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Direct and Indirect Interactions between Calcineurin-NFAT and MEK1–Extracellular Signal-Regulated Kinase 1/2 Signaling Pathways Regulate Cardiac Gene Expression and Cellular Growth

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MEK1, a member of the mitogen-activated protein kinase (MAPK) cascade that directly activates extracellular signal-regulated kinase (ERK), induces cardiac hypertrophy in transgenic mice. Calcineurin is a calcium-regulated protein phosphatase that also functions as a positive regulator of cardiac hypertrophic growth through a direct mechanism involving activation of nuclear factor of activated T-cell (NFAT) transcription factors. Here we determined that calcineurin-NFAT and MEK1-ERK1/2 signaling pathways are interdependent in cardiomyocytes, where they directly coregulate the hypertrophic growth response. For example, genetic deletion of the *calcineurin Aβ* gene reduced the hypertrophic response elicited by an activated MEK1 transgene in the heart, while inhibition of calcineurin or NFAT in cultured neonatal cardiomyocytes also blunted the hypertrophic response driven by activated MEK1. Conversely, targeted inhibition of MEK1-ERK1/2 signaling in cultured cardiomyocytes attenuated the hypertrophic growth response directed by activated calcineurin. However, targeted inhibition of MEK1-ERK1/2 signaling did not directly affect calcineurin-NFAT activation, nor was MEK1-ERK1/2 activation altered by targeted inhibition of calcineurin-NFAT. Mechanistically, we show that MEK1-ERK1/2 signaling augments NFAT transcriptional activity independent of calcineurin, independent of changes in NFAT nuclear localization, and independent of alterations in NFAT transactivation potential. In contrast, MEK1-ERK1/2 signaling enhances NFAT-dependent gene expression through an indirect mechanism involving induction of cardiac AP-1 activity, which functions as a necessary NFAT-interacting partner. As a second mechanism, MEK1-ERK1/2 and calcineurin-NFAT proteins form a complex in cardiac myocytes, resulting in direct phosphorylation of NFATc3 within its C terminus. MEK1-ERK1/2-mediated phosphorylation of NFATc3 directly augmented its DNA binding activity, while inhibition of MEK1-ERK1/2 signaling reduced NFATc3 DNA binding activity. Collectively, these results indicate that calcineurin-NFAT and MEK1-ERK1/2 pathways constitute a codependent signaling module in cardiomyocytes that coordinately regulates the growth response through two distinct mechanisms.

The physiologic and pathological growth of the myocardium is typically initiated by membrane-bound receptors that promote intracellular signaling through multiple GTPase proteins, kinases, and phosphatases. One such regulator is the calcium-calmodulin-activated protein phosphatase calcineurin (PP2B) and its downstream transcriptional target, nuclear factor of activated T cells (NFAT). Calcineurin is a serine/threonine-specific phosphatase that is uniquely activated by sustained elevations in intracellular calcium (12, 16, 26). Calcineurin is comprised of a 59- to 63-kDa catalytic A (CnA) subunit and a 19-kDa calcium binding B (CnB) subunit. Three mammalian *CnA* catalytic genes (α, β, γ) and two *CnB* regulatory genes (*B1*, *B2*) have been identified in vertebrates. The *CnA*α, *A*β, and *B1* gene products are each expressed in a ubiquitous pattern throughout the body, while *CnA*γ and *B2* expression are more restricted to a smaller subset of tissues, such as brain and testis (9, 25, 36, 51). Once activated, calcineurin directly dephosphorylates NFAT transcription factors within the cytoplasm, promoting their translocation into the nucleus, where they participate in the transcriptional induction of various immune response genes in T lymphocytes as well as genes with diverse functions in other cell types (22). There are four calcineurin-regulated NFAT transcription factors, NFATc1 to NFATc4, each of which is expressed in the myocardium (56).

Calcineurin-NFAT was originally implicated as a hypertrophic signaling pathway based on expression of activated forms of each protein in the hearts of transgenic mice (two- to threefold increase in heart size) (33), a result also confirmed in primary cultures of neonatal cardiomyocytes (15). To address the necessary function of calcineurin-NFAT signaling in the heart, the noncompetitive calcineurin-inhibitory domains from the calcineurin-interacting proteins cain/Cabin-1 and AKAP79 were employed (11, 28, 49). Adenovirus expressing the inhibitory domains from these proteins blocked calcineurin activity and attenuated phenylephrine- and angiotensin II-induced hypertrophy in cultured cardiomyocytes (50). More recently, transgenic mice were generated that express the calcineurin-inhibitory domains of cain or AKAP79 (13). These transgenic

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mice demonstrated a significant reduction in pressure overload (aortic banding) and agonist-induced (isoproterenol infusion) cardiac hypertrophy (13). Calcineurin activity is also negatively regulated by the modulatory calcineurin-interacting proteins (MCIP/calcipressin/DSCR1/ZAKI-4), which are highly expressed in the heart (17, 44). Transgenic mice expressing the calcineurin-inhibitory domain from MCIP1 have also been recently characterized and shown to have reduced cardiac hypertrophy in response to stress stimulation (45). Transgenic mice expressing a dominant-negative (dn) mutant of calcineurin within the heart also showed reduced cardiac hypertrophy to pressure overload stimulation (61). Lastly, *CnA*β null mice were generated and shown to have a blunted cardiac hypertrophic growth response following pressure overload, isoproterenol infusion, or angiotensin II infusion (8).

The mitogen-activated protein kinase (MAPK) signaling pathways consists of a sequence of successively acting kinases that ultimately result in the dual phosphorylation and activation of terminal kinases, such as p38, c-Jun N-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERK) (48, 54). Once activated, p38, JNKs, and ERKs each phosphorylate a wide array of intracellular targets that include numerous transcription factors resulting in the reprogramming of cardiac gene expression as part of the hypertrophic program. Gain-of-function and loss-of-function analyses have directly implicated the MEK1-ERK1/2 pathway in regulating the cardiac growth response. For example, using antisense oligonucleotides, ERK signaling was shown to be necessary for effective phenylephrine-induced cardiomyocyte hypertrophy in culture (19). Similarly, the MEK1 inhibitor U0126 blocked both endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy in vitro (59). Adenoviral-mediated gene transfer of either a dominant-negative (dn) MEK1 or Raf-1dn cDNA into cultured neonatal cardiomyocytes also effectively blocked endothelin-1 and phenylephrine-induced hypertrophy (52, 59). With respect to gain-of-function approaches, transgenic mice were generated containing an activated MEK1 cDNA under the transcriptional control of the cardiac-specific α-myosin heavy chain promoter (6). These transgenic mice demonstrated a highly specific activation of only ERK1/2 in the heart (not p38 or JNK1/2) that was associated with pronounced cardiac hypertrophy, indicating that the MEK1-ERK1/2 signaling pathway is sufficient to induce cardiomyocyte growth in vivo.

Cross-talk between MEK1-ERK1/2 and calcineurin-NFAT signaling pathways has been suggested in the literature. For example, mice expressing the activated calcineurin transgene showed enhanced ERK1/2 activation in the heart (14), while isoproterenol stimulation of cardiac myocytes was shown to activate ERK1/2 signaling through a mechanism involving calcineurin (62). Antithetically, Ras activation through MEK1-ERK1/2 was associated with increased NFATc4 nuclear translocation and transcriptional activity in cardiac myocytes (23). This latter result is also consistent with an observation made in T lymphocytes whereby Vav signaling was coupled to Ras-MEK-ERK activation, which in turn promoted NFAT activation (53). Likewise, dominant-negative and constitutively active MEK1 mutants blocked and induced NFAT activation in T lymphocytes, respectively (18). These results suggest that MEK1-ERK1/2 signaling is capable of enhancing calcineurin-NFAT signals, although the mechanism underlying these observations remains largely uncharacterized. In contrast to studies showing synergy between calcineurin-NFAT and MEK1-ERK1/2 signaling pathways, ERK1 can also directly phosphorylate NFATc1 in vitro, resulting in inhibition of nuclear translocation and transcriptional activity in COS cells (41), suggesting a more complex level of regulation between these two pathways.

MATERIALS AND METHODS

Mice. Activated MEK1 transgenic mice under the control of the cardiac-specific α-myosin heavy chain promoter, *CnA*β gene-targeted mice, and NFAT-luciferase reporter transgenic mice have been previously described (6, 8, 55). The Institutional Animal Care and Use Committee approved all experiments involving animals.

Cell culture. Primary neonatal rat cardiomyocytes were prepared from hearts of 1- to 2-day-old Sprague-Dawley rat pups as previously described (14). After separation from fibroblasts, enriched cardiomyocytes were plated on 1% gelatin-coated 12-well plates for transfection and luciferase assays or on 6-cm-diameter dishes for all other experiments. Cells were grown in M199 medium containing 100 U of penicillin-streptomycin/ml and 2 mM L-glutamine without serum for 24 h before transfection or infection. Cardiomyocytes were transfected with Fugene-6 reagent (Roche Applied Sciences, Indianapolis, Ind.). Cultures were harvested 48 h after transfection or infection, and luciferase assays were preformed as described previously (30). Transfections employed a plasmid encoding β-galactosidase, from which enzymatic activity assays were performed to internally normalize luciferase activity data. At harvest, cells were resuspended in 50 μl of lysis buffer, and 20 μl was used for luciferase activity determination. Adenoviral infections were performed as previously described at a multiplicity of infection of 10 to 50 PFU per ml (14).

***CnB1* null fibroblasts.** Mouse embryonic fibroblasts (MEFs) were isolated at embryonic day 12.5 from *CnB1* flox/flox mice (37). MEFs were cultured according to the 3T3 protocol (300,000 cells per plate split every 3 days) until they reached the senescence crisis (~3 months). The spontaneously transformed clones that survived were pooled to avoid clone-specific artifacts and were infected with retroviruses encoding either green fluorescent protein (GFP)-Cre or GFP alone. Efficiency of infection was greater than 90%. Cells were sorted by fluorescence-activated cell sorting, and Cre-positive cells were collected, expanded, and used for further experimentation.

