Requirement of Nuclear Factor of Activated T-cells in Calcineurin-mediated Cardiomyocyte Hypertrophy*

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The calcium-activated phosphatase calcineurin has been implicated as a critical intracellular signal transducer of cardiomyocyte hypertrophy. Although previous data suggested the nuclear factor of activated T-cells (NFAT) as its sole transcriptional effector, the absolute requirement of NFAT as a mediator of calcineurin signaling has not been examined in the heart. We therefore investigated the expression and activation profile of NFAT genes in the heart. Four members (NFATc1–c4) are expressed in cardiomyocytes, elicit nuclear translocation upon calcineurin activation, and are able to drive transactivation of calcium promoter luciferase constructs. To define the necessary function of NFAT factors as hypertrophic transducers, a dominant negative NFAT construct was created, encompassing part of the N-terminal region of NFATc4 containing a conserved calcineurin-binding motif. Cotransfection of this construct dose-dependently abrogated promoter activation, irrespective of the NFAT isoform used, whereas a control construct with the calcineurin-binding motif mutated displayed no such effects. Adenoviral gene transfer of dominant negative NFAT rendered cardiomyocytes resistant toward all aspects of calcineurin or agonist-induced cardiomyocyte hypertrophy, whereas adenoviral gene transfer of the control construct had no discernable effect on these parameters. These results indicate that multiple NFAT isoforms are expressed in cardiomyocytes where they function as necessary transducers of calcineurin in facilitating cardiomyocyte hypertrophy.

Heart failure is a leading cause of morbidity and mortality in industrial countries, affecting over 10 million Americans and Western Europeans, with a 5-year mortality approaching 50% despite current medical therapy (1). These mortality figures reflect the lack of biologically efficacious therapies directed against the underlying disease processes that lead to maladaptive left ventricular remodeling and, ultimately, failure itself. In response to a plethora of intra- and extracardiac stimuli, cardiomyocytes exhibit cellular enlargement or hypertrophy as a compensatory adaptation to increased ventricular wall stress (2). However, sustained cardiac hypertrophy is the single most important risk factor for the development of heart failure (4, 5). Because intracellular signaling pathways are thought to both initiate and perpetuate the cardiac hypertrophic response and its transition to dilated failure, recent investigation has attempted the identification of key regulatory factors with the goal of defining novel therapeutic targets (3).

One recently characterized intracellular signaling pathway that links extracellular stimuli to a hypertrophic transcriptional response employs the phosphatase calcineurin and its downstream transcriptional effector nuclear factor of activated T-cells (NFAT).2 Four of the five NFAT proteins (NFATc1, NFAT2, or NFATc; NFATc2, NFAT1, or NFATp; NFATc3, NFAT4, or NFATx; and NFATc4 or NFAT3) reside in the cytoplasm in unstimulated cells but quickly translocate to the nucleus in response to stimulation that promote Ca2+-mobilization (6). The Ca2+-calmodulin-activated phosphatase calcineurin physically interacts with NFAT members within the cytoplasm, where it directly dephosphorylates multiple serine residues within the N-terminal regulatory domain of NFAT, resulting in the unmasking of two nuclear localization sequences required for nuclear import (7–9).

Calcineurin-NFAT signaling has been implicated as a critical regulator of the cardiomyocyte hypertrophic growth response. Molkentin et al. (10, 11) generated several lines of transgenic mice expressing activated mutants of either calcineurin or NFATc4 in a cardiac-selective manner, which developed robust hypertrophy that quickly transitioned to ventricular dilation and overt heart failure. The identification of calcineurin as a signaling factor has attracted considerable interest, in part due to the demonstration that the calcineurin inhibitory drugs cy-
closporin A and FK506 were shown to abrogate the cardiomyopathic response in several, but not all, rodent models of congenital and acquired forms of hypertrophic heart disease (reviewed in Refs. 12 and 13). A central role for calcineurin in the cardiac hypertrophic response was substantiated by the observation that hearts from transgenic mice expressing either MC1P1, a dominant negative calcineurin mutant, or the calcineurin inhibitor, the cyclophilin CypD, were largely resistant to pleiotropic, hypertrophic stimuli (14–16). More recently, calcineurin Aβ6 gene-targeted mice were generated and shown to be defective in mounting a cardiac hypertrophic response due to pressure overload or agonist infusion (17). Although a large number of studies have convincingly demonstrated the importance of calcineurin as a hypertrophic mediator, the importance of the downstream NFAT factors has not been evaluated in cardiomyocytes.

