PICOT Attenuates Cardiac Hypertrophy by Disrupting Calcineurin–NFAT Signaling

Dongtak Jeong,* Ji Myoung Kim,* Hyeseon Cha, Jae Gyun Oh, Jaeho Park, Soo-Hyeon Yun, Eun-Seon Ju, Eun-Seok Jeon, Roger J. Hajjar, Woo Jin Park

Abstract—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. **(*Circ Res*. 2008;102:711-719.)**

Key Words: cardiac hypertrophy ■ PICOT ■ muscle LIM protein ■ calcineurin ■ NFAT

The myocardium undergoes hypertrophic growth in response to a variety of pathological insults, including hypertension, ischemic heart disease, valvular insufficiency, and cardiomyopathy. At the single-cell level, cardiac hypertrophy is characterized by increases in cell diameter and length and by alterations in gene expression.[1,2] Although cardiac hypertrophy is thought to be initially beneficial by maintaining and/or augmenting cardiac output, a sustained hypertrophic state leads to deleterious alterations in left ventricular architecture and is a leading predictor for the development of arrhythmias, heart failure, and sudden death.[1] Therapies that are directed at inhibiting or reversing cardiac hypertrophy are therefore of significant clinical value. Intensive investigations in the past have revealed crosstalk between multiple parallel prohypertrophic signaling pathways that comprise a complex hypertrophy signaling network (reviewed previously[3–5]). In addition, potential antihypertrophic and inhibitory feedback signaling pathways have been discovered, adding further complexity to the regulatory network.[6] Augmenting these negative signaling pathways may be a viable antihypertrophic strategy.

We have previously shown that PICOT (protein kinase [PK]C–interacting cousin of thioredoxin) activity constitutes a negative feedback loop for cardiac hypertrophy.[7] PICOT expression is upregulated on hypertrophic stimulation and, in turn, abrogates the development of cardiac hypertrophy. In addition, PICOT overexpression significantly enhances ventricular function and cardiomyocyte contractility. With these unusual characteristics, antihypertrophic and positive inotropic activities in the same molecule, PICOT appears to be a potential modality for preventing cardiac hypertrophy and heart failure. However, the detailed molecular mechanisms

Original received May 31, 2007; resubmission received October 12, 2007; revised resubmission received January 3, 2008; accepted January 29, 2008.

From the Global Research Laboratory and Department of Life Science (D.J., J.M.K., H.C., J.G.O., W.J.P.), Gwangju Institute of Science and Technology, Gwangju, Korea; Cardiovascular Institute (J.P., R.J.H.), Mount Sinai School of Medicine of New York University; and Department of Medicine (S.-H.Y., E.-S. Ju, E.-S. Jeon), Sungkyunkwan University School of Medicine, Cardiac and Vascular Center, Samsung Medical Center, Seoul, Korea.

*Both authors contributed equally to this work.

Correspondence to Woo Jin Park, Department of Life Science, Gwangju Institute of Science and Technology (GIST), 1 Oryong-dong, Buk-gu, Gwangju, 500-712, Korea. E-mail wjpark@gist.ac.kr

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.107.165985**

underlying the 2 distinct activities of PICOT have not been determined.

In this study, we examined the signaling pathways associated with PICOT by isolating PICOT binding partners. Our results reveal that PICOT inhibits cardiac hypertrophy largely by blocking the calcineurin–NFAT signaling pathway via competitive binding to muscle LIM protein (MLP).

Materials and Methods

An expanded Materials and Methods section is in the online data supplement, available at http://circres.ahajournals.org.

Cell Culture and Hypertrophic Stimulation

Primary cultures of cardiomyocytes from 1- to 3-day-old Sprague–Dawley rats were prepared as described.[8] Briefly, ventricular tissue was enzymatically dissociated, and the resulting cell suspension was enriched for cardiomyocytes using Percoll (Amersham Pharmacia) step gradients. Cells were plated onto collagen-coated culture dishes or cover slips and cultured in cardiomyocyte culture medium (DMEM supplemented with 10% FBS, 2 mmol/L L-glutamate, and 100 μmol/L 5-bromodeoxyuridine; GIBCO-BRL). To induce hypertrophy, cardiomyocytes were cultured in serum-free medium for at least 24 hours and then treated with 100 μmol/L phenylephrine (PE) for 24 to 40 hours.

Generation of the Recombinant Adenoviruses AdPICOT, AdPICOT-N, and AdPICOT-C

The AdEasy XL Adenoviral Vector System (Stratagene) was used to generate recombinant adenoviruses. Amino-terminal hemagglutinin (HA)-tagged full-length, amino-terminal, and carboxyl-terminal rat PICOT cDNAs were subcloned into the pShuttle-IRES-hrGFP2 vector. The linearized shuttle vectors were then recombined in *Escherichia coli* strain BJ5183 (Stratagene) with a serotype 5 first-generation adenoviral backbone, AdEasy-1 (Stratagene). Successfully recombined viral backbones were transformed into AD293 cells (Stratagene) and grown in large quantities. Adenovirus was purified by standard CsCl ultracentrifugation and desalting. Viral titers were determined using the plaque assay. Cardiomyocytes were infected with recombinant adenoviruses for 2 hours at a multiplicity of infection of 50 to 100 and incubated for an additional 24 to 48 hours to ensure transgene expression. AdMLP was kindly provided by Dr Masahiko Hoshijima (University of California, San Diego).

Generation of Glutathione *S*-Transferase Fusion Proteins and Glutathione *S*-Transferase Pull-Down Assays

The cDNAs encoding the full-length, amino-terminal, and carboxyl-terminal halves of mouse PICOT and the full-length, amino-terminal, and carboxyl-terminal halves of mouse MLP were subcloned into the bacterial glutathione *S*-transferase (GST) fusion vector pGEX-4T-1 (Amersham). Expression and purification of the GST fusion proteins was performed according to the protocols of the manufacturer. Cell lysates (500 μg) from HEK293 cells that had been transfected with various expression vectors were incubated with 50 μg of the purified GST fusion proteins that had been premixed with 20 μL of glutathione–Sepharose 4B (Amersham). Incubations were performed in GST binding buffer (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% Triton X-100, 50 μmol/L ZnCl, 1 mmol/L phenylmethylsulfonyl fluoride, 50 μg/mL aprotinin, 50 μg/mL leupeptin, and 50 μg/mL pepstatin) overnight at 4°C. The glutathione beads were then washed 4 times with binding buffer and analyzed by SDS-PAGE and Western blotting.

