Inhibition of Nuclear Import of Calcineurin Prevents Myocardial Hypertrophy

Matthias Hallhuber, Natalie Burkard, Rongxue Wu, Mamta H. Buch, Stefan Engelhardt, Lutz Hein, Ludwig Neyses, Kai Schuh, Oliver Ritter

Abstract—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 importinβ, 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, [3H]-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. (Circ Res. 2006;99:626-635.)

Key Words: angiotensin II • calcineurin • gene regulation • hypertrophy • NF-AT • nuclear-localizing signals

The calcineurin/nuclear factor of activated T cells (NF-AT) signaling cascade is a crucial transducer of cellular function. It has recently emerged that in addition to the transcription factor NF-AT, the phosphatase calcineurin is also translocated to the nucleus.1–4 Our traditional understanding of calcineurin activation via sustained high Ca2+ levels5,6 was also advanced by recent findings from our laboratory that showed that calcineurin is activated by proteolysis of the C-terminal autoinhibitory domain.7 This leads to the constitutive activation and nuclear translocation of calcineurin. Calcineurin is therefore not only responsible for dephosphorylating NF-AT in the cytosol, thus enabling its nuclear import, but its presence in the nucleus is also significant in ensuring the full transcriptional activity of NF-AT.7

The formation of complexes between transcription factors and DNA regulates the transcriptional process. Therefore, the time that transcription factors remain nuclear is a major determinant of transcriptional activity. The movement of proteins more than 40 kDa into and out of the nucleus is governed by the nuclear pore complex (NPC), a multisubunit structure embedded in the nuclear envelope.8 Transcription factors and enzymes that regulate the activity of these proteins are shuttled across the nuclear envelope by proteins that recognize nuclear localization signals (NLS) and nuclear export signals (NESs) within these transcription factors. The positively charged NLSs are bound by importins α and/or β (also called karyopherins), which tether cargo to the cytosolic face of the NPC and facilitate translocation of proteins into the nucleus. Likewise, the Crm1 protein, also referred to as exportin, mediates the transfer of proteins out of the nucleus.9 The ability of the nuclear import and export machinery to access a NLS or NES is often dictated by signaling events that expose or mask these regulatory sequences.10

In this study, we investigated the precise mechanisms of calcineurin nuclear import and export.

Materials and Methods

Expression Constructs

Eptite tagged derivatives of calcineurin Aβ, containing N-terminal enhanced green fluorescent protein (EGFP), were generated using the mammalian expression vector pEGFP-C3 (BD Biosciences/Clontech). The following C-terminal truncated mutants were amplified by PCR and cloned into the XbaI and XhoI sites of the pEGFP-C3 plasmid: CnAβ(1 to 525), CnAβ(1–485). CnAβ(1 to

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Nuclear Import of Calcineurin

Preparation of Neonatal Rat Cardiomyocytes and Cell Culture Experiments

Neonatal rat cardiomyocytes of Wistar rats (Harlan-Winkelmann, Borchen, Germany) were isolated as described previously. Cells were resuspended in minimum essential medium (MEM) with 1% fetal calf serum (FCS; MEM/1). HeLa cells were cultured in DMEM with 10% FCS and 100 U/mL penicillin and 100 μg/mL streptomycin. All supplements were obtained from Sigma-Aldrich.

Transfection of Cell Cultures and Treatment of Cell Cultures

Neonatal rat cardiomyocytes were transfected with Lipofectamine (Invitrogen Life Technologies), 48 hours after preparation, on 6-well plates at a density of 1 × 10⁶ cells per well or chamber slides at a density of 70 000 cells per cavity. Transfections were performed as described by the manufacturer. Cells were treated according to the respective experiments with the following chemicals: angiotensin II (Ang II) (10⁻⁶ mol/L), phenylephrine (PE) (10⁻⁶ mol/L), calpeptin (10 μmol/L), leptomycin B (LMB) (1 μmol/L), and NLS or control peptides (1 μmol/L). HeLa cells were transfected 24 hours after trypsinisation with GenePorter 2 (Gene Therapy Systems) on 100-mm dishes, 6-well plates, or chamber slides at a confluence of 70% to 80%.

Calcineurin Enzymatic Activity and Protein Synthesis

Calcineurin-dependent NF-AT activity was determined using a luciferase assay according to the protocol of the manufacturer (Promega). The NF-AT reporter plasmid pNP1-luci was used, which contains the II-2 promoter in front of luciferase, whereas the promoter in the control plasmid pNP1-luci is in the reverse direction. To determine the CnA phosphatase activity, a commercial kit (CnA phosphatase assay AK-816; Biomol, Hamburg, Germany) was used as described previously, with minor modifications. The RII-phosphopeptide (Biomol) was used as a specific substrate for calcineurin (PP2B). For measurement of cellular protein synthesis, the amount of incorporated [3H]-marked leucine was measured using a β-counter in counts per milliliter per minute (cpm). The change in protein synthesis is expressed as a percentage of the cpm:DNA concentration ratio in unstimulated cells, which was taken as 100%.

Western Blotting, Comimmunoprecipitation, and Immunostaining

Proteins were visualized with the ECL kit (GE Healthcare) according to the instructions of the manufacturer. To analyze brain natriuretic peptide (BNP) expression an anti-BNP antibody was used (1:200; Biotrend, 1505-0639). For coimmunoprecipitation (Co-IP) experiments, HeLa cells were used according to a standard protocol (Immunoprecipitation Starter Pack, GE Healthcare). Protein/antibody complexes were precipitated with a mixture of 25 μL of protein A and protein G Sepharose beads for 1 hour at 4°C. To detect the CnA fragments, an anti-GFP (1:500; ab5450, Abcam) antibody was used. The subcellular distribution of calcineurin was determined by immunostaining (Figure IVB in the online data supplement). Antibodies used were anti-CnA antibody (StressGen, SPA-610), tropomin I–specific antibody (Santa Cruz Biotechnology, sc15368), anti-FLAG antibody (Acris, DF3002), and Cy3/Cy2-labeled goat anti-rabbit IgG (The Jackson Laboratory) (each 1:500).