Adenoviral constructs and expression vectors. AdMEK1, AdMEK1dn, AdCain, AdΔCnA, AdNFATc3, AdNFAT-Luc, Adβgal, AdMKK6, AdMKK7, AdVIVIT, and AdNFATc1-GFP have been previously described (3, 5, 6, 29, 32, 42, 55). AdVIVIT was a gift of Susan D. Kraner and Chris Norris, University of Kentucky, Lexington, and AdNFATc1-GFP was a gift of Martin Schneider, University of Maryland. An MKP3 cDNA was subcloned into the pAC-CMVpLpA vector to generate AdMKP3 as described previously (14). AdTAM67 was a gift of Rosa Serra (University of Alabama—Birmingham). Reporter plasmids of atrial natriuretic factor (ANF)-luciferase (ANF-Luc) and Gal4-Luc were previously reported (27, 30). NFATc3 deletion domains were PCR amplified from human NFATc3 and cloned into the EcoRI and SalI sites of pM1 (Gal4-DNA binding domain-containing vector). The constitutively nuclear (Δ) NFATc3 adenovirus was made by cloning human NFATc3 from amino acids 400 to 1075 into the HindIII site of the pAC-CMVpLpA vector.

GST-NFATc3 fusion proteins. To generate fusion proteins between glutathione *S*-transferase (GST) and NFATc3, DNA sequence encoding amino acids 1 to 400, 400 to 700, or 700 to 1075 of NFATc3 was amplified by PCR and subcloned into pGEX-4T-1 (Amersham Pharmacia Biotech). All fusion proteins were expressed in *Escherichia coli* BL21 cells, precipitated with glutathione-Sepharose beads, and eluted with reduced glutathione (10 mM in 50 mM Tris, pH 8.0). The purity and concentration of each fusion protein were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using bovine serum albumin standards.

In vitro phosphorylation assays. In vitro phosphorylation of GST-NFATc3 fusion proteins was carried out at 30°C for 20 min using 25 ng of activated ERK2 (Upstate Biotechnology) in a buffer containing 20 mM morpholinepropanesulfonic acid (pH 7.2), 25 mM α-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol supplemented with 75 mM $MgCl_2$, 500 μM ATP, and 10 μCi of [γ-^{32}P]ATP. Five-hundred nanograms of each NFATc3 fusion protein or 10 μg of myelin basic protein (MBP) (positive control) was used to examine ERK2-induced phosphorylation. Reactions were separated by SDS-PAGE. Parallel phosphorylation reactions were performed in the ab-

sence of [γ-^{32}P]ATP and subjected to Coomassie brilliant blue staining as control.

Gel mobility shift assays. Gel mobility shift assays were performed using a double-stranded oligonucleotide containing NFAT motifs from the interleukin-4 (IL-4) promoter as previously described (33). Briefly, 30 μg of cardiomyocyte lysates was incubated with 50,000 cpm of ^{32}P-labeled double-stranded oligonucleotide, 1 μg of poly(dI-dC)-(dI-dC), and buffer (12 mM HEPES [pH 7.9], 4 mM Tris [pH 7.9], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, and 2 μg of aprotinin, leupeptin, and pepstatin/ml) at room temperature for 20 min in a 20-μl volume. A nondenaturing 5% polyacrylamide gel with 0.5× Tris-borate-EDTA (TBE) was used to resolve the bound protein complexes from the free probe.

Immunoprecipitation assays. Neonatal rat cardiomyocytes were infected with or without specific adenoviruses 48 h prior to generation of protein lysates, or adult mouse hearts were used to generate protein lysates (from wild-type or the indicated transgenic lines). Cells or tissue were harvested in lysis buffer as previously described and then incubated with the indicated antibodies and protein A-Sepharose beads in binding buffer overnight at 4°C (30, 42). The beads were washed extensively with binding buffer, and the proteins were resolved on an SDS–7.5% PAGE for subsequent Western blotting with the indicated antibodies. Immunoprecipitation from heart extracts consisted of 500 μg of protein as starting material.

Immunoblotting analysis. The generation of protein extracts from cultured cardiomyocytes or heart tissue and their subsequent immunoblotting has been described previously (14). Antibodies included MEK1 (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.), phospho-ERK1/2, ERK1/2, phospho-Elk-1, Elk-1, phospho-AKT (Cell Signaling Technology, Beverly, Mass.), GAPDH (Research Diagnostics Inc., Flanders, N.J.), calcineurin B1 (Sigma, St. Louis, Mo.), and pan-calcineurin A (Chemicon International, Inc., Temecula, Calif.).

Hypertrophy assessment in neonatal rat cardiomyocytes. For calculation of cell sizes, neonatal rat cardiomyocytes were infected with the indicated adenoviruses for 48 h. Cardiomyocytes were visualized with a 1:400 dilution of tetramethyl rhodamine isocyanate (TRITC)-conjugated phalloidin. Surface area was determined with the image analysis software NIH 1.63. Cells from randomly selected fields in three independent experiments were examined, and the surface area was compared to that of control infected cells (400 cells each).

Cardiac hypertrophy measurements in vivo. Mice from all genotypes were anesthetized with isoflurane, and echocardiography was performed using a Hewlett Packard 5500 instrument with a 15-MHz microprobe. Echocardiographic measurements were taken on M mode in triplicate from four or five separate mice per group. Hearts were collected at the indicated times, fixed in 3.7% formaldehyde containing phosphate-buffered saline (PBS), and embedded in paraffin. Serial 7-μm heart sections from each group were analyzed. Samples were stained with hematoxylin and eosin, Masson's trichrome, or wheat germ agglutinin-TRITC conjugate at 50 μg/ml to accurately identify sarcolemmal membranes so that cellular diameters could be quantified.

Statistical analysis. The results are presented as means ± standard errors of the means. Data analyses were performed using InStat 3.0 software (GraphPad Software for Science, Inc., San Diego, Calif.).

RESULTS

MEK1 increases NFAT transcriptional activity. In vivo, p38 and JNK members of the MAPK superfamily have been proposed to exert an antihypertrophic effect in the heart through a mechanism involving direct phosphorylation of NFAT transcription factors, thus antagonizing NFAT activity and the subsequent growth response (5, 29). However, activation of the ERK branch of the MAPK family likely plays a distinct role, given the observation that activated MEK1, which specifically activates ERK1/2, actually produces a prominent cardiac growth response in vivo (6). Here we sought to determine the manner in which MEK1-ERK1/2 signaling enhances the cardiac growth response and the potential effects on calcineurin-NFAT activation.

To investigate cross-talk between calcineurin-NFAT and MEK1-ERK1/2 signaling, we first employed adenovirus-mediated gene transfer of activated MEK1 in neonatal rat ventricular cardiomyocytes together with an NFAT-specific reporter

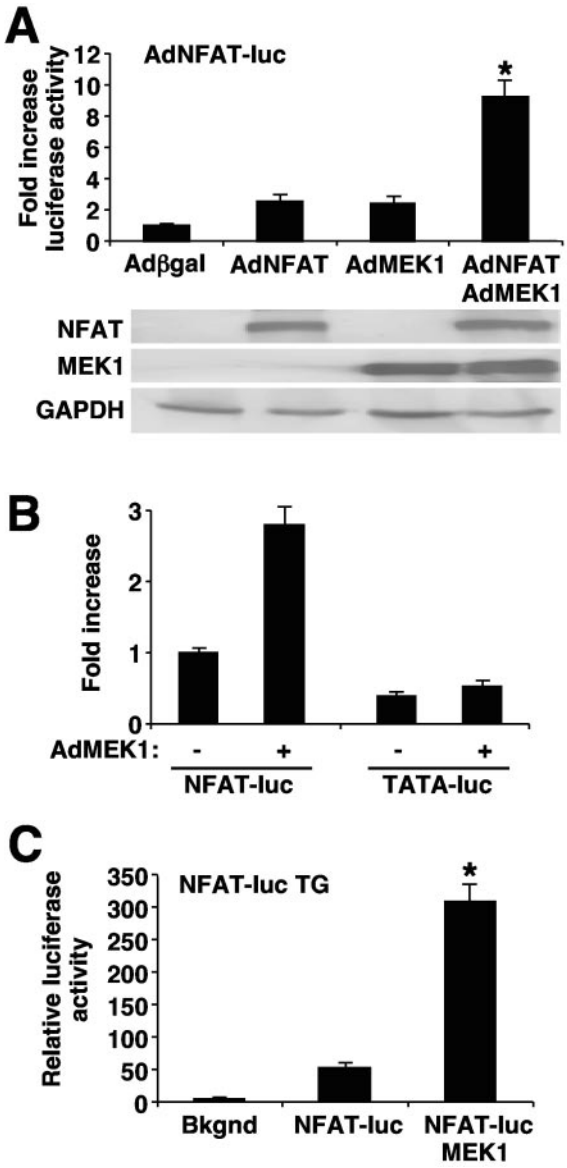

FIG. 1. MEK1 induces NFAT transcriptional activity. (A) Rat neonatal ventricular cardiomyocytes were plated in serum-free medium and were infected 48 h after plating with AdNFAT-luc together with AdNFATc3, AdMEK1, or Adβgal adenovirus. Cell extracts were collected after 48 h, and luciferase assays were performed (*, $P < 0.05$ versus AdMEK1). (B) Neonatal cardiomyocytes were infected with AdMEK1, and after 2 h they were transfected with either a plasmid containing NFAT-luc or an identical construct lacking the NFAT binding sites (TATA-luc). (C) NFAT-luc transgenic ($n = 3$) and NFAT-luc-MEK1 double transgenic ($n = 3$) mice were sacrificed at 6 weeks of age. Wild-type mice were used as background (Bkgnd) controls ($n = 3$) (*, $P < 0.05$ versus NFAT-luc transgenic). Each point was performed in triplicate, and the graph is representative of more than three experiments (A and C).