Calcineurin Phosphatase Activity Assay

The Biomol green calcineurin assay kit (catalog no. AK-816, Biomol) was used to determine the calcineurin phosphatase activity according to the instructions of the manufacturer. The RII phosphopeptide was used as a specific substrate for calcineurin.

NFAT–Luciferase Assays

A luciferase reporter plasmid driven by 3 NFAT binding sites (p3xNFAT-GL) was kindly provided by Dr Jeffery D. Molkentin (University of Cincinnati, Ohio). Assays were performed as described previously with minor modifications.[9] Briefly, the luciferase reporter plasmid was transiently transfected into neonatal cardiomyocytes using the rat cardiomyocyte neonatal nucleofector kit (AMAXA Biosystems) immediately after isolation of the neonatal cardiomyocytes. The transfection efficiency was ≈40% in live cardiomyocytes. After a 24-hour incubation period, neonatal cardiomyocytes were infected with adenoviral PICOT constructs at an multiplicity of infection of 50 and further incubated for 12 hours. To induce hypertrophy, cardiomyocytes were cultured in serum-free medium for at least 24 hours and then treated with 100 μmol/L PE for 40 hours. Luciferase activity was measured using a Lumat LB 9501 luminometer (Berthold).

Quantitative RT-PCR

Total RNA was isolated with TRI reagent (Sigma). Reverse-transcription was performed using ImProm II reverse-transcriptase (Promega) with oligo-dT priming. PCR was performed using an ABI PRISM Sequence Detector System 7500 (Applied Biosystems) with SYBR Green (Takara) as fluorescent and ROX (Takara) as reference dyes. PCR primers used were: atria natriuretic factor, 5′-ACC TGC TAG ACC ACC TGG AGG AG-3′ and 5′-CCT TGG CTG TTA TCT TCG GTA CCG-3′; brain natriuretic peptide, 5′-GCT GCT TTG GGC ACA AGA TAG-3′ and 5′-GGT CTT CCT ACA ACA ACT TCA-3′; myocyte-enriched calcineurin interacting protein (MCIP)1, 5′-TCC AGC TTG GGC TTG ACT GAG-3′ and 5′-ACT GGA AGG TGG TGT CCT TGT C-3′.[10]

Contractility Measurements

Adult rat ventricular myocytes were isolated via enzymatic dissociation, as described previously,[11] and infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C for 12 to 15 hours. Only green fluorescent protein–positive cells were examined. Contraction amplitudes and rates of contraction and relaxation were recorded using a dual excitation spectrofluorometer and a video-edge detection system (IonOptix), as described previously.[7]

Statistics

Where appropriate, the data are expressed as means±SDs. Comparisons of the group means were made using a Student *t* test or 1-way ANOVA with a Bonferroni post test analysis (Statview version 5.0, SAS). $P<0.05$ was considered to be statistically significant.

Results

PICOT Binds to the MLP via Its Carboxyl-Terminal PICOT Homology Domain

The amino-terminal region of PICOT (residues 1 to 143) is highly homologous to thioredoxin and is thus referred to as the thioredoxin homology (TH) domain, whereas the carboxyl-terminal region (residues 145 to 335) contains 2 tandem repeats provisionally termed the PICOT homology (PH) domain (Figure 1A).[12] We generated GST-PICOT fusion proteins, designated GST-PICOT, GST-PICOT-N, and GST-PICOT-C, which contain the full-length, amino-terminal, and carboxyl-terminal regions of PICOT, respectively. Similarly, we generated recombinant adenoviruses that express HA-tagged full-length, amino-terminal, and carboxyl-terminal PICOT, designated AdPICOT, AdPICOT-N, and AdPICOT-C, respectively (Figure I in the online data supplement and Figure 1A). GST pull-down assays revealed that PICOT directly binds to PKCζ, but not to PKCα or PKCε, via

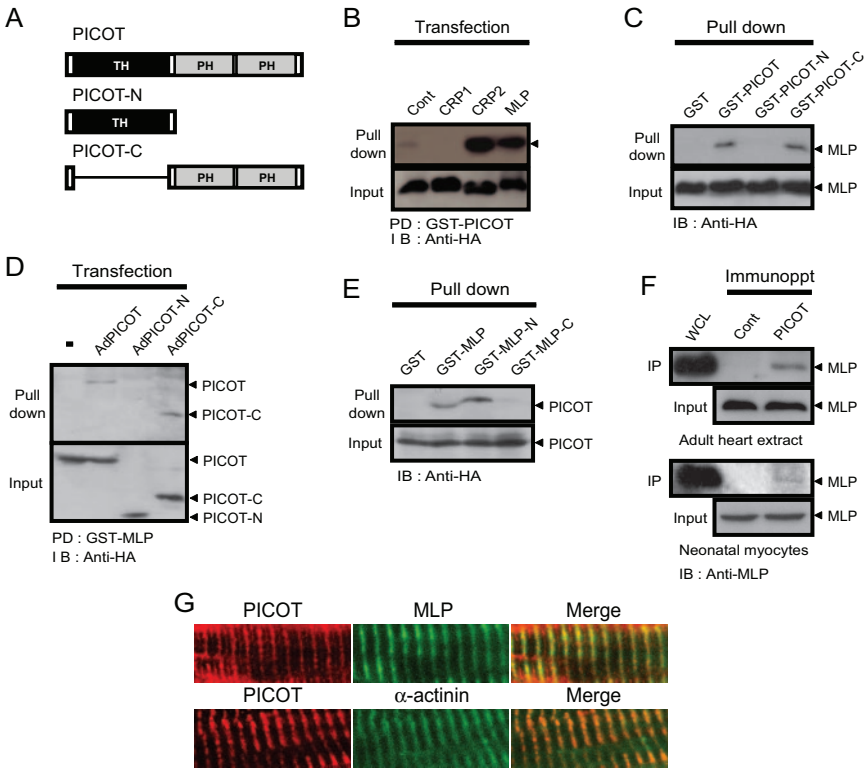

Figure 1. Direct interaction between PICOT and MLP. A, PICOT constructs: full-length PICOT; amino-terminal TH domain of PICOT, PICOT-N; carboxyl-terminal PH domain of PICOT, PICOT-C. B, The GST-PICOT fusion protein was incubated with extracts from NIH-3T3 cells transfected with HA-tagged CRP1, CRP2, and MLP (CRP3) expression constructs. Precipitates were separated by SDS-PAGE and blotted with anti-HA antibody. C, Similar pull-down experiments were carried out with GST-PICOT, GST-PICOT-N, or GST-PICOT-C. D, Conversely, GST-MLP fusion proteins were incubated with extracts from NIH-3T3 cells infected with AdPICOT, AdPICOT-N, or AdPICOT-C. Precipitates were separated by SDS-PAGE and blotted with anti-HA antibody. E, GST-MLP, GST-MLP-N, and GST-MLP-C fusion proteins were generated and incubated with extracts from NIH-3T3 cells infected with AdPICOT. Precipitates were separated by SDS-PAGE and blotted with anti-HA antibody. F, Whole cell lysates (WCL) from rat left ventricle and neonatal cardiomyocytes were immunoprecipitated with anti-PICOT antibody. Precipitates were separated by SDS-PAGE and blotted with anti-MLP antibody. G, Heart sections were coimmunostained with anti-PICOT and anti-MLP antibodies or anti-PICOT and anti-α-actinin antibodies.