Statistics

All data are presented as mean ± SEM. Statistical analyses were performed using Student t test, significance was assigned a value of *P < 0.05 (*) and **P < 0.01 (**) . Nonsignificant differences are expressed as NS.

Results

In Vivo Nuclear Translocation of Calcineurin

We recently identified that posttranslational modification, specifically proteolysis of the autoinhibitory domain (AID), leads to activation of calcineurin and its strong nuclear translocation. The calpain-mediated cleavage of the C-terminal AID and the causative link to myocardial hypertrophy were demonstrated in human myocardial tissue. Here we demonstrate the nuclear translocation of CnA in different animal models of diseased myocardium (Figure 1). In wild-type mice (sham), there was a predominant cytosolic distribution of CnA, whereas in mice that underwent aortic banding or myocardial infarction, we observed a strong nuclear localization of CnA in the hypertrophied myocardium after 4 weeks similar changes could be demonstrated already after 2 days (supplemental Figure III). Nuclear accumulation of calcineurin was observed in 82 ± 13% (P < 0.01) of cardiomyocytes in pathological myocardial hypertrophy. In contrast, nuclear calcineurin was not observed in normal myocardium. For evaluation, >100 cells of 6 animals per group were counted.

Time Course

To assess whether CnA import into the nucleus is a chronic phenomenon or an acute response to a myocardial insult, we investigated the time course of CnA shuttling. A plasmid encoding EGFP-tagged full-length CnAβ was transfected into neonatal rat cardiomyocytes. Cells were stimulated with Ang II (10 μmol/L). Confocal microscopy revealed onset of nuclear translocation of calcineurin after 2 hours. After 4 hours of Ang II stimulation, CnA was predominantly nuclear (Figure 2A). After 6 hours, maximum of intensity of the EGFP-calcineurin signal was seen in the nucleus. We observed nuclear accumulation of calcineurin in >90% of the transfected cells (supplemental Figure 1). Similarly, 2 hours after removal of Ang II from the medium, CnA was homogeneously distributed in the cytosol and the nucleus and after 4 hours, CnA was localized in the perinuclear region. Six hours after removal of the stimulus, CnA was localized completely in the cytosol again (Figure 2B). To protect CnA from calpain-mediated proteolysis, which would cause constitutive activation of CnA and therefore persistent nuclear translocation, all experiments were performed in the presence of a membrane-permeable calpain inhibitor.
Identification of a Nuclear Localization Sequence and the Corresponding Importin

To define the regions of calcineurin that are required for nuclear import different EGFP- or FLAG-tagged calcineurin deletion mutants (Figure 3A) were screened to assess for those that entered the nucleus and those that remained cytosolic. In general, deletion of the autoinhibitory domain led to nuclear translocation and deletion of the region starting with aa 173 (within the putative NLS) prevented calcineurin from entering the nucleus (Figure 3B). Sequence comparisons with known NLSs of other proteins enabled further delineation of the putative NLS region to the sequence from aa 171 to 190 of CnAβ. Fusion of this aa 171 to 190 fragment to the EGFP backbone resulted in translocation of the EGFP/NLS fusion protein into the nucleus, whereas the pure EGFP backbone remained cytosolic. Although full-length CnA resided in the cytosol, it was translocated into the nucleus after Ang II stimulation attributable to uncovering of the catalytic subunit and probably of the putative NLS. In contrast, deletion mutants aa 2 to 173 and aa 3 to 143, both lacking the

Figure 1. Nuclear translocation of calcineurin in the myocardium of different animal models of myocardial disease. Myocardial hypertrophy was induced by subjecting mice to myocardial infarction (MI) or aortic banding (AB). Four weeks postprocedure, a predominately nuclear localization of calcineurin was observed in both models of cardiac stress when compared with sham operated mice. Merged pictures indicate overlay of nuclear (DAPI) and calcineurin staining.

Figure 2. Time course of EGFP-CnA import and export in Ang II–stimulated neonatal rat cardiomyocytes. A, Cardiomyocytes were transfected with EGFP-tagged CnA(1 to 525) and stimulated with Ang II (10 μmol/L). After 4 hours of stimulation, there was predominantly nuclear localization of calcineurin in ~90% of cells. B, After removal of the stimulus calcineurin moved back to the cytosol within 4 hours. Experiments in A and B were performed in the presence of a calpain inhibitor to prevent proteolysis of the CnA autoinhibitory domain, which would leave CnA constitutively nuclear.
Figure 3. Identification of an NLS in calcineurin. A, Schematic drawing of EGFP- and FLAG-tagged CnA deletion mutants. Further details are under Materials and Methods and have been reported previously.\textsuperscript{1,11} B, Subcellular localization of calcineurin deletion mutants in cardiomyocytes. Deletion of the autoinhibitory domain (AID) and presence of a putative NLS were crucial for nuclear import. Fusion of the NLS (aa 171 to 190) fragment to EGFP demonstrated the capability of this NLS to translocate proteins to the nucleus. C, Subcellular localization of CnA after stimulation of cardiomyocytes with Ang II. Full-length calcineurin CnA(1 to 525) was translocated into the nucleus, whereas deletion variants lacking the putative NLS(71 to 190) were not able to enter the nucleus despite stimulation. NLS indicates nuclear localization sequence; NES indicates nuclear export sequence; CnB, calcineurin B binding domain; CaM, calmodulin binding domain. Numbers are corresponding to CnA/H9252 amino acid sequence; EGFP and FLAG indicate tags, respectively.
putative NLS, remained exclusively cytosolic despite Ang II stimulation (Figure 3C).

