, such that the effect of the signaling peptides *in vivo*, alone or as part of a complex, is not known. Thus, suitable, targeted, specific therapeutic agents for the treatment of allergy are not currently available and are potentially complex and difficult to develop.

25 There is therefore a need for, and it would be useful to have, a therapeutic agent

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for the treatment of allergy and related inflammatory conditions, which would block mast cell degranulation and hence the release of histamine, but which would be specifically targeted to the degranulation pathway and which would therefore have few side effects.

Previous work has demonstrated the ability of several peptides to block mast cell degranulation. For example, a novel peptide designated Peptide 2, that was designed and synthesized to include an importation competent signal peptide, as a first segment at the N-terminus (underlined), and the C-terminal sequence of $G\alpha i_3$ as a second segment at the C-terminus AAVALLPAVLLALLAPKNNLKECGLY) inhibited histamine release from activated mast cells (WO 00/78346). Additional active peptides in that disclosure include

Peptide 2-succ: Succinyl-AAVALLPAVLLALLAPKNNLKECGLY;

Peptide 5: AAVALLPAVLLALLAPKENLKDCGLF; and

Peptide 2-cyc:

AAVALLPAVLLALLAPKNNLKECGLY

ε-NH

The present invention is not intended to encompass any of the peptides disclosed and claimed in that application, and they are specifically excluded from the present invention, as are any known peptides according to the principles disclosed hereinbelow.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically indicated to be incorporated herein by reference. In addition, citation of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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SUMMARY OF THE INVENTION

The present invention discloses a therapeutic complex molecule for the specific, direct and targeted treatment of allergies and related inflammatory conditions, which comprises a first segment which is competent for the importation of the complex molecule into mast cells, and a second segment which is able to block or significantly reduce mast cell degranulation and hence the release of histamine. According to a currently preferred embodiment, the first segment comprises a signal peptide, which is competent for importation of the complex into mast cells, while the second segment comprises a biologically active molecule, such as a peptide, which is able to block the G protein-mediated contribution to the mast cell degranulation process. Most preferred embodiments of the present invention will reduce or abolish inflammatory mediators of allergic reactions, including those late phase inflammatory mediators induced by protein kinase activation, as well as inhibiting histamine secretion from mast cells.

According to the present invention, there is provided an anti-allergic agent, comprising a molecule having at least a first segment competent for importation of the molecule into mast cells, and a second segment for having an anti-allergic effect within the mast cells, the first segment being joined to the second segment through a linker.

According to a preferred embodiment of the present invention, the linker is a covalent bond. According to one currently more preferred embodiment of the present invention the covalent bond is a peptide bond.

It is now disclosed that unexpectedly the linker must be of such a nature as to create a conformational constraint at or near the junction between the first segment and the second segment. Preferably the linker must prevent the first segment from being contiguous to the second segment in a linear or an extended conformation. More preferably it will create a bend or a turn. According to certain currently most preferred

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embodiments the conformational constraint is selected from the group consisting of, a proline or proline mimetic, an N-alkylated amino acid, a double bond or triple bond or any other moiety which introduces a rigid bend in the peptide backbone.

In addition to Proline, specific examples of moieties which induce suitable conformations include but are not limited to N-methyl amino acids such as sarcosine; hydroxy proline instead of proline; anthranilic acid (2-amino benzoic acid); and 7-azabicyloheptane carboxylic acid.

The second segment has the anti-allergic effect by at least significantly reducing degranulation of the mast cells. Preferably, the second segment is selected from the group consisting of a peptide, a peptidomimetic, and a polypeptide. More preferably, the second segment is a peptide or peptidomimetic. Also more preferably, the first segment is a peptide or peptidomimetic.

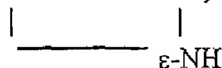
It is now disclosed that the second segment comprising the anti-allergic activity most preferably is a peptide having a cyclic conformation. Preferably the cyclic conformation is stabilized by bonds selected from the group consisting of hydrogen bonds, ionic bonds or covalent bonds.

Preferably, the anti-allergic segment of the molecule is a peptide taken from the C terminal sequence of a G protein, more preferably a G protein involved in exocytosis. Specific examples of useful peptides include $G\alpha i_3$ and $G\alpha t$. Most preferably, the anti-allergic segment of the peptide has an amino acid sequence selected from the group of:

a decapeptide derived from $G\alpha i_3$ having the sequence KNNLKECGLY ;

a decapeptide derived from $G\alpha t$ having the sequence KENLKDCGLF ;

Cyclic $G\alpha i_3$ KNNLKECGLY ;



ε-NH

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KNNLKECGL-para-amino-F; KQNLKECGLY; KSNLKECGLY;

KNNLKEVGLY and KENLKECGLY.

5 Within the scope of the present invention are included all active analogues, homologues and derivatives of these sequences, including but not limited to cyclic derivatives.

Preferably the importation competent segment of the molecule is a peptide taken from a signal peptide sequence. Useful examples thereof include the signal peptide

10 sequence of the Kaposi fibroblast growth factor or a human integrin β3.

According to particularly preferred embodiments of the present invention, the molecule is a peptide having an amino acid sequence selected from the group consisting of:

WALL006: AAVALLPAVLLALLAPKQNLKECGLY

15

WALL007: AAVALLPAVLLALLAPKNNLKEVGLY

WALL008: Succinyl -AAVALLPAVLLALLA-Sar-KNNLKECGLY

20 WALL010: VTVLALGALAGVGVGPKNNLKECGLY

WALL011: Succinyl - AAVALLPAVLLALLAPKSNLKECGLY

WALL012: Succinyl – AAVALLPAVLLALLAPKENLKECGLY

25

WALL013: Succinyl – AAVALLPAVLLALLAPKANLKECGLY

WALL014: Succinyl - AAVALLPAVLLALLAP KNNLKECGL-para-amino-F

30 WALL015: Succinyl – AAVALLPAVLLALLAPKQNLKECGLY

WALL016: Succinyl – AAVALLPAVLLALLAPKNNLKEVGLY

Within the scope of the present invention are included all active analogues,

35 homologues and derivatives of these sequences, including but not limited to cyclic derivatives. In particular, active analogs are intended to include esters, such as but not limited to succinylated derivatives.

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According to another embodiment of the present invention, there is provided a pharmaceutical composition for treating an allergic condition in a subject, comprising as an active ingredient a therapeutically effective amount of an anti-allergic agent, said agent comprising a molecule comprising a first segment competent for importation of the
5 molecule into mast cells, and a second segment having an anti-allergic effect within the mast cells, wherein the first part is connected to the second part via a linker or a direct bond that creates a conformational constraint by forming a bend or turn.

According to certain currently most preferred embodiments the conformational constraint is selected from the group consisting of, a proline or proline mimetic, an N
10 alkylated amino acid, a double bond or triple bond or any other moiety which introduces a rigid bend in the peptide backbone.

According to another preferred embodiment of the present invention, the pharmaceutical composition comprises as an active ingredient a complex peptide having as an anti-allergic segment a peptide having an amino acid sequence selected from the
15 group consisting of:

a decapeptide derived from $G\alpha i_3$ having the sequence KNNLKECGLY ;

a decapeptide derived from $G\alpha t$ having the sequence KENLKDCGLF ;

Cyclic $G\alpha i_3$ KNNLKECGLY ;
20 (cyclized: K linked to Y via ε-NH)

KNNLKECGL-para-amino-Phenylalanine ; KQNLKECGLY;

KSNLKECGLY; KNNLKEVGLY; and KENLKECGLY.
25

Additionally and preferably, the pharmaceutical composition comprises as an active ingredient a complex peptide having an amino acid sequence selected from the

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group consisting of:

WALL 006: AAVALLPAVLLALLAPKQNLKECGLY

WALL 007: AAVALLPAVLLALLAPKNNLKEVGLY

5 WALL 008: Succinyl -AAVALLPAVLLALLA-Sar-KNNLKECGLY

WALL 010: VTVLALGALAGVGVGPKNNLKECGLY

10 WALL 011: Succinyl – AAVALLPAVLLALLAPKSNLKECGLY

WALL 012: Succinyl – AAVALLPAVLLALLAPKENLKECGLY

WALL 013: Succinyl – AAVALLPAVLLALLAPKANLKECGLY

15 WALL 014:Succinyl - AAVALLPAVLLALLAP KNNLKECGL-para-amino-F

WALL 015: Succinyl – AAVALLPAVLLALLAPKQNLKECGLY

20 WALL016: Succinyl – AAVALLPAVLLALLAPKNNLKEVGLY

Within the scope of the present invention are included all active analogues, homologues and derivatives of these sequences, including but not limited to cyclic derivatives.

25 According to still another embodiment of the present invention, there is provided a method for treating an allergic condition in a subject, comprising the step of administering a therapeutically effective amount of an anti-allergic agent to the subject, said agent comprising a molecule having at least a first segment competent for importation of the molecule into mast cells, and a second segment for having an anti-

30 allergic effect within the mast cells, wherein the first part is connected to the second part via a linker or a direct bond that creates a conformational constraint by forming a bend or turn.

According to certain currently most preferred embodiments the conformational constraint is selected from the group consisting of, a proline or proline mimetic, an N

35 alkylated amino acid, a double bond or triple bond or any other moiety which introduces a

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rigid bend in the peptide backbone.

In addition to proline, specific examples of moieties which induce suitable conformations include but are not limited to N-methyl amino acids such as sarcosine; hydroxy proline; anthranilic acid (2-amino benzoic acid); and 7-azabicyloheptane
5 carboxylic acid.

Preferably, the allergic condition is selected from the group consisting of nasal allergy, an allergic reaction in an eye of the subject, an allergic reaction in the skin of the subject, acute urticaria, psoriasis, psychogenic or allergic asthma, interstitial cystitis, bowel diseases, migraines, and multiple sclerosis.

10 A preferred route of administration is oral, but alternative routes of administration include, but are not limited to, intranasal, intraocular, sub-cutaneous and parenteral administration. More preferably, the therapeutic agent is administered by topical administration. Most preferably, the topical administration is to the skin of the subject. According to an alternative preferred embodiment of the present invention, the therapeutic
15 agent is administered intranasally or by inhalation.

In addition to inhibiting histamine release, it is now disclosed that peptides according to the present invention unexpectedly also inhibit the activation of protein tyrosine kinases (PTKs) and mitogen activated protein kinases (MAPKs). Activation of these protein kinases was demonstrated previously as a crucial event, leading to activation
20 of the late phase inflammatory reactions such as synthesis *de novo* of leukotrienes and prostaglandins.

According to yet another embodiment of the present invention, there is thus provided a method for preventing late phase inflammatory responses induced by protein kinase activation, comprising the step of administering a therapeutically effective amount
25 of an anti-allergic agent to the subject, said anti-allergic agent comprising a molecule

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having at least a first segment competent for importation of said molecule into mast cells, and a second segment for having an anti-allergic effect within said mast cells, said first segment being joined to said second segment through a linker, said linker providing a bend or turn at or near the junction between the segments.

5 According to yet another embodiment of the present invention, there is provided a method for promoting importation of an anti-allergic peptide into a cell of a subject *in vivo*, the method comprising the steps of:

(a) attaching to the anti-allergic peptide a leader sequence, the leader sequence being a peptide, via a linker or a direct bond which forms a bend or a turn, to form a

10 complex peptide or peptidomimetic molecule;

(b) administering the complex peptide or peptidomimetic molecule to the subject; and

(c) importing the complex molecule into the cell through the leader sequence, such that the anti-allergic peptide is imported into the cell.

15

Hereinafter, the term "biologically active" refers to molecules, or complexes thereof, which are capable of exerting an effect in a biological system. Hereinafter, the terms "fragment" or "segment" refer to a portion of a molecule or a complex thereof, in

20 which the portion includes substantially less than the entirety of the molecule or the complex thereof.

Hereinafter, the term "amino acid" refers to both natural and synthetic molecules which are capable of forming a peptide bond with another such molecule. Hereinafter, the term "natural amino acid" refers to all naturally occurring amino acids, including both

25 regular and non-regular natural amino acids. Hereinafter, the term "regular natural amino

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acid" refers to those alpha amino acids which are normally used as components of a protein. Hereinafter, the term "non-regular natural amino acid" refers to naturally occurring amino acids, produced by mammalian or non-mammalian eukaryotes, or by prokaryotes, which are not usually used as a component of a protein by eukaryotes or
5 prokaryotes. Hereinafter, the term "synthetic amino acid" refers to all molecules which are artificially produced and which do not occur naturally in eukaryotes or prokaryotes, but which fulfill the required characteristics of an amino acid as defined above. Hereinafter, the term "peptide" includes both a chain of a sequence of amino acids, whether natural, synthetic or recombinant. Hereinafter, the term "peptidomimetic"
10 includes both peptide analogues and mimetics having substantially similar or identical functionality thereof, including analogues having synthetic and natural amino acids, wherein the peptide bonds may be replaced by other covalent linkages.

BRIEF DESCRIPTION OF THE DRAWINGS

15 The invention is herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 is a graph of the dose response of peptide WALL006 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 2 is a graph of the dose response of peptide WALL015 (a) on histamine
20 secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 3 is a graph of the dose response of peptide WALL011 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 4 is a graph of the dose response of peptide WALL012 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

25 FIG. 5 is a graph of the dose response of peptide WALL013 (a) on histamine

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FIG. 6 is a graph of the dose response of peptide WALL005 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 7 is a graph of the dose response of peptide WALL014 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

5 FIG. 8 is a graph of the dose response of peptide WALL007 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 9 is a graph of the dose response of peptide WALL016 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 10 is a graph of the dose response of peptide WALL004 (a) on histamine

10 secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 11 is a graph of the dose response of peptide WALL008 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 12 is a graph of the dose response of peptide WALL009 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

15 FIG. 13 is a graph of the dose response of peptide WALL010 (a) on histamine secretion; and (b) on compound 48/80 induced histamine release from intact mast cells.

FIG. 14 is a graph of the dose response of peptide WALL023 on compound 48/80 induced histamine release from intact mast cells.

FIG. 15 demonstrates protein tyrosine kinase (PTK) activation induced by

20 compound 48/80 (A) or H_2O_2/VO_3 (B), followed by treatment with peptide 2.

FIG. 16 demonstrates mitogen activated protein kinase (MAPK) activation induced by compound 48/80 (A) or H_2O_2/VO_3 (B), followed by treatment with peptide 2.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention discloses a therapeutic complex molecule for the specific, direct and targeted treatment of allergies and related inflammatory conditions, which comprises molecules having at least a first segment which is competent for the
5 importation of the complex into mast cells, and a second segment which is able to block or significantly reduce mast cell degranulation and hence the release of histamine.

It is now disclosed that the linker is a crucial element of the present invention, and that it must impose certain conformational constraints at or near the junction of the two segments of the molecule. The first segment is connected to the second segment through a
10 linker or a direct bond, the linker creating a conformational constraint, by forming a bend or turn. According to certain currently most preferred embodiments the conformational constraint is selected from the group consisting of, a proline or proline mimetic, an N alkylated amino acid, a double bond or triple bond or any other moiety which introduces a rigid bend into the peptide backbone.

15 In addition to proline, specific examples of moieties which induce suitable conformations include but are not limited to N-methyl amino acids such as sarcosine, hydroxy proline, anthranilic acid (2-amino benzoic acid) and 7-azabicyloheptane carboxylic acid.

The first segment is a molecule, preferably a peptide or a peptidomimetic, and
20 more preferably a signal peptide. A signal peptide is a peptide which is capable of penetrating through the cell membrane, to permit the exportation and/or importation of proteins or peptides. As used herein, suitable signal peptides are those which are competent for the importation of proteins, peptides or other molecules into the cell. Such signal peptides generally feature approximately 10-50 amino acids, of which the majority
25 are typically hydrophobic, such that these peptides have a hydrophobic, lipid-soluble

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portion. Preferably, signal peptides are also selected according to the type of cell into which the complex is to be imported, such that signal peptides produced by a particular cell type, or which are derived from peptides and/or proteins produced by that cell type, can be used to import the complex into cells of that type. Examples of such signal peptides are described above and are also disclosed in US 5,807,746, incorporated by reference as if fully set forth herein for the teachings regarding signal peptides.

The second segment is a molecule which has an anti-allergic effect, preferably by preventing mast cell degranulation, and hence the release of histamine from these mast cells. The molecule is preferably a peptide, and more preferably a peptide derived from the C terminal sequence of $G\alpha i_3$, which appears to mediate the peptidergic pathway leading to exocytosis in mast cells. Alternatively, the second segment is selected from the group consisting of a peptidomimetic, a polypeptide, or a protein.

The linker which connects the first segment to the second segment is preferably a covalent bond. Conveniently, the covalent bond may be a peptide bond if at least one of the first and second segments is a peptide. It is now disclosed that the linker is a crucial element of the present invention, and that it must impose certain conformational constraints at or near the junction of the two segments of the molecule.

The first part is connected to the second part via a linker or a direct bond that creates a conformational constraint by forming a bend or turn. According to certain currently most preferred embodiments the conformational constraint is selected from the group consisting of, a proline or proline mimetic, an N alkylated amino acid, a double bond or triple bond or any other moiety which introduces a rigid bend in the peptide backbone.

In addition to proline, specific examples of moieties which induce suitable conformations include but are not limited to N-methyl amino acids such as sarcosine,

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hydroxy proline, anthranilic acid (2-amino benzoic acid) and 7-azabicyloheptane carboxylic acid.

A range of methods of creating suitably constrained conformations at or near the junction of the complex molecules of the invention are well known in the art. Classical
5 methods of introducing conformational constraints include structural alteration of amino acids or introduction of bonds other than a flexible peptide bond. In addition to other modes of conformational restriction, such as configurational and structural alteration of amino acids, local backbone modifications, short-range cyclization, medium and long range cyclizations [Hruby, V. J., Life Sci. 31, 189 (1982); Kessler, H., Angew. Chem. Int.
10 Ed. Eng.,21, 512 (1982); Schiller, P. W., in The Peptides, Udenfriend, S., and Meienhofer, J. Eds., Volume 6 p. 254 (1984); Veber, D. F. and Freidinger, R. M., Trends in Neurosci. 8, 392 (1985); Milner-White, E. J., Trends in Pharm. Sci. 10, 70 (1989)] are useful to optimize the active conformations of the peptides according to the invention.

Therapeutically active peptides are cyclized to achieve metabolic stability, to
15 increase potency, to confer or improve selectivity and to control bioavailability. The possibility of controlling these important pharmacological characteristics through cyclization of linear peptides prompted the use of medium and long range cyclization to convert natural bioactive peptides into peptidomimetic drugs, as is known in the art. Cyclization also brings about structural constraints that enhance conformational
20 homogeneity and facilitates conformational analysis [Kessler, H., Angew. Chem. Int. Ed. Eng., 21, 512 (1982)]. Moreover, the combination of structural rigidification-activity relationship studies and conformational analysis gives insight into the biologically active conformation of linear peptides.

The present invention also discloses methods for treating allergies. Hereinafter,
25 the term "treatment" includes both the prevention of the allergic condition, as well as the

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substantial reduction or elimination of allergic symptoms. Allergic conditions for which the therapeutic agents of the present invention are useful include, but are not limited to, nasal allergy, irritation or allergic reactions in the eyes, allergic reactions in the skin including any type of allergen-induced rash or other skin irritation or inflammation, acute urticaria, psoriasis, psychogenic or allergic asthma, interstitial cystitis, bowel diseases, migraines, and multiple sclerosis.

Such treatment may be performed topically, for example for skin allergies and allergic reactions, including but not limited to, contact dermatitis in reaction to skin contact with an allergen; reactions to insect bites and stings; and skin reactions to systemic allergens, such as hives appearing after a food substance has been ingested by the subject. Alternatively and/or additionally, such treatment may be performed by systemic administration of the therapeutic complex. A preferred route of administration is oral, but alternative routes of administration include, but are not limited to, intranasal, intraocular, sub-cutaneous and parenteral administration. Other routes of administration, and suitable pharmaceutical formulations thereof, are described in greater detail below.

As noted previously, in a certain currently most preferred embodiment of the present invention, the first and the second segments are both peptides, which are joined with a peptide bond.

In the present invention, novel peptides were tested, designated as peptides:

WALL004: AAVALLPAVLLALLAAKNNLKECGLY

WALL005: AAVALLPAVLLALLAPKNNLKECGL-para-amino-F

WALL006: AAVALLPAVLLALLAPKQNLKECGLY

WALL007: AAVALLPAVLLALLAPKNNLKEVGLY

WALL008: Succinyl-AAVALLPAVLLALLA-Sar-KNNLKECGLY

WALL010: VTVLALGALAGVGVGPKNNLKECGLY

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WALL011: Succinyl - AAVALLPAVLLALLAPKSNLKECGLY

WALL012: Succinyl – AAVALLPAVLLALLAPKENLKECGLY

WALL013: Succinyl - AAVALLPAVLLALLAPKANLKECGLY

WALL014:Succinyl - AAVALLPAVLLALLAP KNNLKECGL-para-amino-F

WALL015: Succinyl – AAVALLPAVLLALLAPKQNLKECGLY

WALL016: Succinyl – AAVALLPAVLLALLAPKNNLKEVGLY

WALL023: AAVALLPAVLLALLAPYLGCEKLNNK

Previously (WO 00/78346) it has been shown that certain peptides designed and synthesized to include distinct importation competent signal peptides as a first segment at the N-terminus and the C-terminal sequences of $G\alpha i_3$ or $G\alpha t$ at the C-terminus as a second segment were active and are listed as follows:

Peptide 2: AAVALLPAVLLALLAPKNNLKECGLY

Peptide 2-succ: Succinyl-AAVALLPAVLLALLAPKNNLKECGLY

Pep 5: AAVALLPAVLLALLAPKENLKDCGLF

Peptide 2-Cyc:

AAVALLPAVLLALLAPKNNLKECGLY

ε-NH

In addition, the following peptide was previously synthesized and shown to be inactive: VTVLALGALAGVGVGKNNLKECGLY

This peptide was previously designated as Peptide 1 and is designated herein below as WALL009 for the sake of comparison to the novel peptides of the invention.

These known peptides disclosed and claimed previously are explicitly excluded from the present invention.

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The novel peptides were examined *in-vitro* for their ability to block compound 48/80 induced histamine secretion from purified rat peritoneal mast cells. Peptides which are active in this screening could therefore be useful for mast cell dependent allergies. Such allergies include but are not limited to those in which mast cell degranulation is mediated through the IgE-independent pathway from which the second segment of the above peptides was taken. Examples of such allergies include but are not limited to neurogenic inflammation in the skin and elsewhere, including but not limited to, acute urticaria, psoriases, psychogenic asthma, interstitial cystitis, bowel diseases, migraines, and multiple sclerosis.

The principles of the present invention are illustrated herein with the following examples, which are to be construed in a non-limitative manner. The skilled artisan will appreciate that many modifications and variations of the specific embodiments exemplified are possible within the scope of the present invention.

EXAMPLE 1
TESTING OF PEPTIDES *IN VITRO*

Peptides of the present invention, as described above, were tested *in vitro* for their ability to block histamine secretion from mast cells. Rat peritoneal mast cells were chosen as the experimental model, since it was previously shown that both rat peritoneal and human skin mast cells release histamine in response to substance P by an IgE-independent mechanism (Devillier et al., 1986; Foreman 1987a,b; Columbo et al., 1996). It was also demonstrated that the same peptidergic pathway is involved in both rat peritoneal and human cutaneous mast cells (Mousli et al., 1994; Emadi-Khiav et al., 1995).

Compound 48/80 was chosen as the allergen since it is one of the polycationic compounds, collectively known as the basic secretagogues of mast cells. Compound 48/80 has been shown to induce degranulation of human mast cells. In particular, it is

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very active on skin mast cells. Compound 48/80 has been used as a diagnostic agent *in vivo* to assess the release ability of human mast cells, to determine the effectiveness of drugs against chronic urticaria and to study itch and flare responses in atopic dermatitis (Kivity et al., 1988; Goldberg et al., 1991). Therefore inhibition of compound 48/80 induced histamine release is applicable and relevant to prevention of allergy induced by other basic secretagogues such as substance P, snake, bee and wasp venoms, bacterial toxins and certain drugs such as opiates.

The ability of each of the tested peptides to inhibit mast cell degranulation, when induced compound 48/80, was then tested. The experimental methods were as follows.

MATERIALS AND METHODS

Peptide synthesis

Peptides were synthesized by PolyPeptide laboratories (Wolfenbuttel, Germany). Peptides were synthesized by the solid phase methodology and supplied at > 95% purity. The correct composition and purity of the peptide were verified by HPLC, mass spectrometry and amino acid analysis. Lyophilized peptides were kept at $-20\ ^0$C. Peptides stock solutions (5 mg/ml in 10% dimethylsulfoxide (DMSO) in H_2O) were freshly prepared for each experiment.

Isolation and purification of mast cells

Mast cells from the peritoneal cavity of C.R rats were isolated in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 20 mM Hepes, 1.0 mM $CaCl_2$, 5.6 mM glucose, 1 mg/ml BSA, pH 7.2) and purified over a Ficoll gradient. A suspension of washed peritoneal cells was placed over a cushion of 30% Ficoll 400 (Pharmacia Biotech.) in buffered saline containing 0.1% BSA, and centrifuged at 150xg for 15 min. The purity of mast cells recovered from the bottom of the tube was > 90%, as assessed by toluidine blue staining.

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Triggering histamine secretion from intact cells

Purified mast cells (10^5 cells/0.5ml in duplicates) were incubated in Tyrode buffer with buffer or with desired concentrations of the indicated peptide for 2 h at 37^0C. Histamine secretion was subsequently stimulated by 0.1 μg/ml of compound 48/80 (Sigma) dissolved in Tyrode buffer. Incubation with compound 48/80 was carried out for 20 min at 37^0C. The reaction was terminated by placing the tubes on ice. The cells were sedimented by centrifugation at a brief spin (12,000g x 20s) and the supernatants were collected. The amount of histamine release was determined as previously described (Aridor et al., 1990). Briefly, cell pellets were lysed using 0.1 ml of 0.1N NaOH and the volume of each sample was adjusted to 0.5 ml by H_2O. Histamine content was assayed using the O-phthalaldehyde (OPT) fluorimetric method (Shore et al., 1959). Aliquots of 0.4 ml from the supernatants and cell lysates were incubated with 1.6 ml H_2O, 0.4 ml 1N NaOH and 0.1 ml of 10 mg/ml OPT in methanol, for 4 min. at room temperature. The reaction was terminated by the addition of 0.2 ml 3N HCl. Samples were centrifuged at 150xg for 5 min. and 0.2 ml samples were transferred to a 96 well plate. The histamine spectrofluorimetric assay was run in microplates using a microplate reader (FL-600, Biotek Instruments Winooski, VT, USA). Samples were excited by light at 340nm and read at 440nm. Histamine release was calculated as the percentage of total histamine content (supernatant / pellet + supernatant) in each sample. Each data point represents the average of duplicate measurements. The spontaneously released histamine was subtracted. Statistical analysis and plotting were done with Excel® (Microsoft Ltd., Washington, USA).

Activation of PTK (protein tyrosine kinase) and MAP Kinase

Purified mast cells (10^5 cells/0.5ml) were incubated in Tyrode's buffer in the presence of 0.1 mM vanadate in the absence or presence of 600 μg/ml of peptide 2 for 1h

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at 37^0C. The cells were triggered by 5 μg/ml of compound 48/80 (Sigma) dissolved in Tyrode's buffer or by H_2O_2/VO_3 for 20-min incubation period at 37^0C. At the end of incubation, the cells were sedimented and cells extracts were prepared. The samples were resolved by SDS/10% PAGE and immunoblotted with anti-phospho-Tyr and anti active
5 MAPK antibodies.

EXAMPLE 2
PEPTIDE MODIFICATIONS

Results disclosed previously (International Patent Application serial no. WO
10 00/78346) have demonstrated the ability of several peptides to block mast cell degranulation. For example, a novel peptide, that was designed and synthesized to include an importation competent signal peptide, as a first segment at the N-terminus (underlined), and the C-terminal sequence of $G\alpha i_3$ as a second segment at the C-terminus (AAVALLPAVLLALLAPKNNLKECGLY) inhibited histamine release from activated
15 mast cells.

The present invention is based on results of structure activity relationship studies using several novel peptides, in which point mutations or chemical modifications were introduced. These novel peptides were designed and tested to achieve the following aims:

Aim I: To improve biological efficacy.

20 Aim II: To increase peptide stability and/or solubility.

Aim III: To define amino acid residues which are essential for activity and therefore cannot be replaced without loss of activity.

Aim IV: To determine the structure/function relationships.

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To address these aims, we have synthesized novel peptides as demonstrated below.

1. Peptide WALL006 Computer modeling of the active molecule have demonstrated that the Asparagine at position 18 of the peptide, which is position 2 of the
5 active anti-allergic sequence, is important in order to preserve the cyclic 3-D structure of the active anti-allergic moiety within the peptide. According to the computerized model, a hydrogen bond links this Asparagine with the Tyrosine residue at position 26 (International Patent application WO 00/78346). To test this hypothesis, the Asparagine residue at position 18, was replaced by Glutamine to form peptide WALL006.

10 Peptide WALL006: AAVALLPAVLLALLAPKQNLKECGLY

In this example we have shown that replacement of the Asn with Glutamine (peptide WALL006) resulted in an active, though less potent peptide.

Further substitutions included replacing this Asparagine with Serine, Alanine, or
15 Glutamic acid as well as replacing the tyrosine at position 28 with Para-Amino-Phenyl alanine. All the mutated peptides were also synthesized with a succinyl group linked to their N-terminus in order to increase their solubility. The purpose of these substitutions was to evaluate the contribution of the putative hydrogen bond between the amino acid at position 18 and the Tyrosine residue and to compare the activities of peptides carrying at
20 position 18 either a neutral, or polar or charged amino acid.

2. Peptide WALL015 This sequence is identical to WALL006 but includes a Succinyl group at the N- terminus.

Peptide WALL 015: Succinyl-AAVALLPAVLLALLAPKQNLKECGLY

25

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3. Peptide WALL 011 The Asparagine residue at position 18, was replaced by Serine to form peptide WALL011.

Peptide WALL 011: Succinyl-AAVALLPAVLLALLAPKSNLKECGLY

5 **4. Peptide WALL 012** The Asparagine residue at position 18, was replaced by Glutamic Acid to form peptide WALL012.

Peptide WALL 012: Succinyl-AAVALLPAVLLALLAPKENLKECGLY

5. Peptide WALL 013 The Asparagine residue at position 18, was replaced by

10 Alanine to form peptide WALL013.

Peptide WALL 013: Succinyl-AAVALLPAVLLALLAPKANLKECGLY

***6. Peptide* WALL005** The Tyrosine residue at the C-terminal end of the peptide, at position 26, was replaced by para-amino-phenylalanine, which can also

15 form a hydrogen bond, in a similar fashion to the OH group in Tyrosine, to form peptide WALL005.

Peptide WALL005: AAVALLPAVLLALLAPKNNLKECGL-para-amino-F

7. Peptide WALL 014 A succinylated form of peptide WALL005.

20 Peptide WALL 014: Succinyl-AAVALLPAVLLALLAPKNNLKECGL-para-amino-F

8. Peptide WALL007 - In an attempt to improve peptide efficacy and also to avoid possible oxidation of the peptide, and thereby to increase its stability, the cysteine

25 residue at position 23 was replaced by valine, to form peptide WALL007.

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Peptide WALL007: AAVALLPAVLLALLAPKNNLKEVGLY

9. Peptide WALL016 - to the succinylated form of WALL007.

Peptide WALL016: Succinyl -AAVALLPAVLLALLAPKNNLKEVGLY

5

In order to assess the importance of the linkage between the two parts of the complex peptide and especially the importance for biological activity of the proline residue as the point of junction between the importation segment and the functional moiety, the following peptides were synthesized and tested.

10

10. Peptide WALL004 - The proline at position 16, at the point of junction between the importation segment and the functional moiety, was replaced by Alanine, to form peptide WALL004.

Peptide WALL004: AAVALLPAVLLALLAAKNNLKECGLY

15

To establish the importance of the rigid turn or bend as provided by the Proline three additional peptides were synthesized and tested for biological activity:

11. Peptide WALL008 In which Sarcosine replaces the Proline. The addition

20 of Succinyl again is to increase solubility.

Peptide WALL008: Succinyl-AAVALLPAVLLALLA-Sar-KNNLKECGLY

12. Peptide WALL009 This is a sequence that was shown previously to be inactive (disclosed in WO 00/78346), but contains the same active anti-allergic sequence

25 (last 10 amino acids) and has no solubility problems.

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Peptide WALL009: VTVLALGALAGVGVGKNNLKECGLY

13. Peptide WALL010 This is the same inactive sequence as in WALL009, but this novel peptide includes a Proline residue that is now connecting the leader sequence
5 to the active sequence. This peptide was synthesized to test whether inclusion of a rigid amino acid (proline) that forms a bend at the junction of the two segments may convert it into an active peptide.

Peptide WALL010: VTVLALGALAGVGVGPKNNLKECGLY

10

14. Peptide WALL023: In order to create a peptide that could serve as negative control to the active sequence of $G\alpha i_3$, the last 10 amino acids of peptide 2 were replaced by an anti-sense sequence.

Peptide WALL023: AAVALLPAVLLALLAPYLGCEKLNNK

15

EXPERIMENTAL RESULTS

1) Peptide WALL006: AAVALLPAVLLALLAPKQNLKECGLY

Incubation of purified intact mast cells *in vitro* with increasing concentrations of
20 Peptide WALL006 did not result in histamine secretion. In fact, incubation with the peptide resulted in inhibition of the basal level of histamine secretion, when compared to control cells (illustrated in Figure 1A). These results have indicated that Peptide WALL006 is unlikely to cause allergic side effects. Next, this peptide was tested for its ability to block compound 48/80 induced histamine secretion. For this purpose, mast cells
25 were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80.

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As shown in Figure 1B, peptide WALL006 blocked compound 48/80 induced histamine secretion in a dose dependent manner, with IC_{50} value of 560μg/ml and maximal inhibition of 57% at concentration of 600 μg/ml.

These results demonstrate that substitution of the Asparagine residue at position
5 18 with Glutamine, resulted in an active peptide, which inhibits histamine secretion from isolated mast cells. However Peptide WALL006, while still active, is less potent than the original, unmodified peptide (Peptide 2 in Patent application WO 00/78346).

2) Peptide WALL015: Succinyl-AAVALLPAVLLALLAPKQNLKECGLY

Incubation of purified intact mast cells *in vitro* with increasing concentrations of
10 peptide WALL015 did not result in histamine secretion (Fig 2A). In fact, incubation with the peptide resulted in minor inhibition of the basal level of histamine secretion, when compared to control cells (illustrated in Figure 2A). These results have indicated that peptide WALL015 is unlikely to cause allergic side effects. Next, this peptide was tested for its ability to inhibit the histamine secretion induced by compound 48/80. For this
15 purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 2B, peptide WALL015 blocked compound 48/80 induced histamine secretion in a dose dependent manner, with IC_{50} values of 300 μg/ml and maximal inhibition of 75% at concentration of 600 μg/ml.