(55). Expression of activated MEK1 was previously shown to only activate ERK1/2 and not p38 or JNK1/2 (6). Singular expression of activated MEK1 or full-length NFATc3 each induced an approximately twofold increase in NFAT transcriptional activity (Fig. 1A). However, coinfection of activated MEK1 with NFATc3 induced a synergistic increase in NFAT

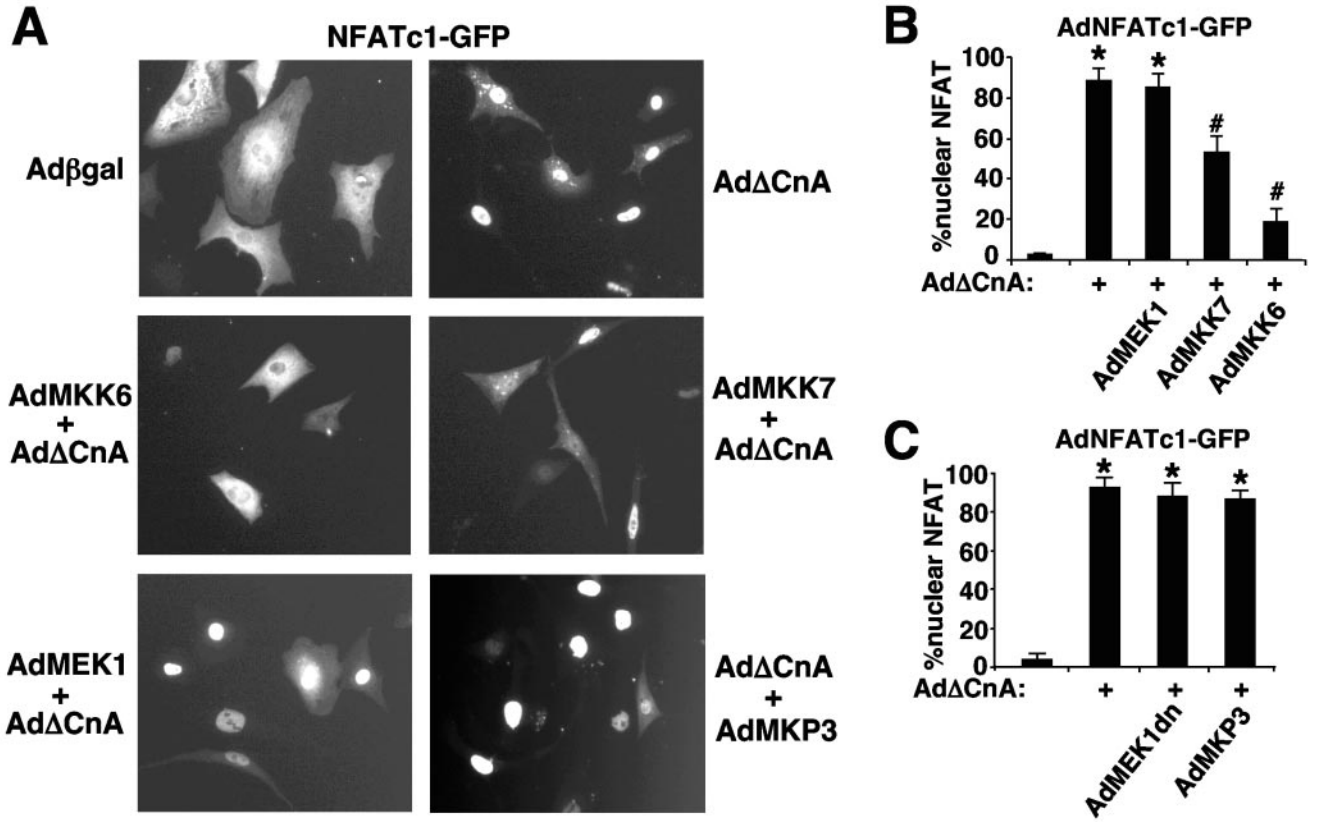

FIG. 2. MEK1 does not affect NFAT nuclear localization. (A) Neonatal cardiomyocytes were infected with AdNFATc1-GFP and the indicated recombinant adenoviruses. After 48 h, cells were fixed and photographed. (B and C) Nuclear localization of NFATc1 was quantified by counting four randomly selected fields containing ~100 cells (400 cells for each experimental point). Cells were considered positive when they essentially showed exclusive nuclear localization. The pictures and graphs are representative of three independent experiments (*, $P < 0.05$ versus AdNFATc1-GFP alone; #, $P < 0.05$ versus AdNFATc1-GFP plus AdΔCnA).

transcriptional activity (~10-fold) (Fig. 1A). Coinfection between AdNFAT and AdMEK1 did not reduce expression of either construct, as measured by Western blotting of the same samples, indicating specific synergy (Fig. 1A, lower panel). An identical reporter that simply lacked the nine multimerized NFAT sites but contained the same minimal promoter region failed to show MEK1-inducible expression, indicating that the observed synergy was mediated through the NFAT sites (Fig. 1B). Moreover, coexpression of MEK1 with NFAT also failed to significantly induce the minimal TATA-luciferase reporter, further validating the specificity of the observed effect (data not shown). To extend our observations within the adult heart, a potentially more physiologic setting, we crossed mice containing the cardiac-specific activated MEK1 transgene with mice containing the NFAT-luciferase reporter transgene (the same reporter construct used in the cell culture experiments). At 6 weeks of age, double transgenic mice showed a significant increase in NFAT-luciferase activity in the heart compared to that of single-NFAT-luciferase transgenic mice ($P < 0.05$) (Fig. 1C). Collectively, these results suggest that MEK1-ERK1/2 signaling specifically enhances NFAT transcriptional activity in vitro and in vivo.

To investigate the potential mechanisms whereby MEK1-ERK1/2 signaling might alter NFAT transcriptional activity in cardiac myocytes, we first investigated NFAT subcellular localization. We have previously observed that JNK and p38 activation directly antagonizes NFAT nuclear localization in cardiomyocytes through a mechanism involving phosphorylation of the N-terminal regulatory domain in NFAT proteins (5, 29). Of interest, ERK1 was also reported to directly phosphorylate NFATc1 in COS cells, similarly inhibiting its activity and nuclear localization (41). However, in cardiomyocytes infected with an adenovirus encoding an NFATc1-GFP fusion protein, MEK1-ERK1/2 activation did not alter calcineurin-induced NFATc1 nuclear shuttling, in contrast to the prominent inhibitory effect associated with p38 (AdMKK6) and JNK1/2 (AdMKK7) activation (Fig. 2A and B). Specifically, coinfection of AdNFATc1-GFP with an adenovirus expressing activated calcineurin (AdΔCnA) or activated calcineurin with activated MEK1 (AdMEK1) resulted in 89 and 84% of cardiomyocytes containing nuclear NFATc1-GFP, respectively ($P < 0.05$). In contrast, coinfection with AdMKK7 or AdMKK6 resulted in only 53 and 19% of myocytes with nuclear localization of NFATc1-GFP, representing a significant reduction in shuttling ($P < 0.05$). A nearly identical profile was also observed in cardiomyocytes infected with AdNFATc3-GFP in conjunction with AdMEK1, AdMKK7, and AdMKK6 (data not shown). We also observed that inhibition of MEK1-ERK1/2 signaling in cardiomyocytes, using either MEK1dn or the ERK1/2 specific dual-specificity phos-

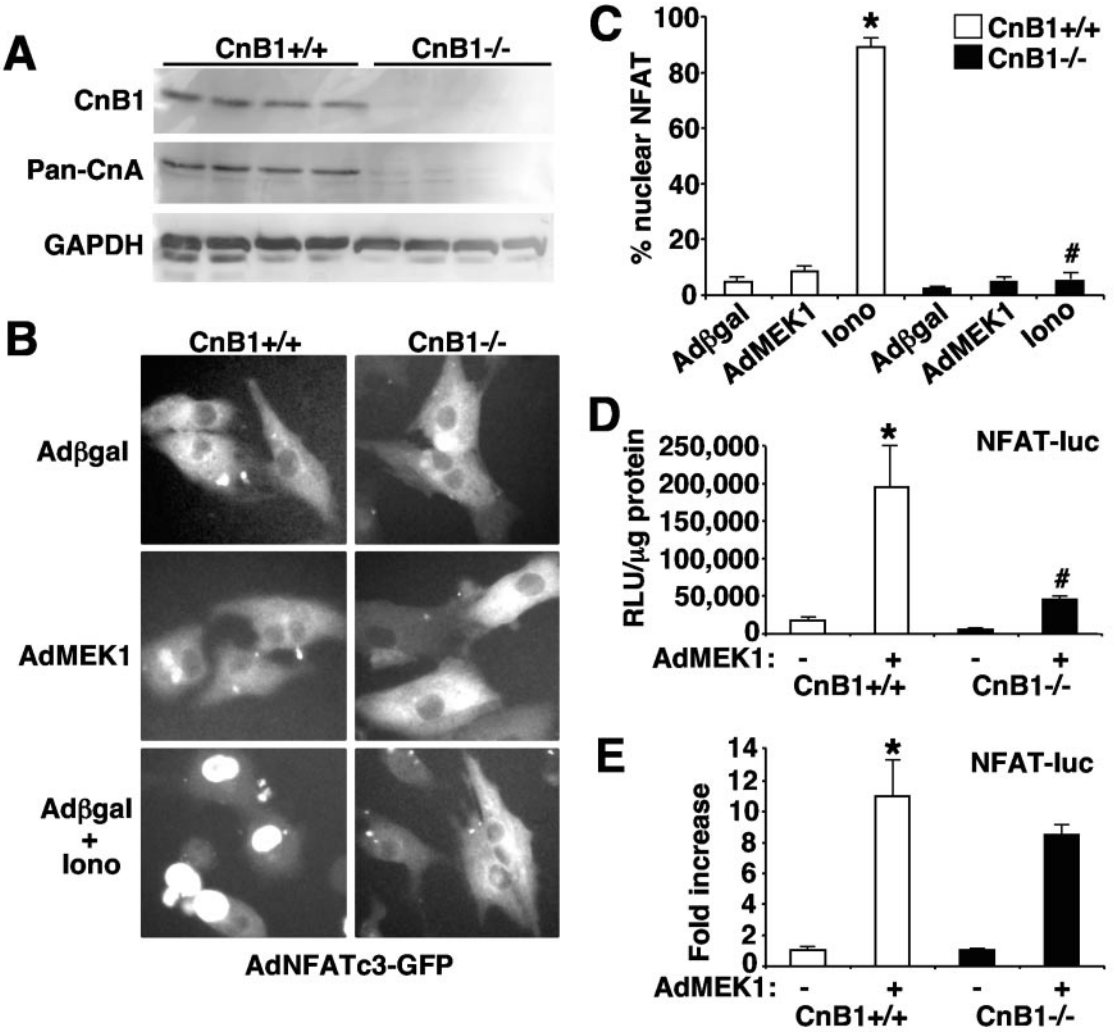

FIG. 3. MEK1-induced NFAT transcriptional activity is independent of calcineurin. (A) *CnB1*-deleted mouse embryonic fibroblasts were obtained as described in Materials and Methods. Cell extracts were analyzed by Western blotting with specific antibodies for CnB1. The same membrane was stripped and reprobed with antibodies for pan-CnA and GAPDH. (B and C) $CnB1^{+/+}$ and $CnB1^{-/-}$ MEFs were infected with AdNFATc3-GFP, with or without AdMEK1, and treated with either ionomycin (iono) (1 μM) or solvent (dimethyl sulfoxide) for 30 min. MEFs were fixed and analyzed for NFAT nuclear localization (∗, $P < 0.05$ versus Adβgal in $CnB^{+/+}$ MEFs; #, $P < 0.05$ versus ionomycin in $CnB1^{+/+}$ MEFs). (D) $CnB1^{+/+}$ and $CnB1^{-/-}$ MEFs were infected with AdNFAT-luc in the presence or absence of AdMEK1. Luciferase data are expressed as total relative light units (RLU)/microgram of protein normalized internally to a β-galactosidase expression vector (E) or as fold increase compared to their controls. Each point was performed in triplicate, and the graph is representative of at least three experiments (∗, $P < 0.05$ for $CnB1^{+/+}$ AdMEK1 versus $CnB1^{+/+}$ Adβgal; #, $P < 0.05$ for $CnB1^{-/-}$ AdMEK1 versus $CnB1^{+/+}$ AdMEK1).