its amino-terminal TH domain; this result is consistent with previous reports (data not shown).[12]

To identify proteins that interact with the carboxyl-terminal half of PICOT, GST pull-down experiments coupled with mass spectrometric analysis were performed. Purified GST-PICOT-C was incubated with extracts from rat hearts, and proteins that coprecipitated with PICOT-C were separated by SDS-PAGE and stained with Coomassie blue. Mass spectrometric analysis of the distinct protein bands revealed that MLP (also known as CRP3) was associated with PICOT (data not shown). MLP was particularly of interest because it was previously shown to function as a component of the stretch sensor machinery and defects in MLP lead to dilated cardiomyopathy and heart failure.[13,14] To verify that PICOT directly interacts with MLP, a series of pull-down assays was conducted. Purified GST-PICOT was incubated with extracts from NIH-3T3 cells that were transfected with HA-tagged CRP1, CRP2, and MLP (CRP3) expression vectors. The resulting precipitates were separated by SDS-PAGE, transferred to poly(vinylidene difluoride), and blotted with an anti-HA antibody. The results showed that PICOT specifically interacted with CRP2 and MLP, but not with CRP1 (Figure 1B). A similar pull-down experiment showed that MLP coprecipitated with PICOT-C but not with PICOT-N (Figure 1C). Conversely, purified GST-MLP was incubated with extracts from NIH-3T3 cells that were infected with AdPICOT, AdPICOT-N, and AdPICOT-C. Western blotting of the resulting precipitates revealed that PICOT and PICOT-C, but not PICOT-N, coprecipitated with MLP (Figure 1D). GST-MLP fusion proteins containing full-length MLP, the amino-terminal, or carboxyl-terminal halves of MLP were generated and designated as GST-MLP, GST-MLP-N, and GST-MLP-C, respectively. A pull-down experiment performed with these fusion proteins revealed that MLP-N, but not MLP-C, binds to PICOT (Figure 1E). Notably, PICOT and MLP were coimmunoprecipitated from lysates of adult rat left ventricle and neonatal cardiomyocytes, implying that the interaction between PICOT and MLP is physiologically relevant (Figure 1F). Finally, PICOT and MLP colocalized in the Z-disc when adult heart sections were immunostained with anti-PICOT and anti-MLP antibodies. PICOT colocalized also with a known Z-disc protein, α-actinin (Figure 1G). These data indicate that PICOT binds to MLP through a direct interaction between the carboxyl-terminal PH domain of PICOT and the amino-terminal half of MLP.

PICOT Inhibits PE-Induced Activation of Calcineurin–NFAT Signaling

MLP is required for calcineurin–NFAT signaling at the sarcomeric Z-disc.[15] Our finding that PICOT directly interacts with MLP raised the possibility that PICOT may affect calcineurin–NFAT signaling. To test this hypothesis, calcineurin phosphatase activity was measured in cardiomyocytes that were stimulated with PE, an agonist for cardiac hypertrophy, and infected with either AdLacZ or AdPICOT. Adenoviral infection preceded PE stimulation by 24 hours to allow the adequate expression of β-galactosidase (control) or PICOT. Calcineurin phosphatase activity significantly increased in cardiomyocytes that were infected with AdLacZ ($P<0.05$) in response to PE treatment. However, AdPICOT infection blunted the increase in calcineurin activity induced by PE (Figure 2A). Activated calcineurin directly binds to NFAT transcription factors, resulting in NFAT dephosphor-