In the present study we demonstrate the presence of all four calcineurin-sensitive members of the NFAT family (NFATc1, -c2, -c3, and -c4) in the ventricular cardiomyocyte cell lineage. All four isoforms displayed calcineurin-dependent nuclear translocation and the ability to transactivate cardiac promoters. To simultaneously inhibit all myocardial NFAT factors in an effort to effectively examine their necessary function as hypertrophic transducers, a dominant negative NFAT strategy was developed. Dominant negative NFAT dose-dependently abrogated calcineurin-NFAT-dependent transactivation of MC1P1 and BNP promoter luciferase constructs. Adenoviral-mediated gene transfer of dominant negative NFAT in cultured cardiomyocytes efficiently inhibited calcineurin- and agonist-induced cardiomyocyte hypertrophy. Taken together, these data demonstrate a previously unexplored level of redundancy of the downstream targets of calcineurin and establish their requirement in pathophysiological signaling in the cardiomyocyte.

EXPERIMENTAL PROCEDURES

**Reporter Constructs and Expression Vectors**—Expression vectors containing a constitutively activated mutant of calcineurin Aβ6 (CnAβ6), NFATc1, -c2, -c3, or -c4 were described previously (10, 11). pEFBOS-HA-NFATp, a vector containing an N-terminal HA-tagged full-length murine NFATc2, was generously provided by Laurie Glimcher (Harvard, Boston, MA). pcG-GATA-4, a vector with full-length rat GATA-4 was generously provided by Antonso Moorman (Academic Medical Center, Amsterdam), pcDNA3-NFATc1/Ala (mut), an expression vector containing the first 130 N-terminal amino acids of human NFATc4 with the conserved PIIXIT box mutated to Ala residues (AXAXAXA) was described previously and a generous gift from Dr. Roger Davis (Harvard, Boston, MA). A constitutively activated FLAG-tagged NFATc3 clone was PCR-generated (fw, 5′-GGTGGCTGACGCCCTGCTTCC and rv, 5′-TTAGAAGCCCA TCACGTCTTCC) and lacks the first 315 N-terminal amino acids of the published human NFATc3/NFATx sequence (PubMed U85429). The isoform-specific probes were randomly selected from the published sequences (Refs. 12 and 13). A central role for calcineurin in the ventricular cardiomyocyte cell lineage of the murine ventriculocytes was analyzed by RT-PCR using primers specific for the individual NFAT isoforms as described before (22). Northern blot hybridizations on size-fractionated total RNA (10 μg) from indicated tissues were performed as described previously (22). To obtain probes specific for the NFAT isoforms, the mRNA sequences of murine NFATc1 through c4 were aligned (using ClustalW software) and primers designed for the 3′-untranslated regions showing no or minimal overlap (primers: NFATc1 fw, 5′-GGATGCTGAACCGTGAGGC and rv, 5′-GCCCAACCGACGTGGGTG; NFATc2 fw, 5′-ATGCTCTTGA TTAAATCAAGG and rv, 5′-TAACTGGAAGACGAAG; NFATc3 fw, 5′-GATGCTGAGGAGACACCTCC and rv, 5′-CAGTAAAGGCCTGCTTCC and rv, 5′-TCCTCCTTCTCC) were used to amplify the NFAT isoforms. The isoform-specific probes were randomly labeled with [32P]dCTP (E. I. du Pont de Nemours & Co, NV, Brussels, Belgium), added to the blots and incubated in Rapid Hyb hybridization solution (Amersham Biosciences) at 58 °C. Stringent post-hybridization wash conditions were used. Filters were exposed to phosphorimaging screens (Bio-Rad) and analyzed using Quantityl (Bio-Rad) and Adobe Photoshop 6.0 software. The intensity of the 1.8 S ribosomal RNA band detected with a radiolabeled 18 S probe was used as a quantitative control.