phatase MAPK phosphatase 3 (MKP3), had no effect on calcineurin-induced NFAT nuclear translocation (Fig. 2C). Similarly, ionomycin-induced NFAT translocation was unaffected by MEK1-ERK1/2 inhibition (data not shown). Collectively, these results indicate that MEK1-ERK1/2 signaling does not significantly oppose or promote calcineurin-NFAT activation or NFAT nuclear localization in cardiomyocytes.

MEK1-induced NFAT transcriptional synergy is calcineurin independent. NFAT activity is typically regulated by calcineurin-mediated dephosphorylation within the cytoplasm, resulting in nuclear translocation and the activation of NFAT-responsive genes. However, it remains formally possible that NFAT activation could be achieved, in part, through one or more calcineurin-independent mechanisms. To gain further insight into the potential mechanisms whereby MEK1-ERK1/2 signaling might enhance NFAT transcriptional responsiveness, we employed a unique cellular model that lacks calcineurin protein. Specifically, mouse embryonic fibroblasts were isolated from homozygous *CnB1 loxP*-targeted embryos (37) and subjected to virus-mediated transfer of Cre to inactivate the locus in all cells. Western blotting confirmed a greater than 98% loss of CnB1 protein (Fig. 3A). Interestingly, loss of CnB1 promoted a destabilization and loss of nearly all CnA protein, similar to a recent observation made with skeletal muscle (38).

Using calcineurin-deleted MEFs, we examined if MEK1-induced NFAT activity was completely dependent on calcineurin. Wild-type control MEFs and *CnB1* null MEFs were infected with AdNFATc1-GFP and stimulated with either MEK1 or ionomycin (Fig. 3B). In control cells, ionomycin treatment resulted in a robust translocation of NFATc1 to the

nucleus ($P < 0.05$), while *CnB1* null cells were completely refractory in NFATc1 translocation ($P < 0.05$) (Fig. 3C). This result confirms that calcineurin is the primary positive regulator of NFAT translocation in mammalian cells. With respect to MEK1-ERK1/2 signaling, NFAT nuclear localization did not significantly change upon MEK1 activation in wild-type (8.3% nuclear compared to 4.7% control) or *CnB1* null MEFs (4.7% nuclear compared to 2.3% control), suggesting that MEK1-ERK1/2 signaling alters NFAT activation independent of calcineurin and nuclear translocation of NFAT.

To further examine the potential mechanism whereby MEK1-ERK1/2 signaling positively influences NFAT transcriptional activation, *CnB1* null and wild-type MEFs were infected with an adenovirus containing the NFAT-luciferase reporter cassette. As anticipated, loss of calcineurin led to a decrease in NFAT transcriptional activation in both control MEFs and MEFs infected with AdMEK1 (Fig. 3D). However, if the data are normalized to the reduction in baseline activation without stimulation, there are no significant differences between wild-type and *CnB1* null MEFs in fold NFAT activation (11-fold increase in wild-type compared to ~9-fold increase in *CnB1* null MEFs) (Fig. 3E). This observation suggests that while the absolute magnitude of MEK1-induced NFAT activity is strictly dependent on NFAT nuclear content regulated by calcineurin, MEK1-ERK1/2 signaling can still enhance activation of the limited fraction of NFAT contained within the nucleus, thus suggesting a more proximal transcriptional mechanism of action. Similar overall results were observed in wild-type and *CnB1* null MEFs infected with AdNFATc3-GFP (data not shown).

MEK1-ERK1/2 signaling does not directly regulate NFAT transactivation. The transcriptional potency of NFAT factors was previously shown to be regulated by protein kinase Cζ (PKCζ)-mediated phosphorylation within the transcriptional activation domain of NFATc1-c2 (46), suggesting a possible mechanism whereby MEK1-ERK1/2 signaling might also augment NFAT activity once in the nucleus. To examine this potential mechanism, a series of Gal4 DNA binding domain fusion constructs was generated consisting of three consecutive domains that encompass the entire NFATc3 protein (Fig. 4A). Cultured cardiomyocytes were transfected with a Gal4-dependent luciferase reporter plasmid together with each of the Gal4-NFATc3 fusion vectors in the presence or absence of activated MEK1. Even though the N1 and N3 regions of NFATc3 contained a transcriptional activation domain (TAD), neither was augmented by MEK1-ERK1/2 signaling (Fig. 4B). These results suggest that MEK1-ERK1/2 signaling does not enhance transcriptional activity within the nucleus through an effect on the transactivation domains of NFATc3.

MEK1-ERK1/2 synergy with NFAT depends, in part, on AP-1. In general, increased transcriptional activity can be achieved through a mechanism involving direct cooperation between dissimilar transcription factors that together form large complexes that more efficiently recruit basal transcriptional machinery. With regard to NFAT factors, AP-1 has been previously shown to function as a critical transcriptional cofactor involved in the induction of immune response genes (22). While the NFAT site used in our multimerized reporter construct does not directly bind AP-1 (NFAT-only site from the interleukin-4 promoter), this site can still be modulated by AP-1 through a protein-protein interaction (43). Thus, we

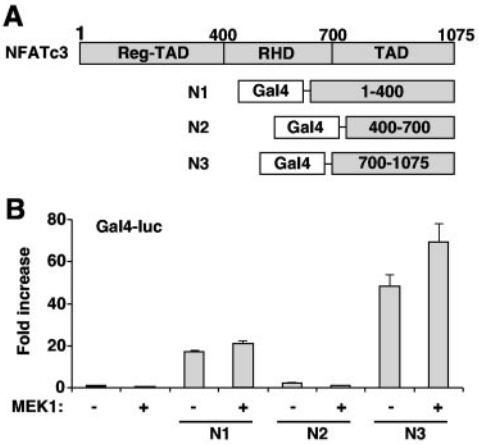

FIG. 4. MEK1 does not increase NFAT transactivation potential. (A) Schematic representation of NFATc3 and the different Gal4 fusion proteins that were generated. (B) A Gal4-dependent luciferase reporter construct (1.0 μg) was cotransfected into neonatal cardiomyocytes with expression vectors (0.25 μg) encoding Gal4DBD-NFATc3 N1-N3 fusion mutants with or without a plasmid encoding constitutively active MEK1 (0.25 μg). After 48 h, luciferase assays were performed. Each point was performed in triplicate, and the graph is representative of at least three experiments.

hypothesized that MEK1-ERK1/2 signaling might enhance NFAT transcriptional potency through an interacting cofactor that is regulated by ERK1/2 phosphorylation, such as AP-1. To investigate this possibility, we transfected cardiomyocytes with the NFAT-luciferase reporter plasmid, plasmids encoding activated MEK1, constitutively nuclear NFATc3, and a cJun-dn (TAM67, which blocks AP-1 [1]). A constitutively active mutant of NFATc3 was used so as to eliminate any minor influence associated with nuclear content of NFAT. The data show that MEK1 still induced a further approximately twofold activation of the NFAT-luciferase reporter in conjunction with constitutively active NFATc3 (Fig. 5A). However, cotransfection with the AP-1-dn expression plasmid (TAM67) largely blocked MEK1-induced NFATc3 activation (Fig. 5A). As an important control, we also assessed AP-1 activity itself by using an AP-1-dependent luciferase reporter plasmid. MEK1 cotransfection induced AP-1 activity ~3.3-fold, and this activation was completely blocked by TAM67, confirming the specificity of the assay (Fig. 5B). MEK1-induced activation of AP-1 was also blocked with the MEK1 inhibitor U0126 and by overexpression of MKP3 (Fig. 5B), confirming that MEK1 directly activates AP-1 through ERK1/2, as previously demonstrated in fibroblasts (34).