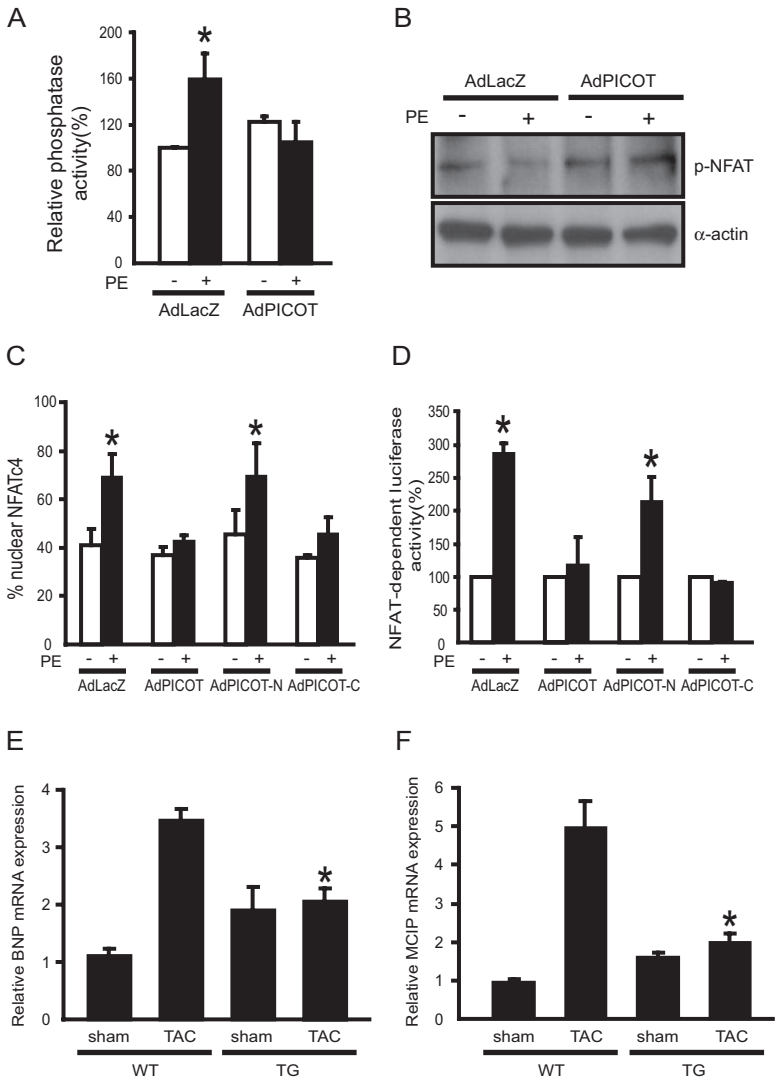

Figure 2. Effect of PICOT on calcineurin–NFAT signaling. Neonatal cardiomyocytes that had been infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C were treated for 24 hours in the presence or absence of PE (100 μmol/L). A, Cell lysates (3 μg) were subjected to calcineurin phosphatase activity assays (n=3). *$P<0.05$ against no PE treatment. B, Cell lysates (50 μg) were separated by SDS-PAGE and blotted with anti-NFATc4 and anti–phospho-NFATc4 antibodies. C, Nuclear localization of NFATc4 was assessed by immunostaining with anti-NFATc4 antibody. Representative cytoplasmic and nuclear staining of NFATc4 is shown in supplemental Figure II. Data are given as percentages of cells with nuclear localization of NFATc4. Approximately 100 cardiomyocytes were examined. *$P<0.05$ against no PE treatment. D, Neonatal cardiomyocytes were transfected with p3xNFAT-GL, a luciferase reporter plasmid driven by 3 NFAT binding sites, and infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C. The cells were further incubated for 24 hours in the presence or absence of PE (100 μmol/L). Cell lysates (20 μg) were subjected to luciferase activity assays. Data represent the luciferase activity in PE-treated cardiomyocytes as a percentage of that in non-PE–treated controls (n=3). *$P<0.05$ against no PE treatment. E and F, WT and PICOT TG mice were subjected either to sham operation (sham) or to transverse aortic constriction (TAC) for 2 weeks. The relative levels of brain natriuretic peptide (BNP) and MCIP1 transcripts in left ventricles were determined by quantitative RT-PCR (n=3). *$P<0.01$ against WT/TAC.

ylation and nuclear translocation.[16,17] NFAT was significantly dephosphorylated by PE in cardiomyocytes infected with AdLacZ. However, NFAT dephosphorylation was unaltered by PE in cardiomyocytes that were infected with AdPICOT, implying that PICOT inhibits the PE-induced increase in calcineurin activity (Figure 2B). The subcellular localization of NFAT in neonatal cardiomyocytes was examined by immunostaining (supplemental Figure II). Nuclear localization of NFAT was observed in approximately 40% of unstimulated cardiomyocytes. However, treatment with PE increased the percentage of cardiomyocytes with nuclear NFAT staining to approximately 70%. Infection of cardiomyocytes with AdPICOT completely abrogated the PE-induced NFAT nuclear translocation, whereas the control AdLacZ had no effect. Infection with AdPICOT-C, but not AdPICOT-N, was effective in blocking NFAT nuclear translocation to a level comparable to that observed with AdPICOT (Figure 2C). To examine whether the alterations in NFAT nuclear translocation affected NFAT transcriptional activity, cardiomyocytes were transfected with a luciferase reporter plasmid driven by 3 NFAT binding sites.[9] Treatment with PE significantly increased NFAT transcriptional activity. Infections with AdPICOT and AdPICOT-C completely inhibited PE-induced NFAT activation, whereas infection with AdLacZ or AdPICOT-N did not (Figure 2D). Hypertrophic marker genes encoding brain natriuretic peptide and MCIP1 are known to be direct transcriptional targets of NFAT in cardiomyocytes.[18,19] Pressure overload markedly elevated the expression level of brain natriuretic peptide and MCIP1, which was significantly blunted in the hearts of PICOT transgenic (TG) mice (Figure 2E and 2F). These data indicate that PICOT inhibits PE-induced increases in calcineurin activity and NFAT nuclear translocation and transcriptional activation and that the carboxyl-terminal PH domain is sufficient for this inhibition.

PICOT Interferes With the MLP–Calcineurin Interaction

MLP directly associates with calcineurin and this interaction is reported to be critical for calcineurin–NFAT signaling.[15] To further characterize the mechanism underlying the inhibitory action of PICOT on the agonist-induced activation of the calcineurin–NFAT signaling pathway, we examined whether PICOT affects the physical interaction between MLP