**Western Blot Analysis**—The method used is a minor modification of a recently described protocol (23, 24). In brief, protein extracts were lysed in ice-cold buffer (0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 2 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride (Sigma), 2 μg/ml soybean trypsin inhibitor). Protein concentration in lysates was determined using a protein dye assay (Bio-Rad) followed by separation on gradient gels (Bio-Rad), and transferred to polyvinylidene difluoride membrane (Bio-Rad). Filters were blocked for 1 h at room temperature using 10% nonfat dry milk dissolved in Tris-buffered saline with 0.1% Tween-20 (Sigma). Anti-NFAT antibodies included rabbit polyclonal anti-NFATc1 (Santa Cruz, H-110), mouse monoclonal anti-NFATc2 (Santa Cruz, 4G6-G5), rabbit polyclonal anti-NFATc3 (Santa Cruz, M75), rabbit polyclonal anti-NFATc4 (Santa Cruz, H-74), and mouse monoclonal anti-FLAG (Sigma, F-3185). Anti-NFATc1–c3 were diluted 1:200, and anti-NFATc4 was diluted 1:1 000 in blocking buffer (5% nonfat dry milk dissolved in Tris-buffered saline with 0.1% Tween-20). Membranes were incubated with primary antibodies overnight at 4 °C. Secondary antibodies included swine anti-rabbit peroxidase or rabbit anti-mouse peroxidase (DOKA, Denmark) and were used at a dilution of 1:2000 in blocking buffer and incubated for 2 h at room temperature.

Signals were detected with an Enhanced Chemiluminescence kit (ECL,
Amersham Biosciences) and analyzed using Adobe Photoshop 6.0 software.

**Generation of Recombinant, Replication-deficient Adenoviruses**—The adenovirus expressing β-galactosidase with a nuclear localization signal (Adβgal) was a generous gift from Mark Sussman (Children’s Hospital, Cincinnati, OH). The adenovirus expressing an activated mutant of calcineurin (AdCaN) was described and characterized previously (11, 24). AdNPAT-c4(IXMIIH) and AdNPAT-c4(Ala mut), replication-deficient adenoviruses expressing either FLAG-tagged NPAT-c4(IXMIIH) or NPAT-c4(Ala mut), were generated by subcloning PCR-amplified fragments (fw, 5′-CCAGAAGTGTGAAGC, rv, 5′-ATGATCATTATTCTA) and fw, 5′-AGCGGGCAGCCACATG; rv, 5′-GACATGTAGGACA-CTAT, respectively) as XbaI fragments into the adenoviral shuttle vector pACCMVlpA, using either pCEC-NPAT-c4(IXMIIH) or pCDNA-NPAT-c3(Ala mut) as templates. The recombinant shuttle vectors were cotransfected with pM17 in HEK 293 cells to produce initial recombinant adenoviruses. Procedures for plaque purification, expansion, and titrating the replication-deficient adenovirus and infection of cardiomyocytes were performed as described previously (11, 24).

Cardiomyocytes were infected with indicated adenoviruses at an m.o.i. of 100 for 2 h and cultured in serum-deficient medium with or without FGF2. Western blots were probed for 18 h to verify quality and equal loading of RNA (data not shown). Collectively, these data indicate that transcripts for all four calcineurin-regulated NFAT factors are present in cardiomyocytes, with those for NFATc3 and NFATc4 being present at the highest levels.

To verify whether these transcripts were also efficiently translated into their respective protein products, a series of Western blots were performed on total protein lysate from cultured neonatal rat ventriculocytes using isoform-specific antibodies. Tissue extracts of rat brain and rat thymus (data not shown) served as positive controls. Fig. 1C demonstrates that signals for all four calcineurin-regulated NFAT factors were obtained in cardiomyocytes, albeit at differing intensity, and with products ranging in mass from 70 to 200 kDa. For NFATc1 and NFATc2, discrete protein signals were observed, whereas for NFATc3 and NFATc4 multiple bands in the range of 100 to 200 kDa were observed. This may reflect generation of proteins by alternatively spliced transcripts (see Fig. 1B) and/or by differential phosphorylation states of the NFATc3 and NFATc4 proteins. Although the different affinities of the separate antibodies used do not allow for direct comparison of signal intensity, it is interesting to note that the relatively higher intensity of the protein signals for NFATc3 (Fig. 1C) correlates with its relatively high signal in the Northern blot analysis (Fig. 1B). Conclusively, RT-PCR, Northern blot, and Western blot analyses all point toward the existence of all four calcineurin-regulated NFAT isoforms in the ventricular cardiomyocyte.

