Calcineurin is a serine/threonine protein phosphatase that plays a critical role in many physiologic processes, such as T-cell activation, apoptosis, skeletal myocyte differentiation, and cardiac hypertrophy. We determined that active MEKK3 was capable of activating calcineurin/nuclear factor of activated T-cells (NFAT) signaling in cardiac myocytes and reprogramming cardiac gene expression. In contrast, small interference RNA directed against MEKK3 and a dominant negative form of MEKK3 caused the reduction of NFAT activation in response to angiotensin II in cardiac myocytes. Genetic studies showed that MEKK3-deficient mouse embryo fibroblasts failed to activate calcineurin/NFAT in response to angiotensin II, a potent NFAT activator. Conversely, restoring MEKK3 to the MEKK3-deficient cells restored angiotensin II-mediated calcineurin/NFAT activation. We determined that angiotensin II induced MEKK3 phosphorylation. Thus, MEKK3 functions downstream of the AT1 receptor and is essential for calcineurin/NFAT activation. Finally, we determined that MEKK3-mediated activation of calcineurin/NFAT signaling was associated with the phosphorylation of modulatory calcineurin-interacting protein 1 at Ser108 and Ser112. Taken together, our studies reveal a previously unrecognized novel essential regulatory role of MEKK3 signaling in calcineurin/NFAT activation.

Calcineurin is a calcium/calmodulin-dependent serine/threonine protein phosphatase originally isolated from brain extracts. It is composed of a catalytic subunit (calcineurin A (CnA)) and a regulatory subunit (calcineurin B). Calcineurin is activated upon binding of calcineurin B and calmodulin to CnA. Calmodulin binding displaces an autoinhibitory domain located at the C-terminal portion of CnA, which otherwise masks the active site. The most thoroughly characterized calcineurin substrate proteins are members of the nuclear factor of activated T-cells (NFAT) family of transcription factors (1–3). Calcineurin, as well as to chronic isoproterenol administration, pressure overload, and exercise (27, 33). A variety of pathologic stimuli, including myocardial infarction, hypertension, contractile abnormalities, and pressure overload, elicit the cardiac hypertrophic response. Autocrine and paracrine signaling pathways involving angiotensin II (Ang II), endothelin-1, and activators of the adrenergic system also contribute to myocyte hypertrophy. Pathological hypertrophy is associated with maladaptive changes in cardiac contractility and calcium handling, cardiac fibrosis, myocyte apoptosis, and sudden death from arrhythmias. Thus, deciphering the details of the signaling pathways that convey hypertrophic stimuli is an important problem with therapeutic relevance (14, 15). Among numerous signaling pathways leading to cardiac hypertrophy, calcineurin is an especially effective inducer of cardiac growth through control of NFAT activity (1, 7, 13, 16–18). Knock-out mice lacking the catalytic subunit, CnA, show a diminished hypertrophic response to hypertrophic stimuli (19). Inhibition of calcineurin activity with cyclosporin A or FK506 blocks cardiac hypertrophy in response to pressure overload, β-adrenergic stimulation, and sarcomere dysfunction (20, 21). FK506 and cyclosporin A exert their effects by promoting the formation of complexes between CnA and FKBP12 or cyclophilin A, respectively (22). Several endogenous protein inhibitors of calcineurin have been identified, including AKAP79, Cabin/Cain, the calcineurin B homology protein (23–25), and modulatory calcineurin-interacting protein 1 (MCIP1) (26–29).