MEK1-ERK1/2 directly regulates NFAT DNA binding activity. While activation of AP-1 serves as an important mechanism for augmenting NFAT-dependent transcription downstream of MEK1-ERK1/2 signaling, we were also interested in examining other potential mechanisms of action. Here we observed that NFATc3 DNA binding activity could be altered in coordination with MEK1-ERK1/2 signaling. Cultured cardiomyocytes were infected with an adenovirus encoding the constitutively nuclear mutant of NFATc3 (amino acids 400 to 1075, lacking the N-terminal regulatory domain) in conjunction with adenoviruses that affected MEK1-ERK1/2 signaling. Protein extracts were generated, and an NFAT-specific gel

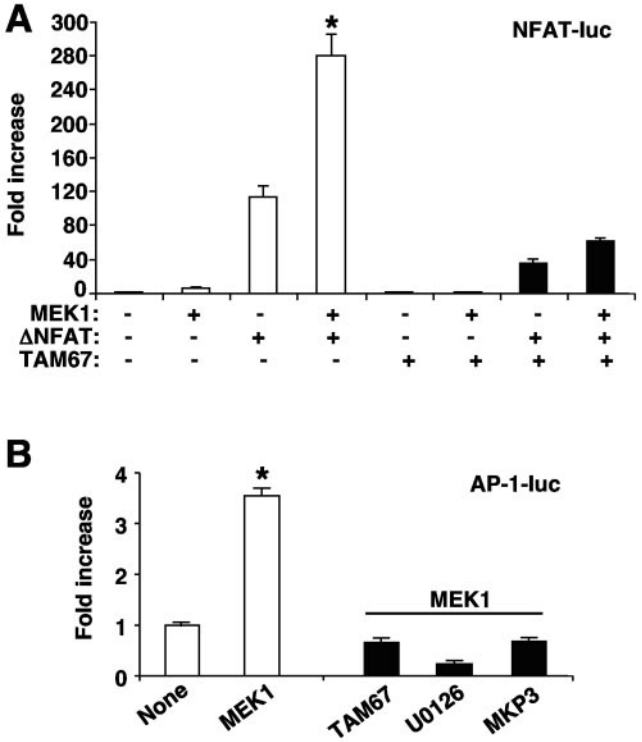

FIG. 5. MEK1 activates NFAT through AP-1 signaling. (A) Neonatal cardiomyocytes were infected with AdNFAT-luc to assay for NFAT transcriptional activity, together with an adenovirus encoding constitutively active NFATc3 (ΔNFAT) in the presence or absence of AdMEK1. The same experimental design was repeated in the presence of an AP-1 dominant-negative TAM67-expressing adenovirus (*, $P < 0.05$ for MEK1/caNFAT versus caNFAT). (B) Cardiomyocytes were infected with the indicated viruses, and after 2 h they were transfected with an AP-1-luciferase reporter vector. After 48 h, luciferase assays were performed. Each point was performed in triplicate, and the graph is representative of at least three experiments. U0126 (20 μM) was added 6 h before harvesting the cells.

mobility shift assay was performed. The data demonstrate that ERK1/2 activation reproducibly augmented ΔNFATc3 DNA binding activity by approximately twofold (Fig. 6A). More remarkably, inhibition of ERK1/2 signaling with MEK1dn or MKP3 dramatically attenuated ΔNFATc3 DNA binding without an alteration in NFAT protein expression, as assessed by Western blotting (Fig. 6A). Identical results were obtained in two additional independent experiments.

The dramatic and consistent alteration in NFATc3 DNA binding activity in association with altered ERK1/2 activity suggested a more direct regulatory relationship, such as might be mediated by direct phosphorylation. Indeed, using bacteria-purified GST-NFATc3 fragments that span the entire protein, in conjunction with bacterially purified and activated ERK2, a direct phosphorylation event was observed on both the N (amino acids 1 to 400) and C termini (amino acids 700 to 1075) of NFATc3 but not the region spanning the rel homology domain that mediates DNA binding (amino acids 400 to 700), an observation that is consistent with a lack of a MAPK consensus binding site in this region (Fig. 6B). Similarly, immunoprecipitation of total ERK2 protein from cardiomyocyte protein lysates, which would include any associated accessory kinases in addition to ERK2, also failed to show phosphorylation of the rel homology domain of NFATc3, while the C terminus was robustly phosphorylated (data not shown). That the N terminus of NFATc3 was phosphorylated by ERK2 was not unexpected, given the known enrichment of kinase-interacting motifs and phosphate acceptor sites in this domain. However, because this N-terminal regulatory region of NFATc3 was not included in the DNA binding assays presented above,

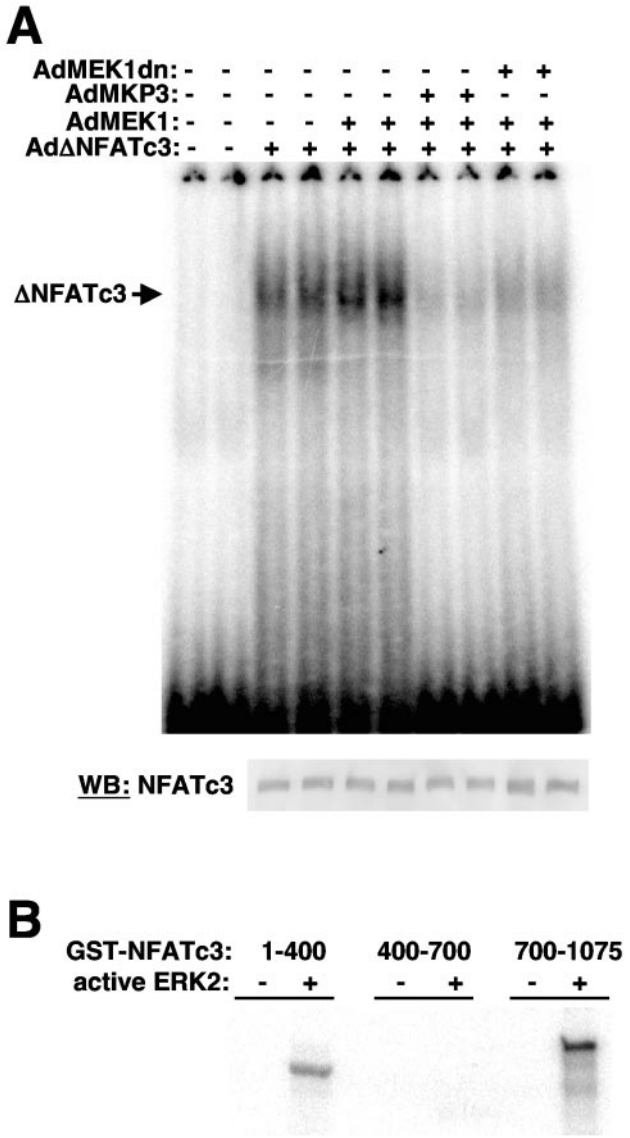

FIG. 6. NFATc3 DNA binding activity is regulated by ERK1/2. (A) Gel mobility shift assay with an NFAT-specific DNA binding site from the interleukin-4 promoter, incubated with cardiomyocyte protein extracts generated from cells infected 24 h prior with the indicated adenoviruses. The lower panel displays a control Western blot (WB) for the activated NFATc3 mutant protein that was overexpressed in each of the indicated reactions. (B) SDS-PAGE of an in vitro kinase reaction between bacterially purified and activated ERK2 and the indicated GST-NFAT fusion protein fragments, also generated in bacteria. Equivalent amounts of protein were loaded in all reactions as assessed by Coomassie brilliant blue staining (data not shown). Identical results were obtained in three independent experiments.

its suggested that phosphorylation within the C terminus of NFATc3 might be responsible for modulating DNA binding activity. That phosphorylation outside the DNA binding domain itself can alter the affinity of a transcription factor for DNA was previously suggested for serum response factor (SRF) (24). Collectively, these results suggest that MEK1-ERK1/2 signaling regulates NFAT transcriptional activation through a mechanism involving direct phosphorylation of NFATc3, which affects DNA binding activity.

Calcineurin-NFAT and MEK1-ERK2 form a complex in cardiomyocytes. The observation that ERK2 directly phosphorylates NFATc3 in vitro suggested that these factors might form a stable complex in vivo. To address this issue a series of coimmunoprecipitation experiments were performed from cardiomyocytes infected with combinations of recombinant adenoviruses encoding CnA, NFATc3, activated MEK1, or wild-type ERK2. Not all combinations were performed, given that CnA is known to directly interact with NFAT transcription factors and that MEK1 is known to directly interact with ERK1/2. Here we observed that immunoprecipitation of CnA brought down ERK2 or MEK1 from cardiomyocytes overexpressing each factor (Fig. 7A). As a control, equivalent amounts of CnA protein was immunoprecipitated in those reactions containing overexpressed CnA (Fig. 7A). As expected, immunoprecipitation of CnA specifically identified NFATc3 as an interacting partner, while immunoprecipitation of ERK2 resulted in the specific isolation of NFATc3 when both proteins were overexpressed (Fig. 7B). Finally, these observations in adenovirus-infected cardiomyocytes were extended to the adult heart by performing immunoprecipitation for endogenous CnA from transgenic mice overexpressing wild-type ERK2 or mice expressing the combination of activated MEK1 with wild-type ERK2 (Fig. 7C). The data demonstrate that endogenous CnA forms a complex with ERK2 in the adult heart and that activation of ERK2 with MEK1 results in even greater complex formation (Fig. 7C). Collectively, these results support the contention that MEK1-ERK1/2 signaling enhances NFAT transcriptional potency through a direct mechanism involving the prior formation of a multisubunit complex, which could then alter the DNA binding characteristics of NFAT.

The calcineurin-NFAT pathway is necessary for MEK1-induced hypertrophy in vitro. The results discussed above showed two distinct mechanisms of transcriptional interdependence between MEK1-ERK1/2 and calcineurin-NFAT signaling in cardiomyocytes, although the biological effects associated with this interdependence were uncertain. To evaluate functional significance, cultured neonatal cardiomyocytes were used to model aspects of the cardiac hypertrophic growth program. As previously reported, expression of activated MEK1 by adenovirus-mediated gene transfer induced a prominent hypertrophic response compared to that of control myocytes infected with a β-galactosidase-expressing adenovirus (Fig. 8A and B). In contrast, coexpression of the calcineurin inhibitory protein domain from cain or the NFAT-selective inhibitory peptide VIVIT each significantly reduced MEK1-induced cardiomyocyte growth (Fig. 8A and B). Expression of cain or VIVIT alone did not compromise basal cardiomyocyte health or otherwise induce apoptosis (Fig. 8A and B and data not shown).