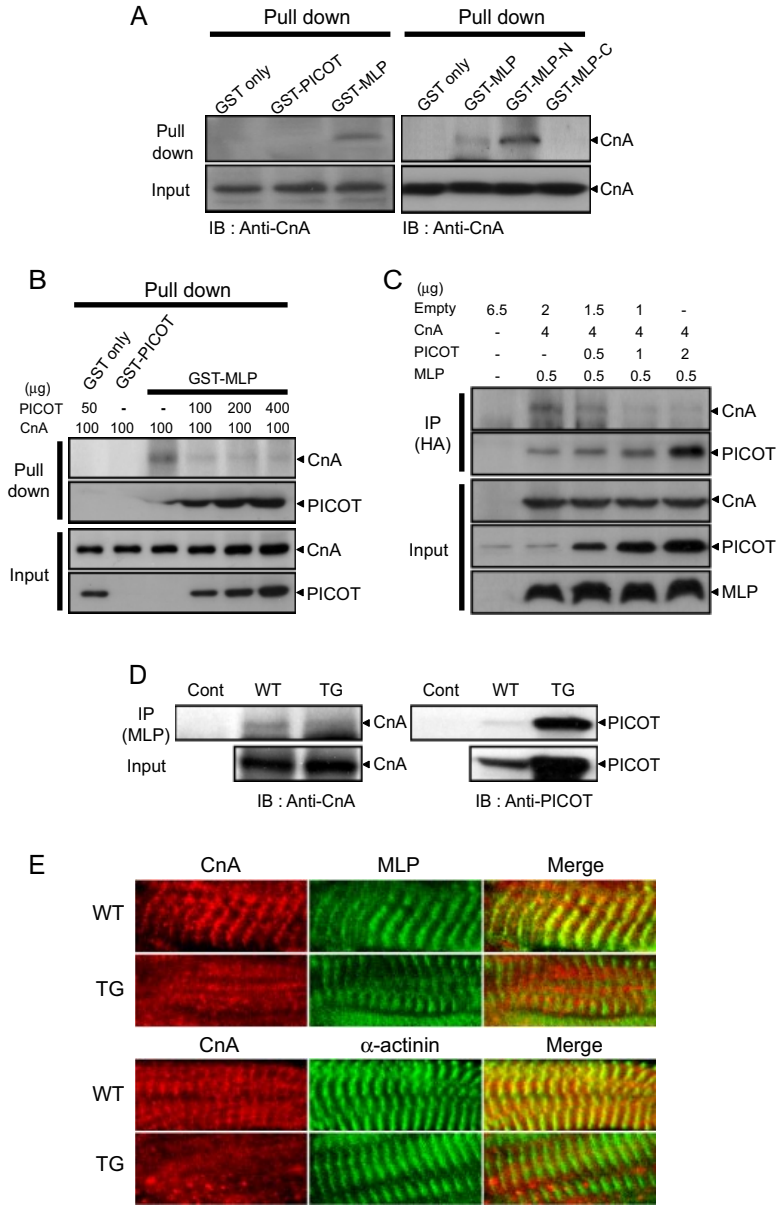

Figure 3. Competitive binding of PICOT and calcineurin to MLP. A, GST-PICOT and GST-MLP fusion proteins were incubated with extracts from NIH-3T3 cells that had been transfected with calcineurin expression constructs (left). GST-MLP, GST-MLP-N, and GST-MLP-C were used for similar pull-down assays (right). Precipitates were separated by SDS-PAGE and immunoblotted with anti-calcineurin antibody. B, GST-MLP was incubated with extracts (100 μg) from NIH-3T3 cells that had been transfected with a calcineurin expression construct in the presence of varying amounts of extracts (0 to 400 μg) from NIH-3T3 cells transfected with a PICOT expression construct. Precipitates were separated by SDS-PAGE and immunoblotted with anti-calcineurin and anti-PICOT antibodies. C, NIH-3T3 cells were transfected with HA-MLP expression vector (0.5 μg), calcineurin expression vector (4 μg), and varying amounts of PICOT expression vector (0 to 2 μg). Cell lysates were prepared and subjected to immunoprecipitation with anti-HA antibody. Precipitates were separated by SDS-PAGE and blotted with anti-calcineurin and anti-PICOT antibodies. D, Whole cell lysates from left ventricles of WT and PICOT TG mice were immunoprecipitated with anti-MLP antibody. Precipitates were separated by SDS-PAGE and blotted with anti-calcineurin antibody (left) or with anti-PICOT antibody (right). E, Left ventricular tissue sections from WT and TG mice were coimmunostained with anti-calcineurin and anti-MLP antibodies and anti-calcineurin and anti-α-actinin antibodies and then analyzed by confocal microscopy.

and calcineurin. GST-PICOT or GST-MLP was incubated with extracts from NIH-3T3 cells that had been transfected with a calcineurin expression vector. Immunoblotting of the resulting precipitates revealed that calcineurin directly binds to MLP, but not to PICOT (Figure 3A). Additional pull-down experiments indicated that the amino-terminal half of MLP was sufficient for calcineurin binding (Figure 3A). In fact, the amino-terminal half alone bound to calcineurin more effectively than full-length MLP, suggesting that the carboxyl-terminal half of MLP may hinder this interaction. It is also intriguing to note that both PICOT and calcineurin bind to the amino-terminal half of MLP. This may support a notion that PICOT and calcineurin competitively bind to MLP. To test this hypothesis, increasing amounts of PICOT-containing cell lysates (0 to 400 μg) were mixed with calcineurin-containing cell lysates (100 μg), and then a pull-down experiment was performed with GST-MLP. The amount of calcineurin that was pulled-down by MLP was reduced in proportion to the amount of PICOT that was added (Figure 3B). NIH-3T3 cells were transfected with fixed amounts of HA-MLP (0.5 μg) and calcineurin (4 μg) expression vectors and with varying amounts of PICOT expression vector (0 to 2 μg). The expression of HA-MLP, calcineurin, and PICOT was verified by Western blotting (input), and then the cell lysates were immunoprecipitated with anti-HA antibody. The amount of calcineurin that coprecipitated with HA-MLP was reduced in proportion to the amount of PICOT that was present in cell lysates (Figure 3C). Calcineurin was coimmunoprecipitated with MLP from lysates of wild-type (WT) hearts, but not from lysates of PICOT TG hearts (Figure 3D). These results indicate that PICOT interferes with the interaction between MLP and calcineurin by competitively binding to MLP.