**Nuclear Translocation of All Cardiac NFAT Isoforms upon Calcineurin Activation**—NFAT transcription factors are dephosphorylated upon activation of the Ca²⁺/CaM-dependent phosphatase calcineurin, resulting in unmasking of their nuclear localization signals permitting nuclear import. To verify that NFATc isoforms could be activated by calcineurin in cardiac myocytes, we performed immunocytochemistry for each NFAT factor at baseline or after infection with an adenovirus expressing a constitutively activated form of calcineurin (AdCnA) or after stimulation with Endo-1. Cardiomyocytes were 4′,6-diamidino-2-phenylindole-stained to visualize the nuclei and facilitate observation of nuclear localization (see **Fig. 2B, D, F, and H, left and right panels**). NFATc1, -c2, and -c3 were easily detectable using their respective antibodies and displayed a predominant cytosolic localization in unstimulated cardiomyocytes (Fig. 2A, left and right panels, NFATc1 and -c3, and data not shown for NFATc2). AdCnA infection resulted in nuclear accumulation of each NFAT isoform in nearly 100% of the myocytes evaluated. Stimulation with the agonist Endo-1 for 12 h resulted in efficient NFATc1 and c3 nuclear translocation in about 70% of cardiomyocytes (Fig. 2E, left and right panels). Similar findings were obtained for NFATc2 (data not shown). To control for the specificity of the antibodies used, the primary isoform-specific antibody was omitted, which resulted in background fluorescence (Fig. 2G, left and right panels). All cells examined were also positive for sarcomeric actin using phalloidin staining, thereby confirming their identity as cardiomyocytes (data not shown). These results indicate that NFATc1, -c2, and -c3 are equally sensitive to endogenous calcineurin activation as demonstrated previously for NFATc4 (10) and point toward a potential contri-
All Cardiac NFAT Isoforms Participate in MCIP1 Induction—To explore whether the calcineurin-mediated, nuclear import of the cardiac NFAT isoforms was associated with their ability to participate in transcriptional activity of cardiac-specific, calcineurin-responsive promoters, a series of transient cotransfection assays were carried out. Recently, a novel gene was characterized that is present at low levels under physiological conditions in the heart but undergoes dramatic up-regulation following calcineurin activation in the heart (15, 18, 25). Remarkably, the gene product itself is a highly specific inhibitor of calcineurin, and the gene was therefore designated myocyte-enriched calcineurin inhibitory protein-1 (MCIP1). It is thought that MCIP1 participates in a negative feedback loop to prevent the deleterious effects of unrestrained activation of the enzyme in the ventricular myocyte (15, 18, 25). Analysis of the gene structure revealed intron 3 to harbor multiple NFAT consensus sites and was found to be uniquely sensitive to calcineurin-NFAT activation (18). Cotransfection of hMCIP1-(Int3)Luc with expression vectors for the individual NFAT isoforms only slightly induced transcriptional activity (Fig. 3). Addition of a construct expressing a constitutively activated mutant of calcineurin (ΔCnA) increased this induction several-
fold for all isoforms, ranging from 3.5-fold for NFATc4 to over 20-fold for NFATc2 (Fig. 3). Taken together, these results suggest that all myocardial NFAT members are able to induce transcriptional activation of the human MCIP1 calcineurin-responsive enhancer region.