These results demonstrated that a peptide sequence identical to WALL006 that
20 includes an addition of a Succinyl at the N- terminus, can serve as a more efficient blocker of histamine secretion from mast cells, as compared to the non-succinylated form.

3) Peptide WALL011: Succinyl-AAVALLPAVLLALLAPKSNLKECGLY

Incubation of purified intact mast cells *in vitro* with increasing concentrations of peptide WALL011 did not result in histamine secretion (Fig 3A). Moreover, incubation

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with the peptide at concentration of 400-600 μg/ml resulted in a minor inhibition of the basal level of histamine secretion, when compared to control cells (illustrated in Figure 3A). These results have indicated that peptide WALL011 is unlikely to cause allergic side effects. Next, this peptide was tested for its ability to inhibit the histamine secretion
5 induced by compound 48/80. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 3B, peptide WALL011 inhibited compound 48/80 induced histamine secretion in a dose dependent manner, with IC_{50} values of 160 μg/ml and maximal inhibition of 87% at concentration of 600 μg/ml.

10 These results demonstrate that substitution of Asparagine residue at position 18 in the peptide sequence with Serine, resulted in an active peptide, which inhibits histamine secretion from isolated mast cells.

4) Peptide WALL012: Succinyl-AAVALLPAVLLALLAPKENLKECGLY

Incubation of purified intact mast cells *in vitro* with increasing concentrations of
15 peptide WALL012 did not result in histamine secretion (Fig 4A). Furthermore, incubation with the peptide resulted in a minor inhibition of the basal level of histamine secretion, when compared to control cells (illustrated in Figure 4A). These results have indicated that peptide WALL012 is unlikely to cause allergic side effects. Next, this peptide was tested for its ability to inhibit the histamine secretion induced by compound 48/80. For
20 this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 4B, peptide WALL012 blocked compound 48/80 induced histamine secretion in a dose dependent manner, with IC_{50} values of 320 μg/ml and maximal inhibition of 97.5% at concentration of 600 μg/ml.

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These results demonstrate that substitution of Asparagine residue at position 18 with Glutamic acid, resulted in an active peptide, which inhibits histamine secretion from isolated mast cells.

5) Peptide WALL013: Succinyl-AAVALLPAVLLALLAPKANLKECGLY

5 Incubation of purified intact mast cells *in vitro* with increasing concentrations of peptide WALL013 did not result in histamine secretion (Fig 5A). In fact, incubation with the peptide resulted in a minor inhibition of the basal level of histamine secretion, when compared to control cells (illustrated in Figure 5A). These results have indicated that peptide WALL013 is unlikely to cause allergic side effects. Next, this peptide was tested 10 for its ability to inhibit histamine secretion induced by compound 48/80. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 5B, peptide WALL013 blocked compound 48/80 induced histamine secretion in a dose dependent manner, with IC_{50} values of 245 μg/ml and maximal inhibition of 100% at concentration of 600 μg/ml.

15

Results obtained with peptides WALL006, WALL011, WALL012 WALL013 and WALL015 demonstrate that replacement of the Asparagine at position 18 with one of the following: Glutamine, Serine, Glutamic acid or Alanine result in active peptides that significantly inhibit histamine secretion from mast cells. Since Asparagine, Glutamine, 20 Serine, and Glutamic acid are capable of forming a hydrogen bond with the tyrosine residue located at the C-terminal end of the peptide, it is suggested that the formation of a cyclic three-dimensional structure might be mediated by this bond. However since Alanine is not capable of forming a hydrogen bond and yet results in an active peptide we assume that other connections are also involved and contribute to the formation of the 25 active cyclic three-dimensional structure.

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6) Peptide WALL005: AAVALLPAVLLALLAP NNLKECGL-para-amino-F

Incubation of purified intact mast cells *in vitro* with increasing concentrations of peptide WALL005 resulted in histamine secretion (Fig 6A). These results have indicated
5 that peptide WALL005 is likely to cause allergic side effects. Next, this peptide was tested for its ability to inhibit histamine secretion induced by compound 48/80. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 6B, peptide WALL005 had no effect on compound 48/80 induced histamine secretion. These results indicate that
10 replacement of the tyrosine residue at the C-terminus with para-amino-F interferes with the activity of the peptide. Since peptide WALL005 had severe solubility problems, peptide aggregation may have accounted for the observed effects. Therefore, the activity of the succinylated form of this peptide was tested as well.

15 **7) Peptide WALL014**: Succinyl-AAVALLPAVLLALLAPKNNLKECGL-para-amino-F

Peptide WALL014 is identical to peptide WALL005 except for an additional Succinyl group at the N- terminus.

Incubation of purified intact mast cells *in vitro* with increasing concentrations of
20 peptide WALL014 did not result in histamine secretion (Fig 7A). These results have indicated that peptide WALL014 is unlikely to cause allergic side effects. Next, this peptide was tested for its ability to inhibit histamine secretion induced by compound 48/80. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 7B, peptide
25 WALL014 blocked compound 48/80 induced histamine secretion in a dose dependent

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manner, with IC_{50} values of 230 μg/ml and maximal inhibition of 83% at concentration of 600 μg/ml.

These results indicate that a soluble peptide, in which the Tyrosine residue at the C-terminal position 26 was replaced with para-amino-F, maintains its biological activity,
5 that is to block histamine release induced by c48/80 and it has no side effects by itself.

These results may suggest that maintaining the biological activity of the peptide requires a C-terminal amino acid which includes an aromatic ring and a hydrogen bond forming head group.

8) Peptide WALL007: AAVALLPAVLLALLAPKNNLKEVGLY

10 Incubation of purified intact mast cells *in vitro* with increasing concentrations of Peptide WALL007 did not result in histamine secretion. In fact, incubation with the peptide resulted in inhibition of the basal level of histamine secretion, when compared to control cells (illustrated in Figure 8A). These results have indicated that Peptide WALL007 is unlikely to cause allergic side effects. Next, this peptide was tested for its
15 ability to block compound 48/80 induced histamine secretion. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80.

As shown in Figure 8B peptide WALL007 blocked compound 48/80 induced histamine secretion in a dose dependent manner. A potent inhibition was already
20 demonstrated at a concentration of 400 μg/ml, while maximal inhibition was demonstrated at a concentration of 600 μg/ml. Under these conditions, the level of histamine secretion was lower than the basal level of histamine secretion in control cells.

These results demonstrate that substitution of the cysteine residue at position 23 with valine, while reducing the risk of possible oxidation of the peptide, increases peptide

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efficacy. The IC_{50} was reduced from 400 μg/ml for the unmodified peptide (Peptide 2 in Patent application WO 00/78346) to 230 μg/ml for peptide WALL007 as shown in Figure 8B. Therefore peptide WALL007 (AAVALLPAVLLALLAPKNNLKEVGLY) is a novel potent inhibitor of mast cell degranulation.

5 From these results it would appear that the amino acid located at position 23 can be replaced by Valine demonstrating improved efficacy. However, we have previously shown that substitution of the cysteine residue with serine, that formed the sequence AAVALLPAVLLALLAPKNNLKESGLY, resulted in loss of activity of the entire peptide (Patent application WO 00/78346). Therefore, we claim that an active peptide,
10 which inhibits mast cell degranulation, should contain at position 23 Cysteine or a stable isosteric residue which is not prone to oxidation or any chemical modification, such as Valine , as an essential condition for peptide activity

9) Peptide WALL016: Succinyl- AAVALLPAVLLALLAPKNNLKEVGLY

Incubation of purified intact mast cells *in vitro* with increasing concentrations of
15 peptide WALL016 did not result in histamine secretion (Fig 9A). In fact, incubation with the peptide resulted in inhibition of the basal histamine secretion, when compared to control cells (illustrated in Figure 9A). These results have indicated that peptide WALL016 is unlikely to cause allergic side effects. Next, this peptide was tested for its ability to inhibit histamine secretion induced by compound 48/80. For this purpose, mast
20 cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 9B, peptide WALL016 blocked compound 48/80 induced histamine secretion in a dose dependent manner, with IC_{50} values of 295 μg/ml and maximal inhibition of 79.6% at a concentration of 600 μg/ml.

These results indicate that replacement of the Cysteine residue at position 23 with
25 valine, in conjunction with the addition of a succinyl residue at the N-terminus of the

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peptide, results in an active peptide demonstrating the ability to block histamine secretion from mast cells.

The next set of peptides were synthesized and analyzed in order to demonstrate the importance of the type of linkage which connects between the two segments of the
5 complex peptide, that is the connection between the importation and the functional sequences. In particular, to assess the importance for biological activity of the proline residue as the point of junction between the importation segment and the functional moiety.

10) Peptide WALL004: AAVALLPAVLLALLAAKNNLKECGLY

10 Incubation of purified intact mast cells *in vitro* with increasing concentrations of Peptide WALL004 resulted in moderate histamine secretion, especially at a peptide concentration of exceeding 200 μg/ml (demonstrated in Figure 10A). These results suggested that this peptide is likely to cause only minor or no allergic side effects and can therefore serve as a potential inhibitor of mast cell exocytosis.

15 The peptide was also tested for its ability to block compound 48/80 induced histamine secretion. Mast cells were incubated with increasing concentrations of the peptide, followed by induction of histamine secretion by compound 48/80.

As shown in Figure 10B, throughout the range of concentrations tested, peptide WALL004 failed to block histamine secretion induced by compound 48/80. These results
20 demonstrate that substitution of the proline residue at position 16 with alanine caused a complete loss of the desired activity of the peptide. Therefore, we suggest that the amino acid proline, or any other natural or non-natural amino acid or covalent bond or moiety that would link covalently the importation segment (competent for cell penetration) with the functional segment (active in reducing or abolishing mast cell degranulation) in a
25 manner, which gives rise to a bend or turn, is essential for the maintenance of the desired

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peptide activity. Examples include proline mimetics, N-alkylated or N-methylated amino acids at this position, double or triple bonds or the like.

In addition to proline, specific examples of moieties which induce suitable conformations include but are not limited to N-methyl amino acids such as sarcosine; 5 hydroxy proline; anthranilic acid (2-amino benzoic acid); and 7-azabicyloheptane carboxylic acid.

11) Peptide WALL008: Succinyl-AAVALLPAVLLALLA-Sar-KNNLKECGLY

Incubation of purified intact mast cells *in vitro* with increasing concentrations of peptide WALL008 did not result in histamine secretion. In fact, incubation with the 10 peptide resulted in inhibition of the basal level of histamine secretion, when compared to control cells (illustrated in Figure 11A). These results have indicated that peptide WALL008 is unlikely to cause allergic side effects. Next, this peptide was tested for its ability to block compound 48/80 induced histamine secretion. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with 15 compound 48/80. As shown in Figure 11B, peptide WALL008 blocked compound 48/80 induced histamine secretion in a dose dependent manner, with IC_{50} values at concentration of 220 μg/ml and maximal inhibition of 98.7% at concentration of 600 μg/ml.

These results demonstrate that substitution of the proline residue at position 16 in the peptide sequence with sarcosine, which, like the proline residue, introduces a 20 conformational constraint in the peptide backbone, results in an active peptide, which inhibits histamine secretion from isolated mast cells.

12) Peptide WALL009: VTVLALGALAGVGVGKNNLKECGLY

Incubation of purified intact mast cells *in vitro* with increasing concentrations of peptide WALL009 resulted in histamine secretion as a function of the peptide 25 concentration (Fig 12A). These results have indicated that peptide WALL009 is a potent

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secretagogue of mast cells which is likely to cause allergic side effects. This peptide was also tested for its ability to block compound 48/80-induced histamine secretion. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 12B, peptide WALL009 did not
5 inhibit histamine release, induced by compound 48/80.

These results confirm our previous results (peptide 1 in WO 00/78346) demonstrating that peptide WALL009, which includes the leader motif of the signal sequence within human integrin β3, and the C-terminal sequence of $G\alpha i_3$, with no proline residue linking these two parts is inactive.

10 **13) Peptide WALL010:** VTVLALGALAGVGVGPKNNLKECGLY

Incubation of purified intact mast cells *in vitro* with increasing concentrations of peptide WALL010 resulted in histamine secretion as a function of peptide concentration (Fig 13A). These results have indicated that peptide WALL010 is a potent secretagogue of mast cells and is therefore likely to cause allergic side effects. Next, this peptide was
15 tested for its ability to block compound 48/80 induced histamine secretion. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their trigger with compound 48/80. As shown in Figure 13B, peptide WALL010 did demonstrate a mild inhibition of histamine release, induced by compound 48/80, with maximal inhibition of 16.7% at a concentration of 200μg/ml.

20 These results demonstrate that the addition of the proline residue, at the point of junction between the importation segment and the functional moiety has succeeded in converting an inactive peptide (WALL009), which by itself exhibited mast cell secretagogue activity, into an active peptide capable of inhibiting histamine secretion induced by compound 48/80. In this case the ability of the active sequence to inhibit
25 histamine secretion, might be masked by the secretagogue activity of the leader sequence

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(as demonstrated in peptide WALL010), therefore resulting in only mild inhibition and efficacy. Nevertheless, it is evident that the addition of the proline residue at the point of linkage between the importation segment and the functional segment resulted in a significant shift in the peptide activity from a potent mast cell secretagogue into an
5 inhibitor of histamine secretion.

14) Peptide WALL023: AAVALLPAVLLALLAPYLGCEKLNNK

Incubation of purified intact mast cells *in vitro* with 600 μg/ml of peptide WALL023 did not result in histamine secretion. These results have indicated that peptide WALL023 is unlikely to cause allergic side effects. Next, this peptide was tested for its
10 ability to inhibit histamine secretion induced by compound 48/80. For this purpose, mast cells were incubated with increasing concentrations of the peptide, prior to their being triggered with compound 48/80. As shown in Figure 14, peptide WALL023 had no effect on compound 48/80 induced histamine secretion.

These results indicate that the peptide that comprises the non-active sequence of
15 Gi_3 (anti-sense sequence) is not able to inhibit the histamine secretion induced by compound 48/80, indicating that blocking the histamine release, induced by compound 48/80 is specific and is dependent on Gi_3 activation.

15) Inhibition of late phase inflammatory responses via protein kinases

Experiments were conducted in order to demonstrate specific inhibition by
20 peptides of the invention of protein tyrosine kinases (PTKs) and the Mitogen-activated protein kinases (MAPKs) activation after exposure to basic secretagogues. Purified intact mast cells were incubated with 600 μg/ml of peptide 2 and the activation of protein tyrosine kinase (PTK) and Mitogen-activated protein kinase (MAPK) was validated. The results demonstrate an inhibition by Peptide 2 of PTKs and MAPKs activation induced by
25 compound 48/80 (fig 15A and 16A), a basic secretagogue that activates directly the

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pertussis toxin sensitive Gi_3. In contrast, peptide 2 did not inhibit the activation of protein tyrosine kinases and MAPKs induced by H_2O_2/VO_3 (Fig 15B, and 16B) that stimulates protein tyrosine phosphorylation in a pertussis toxin insensitive fashion by inhibiting protein tyrosine phosphatases.

5 These results indicate that peptide 2 inhibits, in addition to histamine release, the activation of PTKs and MAPKs induced by basic secretagogues. Activation of these protein kinases was demonstrated previously as a crucial event, leading to activation of the late phase inflammatory reaction such as synthesis *de novo* of leukotrienes and prostaglandins. Therefore, our results indicate that this peptide inhibited also the pathway 10 that contributes to the *de novo* production of inflammatory mediators such as leukotrienes and prostaglandins. We have also demonstrated that peptide 2 inhibited a specific pertussis toxin sensitive activation of PTKs and MAPKs that can be dependent on Gi_3 activation.

15 The aforementioned results, demonstrated by peptides WALL004, WALL008, WALL009 and WALL010 confirm that the linker is a crucial element of the present invention, whereby the linker must impose conformational constraints at or near the junction of the two segments of the molecule to yield a biologically active entity. Therefore, the first segment must be connected to the second segment through a linker or 20 a direct bond, whereby the linker creates a conformational constraint, by forming a bend or turn. Examples include but are not limited to, residues such as proline, or proline mimetic or N-methyl amino acids such as sarcosine or any other moiety which introduces a rigid bend into the peptide backbone.

Table 1 summarizes the results obtained in the *in vitro* system.

25

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Table 1: Summary of *in vitro* experiments monitoring histamine secretion from isolated mast cells, following incubation with the following peptides

Peptide	Sequence	Secretagogue*	Inhibitor**	IC_{50} (μg/ml)	Remarks
WALL006	AAVALLPAVLLALLAPKQNLKECGLY	-	+	560	solubility problem
WALL015	Succinyl-AAVALLPAVLLALLAPKQNLKECGLY	-	++	300	Good solubility
WALL011	Succinyl-AAVALLPAVLLALLAPKSNLKECGLY	-	+++	160	Good solubility
WALL012	Succinyl-AAVALLPAVLLALLAPKENLKECGLY	-	+++	320	Good solubility
WALL013	Succinyl-AAVALLPAVLLALLAPKANLKECGLY	-	+++	245	Good solubility
WALL005	AAVALLPAVLLALLAPKNNLKECGL-para- amino-F	+	-	-	Solubility problem
WALL014	Succinyl-AAVALLPAVLLALLAPKNNLKECGL-para-amino-F	-	+++	230	Good solubility
WALL007	AAVALLPAVLLALLAPKNNLKEVGLY	-	+++	230	Solubility problem
WALL016	Succinyl-AAVALLPAVLLALLAPKNNLKEVGLY	-	++	295	Good solubility
WALL004	AAVALLPAVLLALLAKNNLKECGLY	-/+	-	-	Solubility problem
WALL008	Succinyl-AAVALLPAVLLALLA-Sar-KNNLKECGLY	-	+++	220	Good solubility
WALL009	VTVLALGALAGVGVGKNNLKECGLY	+	-	-	Good solubility
WALL010	VTVLALGALAGVGVGPKNNLKECGLY	+	- / +	-	Good solubility
WALL023	AAVALLPAVLLALLAPYLGCEKLNNK	_	_	_	Good solubility

* Histamine secretion following incubation of mast cell with different concentrations of each peptide: - No side effect of histamine secretion. + Peptide that induce histamine secretion (Secretagogue).

** Extent of inhibition of histamine secretion from mast cells, followed by incubation with different concentrations of each peptide and induction of the allergic

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reaction. +++ Potent inhibitor (≥80% inhibition), ++ Moderate inhibitor (50% - 70% inhibition), + Poor inhibitor (≤ 50% inhibition), - No inhibition.

5

EXAMPLE 3
TESTING THE EFFECTS OF THE TREATMENT OF THE PRESENT INVENTION *IN VIVO*

The ability of peptides according to the present invention to block allergic reaction *in vivo* was tested on the skin of rats by using compound 48/80 as the allergen. Peptides
10 WALL007, WALL008, WALL012, WALL013, WALL014, WALL015 and WALL016 that were demonstrated to be effective in vitro, are shown to effectively block the allergic response *in vivo*.

WALL007: AAVALLPAVLLALLAPKNNLKEVGLY
15
WALL008: Succinyl -AAVALLPAVLLALLA-Sar-KNNLKECGLY

WALL012: Succinyl – AAVALLPAVLLALLAPKENLKECGLY
20
WALL013: Succinyl - AAVALLPAVLLALLAPKANLKECGLY

WALL014: Succinyl - AAVALLPAVLLALLAP KNNLKECGL-para-amino-F

WALL015: Succinyl – AAVALLPAVLLALLAPKQNLKECGLY
25
WALL016: Succinyl – AAVALLPAVLLALLAPKNNLKEVGLY

The experimental method is described below.

30

MATERIALS AND METHODS

The hair of the abdominal area of CR rats was carefully removed with an electric clipper and a depilatory cream. In each animal the abdominal area was divided to six equal zones that were marked by pen. Each zone was either subjected to peptide treatment or served as a control. The peptide was injected intradermally as follows: 20 µl

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of peptide solution at different concentrations (dissolved in 10% DMSO in DDW) were injected intradermally to an indicated abdominal area using a 27-gauge sterile needle.

Skin tests were performed 0.5, or 1 hour following application of the peptide. Skin tests were performed by injecting intradermally 20 μl of the allergen (0.1 mg/ml
5 compound 48/80 dissolved in DDW) or DDW alone (Vehicle), into the center of each marked area on the abdominal skin using a 27-gauge sterile needle. The allergic response was monitored by outlining with a marker the wheals which developed in response to allergen or vehicle treatment.

To quantitate the skin test results, the marker signs were transferred onto paper
10 with scotch tape. The areas of the wheals were outlined and calculated by a computerized software (NIH-Image).

EXPERIMENTAL RESULTS

The area of the wheals which developed in response to topical application of the
15 test peptide followed by compound 48/80 or saline injection are recorded.

Tables 2-8 presents the mean areas of the wheals, which developed in response to intradermal injection of each of the tested peptides, followed by either compound 48/80 or DDW injection applied after 0.5 or 1 hour. Two doses were tested for each peptide - 20 and 200 μg (injection of 20 μl from a stock solution of 1 mg/ml or 10 mg/m respectively).
20 Mean wheal areas were calculated for each treatment and the significance of the results was determined using student's T-test.

Table 2 The results presented in Table 2 demonstrate that intradermal injection of **Peptide WALL007** reduced the allergic reaction in a dose dependent manner, reaching
25 significant inhibition when administered 0.5 or 1 hour before the allergic induction. These

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results therefore indicate that **Peptide WALL007** has the potential to block allergic reactions in vivo

Table 3 The results presented in Table 3 demonstrate that intradermal injection of

5 **Peptide WALL016** reduced compound 48/80 induced allergic reaction in a dose dependent manner, reaching significant inhibition at both 0.5 and 1 hour before the allergic induction. These results therefore indicate that **Peptide WALL0016** has the potential to block allergic reactions in vivo.

10 Table 4 The results presented in Table 4 demonstrate that intradermal injection of **Peptide WALL008** reduced the allergic reaction in a dose dependent manner, reaching significant inhibition at 0.5 hour before the allergic induction. These results therefore indicate that **Peptide WALL008** has the potential to block allergic reactions in vivo. .

15 Table 5 The results presented in Table 5 demonstrate that intradermal injection of **Peptide WALL012** reduced compound 48/80-induced allergic reactions in a dose dependent manner, reaching significant inhibition at 0.5 hour before the allergic induction. Therefore, **Peptide WALL012** has the potential to block allergic reactions in vivo.

20

Table 6 The results presented in Table 6 demonstrate that intradermal injection of **Peptide WALL013** significantly reduced compound 48/80-induced allergic reaction at concentrations of 1 mg/ml and 10 mg/ml, when applied 0.5 hour before induction of the allergic reaction. Peptide WALL013 therefore has the potential to block allergic reactions

25 in vivo.

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A representative experiment (depicted in Table 7) demonstrates that intradermal injection of Peptide **WALL015** blocked compound 48/80-induced allergic reaction in vivo. **Peptide WALL015** reduced the allergic reaction at concentration of 1 and 10 1 and 10 mg/ml , when applied 0.5 hour before induction of the allergic induction.

5

The results presented in Table 8 demonstrate that intradermal injection of **Peptide WALL014** *significantly reduced the allergic reaction evoked by compound 48/80* at a concentration of 1 mg/ml, when applied 0.5 hour before induction of the allergic reaction. In contrast, when applied 1 hour before compound 48/80, no significant inhibition was

10 demonstrated (data not shown). **Peptide WALL014** therefore has the potential to block allergic reactions in vivo.

It is noteworthy that intradermal injection of each peptide alone exerted no stimulatory effect on the cutaneous allergic reactions, thus indicating that each compound by itself is not allergenic (see Tables 2-8).

15

Table 2: Mean Wheal Area (mm^2±STD) in Response to Intradermal Injection of Peptide WALL007 followed by compound 48/80.

20

A. Intradermal injection of Peptide WALL007, 0.5 hour before allergic induction

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	74.5 ± 18.0 (n=12)	76.5 ± 14.1 (n=12)	74.5 ± 26.4 (n=12)
Compound 48/80	141.4 ± 29.7 (n=12)	116.2 ± 22.8*(n=12)	99.0 ± 32.4** (n=12)

25 **B. Intradermal injection of Peptide WALL007, 1 hour before allergic induction**

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	67.5 ± 20.5 (n=14)	62.4 ± 11.1 (n=15)	65.9 ± 10.0 (n=11)
Compound 48/80	113.4 ± 30.5 (n=14)	86.7 ± 21.8**(n=15)	95.4 ± 21.2*(n=12)

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* $p < 0.05$ as compared to positive control group (Compound 48/80).
** $p < 0.01$ as compared to positive control group (Compound 48/80)
All vehicle groups are significantly different form the positive control groups (Compound 48/80, $p<0.01$)

5

Table 3: Mean Wheal Area (mm^2±STD) in Response to Intradermal Injection of Peptide WALL016 followed by compound 48/80.

10

A. Intradermal injection of Peptide WALL016, 0.5 hour before allergic induction

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	88.2 ± 19.6 (n=5)	66.4 ± 12.5 (n=5)	72.6 ± 16.2 (n=5)
Compound 48/80	142.6 ± 39.3 (n=5)	104.2 ± 21.6* (n=5)	1.2 ± 7.9 **(n=5)

15

B. Intradermal injection of Peptide WALL016, 1 hour before allergic induction

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	79.3 ± 24.3 (n=6)	62.8 ± 14.6 (n=6)	69.3 ± 17.0 (n=6)
Compound 48/80	151.4 ± 17.0 (n=6)	96.3 ± 15.6**(n=6)	82.1 ± 27.2**(n=6)

* $p < 0.05$ as compared to positive control group (Compound 48/80).
** $p < 0.01$ as compared to positive control group (Compound 48/80).
All Vehicle groups are significantly different form the positive control groups
20 (Compound 48/80) at 0.5 hour- $p < 0.05$, and at 1 hour - $p<0.01$.

Table 4: Mean Wheal Area (mm^2±STD) in Response to Intradermal
25 **Injection of Peptide WALL008 followed by compound 48/80.**

Intradermal injection of PeptideWALL008, 0.5 hour before allergic induction

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	63.7 ± 16.8 (n=4)	79.9 ± 22.3 (n=4)	72.8 ± 13.6 (n=4)
Compound 48/80	128.0 ± 5.7 (n=4)	104.1 ± 18.5*(n=4)	73.7 ± 12.2**(n=4)

* $p < 0.05$ as compared to positive control group (Compound 48/80).
30 ** $p < 0.01$ as compared to positive control group (Compound 48/80)
All vehicle groups are significantly different form the positive control groups (Compound 48/80, $p<0.01$).

35

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Table 5: Mean Wheal Area ($mm^2 \pm STD$) in Response to Intradermal Injection of Peptide WALL0012 followed by compound 48/80.

5

Intradermal injection of PeptideWALL012, 0.5 hour before allergic induction

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	70.0 ± 13.7 (n=5)	71.6 ±13.8 (n=5)	66.7 ± 10.1 (n=5)
Compound 48/80	128.1 ± 14.5 (n=5)	104.8 ± 12.6 *(n=5)	75.1 ± 8.5 (n=5) **

* $p < 0.05$ as compared to positive control group (Compound 48/80).
** $p < 0.01$ as compared to positive control group (Compound 48/80)
10 All vehicle groups are significantly different form the positive control groups (Compound 48/80, $p < 0.01$)

Table 6: Mean Wheal Area ($mm^2 \pm STD$) in Response to Intradermal Injection of
15 **Peptide WALL013 followed by compound 48/80.**

Intradermal injection of Peptide WALL013, 0.5 hour before allergic induction

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	72.4 ± 16.2 (n=4)	74.7 ± 12.0 (n=4)	77.3 ±13.5 (n=4)
Compound 48/80	146.4 ± 28.5 (n=4)	89.7 ± 13.7 (n=4) **	96.4 ± 28.4 (n=4)*

20 * $p < 0.05$ as compared to positive control group (Compound 48/80).
** $p < 0.01$ as compared to positive control group (Compound 48/80)
All vehicle groups are significantly different form the positive control groups (Compound 48/80, $p < 0.01$).

25

Table 7: A Representative Experiment Demonstrating the Wheal Area (mm^2) in Response to Intradermal Injection of Peptide WALL015 followed by compound 48/80.

30

Intradermal injection of Peptide WALL015, 0.5 hour before allergic induction

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	98.3	110.4	89.7
Compound 48/80	151.5	100.1	75.3

35

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Table 8: Mean Wheal Area ($mm^2 \pm STD$) in Response to Intradermal Injection of Peptide WALL014 followed by compound 48/80.

5

Intradermal injection of Peptide WALL014, 0.5 hour before allergic induction

	Peptide Concentration (mg/ml)		
	0	**1**	**10**
Vehicle	96.6 ± 8.6 (n=2)	87.2 ± 1.9 (n=2)	100.1 ± 38.9 (n=2)
Compound 48/80	153.7 ± 13.4 (n=2)	108.1 ± 15.0*(n=2)	91.5 ± 34.9 (n=2)

* $p < 0.05$ as compared to positive control group (Compound 48/80).

10

The in vivo results demonstrated above, further reinforce the *in vitro* results, demonstrating that the active peptides according to the invention have the potential to also block allergic reactions *in vivo*, such as the cutaneous allergic reactions.

15

EXAMPLE 4
METHODS AND COMPOSITIONS FOR ADMINISTRATION

The peptides of the present invention, and their homologues or related compounds, hereinafter referred to as the "therapeutic agents of the present invention", can
20 be administered to a subject by various routes of administration, which are well known in the art. Hereinafter, the term "therapeutic agent" includes a peptide as previously defined, in particular peptides exemplified herein and/or homologues, analogues or mimetics thereof, or any biologically active substance having a substantially similar effect as previously defined.

25 Hereinafter, the term "subject" refers to the human or lower animal to whom the therapeutic agent is administered. For example, administration may be done topically (including ophthalmically, vaginally, rectally, intranasally and by inhalation), orally, or parenterally, for example by intravenous drip or intraperitoneal, subcutaneous, or

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intramuscular injection.

Formulations for topical administration may include but are not limited to lotions, ointments, gels, creams, suppositories, drops, liquids, sprays and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like
5 may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, sachets, capsules or tablets. Thickeners, diluents, flavorings, dispersing aids, emulsifiers or binders may be desirable.

Formulations for parenteral administration may include but are not limited to
10 sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

Dosing is dependent on the severity of the symptoms and on the responsiveness of the subject to the therapeutic agent. Persons of ordinary skill in the art can easily determine optimum dosages, dosing methodologies and repetition rates.

15

EXAMPLE 5
METHOD OF TREATMENT OF ALLERGIC CONDITIONS

As noted above, the therapeutic agents of the present invention have been shown to be effective inhibitors of the allergic process by blocking mast cell
20 degranulation, thereby preventing and/or alleviating an allergenic condition. The following example is an illustration only of a method of treating an allergenic condition with the therapeutic agent of the present invention, and is not intended to be limiting.

The method includes the step of administering a therapeutic agent, in a pharmaceutically acceptable carrier as described in Example 4 above, to a subject to be
25 treated. The therapeutic agent is administered according to an effective dosing

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methodology, preferably until a predefined endpoint is reached, such as the absence of a symptom of the allergenic condition in the subject, or the prevention of the appearance of such a symptom in the subject.

Allergic conditions for which the therapeutic agents of the present invention are useful include, but are not limited to, nasal allergy, irritation or allergic reactions in the eyes, allergic reactions in the skin including any type of allergen-induced rash or other skin irritation or inflammation, acute urticaria, psoriasis, psychogenic or allergic asthma, interstitial cystitis, bowel diseases, migraines, and auto-immune diseases such as multiple sclerosis.

EXAMPLE 6
METHODS FOR MANUFACTURING
THE THERAPEUTIC COMPLEX OF THE PRESENT INVENTION

The therapeutic complex of the present invention can be manufactured in various ways. For example, if the therapeutic complex includes a peptide for at least one the first segment and the second segment, or if the entire therapeutic complex is a peptide, then such a peptide could be manufactured by peptide synthetic methods which are well known in the art.

Alternatively, such a peptide could be produced by linking the signal sequence and the biologically active moiety through laboratory techniques for molecular biology which are well known in the art.

By way of illustration , as a non-limitative example, a recombinant fusion protein could be prepared which would feature the peptide permeabilization sequence in the N-terminus and the C-terminal moiety of $G\alpha_t$ or $G\alpha_{i3}$, preferably including the last 10 amino acids, for production in bacteria. For this purpose, DNA sequences coding for the desired peptides are amplified by PCR and purified. After sequence verification, these DNA

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sequences are ligated and cloned in an appropriate vector. The resulting recombinant plasmid is expressed in E. coli and the recombinant proteins purified from bacterial extracts.

EXAMPLE 7

5 CONFORMATIONAL ANALYSIS AND COMPUTATIONAL PROTOCOLS

Conformation Sampling

As a full enumeration of all the possible conformations of a 10-residues peptide is impractical, a sampling procedure must be applied in order to generate a representative sample of the molecule's conformation space. Many methods are available for sampling 10 molecular conformations, each harboring advantages and limitations. The sampling procedure adopted for the present study stems from the tendency to get the most stable conformation of a peptide at physiological pH with reasonable time. To accomplish this goal a two-step sampling procedure was applied. First, conformations are sampled from a high temperature molecular dynamics trajectory at 1000 K. Then each of the sampled high 15 temperature conformations is gradually annealed down to 300 K using molecular dynamics. After the cooling step the energy of each conformation was quenched by direct minimization. The annealed and minimized conformations constitute the conformation sample of that molecule. The gradual annealing guarantees that the resulting conformations will indeed be on the 300 K manifold (i.e., are accessible at 300 K), while 20 the high temperature sampling allows us to cross high-energy barriers.

Technically, each sampling procedure starts with a 500 ps molecular dynamics trajectory at 1000 K (simulated using 2 fs timesteps). Conformations are sampled along the high temperature trajectory every 1 ps, resulting in a total of 500 conformations. Short molecular dynamic trajectories (simulated at 1 fs timesteps) are then applied to cool each 25 of the high temperature conformations down to 300K (temperature decreases at 100 K

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steps). Following the cooling phase each structure is minimized by a combined protocol consisting of 200 Steepest Decent steps followed by Adopted Basis Newton-Raphson (ABNR) minimization until a total gradient of 0.01 is reached. The representation of the molecular dynamics and the various energy calculations were performed with the
5 CHARMM program and the CHARMM all atom forcefield. No explicit water molecules were included, no energy cutoffs were applied and a distance dependent dielectric constant was used. In each conformational sample the conformation with the lowest energy was selected to represent the most stable conformation of the sequence.