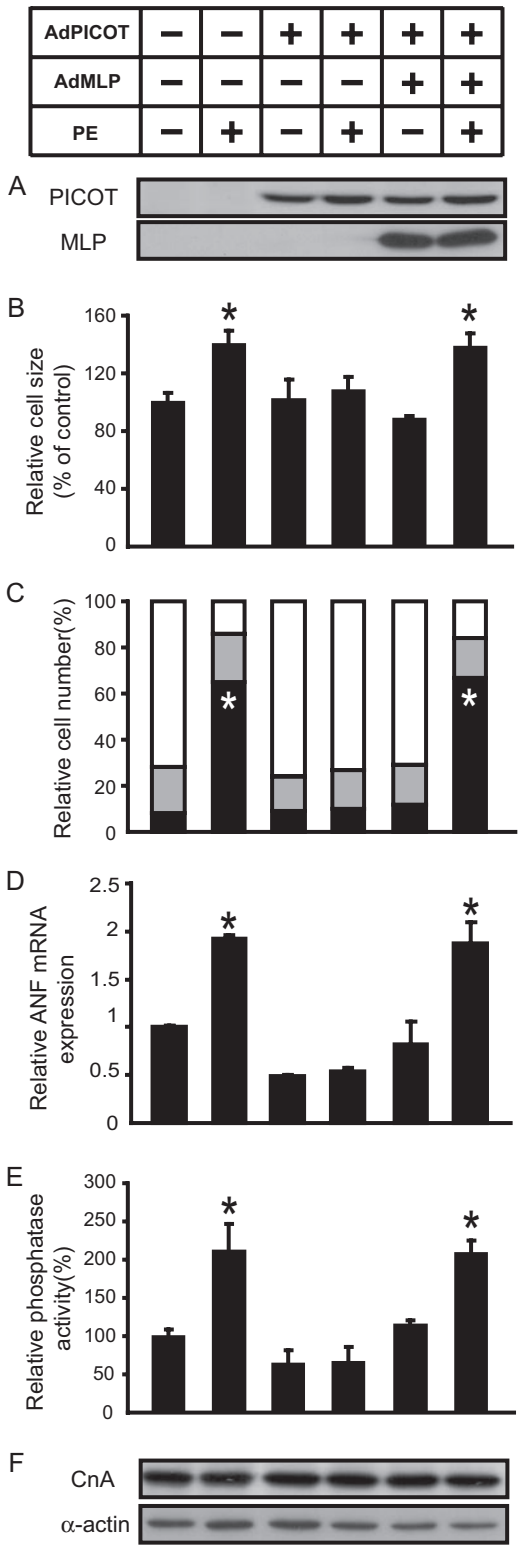

Figure 4. The antihypertrophic activity of PICOT nullified by replenishment of MLP. Neonatal cardiomyocytes were infected with AdPICOT alone or coinfected with AdPICOT and AdMLP and further stimulated with PE (100 μmol/L) for 24 hours. A, Cell lysates (50 μg) were separated by SDS-PAGE and blotted with anti-PICOT or anti-MLP antibodies. B, The cell surface area of cardiomyocytes was measured using i-solution software (n=100 cells each group). *$P<0.05$ against controls. C, Sarcomeric reorganization of cardiomyocytes was visualized by phalloidin staining (pictures are not shown). The morphology of myofibrils was semiquantitatively scored based on the area occupied by the reorganized sarcomere relative to the total cell area as follows: less than one-third of the cell area (white bar); more than one-third but less than two-thirds of the cell area (gray bar); more than two-thirds of the cell area (black bar) (n=50 for each group). *$P<0.05$ against controls. D, The relative expression levels of atria natriuretic factor (ANF) were determined by quantitative RT-PCR (n=3). *$P<0.01$ against controls. E, Cell lysates (3 μg) were subjected to calcineurin phosphatase activity assays (n=3). *$P<0.05$ against controls. F, Cell lysates (50 μg) were separated by SDS-PAGE and blotted with anti-calcineurin antibodies.

Anchorage of calcineurin at the Z-disc is essential for the coordinated dephosphorylation of NFAT.[20,21] It has also been shown that calcineurin is dislocated from the Z-disc in MLP$^{+/-}$ mice.[15] We, therefore, assessed the localization of calcineurin in the hearts of WT and PICOT TG mice by immunohistochemistry. The expression pattern of calcineurin indicated that calcineurin colocalizes with MLP and α-actinin in WT hearts. However, the prominent striated expression pattern of calcineurin was significantly lost in PICOT TG hearts (Figure 3E), indicating that PICOT induces the dislocation of calcineurin from the Z-disc. Immunostaining with anti-MLP and anti–α-actinin revealed prominent striation in both WT and PICOT TG mice, excluding gross Z-disc abnormalities in PICOT TG mice (Figure 3E).

If our model is correct, replenishment of MLP should nullify the inhibitory activity of PICOT in calcineurin–NFAT signaling. To test this hypothesis, cardiomyocytes were infected with AdPICOT or coinfected with AdPICOT and AdMLP in the presence or absence of PE. The expression of PICOT and MLP was confirmed by Western blotting (Figure 4A). Whereas infection with AdPICOT alone inhibited PE-induced hypertrophy, coinfection with AdMLP and AdPICOT completely restored the AdPICOT-mediated repressed hypertrophy, as assessed by increased cell size, enhanced sarcomeric rearrangement, and upregulation of a hypertrophic marker gene, atria natriuretic factor (Figure 4B through 4D). The calcineurin phosphatase activity that was inhibited by infection with AdPICOT was also restored by coinfection with AdMLP (Figure 4E). Calcineurin expression levels were essentially unaltered (Figure 4F).

The Carboxyl-Terminal PH Domain of PICOT Is Sufficient in Inhibiting Cardiomyocyte Hypertrophy

The above data indicate that PICOT inhibits activation of the calcineurin–NFAT signaling pathway by competitively binding to MLP through its carboxyl-terminal PH domain. We therefore examined whether inhibition of calcineurin–NFAT signaling by the PH domain correlated with the inhibition of cardiomyocyte hypertrophy. Neonatal cardiomyocytes were infected with AdPICOT, AdPICOT-N, AdPICOT-C, or AdLacZ and were then further stimulated with PE. PE treatment significantly increased the size of AdLacZ-infected, as well as uninfected, cardiomyocytes. In contrast, the increase in cell size was completely abrogated by AdPICOT or AdPICOT-C infection and partially abrogated by AdPICOT-N infection

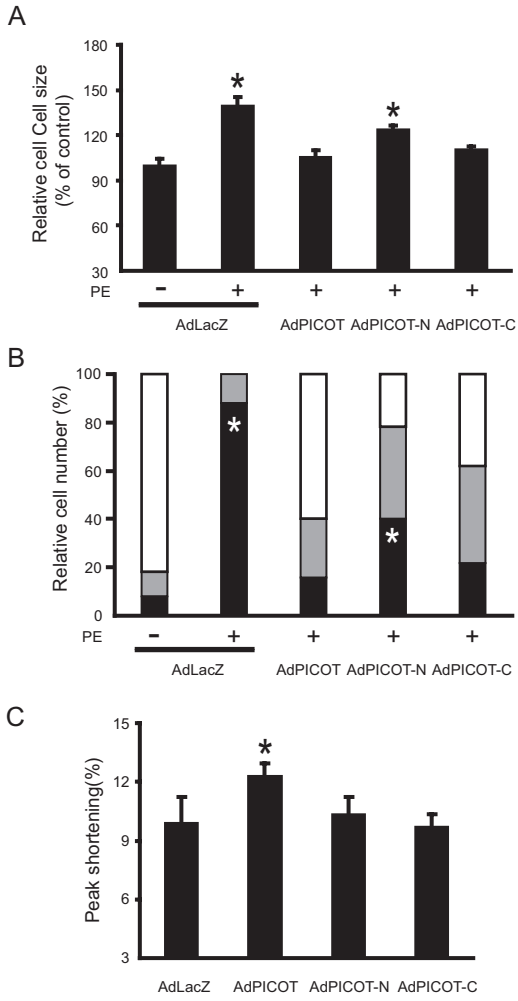

Figure 5. Dissection of the antihypertrophic and inotropic effects of PICOT. A, Neonatal cardiomyocytes that had been infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C were treated for 24 hours in the presence or absence of PE (100 μmol/L). The cell surface area of cardiomyocytes was measured using i-solution software (n=100 cells each group). *$P<0.05$ against controls. B, Sarcomeric reorganization of cardiomyocytes was visualized by phalloidin staining. The morphology of myofibrils was semiquantitatively scored based on the area occupied by the reorganized sarcomere relative to the total cell area as indicated in Figure 4C (n=50 for each group). *$P<0.05$ against controls. C, Isolated adult cardiomyocytes were infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C and the extent of cell shortening was determined (AdLacZ, n=25; AdPICOT, n=27; AdPICOT-N, n=37; AdPICOT-C, n=14). *$P<0.05$ against controls.