Importinβ has been shown to bind the “nonclassical” NLS of different cargo proteins. Interactions between the CnA mutants and importinβ were therefore assessed to determine whether the functionally defined NLS physically interacts with importinβ. As demonstrated by Co-IP, importinβ binds to full-length calcineurin (CnA[1 to 525]) and also to the deletion mutants CnA(1 to 415) and CnA(1 to 44) (Figure 4A).

To demonstrate further that the identified NLS in CnA is essential for the nuclear import of calcineurin, a peptide competition assay was used to prevent importinβ/CnA binding. A peptide containing the putative NLS sequence of calcineurin (AAVALLPAVLAALAAKOECKIKYSERV) was synthesized and added to the medium. (Small capital letters give N-terminal extension to increase membrane permeability; NLS sequence is underlined.) In control experiments, a nonsense peptide (control peptide) (AAVALLPAVLAALAAAQECALAYSEYV) was used. Addition of the synthetic NLS peptide (1 μmol/L) saturated the binding domain of importinβ, for CnA and, therefore, prevented CnA binding to importinβ.

Specifically, the interaction domain was mapped to the region aa 171 to 190 as evidenced by the ability of the NLS peptide to abolish the interaction between importinβ and CnA completely. These data indicate that the NLS identified by functional analyses also mediates physical interactions between importinβ and calcineurin (Figure 4A).

Inhibition of this interaction suppressed nuclear import of a constitutively active calcineurin mutant (CnA[1 to 415]). The noninhibitory control peptide (1 μmol/L) did not interfere with the calcineurin/importin binding; accordingly, nuclear translocation of CnA was not inhibited (Figure 4B). The results were identical in cells treated with Ang II (10 μmol/L) and with the α-adrenergic receptor agonist phenylephrine (10 μmol/L).

Detection of a Nuclear Export Signal

To screen the calcineurin sequence for nuclear export signals we used the NetNES 1.1 server (http://www.cbs.dtu.dk/services/NetNES). This program predicts leucine-rich NESs in eukaryotic proteins. Our input was the C terminus downstream of aa 410 of CnA. In this region, a typical NES was predicted between aa 420 and 434 (Figure 3A). To exactly identify the sequence in CnA that controls nuclear export,

![Figure 4](image-url)
serial carboxy-terminally truncated CnA mutants with an N-terminal EGFP tag were generated and examined by confocal microscopy (Figures 3A and 5A). Experiments were performed in the presence of a calpain inhibitor to prevent calpain induced cleavage of the AID and to ensure functional integrity of calcineurin. Cells were stimulated with Ang II for 12 hours to achieve nuclear entry of CnA, followed by removal of the stimulus to promote nuclear export. Full-length CnA(1 to 525) was relocalized exclusively to the cytosol of transfected cardiomyocytes after removal of the stimulus. An extended deletion variant (1 to 415) was not able to leave the nucleus any more (Figure 5A).

These results suggest that sequences in the region downstream of aa 415 regulate nuclear export. Consistent with these findings and sequence comparisons with known NES sites, a CnA mutant lacking aa 420 to 434 remained exclusively nuclear after removal of the stimulus. Inhibition of calpain did not influence this result as the calpain cleavage site (at aa 424) was deleted in this mutation variant (Figure 5A).

To address whether CnA nuclear export is mediated by the export protein Crm1, experiments using the Crm1-specific inhibitor, 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 Crm1-mediated export suppressed nuclear export of CnA. Interestingly, LMB alone caused nuclear accumulation of CnA after 48 hours, indicating permanent shuttling of CnA across the nuclear membrane.

Figure 5. Nuclear export signal in calcineurin. A, Cardiomyocytes were transfected with different EGFP-tagged calcineurin deletion mutants (see Figure 3A). Cells were stimulated with Ang II for 12 hours (to promote nuclear import), and the stimulus was subsequently removed for 12 hours (to promote nuclear export). Calpeptin was present throughout to prevent proteolysis of the autoinhibitory domain of calcineurin. A truncated deletion mutant lacking the C-terminal part of calcineurin (CnA[1 to 415]) was not able to leave the nucleus. A CnA mutant with targeted disruption of the NES was also not able to leave the nucleus any more after application/removal of the stimulus. B, Crm1 mediates nuclear export of CnA. EGFP-tagged CnA(1 to 525) was transfected into cardiomyocytes. Cells were stimulated as described for A. When LMB, a specific inhibitor of Crm1, was added, CnA(1 to 525) remained nuclear even when the hypertrophic stimulus was removed. Without additional treatment, LMB alone caused nuclear accumulation of CnA after 48 hours, indicating permanent shuttling of CnA across the nuclear membrane.