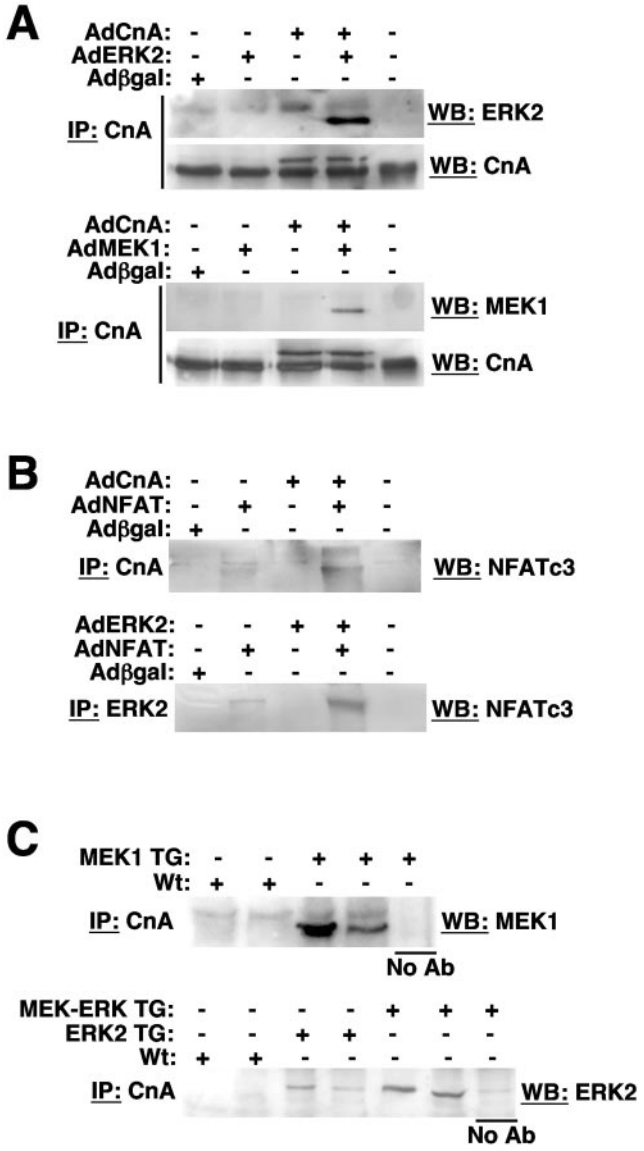

FIG. 7. Calcineurin-NFATc3-MEK1-ERK2 form a complex in vivo. (A) Western blots (WB) for ERK2, CnA, or MEK1 following immunoprecipitation (IP) of CnA from the indicated cardiomyocyte cell extracts, generated from cell cultures previously subjected to adenoviral mediated gene transfer of the indicated constructs. The last lane contains a nonspecific immunoglobulin G (IgG) antibody (Ab). (B) Western blots for NFATc3, following immunoprecipitation of CnA or ERK2 from the indicated cardiomyocyte cell extracts, generated from cell cultures previously subjected to adenovirus-mediated gene transfer of the indicated constructs. The last lane contains a nonspecific IgG antibody. (C) Western blot for activated MEK1 or ERK2 following immunoprecipitation for endogenous CnA from hearts of mice that were either wild-type (Wt), ERK2 transgenic (TG), or ERK2-MEK1 double transgenic. The position of a no-antibody control is also shown. Similar results were observed in three independent experiments.

Induction of the ANF promoter was also evaluated to further investigate the underlying transcriptional effects associated with the hypertrophic growth response. Cotransfection of activated MEK1 induced transcriptional activation of the ANF-luciferase reporter, which was inhibited by expression of the calcineurin inhibitory domain from cain or with cyclospor-

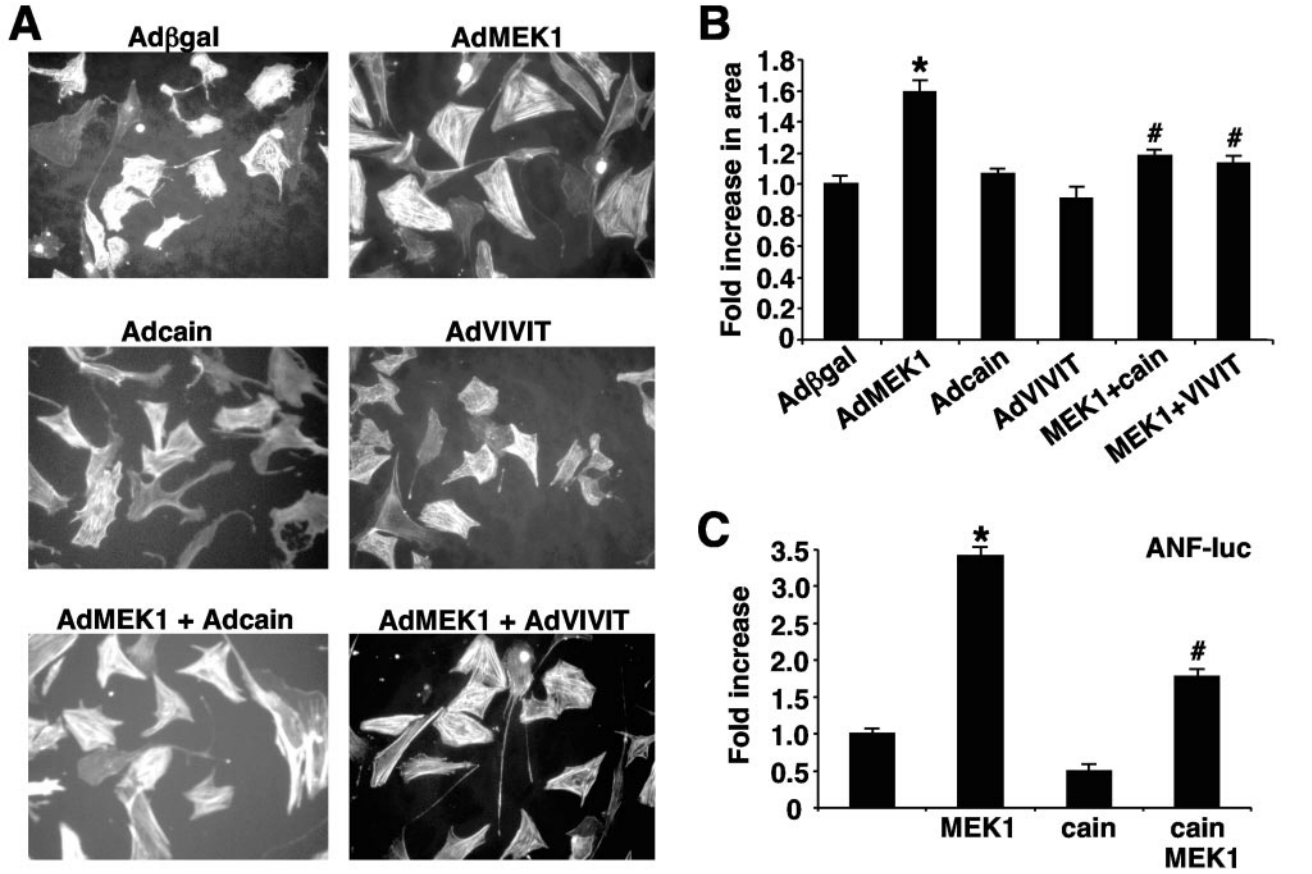

FIG. 8. Calcineurin-NFAT signaling is necessary for MEK1-induced hypertrophy in vitro. (A) Representative immunocytochemical analysis of actin-stained (phalloidin) neonatal cardiomyocytes treated as indicated. (B) Measurement of cell surface area of neonatal cardiomyocytes infected with the indicated adenoviruses in serum-free media for 48 h ($n = 3$ independent experiments, at least 400 cells measured in each experiment). (C) ANF promoter activity from cultured cardiomyocytes infected with the indicated adenovirus for 48 h. (*, $P < 0.05$ versus Adβgal; #, $P < 0.05$ versus AdMEK1).

ine A treatment (Fig. 8C and data not shown). Collectively, these data suggest that MEK1-ERK1/2-induced hypertrophy requires functionality in calcineurin-NFAT signaling, supporting the proposed interdependence between these two pathways.

MEK1-ERK1/2 signaling is necessary for calcineurin-NFAT-induced hypertrophy in vitro. While efficient calcineurin-NFAT signaling was required for MEK1-ERK1/2 pathway-mediated hypertrophy, it was also of interest to investigate the reciprocal relationship, thus extending the proposed interdependency between these two pathways. To this end, neonatal cardiomyocytes were induced to hypertrophy in culture by expression of activated calcineurin (AdΔCnA), characterized by increased sarcomeric organization and an increase in cell area ($P < 0.05$) (Fig. 9A and B) as previously observed (50). Expression of MEK1dn or MKP3 significantly reduced myocyte growth driven by activated calcineurin ($P < 0.05$) (Fig. 9A and B). Because MKP3 is highly specific for ERK1/2 (no alterations were observed in p38 or JNK activation), these results further support a more proximal effect that involves an ERK1/2-dependent transcriptional mechanism. Indeed, MEK1dn and MKP3 each significantly reduced MEK1-activated transactivation of the NFAT-dependent luciferase reporter and the ANF-luciferase reporter (Fig. 9C and D). These later results further suggest that calcineurin-NFAT-regulated transcriptional induction of the hypertrophic program requires input from MEK1-ERK1/2 signaling.

The calcineurin-NFAT pathway is necessary for MEK1-induced hypertrophy in vivo. While cultured neonatal cardiomyocytes are a widely used model system for investigating the cell growth response, they do not always model growth effects observed in the adult heart. In order to investigate whether our results could be reproduced in a more physiologic setting, cardiac-specific MEK1 transgenic mice were crossed with mice deficient in *CnA*β, a model with reduced calcineurin activity in the heart (8). We first assessed the integrity of the activated MEK1 transgene in the heart by Western blotting for MEK1, phosphorylated ERK1/2, and phosphorylated Elk-1 from cardiac protein extracts (Fig. 10A). As expected, the MEK1 transgenic mice expressed abundant MEK1 protein, which was associated with a sustained activation of ERK1/2 and Elk-1 in both wild-type and $CnA\beta^{-/-}$ mice (Fig. 10A). As a control, overexpression of MEK1 did not alter GAPDH levels in the heart, showing equal loading, nor was serine 473 phosphorylation in Akt altered (Fig. 10A and data not shown).