Synergistic Activation of the BNP Gene by NFAT and GATA4—It was previously demonstrated that the BNP gene promoter is regulated by a distal NFAT sequence element in cooperation with calcineurin and GATA4 (10). To assess whether the additional cardiac NFAT members are also capable of synergizing with GATA4 in regulating this promoter, the hBNP(1800)Luc reporter was tested in the presence or absence of GATA4, ΔCnA, and expression vectors for the individual NFAT isoforms. GATA4 alone markedly up-regulated hBNP(1800)Luc to about 17-fold over baseline (Fig. 4A), confirming the previously documented sensitivity of this gene to GATA factors (26, 27). Each individual NFAT isoform demonstrated relatively weak activation in the presence of ΔCnA (Fig. 4A). In contrast, cotransfection of any single NFAT isoform in the presence of ΔCnA and GATA4 resulted in robust up-regulation of hBNP(1800)Luc, ranging from 25- to 50-fold induction depending upon the NFAT member studied (Fig. 4A). These results indicate that all NFAT members can participate in synergistic activation of the BNP gene in conjunction with GATA4.

To test the specificity of this interaction and whether functional binding sites for either factor are required in the synergistic activation of the BNP reporter, a series of hBNP(1800)promoter-luciferase mutants were generated and examined. As a control, the activity of the wild-type hBNP(1800)Luc is shown at the left panel in Fig. 4B. The two GATA binding sites centered around ΔCnA and GATA4 resulted in robust up-regulation of hBNP(1800)Luc, ranging from 25- to 50-fold induction depending upon the NFAT member studied (Fig. 4A). These results indicate that all NFAT members can participate in synergistic activation of the BNP gene in conjunction with GATA4.

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NFAT transcription factor family are present in cardiomyocytes, a dominant inhibitory strategy was developed that targets NFAT activation. Such a strategy would bypass gene redundancy issues to permit evaluation of the role of NFAT as a cardiac responsive promoter, regardless of the NFAT isoform studied.

To test whether our dominant inhibitory NFATc4(PXXIXIT) construct was dependent upon the presence of an intact PXXIXIT box and to exclude issues regarding cytotoxicity, a similar N-terminal NFATc4 construct, NFATc4(Ala mut), was included in cotransfection assays. In this construct, the conserved PXXIXIT box residues were mutated to Ala residues to generate an AAXAXAA box, which is now ineffective in blocking calcineurin interaction. As anticipated, coexpression of this mutant construct displayed no inhibitory effect on NFAT-mediated induction of the hMCIP1(1nt3)/Luc reporter (Fig. 5B). These data indicate that the PXXIXIT box mediates the dominant negative action of our NFATc4(PXXIXIT) construct.

**Requirement of NFAT in Cardiomyocyte Hypertrophy**—To investigate the requirement of NFAT activation in calcineurin-mediated cardiomyocyte hypertrophy, we generated two replication-deficient adenoviral vectors expressing either the dominant negative N-terminal NFAT construct NFATc4(PXXIXIT) or the control construct NFATc4(Ala mut) under control of the CMV promoter (Fig. 6A). Infection of COS-7 cells with either AdNFATc4(PXXIXIT) or AdNFATc4(Ala mut) at an m.o.i. of 100 resulted in robust expression of polyepitope fragments of ~19 and ~16 kDa, respectively, which were easily detectable on the basis of their FLAG-immunoreactivity (lanes 2 and 3, Fig. 6B). Conversely, Ad2gag infection resulted in the absence of any proteins reactive for the anti-FLAG antibody (lane 1, Fig. 6B). Taken together, these results demonstrate that AdNFATc4(PXXIXIT) and AdNFATc4(Ala mut) are correctly expressed and should represent an effective way to inhibit NFAT activity in cultured neonatal cardiomyocytes.