Figure 6. To investigate the relative importance of the NES for nuclear export, an EGFP-tagged CnA mutant with targeted deletion of the NES region was transfected into cardiomyocytes. In resting conditions, the deletion mutant resided in the cytosol. Stimulation resulted in nuclear translocation. After removal of the stimulus, the CnA(Δ420 to 434) variant remained nuclear, confirming the necessity of the NES for CnA export.
In vivo studies of pathological myocardial hypertrophy show that proteolysis of the calcineurin autoinhibitory domain at aa 424 results in a constitutively active calcineurin mutant lacking both the AID (aa 468 to 490) and the NES (aa 423 to 433). 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 an EGFP-tagged CnA mutant with the deletion of the NES, CnA(Δ420 to 434), was investigated. 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, we conclude that the AID not only blocks the catalytic activity of CnA but also masks the NLS. Removal of the AID via a conformational change in calcineurin following Ca²⁺ activation or by proteolysis of the AID 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 a nuclear localization of the CnA(Δ420 to 434) mutant, as the lack of the NES made it impossible for Crm1 to interact with CnA and to

Figure 7. Functional consequences of inhibition of CnA nuclear import; neonatal rat cardiomyocytes were incubated with a peptide mimicking the NLS sequence of CnA (NLS peptide) and stimulated with Ang II (10 μmol/L). Saturation of the CnA/importinβ₁ binding capacity by the NLS peptide prevented nuclear import of CnA. Control experiments were performed with a control peptide (ctr) (a nonsense peptide) at equal concentrations. A, Phosphatase activity of CnA was not influenced by the synthetic NLS peptide (1 μmol/L) as measured using a specific phosphosubstrate of CnA. In contrast, transcriptional activity of the calcineurin/NF-AT complex was suppressed by the inhibitory peptide (1 μmol/L) in cardiomyocytes stimulated with Ang II or PE (each 10 μmol/L). Transcriptional activity was assessed using a NF-AT luciferase reporter plasmid. B, Development of myocardial hypertrophy, assessed by measuring protein synthesis and cell size, was also suppressed by the NLS peptide (1 μmol/L). C, Molecular markers of hypertrophy (expression of BNP) were suppressed by the use of the inhibitory peptide. Top, Representative BNP Western blot of cardiomyocyte lysate stimulated with Ang II and treated with the NLS or the control peptide. Bottom, Relative expression levels of GAPDH (loading control). D, Dose-dependent decrease of NF-AT transcriptional activity in cardiomyocytes when treated with the NLS peptide. The values at higher concentrations are less than background of untreated cells, suggesting a toxic or osmotic effect of the peptide.
transport it back to the cytosol (Figure 6). 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 importinβ, and loss of the NES hinders nuclear export via Crm1 mediated mechanisms.

Inhibition of Myocardial Hypertrophy by a NLS Corresponding Peptide

We examined phosphatase activity, transcriptional activity, protein synthesis, cell size, and markers of myocardial hypertrophy in response to the peptide-related inhibition of CnA nuclear import. Phosphatase activity was assessed using a specific substrate (R11) for CnA.14 Cardiomyocytes were stimulated with Ang II (10 μmol/L), and CnA phosphatase activity was measured in the presence of the NLS peptide (1 μmol/L) or a nonsense control peptide (1 μmol/L). Total CnA phosphatase activity was not affected by inhibition of the access of importinβ to the CnA NLS (298±9% versus 270±11%; n=8; P>0.05). Additionally, we assessed NF-ATc2 phosphorylation status because NF-AT is the physiological substrate for calcineurin. In cells that were stimulated with Ang II, there was an increase in dephosphorylated NF-ATc2 (120 kDa) compared with control cells. Addition of the NLS peptide had no significant effect on NF-ATc2 dephosphorylation. This indicates that the NLS peptide had no impact on phosphatase activity of calcineurin (supplemental Figure II). In contrast, transcriptional activity of the CnA/NF-AT signaling pathway was decreased significantly by the NLS peptide in cardiomyocytes stimulated with Ang II (10 μmol/L) (227±11% versus 133±8%; n=8; P<0.05) or with PE (10 μmol/L) (189±10% versus 91±7%; n=8; P<0.05) (Figure 7A). Similarly, myocardial hypertrophy, as evidenced by protein synthesis (159±9% versus 111±11%; n=8; P<0.05) and cell size (1180±91 μm² versus 744±65 μm²; n=8; P<0.05) (Figure 7B), was suppressed by the NLS peptide. To further investigate the inhibitory effect of the NLS peptide, the expression of brain natriuretic peptide (BNP) as a molecular marker of myocardial hypertrophy was measured. In cardiomyocytes stimulated with Ang II to induce myocardial hypertrophy, the NLS peptide significantly reduced the expression of BNP (163±11% versus 88±8%; n=8; P<0.05) (Figure 7C). Transcriptional activity detected by an NF-AT luciferase reporter plasmid was decreased when nuclear import of CnA was blocked by the NLS peptide in a dose-dependent manner (Figure 7D).

These data indicate that despite full CnA phosphatase activity, CnA was unable to form effective transcriptional complexes. Full transcriptional activity of CnA/NF-AT is achieved only in the presence of nuclear calcineurin. Thus it is clear that calcineurin nuclear translocation is a prerequisite to the formation of effective NF-AT transcriptional complexes (Figure 8).

Discussion

The calcineurin/NF-AT signaling cascade is crucial for T-cell activation and for the development of myocardial hypertrophy. After activation, NF-AT nuclear localization is directly induced by calcineurin-mediated dephosphorylation of multiple conserved serine residues in the N terminus of these proteins, revealing a nuclear localization signal.16 Once dephosphorylated, NF-AT translocates into the nucleus and the transcriptional process begins.