MCIP1 was first cloned as the product of the Down syndrome critical region 1 (DSCR1) gene on chromosome 21 (30). MCIP1 is expressed at the highest levels in striated muscles and brain (26). MCIP orthologs have also been identified in other eukaryotes, including yeast (3, 31) and the pathogenic fungus Cryptococcus neoformans (32). MCIP1 interacts with the catalytic subunit of calcineurin (CnA) and inhibits its phosphatase activity (26). MCIP1 shares a structural motif with NFAT termed the SP repeat domain. Peptides derived from the SP repeat domain of human MCIP1 inhibit calcineurin activity (27, 28). These findings support a competitive antagonist function for MCIP1 with respect to calcineurin action on NFAT proteins. The function of MCIP1 as a suppressor of calcineurin activity has been defined largely from studies of MCIP1 overexpression and supports the hypothesis that MCIP1 antagonizes the prohypertrophic actions of calcineurin. For example, cardiac overexpression of MCIP1 in transgenic mice diminished hypertrophy in response to overexpression of a constitutively active form of calcineurin, as well as to chronic isoproterenol administration, pressure overload, and exercise (27, 33). MCIP1 gene expression is also up-reg-
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ulated in response to calcineurin signaling, suggesting that MCIP1 functions in a feedback inhibition loop to suppress calcineurin activity (34). MCIP1 can be phosphorylated in vitro by p43/44 mitogen-activated protein kinase (MAPK), and this phosphorylation is necessary for efficient phosphorylation by glycogen synthase kinase 3 (35). In vitro studies have also shown that phosphorylated MCIP1 itself can serve as a substrate for calcineurin (35). More recent evidence indicates that phosphorylation of RCN1, a yeast ortholog of MCIP1, enhances calcineurin activity (36), although the mechanism of calcineurin activation was unclear. Nevertheless, these findings suggest that calcineurin signaling may be regulated through phosphorylation of MCIP1 by protein kinases. To test this hypothesis, we sought initially to identify candidate kinases that activate calcineurin-NFAT signaling and then to determine whether calcineurin/NFAT signaling is regulated through MCIP1 phosphorylation by the candidate kinases.

MATERIALS AND METHODS

Mammalian Expression Constructs and Reporter Constructs—Plasmids for HA-tagged or non-HA-tagged MEKK3 (wild type (WT), constitutively active (CA), and dominant negative (DN)) have been described (5, 20–23). In detail, the wild type HA-tagged MEKK3 expression vector, SRa3HA-MEKK3, was constructed by cloning the NcoI fragment, which contains the first methionine codon of MEKK3. The constitutively active HA-tagged MEKK3 expression vector, SRa3HA-MEKK3(CA), was generated by cloning the SalI/EcoRI fragment, which contains the first methionine codon of MEKK3. The dominant negative MEKK3 expression vector, SRa3HA-MEKK3(DN), was generated by a Lys to Met mutation to destroy the ATP binding site through PCR-directed mutagenesis. The mutant cDNA was subcloned into the SRa3HA vector, as described for SRa3HA-MEKK3.

Adss1/CAT and mAdss1/CAT have been previously described (37). c-Fos-Luc, β-myosin heavy chain (MHC)-Luc, and skeletal α-actin-Luc have been described (37). The 4× NFAT-luciferase construct was purchased from Clontech. Myc-tagged MCIP1 has been described (29). Mutants of MCIP1 were generated as follows. An alanine was substituted for the serine at positions 108 and 112 using a PCR-directed mutagenesis technique as described previously (38).

MEKK3 Small Interference RNA—The mammalian expression vector, pSUPER (OligoEngine, Seattle, WA) was used for expression of MEKK3 siRNA in the cells. For expressing short hairpin RNA, one siRNA targeting MEKK3 with the following sense and antisense sequences was fused to pSUPER-MEKK3: 5′-GATCCCCGGCAAGAGGTGAGTGCTCTTTCAAGAGAGACCTCACCCTTCTGCTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAGAGAACAG-GAGGTGAGTGCTCTTTCAAGAGAGACCTCACCCTTCTGCTTTTGGGG-3′. The two oligonucleotides were annealed and subcloned into pSUPER vector digested with HindIII and BglII.

Antibodies—Anti-HA, anti-Myc, and anti-CnA were purchased from Sigma. Anti-MEKK3 and anti-phosphoserine were purchased from Upstate Cell Signaling Solutions (Charlottesville, VA).

Cell Culture—Neonatal rat cardiac myocytes (1–2 days old) were isolated as described previously (37) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (HyClone, Logan, UT). Cells were plated in 6-well dishes (21 cm²/well) at a density of $6 \times 10^5$ cells/well for transient transfection experiments (37). After 24 h, for some experiments, the cells were rinsed and then maintained for the remainder of the experiment in serum-free Dulbecco’s modified Eagle’s medium, supplemented with 1% bovine serum albumin with or without Ang II treatment. CHO-AT1 cells were cultured as described previously (39) and plated at 1.5×10⁶ in 10-cm wells in RPMI 1640 medium supplemented with 5% fetal bovine serum. The suspended cells were transiently transfected with various expression plasmids. 48 h after transfection, cellular extracts were collected and used for Western blot analysis and immunoprecipitation assays.