Cardiomyocytes were first infected with the control adenovirus Ad2gag (Fig. 6C, panels A–C), AdNFATc4(PXXIXIT) (Fig. 6C, panels D–F), or AdNFATc4(Ala mut) (Fig. 6C, panels G–I). After 24 h, the cultured cells were then stimulated with the hypertrophic agonist cardiotrophin-1 (CT-1) or Endo-1 (data not shown), by infection with the activated calcineurin-expressing adenovirus, or left untreated for 24 h (Fig. 6C). The data demonstrate that only AdNFATc4(PXXIXIT) infection prevented cardiomyocyte hypertrophy in response to AdCnA or CT-1 (Fig. 6C, panels E and F). Adenoviral infection with either Ad2gag (Fig. 6C, panels B and C) or the control adenovirus AdNFATc4(Ala mut) (Fig. 6C, panels H and I) had no discernable effects on either AdCnA or agonist-induced sarcomeric deposition and cellular enlargement. Importantly, neither Ad2gag, AdNFATc4(PXXIXIT), nor AdNFATc4(Ala mut) infection induced cardiomyocyte apoptosis nor did it affect the morphology (Fig. 6C, panels J–L).
Counting of cardiomyocyte hypertrophy was performed by video edge detection on large numbers of myocytes (Fig. 6D). Serum-free (SF) cultured cardiomyocytes were found to have a similar cell surface area (1153 ± 208 vs. 1100 ± 87 μm²) as AdGal-infected, SF-cultured cardiomyocytes (1061 ± 112 μm²). In agreement with previous studies, AdCnA, CT-1, or Endo-1 treatment (20, 24) resulted in a more than 2-fold increase in cell surface area (2295 ± 112, 2274 ± 104, and 2072 ± 151 μm², respectively, p < 0.01 versus SF). Prior infection with AdGal and subsequent stimulation with AdCnA, CT-1, or Endo-1 resulted in comparable cardiomyocyte hypertrophy as previous SF-cultured myocytes (2080 ± 89, 2455 ± 155, and 2347 ± 118 μm², respectively, p < 0.01 versus SF). AdNFATc4(PXIXIT) infection completely abrogated the prohypertrophic effects of AdCnA, CT-1, or Endo-1 treatment to 1147 ± 52, 1193 ± 56, and 1184 ± 92 μm², respectively (p < 0.01 versus AdCnA, CT-1, and Endo-1, p = NS versus SF). In sharp contrast, AdNFATc4(Ala mut) infection prior to AdCnA infection or treatment with CT-1 or Endo-1 had no effects on the morphological alterations of these prohypertrophic stimuli (2384 ± 116, 2905 ± 224, and 2476 ± 171 μm², respectively, p = NS versus AdCnA, CT-1, or Endo-1). The data demonstrate that adenoviral dominant negative NFAT transfer was able to prevent the hypertrophic remodeling of cardiomyocytes following calcineurin activation.

Increased ANF expression is a hallmark of cardiac hypertrophy and is readily detected by immunocytochemistry as perinuclear staining (31, 32). Serum-free cultured cardiomyocytes infected with either AdGal, AdNFATc4(PXIXIT), or AdNFATc4(Ala mut) were stimulated with agonist or the activated calcineurin-expressing adenovirus and scored for the numbers of cells with perinuclear ANF expression (Fig. 6C). The data demonstrate that only AdNFATc4(PXIXIT) infection blocked ANF expression in response to the prohypertrophic stimuli investigated (Fig. 6C, panels E and F). Taken together, the results indicate that dominant negative NFAT abrogated...
Requirement of NFAT in Cardiomyocyte Hypertrophy

Fig. 6. Adenoviral-mediated gene transfer of dominant negative NFAT abrogates cardiomyocyte hypertrophy. A, schematic representation of dominant negative NFATc4(PIXXIT) and the control NFATc4(Ala mut) constructs expressed as adenoviral vectors. B, Western blot analysis using an anti-FLAG antibody on COS-7 cell lysates infected with either AdFlag, the dominant negative NFAT adenovirus AdNFATc4(PIXXIT), or the control virus AdNFATc4(Ala mut) at an m.o.i. of 100. C, representative images of immunostained cardiomyocytes infected with the indicated adenoviruses either or not in combination with the hypertrophic agonist CT-1. Phalloidin/ANF double staining demonstrates less cellular enlargement, sarcomeric organization, and perinuclear ANF staining in the presence of AdNFATc4(PIXXIT) following AdCnA infection or agonist stimulation. D, cell surface areas were quantified for each of the indicated conditions, demonstrating that inactivation of NFAT signaling abrogates cardiomyocyte hypertrophy in response to an activated calcineurin mutant or stimulation with Endo-1 or CT-1. Data in D represent the mean ± S.E. of three independent experiments. * p < 0.05 versus serum free conditions; † p < 0.05 versus AdFlag followed by AdCnA infection.