10 **Molecular systems**

Four 10-residues peptides analogs were studied. The peptides were with neutral N-terminal and with negative charge at the C-terminal. The initial conformations used in the sampling process of all peptides were the fully extended conformations. However, since the difference between peptide c and peptide b and between peptide d and peptide a is
15 only in one residue an additional sampling was applied on peptides 3 and 4. These additional samplings for peptides c and d were based on the most stable conformation of peptides b and a, respectively.

Peptide a ($G\alpha i_3$): NH_2-Lys-Asn-Asn-Leu-Lys-Glu-Cys-Gly-Leu-Tyr-CO_2^-

Peptide b ($G\alpha i_2$): NH_2-Lys-Asn-Asn-Leu-Lys-Asp-Cys-Gly-Leu-Phe-CO_2^-

20 Peptide c3 ($G\alpha_4$): NH_2-Lys-Glu-Asn-Leu-Lys-Asp-Cys-Gly-Leu-Phe-CO_2^-

Peptide d: NH_2-Lys-Asn-Asn-Leu-Lys-Glu-Ser-Gly-Leu-Tyr-CO_2^-

The effect of solvation was explored only on peptide 1. This simulation was performed using the CHARMM molecular dynamics program. The simulations used 1 fs timesteps, the SHAKE constraints on bonds to hydrogen atoms, a dielectric constant of
25 ε=1, and a 15 Å energy cutoff. The peptides were embedded in a 14 Å sphere of TIP3

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water molecules, using stochastic boundary conditions. The water sphere was added in two steps, each of which involved overlaying a sphere of equilibrated water molecules at a random orientation followed by 20 ps of equilibration at 300 K. In this simulation 305 water molecules were added to the model in the first step, and 6 water molecules were
5 added in the second step, resulting in a total of 311 water molecules. The total number of atoms in this simulation (peptide and water) was 1099 atoms.

Based on these computational methods, it was determined that peptides possessing anti-allergic activity share a cyclic conformation and that extended or linear conformations are inactive. Furthermore, analysis of the complex peptides show that the
10 active species have a bend or turn at or near the junction of the importation competent segment and the anti-allergic segment.

Conformational measurements to confirm the computational analyses, based on NMR technologies and are performed as known in the art.

15 Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and scope of the appended claims.

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WHAT IS CLAIMED IS:

1. An anti-allergic agent, comprising a complex molecule having at least a first segment competent for importation of said molecule into mast cells, and a second segment for having an anti-allergic effect within said mast cells, said first segment being
5 joined to said second segment through a linker, said linker providing a bend or turn at or near the junction between the two segments, with the proviso that the first segment is other than the peptide AAVALLPAVLLALLAP, when the second segment is the anti-allergic decapeptide derived from Gαi3.

10 2. The agent of claim 1, wherein said second segment has said anti-allergic effect by at least significantly reducing degranulation of said mast cells.

3. The agent of claim 2, wherein said second segment is selected from the group consisting of a peptide, a peptidomimetic, or a polypeptide.

15

4. The agent of claim 3, wherein said second segment is a peptide, having a cyclic conformation stabilized by bonds selected from the group consisting of hydrogen bonds, ionic bonds and covalent bonds.

20 5. The agent of claim 4, wherein said first segment is a peptide.

6. The agent of claim 5, wherein said linker is a covalent bond.

7. The agent of claim 6, wherein said covalent bond is a peptide bond.

25

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8. The agent of claim 7, wherein said anti-allergic segment has an amino acid sequence selected from the group consisting of:

a decapeptide derived from $G\alpha i_3$ having the sequence KNNLKECGLY ;

a decapeptide derived from $G\alpha t$ having the sequence KENLKDCGLF ;

5 Cyclic $G\alpha i_3$ KNNLKECGLY ;

ε-NH

KNNLKECGL-para-amino-F; KQNLKECGLY; KSNLKECGLY;

10 KNNLKEVGLY and KENLKECGLY.

9. The agent of claim 7, wherein the anti-allergic segment is a peptide taken

15 from the C terminal sequence of $G\alpha i_3$.

10. The agent of claim 7, wherein said molecule is a peptide having an amino acid sequence selected from the group consisting of

WALL006: AAVALLPAVLLALLAPKQNLKECGLY

20 WALL007: AAVALLPAVLLALLAPKNNLKEVGLY

WALL008: Succinyl-AAVALLPAVLLALLA-Sar-KNNLKECGLY

25 WALL010: VTVLALGALAGVGVGPKNNLKECGLY

WALL011: Succinyl - AAVALLPAVLLALLAPKSNLKECGLY

WALL012: Succinyl – AAVALLPAVLLALLAPKENLKECGLY

30 WALL013: Succinyl – AAVALLPAVLLALLAPKANLKECGLY

WALL014: Succinyl - AAVALLPAVLLALLAP KNNLKECGL-para-amino-F

35 WALL015: Succinyl – AAVALLPAVLLALLAPKQNLKECGLY

WALL016: Succinyl – AAVALLPAVLLALLAPKNNLKEVGLY

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and the active analogues, homologues and derivatives of these sequences, including cyclic derivatives.

11. A pharmaceutical composition for treating an allergic condition in a
5 subject, comprising a therapeutically effective amount of an anti-allergic agent, said anti-allergic agent comprising a complex molecule having at least a first segment competent for importation of said molecule into mast cells, and a second segment for having an anti-allergic effect within said mast cells, said first segment being joined to said second segment through a linker, said linker providing a bend or turn at or near the junction
10 between the two segments, with the proviso that the first segment is other than the peptide AAVALLPAVLLALLAP, when the second segment is the anti-allergic decapeptide derived from $G\alpha i_3$.

12. The composition of claim 11, wherein the allergic condition is selected
15 from the group consisting of nasal allergy, an allergic reaction in an eye of the subject, an allergic reactions in the skin of the subject, acute urticaria, psoriasis, psychogenic or allergic asthma, interstitial cystitis, bowel diseases, migraines, and multiple sclerosis.

13. The composition of claim 11 or 12 further comprising a pharmaceutically
20 acceptable excipient, diluent or carrier.

14. The composition of claim 13, wherein said composition is suitable for topical administration.

25 15. The composition of claim 14, wherein said topical administration is to the

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skin of the subject.

16. The composition of claim 13, wherein said composition is suitable for administration intranasally or by inhalation.

5

17. The composition of claim 11, wherein said second segment has said anti-allergic effect by at least significantly reducing degranulation of said mast cells.

18. The composition of claim 17, wherein said second segment is selected

10 from the group consisting of a peptide, a peptidomimetic, or a polypeptide.

19. The composition of claim 18, wherein said second segment is a peptide, having a cyclic conformation, stabilized by bonds selected from the group consisting of hydrogen bonds, ionic bonds and covalent bonds.

15

20. The composition of claim 19, wherein said first segment is a peptide.

21. The composition of claim 20, wherein said linker is a covalent bond.

20 22. The composition of claim 21, wherein said covalent bond is a peptide bond.

23. The composition of claim 21, wherein said anti-allergic segment has an amino acid sequence selected from the group consisting of

25 a decapeptide derived from $G\alpha i_3$ having the sequence KNNLKECGLY ;

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a decapeptide derived from $G\alpha i_3$ having the sequence KNNLKECGLY ;

a decapeptide derived from $G\alpha t$ having the sequence KENLKDCGLF ;

Cyclic $G\alpha i_3$ KNNLKECGLY ;

5 ε-NH

KNNLKECGL-para-amino-F; KQNLKECGLY; KSNLKECGLY;

KNNLKEVGLY and KENLKECGLY.

10

24. The composition of claim 21, *wherein the anti-allergic segment is a* peptide taken from the C terminal sequence of $G\alpha i_3$.

15 25. The composition of claim 21, wherein said molecule is a peptide having an amino acid sequence selected from the group consisting of

WALL006: AAVALLPAVLLALLAPKQNLKECGLY

WALL007: AAVALLPAVLLALLAPKNNLKEVGLY

20

WALL008: Succinyl -AAVALLPAVLLALLA-Sar-KNNLKECGLY

WALL010: VTVLALGALAGVGVGPKNNLKECGLY

25 WALL011: Succinyl - AAVALLPAVLLALLAPKSNLKECGLY

WALL012: Succinyl – AAVALLPAVLLALLAPKENLKECGLY

WALL013: Succinyl – AAVALLPAVLLALLAPKANLKECGLY

30

WALL014: Succinyl - AAVALLPAVLLALLAP KNNLKECGL-para-amino-F

WALL015: Succinyl – AAVALLPAVLLALLAPKQNLKECGLY

35 WALL016: Succinyl – AAVALLPAVLLALLAPKNNLKEVGLY

and the active analogues, homologues and derivatives of these sequences, including cyclic derivatives.

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26. A method for treating an allergic condition in a subject, comprising the step of administering a therapeutically effective amount of an anti-allergic agent to the
5 subject, said anti-allergic agent comprising a molecule having at least a first segment competent for importation of said molecule into mast cells, and a second segment for having an anti-allergic effect within said mast cells, said first segment being joined to said second segment through a linker, said linker providing a bend or turn at or near the junction between the segments, with the proviso that the first segment is other than the
10 peptide AAVALLPAVLLALLAP, when the second segment is the anti-allergic decapeptide derived from Gαi3.

27. The method of claim 26, wherein the allergic condition is selected from the group consisting of nasal allergy, an allergic reaction in an eye of the subject, an allergic
15 reactions in the skin of the subject, acute urticaria, psoriasis, psychogenic or allergic asthma, interstitial cystitis, bowel diseases, migraines, and multiple sclerosis.

28. The method of claim 27, wherein the step of administering said anti-therapeutic agent is performed by topical administration.

20

29. The method of claim 28, wherein said topical administration is to the skin or the eye of the subject.

30. The method of claim 27, wherein the step of administering said therapeutic
25 agent is performed by inhalation or intranasal administration

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allergic effect by at least significantly reducing degranulation of said mast cells.

32. The method of claim 31, wherein said second segment is selected from the group consisting of a peptide, a peptidomimetic or a polypeptide.

5

33. The method of claim 32, wherein said second segment is a peptide having a cyclic conformation stabilized by bonds selected from the group consisting of hydrogen bonds, ionic bonds or covalent bonds.

10 34. The method of claim 33, wherein said first segment is a peptide.

35. The method of claim 34, wherein said linker is a covalent bond.

36. The method of claim 35, wherein said covalent bond is a peptide bond.

15

37. The method of claim 35, wherein said anti-allergic segment has an amino acid sequence selected from the group consisting of:

a decapeptide derived from $G\alpha i_3$ having the sequence KNNLKECGLY ;

a decapeptide derived from $G\alpha t$ having the sequence KENLKDCGLF ;

20 Cyclic $G\alpha i_3$ KNNLKECGLY ;

KNNLKECGL-para-amino-F; KQNLKECGLY;

25 KSNLKECGLY;KNNLKEVGLY and KENLKECGLY.

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KSNLKECGLY;KNNLKEVGLY and KENLKECGLY.

38. The method of claim 36, wherein the anti-allergic segment is a peptide taken from the C terminal sequence of $G\alpha i_3$.

39. The method of claim 36, wherein said molecule is a peptide having an amino acid sequence selected from the group consisting of:

WALL006: AAVALLPAVLLALLAPKQNLKECGLY

WALL007: AAVALLPAVLLALLAPKNNLKEVGLY

WALL008: Succinyl -AAVALLPAVLLALLA-Sar-KNNLKECGLY

WALL010: VTVLALGALAGVGVGPKNNLKECGLY

WALL011: Succinyl - AAVALLPAVLLALLAPKSNLKECGLY

WALL012: Succinyl – AAVALLPAVLLALLAPKENLKECGLY

WALL013: Succinyl – AAVALLPAVLLALLAPKANLKECGLY

WALL014: Succinyl - AAVALLPAVLLALLAP KNNLKECGL-para-amino-F

WALL015: Succinyl – AAVALLPAVLLALLAPKQNLKECGLY

WALL016: Succinyl – AAVALLPAVLLALLAPKNNLKEVGLY

and the active analogues, homologues and derivatives of these sequences, including cyclic derivatives.

40. A method for preventing late phase inflammatory responses induced by protein kinase activation, comprising the step of administering a therapeutically effective amount of an anti-allergic agent to the subject, said anti-allergic agent comprising a molecule having at least a first segment competent for importation of said molecule into mast cells, and a second segment for having an anti-allergic effect within said mast cells,

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said first segment being joined to said second segment through a linker, said linker providing a bend or turn at or near the junction between the segments.

41. The method of claim 40 wherein the protein kinase activity is a mitogen

5 activated protein kinase.

42. The method of claim 40 wherein the anti allergic agent is according to any one of claims 1-10.

10 43. The method of claim 40 wherein the anti-allergic agent is Peptide 2, Peptide 2-Succ and Peptide 2-Cyc.

44. A method for promoting importation of an anti-allergic peptide into a cell of a subject *in vivo*, the method comprising the steps of:

15 (a) attaching to the anti-allergic peptide a leader sequence, the leader sequence being a peptide, via a linker or a direct bond which forms a bend or a turn, to form a complex peptide or peptidomimetic molecule;

(b) administering the complex peptide or peptidomimetic molecule to the subject; and

20 (c) importing the complex molecule into the cell through the leader sequence, such that the anti-allergic peptide is imported into the cell; with the proviso that the first segment is other than the peptide AAVALLPAVLLALLAP, when the second segment is the anti-allergic decapeptide derived from $G\alpha i_3$.

25

BNSDOCID: <WO_____ 0250097A2_I_>

FIGURE 1A

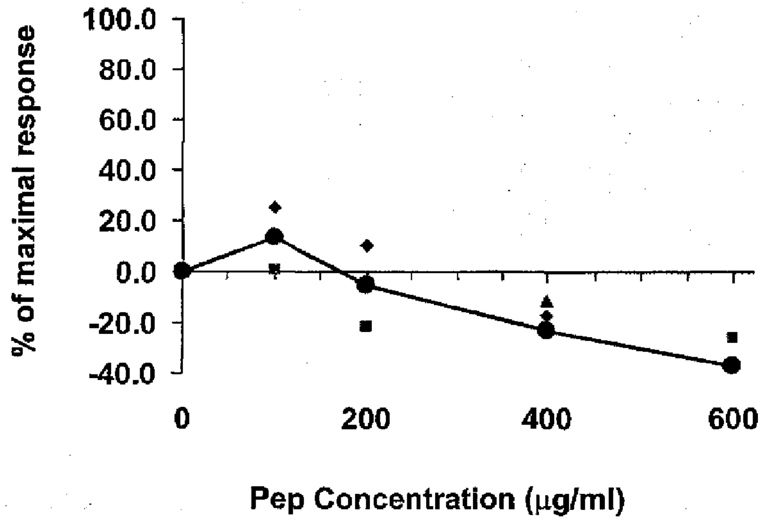


FIGURE 1B

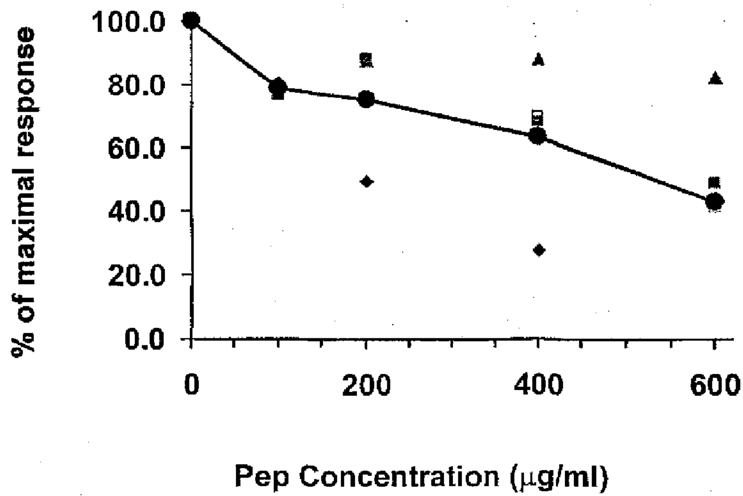


FIGURE 2A

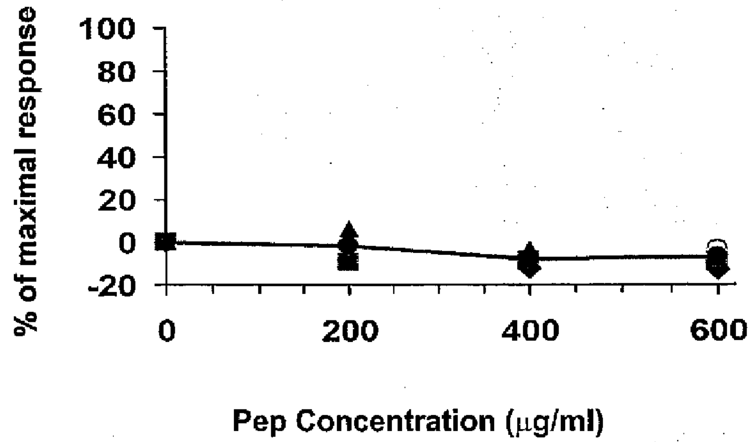


FIGURE 2B

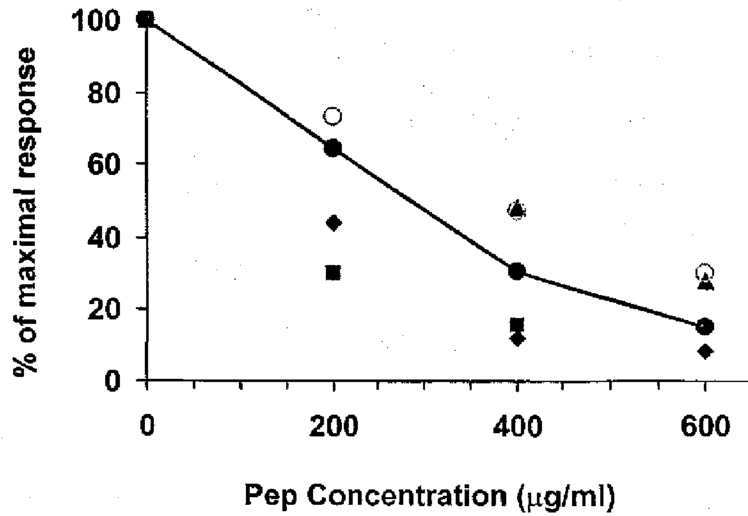


FIGURE 3A

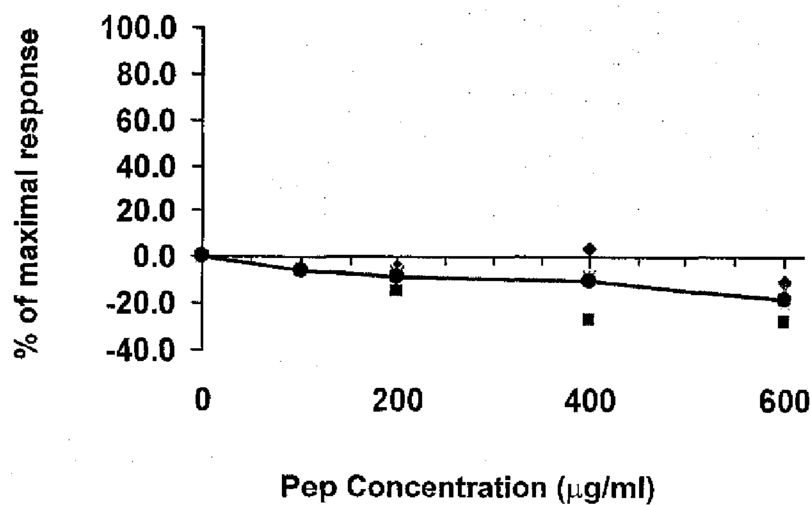


FIGURE 3

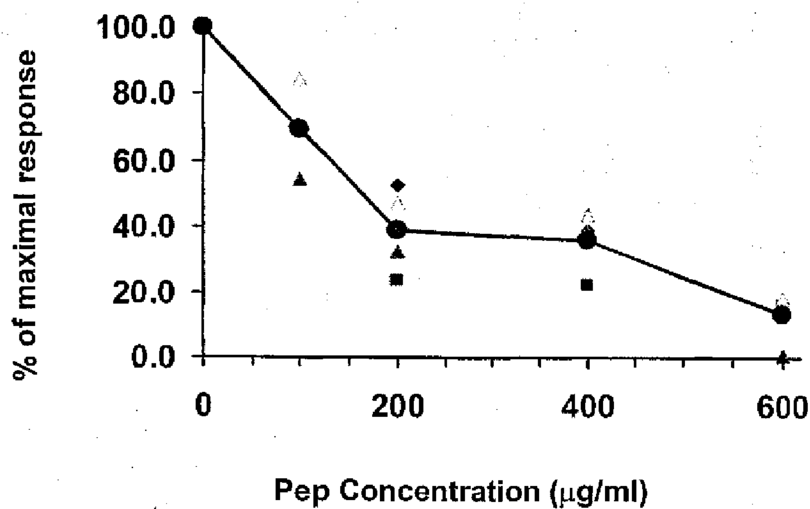


FIGURE 4A

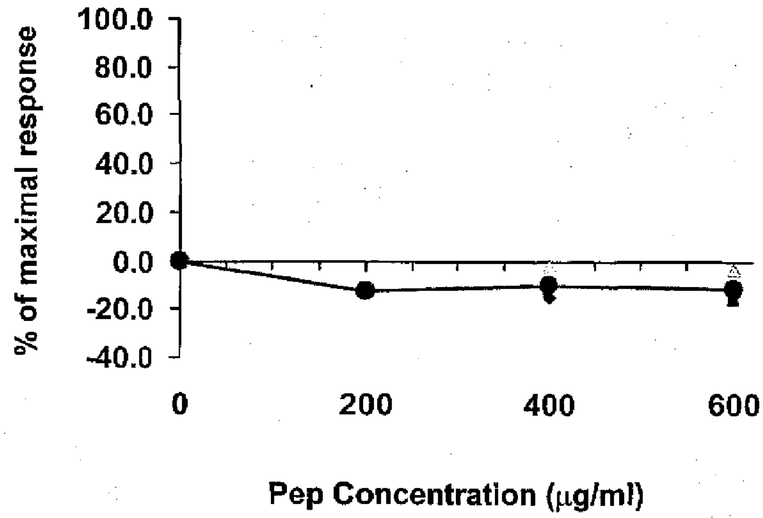


FIGURE 4B

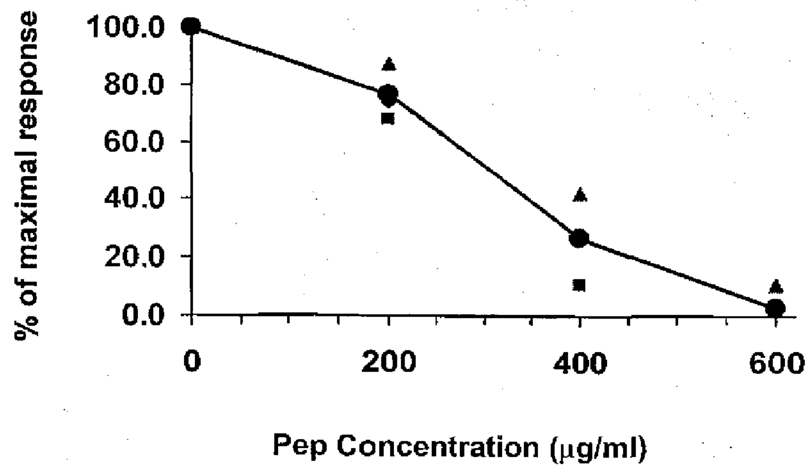


FIGURE 5A

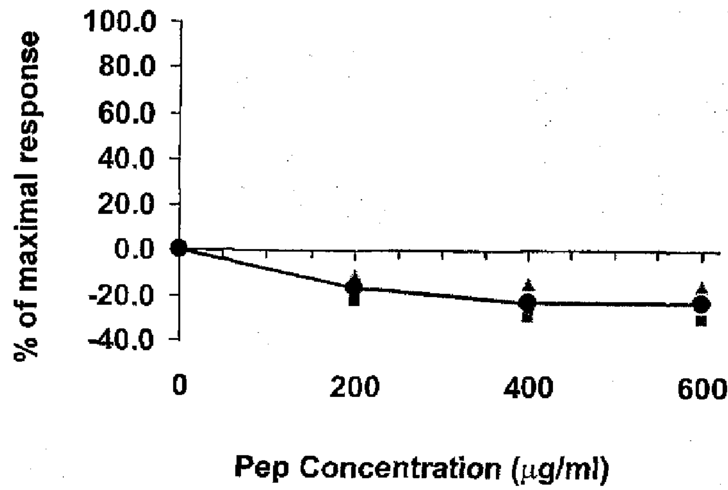
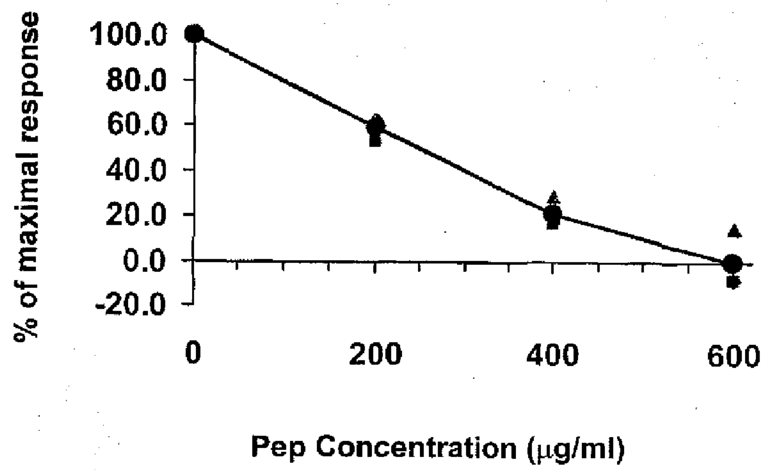