Transient Transfection and Reporter Gene Assays—Primary cardiac myocytes were co-transfected with a 4×NFAT-luciferase, 1.9Adss1/CAT, c-fos-luciferase, β-MHC-luciferase, or skeletal α-actin-luciferase reporter plasmid (1 μg) and various expression plasmids (0.1 μg) that use the cytomegalovirus promoter to drive expression of WT, CA, or DN forms of MEKK3, respectively, as described (37, 40). For some experiments, cells were treated with FK506 or co-transfected with MCIP1 expression vectors in a dosage range of 0.1–1 μg (37). In addition, 4×NFAT-luciferase reporter plasmids were co-transfected with constitutively active MEKK3 in the presence of constitutively active or dominant negative MEK5, MEK6, MEK7, or BMK1 in a dosage range of 0.1–1 μg. The pCMVlacZ plasmid (1 μg) was used as an internal control to monitor transfection efficiency. The data are presented as relative luciferase activity or CAT activity, calculated as the ratio of the activity of luciferase or CAT activity to the activity of β-galactosidase.

Immunoprecipitation and Western Blot Analysis—Full-length Myc-MCIP1 or NFAT3-GFP constructs were transiently co-transfected with wild type or constitutively active forms of HA-MEKK3 into cardiac myocytes or CHO-AT1 cells. Forty-eight hours after transfection, cells were treated with or without Ang II from 0 to 60 min. Following treatment, cells were lysed in immunoprecipitation buffer, consisting of 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitors (Roche Applied Science). The cellular extracts from Mvak-MCIP1- and HA-MEKK3-transfected cells were precipitated with specific antibodies, respectively, with rotation overnight at 4 °C. Immune complexes were recovered with protein A-Sepharose beads, separated by SDS-PAGE, and probed with specific antibodies, respectively. For NFAT-GFP transfection, the nuclear, cytosolic, and total cellular extracts were isolated, and Western blot analysis was performed as described previously (37).

Alkaline Phosphatase Treatment—Rat neonatal cardiac myocytes were transfected with an expression construct encoding murine MCIP1 with Myc tag or wild type MEKK3 with HA tag. 48 h after transfection, cells were treated with or without Ang II from 0 to 60 min. Then cellular extracts were collected as described above. Lysates were treated with 1 unit of calf alkaline phosphatase (Roche Applied Science) per 100 μl of cellular lysates at room temperature for 30 min. Samples were processed for Western blot analysis as described above.

Generation of MEKK3−/− Murine Embryo Fibroblasts (MEFs) and Stable Transformants—The matings of MEKK3−/− mice were timed, and the embryos generated were dissected at embryonic day 9.5, as described (41). Yolk sacs were isolated and genotyped with the use of the polymerase chain reaction; each individual embryo was homogenized and the embryos generated were dissected at embryonic day 9.5, as

CHO-AT1 cells were cultured as described previously (39) and plated at 1.5×10⁶ in 10-cm wells in RPMI 1640 medium supplemented with 5% fetal bovine serum. The suspended cells were transiently transfected with various expression plasmids. 48 h after transfection, cellular extracts were collected and used for Western blot analysis and immunoprecipitation assays.

Transient Transfection and Reporter Gene Assays—Primary cardiac myocytes were co-transfected with a 4×NFAT-luciferase, 1.9Adss1/CAT, c-fos-luciferase, β-MHC-luciferase, or skeletal α-actin-luciferase reporter plasmid (1 μg) and various expression plasmids (0.1 μg) that use the cytomegalovirus promoter to drive expression of WT, CA, or DN forms of MEKK3, respectively, as described (37, 40). For some experiments, cells were treated with FK506 or co-transfected with MCIP1 expression vectors in a dosage range of 0.1–1 μg (37). In addition, 4×NFAT-luciferase reporter plasmids were co-transfected with constitutively active MEKK3 in the presence of constitutively active or dominant negative MEK5, MEK6, MEK7, or BMK1 in a dosage range of 0.1–1 μg. The pCMVlacZ plasmid (1 μg) was used as an internal control to monitor transfection efficiency. The data are presented as relative luciferase activity or CAT activity, calculated as the ratio of the activity of luciferase or CAT activity to the activity of β-galactosidase.