The biological activity of transcription factors is in part regulated by their intracellular localization. In the case of the calcineurin/NF-AT signaling cascade this means inactive (hyperphosphorylated) NF-AT resides in the cytosol and activated (dephosphorylated) NF-AT resides in the nucleus. However, it has also been demonstrated by our group and others that full transcriptional activity of the calcineurin/NF-AT pathway is achieved only when calcineurin is also translocated into the nucleus. The nuclear half-life of NF-AT alone is very short. In the absence of active calcineurin, it is rapidly transported back into the cytoplasm within minutes.4 In this study, we investigated the mechanisms leading to nuclear import and export of calcineurin.

The active transport of proteins into the nucleus requires an array of proteins including nuclear cargo or carrier proteins (called importins or karyopherins, respectively), which in many instances make the primary contact with the classical NLSs of the imported protein.17 Classical NLSs consist of 5 to 11 amino acids. When importinα binds to the target protein that contains the classical NLS, the complex interacts with accessory proteins such as importinβ and the small GTP-binding protein Ran. This complex binds to the nuclear pore and is then transported through it in an energy-dependent manner. Nonclassical NLSs can bind directly to importinβ, initiating nuclear transport through the nuclear pore complex.18 Similarly, NESs are responsible for binding to export proteins, so-called exportins. Exportins transport their target proteins across the nuclear envelope back into the cytosol. A number of proteins that shuttle across the nuclear membrane have been identified using Crm1 as the export shuttle (eg, NF-AT). Here, we have identified a NLS and a NES in the calcineurin sequence. We have also identified the respective carrier proteins for calcineurin shuttling across the nuclear membrane. Importinβ is responsible for the nuclear import, whereas the export protein Crm1 is required for nuclear export of calcineurin. These findings identify a potentially novel therapeutic strategy to inhibit myocardial hypertrophy. Inhibition of the calcineurin/importinβ interaction would prevent nuclear translocation of calcineurin and subsequently inhibit the full transcriptional activity of the calcineurin/NF-AT signaling pathway. Similar approaches, such as inhibition of the nuclear factor κB/importin interaction19–20 and calcineurin/NF-AT interaction21 by competitive peptides, have already been successfully proven. Using this strategy, we synthesized a peptide comprising 12 amino acids that mimicked the NLS sequence of calcineurin and an N-terminal peptide extension of additional 15 amino acids to increase membrane permeability. This peptide was able to suppress calcineurin/importinβ interaction, which subsequently prevented calcineurin nuclear import. The physiological result was blunting of NF-AT transcriptional activity and inhibition of the development of myocardial hypertrophy. In contrast, calcineurin phosphatase activity was unaffected, although assessment of calcineurin phosphatase activity in...
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calcineurin with the glycogen synthase kinase 3

act as a transcriptional coactivator. However, competition by

NF-AT factors, enabling Crm1-mediated nuclear export.23–26

activated protein kinase kinase 1 (MEKK1) (indirectly), all

kinase I (CK1), protein kinase A (PKA), and mitogen-acti-

vated protein kinase JNK) and p38 mitogen-activated protein kinases, casein

phosphatase function also increase transcriptional activity of

major task of nuclear calcineurin, as CnA mutants devoid of

at least to prevent rephosphorylation is unlikely to be the

first required to export calcineurin, so that in a second round

Crm1 can access the NESs of NF-AT and subsequently

procceed with its nuclear export. This mechanism may be

vivo is often imprecise. As a surrogate the NLS peptide had

no impact of NF-AT dephosphorylation.

These results demonstrate that inhibition of the calcineurin/importin interaction by interfering peptides is an effective tool to suppress calcineurin signaling. These results however raise the following question: What is the precise role of calcineurin in the nucleus? The transcriptional effector of the CnA/NF-AT system is NF-AT through its DNA-binding domain. NF-AT factors share an imperfect Rel homology domain that is only capable of weak DNA binding in the monomeric or dimeric state. To strengthen NF-AT/DNA interactions, these factors prefer to interact cooperatively with other nuclear transcription factors such as AP-1 (c-Jun/c-Fos), GATA-4, and MEF-2.22 Therefore, calcineurin may act as a transcriptional coactivator. However, competition by calcineurin with the glycogen synthase kinase 3β (GSK3β) to ensure further dephosphorylation of NF-AT in the nucleus or at least to prevent rephosphorylation is unlikely to be the major task of nuclear calcineurin, as CnA mutants devoid of phosphatase function also increase transcriptional activity of the CnA/NF-AT signaling pathway when translocated to the nucleus.7 Also, multiple other kinases beside glycogen synthase kinase 3β (GSK-3β) such as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases, casein kinase 1 (CK1), protein kinase A (PKA), and mitogen-activated protein kinase kinase 1 (MEKK1) (indirectly), all promote rephosphorylation of the serine-rich N terminus of NF-AT factors, enabling Crm1-mediated nuclear export.23–26

Another model of competition between CnA and Crm1 for the nuclear export sequence of NF-AT has also been proposed. It has previously been demonstrated that the nuclear export protein Crm1 is capable of transporting NF-AT out of the nucleus. A constitutive nuclear calcineurin will shift Crm1 off the NES of NF-AT and leave the CnA/NF-AT complex nuclear, thereby enhancing transcriptional output.7,27 Our data show that Crm1 not only exports NF-AT but also calcineurin from the nucleus. To interrupt transcriptional activity of the calcineurin/NF-AT signaling cascade, Crm1 is first required to export calcineurin, so that in a second round Crm1 can access the NESs of NF-AT and subsequently proceed with its nuclear export. This mechanism may be prevented in myocardial hypertrophy by the proteolysis of calcineurin by calpain at aa 424, resulting in a loss of the autoinhibitory domain including the NES. In this scenario, calcineurin remains nuclear because it is inaccessible to the export protein Crm1. These observations suggest that calcineurin function in the nucleus is largely driven via its anti-Crm1 as opposed to anti-GSK3β effects.