FIGURE 5B



SDOCID: <WO_____0250097A2_I_>

FIGURE 6A

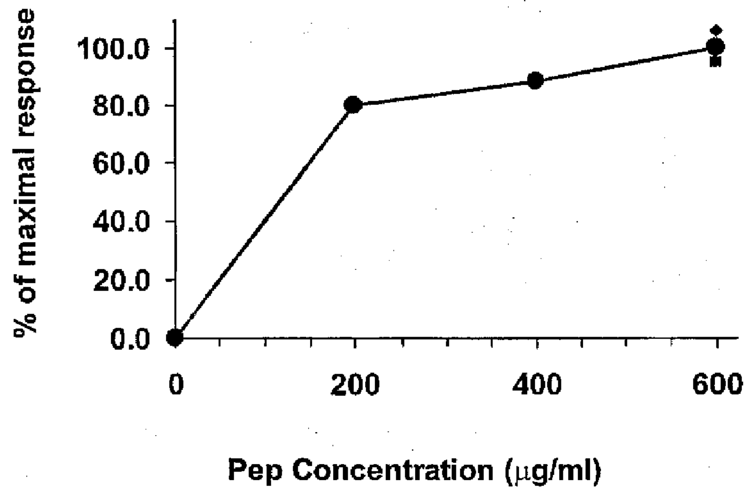


FIGURE 6B

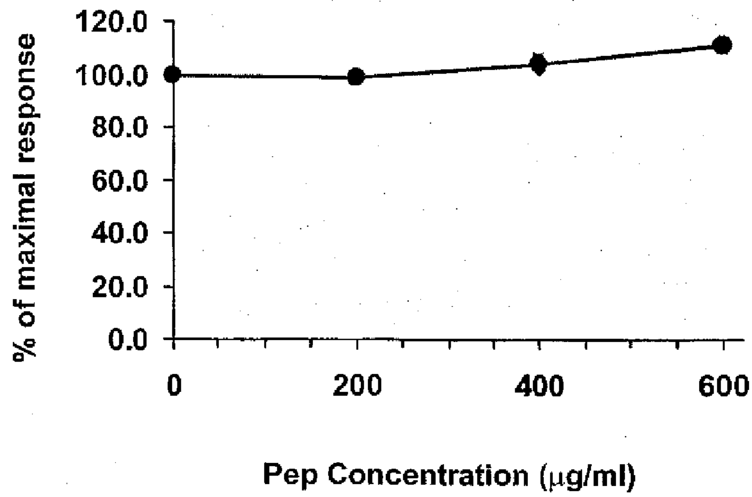


FIGURE 7A

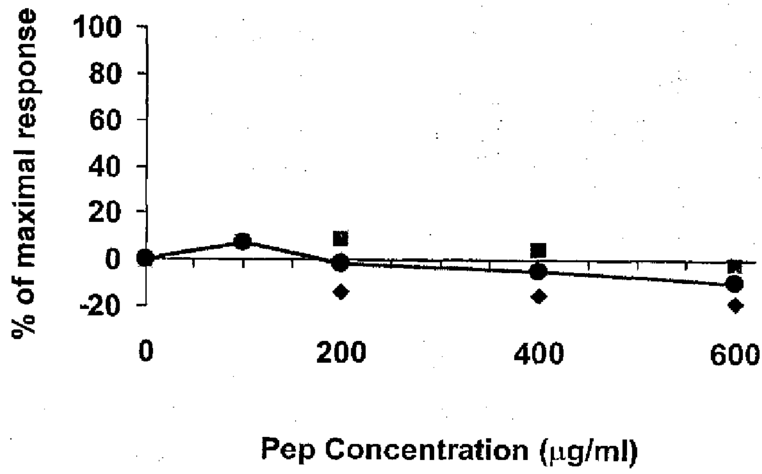


FIGURE 7B

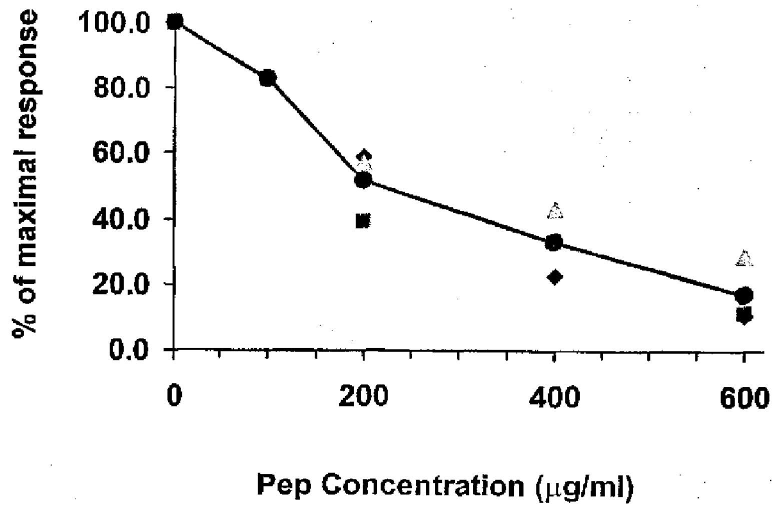


FIGURE 8A

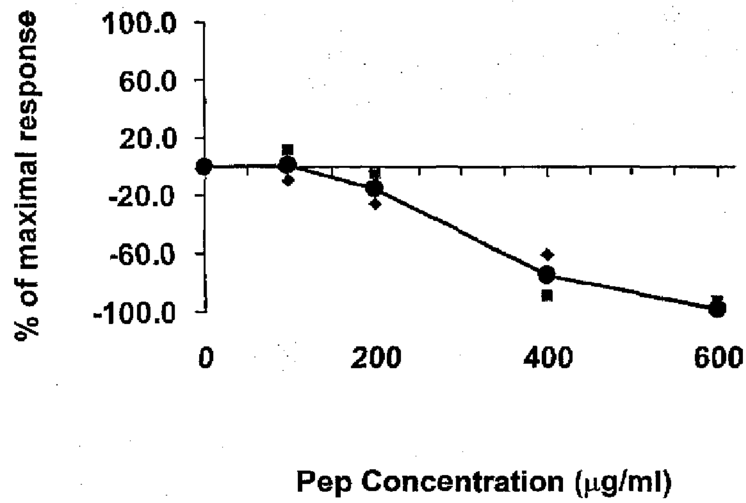


FIGURE 8B

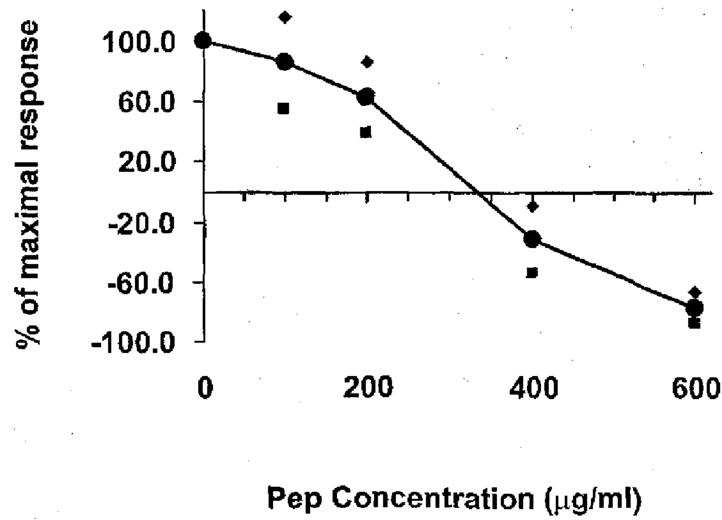


FIGURE 9A

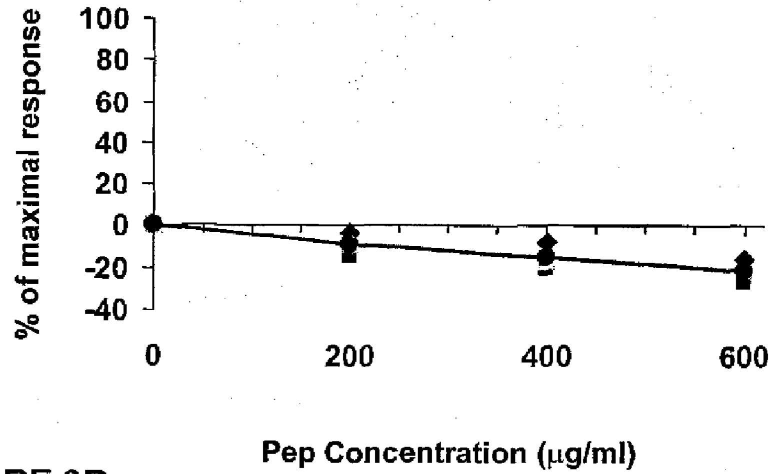


FIGURE 9B

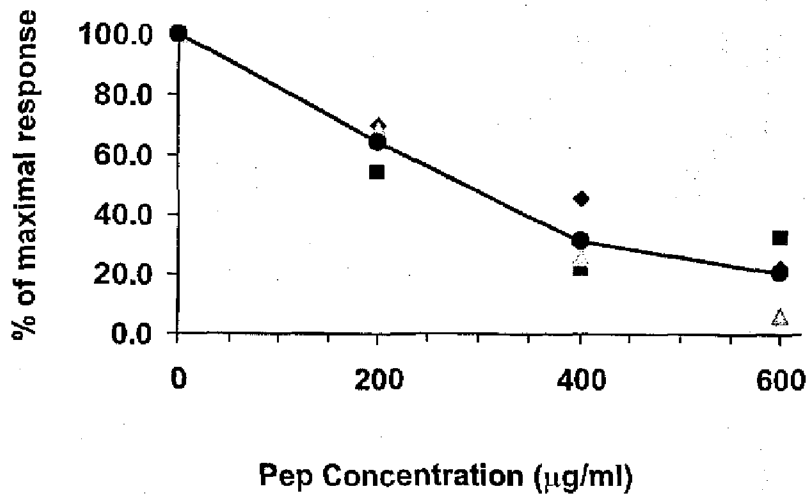


FIGURE 10A

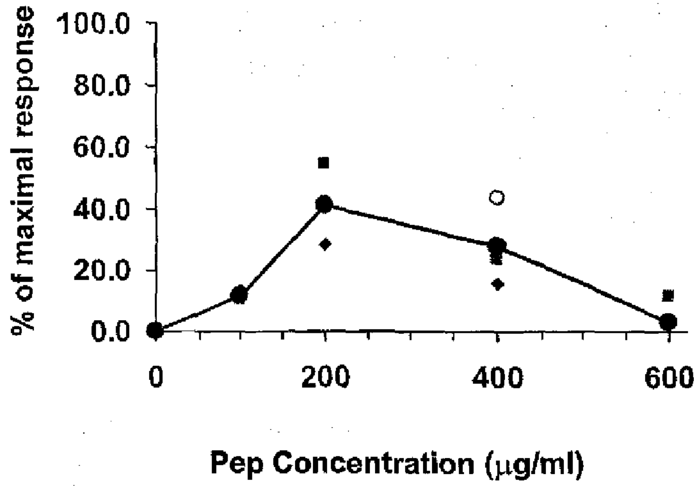


FIGURE 10B

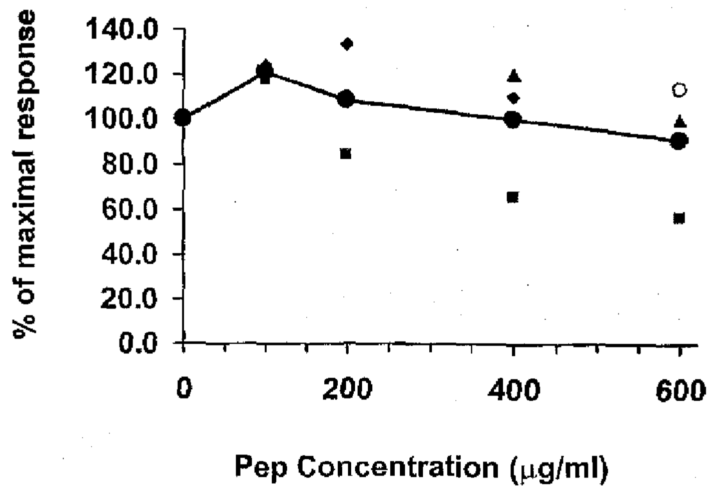


FIGURE 11A

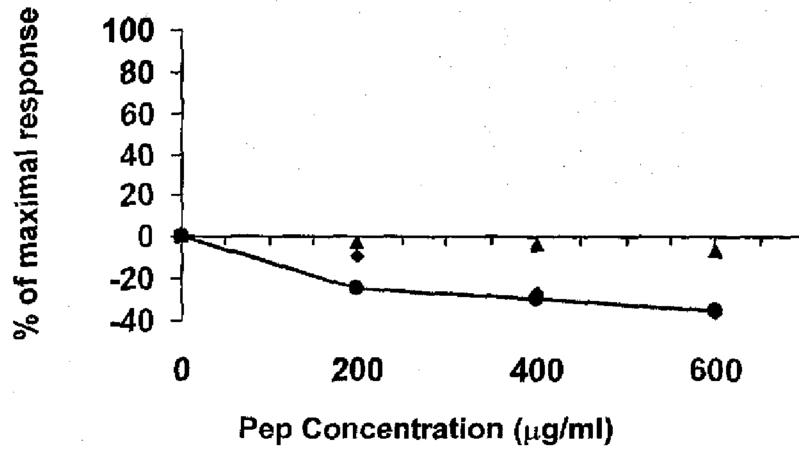


FIGURE 11B

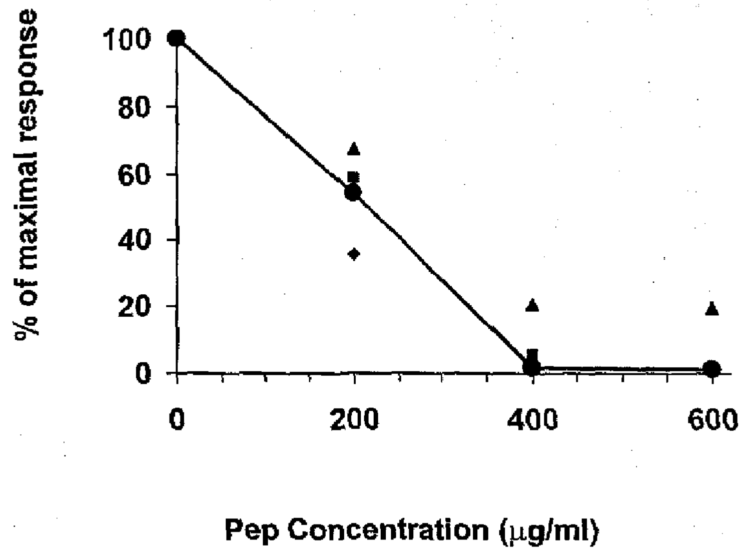


FIGURE 12A

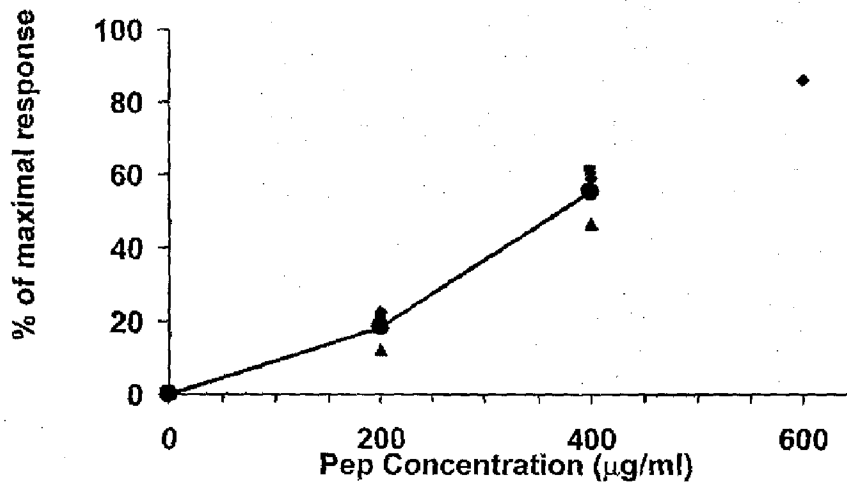
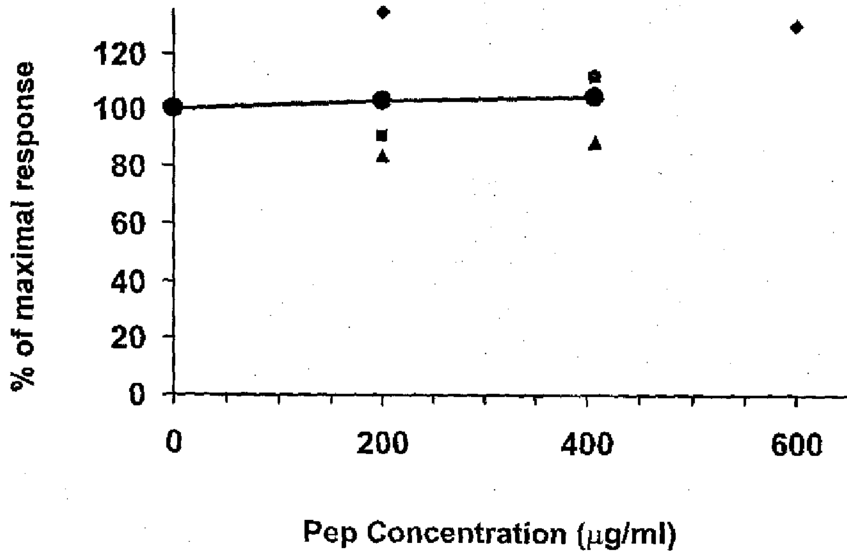


FIGURE 12B



BNSDOCID: <WO_____0250097A2_I_>

FIGURE 13A

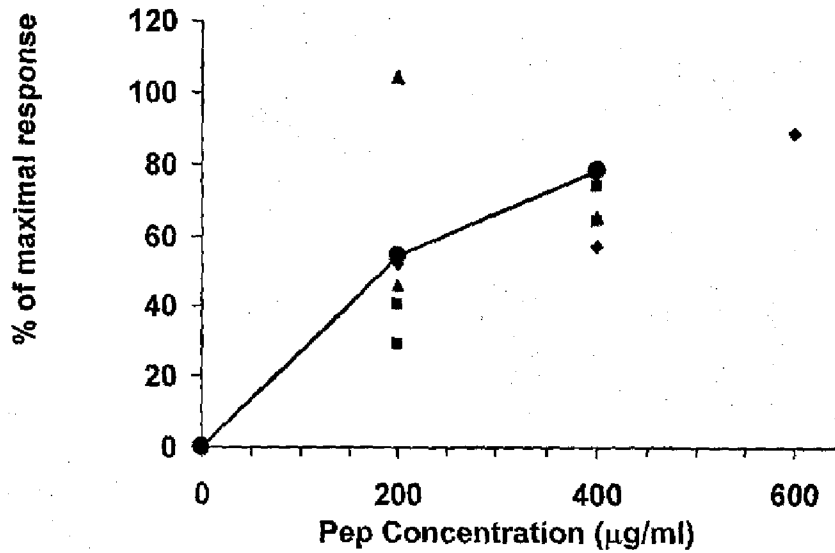
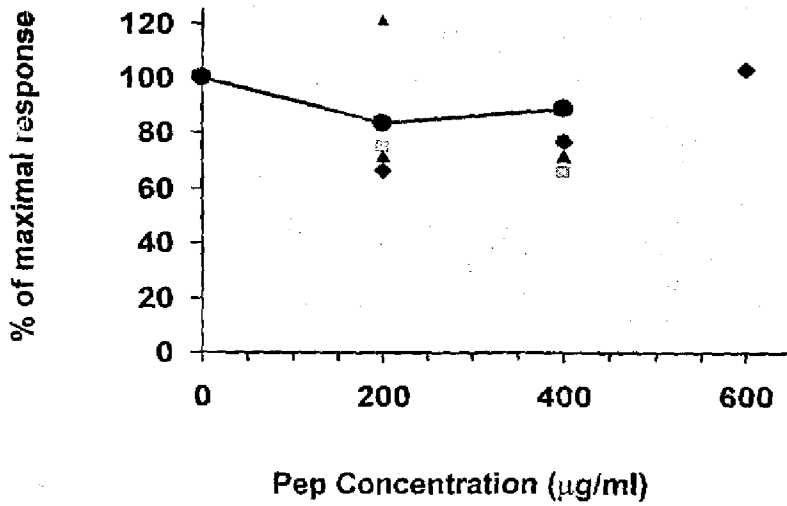


FIGURE 13B



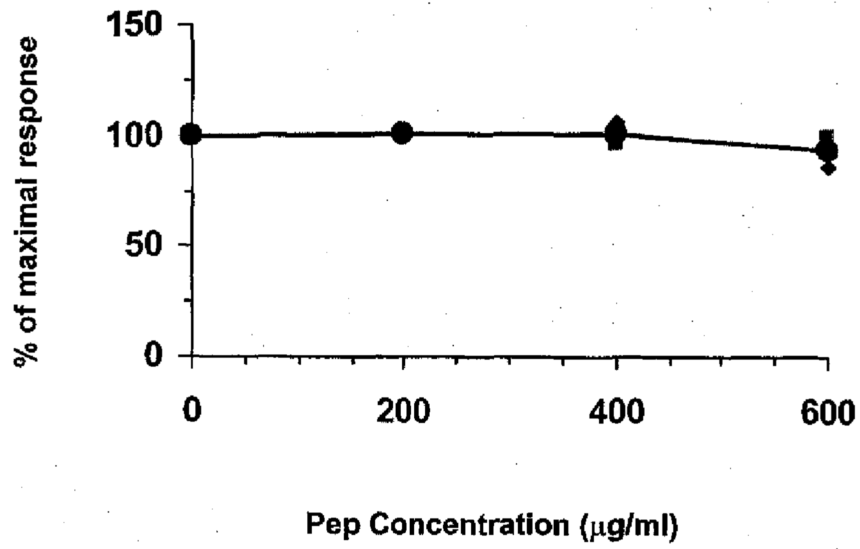


FIGURE 14

FIGURE 15A

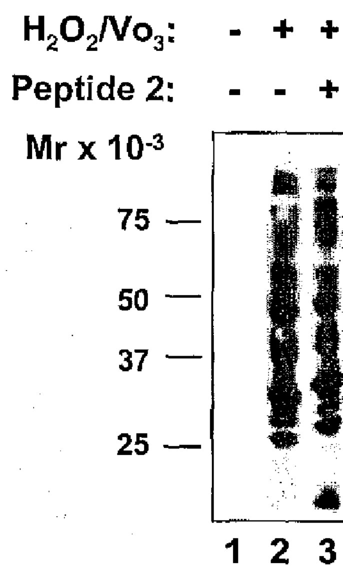
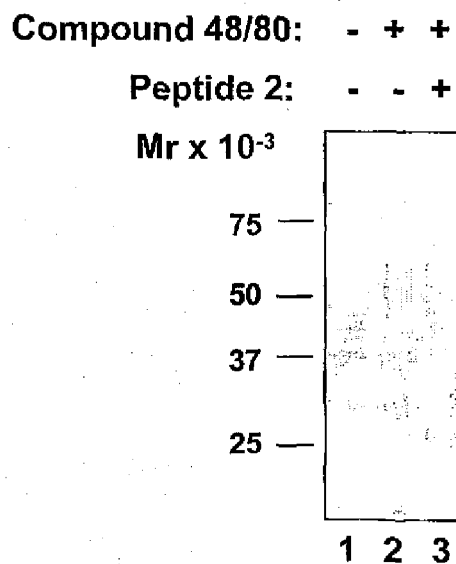


FIGURE 15B



INSDOCID: <WO_______0250097A2_I_>

FIGURE 16A

H_2O_2/Vo_3:	-	+	+
Peptide 2:	-	-	+

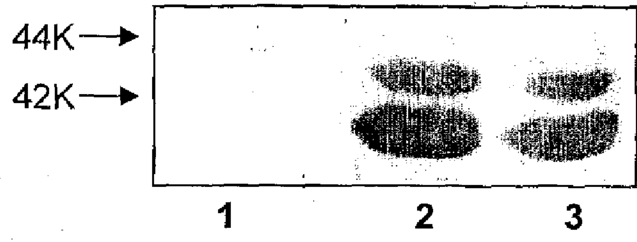
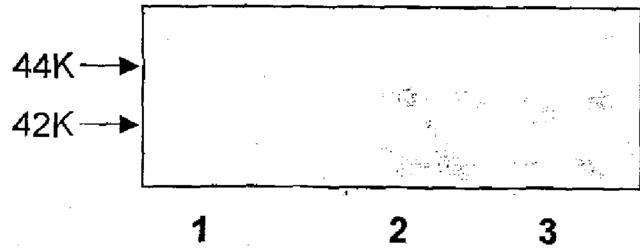


FIGURE 16B

Compound 48/80:	-	+	+
Peptide 2:	-	-	+



Electronic Acknowledgement Receipt

EFS ID:	4258149
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	OLIVER RITTER
Customer Number:	22506
Filer:	Ajay A. Jagtiani/g mills
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	ROTE-0007-UT1
Receipt Date:	10-NOV-2008
Filing Date:	
Time Stamp:	11:58:02
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement Letter	111008_ROTE0007UT1_AsFiled_IDS.pdf	108349 f36eb78d819e7ffa0d29e3e78b6bf772f9f5c18b	no	2

Warnings:

Information:

2	Information Disclosure Statement (IDS) Filed (SB/08)	111008_ROTE0007UT1_AsFiled_SB08_Forms.pdf	150309 227d6247465469bce2de9ecff5e49dcabc136ad6	no	3
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3	Foreign Reference	WO_00_028011.pdf	6166655 f4daba92eed257a582d6dacb7f7de9a3e59114a0	no	117
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5	NPL Documents	Anonymous_Calcineurin_A_Antibodies_2005.pdf	25075 1ef028f1715b9b33c96102b89e52844a1db93331	no	1
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7	NPL Documents	Burkard_Targeted_Proteolysis_2005.pdf	469119 889643a584957cacfa72af2d1f8d49bcb8f1dfb1	no	10
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8	NPL Documents	Cyert_Regulation_of_Nuclear_2001.pdf	97521 016bacfc1b878facbd69178e65be7a9121628376	no	4
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9	NPL Documents	Dolmetsch_Differential_Activation_1997.pdf	940297 b814abb2f9b4bfc2c0f03001fb1b643b631019d1	no	4
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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Acknowledgement Receipt

EFS ID:	4258278
Application Number:	12162135
International Application Number:	
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	OLIVER RITTER
Customer Number:	22506
Filer:	Ajay A. Jagtiani/g mills
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	ROTE-0007-UT1
Receipt Date:	10-NOV-2008
Filing Date:	
Time Stamp:	12:10:49
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	NPL Documents	Fornerod_CRM1_is_an_Export_1997.pdf	1260261 af019546bd4556469c5eb336b510c6f7127193d9	no	10

Warnings:

Information:

2	NPL Documents	Kutay_Export_of_Importin_1997.pdf	1635873 98d795f967264f82a2742c3805a6774eb12b1a87	no	11
Warnings:					
Information:					
3	NPL Documents	Ritter_Calcineurin_in_Human_2002.pdf	186483 15c51d77d8f32cb338d931c3f220c8756981118d	no	6
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Information:					
4	NPL Documents	Shibasaki_Role_of_Kinases_1996.pdf	375932 6a890776f3cec03de893bd5d983791d437fc9f4e	no	3
Warnings:					
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5	NPL Documents	Timmerman_Rapid_shuttling_1996.pdf	805436 f76b48aec0e8a0ce10946e6185b76e067e4e587c	no	4
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6	NPL Documents	Wilkins_Calcineurin_NFAT_2004.pdf	504091 e4cb966409fa254dc83924b2b46461baef3dce9c	no	10
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Information:					
7	NPL Documents	Wu_Critical_Role_of_Calpain_2004.pdf	715749 98c2f3a91b3c828c0d6df18dcdff66915184a812	no	12
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8	NPL Documents	Zhu_NF_AT_Activation_1999.pdf	165831 6dc22371484da6059579e54d67a06fb5dd36f90a	no	5
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Warnings:					
Information:					
		Total Files Size (in bytes):	5906376		

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New Applications Under 35 U.S.C. 111
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National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

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U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
12/162,135	OLIVER RITTER	ROTE-0007-UT1

22506
JAGTIANI + GUTTAG
10363-A DEMOCRACY LANE
FAIRFAX, VA 22030

INTERNATIONAL APPLICATION NO.	
PCT/EP07/00643	
I.A. FILING DATE	PRIORITY DATE
01/25/2007	01/27/2006

CONFIRMATION NO. 8455
371 FORMALITIES LETTER

OC000000032417190

Date Mailed: 10/03/2008

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as a Designated Office (37 CFR 1.494):

- Indication of Small Entity Status
- Priority Document
- Copy of the International Application filed on 07/25/2008
- Copy of the International Search Report filed on 07/25/2008
- Preliminary Amendments filed on 07/25/2008
- U.S. Basic National Fees filed on 07/25/2008
- Assignment filed on 07/25/2008

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.
- A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000). Applicant must provide an initial computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). If applicant desires the sequence listing in the instant application to be identical with that of another application on file in the U.S. Patent and Trademark Office, such request in accordance with 37 CFR 1.821(e) may be submitted in lieu of a new CRF.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 32 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

FORM PCT/DO/EO/905 (371 Formalities Notice)

For questions regarding compliance to 37 CFR 1.821-1.825 requirements, please contact:

- **For Rules Interpretation, call (571) 272-0951**
- **For Patentin Software Program Help, call Patent EBC at 1-866-217-9197 or directly at 703-305-3028 / 703-308-6845 between the hours of 6 a.m. and 12 midnight, Monday through Friday, EST.**
- **Send e-mail correspondence for Patentin Software Program Help @ ebc@uspto.gov**

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
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JOHN L ANDERSON

Telephone: (703) 308-9140 EXT 211

DO/EO WORKSHEET

U.S. Appl. No. 12/162135 International Appl. No. EP2007/000643

Application filed by : ☐ 20 months ☐ 30 months

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Publication Date : Aug 2, 2007
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INTERNATIONAL APPLICATION PAPERS IN THE APPLICATION FILE :

- ☑ International Application *(RECORD COPY)*
- ☐ Article 19 Amendments
- ☐ PCT/IB/331
- ☐ PCT/IPEA/409 IPER (PCT/IPEA/416 on front)
- ☐ Annexes to 409
- ☐ Priority Document (s) No. ______
- ☐ International Appl. on Double Sided Paper *(COPIES MADE)*
- ☐ Request form PCT/RO/101
- ☑ PCT/ISA/210 - Search Report
- ☐ Search Report References
- ☐ Other : ______

RECEIPTS FROM THE APPLICANT *(other than checked above)* **:**

- ☑ Basic National Fee *(or authorization to charge)*
- ☑ Description
- ☑ Claims
- ☑ Words in the Drawing Figure(s) - (# of dwgs. 9)
- ☐ Article 19 Amendments
  - ☐ english transl. of annexes NOT present
  - ☐ entered ☐ not entered :
    - ☐ not a page for page substitution
    - ☐ other : ______
- ☐ Annexes to 409
  - ☐ english transl. of annexes NOT present
  - ☐ entered ☐ not entered :
    - ☐ not a page for page substitution
    - ☐ other : ______
- ☑ Preliminary Amendment(s) Filed on : 1. July 25, 2008 2. ______ 3. ______
- ☐ Information Disclosure Statement(s) Filed on : 1. ______ 2. ______ 3. ______
- ☑ Assignment Document
- ☐ Power of Attorney/ Change of Address
- ☐ Substitute Specification Filed on : 1. ______ 2. ______
- ☑ Small Entity
- ☐ Oath/ Declaration (executed)
  - ☐ surcharge was paid at the time of filing
- ☐ DNA Diskette ☐ Sequence Listing
- ☐ Other : 1. ______ 2. ______

NOTES : ☑ I.A. used as Specification ☐ Other : Seq Chain pg. 15, 21, 22, (NO CRF [illegible])

35 U.S.C. 371 - Receipt of Request (PTO-1390)	July 25, 2008
Date Acceptable Oath/Declaration Received	
Date of Completion of requirements under 35 U.S.C. 371	
102(e) Date	
Date of Completion of DO/ EO 903 - Notification of Acceptance	
Date of Completion of DO/ EO 905 - Notification of Missing Requirements	Oct 2, 2008
Date of Completion of DO/ EO 906 - Notification of Missing 102(e) Requirements	
Date of Completion of DO/ EO 907 - Notification of Acceptance for 102(e) Date	
Date of Completion of DO/ EO 909 - Notification of Abandonment	
Date of Completion of DO/ EO 911 - Application Accepted Under 35 U.S.C. 111	
Date of Completion of DO/ EO 916 - Notification of Defective Response	
Date of Completion of DO/ EO 910 - Notification to Comply w/ Seq. Requirements	

PATENT APPLICATION FEE DETERMINATION RECORD

Effective September 30,2007

Application or Docket Number

12/162135

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)	SMALL ENTITY TYPE RATE	FEE	OR	OTHER THAN SMALL ENTITY RATE	FEE
U.S. NATIONAL STAGE FEES							
BASIC FEE	SMALL ENT. = $155	LARGE ENT. = $ 310	BASIC FEE	155	OR	BASIC FEE	
EXAMINATION FEE	Satisfies PCT Article 33(1)-(4) = $ 50 / $ 100	All other situations = $ 100 / $ 200	EXAM. FEE	105		EXAM. FEE	
SEARCH FEE	U.S. is ISA = $ 50 / $ 100 ALL other countries = $ 205 / $ 410	ALL other situations = $ 255 / $ 510	SEARCH FEE	205		SEARCH FEE	
FEE FOR EXTRA SPEC. PGS.	minus 100 =	/ 50 =	X$130			X$260	
TOTAL CHARGEABLE CLAIMS	12 minus 20 =	* —	X $ 25 =		OR	X $ 50 =	
INDEPENDENT CLAIMS	7 minus 3 =	* 4	X $ 105 =	420	OR	X $ 210=	
MULTIPLE DEPENDENT CLAIM PRESENT		— ☐	$185		OR	$370	
			TOTAL		OR	TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

AMENDMENT A	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE	ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	ADDITIONAL FEE
Total	*	Minus	**	=	X $ 25 =		OR	X $ 50 =	
Independent	*	Minus	***	=	X$105		OR	X$210	
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐					$185		OR	$370	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT B	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE	ADDITIONAL FEE	OR	RATE	ADDITIONAL FEE
Total	*	Minus	**	=	X $ 25 =		OR	X $ 50 =	
Independent	*	Minus	***	=	X$105		OR	X$210	
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐					$185		OR	$370	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than '20', enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than '3', enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET
(FOR USE WITH FORM PTO-875)

SERIAL NO. 12/162135

FILING DATE

APPLICANT(S)

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
1	1		1			
2		1		1		
3		2		1		
4		(1)		1		
5	1		1			
6	1		1			
7	1		1			
8	1		1			
9		3		1		
10	1		1			
11	1		1			
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TOTAL IND.	7	↓	7	↓		↓
TOTAL DEP.	8	←	5	←		←
TOTAL CLAIMS	15		12			

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
51						
52						
53						
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100						
TOTAL IND.		↓		↓		↓
TOTAL DEP.		←		←		←
TOTAL CLAIMS						

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 10/02/2008

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Credit Card Refund Total: $50.00

American Express XXXXXXXXXXXX2006

FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER

ROTE-0007-UT1

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP2007/000643	**25 January 2007**	**27 January 2006**

TITLE OF INVENTION

PEPTIDE FOR INHIBITION OF CALCINEURIN

APPLICANT(S) FOR DO/EO/US

Oliver RITTER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

- ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
- ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
- ☐ This express request to begin national examination procedures (35 U.S.C. 371(f). The submission must include items (5), (6), (9) and (21) indicated below.
- ☐ The US has been elected (Article 31).
- ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - ☒ is attached hereto (required only if not communicated by the International Bureau).
  - ☐ has been communicated by the International Bureau.
  - ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
- ☒ A English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - ☒ is attached hereto.
  - ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
- ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - ☐ are attached hereto (required only if not communicated by the International Bureau).
  - ☐ had been communicated by the International Bureau.
  - ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - ☐ have not been made and will not be made.
- ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
- ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20. below concern document(s) or information included:

- ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
- ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
- ☒ A preliminary amendment.
- ☒ An Application Data Sheet under 37 CFR 1.76.
- ☐ A substitute specification.
- ☐ A power of attorney and/or address letter.
- ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 132 and 37 CFR 1.821-1825
- ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4)
- ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
- ☐ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.7)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
	PCT/EP2007/000643	**ROTE-0007-UT1**

The following fees are submitted:

☒ a) Basic national fee.....$310.00

☒ b) Examination fee $210.00

☒ c) Search fee.....$410.00

	CALCULATIONS	PTO USE ONLY
TOTAL OF ABOVE CALCULATIONS =	**930.00**	
☐ Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing or computer program listing filed in an electronic medium). The fee is **$250** for each additional 50 sheets of paper or fraction thereof.		

TOTAL SHEETS	EXTRA SHEETS	NUMBER EXTRA (of each additional 50 or fraction thereof rounded up to a whole number)	RATE	CALCULATIONS	PTO USE ONLY
-100 =	50 =		x $260.00		
Surcharge of **$130.00** for furnishing the oath or declaration later than 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				**130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	CALCULATIONS	PTO USE ONLY
Total claims	**12** - 20 =	**0**	x $50.00		
Independent claims	**7** - 3 =	**4**	x $210.00	**840.00**	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $370.00		
SUBTOTAL =				**2,000.00**	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.					
SUBTOTAL =				**1,115.00**	
Processing fee of **$130.00** for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +					
TOTAL NATIONAL FEE =				**1,115.00**	
Fee for recording the enclose assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property					
TOTAL FEES ENCLOSED =				**1,115.00**	
				Amount to be refunded:	**$**
				Amount to be charged:	**$**

☐ A check in the amount of **$** ________ to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. **10-0233** in the amount of ________ to cover the above fees.

A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment To Deposit Account No. **10-0233** A duplicate coy of this sheet is enclosed.

d. ☒ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030
Customer No.: 22506

/Ajay A. Jagtiani/Reg. No. 35,205/
SIGNATURE

AJAY A. JAGTIANI — JULY 25, 2008
NAME — DATE

REGISTRATION NO.: **35,205**

Application Data Sheet

Application Information

Application number::	To be assigned
Filing Date::	July 25, 2008
Application Type::	Regular
Subject Matter::	371 Application
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	
Computer Readable Form (CRF)?::	
Number of copies of CRF::	
Title::	PEPTIDE FOR INHIBITION OF CALCINEURIN
Attorney Docket Number::	ROTE-0007-UT1
Request for Early Publication?::	NO
Request for Non-Publication?::	NO
Suggested Drawing Figure::	1
Total Drawing Sheets::	9
Small Entity?::	YES
Latin name::	
Variety denomination name::	
Petition included?::	
Petition Type::	
Licensed US Govt. Agency::	
Contractor Grant Numbers::	
Secrecy Order in Parent Appl.?::	NO

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	Germany
Status::	Full Capacity
Given Name::	Oliver
Middle Name::	
Family Name::	RITTER
City of Residence::	
State or Province of Residence:	
Country of Residence::	
Street of mailing address::	Rembrandtstrasse 4
City of mailing address::	Wuerzburg
State or Province of Residence:	Wuerzburg
Country of mailing address::	Germany
Postal or Zip Code of mailing address::	97076

Correspondence Information

Correspondence Customer Number::	22506
Name::	Jagtiani + Guttag, LLLP
Street of mailing address::	10363-A Democracy Lane
City of mailing address::	Fairfax
State or Province of mailing address::	VA
Country of mailing address::	U.S.A.
Postal or Zip Code of mailing address::	22030
Phone number::	(703) 591-2664
Fax Number:	(703) 591-5907
E-Mail address::	iplaw@jagtiani.com

Representative Information

Representative Customer Number::	22506	

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::
Europe	06090014.9	January 27, 2006	Yes
WIPO	PCT/EP2007/000643	January 25, 2007	Yes

Assignee Information

Assignee name::	Julius-Maximillans-Universitaet Wuerzburg
Street of mailing address::	Sanderring 2
City of mailing address::	97070 Wuerzburg
State or Province of Mailing Address:	
Country of mailing address::	GERMANY
Postal or Zip Code of mailing address::	

Docket No. ROTE-0007-UT1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: RITTER, Oliver	)	Confirmation No: Unassigned
	)	
Application No.: New Application	)	Group Art Unit: Unassigned
	)	
Filed: Concurrently Herewith	)	Examiner: Unassigned

For: PEPTIDE FOR INHIBITION OF CALCINEURIN

United States Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

PRELIMINARY AMENDMENT

Sir:

Prior to an examination on the merits, please amend the above-referenced application as follows:

Amendments to the Claims begin on page 2 of this paper.

Remarks/Arguments begin on page 5 of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings, of claims in the application:

Listing of Claims

1. (ORIGINAL) A method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:
   a) providing a test sample comprising at least one cell;
   b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10;
   c) determining an interaction between the peptide of SEQ ID NO: 1, 2, 5-10 and cellular calcineurin;

   wherein a primarily nuclear localization of a complex comprising calcineurin and the peptide of SEQ ID NO: 1, 2, 5-10 indicates susceptibility for a myocardial and/or immunological disorder.
2. (ORIGINAL) The method of claim 1, wherein the peptide of SEQ ID NO: 1, 2, 5-10 is chemically, biologically and/or physically labeled.
3. (CURRENTLY AMENDED) The method of claim 1 ~~or 2~~, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyopathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.
4. (CURRENTLY AMENDED) The method of ~~any of any of~~ claims 1 ~~to 3~~, wherein the disorder is a T-cell associated disorder.
5. (ORIGINAL) A kit comprising the peptide of SEQ ID NO: 1,2, 5-10 for diagnosing susceptibility for a myocardial and/or immunological disorder.
6. (ORIGINAL) A therapeutic agent comprising the peptide of SEQ ID NO: 1, 2, 5-10.
7. (ORIGINAL) A therapeutic agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5-10.
8. (ORIGINAL) A therapeutic agent comprising a peptide of at least 90% preferably at least

95%, more preferred at least 98%, still more preferred 100% identity of the peptide of SEQ ID NO: 1, 2, 5-10; wherein he peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

9. (CURRENTLY AMENDED) The therapeutic agent of ~~any of the claims 6 to 8~~ claim 6, further comprising a pharmaceutically acceptable carrier.

10. (ORIGINAL) An antibody directed against the peptide of SEQ ID NO: 1, 2, 5-10.

11. (CURRENTLY AMENDED) A method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:

a) providing a test sample comprising at least one cell;

b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10;

c) determining an interaction between the peptide of SEQ ID NO: 1, 2, 5-10 and cellular calcineurin; and

d) ~~Use of~~ using the peptide of SEQ ID NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

12. (CURRENTLY AMENDED) The ~~use~~ method of claim 11, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

REMARKS

Applicants respectfully request that the above-referenced application be reconsidered, as amended. Claims 1 through 12 are currently pending.

It is respectfully requested that the foregoing Preliminary Amendment be entered prior to examination of the application. Applicants have amended the claims to remove multiple dependencies and to place the application in condition for examination and allowance. No new matter has been added.

Should the Examiner feel that there are any issues outstanding after consideration of this response, the Examiner is invited to contact the undersigned to expedite prosecution of the application.

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance, and favorable action is respectfully solicited.

Respectfully submitted,

/Ajay A. Jagtiani/Reg. No. 35,205
Ajay A. Jagtiani
Attorney for Applicant(s)
Reg. No.: 35,205

Customer No. 22506

Date: July 25, 2008

Patent Administrator
Jagtiani + Guttag, LLLP
10363-A Democracy Lane
Fairfax, VA 22030
Telephone: (703) 591-2664
Facsimile: (703) 591-5907
CUSTOMER NO: 22506

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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WO 2007/085455 A1

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G01N 33/50 (2006.01) *G01N 33/68* (2006.01)

(21) International Application Number:
PCT/EP2007/000643

(22) International Filing Date: 25 January 2007 (25.01.2007)

(25) Filing Language: English

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(30) Priority Data:
06090014.9 27 January 2006 (27.01.2006) EP

(71) Applicant *(for all designated States except US)*: **JULIUS-MAXIMILIANS-UNIVERSITÄT WÜRZBURG** [DE/DE]; Sanderring 2, 97070 Würzburg (DE).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **RITTER, Oliver** [DE/DE]; Rembrandtstrasse 4, 97076 Würzburg (DE).