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resistant clones were collected and are referred to as the MEKK3−/− cells herein.

Calcineurin Activity Assay—Cardiac tissue was minced finely and homogenized in lysis buffer as described above, followed by further disruption by sonication. Lysates then were cleared by centrifugation. Free phosphate was removed by passing the lysate through a Micro Bio 6 desalting column (Bio-Rad). Calcineurin activity was measured by using the synthetic phosphorylated RII peptide (Biomol, Plymouth Meeting, PA). The amount of free phosphate released was measured with molybdate dye solution (Promega). Calcineurin-specific phosphatase activity was measured as the amount inhibited by cyclosporin A. Western blots to detect calcineurin protein were performed with the above lysates by using a calcineurin A antibody (Sigma).

Statistical Analysis—All values are expressed as mean ± S.E. Data were analyzed for statistical significance using GraphPad Prism software. Statistical significance was determined by Student’s *t* test or an analysis of variance test. A value of *p* < 0.05 was interpreted to mean that observed experimental differences were statistically significant.

RESULTS

MEKK3 Is Capable of Activating Calcineurin/NFAT Signaling in Cardiac Myocytes—To identify protein kinases capable of activating calcineurin/NFAT signaling, we co-transfected an NFAT-luciferase reporter construct along with constructs encoding a candidate protein kinase into rat neonatal cardiac myocytes. Following co-transfection, we monitored NFAT activation by measuring luciferase activity in cell extracts. Using this screening assay, we found that a constitutively active form of MEKK3, a member of the MAP3K family of protein kinases, activated the NFAT-luciferase reporter construct ~25–30-fold (Fig. 1A). Next, we showed that FK506, a calcineurin inhibitor, completely inhibited NFAT activation by MEKK3(CA), indicating that calcineurin is involved in MEKK3-mediated NFAT activation (Fig. 1A). In addition, we found that MCIP1 inhibited MEKK3(CA)-mediated NFAT activation in a dosage-dependent way (Fig. 1B). The Western blot analysis using anti-HA antibody indicated that the expression of various forms of HA-tagged MEKK3 was at the same level (the insets in Fig. 1, A and B). The inhibitory effects of FK506 and MCIP1 on NFAT activation by MEKK3(CA) indicated that MEKK3(CA)-induced NFAT activation is mediated through calcineurin signaling.

MEKK3 Activates the Cardiac Hypertrophic Transcriptional Response—To determine whether MEKK3 is capable of inducing the cardiac hypertrophic transcriptional response, we evaluated the effect of MEKK3 on the activation of a number of genes associated with cardiac hypertrophy. For this purpose, we co-transfected cardiac myocytes with c-fos, β-MHC, and skeletal α-actin-luciferase reporter constructs with or without constitutively active MEKK3. We found that the transcriptional activities of c-fos, β-MHC, and skeletal α-actin reporter genes were induced by constitutively active MEKK3, and that the induction was inhibited by FK506 or MCIP1 (Fig. 2A). These results demonstrate that MEKK3 functions upstream of calcineurin-NFAT signaling to induce the cardiac hypertrophic transcriptional response.
Similarly, Western blot analysis confirmed equal expression levels of for Ang II-mediated NFAT activation in rat neonatal cardiomyocytes.

MEKK3 Functions Downstream of the Angiotensin Receptor to Mediate NFAT Activation—Angiotensin II is a potent calcineurin/NFAT activator. To determine whether MEKK3 acts downstream of the AT1 receptor and is needed for calcineurin/NFAT activation in cardiomyocytes, we used DN MEKK3 and siRNA specific for MEKK3 to knock down endogenous MEKK3 expression levels. For these experiments, NFAT-luciferase reporter constructs were introduced into rat neonatal cardiomyocytes along with expression plasmids encoding MEKK3(CA). Western blot analysis (Fig. 3D) showed that only wild type MEKK3 induced NFAT nuclear translocalization from cytosol into the nucleus following Ang II treatment, whereas both dominant negative MEKK3 and siRNA specific for MEKK3 reduced Ang II-induced NFAT-GFP nuclear translocalization. In contrast, constitutively active MEKK3 induced NFAT nuclear translocalization, as did controls with Ang II and Ang II with wild type MEKK3 transfection (Fig. 3, B and C). Thus, our results indicate that MEKK3 is essential for Ang II-mediated NFAT translocalization from cytosol into nucleus.