While wild-type and $CnA\beta^{-/-}$ mice showed identical ERK1/2 and Elk-1 activation in the heart, indicating similar signaling efficacies, $CnA\beta^{-/-}$ mice showed a remarkable attenuation in

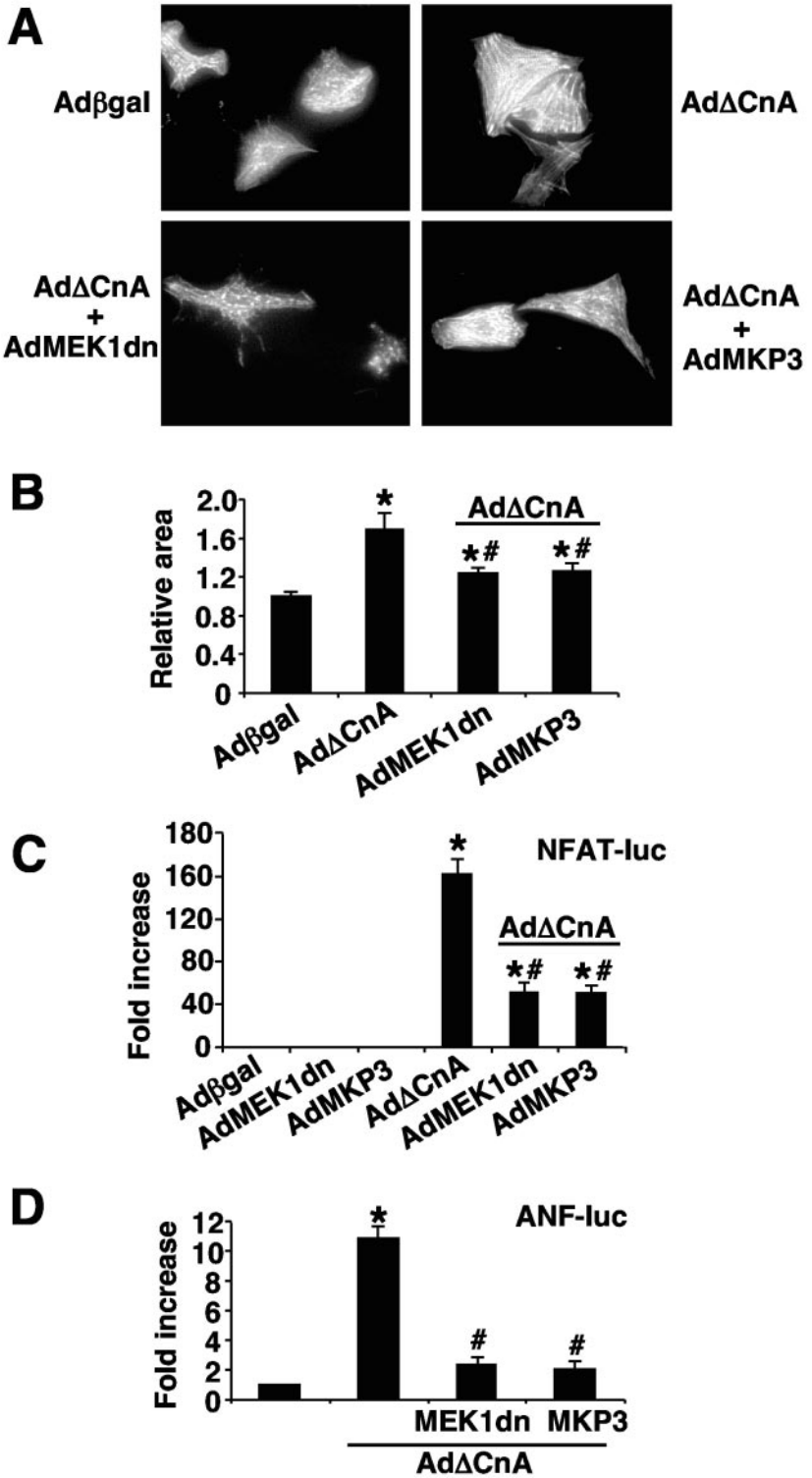

FIG. 9. MEK1-ERK1/2 signaling is necessary for a full calcineurin-induced hypertrophic response. (A) Representative immunocytochemical analyses of actin-stained (phalloidin) neonatal cardiomyocytes treated as indicated. (B) Measurement of cell surface area of neonatal cardiomyocytes infected with the indicated adenoviruses in serum-free media for 48 h ($n = 3$ independent experiments, at least 400 cells measured in each experiment). (C) Neonatal cardiomyocytes were infected with AdNFAT-luc and the indicated adenoviruses, and luciferase assays were performed 48 h afterwards. (D) ANF promoter activity was measured after transfection with an ANF-luciferase promoter vector (0.25 μg), followed by infection with the indicated adenoviruses. (*, $P < 0.05$ versus Adβgal; #, $P < 0.05$ versus AdΔCnA alone).

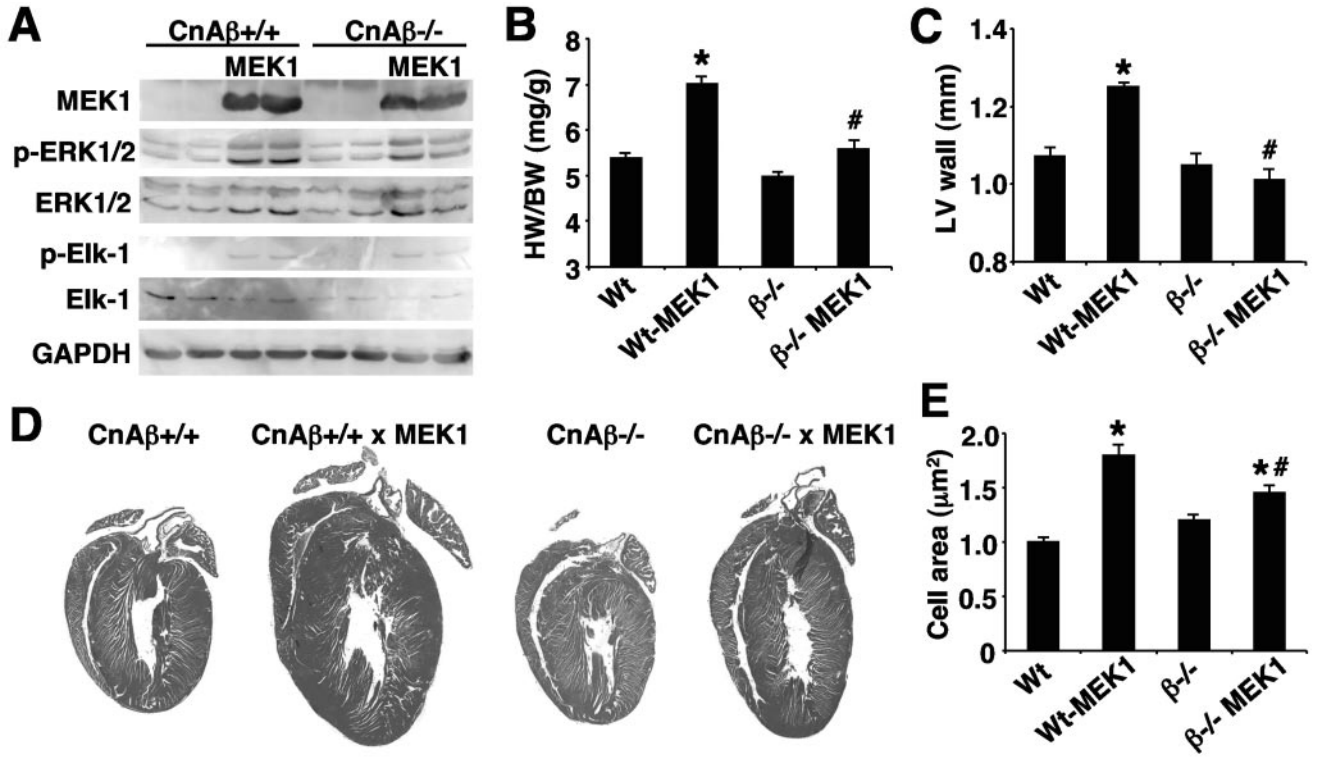

FIG. 10. *CnA*β gene targeting reduces MEK1-induced cardiac growth. (A) Western blot analysis of MEK1-ERK1/2 pathway phosphorylation in the hearts of 2-month-old MEK1 transgenic mice (FVB strain) crossed into the *CnA*β wild-type (Wt) or null backgrounds. (B) Measurement of heart-to-body weight ratios and (C) echocardiography-measured left ventricular posterior wall thickness (LV wall) show a reduction of cardiac hypertrophy by *CnA*β gene targeting. Four or five mice were analyzed in each group. (D) Gross histological analysis and (E) myocyte cross-sectional areas measurements ($n = 400$ cells per section) also revealed a reduction in hypertrophic growth associated with expression of activated MEK1 in the heart by *CnA*β gene disruption. (*, $P < 0.05$ versus wild type; #, $P < 0.05$ versus Wt-MEK1).

cardiac hypertrophy induced by the activated MEK1 transgene at 8 weeks of age. Specifically, analysis of heart weight normalized to body weight showed a 60% reduction in MEK1-induced hypertrophic growth in the absence of *CnA*β ($P < 0.05$) (Fig. 10B). Similarly, echocardiography showed a significant reduction in left ventricular free-wall thickness ($P < 0.05$) (Fig. 10C). Finally, histological analysis revealed a noticeable reduction in macroscopic heart size (Fig. 10D) and in myocyte cross-sectional areas measured from wheat germ agglutinin-TRITC-stained microscopic images (Fig. 10E). Collectively, these results indicate that MEK1-induced cardiac hypertrophy requires effective calcineurin-NFAT signaling in vivo, further supporting the codependency between these two parallel signaling pathways.