As import always precedes export, the inhibition of CnA nuclear import by peptide competition for the binding of the nuclear import protein importinβ presents a sophisticated approach to abolishing the deleterious effects of exaggerated NF-AT transcriptional activity. Nevertheless, assessment of the specific action of the NLS peptide on the calcineurin/NF-AT interaction must be performed before further experiments can be undertaken in vivo.

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Disclosures

None.

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Material and Methods

Expression constructs
Epitope tagged derivatives of calcineurin Aβ, containing N-terminal EGFP, were generated using the mammalian expression vector pEGFP-C3 (BD Bioscience Clontech). For cloning different calcineurin mutants the mammalian expression vector pCMV-Sport6 containing the directionally cloned cDNA of human calcineurin Aβ was used (Invitrogen). The following C-terminal truncated mutants have been amplified by PCR, digested with XbaI and XhoI and cloned into the Xbal and XhoI sites of the described plasmid: CnAβ(1-525), CnAβ(1-485), CnAβ(1-465), CnAβ(1-445), CnAβ(1-425), CnAβ(1-415) and CnAβ(171-190). For all these truncated mutants different reverse oligonucleotides were used. Each primer had its specific binding site a few amino acids (aa) up to the N-terminus. The forward primer, binding at the N-terminus of calcineurin, was the same for each one apart from the CnA(171-190) mutant. For this derivative primers bound upstream aa 171 (forward) and downstream of aa 190 (reverse), respectively. The internal mutant CnAβ(Δ420-434) was cloned into the XbaI and XhoI sites of pEGFP-C3 by a two step strategy. For deleting the short area from aa 420 to 434 two fragments were amplified by PCR. After ligation the whole fragment was cloned into the XbaI and XhoI sites of pEGFP-C3. The generation of the FLAG-tagged calcineurin derivatives has been described previously¹. For a graphical view look at Figure 3A.

NLS peptide and ctr peptide
The NLS peptide (sense) and the ctr peptide (nonsense) were synthesized by Genosphere Biotechnologies (Paris, France). To improve the import into the cells a hydrophobic membrane permable sequence (MPS)² is attached at the N-terminus. The NLS peptide mimicked the amino acid sequence of calcineurin Aβ from aa 172 to 183 whereas the amino acid sequence of the ctr peptide is modified. The positive charged aa (position 172, 176, 178 and 182) are replaced by uncharged amino acids (alanin and tyrosine). During the experiments the final concentration of these peptides was 1 µmol/L. The amino acid sequences are:

NLS peptide: AAVALLPAVLLALLAKQECKIKYSERV
ctr peptide: AAVALLPAVLLALLAAQECAIAYSEYV

Myocardial infarction and aortic banding in mice
After an adequate anesthesia and fixing the mouse in a supine position with tape a 5-0 ligature was placed behind the front upper incisors and pulled so that the neck is slightly extended. After retracting and holding the tongue a 20-G i/v catheter was inserted into the trachea and attached to the mouse ventilator via the Y-shaped connector. Ventilation (100 % oxygen) was performed with a tidal volume of 200 µl and a respiratory rate of 133/min. Following desinfection with betadine solution and 70 % ethyl alcohol the chest cavity was opened through an incision over the left fourth intercostal space. The heart was exposed and the pericardial sac opened and pulled apart to identify the left anterior descending (LAD) artery. Ligation was performed with a 7-0 silk suture passed with a tapered needle underneath the LAD artery, approximately 1 – 2 mm beneath the tip of the left auricle. Occlusion was confirmed by pallor of the anterior wall of the left ventricle. Dropping 1 % lidocaine on the apex of the heart prevented arrhythmia. Lungs were overinflated and the chest cavity, muscles and skin were closed.

Chronic pressure-overload LV hypertrophy was induced by aortic banding (AB) in wild type (WT) mice. After anesthetizing with tribromoethanol / amylene hydrate (Avertin; 2.5 % wt/vol, 6 µL/g body weight; i.p.) a topical depilatory agent was applied to the neck and chest, and the area was cleaned with betadine and alcohol. Mice were placed in a supine position, a horizontal skin incision 0.5 – 1.0 cm in length was made at the level of the suprasternal notch. The thyroid gland was retracted, and a 2 – 3 mm longitudinal cut was made in the proximal portion of the sternum. This allowed uncovering the aortic arch under low-power magnification. A wire with a snare on the end was passed under the aorta between the origin of the right innominate and left common carotid arteries. A 6-0 silk suture was snared with the wire and pulled back around the aorta. A 27-gauge needle was then placed next to the aortic arch, and the suture was snugly tied around the needle and the aorta. After ligation, the needle was quickly removed. The skin was closed, and mice were allowed to recover on a warming pad until they were fully awake. The sham procedure was identical but without aortic ligation.

**Preparation of neonatal rat cardiomyocytes and cell culture experiments**

Neonatal rat cardiomyocytes of Wistar rats were isolated as described previously. Cells were resuspended in minimum essential medium (MEM) with 1 % fetal calf serum (FCS) (MEM/1). After preplating, the supernatant (cardiomyocytes) was recovered, and cells were plated in MEM/5 on 6-well plates at a density of 1 x 10⁶ cells per well or on chamber slides at a density of 700,000 cells per chamber well. Medium for cardiomyocytes contained 5-bromo-2'-deoxyuridine (0.1 mmol/L) to suppress fibroblast growth. Contamination of cardiomyocyte
cultures with non-cardiomyocytes was below 10 % as regularly determined by immunohistochemical stainings for troponin I (suppl. figure 4).