(74) Agent: **ELBEL, Michaela**; Rothkopf Theobald Elbel, Isatorplatz 5, 80331 München (DE).

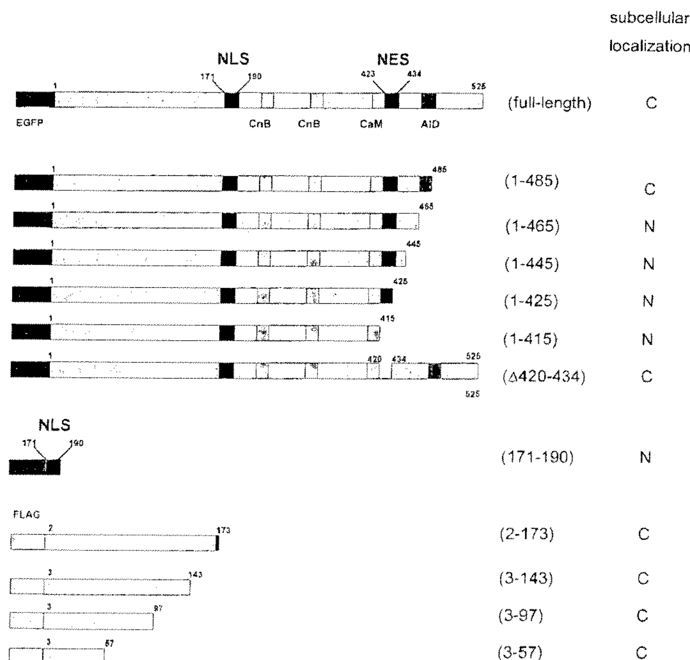
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PEPTIDE FOR INHIBITION OF CALCINEURIN



(57) **Abstract:** The present invention relates to a method for diagnosing susceptibility for a myocardial and/or immunological disorder disorder, a kit and a therapeutic agent comprising a peptide of SEQ ID NO: 1 or 2 and uses thereof.

Peptide for Inhibition of Calcineurin

Field of the invention

5 The present invention relates to a method for diagnosing susceptibility for a myocardial and/or immunological disorder, a kit, an antibody and a therapeutic agent comprising a peptide of SEQ ID NO: 1, 2, 5-10 and uses thereof.

Background of the invention

10 The movement of proteins over ~40 kDa into and out of the nucleus is governed by the nuclear pore complex (NPC), a multi subunit structure embedded in the nuclear envelope (Jans et al., 2000). Transcription factors and enzymes that regulate the activity of these proteins are shuttled across the nuclear envelope by proteins that recognize nuclear localization signals/sequences (NLS) and nuclear export 15 signals (NES). Positively charged NLSs are bound by importins α and/or ß – also known as karyopherins – which tether cargo to the cytosolic face of the nuclear pore complex and facilitate translocation of proteins into the nucleus. The CRM1 protein, also referred to as exportin, mediates the transfer of proteins out of the nucleus (Fornerod et al., 1997), although CRM1-independent mechanisms for nu- 20 clear export exist (Kutay et al., 1997). The ability of nuclear import and export machinery to access a NLS or NES is often dictated by signalling events that lead to the exposure or masking of these regulatory sequences (Cyert, 2001). This may occur through direct modification of the target protein or via modification of an associated factor.

25

The signalling cascade of calcineurin (CnA) and the nuclear factor of activated T cells (NF-ATc) is a crucial transducer of cellular function. NF-ATc is an ubiquitous transcription factor but has particular relevance in cytokine expression and the development of pathologic myocardial hypertrophy. The formation of complexes 30 between transcription factors and DNA is crucial for the transcriptional process. Therefore, the time that transcription factors remain nuclear is a major determi-

nant for transcriptional activity. It was shown that that in addition to the transcription factor NF-ATc, the phosphatase calcineurin is also translocated to the nucleus (Burkhard et al., 2005; Frey et al., 2000; Zou et al., 2001; Shibasaki et al., 1996). Calcineurin is therefore not only responsible for dephosphorylating NF-ATc, thus enabling its nuclear import; its presence in the nucleus is also significant in ensuring the full transcriptional activity of NF-ATc (Zhu et al., 1999). The traditional understanding of calcineurin activation via sustained high Ca^{2+}-levels (Timmerman et al., 1996; Dolmetsch et al., 1997) was advanced by findings of the inventor that calcineurin is activated by proteolysis of the C-terminal auto-inhibitory domain, which leads to a constitutively nuclear translocation of calcineurin (Burkhard et al., 2005).

Calcineurin is known to suppress the immune system, and various pharmaceutical compositions are available from commercial sources. Calcineurin inhibitors belong to the group of cyclosporine, tacrolimus (Protopic®, Prograf®) and pimecrolimus. Indications are psoriasis, atopic dermatitis, rheumatism and allergies, to name a few.

US patent application No. 2003/0045679 A1 to Crawford describes compositions which are useful for inhibiting and potentiating the activity of cellular calcineurin. These compositions include peptides, peptide analogs and whole proteins. They can be used to treat calcineurin related pathologies such as cardiac, brain, immune system and developmental abnormalities.

All known calcineurin inhibitors show strong side effects like high blood pressure, renal disorders and viral and bacterial infections. The later two are due to their general immunosuppressive properties. Thus there is a still existing need for a pharmaceutical composition for the treatment of calcineurin related disorders, like myocardial disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

The solution to this problem is achieved by providing the embodiments characterized by the claims, and described further below.

5 **Summary of the invention**

The present invention is directed to a method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of (a) providing a test sample comprising at least one cell; (b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10; (c) determining an interaction between the peptide of SEQ
10 ID NO: 1 or 2 and cellular calcineurin; wherein a primarily nuclear localization of a complex comprising calcineurin and the peptide of SEQ ID NO: 1, 2, 5-10 indicates susceptibility for a myocardial and/or immunological disorder.

Further, the present invention is directed to a kit comprising the peptide of SEQ ID
15 NO: 1, 2, 5-10 for diagnosing a myocardial and/or immunological disorder.

The present invention also concerns a therapeutic agent comprising the peptide of SEQ ID NO: 1, 2, 5-10, a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5-10 or a peptide of at least 90 % identity of the peptide of SEQ ID NO: 1, 2,
20 5-10. The peptide inhibits substantially the translocation, transport or shuffling of calcineurin and its derivates from the cytoplasm to the nucleus of a cell.

Further the present invention is directed to an antibody against the peptide of SEQ ID NO: 1, 2, 5-10.

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Moreover, the present invention is also directed to the use of the peptide of SEQ ID NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

30 **Brief description of the drawings**

Figure 1 shows the identification of a nuclear localization signal (NLS) in calcineurin (CnA). A schematic drawing of green fluorescent protein (GFP)- and FLAG-tagged CnA deletion mutants is given. The subcellular localization is indicated (c = primarily cytoplasmatic; n = primarily nuclear). NLS = nuclear localization signal; NES = nuclear export sequence; CnB = calcineurin B binding domain; CaM = calmodulin binding domain; AID = auto-inhibitory domain; numbers correspond to CnAß amino acid sequence; EGFP and FLAG indicate tags.

Figure 2 shows interactions of calcineurin (CnA) with importin ß1. Various deletion mutants were transfected into HeLa cells. Immunoprecipitation of GFP-tagged full length CnA and GFP-NLS fusion protein was performed with a GFP-antibody; the FLAG-tagged CnA 2-173 and CnA 3-143 mutants were precipitated with a FLAG-antibody. Immunodetection was performed with an importin ß1 antibody. Full length calcineurin interacted with importin ß1 as demonstrated by co-immunoprecipitation whereas the truncated CnA-mutants, lacking the NLS, did not co-immunoprecipitate with importin.

Figure 3 shows the results of HeLa cells, which were transfected with the indicated GFP-tagged CnA mutants. Immunoprecipitation of the CnA/importin complexes was performed with importin ß1 antibodies, detection with a GFP antibody. Addition of the NLS peptide (SEQ ID NO: 2) abrogated calcineurin interaction with importin ß1.

Figure 4 shows nuclear export signals (NES) in calcineurin (CnA). CnA lacking the NES (Δ420-434) was resistant to proteolysis by calpain. GFP-tagged full length CnA and CnA (Δ420-434) were transfected into HeLa cells. Whole cell lysates were incubated with calpain I for 30 min and the lysates run on a 5 % gel. Western blot analysis for GFP revealed proteolysis of full length CnA.

Figure 5 shows functional consequences of inhibition of calcineurin (CnA) nuclear import. Neonatal rat cardiomyocytes were incubated with a peptide mimicking the

NLS sequence of CnA (SEQ ID NO:2) and stimulated with Ang II (100 μM). The peptide saturated CnA/importin ß1 binding capacity. Nuclear import of CnA was therefore prevented. Control experiments were performed with a non-sense peptide SEQ ID NO: 3).

5

Figure 5A top shows that phosphatase activity of CnA was not influenced by the synthetic NLS peptide (SEQ ID NO: 2) as measured using a specific phosphor substrate of CnA.

10 **Figure 5A bottom** shows that transcriptional activity of the calcineurin/NF-ATc complex was suppressed by the inhibitory NLS peptide (SEQ ID NO: 2). Transcriptional activity was assessed with a NF-ATc luciferase reporter plasmid.

Figure 5B shows that the development of myocardial hypertrophy was also suppressed by the inhibitory NLS peptide (SEQ ID NO: 2) as demonstrated by protein synthesis **(Figure 5B top)** and cell size (magnification 600 x) **(Figure 5B bottom)**.

15

Figure 5C shows that B-type natriuretic peptide (BNP), a molecular marker of hypertrophy, is suppressed by the use of the inhibitory NLS peptide (SEQ ID NO: 2).

20

Figure 5D shows the dose dependent decrease of NF-ATc transcriptional activity when treated with the NLS peptide of SEQ ID NO: 2. At higher concentrations (>1 μM, indicated by asterisk) a toxic effect is possible since values are below background levels of untreated cardiomyocytes (ctr = control).

25

Figure 6 shows T cells, which were stimulated with spleen dendritic cells as antigen stimulus. Bars indicate protein synthesis (H3-thymidin incorporation). When T cells were stimulated (spleen-DC) and simultaneously treated with the blocking peptide (NLS), protein synthesis was inhibited significantly.

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Figure 7A shows the blocking peptide (NLS), which prevented an increase in heart weight in mice that were treated with Ang II for 4 weeks.

Figure 7B shows the treatment of mice with the hypertrophic stimulus angiotensin for 4 weeks resulted in an upregulation of ß-MHC as molecular marker of hypertrophy. The NLS peptide was able to suppress the development of myocardial hypertrophy in vivo and therefore prevented expression of this marker.

Figure 8 shows the treatment of mice with implanted mini-osmotic pumps with AngII. This treatment caused dilation of the left ventricle (left ventricular end diastolic diameter, LVEDD). When the mice were treated with the NLS peptide dilation could prevented. A control peptide did not prevent left ventricular dilation.

Figure 9 shows the blocking peptide (NLS), which did not prevent nuclear accumulation of selected transcription factors (cJUN, ERK 1/2, GATA4, NFAT2) in cardiomyocytes that were stimulated with phenylephrin.

Detailed description of the invention

The inventor has surprisingly found that a peptide which mimics the nuclear localization signal of calcineurin (SEQ ID NO: 2) successfully prevents the translocation of calcineurin from the cellular cytoplasm to the nucleus. Without intending to be bound by any theory, it is believed that the NLS peptide (SEQ ID NO: 2) binds to importin, thereby preventing complex formation between calcineurin and importin. However, complex formation between calcineurin and importin is necessary for transporting calcineurin to the nucleus, where calcineurin together with NF-ATc then act as activated transcription factor due to the constant presence of calcineurin. The NLS peptide (SEQ ID NO: 2) efficiently inhibits complex formation and thereby blocks entry of calcineurin into the nucleus. It is further believed that that the auto-inhibitory domain (AID) not only blocks the catalytical activity of calcineurin but also masks the nuclear localization signal. Removal of the AID via a

conformational change in calcineurin following Ca^{2+} activation or by proteolysis of the auto-inhibitory domain leads to exposure of the nuclear localization signal and resultant nuclear translocation of calcineurin.

5 Sequences with SEQ ID NO: 5-10 (see section Examples) also proved to behave similar like SEQ ID NO: 1 and can be equally used in the present invention.

Myocardial disorders and immunological disorder are associated with altered transcription patterns of NF-ATc downstream targets. Therefore, the NLS peptide may 10 be used as a diagnostic tool for assessing the susceptibility for a myocardial and/or immunological disorder.

In a first aspect, the present invention is directed to a method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:
15 a) providing a test sample comprising at least one cell;
b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10;
c) determining an interaction between the peptide of SEQ ID NO: 1, 2, 5-10 and cellular calcineurin;

wherein a primarily nuclear localization of a complex comprising calcineurin and 20 the peptide of SEQ ID NO: 1, 2, 5-10 indicates susceptibility for a myocardial and/or immunological disorder.

As used herein the term susceptibility means predisposition or likelihood for the development of a myocardial and/or immunological disorder. The term disorder 25 means a dysfunction, a malfunction, a disease or a medical condition. The term peptide includes linear peptides, cyclic peptides and peptide analogs, derivates and salts thereof. Cyclic peptides are particularly preferred. Peptides forming a cyclic peptide may be closed head to tail, head to one of the side chains or tail to one of the side chains. Any modification can be employed as long as the peptide 30 retains its ability to prevent entry of calcineurin into the nucleus of a cell. The peptides of the present invention can be incorporated in a structure comprising more

amino acids, and the peptides comprise preferably about 60 amino acids, more preferred about 30 to 40 amino acids, most preferred about 20 to 30 amino acids.

The test sample can be derived from an animal, in particular a mammal, preferably from a human, more preferably from a patient who is suspected to be susceptible of a myocardial and/or immunological disorder.

Likewise, SEQ ID NO: 1, 2, 5-10 can be used for a method for diagnosing further calcineurin related disorders, like myocardial disorders, immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

In a preferred embodiment the peptide of SEQ ID NO: 1, 2, 5-10 is chemically, biologically and/or physically labeled. Examples for labeling peptides are dyes, like streptavidin-biotin labeling, fluorescent labeling, labeling by antibodies and radioactive labeling.

In a further preferred embodiment the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack. The immunological disorder is selected from the group consisting of transplant rejection and immune suppression. It is also possible to prevent restenosis after stent implantation by means of the NLS peptide of the present invention.

In a particularly preferred embodiment the disorder is a T-cell associated disorder. Specific examples for T-cell associated disorders are rheumatoid arthritis and forms thereof, psoriasis, psoriasis arthritis, systemic lupus erythematodes, vasculitis, other mixed connective tissue diseases, Sjögren syndrome, uveitis, inflammatory bowl diseases like Colitis ulcerosa and Morbus Crohn, intrinsic/extrinsic

asthma, graft versus host reaction and transplant rejection (prevention of organ rejection and treatment of chronic rejection)

Also diagnostic purposes with respect to T-cells are enclosed. The peptide may be used in a diagnostic kit to test activation status of T-cells from patients. This may be used to monitor disease progress or therapeutic success in e.g. immunosuppressed patients with transplanted organs.

In a second aspect the invention provides a kit comprising the peptide of SEQ ID NO: 1, 2, 5-10 for diagnosing susceptibility for a myocardial and/or immunological disorder. Likewise, SEQ ID NO: 1, 2, 5-10 can be used for a kit for diagnosing further calcineurin related disorders, like myocardial disorders, immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

In another aspect the present invention concerns a therapeutic agent comprising the peptide of SEQ ID NO: 1, 2, 5-10 and a therapeutic agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5-10.

The term nucleic acid, as used herein, includes DNA molecules, like cDNA and genomic DNA, RNA molecules, like mRNA, analogs of DNA and RNA which are generated by nucleotide analogs and derivates, fragments and homologs thereof. The nucleic acid can be single stranded or double stranded, however double stranded DNA molecules are preferred.

In another aspect, the present invention covers a therapeutic agent comprising a peptide of at least 90 %, preferably at least 95 %, more preferred at least 98 %, still more preferred 100 % identity of the peptide of SEQ ID NO: 1, 2, 5-10; wherein the peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell. Conserved amino acid substitutions are cov-

ered by the present disclosure. The peptide of SEQ ID NO: 2 comprises three lysine amino acids (K) at positions 1, 5 and 7. When all lysine residues (K) are substituted by alanine (A) the peptides does no longer inhibit entry of calcineurin to the nucleus. However individual lysine residues may be substituted as long as the 5 peptide prevents it ability to block the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

In a preferred embodiment the therapeutic agent further comprises a pharmaceutically acceptable carrier, like fillers and excipients. Various application forms of 10 the peptides are possible. Cremes and ointments can be applied topically. In stent technology, the peptide can be used to cover a stent, which is then implanted to a patient in need thereof. Coronary stents are particularly preferred. Also intravascular pumps, in particular mini pumps can be used for delivery of the peptide to a patient.

15

In a further aspect, the invention is directed to an antibody against the peptide of SEQ ID NO: 1, 2, 5-10.

In another aspect, the present invention covers the use of the peptide of SEQ ID 20 NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

In a preferred embodiment the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive 25 cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack. The immunological disorder is selected from the group consisting of a transplant rejection and immune suppression.

30 In yet another aspect the present invention is directed to the use of the peptide of SEQ ID NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic

and/or therapeutic treatment of calcineurin related disorders, like myocardial disorders, immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

A more complete understanding of the present invention can be obtained by reference to the following examples, which are provided for the purpose of illustration only and are not intended to limit the scope of the invention.

Examples

A) Material and Methods

The following materials and methods were employed:

Cell culture

Neonatal rat cardiomyocytes of Wistar rats were isolated as described previously (Ritter et al., 2003). Cells were resuspended in minimum essential medium/1 % FCS. After preplating, the cardiomyocyte containing supernatant was recovered, and cells were plated in minimum essential medium on 6-well plates at a density of $1x10^6$ cells per well. The medium for cardiomyocytes contained 5-bromo-2'-deoxyuridine (0.1 mmol/L) to suppress fibroblast growth. Fibroblast contamination of cardiomyocyte cultures was between 4 % and 7 % as regularly determined by immunohistochemical staining for troponin T. HeLa cells were grown in DMEM/5 % FCS (Sigma). 48 hours after preparation, cells were stimulated with 10 nmol/L angiotensin II (Ang II). Cells were harvested 24 hours after stimulation. Concentrations were as follows: Ang II: 10 µM, calpeptin: 10 µM, NLS peptide and control peptide: 1 µM, respectively.

Calcineurin (CnA) activity

NF-ATc reporter plasmid comprised the Il-2 promoter followed by luciferase. Luciferase activity was determined according to the manufacturer's protocol

(Promega). CnA phosphatase activity was determined using a commercial kit (CnA kit assay, Biomol) that measures effect on a specific CnA phosphosubstrate. Free PO_4 was indicated by a malachite green dye. The use of this kit has been described previously (Ritter et al., 2002).

5

Plasmids

Epitope-tagged derivatives of calcineurin Aβ containing amino-terminal EGFP were generated using the mammalian expression vector pEGFP-C3 (BD Bioscience Clontech). For cloning of calcineurin mutants the mammal-
10 ian expression vector pCMV-Sport6 containing the directionally cloned cDNA of human calcineurin Aβ was used (Invitrogen). The following mutants have been amplified by PCR, digested with XbaI and XhoI and cloned into the XbaI and XhoI sites of the described plasmid: CnAβ(full), CnAβ 1-415, CnAβ 1-425, CnAβ 1-445, CnAβ 1-465, CnAβ 1-485 and CnAβ 171-
15 190. For these truncated mutants different reverse oligonucleotides were designed with subsequent N-terminal binding sites. The forward primer, binding at the N-terminus of calcineurin, was identical for each plasmid apart from the CnA(171-190) mutant. For this derivative primers are bound upstream of aa 171 and downstream of aa 190, respectively. Two internal
20 mutants CnAβ(Δ420-434) and CnAβ(Δ420-445) were cloned into the XbaI and XhoI sites of pEGFP-C3 using a two step strategy. For deleting the short areas from aa 420 to 434 and from aa 420 to 445 two fragments have been amplified. After ligation of these two fragments the resulting whole fragment was cloned into the XbaI and XhoI sites of pEGFP-C3. FLAG-
25 tagged calcineurin derivatives were kindly provided by the group of Ludwig Neyses (Divison of Cardiology, The University of Manchester).

Immunofluorescence and immunoprecipitation

The subcellular distribution of calcineurin was determined by immunofluo-
30 rescence stainings. The primary antibodies used in fluorescence staining are described elsewhere (Burkhard et al., 2005). Secondary antibodies

were Cy-3–labeled sheep anti-rabbit IgG or Cy2-conjugated mouse anti-goat IgG (Jackson Laboratories).

B) Results

5 The inventor's group demonstrated that posttranslational modification, specifically proteolysis of the auto-inhibitory domain (AID) of calcineurin leads to its activation and strong nuclear translocation (Burkhard et al., 2005). The calpain mediated cleavage of the c-terminal AID and the causative link to myocardial hypertrophy was demonstrated in human myocardial tissue.

10

In this invention the inventor demonstrates the prominent nuclear translocation of CnA in different animal models of diseased myocardium. In wild-type mice a predominantly cytosolic distribution of CnA was observed, whereas in mice that underwent aortic banding or myocardial infarction a strong nu-
15 clear localization of CnA in the hypertrophied myocardium was observed.

This is consistent with observations of nuclear import of calcineurin in cell culture models (Burkhard et al., 2005; Frey et al., 2000; Zou et al., 2001; Zhu et al., 1999). However, the role of calcineurin within the nucleus was
20 not been investigated before and the precise mechanism leading to nuclear import and export remains unclear.

Reversible nuclear localization of calcineurin after angiotensin stimulation

25 To assess whether CnA import into the nucleus is a chronic phenomenon or an acute response the inventor investigated the time course of CnA shuttling. A GFP tagged calcineurin plasmid encoding full length CnAß was transfected into neonatal rat cardiomyocytes. Cells were stimulated with Ang II at 10 μM. Confocal microscopy revealed onset of nuclear transloca-
30 tion of calcineurin after 2h. After 4h of Ang II stimulation CnA was predominantly nuclear. After 6h maximum of intensity of the GFP-calcineurin signal

was in the nucleus. Similarly, 2h after removal of Ang II from the medium there was a homogenous distribution of CnA in the cytosol and the nucleus and after 4h there was perinuclear localization of CnA. 6h after removal of the stimulus calcineurin localised completely in the cytosol again. To prevent CnA from calpain mediated proteolysis, which would cause constitutive activation of CnA and therefore persistent nuclear translocation, all experiments where performed in the presence of a membrane permeable calpain inhibitor (Burkhard et al., 2005). Calcineurin is very sensitive to pathological stimuli in cardiomyocytes and is able to induce a response within a few hours after the initial stimulation.

Construction of NLS peptide mutants

The transport of proteins into the nucleus relies on nuclear localization signal/sequences (NLS) which are bound by importins; the complex of cargo protein and importin is then transported into the nucleus. To define the regions of calcineurin that are required for nuclear import different GFP- or FLAG-tagged calcineurin deletion mutants (Figure 1) were screened to assess those that entered the nucleus and those that remained cytosolic. In general, deletion of the auto-inhibitory domain led to nuclear translocation and deletion of the region starting amino acid 173 (within the putative NLS) prevented calcineurin from entering the nucleus. The mutants 2-173 and 3-143 may have reduced catalytic phosphatase activity as the catalytic subunit of CnA extends to amino acid 333. Therefore failure to translocate may in theory be a result of decreased phosphatase activity. Previous work however has demonstrated that catalytically inactive CnA mutants are also translocated into the nucleus (Shibasaki et al., 1996). Sequence comparisons with known NLS of other proteins enabled further delineation of the putative NLS region to the sequence 171-190. Fusion of this 171-190 fragment to the GFP backbone resulted in translocation of the GFP/NLS fusion protein into the nucleus, whereas the pure GFP backbone remained cytosolic. Whilst full length CnA resides in the cytosol, it was translocated into the

nucleus after Ang II stimulation due to removal of the auto-inhibitory domain from the catalytic subunit and probably from the putative NLS. In contrast, deletion mutants 2-173 and 3-143 which both lacked the putative NLS remained exclusively cytosolic despite Ang II stimulation.

5

Interaction between calcineurin mutants and importin

Importin ß1 has been shown to bind the NLS of different cargo proteins (Pemberton et al., 2005). Interactions between CnA mutants and importin ß1 were assessed to determine whether the functionally defined NLS

10 physically interacts with importin ß1. As demonstrated by co-immunoprecipitation experiments importin ß1 displayed good affinity for full length calcineurin and also the deletion mutant CnA 1-415. Specifically, the interaction domain was mapped to the region 171-190 as evidenced by the ability of a fusion protein consisting of GFP and the 171-190 fragment to

15 co-precipitate importin ß1. However, both deletion mutants 1-173 and 1-143 completely abolished the interaction between importin ß1 and CnA. These data indicate that the NLS identified by functional analyses also mediate interactions between importin ß1 and calcineurin (Figure 2).

20 **Peptide competition assay**

To demonstrate further that the identified NLS in CnA is essential for the nuclear import of calcineurin, a peptide competition assay to prevent importin ß1/CnA binding was used. A peptide with the putative NLS sequence of calcineurin (AAVALLPAVLLALLAKQECKIKYSERV – SEQ ID NO: 1) was

25 synthesised and added to the medium (the first 15 letters give N-terminal extension to increase membrane permeability, NLS sequence is underlined). However, the peptide facilitating membrane permeability is not critical, and an alternative membrane anker, harbouring two alanine residues instead of leucine residues at positions 11 and 14, respectively, can also be

30 used (AAVALLPAVLAALAA – SEQ ID NO: 4). In control experiments a non sense peptide (AAVALLPAVLLALLAAQECAIAYSEYV – SEQ ID NO: 3)

was used. Addition of the synthetic NLS peptide saturated the binding domain of importin ß1 for CnA and therefore prevented CnA binding to importin ß1. Inhibition of this interaction suppressed calcineurin nuclear import. The non-inhibitory control peptide did not interfere with the calcineurin/importin binding; accordingly, nuclear translocation of CnA was not inhibited. Also the NLS peptide abrogated the interaction of calcineurin and importin ß1 as demonstrated in co-immunoprecipitation assays (Figure 3).

Nuclear export control sequences

To identify sequences in CnA that control nuclear export serial carboxy-terminal truncation mutants with an N-terminal GFP tag of CnA were generated and examined by confocal fluorescence microscopy. Experiments were performed in the presence of a calpain inhibitor to prevent calpain induced cleavage of the auto-inhibitory domain (AID) and to ensure functional integrity of calcineurin. Cells were stimulated with Ang II for 12 h to achieve nuclear entry of CnA, subsequently the stimulus was removed to promote nuclear export. Full-length CnA (amino acids 1–524) was re-localized exclusively to the cytosol of transfected cardiomyocytes after removal of the stimulus. An extended deletion variant (1-415) was not able to leave the nucleus any more.

These results demonstrated that sequences in the region c-terminal to amino acid 415 regulate nuclear export. Consistent with these findings and sequence comparisons with known NES sites, a CnA mutant lacking amino acids 420-434 remained exclusively nuclear after removal of the stimuli. Inhibition of calpain did not influence this result as the calpain cleaveage site (at 424) was deleted in this mutation variant.

As different cleavage sites in the calcineurin sequence (Wu et al., 2004) have been described for calpain, the inventor assessed whether the NES

sequence (423-434) really contains the calpain cleavage site. Therefore, the GFP-tagged calcineurin mutant lacking the NES domain was used for further experiments. The plasmid was transfected into HeLa cells and lysates of these cells expressing this deletion variant were incubated with calpain I. Western Blot analysis was used to demonstrate that full length CnA is proteolysed by calpain whereas CnAΔ423-434 is resistant to calpain mediated proteolysis (Figure 4).

Mechanism of nuclear export of calcineurin

A number of proteins that shuttle across the nuclear membrane have been identified using CRM1 as the export shuttle. Some are transcription factors like NF-ATc that confer pro-hypertrophic actions. Others act within counter-regulatory pathways that repress cardiac hypertrophic growth such as the transcriptional repressor HDAC5 (McKinsey et al., 2000). To address whether CnA nuclear export is mediated by CRM1, experiments using the CRM1 specific inhibitor, leptomycin B (LMB), were performed. Agonist-dependent nuclear import of full length CnA was achieved by Ang II stimulation. Calpeptin was added to prevent proteolysis of CnA. The addition of LMB to prevent CRM 1 mediated export indeed suppressed nuclear export of CnA. Together, these findings demonstrate that LMB confines CnA to the nucleus in the cardiomyocyte by blocking its nuclear export. This supports the hypothesis that nucleo-cytoplasmic shuttling of CnA is coupled to a NES between amino acids 423-434 and is mediated by CRM1.

Nuclear accumulation of calcineurin

In vivo studies of pathological myocardial hypertrophy showed that proteolysis of the calcineurin auto-inhibitory domain at amino acid 424 results in a constitutively active calcineurin mutant lacking both the AID (aa 468-490) and the NES (423-434). To determine whether loss of the AID or disruption of the NES is responsible for strong nuclear accumulation of CnA the nuclear import and export qualities of a GFP tagged CnA mutant with a dele-

tion of the NES was investigated. Cells were transfected with CnAΔ423-434. In this case calcineurin resided in the cytosol. Stimulation of the transfected cells with Ang II resulted in subsequent translocation of CnA into the nucleus. Based on these results the inventor concluded that the AID not only blocks the catalytical activity of CnA but also masks the NLS. Removal of the AID via a conformational change in calcineurin following Ca^{++} activation or by proteolysis of the auto-inhibitory domain leads to exposure of the NLS and resultant nuclear translocation of CnA.

Subsequent removal of the stimulating Ang II agent from the medium resulted in the CnAΔ423-434 mutant remaining nuclear, as the lack of the NES made it impossible for CRM1 to interact with CnA and to transport it back to the cytosol. The loss of the C-terminal part of CnA would therefore appear to regulate nuclear shuttling of CnA at the level of both nuclear import and export. Deprivation of the AID promotes import via importin ß1, and loss of the NES hinders nuclear export via CRM1 mediated mechanisms.

A peptide corresponding to the calcineurin NLS inhibited CnA nuclear import and maintained the overall structure of CnA. The suppression of calcineurin transport into the nucleus is important for the NF-ATc transactivational status; this peptide is therefore useful as a tool to suppress myocardial hypertrophy.

Markers for myocardial hypertrophy

Phosphatase activity, transcriptional activity, protein synthesis, cell size and makers of myocardial hypertrophy in response to the peptide related inhibition of CnA nuclear import were examined. Phosphatase activity was assessed employing a specific substrate (RII) for can (Ritter et al., 2002). Cardiomyocytes were stimulated with Ang II (10 µM), and CnA phos-

phatase activity was measured in the presence of the NLS peptide or a non sense control peptide. Total CnA phosphatase activity was not affected by inhibition of the access of importin ß1 to the CnA NLS (289±17 % vs. 273±11 %, n = 8, p = not significant). In contrast, transcriptional activity of
5 the CnA/NF-ATc signalling pathway was decreased significantly by the NLS peptide (463±11 % vs. 123±8 %, n=8, $p<0.05$) (Figure 5A top). Similarly, myocardial hypertrophy, as evidenced by protein synthesis (707±21 % vs. 133±12 %, n=8, $p<0.05$), cell size (1191±91 μm^2 vs. 728±65 μm^2, n=8, $p<0.05$) (Figure 5B), and expression of brain natriuretic peptide (BNP)
10 (163±11 % vs. 88±8 %, n=8, $p<0.05$) (Figure 5C), were suppressed by the NLS peptide. Transcriptional activity of a NF-ATc luciferase reporter plasmid was decreased when nuclear import of CnA was blocked by the NLS peptide in a dose dependent manner (Figure 5D).

15 These data indicated that despite full CnA phosphatase activity, CnA was unable to form effective transcriptional complexes. Activated calcineurin in itself does not appear to be sufficient to induce hypertrophy. Full transcriptional activity of CnA/NF-ATc is only possible in the presence of nuclear calcineurin. It is thus clear that calcineurin nuclear translocation is a pre-
20 requisite to the formation of effective NF-ATc transcriptional complexes.

T-cell experiments

The inventor above presented evidence, that inhibition of the interaction of the phosphatase calcineurin and its importin (importin ß1) prevents nuclear
25 translocation of calcineurin. This suppressed activation of the calcineurin/NF-AT signalling cascade (Hallhuber et al.). The above experiments were performed in cell cultures of isolated cardiomyocytes or immortalized cell lines.

30 The inventor now extended the experiments to T-cells and to in vivo experiments in mice. In Figure 6 the inventor demonstrates suppression of T-

cell function using the specific approach of competitive peptide inhibition of the calcineurin/importin interaction. The blocking peptide significantly prevented activation of lymphocytes.

5 In Figure 7 the inventor demonstrates in vivo data. For in vivo use the blocking peptide, that mimics the calcineurin nuclear localization signal (NLS) received a peptide extension to increase membrane permeability and was synthesised as a cyclic peptide to withstand proteolysis of N- and C-terminal proteases. This peptide was administered twice daily at a concentration of 25 mg/kg body weight/day by i.p. injections into mice. The mice were treated with implanted osmotic minipumps to release either angiotensin II or saline as control. Angiotensin II caused myocardial hypertrophy as demonstrated by the heart/body weight ratio and expression of molecular markers of hypertrophy. The blocking peptide was able to prevent the development of myocardial hypertrophy. On a cellular level the inventor could also demonstrate, that the blocking peptide suppressed calcineurin entry into the nucleus in vivo.

Similar results were seen in NFAT Luciferase reporter transgenic mice (mice were received from Wilkins et al. for the experiments). Administration of the NLS peptide in angiotensin II treated mice suppressed Luciferase activity and therefore demonstrated effectiveness of the NLS peptide in this animal model.

From the presented data it is concluded that inhibition of calcineurin/importin interaction and the resulting inhibition of calcineurin nuclear entry is a promising concept to inhibit the calcineurin/NF-AT signalling pathway. For the first time in vivo data and proof of concept in lymphocytes is provided. The NLS peptide can be used as therapeutic tool in further pathological conditions were suppression of calcineurin signalling is promising like treatment of myocardial hypertrophy, immunosuppression in rheu-

matic disease or in organ transplantation to suppress donor organ rejection. Additional therapeutic fields are psoriasis, colitis, allergic diseases as asthma or restenosis of coronary vessels after percutaneous interventions.