ANF expression following calcineurin activation or agonist stimulation in cultured cardiomyocytes.

DISCUSSION

Overlapping Expression of NFAT Isoforms in the Heart—One unexpected finding of the present study is that the ventricular cardiomyocyte population contains all four, calcineurin-sensitive NFAT isoforms described in the literature to date (reviewed in Refs. 6, 33, and 34). All NFAT members of the transcription factor family are expressed in multiple isoforms, generated by alternative splicing (35–38). The results in the present study support this notion (Fig. 1B). The existence of multiple splice isoforms has been shown in detail for NFATc2 and -c4 in T lymphocytes and other cells (35–37), and it has been shown that all spliced isoforms elicit transactivation of NFAT-responsive promoters, albeit with slightly differing efficiencies (38). The observation that cardiomyocytes express each of the four calcineurin-regulated NFAT family members, which themselves undergo differential splicing, justifies the dominant negative strategy employed here to inhibit NFAT-mediated transcriptional activation.

Although initially characterized in T-cells, almost all tissues in the mammalian organism express one or more NFAT family member. For example, NFATc2 is somewhat restricted in expression to immune cells and skeletal muscle, whereas NFATc3 expression is enriched in thymocytes and skeletal muscle cells but also present at lower levels in various other tissues. NFATc1 and NFATc4 appear to be expressed in a more ubiquitous pattern (6, 39–44), where they influence development, proliferation, and differentiation of a number of mammalian tissues (33, 45). The data in the present study confirm this ubiquitous expression pattern of NFAT members throughout several muscle types, supporting the function of the calcineurin-NFAT signaling pathway regulating cardiac hypertrophy, skeletal muscle myogenesis, and fiber-type specification, and smooth muscle cell proliferation and vessel remodeling (33, 45–50).

Crucial Role for NFAT Signaling in Cardiomyocyte Hypertrophy—The NFAT dominant inhibitory approach employed here specifically blocked the ability of calcineurin or agonist stimuli to promote nuclear accumulation and transcriptional activation of endogenous or overexpressed NFAT factors. Our approach utilized overexpression of the NFAT N-terminal calcineurin-docking domain containing the conserved sequence Pro-Xaa-Ile-Xaa-Ile-Thr (PIXXIT box) (51). It should be noted that NFATc4(PIXXIT), the dominant negative NFAT construct used in the present study, encompasses aa residues 3–191 of human NFATc4, whereas the “internal control” construct, NFATc4(Ala mut), was slightly shorter and encompasses residues 2–130 of human NFATc4. Although it would have been formally more correct to use dominant negative and control constructs of the same length, it is highly unlikely that the differing phenotypic effects observed between the dominant negative construct and the control construct may be due to this slight difference in length. Indeed, Chow et al. (28) have clearly demonstrated that the dominant inhibitory action of N-terminal portions of NFAT only depends upon the presence of the PIXXIT box, which encompasses residues 114–119 in human NFATc4, rendering the relative length of truncated NFAT constructs beyond residue 119 in this particular context irrelevant.

To address whether NFAT signaling is required for (calcineurin-mediated) cardiomyocyte hypertrophy, two model systems are routinely employed: cultured cardiomyocytes and genetically altered mice. In this study we employed adenoviral-mediated gene transfer in cultured cardiomyocytes to circumvent potential difficulties associated with gene targeting such as isoform redundancy or compensatory changes in gene ex-
pression. For example, gene targeting of individual NFAT family members did not reveal a widespread defect in the ability of T cells to proliferate or generate cytokines such as interleukin-2 (28, 52–55), even though the calcineurin-NFAT paradigm was established as a regulator of interleukin-2 gene transcription. Germaine to our study, Chow et al. (28) established the involvement of NFAT activity in regulating interleukin-2 expression using a similar dNFAT molecule. This dNFAT molecule selectively inhibited NFAT transcription activity by interfering with the activation-induced nuclear import of NFAT, and the active component of this inhibitor corresponds to the PXIXIT box located in the conserved N-terminal homology region of NFAT (28).