HeLa cells (human epithelial cells from a fatal cervical carcinoma; Henrietta Lacks, 1951) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10 % FCS and 100 U/ml penicillin and 100 µg/ml streptomycin. For diverse experiments the cells were plated on 100 mm dishes, 6-well plates or chamber slides. All supplements were obtained from Sigma-Aldrich.

Transfection of cell cultures and cell treatment

Neonatal rat cardiomyocytes were transfected with Lipofectamine (Invitrogen Life Technologies), forty-eight hours after preparation, on 6-well plates at a density of 1 x 10^6 cells per well or chamber slides at a density of 700,000 cells per well. The transfection was performed as described by the manufacturers with the following plasmids: pEGFP-C3 – CnA(full), (1-485), (1-465), (1-445), (1-425), (1-415), (171-190), (Δ420-434); pEf-FLAG – CnA(2-173), p3xFlag-CMV7.1 – CnA(3-143), (3-97), (3-57); pNP1-luci and pNP3-luci. After incubation for three hours at 37°C in the CO₂-incubator (0,9 %) following a medium change to MEM/1, the cells were treated according to the respective experiments with the following chemicals: angiotensin II (Ang II; 10 µmol/L), phenylephrine (PE; 10 µmol/L), calpeptin (10 µmol/L), leptomycin B (LMB; 1 µmol/L) and NLS or ctr peptides (1 µmol/L). All cells were harvested twenty-four hours after stimulation and peptide treatment with low salt buffer (1 % IGEPAL CA-630, 50 mM Tris (pH 8.0), 1 mM PMSF) or visualized by microscopy.

HeLa cells were transfected twenty-four hours after trypsinization with GenePorter2 (Gene Therapy Systems) on 100 mm dishes, 6-well plates or chamber slides at a confluency of 70 – 80 %. The performance was performed according to the manufacturer’s manual. After three hours incubation at 37°C in the CO₂-incubator (5 %) the booster 2 reagent was added at a concentration of 1 : 100 and incubated in turn for twenty-four hours. Cells were harvested with low salt buffer or visualized by confocal microscopy.

Calcineurin enzymatic activity

The activity of calcineurin was determined using a luciferase assay according to the manufacturer’s protocol (Promega). The NF-AT reporter plasmid pNP3-luci was used which contains the Il-2 promotor in forward direction whereas the promotor in a control plasmid pNP1-luci is in the reverse direction. The plasmids were transfected to cardiomyocytes as described above on 6-well plates. After cell treatment for twenty-four hours cells were harvested with 1 x luciferase cell culture lysis reagent (25 mM Tris phosphate (pH 7.8),
2 mM DTT, 2 mM 1,2-diamino-cyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton® X-100). After rocking the 6-well plates for 10 min cells were harvested and centrifuged for 5 min at 8,000 g. The luciferase activity was measured for 10 seconds using a luminometer by adding 20 µl cell lysate to 100 µl of pre-warmed (37°C) 1 x luciferase assay reagent. During the measuring time the relative light units (RLU) were measured and transformed to relative values (rel. %). For determination of the CnA phosphatase activity, a commercial kit (CnA kit assay; Biomol) was used as described previously with minor modifications. The RII-phosphopeptide (Biomol) was used as a specific substrate for calcineurin (PP2B). Nonetheless the phospho group is cleaved by other competing phosphatases. Thus a few conditions have been preserved to discriminate between the contributions of other phosphatases. PP2B requires Ca$^{2+}$ for its activity. For this reason “EGTA buffer” sample represents total phosphatase activity apart from PP2B. Okadaic acid (“OA”) inhibits protein phosphatases 1 and 2A (PP1, PP2A) but not PP2B and PP2C. Finally, “OA + EGTA buffer” inhibits PP1, PP2A and PP2B, but not PP2C. The detection of free phosphate released from RII by calcineurin was based on the malachite green dye reaction at OD$_{620}$nm. For valuating the data “background” was subtracted from each sample and calcineurin activity was determined as phosphate released from RII in the presence of “OA” minus phosphate released in the presence of “OA + EGTA buffer”. For a detailed description we refer to the users manual (CnA kit assay AK-816; Biomol, Hamburg, Germany). Alternatively, phosphorylation status of NF-AT was measured to assess calcineurin enzymatic activity. The method was described earlier. The antibody was directed against NF-ATc2 (Abcam, ab2722).

Western blotting, co-immunoprecipitation (Co-IP) and immunostainings

For all Western blots cells were plated on 100 mm dishes and scraped into 200 µl RIPA buffer (150 mM NaCl, 1% IGE PAL CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM PMSF). Protein concentrations were determined using the Bradford assay. Equal amounts of total protein were denatured in Laemmli buffer, separated on 7.5 and 15% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were imaged with ECL kit (GE Healthcare) according to manufacture’s instructions. Analyzing BNP expression an anti-BNP antibody was used (1:200; Biotrend, 1505-0639). Second antibody was HRP-conjugated.