5

Further alternative nuclear localisation signals and membrane ankers

Above evidence is presented that inhibiton of the interaction of the phosphatase calcineurin and its importin (importin ß1) prevents nuclear translocation of calcineurin. This suppressed activation of the calcineurin/NF-AT signalling cascade (Hallhuber et al. 2006). Previous experiments were performed in cell cultures of isolated cardiomyocytes or immortalized cell lines.

10

The inventor now extended his experiments to in vivo data. Specifically, the inventor was able to show that the blocking peptide prevents myocardial hypertrophy in AngII treated mice as demonstrated by echocardiography (Figure 8).

15

The inventor also analyzed the specificity with the peptide (AAVALLPAVLAALAA KQECKIKYSERV SEQ ID NO: 5). In theory this peptide that mimics the Calcineurin NLS saturates the binding site on importin and therefore might completely block importin function for other transcription factors as well. However, this peptide did not block import of selected transcription factors, that also utilize importin ß1 (such as cJun, GATA4, NFAT2 and ERH 1/2) (Figure 9).

20

25

The inventor also tested different amino acid sequences that are theoretically more resistant to proteolysis for the capacity to inhibit calcineurin/importin interaction, namely:

- AAVALLPAVLAALAA KQEAKIKYSERV (SEQ ID NO: 6)
- AAVALLPAVLAALAA KQECKIKYAERV (SEQ ID NO: 7)
- AAVALLPAVLAALAA KQEAKIKYAERV (SEQ ID NO: 8)

30

-AAVALLPAVLAALAA KAECKIKYSERV (SEQ ID NO: 9)
-AAVALLPAVLAALAA KAEAKIKYSERV (SEQ ID NO: 10)

These peptides have all the same impact: they do block calcineurin/importin interaction as demonstrated in coimmunoprecipitation experiments

Conclusion

The inventor's data show that CRM1 not only exports NF-ATc, but also calcineurin across the nuclear envelope. To interrupt transcriptional activity of the calcineurin/NF-ATc signalling cascade CRM1 is first required to export calcineurin, so that in a second round CRM1 can access the NES of NF-ATc and subsequently proceed with its nuclear export. This mechanism is prevented in myocardial hypertrophy by the proteolysis of calcineurin by calpain at amino acid 424, resulting in loss of the auto-inhibitory domain including the NES. In this scenario calcineurin remains nuclear because it is inaccessible to the export protein CRM1.

As import always precedes export, the inhibition of CnA nuclear import by peptide competition for the binding of the nuclear import protein importin ß1 presents a more sophisticated approach to abolishing the deleterious effects of exaggerated NF-ATc transcriptional activity. Therefore the NLS peptide is useful as a new therapeutic agent for pathologic myocardial hypertrophy.

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Claims

1. A method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:
   a) providing a test sample comprising at least one cell;
   b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10;
   c) determining an interaction between the peptide of SEQ ID NO: 1, 2, 5-10 and cellular calcineurin;

   wherein a primarily nuclear localization of a complex comprising calcineurin and the peptide of SEQ ID NO: 1, 2, 5-10 indicates susceptibility for a myocardial and/or immunological disorder.

2. The method of claim 1, wherein the peptide of SEQ ID NO: 1, 2, 5-10 is chemically, biologically and/or physically labeled.

3. The method of claim 1 or 2, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

4. The method of any of any of claims 1 to 3, wherein the disorder is a T-cell associated disorder.

5. A kit comprising the peptide of SEQ ID NO: 1, 2, 5-10 for diagnosing susceptibility for a myocardial and/or immunological disorder.

6. A therapeutic agent comprising the peptide of SEQ ID NO: 1, 2, 5-10.

7. A therapeutic agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5-10.

8. A therapeutic agent comprising a peptide of at least 90 %, preferably at least 95 %, more preferred at least 98 %, still more preferred 100 % identity of the peptide of SEQ ID NO: 1, 2, 5-10; wherein the peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

9. The therapeutic agent of any of the claims 6 to 8, further comprising a pharmaceutically acceptable carrier.

10. An antibody directed against the peptide of SEQ ID NO: 1, 2, 5-10.

11. Use of the peptide of SEQ ID NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

12. The use of claim 11, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

SEQUENCE LISTING

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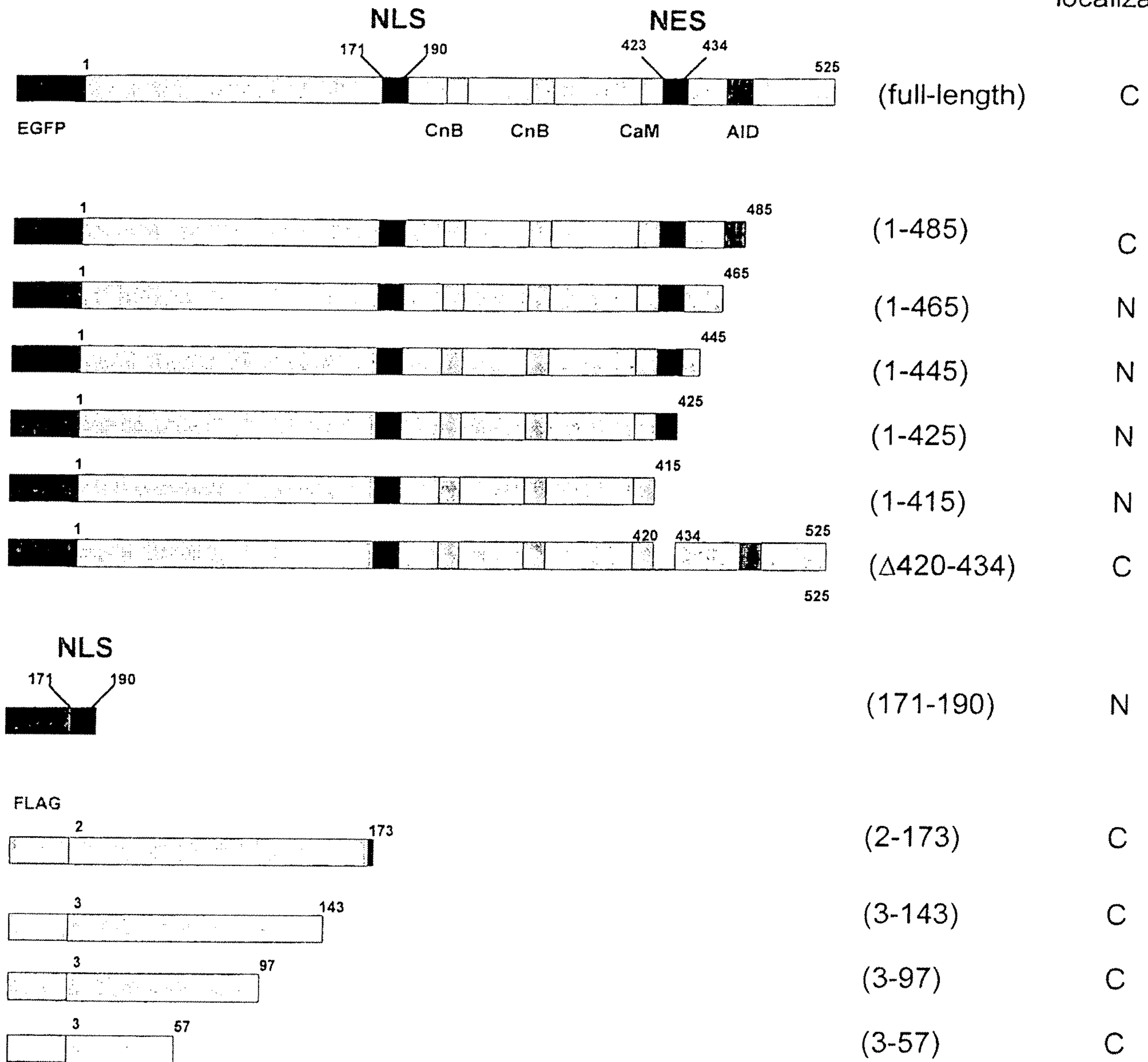

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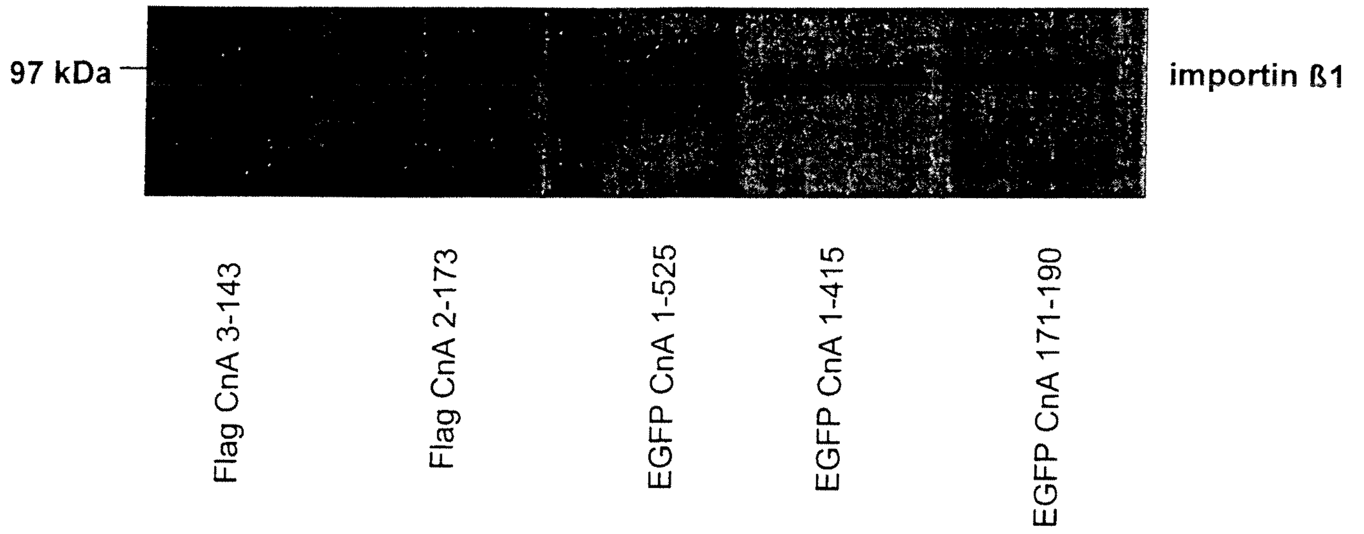

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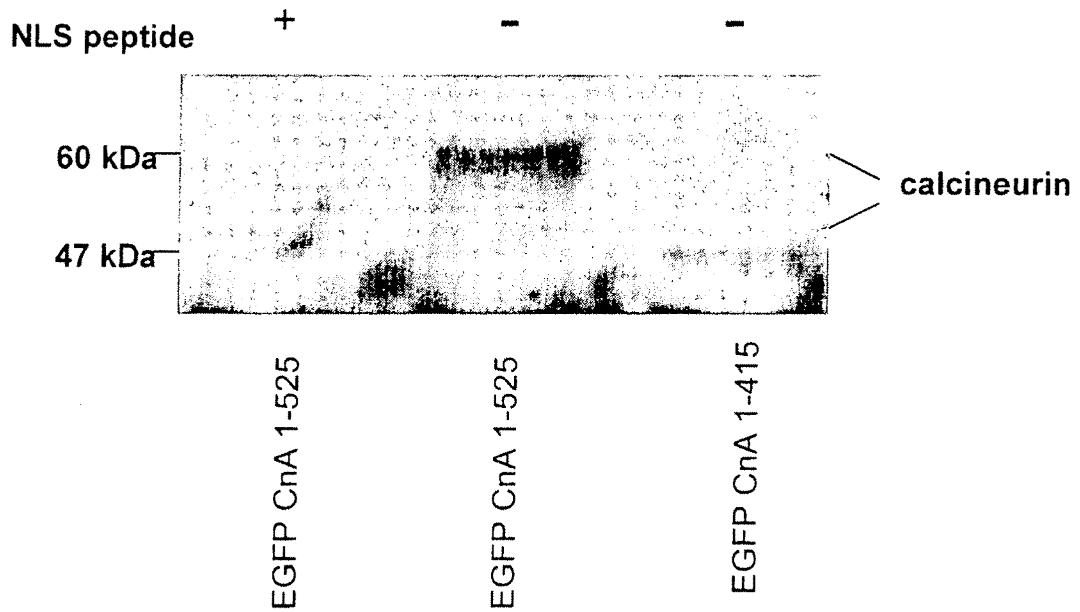

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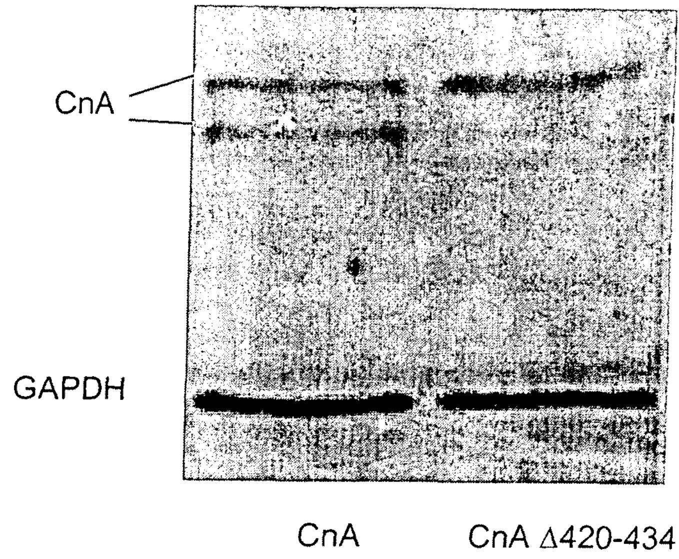

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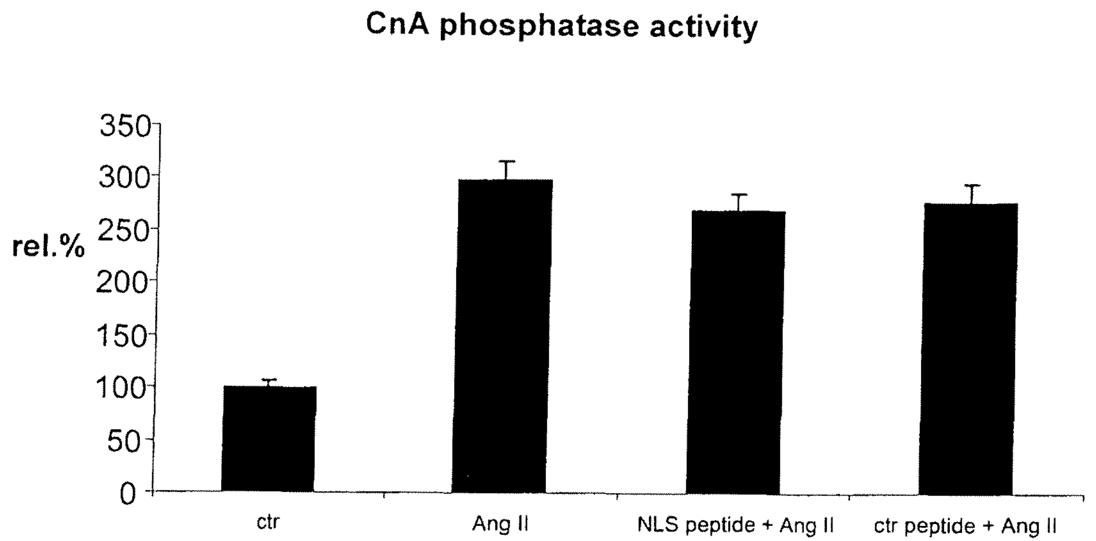

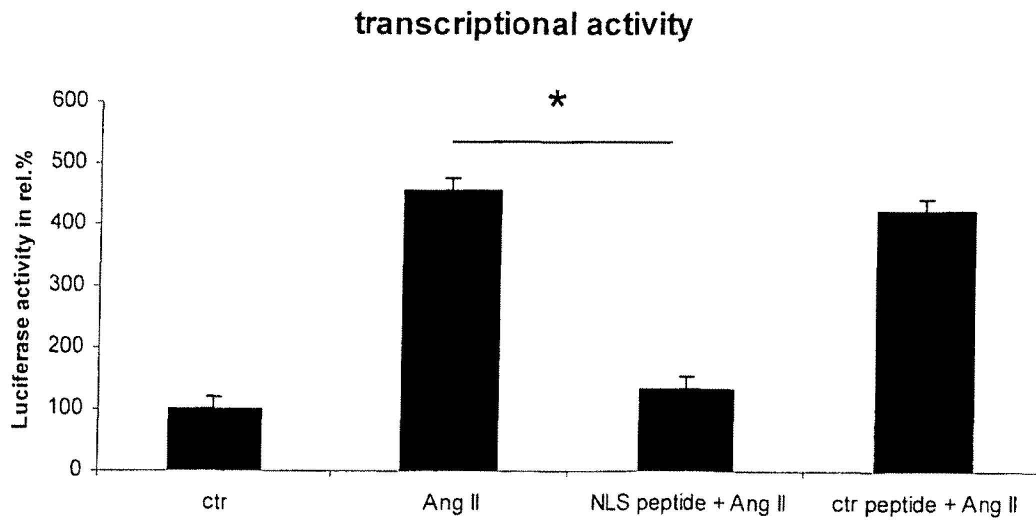

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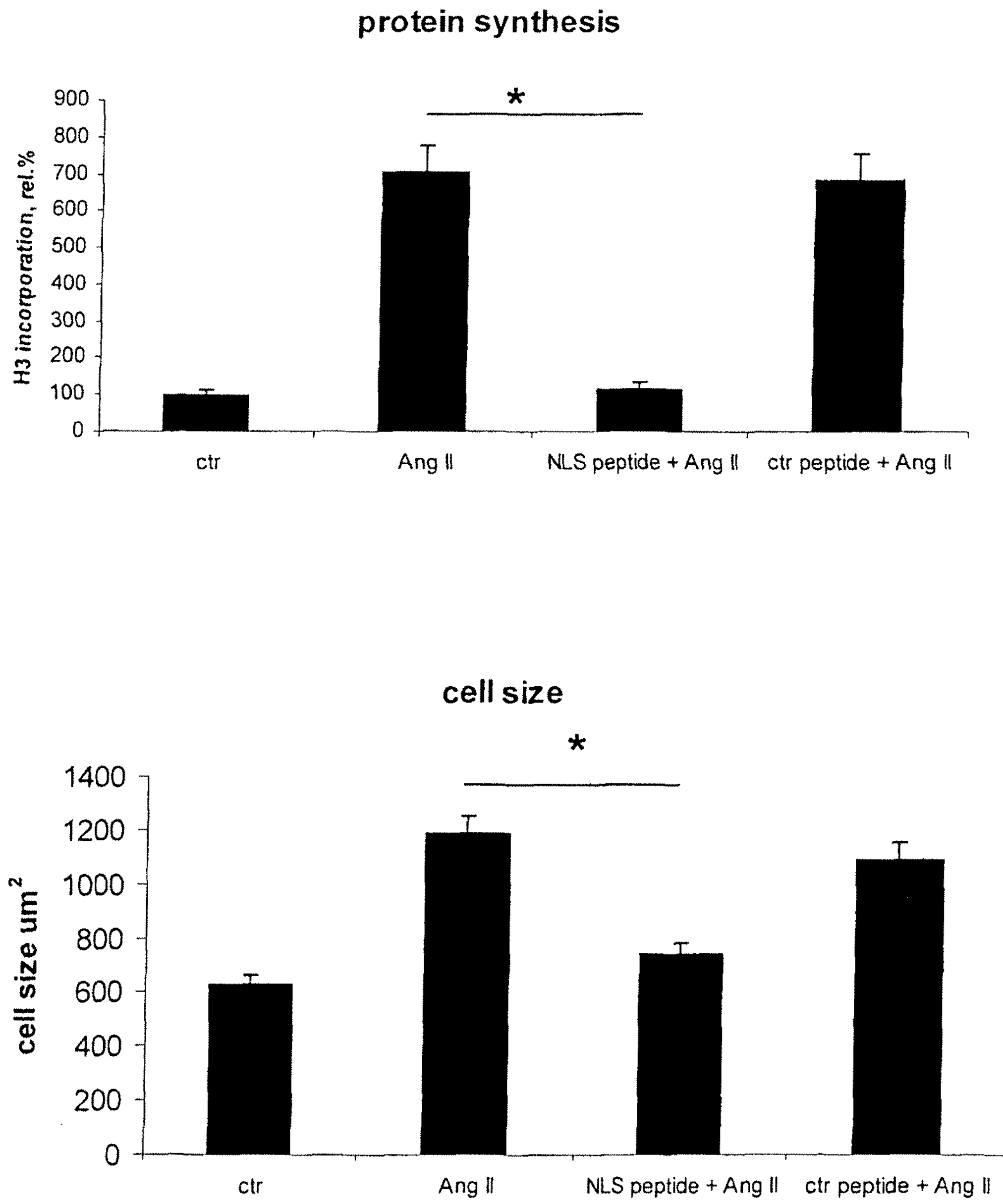

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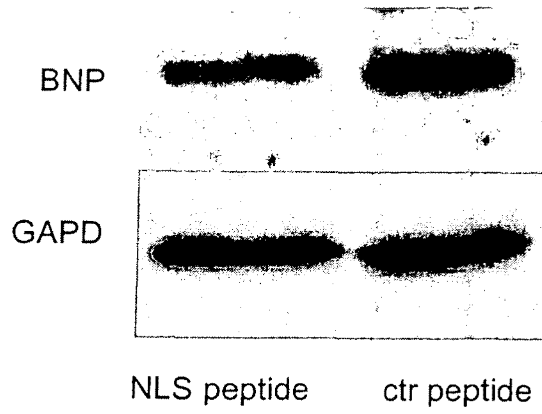

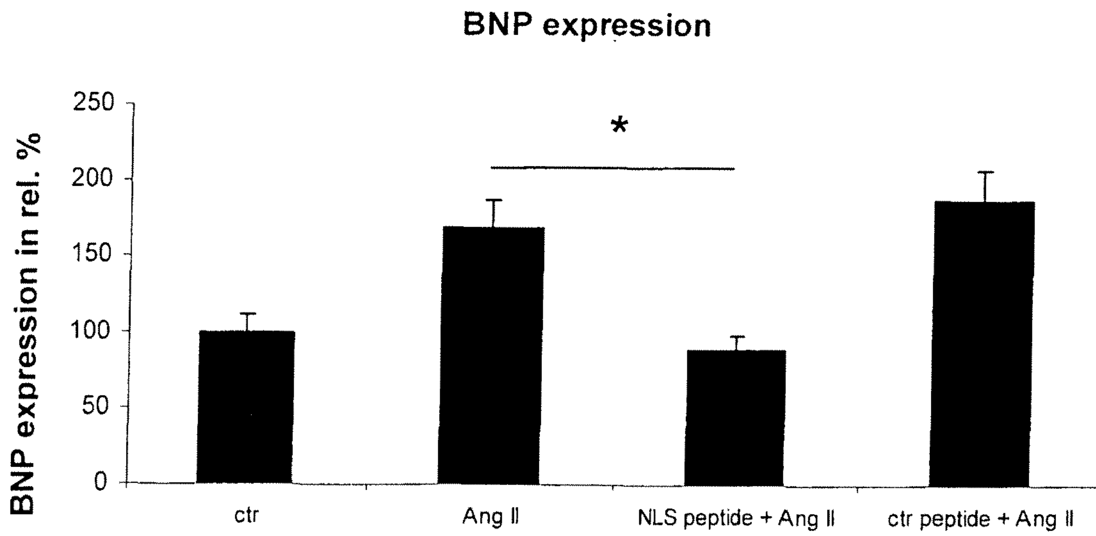

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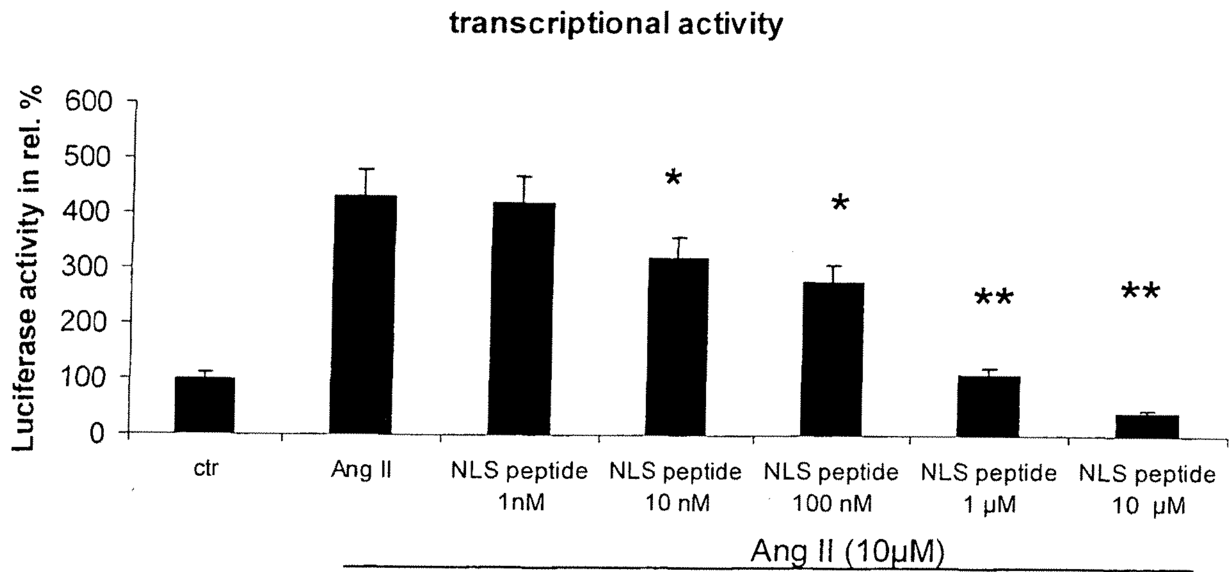

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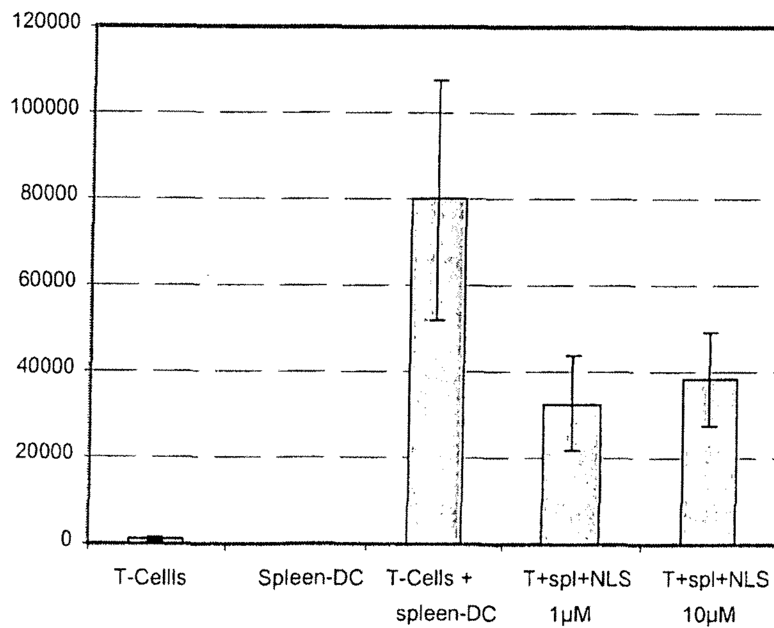

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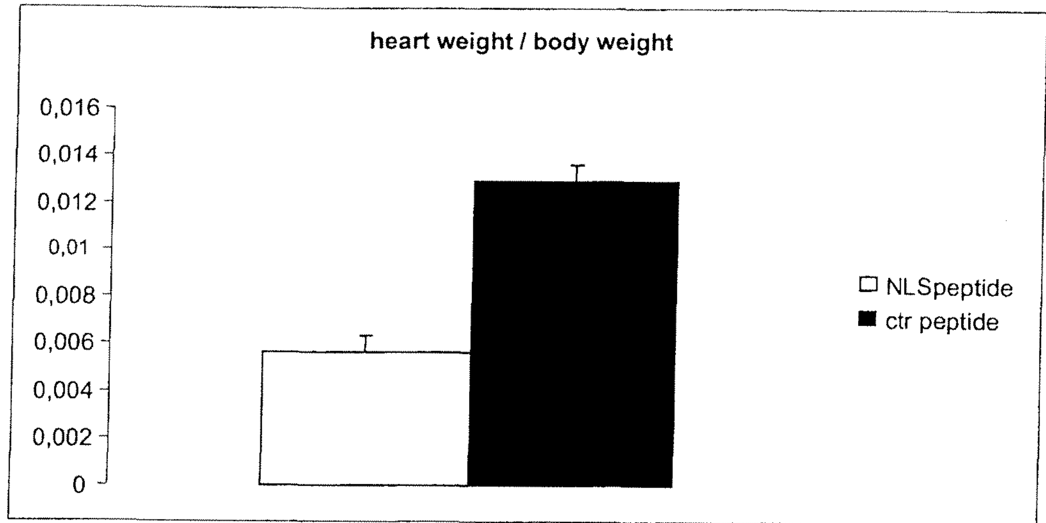

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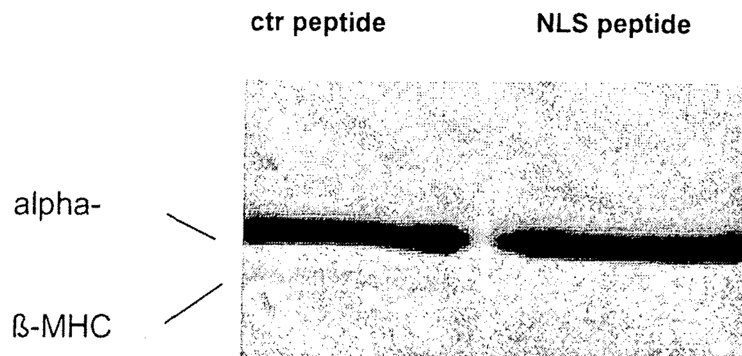

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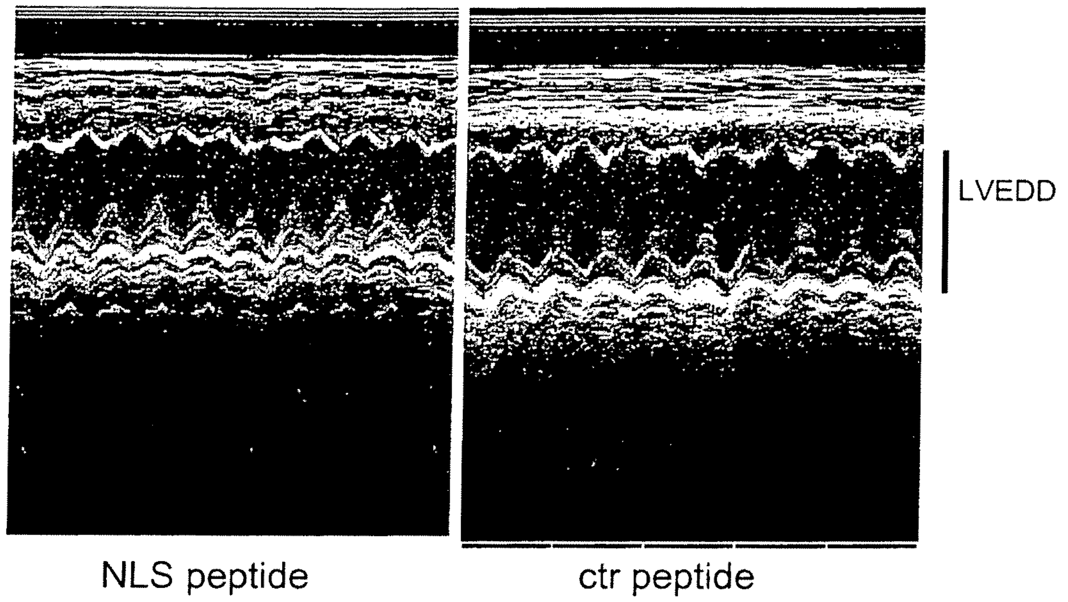

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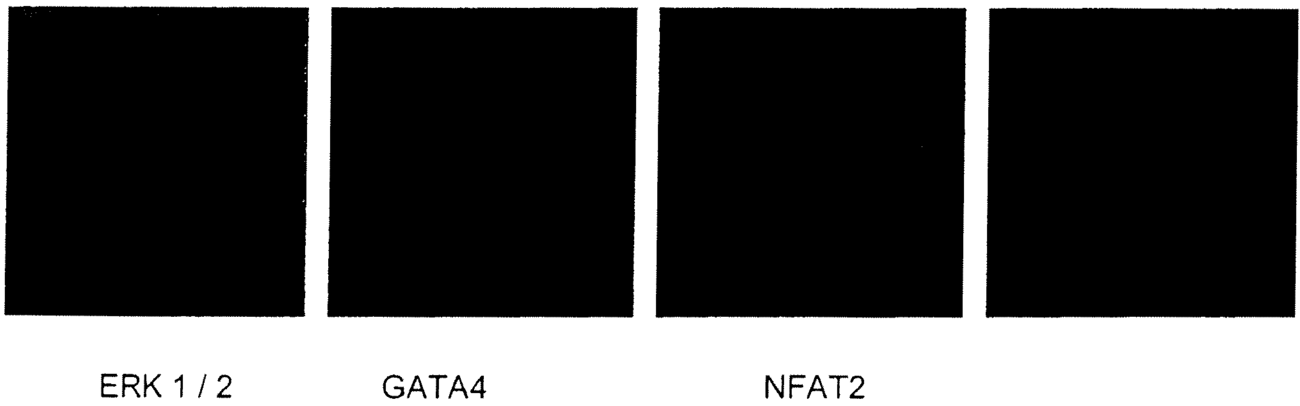

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Figure 9

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First Named Inventor/Applicant Name:	OLIVER RITTER
Filer:	Ajay A. Jagtiani/Susan Mahon
Attorney Docket Number:	ROTE-0007-UT1

Filed as Small Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
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Basic National Stage Fee	2631	1	155	155
Natl Stage Search Fee - all other cases	2632	1	255	255
Natl Stage Exam Fee - all other cases	2633	1	105	105
Pages:				
Claims:				
Independent claims in excess of 3	2614	4	105	420
Miscellaneous-Filing:				
Oath/decl > 30 mo. from priority date	2617	1	65	65

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Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
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Application Number:	12162135
International Application Number:	PCT/EP07/00643
Confirmation Number:	8455
Title of Invention:	PEPTIDE FOR INHIBITION OF CALCINEURIN
First Named Inventor/Applicant Name:	OLIVER RITTER
Customer Number:	22506
Filer:	Ajay A. Jagtiani/Susan Mahon
Filer Authorized By:	Ajay A. Jagtiani
Attorney Docket Number:	ROTE-0007-UT1
Receipt Date:	25-JUL-2008
Filing Date:	
Time Stamp:	09:48:16
Application Type:	U.S. National Stage under 35 USC 371

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Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$1000
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(71) Applicant *(for all designated States except US)*: JULIUS-MAXIMILIANS-UNIVERSITÄT WÜRZBURG [DE/DE]; Sanderring 2, 97070 Würzburg (DE).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: RITTER, Oliver [DE/DE]; Rembrandtstrasse 4, 97076 Würzburg (DE).