(Figure 5A). Sarcomeric rearrangement was assessed by semiquantitative scoring. The ratio of cardiomyocytes with well-organized sarcomeres (more than two-thirds of cell area) increased from 8% to 88% on PE treatment in AdLacZ-infected cardiomyocytes, whereas the increases were only 16% and 22% in AdPICOT and AdPICOT-C infected cardiomyocytes, respectively, and 40% in AdPICOT-N infected cardiomyocytes (Figure 5B). These data indicate that PICOT-C inhibits cardiac hypertrophy as efficiently as full-length PICOT, and suggest that PICOT inhibits cardiac hypertrophy largely by abrogating calcineurin–NFAT signaling through its carboxyl-terminal PH domain.

We have previously shown that PICOT enhances cardiomyocyte contractility.[7] Isolated adult rat cardiomyocytes were infected with AdPICOT, AdPICOT-N, and AdPICOT-C, and the consequential mechanical properties were determined using a dual-excitation spectrofluorometer equipped with a video-edge detection system. Whereas AdPICOT increased cell shortening by ≈25%, neither AdPICOT-N nor AdPICOT-C significantly increased cell shortening (Figure 5C). These results indicate the presence of a separate mechanism for enhancement of contractility by PICOT.

Discussion

PICOT was first identified as a PKCθ-interacting protein in a yeast 2-hybrid screen.[12] It has an amino-terminal TH domain that is highly homologous to the thioredoxin family proteins. PICOT is unlikely to be involved in intracellular redox regulation because the TH domain of PICOT lacks the conserved Cys-Gly-Pro-Cys motif that is essential for catalytic activity. Instead, the TH domain may serve as a structural motif for specific interactions with PKC.[22] In our pull-down experiments using GST-PICOT and GST-PICOT-N fusion proteins, we confirmed that the TH domain binds to PKC (data not shown). The carboxyl-terminal region of PICOT contains 2 tandem repeats of an evolutionarily conserved domain of unknown function, referred to as the PH domain.[12,23] In this study, we found that the PH domain directly binds to MLP. MLP interacts with telethonin, a titin binding protein, at the Z-disc and is critical for cardiac cytoarchitectural organization. MLP-deficiency leads to a selective defect in the mechanical stretch response and eventually to dilated cardiomyopathy and heart failure.[14] Moreover, a human MLP mutation (W4R) that is associated with dilated cardiomyopathy results in a marked defect in MLP–telethonin interactions.[13] It was, therefore, suggested that MLP functions as a pivot for the reception and transduction of mechanical stress signals in the Z-disc. Recently, MLP was found to play an essential role in calcineurin–NFAT signaling pathways, perhaps by anchoring calcineurin to the Z-disc.[15] Calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase, plays an essential role in cardiomyocyte growth and gene expression by promoting dephosphorylation and nuclear translocation of NFAT.[16,17] A number of proteins bind to the catalytic subunit of calcineurin, calcineurin A, and regulate its enzymatic activity, including calcineurin B homologous protein,[24] FKBP38,[25] Cabin/Cain,[26,27] MCIPI1,[21,27a] and a F-box adaptor protein atrogin-1.[28] This study has demonstrated that PICOT competes with calcineurin for binding to MLP and, thus, interferes with the MLP–calcineurin interaction in vitro and in vivo.

We have also shown that disruption of the MLP–calcineurin interaction by PICOT inhibited increases in calcineurin phosphatase activity on PE treatment and also resulted in dislocation of calcineurin from the Z-disc; this result is consistent with a previous report showing that calcineurin is dislocated from the Z-disc in $MLP^{+/-}$ mice.[15] Therefore, sufficient MLP appears to be required both for PE-induced calcineurin phosphatase activation and anchoring

calcineurin to the Z-disc. This notion is partially supported by our data showing that replenishment of MLP suppressed the function of PICOT in inhibiting PE-induced calcineurin phosphatase activation and hypertrophy in cardiomyocytes. It is currently unclear whether anchoring calcineurin to the Z-disc via an interaction with MLP is prerequisite for the induction of calcineurin phosphatase activity or vice versa. It is possible that anchoring calcineurin at the Z-disc may facilitate its access to specific substrates such as NFAT, which is also localized at the Z-disc.[20] The extent to which anchoring calcineurin at the Z-disc contributes to activation of downstream NFAT signaling remains to be determined. In this sense, it is intriguing to note that calsarcin-1, a sarcomeric negative regulator of calcineurin, does not affect the localization of calcineurin at the Z-disc.[10]

We do not rule out another interesting possibility that the molecular events associated with PICOT, MLP, and calcineurin shown in this study are also occurring in nucleus because all 3 of these proteins are known to localize in the nucleus as well as the Z-disc. PICOT was shown to be phosphorylated and translocated to the nucleus in response to oxidative stress in human T cells.[29] MLP was shown to be exclusively nuclear in nonmyogenic cells and in differentiating myogenic cells, whereas it is associated with f-actin in mature myotubes and myocytes.[30] Moreover, MLP was shown to be relocalized to the nucleus in hypertrophied and failing hearts.[31,32] Interestingly, calcineurin is not only capable of dephosphorylating NFAT, but the presence of calcineurin in the nucleus is also important for full NFAT transcriptional activity.[33] The colocalization of PICOT, MLP, and calcineurin in the nucleus, especially in stressed conditions, suggests a scenario that MLP-associated calcineurin, which can be intervened by PICOT, may act as a transcriptional coactivator that is critical for eliciting hypertrophic responses. This hypothesis needs to be tested further.

In conclusion, our study has revealed a novel regulatory point for modulating calcineurin–NFAT signaling which may provide a new therapeutic strategy for the treatment of cardiac hypertrophy and heart failure.

Sources of Funding

During this study, W.J.P. was supported by the Global Research Laboratory Program (M6-0605-00-0001) of the Korean Ministry of Science and Technology. R.J.H. and W.J.P. were supported by NIH grant HL-080498-01.

Disclosures

None.