DISCUSSION

Mechanism whereby MEK1 enhances NFAT transcriptional activity. The molecular mechanisms whereby MEK1-ERK1/2 signaling regulates the cardiac growth response are largely unknown, although the transcription factor GATA4 has been proposed as one potential downstream target (30). By comparison, calcineurin promotes the cardiac growth response through a distinct mechanism that largely relies on the transcription factor NFAT (33, 56). Here we provided evidence that the MEK1-ERK1/2 and calcineurin-NFAT signaling pathways, while parallel and physically distinct, are functionally interconnected in the heart such that both must be actively engaged in order to produce myocyte growth. The functional interconnection was best highlighted by the observation that MEK1-ERK1/2 signaling enhanced NFAT transcriptional activity, both in cultured cells and in whole-animal studies. To date, nearly all described signals that positively alter NFAT transcriptional activity have involved an alteration in calcineurin activity. For example, Ichida and colleagues observed that Ras activation, which directly activates the Raf-1 kinase, hence MEK1-ERK1/2 activation, directly promoted NFAT nuclear localization in cardiomyocytes (23). However, we did not observe any alterations in calcineurin phosphatase activity (data not shown), nor did we observe alterations in NFATc1 or NFATc3 nuclear localization in cardiac myocytes in response to MEK1-ERK1/2 activation. Possible explanations for this discrepancy may reflect the pleiotrophic effects associated with Ras activation, which induces multiple parallel signaling pathways besides Raf-1–MEK1–ERK1/2. Alternatively, Ichida et al. investigated NFATc4 nuclear translocation, while we investigated NFATc1 and NFATc3. Despite these minor differences, our study and that of Ichida et al. identified a similar biologic effect associated with MEK1-ERK1/2 activation: an augmentation in NFAT transcriptional activity. This overall paradigm is supported by previous studies of nonmyocytes. Specifically, Vav signaling in T lymphocytes was coupled to Ras-MEK1-ERK1/2 activation, which in turn promoted NFAT activation (53). Moreover, dominant-negative and constitu-

tively active MEK1 mutants blocked and enhanced NFAT activation in T lymphocytes, respectively (18). In contrast to the reports discussed above, another level of regulation has been suggested whereby ERK1 can directly antagonize NFATc1 nuclear import in COS cells through a mechanism involving direct phosphorylation within the N-terminal regulatory domain of NFAT proteins (41). This paradigm is similar to the well-established roles ascribed to glycogen synthase kinase 3β (GSK3β), JNK1/2, p38, protein kinase A (PKA), and casein kinase 1 (CK1) (4, 10, 20, 41, 47, 58, 60). These five kinases have each been shown to directly phosphorylate the N-terminal regulatory domain in multiple NFAT factors, thus antagonizing calcineurin-mediated dephosphorylation and subsequent nuclear translocation of NFAT. In cardiac myocytes, GSK3β, JNK1/2, and p38 have all been implicated as critical negative regulators of NFAT translocation, thus antagonizing the cardiac hypertrophic response (2, 21). Given these later studies, a paradigm has emerged whereby the JNK1/2 and p38 branches of the MAPK might actually function as overall negative regulators of the cardiac hypertrophic response. Indeed, transgene-mediated expression of activated MKK7 (JNK1/2 activation) or MKK3/6 (p38 activation) in the heart did not produce cardiac hypertrophy but instead induced dilated cardiomyopathy (31, 39, 40). In support of these observations, expression of dominant-negative mutants for MKK3, MKK6, p38α, or JNK1/2 (or *Jnk1/2* gene-targeted mice) each showed enhanced cardiac growth through a mechanism involving increased NFAT activation in vitro and in vivo (5, 29).

In contrast to the role proposed for JNK1/2 and p38 in the heart, expression of activated MEK1 in the heart actually produced a prominent cardiac growth response (6, 7) which, as we showed here, enhances NFAT transcriptional responsiveness. Taken together, these data suggest that ERK1/2 are unlikely to function as physiologic regulators of NFAT nuclear shuttling as described for GSK3β, JNK1/2, and p38 in the heart.

While MEK1-ERK1/2 activation did not alter calcineurin activity or NFAT's subcellular localization, it did enhance NFAT-dependent transcriptional activation in cultured myocytes and in the mouse heart. One mechanism underlying the observed increase in NFAT transcriptional responsiveness is through effects on NFAT partner proteins such as AP-1 and possibly even GATA4 (Fig. 11). Indeed, MEK1-ERK1/2-induced synergy was blocked by the AP-1 inhibitory mutant protein TAM67. ERK1/2 were previously shown to be required for platelet-derived growth factor (PDGF)-regulated proliferation through a mechanism involving direct phosphorylation of c-Fos, which increased AP-1 transcriptional activity (34). In cardiac myocytes, MEK1-ERK1/2 signaling significantly enhanced AP-1 transcriptional activity, which was blocked with TAM67 (Fig. 5B). These data suggest a new level of complexity to NFAT signaling within cardiac cells, which suggests that the physiological outcome of NFAT activation may not depend exclusively on the degree of calcineurin activation but also on interactions with other pathways that secondarily affect NFAT activity through cofactor association. In T lymphocytes, activation of NFAT alone with ionomycin leads to anergy, while a productive immune response is obtained only by costimulation with phorbol myristate acetate, thus recruiting other signaling circuitry, such as MEK1-ERK1/2 and PKC (22).

In parallel, it is also likely that the transcription factor GATA4 participates in the observed synergy between the MEK1-ERK1/2 and calcineurin signaling pathways (Fig. 11). For example, MEK1-ERK1/2-induced cardiomyocyte hypertrophy in culture was significantly attenuated by expression of a dominant-negative GATA4-engrailed fusion protein, suggesting that GATA4 is a necessary transcriptional mediator of this pathway (30). Also to be considered, GATA4 and NFAT were previously shown to directly interact in cardiomyocytes, synergistically activating hypertrophic gene expression (33, 57). Thus, MEK1-ERK1/2 signaling is likely to regulate a diverse array of transcription factors that either directly or indirectly interact with NFAT factors to coordinately regulate the cardiac growth response.

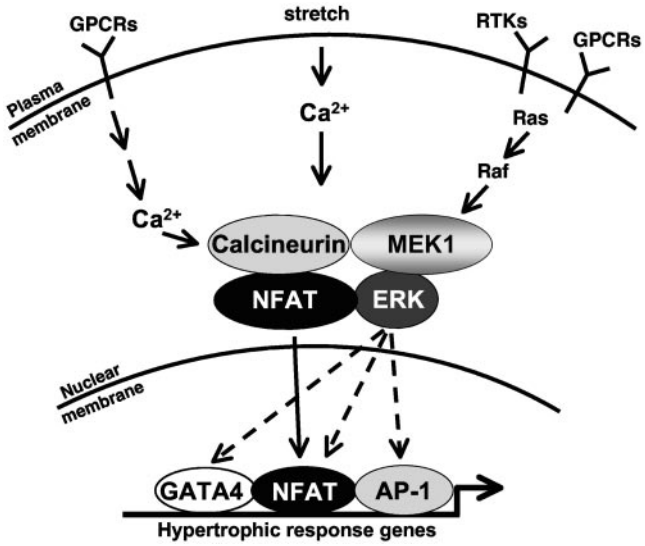

FIG. 11. Model of the calcineurin-NFAT and MEK1-ERK1/2 signaling module. While calcineurin-NFAT and MEK1-ERK1/2 receive input from stimuli that either mobilize intracellular calcium or result in Ras activation, the full effectiveness of either pathway likely requires simultaneous input from G-protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), and other ill-defined stimuli. All four proteins can form a complex in cardiomyocytes, likely serving as a means of coordinating regulation through at least one direct mechanism involving ERK-mediated phosphorylation of NFAT, which does not alter nuclear translocation but instead influences DNA binding activity of NFAT. Once in the nucleus through the sole action of calcineurin, NFAT can regulate gene expression in coordination with ERK signaling through direct effects on AP-1, GATA4, and other cofactors. It is uncertain if the entire four-protein complex can translocate to the nucleus with NFAT or if only ERK becomes associated with NFAT localized at specific DNA binding sites, permitting additional ERK-regulated events on transcription.

While our observations concerning the proposed regulatory mechanism through AP-1 are not novel, considering past observations in lymphocytes, the observation that MEK1-ERK1/2 signaling directly alters the DNA binding activity of NFAT is unique. Hence, a second mechanism was demonstrated, whereby MEK1-ERK1/2 signaling directly regulates calcineurin-NFAT responsiveness through formation of a large protein complex between MEK1-ERK1/2 and calcineurin-NFAT in cardiomyocytes, which likely facilitates the direct phosphorylation of NFAT leading to augmented DNA binding activity (Fig. 11). Thus, coordinate signals through G-protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), or other ill-de-

fined stressors, such as stretch, can promote the hypertrophic growth response of cardiomyocytes in coordination with both calcineurin-NFAT and MEK1-ERK1/2 signaling through a direct physical interaction between these pathways (Fig. 11).

MEK1-ERK1/2 and calcineurin-NFAT pathways coregulate cardiomyocyte hypertrophy. It is becoming increasingly clear that the cardiac growth response utilizes a complex array of distinct intracellular signaling pathways. Moreover, recent evidence has suggested a degree of functional convergence or interdependency between various pathways, such that some operate in concert with one another while others are directly antagonistic. For example, the p38 and JNK1/2 branches of the MAPK pathway antagonize cardiac growth by opposing calcineurin signaling through phosphorylation of NFAT factors within their N-terminal regulatory domain (5, 29). With respect to MEK1-ERK1/2 signaling, β-adrenergic-mediated activation of ERK1/2 and endothelin-1 transcription is blocked in response to calcineurin inhibition (35). Moreover, genetic reduction of calcineurin signaling blocks the onset of cardiac hypertrophy to a broad array of stimuli that are likely mediated by multiple classes of membrane-bound receptors (8). Here we have extended the overall paradigm of coordinated regulation of cardiac growth through the observation that either calcineurin-NFAT- or MEK1-ERK1/2-induced hypertrophy requires the other for an effective response. Blockade of either calcineurin-NFAT signaling (cain or cyclosporine A) or MEK1-ERK1/2 signaling (MKP3 or U0126) did not affect the intrinsic signaling within either pathway itself (phosphatase or kinase activity was unaltered), but it instead resulted in a loss in transcriptional potency induced by each pathway. For example, Elk-1 phosphorylation was not altered in *CnA*β gene-targeted mice, which is a direct transcriptional effector of MEK1-ERK1/2.

While *CnA*β gene disruption attenuated the hypertrophic growth of the myocardium in MEK1 transgenic mice at 8 weeks of age, as these mice aged for significantly longer periods of time the overall effect was lost, suggesting that calcineurin-NFAT-independent mechanisms eventually compensate (data not shown). In the same way, MEK1-ERK1/2 inhibition did not completely block calcineurin-induced hypertrophy in vitro, suggesting some degree of MEK1-ERK1/2 independence within the calcineurin-NFAT signaling circuit. Despite these caveats, our overall model indicates that a productive and timely hypertrophic growth response requires transcriptional cross-talk between MEK1-ERK1/2 and calcineurin-NFAT signaling pathways in the heart. Thus, blockade of either intracellular signal in the heart dramatically affects the orchestration of the entire hypertrophic response and effectively diminishes heart enlargement.

ACKNOWLEDGMENTS

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