(74) Agent: ELBEL, Michaela; Rothkopf Theobald Elbel, Isatorplatz 5, 80331 München (DE).

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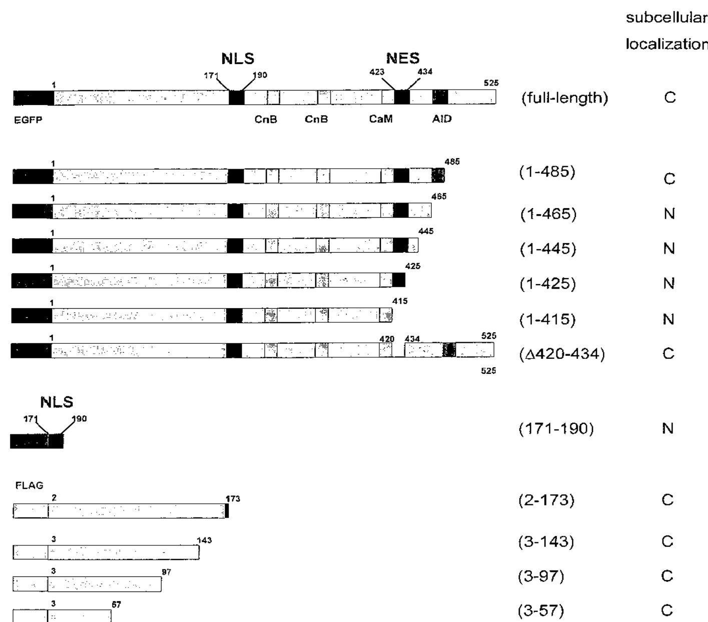
(54) Title: PEPTIDE FOR INHIBITION OF CALCINEURIN



(57) Abstract: The present invention relates to a method for diagnosing susceptibility for a myocardial and/or immunological disorder disorder, a kit and a therapeutic agent comprising a peptide of SEQ ID NO: 1 or 2 and uses thereof.

WO 2007/085455 A1

Peptide for Inhibition of Calcineurin

Field of the invention

5 The present invention relates to a method for diagnosing susceptibility for a myocardial and/or immunological disorder, a kit, an antibody and a therapeutic agent comprising a peptide of SEQ ID NO: 1, 2, 5-10 and uses thereof.

Background of the invention

10 The movement of proteins over ~40 kDa into and out of the nucleus is governed by the nuclear pore complex (NPC), a multi subunit structure embedded in the nuclear envelope (Jans et al., 2000). Transcription factors and enzymes that regulate the activity of these proteins are shuttled across the nuclear envelope by proteins that recognize nuclear localization signals/sequences (NLS) and nuclear export 15 signals (NES). Positively charged NLSs are bound by importins α and/or ß – also known as karyopherins – which tether cargo to the cytosolic face of the nuclear pore complex and facilitate translocation of proteins into the nucleus. The CRM1 protein, also referred to as exportin, mediates the transfer of proteins out of the nucleus (Fornerod et al., 1997), although CRM1-independent mechanisms for nu-20 clear export exist (Kutay et al., 1997). The ability of nuclear import and export machinery to access a NLS or NES is often dictated by signalling events that lead to the exposure or masking of these regulatory sequences (Cyert, 2001). This may occur through direct modification of the target protein or via modification of an associated factor.

25

The signalling cascade of calcineurin (CnA) and the nuclear factor of activated T cells (NF-ATc) is a crucial transducer of cellular function. NF-ATc is an ubiquitous transcription factor but has particular relevance in cytokine expression and the development of pathologic myocardial hypertrophy. The formation of complexes 30 between transcription factors and DNA is crucial for the transcriptional process. Therefore, the time that transcription factors remain nuclear is a major determi-

nant for transcriptional activity. It was shown that that in addition to the transcription factor NF-ATc, the phosphatase calcineurin is also translocated to the nucleus (Burkhard et al., 2005; Frey et al., 2000; Zou et al., 2001; Shibasaki et al., 1996). Calcineurin is therefore not only responsible for dephosphorylating NF-ATc, thus enabling its nuclear import; its presence in the nucleus is also significant in ensuring the full transcriptional activity of NF-ATc (Zhu et al., 1999). The traditional understanding of calcineurin activation via sustained high Ca^{2+}-levels (Timmerman et al., 1996; Dolmetsch et al., 1997) was advanced by findings of the inventor that calcineurin is activated by proteolysis of the C-terminal auto-inhibitory domain, which leads to a constitutively nuclear translocation of calcineurin (Burkhard et al., 2005).

Calcineurin is known to suppress the immune system, and various pharmaceutical compositions are available from commercial sources. Calcineurin inhibitors belong to the group of cyclosporine, tacrolimus (Protopic®, Prograf®) and pimecrolimus. Indications are psoriasis, atopic dermatitis, rheumatism and allergies, to name a few.

US patent application No. 2003/0045679 A1 to Crawford describes compositions which are useful for inhibiting and potentiating the activity of cellular calcineurin. These compositions include peptides, peptide analogs and whole proteins. They can be used to treat calcineurin related pathologies such as cardiac, brain, immune system and developmental abnormalities.

All known calcineurin inhibitors show strong side effects like high blood pressure, renal disorders and viral and bacterial infections. The later two are due to their general immunosuppressive properties. Thus there is a still existing need for a pharmaceutical composition for the treatment of calcineurin related disorders, like myocardial disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

The solution to this problem is achieved by providing the embodiments characterized by the claims, and described further below.

5 **Summary of the invention**

The present invention is directed to a method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of (a) providing a test sample comprising at least one cell; (b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10; (c) determining an interaction between the peptide of SEQ
10 ID NO: 1 or 2 and cellular calcineurin; wherein a primarily nuclear localization of a complex comprising calcineurin and the peptide of SEQ ID NO: 1, 2, 5-10 indicates susceptibility for a myocardial and/or immunological disorder.

Further, the present invention is directed to a kit comprising the peptide of SEQ ID
15 NO: 1, 2, 5-10 for diagnosing a myocardial and/or immunological disorder.

The present invention also concerns a therapeutic agent comprising the peptide of SEQ ID NO: 1, 2, 5-10, a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5-10 or a peptide of at least 90 % identity of the peptide of SEQ ID NO: 1, 2,
20 5-10. The peptide inhibits substantially the translocation, transport or shuffling of calcineurin and its derivates from the cytoplasm to the nucleus of a cell.

Further the present invention is directed to an antibody against the peptide of SEQ ID NO: 1, 2, 5-10.

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Moreover, the present invention is also directed to the use of the peptide of SEQ ID NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

30 **Brief description of the drawings**

Figure 1 shows the identification of a nuclear localization signal (NLS) in calcineurin (CnA). A schematic drawing of green fluorescent protein (GFP)- and FLAG-tagged CnA deletion mutants is given. The subcellular localization is indicated (c = primarily cytoplasmatic; n = primarily nuclear). NLS = nuclear localization signal; NES = nuclear export sequence; CnB = calcineurin B binding domain; CaM = calmodulin binding domain; AID = auto-inhibitory domain; numbers correspond to CnAß amino acid sequence; EGFP and FLAG indicate tags.

Figure 2 shows interactions of calcineurin (CnA) with importin ß1. Various deletion mutants were transfected into HeLa cells. Immunoprecipitation of GFP-tagged full length CnA and GFP-NLS fusion protein was performed with a GFP-antibody; the FLAG-tagged CnA 2-173 and CnA 3-143 mutants were precipitated with a FLAG-antibody. Immunodetection was performed with an importin ß1 antibody. Full length calcineurin interacted with importin ß1 as demonstrated by co-immunoprecipitation whereas the truncated CnA-mutants, lacking the NLS, did not co-immunoprecipitate with importin.

Figure 3 shows the results of HeLa cells, which were transfected with the indicated GFP-tagged CnA mutants. Immunoprecipitation of the CnA/importin complexes was performed with importin ß1 antibodies, detection with a GFP antibody. Addition of the NLS peptide (SEQ ID NO: 2) abrogated calcineurin interaction with importin ß1.

Figure 4 shows nuclear export signals (NES) in calcineurin (CnA). CnA lacking the NES (Δ420-434) was resistant to proteolysis by calpain. GFP-tagged full length CnA and CnA (Δ420-434) were transfected into HeLa cells. Whole cell lysates were incubated with calpain I for 30 min and the lysates run on a 5 % gel. Western blot analysis for GFP revealed proteolysis of full length CnA.

Figure 5 shows functional consequences of inhibition of calcineurin (CnA) nuclear import. Neonatal rat cardiomyocytes were incubated with a peptide mimicking the

NLS sequence of CnA (SEQ ID NO:2) and stimulated with Ang II (100 µM). The peptide saturated CnA/importin ß1 binding capacity. Nuclear import of CnA was therefore prevented. Control experiments were performed with a non-sense peptide SEQ ID NO: 3).

5

Figure 5A top shows that phosphatase activity of CnA was not influenced by the synthetic NLS peptide (SEQ ID NO: 2) as measured using a specific phosphor substrate of CnA.

10 **Figure 5A bottom** shows that transcriptional activity of the calcineurin/NF-ATc complex was suppressed by the inhibitory NLS peptide (SEQ ID NO: 2). Transcriptional activity was assessed with a NF-ATc luciferase reporter plasmid.

Figure 5B shows that the development of myocardial hypertrophy was also suppressed by the inhibitory NLS peptide (SEQ ID NO: 2) as demonstrated by protein synthesis **(Figure 5B top)** and cell size (magnification 600 x) **(Figure 5B bottom)**.

15

Figure 5C shows that B-type natriuretic peptide (BNP), a molecular marker of hypertrophy, is suppressed by the use of the inhibitory NLS peptide (SEQ ID NO: 2).

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Figure 5D shows the dose dependent decrease of NF-ATc transcriptional activity when treated with the NLS peptide of SEQ ID NO: 2. At higher concentrations (>1 µM, indicated by asterisk) a toxic effect is possible since values are below background levels of untreated cardiomyocytes (ctr = control).

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Figure 6 shows T cells, which were stimulated with spleen dendritic cells as antigen stimulus. Bars indicate protein synthesis (H3-thymidin incorporation). When T cells were stimulated (spleen-DC) and simultaneously treated with the blocking peptide (NLS), protein synthesis was inhibited significantly.

30

Figure 7A shows the blocking peptide (NLS), which prevented an increase in heart weight in mice that were treated with Ang II for 4 weeks.

Figure 7B shows the treatment of mice with the hypertrophic stimulus angiotensin for 4 weeks resulted in an upregulation of ß-MHC as molecular marker of hypertrophy. The NLS peptide was able to suppress the development of myocardial hypertrophy in vivo and therefore prevented expression of this marker.

Figure 8 shows the treatment of mice with implanted mini-osmotic pumps with AngII. This treatment caused dilation of the left ventricle (left ventricular end diastolic diameter, LVEDD). When the mice were treated with the NLS peptide dilation could prevented. A control peptide did not prevent left ventricular dilation.

Figure 9 shows the blocking peptide (NLS), which did not prevent nuclear accumulation of selected transcription factors (cJUN, ERK 1/2, GATA4, NFAT2) in cardiomyocytes that were stimulated with phenylephrin.

Detailed description of the invention

The inventor has surprisingly found that a peptide which mimics the nuclear localization signal of calcineurin (SEQ ID NO: 2) successfully prevents the translocation of calcineurin from the cellular cytoplasm to the nucleus. Without intending to be bound by any theory, it is believed that the NLS peptide (SEQ ID NO: 2) binds to importin, thereby preventing complex formation between calcineurin and importin. However, complex formation between calcineurin and importin is necessary for transporting calcineurin to the nucleus, where calcineurin together with NF-ATc then act as activated transcription factor due to the constant presence of calcineurin. The NLS peptide (SEQ ID NO: 2) efficiently inhibits complex formation and thereby blocks entry of calcineurin into the nucleus. It is further believed that that the auto-inhibitory domain (AID) not only blocks the catalytical activity of calcineurin but also masks the nuclear localization signal. Removal of the AID via a

conformational change in calcineurin following Ca^{2+} activation or by proteolysis of the auto-inhibitory domain leads to exposure of the nuclear localization signal and resultant nuclear translocation of calcineurin.

5 Sequences with SEQ ID NO: 5-10 (see section Examples) also proved to behave similar like SEQ ID NO: 1 and can be equally used in the present invention.

Myocardial disorders and immunological disorder are associated with altered transcription patterns of NF-ATc downstream targets. Therefore, the NLS peptide may 10 be used as a diagnostic tool for assessing the susceptibility for a myocardial and/or immunological disorder.

In a first aspect, the present invention is directed to a method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:

15 a) providing a test sample comprising at least one cell;
b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10;
c) determining an interaction between the peptide of SEQ ID NO: 1, 2, 5-10 and cellular calcineurin;

wherein a primarily nuclear localization of a complex comprising calcineurin and 20 the peptide of SEQ ID NO: 1, 2, 5-10 indicates susceptibility for a myocardial and/or immunological disorder.

As used herein the term susceptibility means predisposition or likelihood for the development of a myocardial and/or immunological disorder. The term disorder 25 means a dysfunction, a malfunction, a disease or a medical condition. The term peptide includes linear peptides, cyclic peptides and peptide analogs, derivates and salts thereof. Cyclic peptides are particularly preferred. Peptides forming a cyclic peptide may be closed head to tail, head to one of the side chains or tail to one of the side chains. Any modification can be employed as long as the peptide 30 retains its ability to prevent entry of calcineurin into the nucleus of a cell. The peptides of the present invention can be incorporated in a structure comprising more

amino acids, and the peptides comprise preferably about 60 amino acids, more preferred about 30 to 40 amino acids, most preferred about 20 to 30 amino acids.

The test sample can be derived from an animal, in particular a mammal, preferably from a human, more preferably from a patient who is suspected to be susceptible of a myocardial and/or immunological disorder.

Likewise, SEQ ID NO: 1, 2, 5-10 can be used for a method for diagnosing further calcineurin related disorders, like myocardial disorders, immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

In a preferred embodiment the peptide of SEQ ID NO: 1, 2, 5-10 is chemically, biologically and/or physically labeled. Examples for labeling peptides are dyes, like streptavidin-biotin labeling, fluorescent labeling, labeling by antibodies and radioactive labeling.

In a further preferred embodiment the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack. The immunological disorder is selected from the group consisting of transplant rejection and immune suppression. It is also possible to prevent restenosis after stent implantation by means of the NLS peptide of the present invention.

In a particularly preferred embodiment the disorder is a T-cell associated disorder. Specific examples for T-cell associated disorders are rheumatoid arthritis and forms thereof, psoriasis, psoriasis arthritis, systemic lupus erythematodes, vasculitis, other mixed connective tissue diseases, Sjögren syndrome, uveitis, inflammatory bowl diseases like Colitis ulcerosa and Morbus Crohn, intrinsic/extrinsic

asthma, graft versus host reaction and transplant rejection (prevention of organ rejection and treatment of chronic rejection)

Also diagnostic purposes with respect to T-cells are enclosed. The peptide may be used in a diagnostic kit to test activation status of T-cells from patients. This may be used to monitor disease progress or therapeutic success in e.g. immunosuppressed patients with transplanted organs.

In a second aspect the invention provides a kit comprising the peptide of SEQ ID NO: 1, 2, 5-10 for diagnosing susceptibility for a myocardial and/or immunological disorder. Likewise, SEQ ID NO: 1, 2, 5-10 can be used for a kit for diagnosing further calcineurin related disorders, like myocardial disorders, immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

In another aspect the present invention concerns a therapeutic agent comprising the peptide of SEQ ID NO: 1, 2, 5-10 and a therapeutic agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5-10.

The term nucleic acid, as used herein, includes DNA molecules, like cDNA and genomic DNA, RNA molecules, like mRNA, analogs of DNA and RNA which are generated by nucleotide analogs and derivates, fragments and homologs thereof. The nucleic acid can be single stranded or double stranded, however double stranded DNA molecules are preferred.

In another aspect, the present invention covers a therapeutic agent comprising a peptide of at least 90 %, preferably at least 95 %, more preferred at least 98 %, still more preferred 100 % identity of the peptide of SEQ ID NO: 1, 2, 5-10; wherein the peptide substantially inhibits the translocation of calcineurin from the cytoplasm to the nucleus of a cell. Conserved amino acid substitutions are cov-

ered by the present disclosure. The peptide of SEQ ID NO: 2 comprises three lysine amino acids (K) at positions 1, 5 and 7. When all lysine residues (K) are substituted by alanine (A) the peptides does no longer inhibit entry of calcineurin to the nucleus. However individual lysine residues may be substituted as long as the
5 peptide prevents it ability to block the translocation of calcineurin from the cytoplasm to the nucleus of a cell.

In a preferred embodiment the therapeutic agent further comprises a pharmaceutically acceptable carrier, like fillers and excipients. Various application forms of
10 the peptides are possible. Cremes and ointments can be applied topically. In stent technology, the peptide can be used to cover a stent, which is then implanted to a patient in need thereof. Coronary stents are particularly preferred. Also intravascular pumps, in particular mini pumps can be used for delivery of the peptide to a patient.
15

In a further aspect, the invention is directed to an antibody against the peptide of SEQ ID NO: 1, 2, 5-10.

In another aspect, the present invention covers the use of the peptide of SEQ ID
20 NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

In a preferred embodiment the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive
25 cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack. The immunological disorder is selected from the group consisting of a transplant rejection and immune suppression.

30 In yet another aspect the present invention is directed to the use of the peptide of SEQ ID NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic

and/or therapeutic treatment of calcineurin related disorders, like myocardial disorders, immunological disorders, psoriasis, rheumatism, immune reactions and suppression, e.g. graft versus host reaction and transplant rejection; allergies, asthma, in particular cardiac asthma, bronchial asthma and allergic asthma.

5

A more complete understanding of the present invention can be obtained by reference to the following examples, which are provided for the purpose of illustration only and are not intended to limit the scope of the invention.

10 **Examples**

A) Material and Methods

The following materials and methods were employed:

Cell culture

15 Neonatal rat cardiomyocytes of Wistar rats were isolated as described previously (Ritter et al., 2003). Cells were resuspended in minimum essential medium/1 % FCS. After preplating, the cardiomyocyte containing supernatant was recovered, and cells were plated in minimum essential medium on 6-well plates at a density of 1×10^6 cells per well. The medium for cardiomyocytes contained 5-bromo-2'-deoxyuridine (0.1 mmol/L) to suppress
20 fibroblast growth. Fibroblast contamination of cardiomyocyte cultures was between 4 % and 7 % as regularly determined by immunohistochemical staining for troponin T. HeLa cells were grown in DMEM/5 % FCS (Sigma). 48 hours after preparation, cells were stimulated with 10 nmol/L angiotensin
25 II (Ang II). Cells were harvested 24 hours after stimulation. Concentrations were as follows: Ang II: 10 µM, calpeptin: 10 µM, NLS peptide and control peptide: 1 µM, respectively.

Calcineurin (CnA) activity

30 NF-ATc reporter plasmid comprised the Il-2 promoter followed by luciferase. Luciferase activity was determined according to the manufacturer's protocol

(Promega). CnA phosphatase activity was determined using a commercial kit (CnA kit assay, Biomol) that measures effect on a specific CnA phosphophosubstrate. Free PO_4 was indicated by a malachite green dye. The use of this kit has been described previously (Ritter et al., 2002).

5

Plasmids

Epitope-tagged derivatives of calcineurin Aβ containing amino-terminal EGFP were generated using the mammalian expression vector pEGFP-C3 (BD Bioscience Clontech). For cloning of calcineurin mutants the mammal-
10 ian expression vector pCMV-Sport6 containing the directionally cloned cDNA of human calcineurin Aβ was used (Invitrogen). The following mutants have been amplified by PCR, digested with XbaI and XhoI and cloned into the XbaI and XhoI sites of the described plasmid: CnAβ(full), CnAβ 1-415, CnAβ 1-425, CnAβ 1-445, CnAβ 1-465, CnAβ 1-485 and CnAβ 171-
15 190. For these truncated mutants different reverse oligonucleotides were designed with subsequent N-terminal binding sites. The forward primer, binding at the N-terminus of calcineurin, was identical for each plasmid apart from the CnA(171-190) mutant. For this derivative primers are bound upstream of aa 171 and downstream of aa 190, respectively. Two internal
20 mutants CnAβ(Δ420-434) and CnAβ(Δ420-445) were cloned into the XbaI and XhoI sites of pEGFP-C3 using a two step strategy. For deleting the short areas from aa 420 to 434 and from aa 420 to 445 two fragments have been amplified. After ligation of these two fragments the resulting whole fragment was cloned into the XbaI and XhoI sites of pEGFP-C3. FLAG-
25 tagged calcineurin derivatives were kindly provided by the group of Ludwig Neyses (Divison of Cardiology, The University of Manchester).

Immunofluorescence and immunoprecipitation

The subcellular distribution of calcineurin was determined by immunofluo-
30 rescence stainings. The primary antibodies used in fluorescence staining are described elsewhere (Burkhard et al., 2005). Secondary antibodies

were Cy-3–labeled sheep anti-rabbit IgG or Cy2-conjugated mouse anti-goat IgG (Jackson Laboratories).

B) **Results**

5 The inventor's group demonstrated that posttranslational modification, specifically proteolysis of the auto-inhibitory domain (AID) of calcineurin leads to its activation and strong nuclear translocation (Burkhard et al., 2005). The calpain mediated cleavage of the c-terminal AID and the causative link to myocardial hypertrophy was demonstrated in human myocardial tissue.

10

In this invention the inventor demonstrates the prominent nuclear translocation of CnA in different animal models of diseased myocardium. In wild-type mice a predominantly cytosolic distribution of CnA was observed, whereas in mice that underwent aortic banding or myocardial infarction a strong nu-

15 clear localization of CnA in the hypertrophied myocardium was observed.

This is consistent with observations of nuclear import of calcineurin in cell culture models (Burkhard et al., 2005; Frey et al., 2000; Zou et al., 2001; Zhu et al., 1999). However, the role of calcineurin within the nucleus was

20 not been investigated before and the precise mechanism leading to nuclear import and export remains unclear.

Reversible nuclear localization of calcineurin after angiotensin stimulation

25 To assess whether CnA import into the nucleus is a chronic phenomenon or an acute response the inventor investigated the time course of CnA shuttling. A GFP tagged calcineurin plasmid encoding full length CnAß was transfected into neonatal rat cardiomyocytes. Cells were stimulated with Ang II at 10 µM. Confocal microscopy revealed onset of nuclear transloca-

30 tion of calcineurin after 2h. After 4h of Ang II stimulation CnA was predominantly nuclear. After 6h maximum of intensity of the GFP-calcineurin signal

was in the nucleus. Similarly, 2h after removal of Ang II from the medium there was a homogenous distribution of CnA in the cytosol and the nucleus and after 4h there was perinuclear localization of CnA. 6h after removal of the stimulus calcineurin localised completely in the cytosol again. To prevent CnA from calpain mediated proteolysis, which would cause constitutive activation of CnA and therefore persistent nuclear translocation, all experiments where performed in the presence of a membrane permeable calpain inhibitor (Burkhard et al., 2005). Calcineurin is very sensitive to pathological stimuli in cardiomyocytes and is able to induce a response within a few hours after the initial stimulation.

Construction of NLS peptide mutants

The transport of proteins into the nucleus relies on nuclear localization signal/sequences (NLS) which are bound by importins; the complex of cargo protein and importin is then transported into the nucleus. To define the regions of calcineurin that are required for nuclear import different GFP- or FLAG-tagged calcineurin deletion mutants (Figure 1) were screened to assess those that entered the nucleus and those that remained cytosolic. In general, deletion of the auto-inhibitory domain led to nuclear translocation and deletion of the region starting amino acid 173 (within the putative NLS) prevented calcineurin from entering the nucleus. The mutants 2-173 and 3-143 may have reduced catalytic phosphatase activity as the catalytic subunit of CnA extends to amino acid 333. Therefore failure to translocate may in theory be a result of decreased phosphatase activity. Previous work however has demonstrated that catalytically inactive CnA mutants are also translocated into the nucleus (Shibasaki et al., 1996). Sequence comparisons with known NLS of other proteins enabled further delineation of the putative NLS region to the sequence 171-190. Fusion of this 171-190 fragment to the GFP backbone resulted in translocation of the GFP/NLS fusion protein into the nucleus, whereas the pure GFP backbone remained cytosolic. Whilst full length CnA resides in the cytosol, it was translocated into the

nucleus after Ang II stimulation due to removal of the auto-inhibitory domain from the catalytic subunit and probably from the putative NLS. In contrast, deletion mutants 2-173 and 3-143 which both lacked the putative NLS remained exclusively cytosolic despite Ang II stimulation.

5

Interaction between calcineurin mutants and importin

Importin ß1 has been shown to bind the NLS of different cargo proteins (Pemberton et al., 2005). Interactions between CnA mutants and importin ß1 were assessed to determine whether the functionally defined NLS physically interacts with importin ß1. As demonstrated by co-immunoprecipitation experiments importin ß1 displayed good affinity for full length calcineurin and also the deletion mutant CnA 1-415. Specifically, the interaction domain was mapped to the region 171-190 as evidenced by the ability of a fusion protein consisting of GFP and the 171-190 fragment to co-precipitate importin ß1. However, both deletion mutants 1-173 and 1-143 completely abolished the interaction between importin ß1 and CnA. These data indicate that the NLS identified by functional analyses also mediate interactions between importin ß1 and calcineurin (Figure 2).

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Peptide competition assay

To demonstrate further that the identified NLS in CnA is essential for the nuclear import of calcineurin, a peptide competition assay to prevent importin ß1/CnA binding was used. A peptide with the putative NLS sequence of calcineurin (AAVALLPAVLLALLAKQECKIKYSERV – SEQ ID NO: 1) was synthesised and added to the medium (the first 15 letters give N-terminal extension to increase membrane permeability, NLS sequence is underlined). However, the peptide facilitating membrane permeability is not critical, and an alternative membrane anker, harbouring two alanine residues instead of leucine residues at positions 11 and 14, respectively, can also be used (AAVALLPAVLAALAA – SEQ ID NO: 4). In control experiments a non sense peptide (AAVALLPAVLLALLAAQECAIAYSEYV – SEQ ID NO: 3)

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30

was used. Addition of the synthetic NLS peptide saturated the binding domain of importin ß1 for CnA and therefore prevented CnA binding to importin ß1. Inhibition of this interaction suppressed calcineurin nuclear import. The non-inhibitory control peptide did not interfere with the calcineurin/importin binding; accordingly, nuclear translocation of CnA was not inhibited. Also the NLS peptide abrogated the interaction of calcineurin and importin ß1 as demonstrated in co-immunoprecipitation assays (Figure 3).

Nuclear export control sequences

To identify sequences in CnA that control nuclear export serial carboxy-terminal truncation mutants with an N-terminal GFP tag of CnA were generated and examined by confocal fluorescence microscopy. Experiments were performed in the presence of a calpain inhibitor to prevent calpain induced cleavage of the auto-inhibitory domain (AID) and to ensure functional integrity of calcineurin. Cells were stimulated with Ang II for 12 h to achieve nuclear entry of CnA, subsequently the stimulus was removed to promote nuclear export. Full-length CnA (amino acids 1–524) was re-localized exclusively to the cytosol of transfected cardiomyocytes after removal of the stimulus. An extended deletion variant (1-415) was not able to leave the nucleus any more.

These results demonstrated that sequences in the region c-terminal to amino acid 415 regulate nuclear export. Consistent with these findings and sequence comparisons with known NES sites, a CnA mutant lacking amino acids 420-434 remained exclusively nuclear after removal of the stimuli. Inhibition of calpain did not influence this result as the calpain cleaveage site (at 424) was deleted in this mutation variant.

As different cleavage sites in the calcineurin sequence (Wu et al., 2004) have been described for calpain, the inventor assessed whether the NES

sequence (423-434) really contains the calpain cleavage site. Therefore, the GFP-tagged calcineurin mutant lacking the NES domain was used for further experiments. The plasmid was transfected into HeLa cells and lysates of these cells expressing this deletion variant were incubated with 5 calpain I. Western Blot analysis was used to demonstrate that full length CnA is proteolysed by calpain whereas CnAΔ423-434 is resistant to calpain mediated proteolysis (Figure 4).

Mechanism of nuclear export of calcineurin

10 A number of proteins that shuttle across the nuclear membrane have been identified using CRM1 as the export shuttle. Some are transcription factors like NF-ATc that confer pro-hypertrophic actions. Others act within counter-regulatory pathways that repress cardiac hypertrophic growth such as the transcriptional repressor HDAC5 (McKinsey et al., 2000). To address 15 whether CnA nuclear export is mediated by CRM1, experiments using the CRM1 specific inhibitor, leptomycin B (LMB), were performed. Agonist-dependent nuclear import of full length CnA was achieved by Ang II stimulation. Calpeptin was added to prevent proteolysis of CnA. The addition of LMB to prevent CRM 1 mediated export indeed suppressed nuclear export 20 of CnA. Together, these findings demonstrate that LMB confines CnA to the nucleus in the cardiomyocyte by blocking its nuclear export. This supports the hypothesis that nucleo-cytoplasmic shuttling of CnA is coupled to a NES between amino acids 423-434 and is mediated by CRM1.

25 **Nuclear accumulation of calcineurin**

In vivo studies of pathological myocardial hypertrophy showed that proteolysis of the calcineurin auto-inhibitory domain at amino acid 424 results in a constitutively active calcineurin mutant lacking both the AID (aa 468-490) and the NES (423-434). To determine whether loss of the AID or disruption 30 of the NES is responsible for strong nuclear accumulation of CnA the nuclear import and export qualities of a GFP tagged CnA mutant with a dele-

tion of the NES was investigated. Cells were transfected with CnAΔ423-434. In this case calcineurin resided in the cytosol. Stimulation of the transfected cells with Ang II resulted in subsequent translocation of CnA into the nucleus. Based on these results the inventor concluded that the AID not only blocks the catalytical activity of CnA but also masks the NLS. Removal of the AID via a conformational change in calcineurin following Ca^{++} activation or by proteolysis of the auto-inhibitory domain leads to exposure of the NLS and resultant nuclear translocation of CnA.

Subsequent removal of the stimulating Ang II agent from the medium resulted in the CnAΔ423-434 mutant remaining nuclear, as the lack of the NES made it impossible for CRM1 to interact with CnA and to transport it back to the cytosol. The loss of the C-terminal part of CnA would therefore appear to regulate nuclear shuttling of CnA at the level of both nuclear import and export. Deprivation of the AID promotes import via importin ß1, and loss of the NES hinders nuclear export via CRM1 mediated mechanisms.

A peptide corresponding to the calcineurin NLS inhibited CnA nuclear import and maintained the overall structure of CnA. The suppression of calcineurin transport into the nucleus is important for the NF-ATc transactivational status; this peptide is therefore useful as a tool to suppress myocardial hypertrophy.

Markers for myocardial hypertrophy

Phosphatase activity, transcriptional activity, protein synthesis, cell size and makers of myocardial hypertrophy in response to the peptide related inhibition of CnA nuclear import were examined. Phosphatase activity was assessed employing a specific substrate (RII) for can (Ritter et al., 2002). Cardiomyocytes were stimulated with Ang II (10 µM), and CnA phos-

phatase activity was measured in the presence of the NLS peptide or a non sense control peptide. Total CnA phosphatase activity was not affected by inhibition of the access of importin ß1 to the CnA NLS (289±17 % vs. 273±11 %, n = 8, p = not significant). In contrast, transcriptional activity of the CnA/NF-ATc signalling pathway was decreased significantly by the NLS peptide (463±11 % vs. 123±8 %, n=8, $p<0.05$) (Figure 5A top). Similarly, myocardial hypertrophy, as evidenced by protein synthesis (707±21 % vs. 133±12 %, n=8, $p<0.05$), cell size (1191±91 μm^2 vs. 728±65 μm^2, n=8, $p<0.05$) (Figure 5B), and expression of brain natriuretic peptide (BNP) (163±11 % vs. 88±8 %, n=8, $p<0.05$) (Figure 5C), were suppressed by the NLS peptide. Transcriptional activity of a NF-ATc luciferase reporter plasmid was decreased when nuclear import of CnA was blocked by the NLS peptide in a dose dependent manner (Figure 5D).

These data indicated that despite full CnA phosphatase activity, CnA was unable to form effective transcriptional complexes. Activated calcineurin in itself does not appear to be sufficient to induce hypertrophy. Full transcriptional activity of CnA/NF-ATc is only possible in the presence of nuclear calcineurin. It is thus clear that calcineurin nuclear translocation is a prerequisite to the formation of effective NF-ATc transcriptional complexes.

T-cell experiments

The inventor above presented evidence, that inhibition of the interaction of the phosphatase calcineurin and its importin (importin ß1) prevents nuclear translocation of calcineurin. This suppressed activation of the calcineurin/NF-AT signalling cascade (Hallhuber et al.). The above experiments were performed in cell cultures of isolated cardiomyocytes or immortalized cell lines.

The inventor now extended the experiments to T-cells and to in vivo experiments in mice. In Figure 6 the inventor demonstrates suppression of T-

cell function using the specific approach of competitive peptide inhibition of the calcineurin/importin interaction. The blocking peptide significantly prevented activation of lymphocytes.

In Figure 7 the inventor demonstrates in vivo data. For in vivo use the blocking peptide, that mimics the calcineurin nuclear localization signal (NLS) received a peptide extension to increase membrane permeability and was synthesised as a cyclic peptide to withstand proteolysis of N- and C-terminal proteases. This peptide was administered twice daily at a concentration of 25 mg/kg body weight/day by i.p. injections into mice. The mice were treated with implanted osmotic minipumps to release either angiotensin II or saline as control. Angiotensin II caused myocardial hypertrophy as demonstrated by the heart/body weight ratio and expression of molecular markers of hypertrophy. The blocking peptide was able to prevent the development of myocardial hypertrophy. On a cellular level the inventor could also demonstrate, that the blocking peptide suppressed calcineurin entry into the nucleus in vivo.

Similar results were seen in NFAT Luciferase reporter transgenic mice (mice were received from Wilkins et al. for the experiments). Administration of the NLS peptide in angiotensin II treated mice suppressed Luciferase activity and therefore demonstrated effectiveness of the NLS peptide in this animal model.