In addition, Western blot analysis was performed to determine the expression levels of the cotransfected plasmids encoding various forms of HA-tagged MEKK3 (WT and DN) (Fig. 3A, inset).
MEKK3-deficient MEFs failed to induce calcineurin activity following Ang II treatment. Inset, Western blot analysis to determine the expression of transfected HA-tagged MEKK3 (WT and DN). MEKK3 is essential for Ang II-mediated NFAT nuclear translocation. Rat neonatal cardiac myocytes were co-transfected with NFAT-GFP expression plasmids in the presence or absence of Ang II along with MEKK3(WT), MEKK3(DN), or MEKK3(siRNA) expression plasmids. Western blot analysis of NFAT-GFP from both cytosolic and nuclear compartments was determined in cardiac myocytes treated with Ang II for 30 min. The experiments were repeated three times, and a representative Western blot is shown here. C, densitometric analysis of three Western blots was performed. The results are shown as cytosolic or nuclear NFAT-GFP/total NFAT-GFP protein optical density ratios. Data are expressed as mean \( \pm S.E. \) of three independent determinations.

**Defective Ang II-mediated Calcineurin/NFAT Activation in MEKK3-deficient Mouse Embryo Fibroblasts**—To determine whether MEKK3 is essential for calcineurin activation, we treated wild type and MEKK3-deficient MEFs (42) with Ang II. We found that MEKK3-deficient cells failed to activate the NFAT-luciferase reporter following Ang II treatment (Fig. 4A). In addition, we found that endogenous NFAT activity is significantly lower in MEKK3-deficient cells compared with wild type cells (Fig. 4A). Endogenous NFAT activity and Ang II-mediated NFAT activation were restored by introducing a MEKK3 minigene into MEKK3-deficient cells (these cells are termed MEKK3\(^{\sim/-}\) in Fig. 4A). As expected, the specific activity of calcineurin was induced by Ang II in wild type cells (Fig. 4B), whereas in MEKK3-deficient cells, the endogenous calcineurin activity was significantly lower than wild type. MEKK3-deficient MEFs failed to induce calcineurin activity following treatment with Ang II, whereas calcineurin activity was induced by Ang II in wild type cells. Similarly, MEKK3\(^{\sim/-}\) cells regained the ability to respond to Ang II, resulting in increased calcineurin activity. Western blot analysis showed that there was no difference in calcineurin protein levels in these three types of MEFs. Thus, these results provide strong genetic evidence that MEKK3 is required for Ang II-mediated calcineurin/NFAT activation.

**Activated MEKK3 Induces MCIP1 Phosphorylation**—It is known that in yeast, the phosphorylation of RCN1, an ortholog of MCIP1, leads to activation of calcineurin (36). Therefore, it is possible that MEKK3 signaling activates calcineurin through the phosphorylation of MCIP1. To test this possibility, we monitored the phosphorylation status of Myc-tagged MCIP1 by Western blot analysis following co-transfection with expression constructs encoding wild type, constitutively active, and dominant negative forms of the MEKK3 protein (Fig. 5A). We found that the mobility of Myc-tagged MCIP1 was significantly retarded following co-transfection with constitutively active MEKK3 (Fig. 5A). To determine whether the slower migrating form of MCIP1 was phosphorylated, we immunoprecipitated MCIP1 by anti-Myc antibody and performed Western blot analysis using anti-phosphoserine antibodies. The results (Fig. 5B) show that the slower migrating form of MCIP1 (Fig. 5A) represents MCIP1 protein phosphorylated at serine sites (Fig. 5B). Finally, the slower migrating band was confirmed as a phosphorylated form of MCIP1 by alkaline phosphatase (AP) treatment of the constitutively active MEKK3-transfected cell lysates (Fig. 5D). Taken together, these results demonstrate that active MEKK3 promotes the phosphorylation of MCIP1.