In addition to gene targeting, transgenesis in the mouse could be employed as a means of blocking NFAT activation through overexpression of the dominant negative NFAT protein domain in the heart. However, exhaustive attempts to generate dominant negative NFAT transgenic mice failed, presumably due to embryonic or early post-natal lethal effects associated with complete NFAT inhibition in the heart. Indeed, NFATc3 × NFATc4 double-null mice die during late embryogenesis with severe vascular abnormalities (47). Another documented approach to abrogating NFAT signaling is the use of kinases that directly phosphorylate NFAT transcription factors, thus antagonizing nuclear accumulation. For example, glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase with many targets, including at least two NFAT proteins (55). In addition, GSK-3 has been identified as a critical negative modulator of cardiomyocyte hypertrophy by directly antagonizing the prohypertrophic effects of activated calcineurin (52). Recently, GSK-3 was also proven to be capable of inhibiting hypertrophic signaling in the intact myocardium (53). Transgenic mice expressing a constitutively activated form of GSK-3β in cardiomyocytes displayed a severely blunted hypertrophic response to chronic β-adrenergic stimulation, pressure overload, and the actions of the calcineurin transgene (53). However, GSK-3β also inhibits GATA-4 function in cardiomyocytes (54), suggesting that GSK-3β likely also inhibits the hypertrophic response through NFAT-independent mechanisms. Nevertheless, we favor the interpretation that calcineurin-NFAT signaling is a dominant regulatory pathway for cardiac hypertrophy, and likely the general target underlying the anti-hypertrophic effect of GSK-3β. Indeed, cardiomyocytes infected with adenoviruses expressing truncated forms of either the calcineurin inhibitory protein Cain/cabin-1 or AKAP79 (20), which target and inhibit calcineurin itself (56–58), also showed a severe attenuation of myocyte hypertrophy in vitro. The combined observations suggest a pivotal role for calcineurin-NFAT signaling in cardiomyocyte hypertrophy.

**Cardiac NFAT Signaling: Functional Redundancy or Functional Specification?**—Although this study establishes that NFAT activity is required for both calcineurin as agonist-induced cardiomyocyte hypertrophy, the present data await extrapolation to the in vivo situation. As discussed above, we were unsuccessful in generating cardiac-specific transgenic mice expressing this dominant negative NFAT protein. These observations suggest that NFAT factors are crucial during developmental maturation of the myocardium. However, it is not known if all NFAT factors contribute to the myocyte growth response through a generalized mechanism or if individual isoforms play specific functions. For example, NFATc1 gene-targeted mice die during embryonic development due to defects in heart valve formation (59, 60). With respect to the adult heart and the regulation of hypertrophic growth, we have recently targeted the NFATc4 gene in the mouse. Surprisingly, NFATc4-null mice did not show a defect in their ability to mount a hypertrophic response (10, 61). By contrast, NFATc3-null mice did show a significant attenuation of hypertrophy following diverse stimuli (61). Collectively, these observations suggest that several NFAT isoforms might play critical regulatory roles in the adult myocardium. Indeed, here we observed that NFATc3 is abundantly present in ventricular myocytes (Fig. 1).

Alternatively, it is possible that certain NFAT factors are specified to fulfill various pathophysiological roles in the heart, in addition to or even excluding hypertrophic signaling. For example, we have demonstrated that adenoviral expression of NFATc4 rendered cardiomyocytes less susceptible to staurosporine or oxidative stress-induced apoptosis (11). Moreover, Kakita et al. (2) demonstrated that NFATc1 plays a crucial role in endothelin-1-mediated protection against oxidative stress-induced apoptosis in cardiomyocytes. Because NFATc4 apparently plays a minor role in cardiac hypertrophic signaling (61), yet signals a pro-survival phenotype (11) it is possible that certain NFAT factors have highly specified functions in the heart. To more firmly establish the functional hierarchy or potential inter-isofrom-specific roles between the individual myocardial NFAT members in the heart, generation of mouse models with loxp-flanked alleles for the four documented NFAT genes is warranted.

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