From the presented data it is concluded that inhibition of calcineurin/importin interaction and the resulting inhibition of calcineurin nuclear entry is a promising concept to inhibit the calcineurin/NF-AT signalling pathway. For the first time in vivo data and proof of concept in lymphocytes is provided. The NLS peptide can be used as therapeutic tool in further pathological conditions were suppression of calcineurin signalling is promising like treatment of myocardial hypertrophy, immunosuppression in rheu-

matic disease or in organ transplantation to suppress donor organ rejection. Additional therapeutic fields are psoriasis, colitis, allergic diseases as asthma or restenosis of coronary vessels after percutaneous interventions.

5

Further alternative nuclear localisation signals and membrane ankers

Above evidence is presented that inhibiton of the interaction of the phosphatase calcineurin and its importin (importin ß1) prevents nuclear translocation of calcineurin. This suppressed activation of the calcineurin/NF-AT signalling cascade (Hallhuber et al. 2006). Previous experiments were per-
10
formed in cell cultures of isolated cardiomyocytes or immortalized cell lines.

The inventor now extended his experiments to in vivo data. Specifically, the inventor was able to show that the blocking peptide prevents myocardial
15
hypertrophy in AngII treated mice as demonstrated by echocardiography (Figure 8).

The inventor also analyzed the specificity with the peptide (AAVALLPAVLAALAA KQECKIKYSERV SEQ ID NO: 5). In theory this pep-
20
tide that mimics the Calcineurin NLS saturates the binding site on importin and therefore might completely block importin function for other transcription factors as well. However, this peptide did not block import of selected transcription factors, that also utilize importin ß1 (such as cJun, GATA4, NFAT2 and ERH 1/2) (Figure 9).

25

The inventor also tested different amino acid sequences that are theoretically more resistant to proteolysis for the capacity to inhibit calcineurin/importin interaction, namely:

- AAVALLPAVLAALAA KQEAKIKYSERV (SEQ ID NO: 6)
30
-AAVALLPAVLAALAA KQECKIKYAERV (SEQ ID NO: 7)
-AAVALLPAVLAALAA KQEAKIKYAERV (SEQ ID NO: 8)

-AAVALLPAVLAALAA KAECKIKYSERV (SEQ ID NO: 9)
-AAVALLPAVLAALAA KAEAKIKYSERV (SEQ ID NO: 10)

These peptides have all the same impact: they do block calcineurin/importin
5 interaction as demonstrated in coimmunoprecipitation experiments

Conclusion

The inventor's data show that CRM1 not only exports NF-ATc, but also calcineurin across the nuclear envelope. To interrupt transcriptional activity of
10 the calcineurin/NF-ATc signalling cascade CRM1 is first required to export calcineurin, so that in a second round CRM1 can access the NES of NF-ATc and subsequently proceed with its nuclear export. This mechanism is prevented in myocardial hypertrophy by the proteolysis of calcineurin by calpain at amino acid 424, resulting in loss of the auto-inhibitory domain in-
15 cluding the NES. In this scenario calcineurin remains nuclear because it is inaccessible to the export protein CRM1.

As import always precedes export, the inhibition of CnA nuclear import by peptide competition for the binding of the nuclear import protein importin ß1
20 presents a more sophisticated approach to abolishing the deleterious effects of exaggerated NF-ATc transcriptional activity. Therefore the NLS peptide is useful as a new therapeutic agent for pathologic myocardial hypertrophy.

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Claims

1. A method for diagnosing susceptibility for a myocardial and/or immunological disorder comprising the steps of:
   a) providing a test sample comprising at least one cell;
   b) contacting the cell with a peptide of SEQ ID NO: 1, 2, 5-10;
   c) determining an interaction between the peptide of SEQ ID NO: 1, 2, 5-10 and cellular calcineurin;

   wherein a primarily nuclear localization of a complex comprising calcineurin and the peptide of SEQ ID NO: 1, 2, 5-10 indicates susceptibility for a myocardial and/or immunological disorder.

2. The method of claim 1, wherein the peptide of SEQ ID NO: 1, 2, 5-10 is chemically, biologically and/or physically labeled.

3. The method of claim 1 or 2, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

4. The method of any of any of claims 1 to 3, wherein the disorder is a T-cell associated disorder.

5. A kit comprising the peptide of SEQ ID NO: 1, 2, 5-10 for diagnosing susceptibility for a myocardial and/or immunological disorder.

6. A therapeutic agent comprising the peptide of SEQ ID NO: 1, 2, 5-10.

7. A therapeutic agent comprising a nucleic acid which encodes the peptide of SEQ ID NO: 1, 2, 5-10.

8. A therapeutic agent comprising a peptide of at least 90 %, preferably at least 95 %, more preferred at least 98 %, still more preferred 100 % identity of the peptide of SEQ ID NO: 1, 2, 5-10; wherein the peptide substantially *inhibits the translocation of calcineurin from the cytoplasm to the nucleus of* a cell.

9. The therapeutic agent of any of the claims 6 to 8, further comprising a pharmaceutically acceptable carrier.

10. An antibody directed against the peptide of SEQ ID NO: 1, 2, 5-10.

11. Use of the peptide of SEQ ID NO: 1, 2, 5-10 for manufacturing a therapeutic agent for prophylactic and/or therapeutic treatment of a myocardial and/or immunological disorder.

12. The use of claim 11, wherein the myocardial disorder is selected from the group consisting of hypertrophic cardiomyopathy, in particular hypertrophic obstructive cardiomyophathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, dilated cardiomyopathy, aortic stenosis and heart attack; and the immunological disorder is selected from the group consisting of transplant rejection and immune suppression.

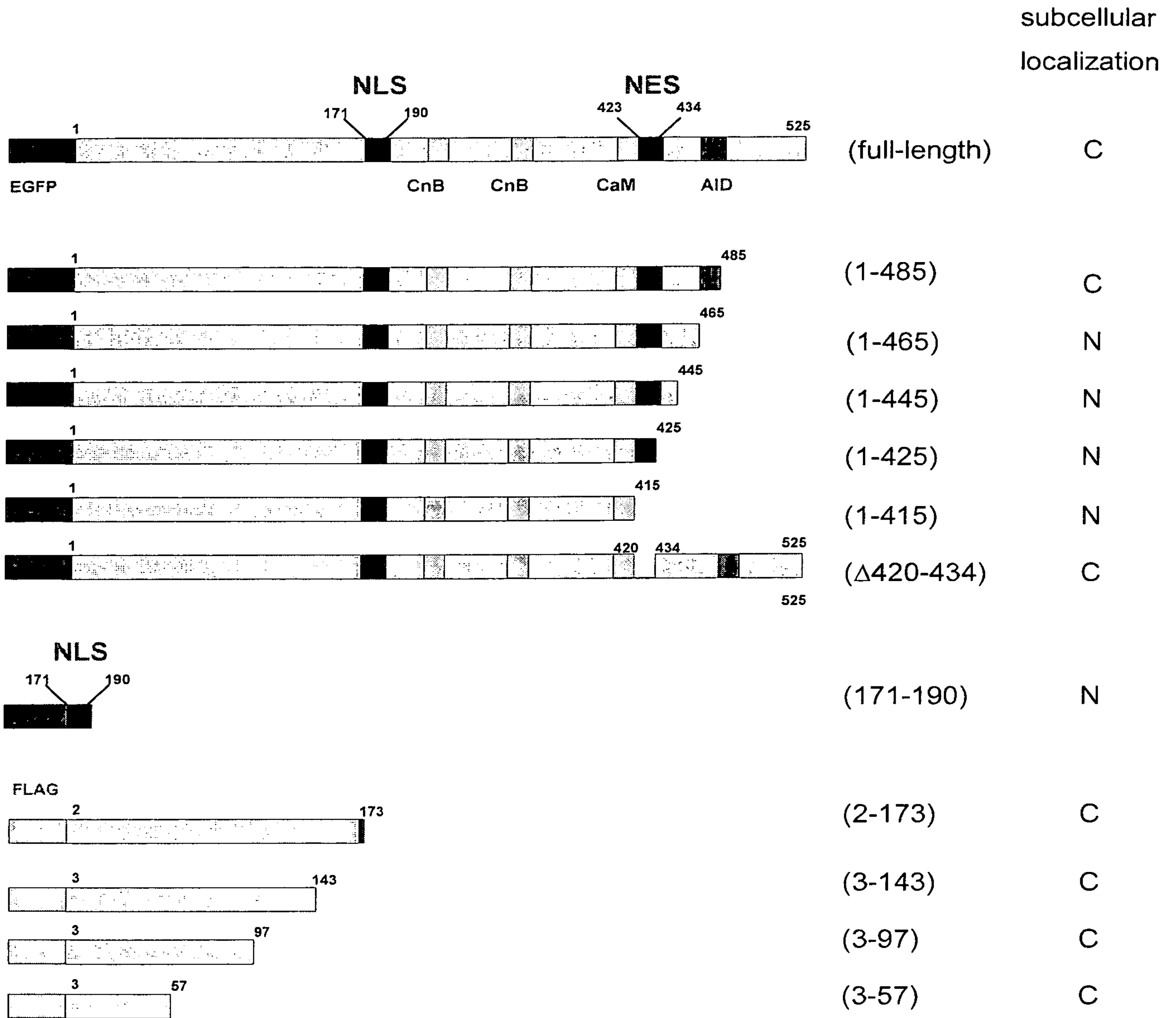
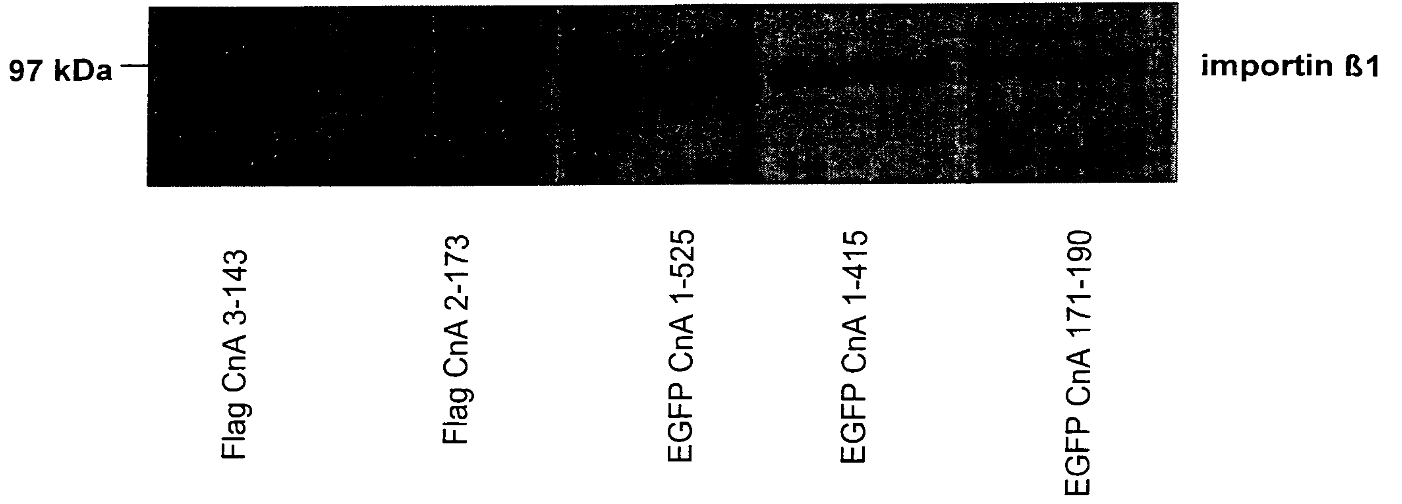


Figure 1



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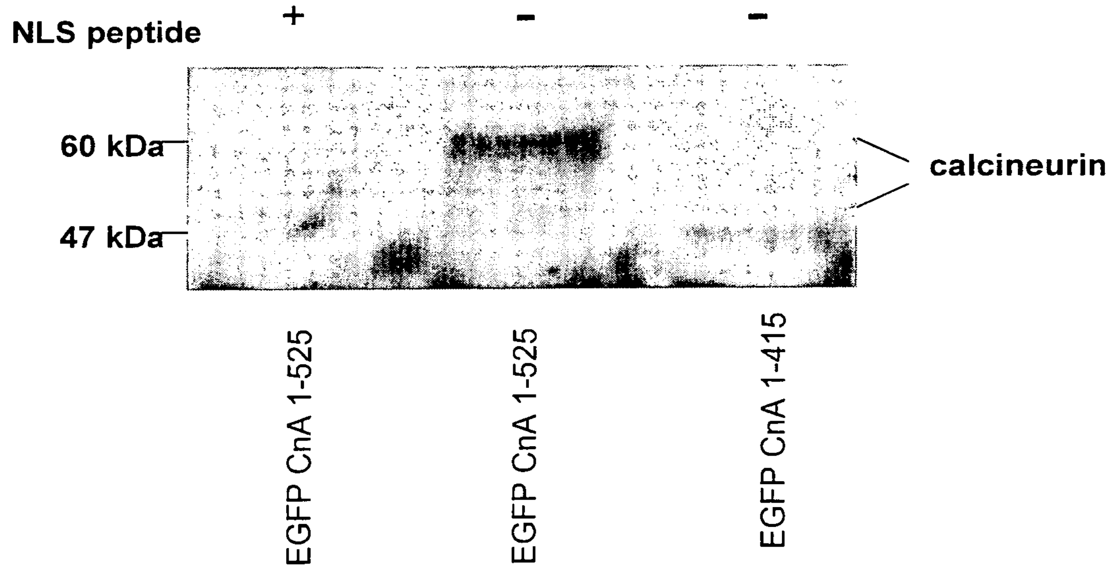

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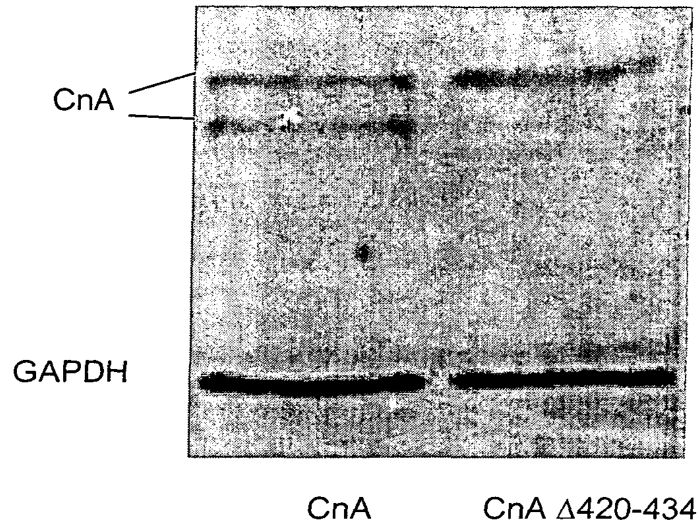

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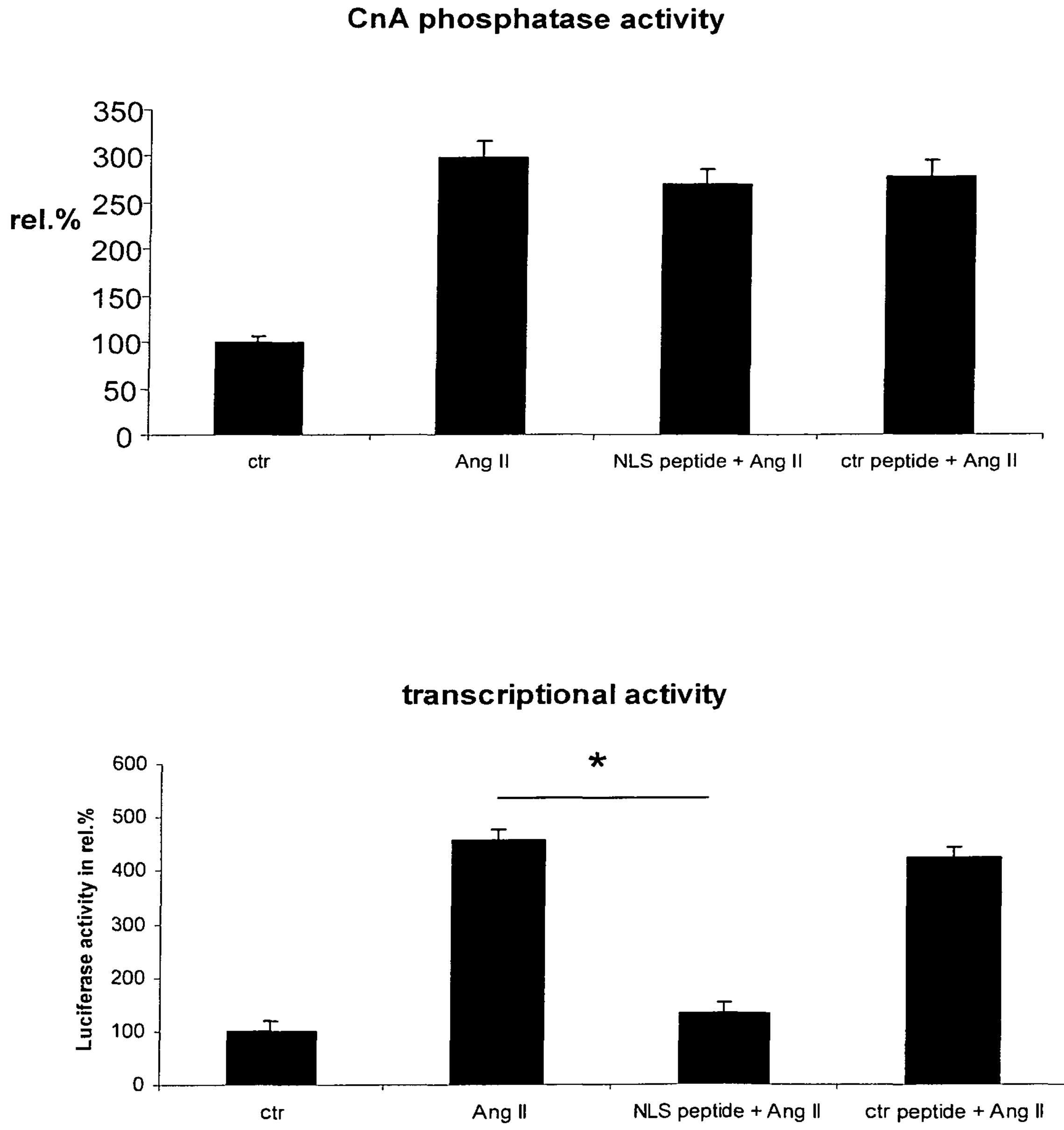


Figure 5A

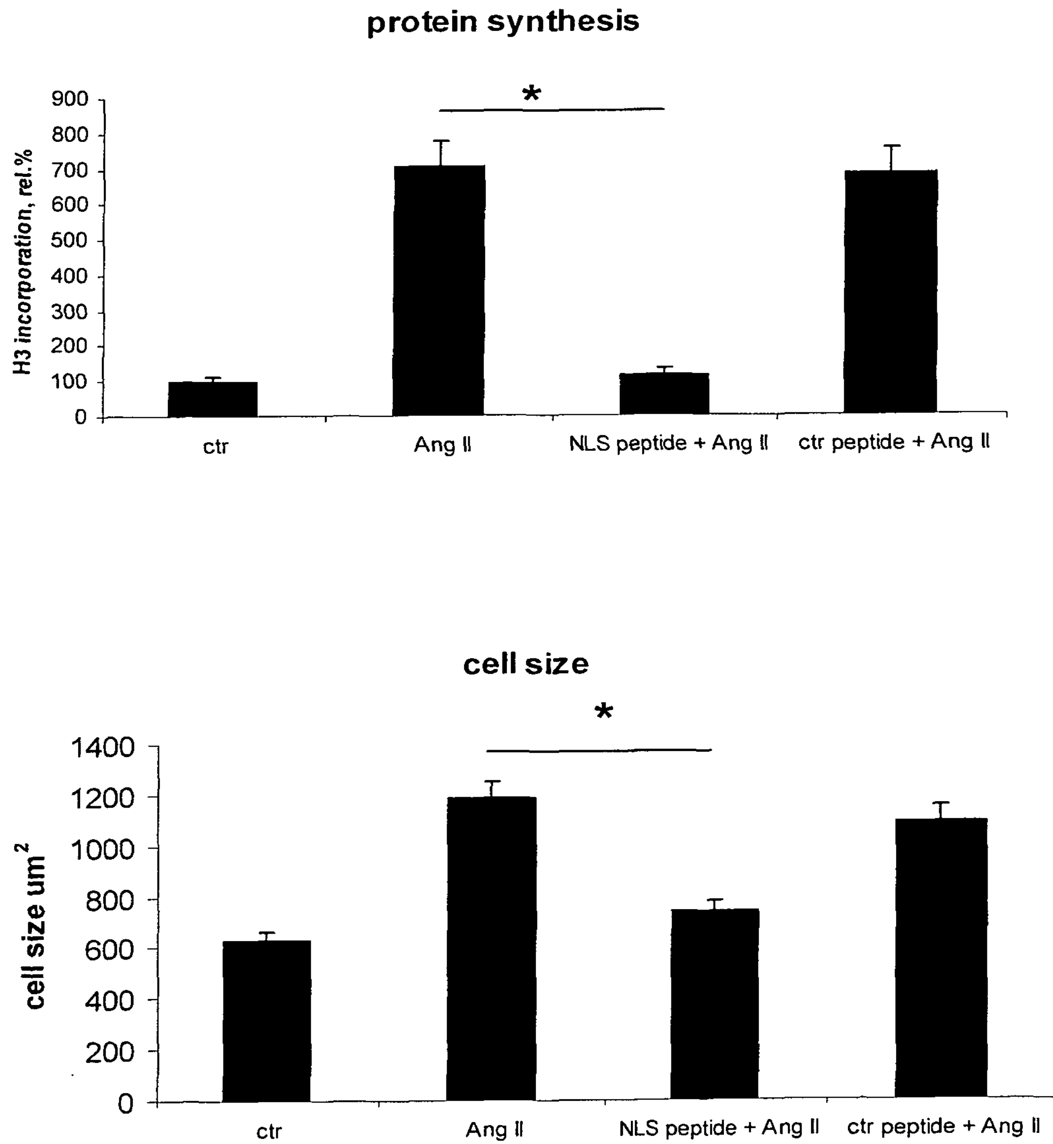
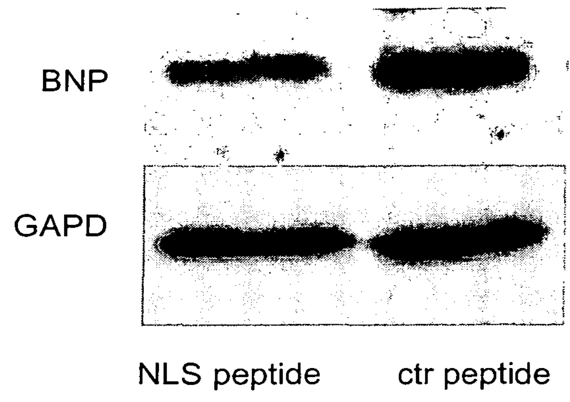


Figure 5B



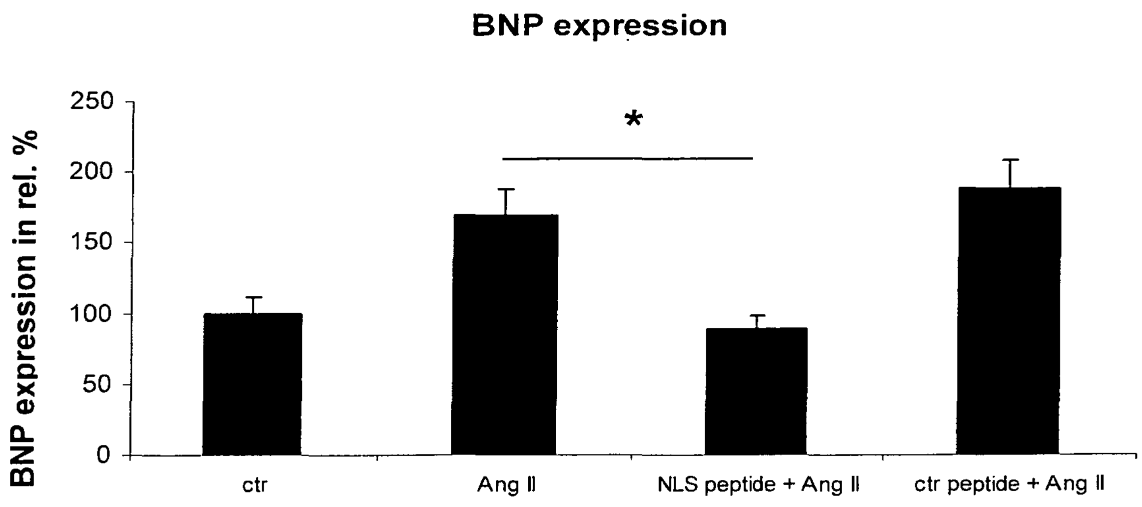
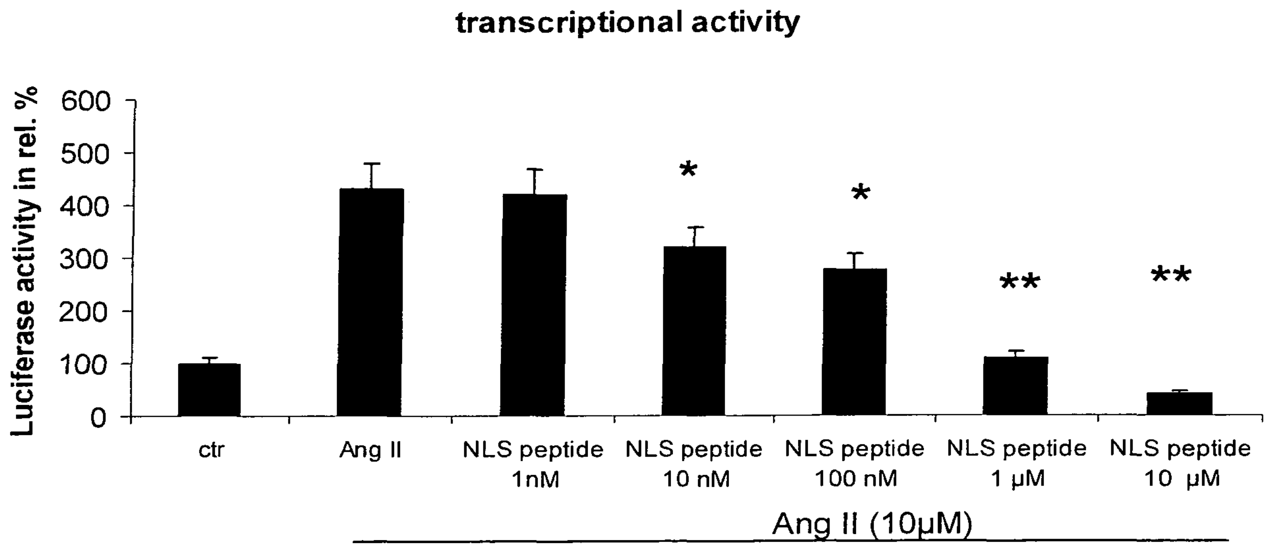


Figure 5C



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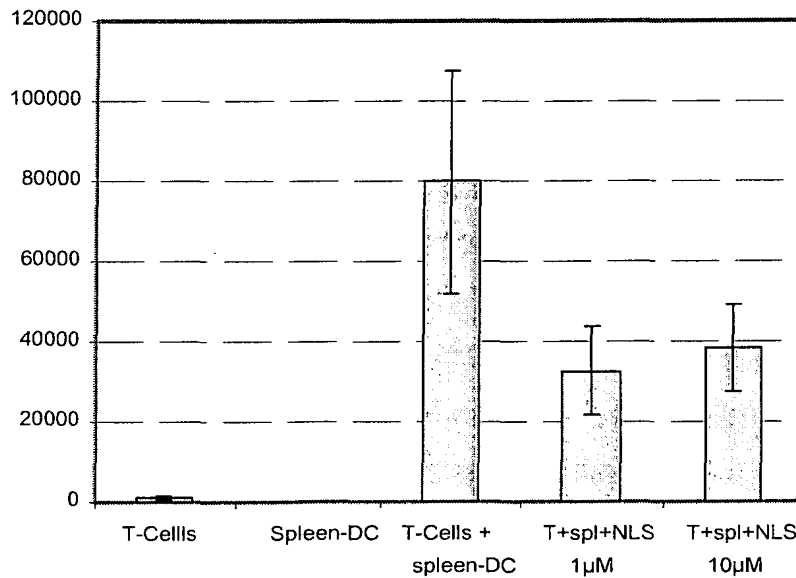

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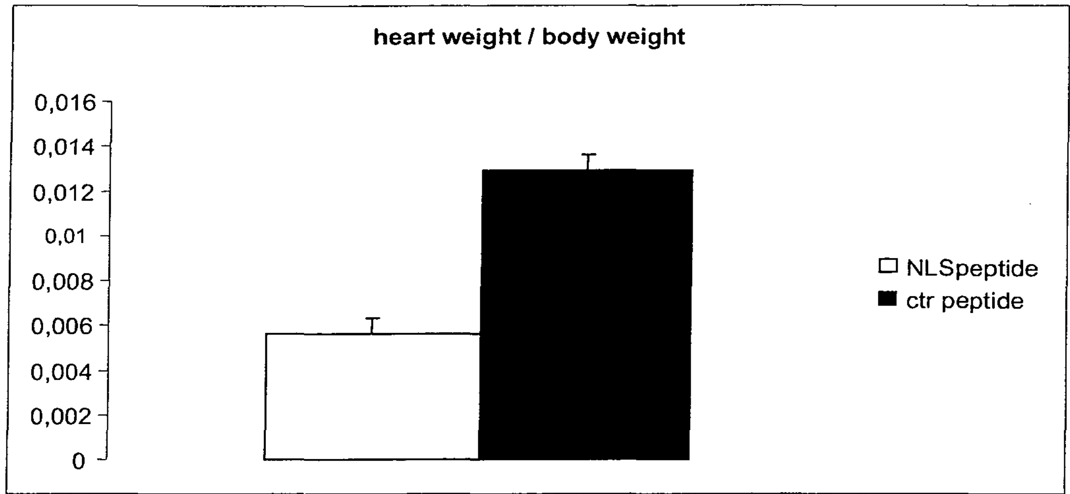


Figure 7A

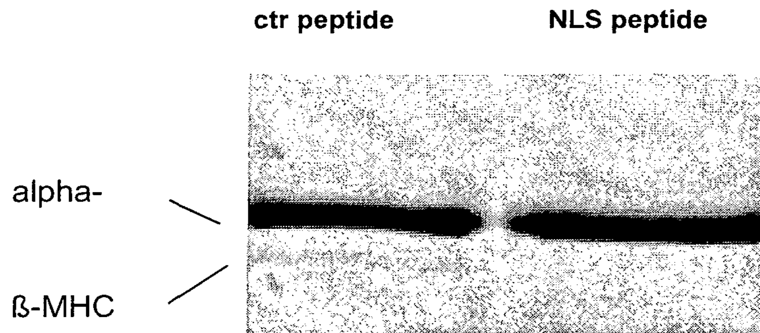
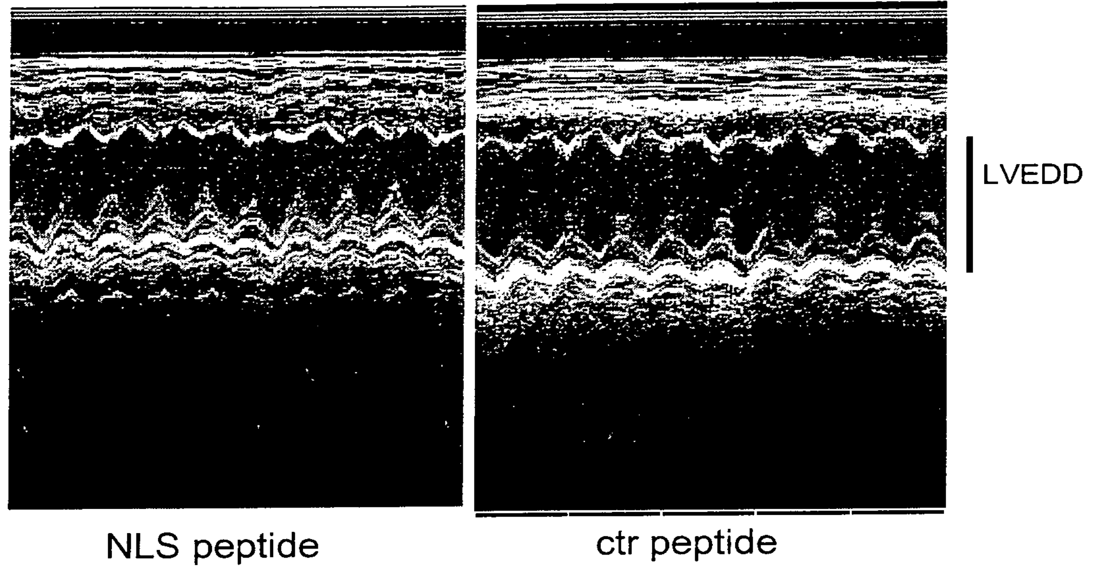


Figure 7B



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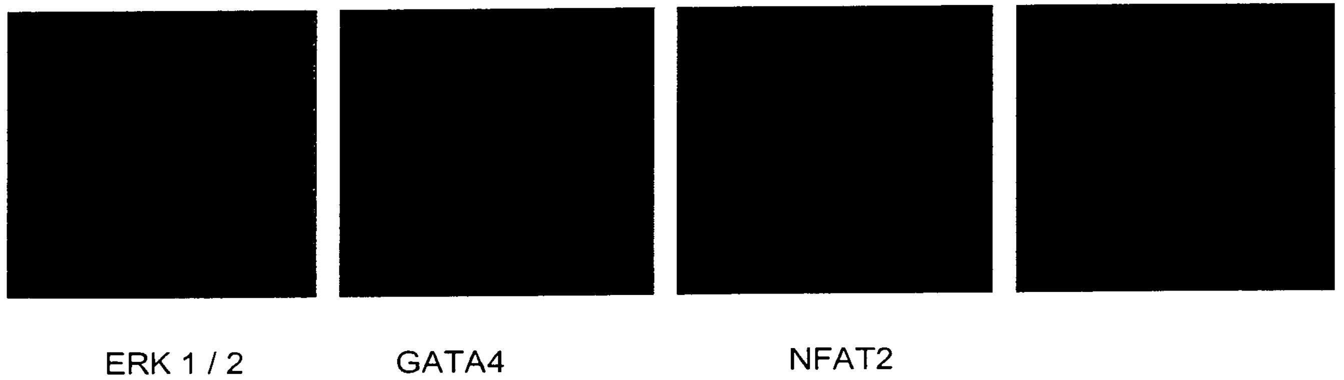

Figure 9

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APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
			TOTAL			TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT 07/25/2008	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 12	Minus	** 20	= 0	X $25 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 7	Minus	***3	= 4	X $105 =	420	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	**420**	OR	TOTAL ADD'L FEE	

AMENDMENT	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
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Legal Instrument Examiner:
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