References

1. Katz AM. Cardiomyopathy of overload. A major determinant of prognosis in congestive heart failure. *N Engl J Med*. 1990;322:100–110.
2. Dorn GW, II, Robbins J, Sugden PH. Phenotyping hypertrophy: eschew obfuscation. *Circ Res*. 2003;92:1171–1175.
3. Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. *N Engl J Med*. 1999;341:1276–1283.
4. Molkentin JD, Dorn IG II. Cytoplasmic signaling pathways that regulate cardiac hypertrophy. *Annu Rev Physiol*. 2001;63:391–426.
5. Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. *Annu Rev Physiol*. 2003;65:45–79.
6. Hardt SE, Sadoshima J. Negative regulators of cardiac hypertrophy. *Cardiovasc Res*. 2004;63:500–509.
7. Jeong D, Cha H, Kim E, Kang M, Yang DK, Kim JM, Yoon PO, Oh JG, Bernecker OY, Sakata S, Le TT, Cui L, Lee YH, Kim do H, Woo SH, Liao R, Hajjar RJ, Park WJ. PICOT inhibits cardiac hypertrophy and enhances ventricular function and cardiomyocyte contractility. *Circ Res*. 2006;99:307–314.
8. Sadoshima J, Jahn L, Takahashi T, Kulik TJ, Izumo S. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *J Biol Chem*. 1992;267:10551–10560.
9. Taigen T, De Windt LJ, Lim HW, Molkentin JD. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. *Proc Natl Acad Sci U S A*. 2000;97:1196–1201.
10. Frey N, Barrientos T, Shelton JM, Frank D, Rutten H, Gehring D, Kuhn C, Lutz M, Rothermel B, Bassel-Duby R, Richardson JA, Katus HA, Hill JA, Olson EN. Mice lacking calsarcin-1 are sensitized to calcineurin signaling and show accelerated cardiomyopathy in response to pathological biomechanical stress. *Nat Med*. 2004;10:1336–1343.
11. Ren J. Attenuated cardiac contractile responsiveness to insulin-like growth factor I in ventricular myocytes from biobreeding spontaneous diabetic rats. *Cardiovasc Res*. 2000;46:162–171.
12. Witte S, Villalba M, Bi K, Liu Y, Isakov N, Altman A. Inhibition of the c-Jun N-terminal kinase/AP-1 and NF-kappaB pathways by PICOT, a novel protein kinase C-interacting protein with a thioredoxin homology domain. *J Biol Chem*. 2000;275:1902–1909.
13. Arber S, Hunter JJ, Ross J Jr, Hongo M, Sansig G, Borg J, Perriard JC, Chien KR, Caroni P. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell*. 1997;88:393–403.
14. Knoll R, Hoshijima M, Hoffman HM, Person V, Lorenzen-Schmidt I, Bang ML, Hayashi T, Shiga N, Yasukawa H, Schaper W, McKenna W, Yokoyama M, Schork NJ, Omens JH, McCulloch AD, Kimura A, Gregorio CC, Poller W, Schaper J, Schultheiss HP, Chien KR. The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. *Cell*. 2002;111:943–955.
15. Heineke J, Ruetten H, Willenbockel C, Gross SC, Naguib M, Schaefer A, Kempf T, Hilfiker-Kleiner D, Caroni P, Kraft T, Kaiser RA, Molkentin JD, Drexler H, Wollert KC. Attenuation of cardiac remodeling after myocardial infarction by muscle LIM protein-calcineurin signaling at the sarcomeric Z-disc. *Proc Natl Acad Sci U S A*. 2005;102:1655–1660.
16. Shaw KT, Ho AM, Raghavan A, Kim J, Jain J, Park J, Sharma S, Rao A, Hogan PG. Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. *Proc Natl Acad Sci U S A*. 1995;92:11205–11209.
17. Loh C, Shaw KT, Carew J, Viola JP, Luo C, Perrino BA, Rao A. Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. *J Biol Chem*. 1996;271:10884–10891.
18. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*. 1998;93:215–228.
19. Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, Olson EN, Bassel-Duby R, Williams RS. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. *Circ Res*. 2000;87:e61–e68.
20. Liu Y, Cseresnyes Z, Randall WR, Schneider MF. Activity-dependent nuclear translocation and intranuclear distribution of NFATc in adult skeletal muscle fibers. *J Cell Biol*. 2001;155:27–39.
21. Vega RB, Bassel-Duby R, Olson EN. Control of cardiac growth and function by calcineurin signaling. *J Biol Chem*. 2003;278:36981–36984.
22. Watson JA, Rumsby MG, Wolowacz RG. Phage display identifies thioredoxin and superoxide dismutase as novel protein kinase C-interacting proteins: thioredoxin inhibits protein kinase C-mediated phosphorylation of histone. *Biochem J*. 1999;343(pt 2):301–305.
23. Isakov N, Witte S, Altman A. PICOT-HD: a highly conserved protein domain that is often associated with thioredoxin and glutaredoxin modules. *Trends Biochem Sci*. 2000;25:537–539.
24. Lin X, Sikkink RA, Rusnak F, Barber DL. Inhibition of calcineurin phosphatase activity by a calcineurin B homologous protein. *J Biol Chem*. 1999;274:36125–36131.

25. Shirane M, Nakayama KI. Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat Cell Biol*. 2003;5: 28–37.
26. Sun L, Youn HD, Loh C, Stolow M, He W, Liu JO. Cabin 1, a negative regulator for calcineurin signaling in T lymphocytes. *Immunity*. 1998;8: 703–711.
27. Lai MM, Burnett PE, Wolosker H, Blackshaw S, Snyder SH. Cain, a novel physiologic protein inhibitor of calcineurin. *J Biol Chem*. 1998; 273:18325–18331.
27a. Rothermel 2001.
28. Li HH, Kedar V, Zhang C, McDonough H, Arya R, Wang DZ, Patterson C. Atrogin-1/muscle atrophy F-box inhibits calcineurin-dependent cardiac hypertrophy by participating in an SCF ubiquitin ligase complex. *J Clin Invest*. 2004;114:1058–1071.
29. Babichev Y, Isakov N. Tyrosine phosphorylation of PICOT and its translocation to the nucleus in response of human T cells to oxidative stress. *Adv Exp Med Biol*. 2001;495:41–45.
30. Arber S, Halder G, Caroni P. Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. *Cell*. 1994; 79:221–231.
31. Ecarnot-Laubriet A, De Luca K, Vandroux D, Moisant M, Bernard C, Assem M, Rochette L, Teyssier JR. Downregulation and nuclear relocation of MLP during the progression of right ventricular hypertrophy induced by chronic pressure overload. *J Mol Cell Cardiol*. 2000;32: 2385–2395.
32. Boateng SY, Belin RJ, Geenen DL, Margulies KB, Martin JL, Hoshijima M, de Tombe PP, Russell B. Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein. *Am J Physiol Heart Circ Physiol*. 2007;292:H259–H269.
33. Hallhuber M, Burkard N, Wu R, Buch MH, Engelhardt S, Hein L, Neyses L, Schuh K, Ritter O. Inhibition of nuclear import of calcineurin prevents myocardial hypertrophy. *Circ Res*. 2006;99:626–635.