For Co-IP experiments HeLa cells were used (because of the higher transfection efficiency compared to cardiomyocytes) according to a standard protocol (Immunoprecipitation Starter
Pack, GE Healthcare). Cells of two 100 mm dishes transfected with EGFP-marked CnAβ mutants were harvested in 1 ml low salt buffer (1 % IGEPAL CA-630, 50 mM Tris (pH 8.0), 1 mM PMSF). After an initial pre-clearing step of one hour at 4°C (500 µl of whole cell lysate with ever 25 µl protein G / A sepharose beads), antigens were coupled overnight at 4°C to 2.5 µg purified antibody anti-importin β1 (Sigma-Genosys, I2534). Protein - antibody complexes were precipitated with a mix of 25 µl protein A and protein G sepharose beads for one hour at 4°C. The saved beads were washed three times with low salt buffer and suspended in 50 µl Laemmli buffer. After denaturation for 5 minutes at 95°C and a following centrifugation step the supernatant was analysed by a SDS-PAGE (8 and 12 % gel). For detecting the CnA fragments, an anti-GFP (1 : 500; Abcam, ab5450) antibody has been used. The species-specific second antibody was HRP-conjugated. For visualizing the ECL kit was used (GE Healthcare).

The subcellular distribution of calcineurin was determined by immunostainings. The polyclonal rabbit anti-CnA antibody (StressGen, SPA-610) was used as the primary antibody at a concentration of 1 : 500. For detecting fibroblasts after preparation of cardiomyocytes a troponin I specific antibody was used (Santa Cruz, sc15368). Secondary antibodies were Cy3–labeled goat anti-rabbit IgG (Jackson Laboratories; 1 : 1.000). For analyzing the cell localization of the FLAG-tagged CnA mutants an anti-FLAG antibody (1 : 500; Acris, DP3002) and the species specific Alexa Fluor 488 nm antibody were used.

**Measurement of cellular protein synthesis**

Neonatal rat cardiomyocytes were prepared as described above. On day 3, the MEM/1 was replaced by a medium containing growth stimulus Ang II (10 µmol/L) and NLS or ctr peptide (1 µmol/L). The cells were then incubated for twenty-four hours. Four hours before ending the incubation period, [3H]-leucine at an activity of 2.5 µCi/mL was added. After incubation cells were washed two times with 1 x PBS, lysed with 1 % SDS and harvested. A small amount of the cell lysate (20 µl) was used to determine DNA concentration using the Hoechst 33258 dye. The protein was precipitated by icecold 10 % TCA (500 µl) for 30 minutes. After centrifugation for 10 minutes at 13.000 g the supernatant was discarded and the pellet resolved in 500 µl 1 % SDS. After incubation for 30 minutes at room temperature, 5 ml scintillation fluid was added and the amount of the incorporated [3H]-marked leucine was measured using a β-counter in counts/ml/min (c.p.m. / CPM). The CPM:DNA concentration ratio was calculated for each sample. The change in protein synthesis is expressed as a
percentage of the CPM : DNA concentration ration in unstimulated cells which was taken as 100%.

**Statistics**

All data are presented as mean ± SEM. Statistical analyses were performed using Student *t* test, significance was assigned a value of $p < 0.05$ (*) and $p < 0.01$ (**). Non-significant differences are expressed as n.s.
**Legend**

Supplementary figure 1)
Representative immunostainings of cardiomyocytes transfected with EGFP-tagged calcineurin mutants. Fields of view were selected to demonstrate several cells in context. **A)** Cardiomyocytes were transfected with the constitutive active calcineurin mutant CnA(1-415). Cells that were transfected successfully displayed nuclear translocation of CnA. **B)** Cells were transfected with full length calcineurin CnA(1-525) and stimulated with Ang II for 12 h. In this case there is one cell without a nuclear translocation of CnA.
After stimulation with Ang II we found a nuclear accumulation of CnA(1-525) in more than 90% of the transfected cells. We obtained the same results for all calcineurin mutants that were expected to be translocated into the nucleus.

Supplementary figure 2)
Assessment of NF-ATc2 dephosphorylation. Cardiomyocytes were stimulated with Ang II for twenty-four hours or left untreated (ctr). Simultaneously cells were treated with the NLS peptide or the control (ctr) peptide. After twenty-four hours cells were harvested and a Western blot was performed with an antibody for NF-ATc2 (Abcam, ab2722). The antibody detected a band of 140 kDa representing phosphorylated NF-ATc2 (NF-ATc2 – “P”) and a 120 kDa band that represents completely dephosphorylated NF-ATc2. In cells that were stimulated with Ang II, there is an increase in dephosphorylated NF-ATc2 (120 kDa) compared to control cells. Addition of the NLS peptide had no significant effect on NF-ATc2 dephosphorylation. This indicates, that the NLS peptide had no impact on phosphatase activity of calcineurin.

Supplementary figure 3)
Nuclear translocation of calcineurin in the myocardium of different animal models of myocardial disease. Mice were subjected to myocardial infarction (MI) or aortic banding (AB). Two days after the procedure a predominately nuclear localization of calcineurin was observed in the myocardium of mice after MI and AB compared to sham operated mice. Merged pictures indicate overlay of nuclear (DAPI) and calcineurin staining.
Supplementary figure 4)

A) Representative figure of neonatal rat cardiomyocyte cultures. Cells were counterstained for troponin I (green, myofibrillar protein) and DAPI (blue, nucleus). Nuclei that are indicated (arrow) are presumably non myocytes. Contamination is ~10 %. B) Representative counterstainings from cardiomyocytes for calcineurin (CnA, green) and troponin I (red). Cells were stimulated for 12 h with phenylephrine (10 µM, PE) or left untreated. Staining for troponin I (Santa Cruz; sc15368) ensures that the cells are myocytes.
References


Supplementary figures

Supplementary figure 1)

A) CnA(1-415)

B) CnA(1-525)

Supplementary figure 2)

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Supplementary figure 3)
Supplementary figure 4)

A)

B)

CnA               troponin

- PE

+ PE