**Ang II Stimulation Leads to MEKK3 and MCIP1 Phosphorylation**—In Fig. 3D, lane 4, we showed that Ang II treatment resulted in the appearance of a slower migrating band. The dominant negative form of MEKK3 (with ATP binding site mutation) abolished the slower migrating band, suggesting that Ang II induces MEKK3 phosphorylation. In Fig. 5, we also found that active MEKK3 induced MCIP1 phosphorylation. Thus, we hypothesize that Ang II induces phosphorylation of both MEKK3 and MCIP1. To test this hypothesis, we monitored MCIP1 and MEKK3 phosphorylation in Ang II-treated cardiac myocytes. Neonatal cardiac myocytes were transfected with both Myc-tagged MCIP1 and HA-tagged wild type MEKK3 with or without Ang II treatment. Extracts from cardiomyocytes were examined by Western blotting using anti-Myc and anti-HA antibodies. The results (Fig. 6A) showed that Ang II induces MCIP1 phosphorylation appearing by 10 min and lasting for up to 60 min. For MEKK3, consistent with the results shown in Fig. 3D, we found that Ang II treatment led to a slower migrating form of MEKK3 appearing by 10 min and lasting for up to 60 min (Fig. 6B). Similarly, the slower migrating band was confirmed to be the phosphorylated MEKK3 by alkaline phosphatase (AP) treatment (Fig. 6C). Thus, we demonstrate that Ang II induces both MCIP1 and MEKK3 phosphorylation.

**MEKK3 Is Required for Ang II-mediated MCIP1 Phosphorylation**—To test whether Ang II-mediated MCIP1 phosphorylation is dependent on MEKK3, we monitored the MCIP1 and MEKK3 phosphorylation...
MEKK3 is essential for Ang II-mediated MC1P1 phosphorylation. A and B, Ang II induces phosphorylation of MCIP1 (A) and slower migrating band of MEKK3 (B). Rat neonatal cardiac myocytes were transfected with expression constructs encoding wild type HA-tagged MEKK3 and Myc-tagged MCIP1 with or without siRNA specific for MEKK3 following Ang II treatment from 0 to 60 min. Western blot analysis was used to determine the phosphorylation state of MCIP1 using anti-Myc antibody (A) and MEKK3 using anti-HA antibody (B). C, Ang II induces MEKK3 phosphorylation. The cellular extracts from the cells transfected and treated with Ang II for 30 min as described in A were treated (+AP) or untreated (−AP) with alkaline phosphatase. Western blot analysis was used to determine the change in migration of MEKK3 following alkaline phosphatase treatment, D and E, wild type MEKK3, mutant MEKK3, and rescued MEKK3 without Ang II treatment. The results also showed siRNA specific for MEKK3 knocking down both nonphosphorylated and phosphorylated MEKK3 (Fig. 6B, lane 5). Therefore, these findings indicate that MEKK3 is required for Ang II-induced MCIP1 phosphorylation in cardiac myocytes.

Next, we used MEKK3-deficient MEFs to determine whether MEKK3 is required for MCIP1 phosphorylation. We examined the effect of Ang II treatment on both MEKK3 and MCIP1 phosphorylation in wild type and MEKK3-deficient mouse embryo fibroblasts. We transfected Myc-tagged MCIP1 constructs into the cells. Using anti-Myc to detect MCIP1 and anti-MEKK3 antibodies, we found that phosphorylation of MCIP1 and MEKK3 was induced by Ang II stimulation in wild type mouse embryonic fibroblasts but was absent in MEKK3-deficient mouse embryo fibroblasts (Fig. 6, D and E). Ang II-induced MEKK3 and MCIP1 phosphorylation was restored in the MEKK3−/− cells carrying a stably integrated MEKK3 expression plasmid (Fig. 6, D and E). Thus, these results provide strong genetic evidence that phosphorylated MEKK3 is required for Ang II-induced MCIP1 phosphorylation.

MEKK3 is an upstream molecule associated with MCIP1 phosphorylation at Ser108 and Ser112. — Earlier in vitro studies showed that serine 108 and serine 112 are phosphorylation sites of murine MCIP1 and that phosphorylation of Ser112 is required for phosphorylation at Ser108 (35). To investigate whether Ser108 and Ser112 are associated with MEKK3-mediated MCIP1 phosphorylation, we co-transfected wild type or various mutant forms of Myc-tagged MCIP1 into CHO-AT1 cells with or without MEKK3(CA). Western blot analysis (Fig. 7A, lane 4) shows that mutation of serine 112 to alanine blocked MEKK3(CA)-induced phosphorylation of MCIP1. This result suggests that MEKK3 mediated
Ser^{112} phosphorylation and is necessary for subsequent phosphorylation of Ser^{108}. However, the S108A MCIP1 mutant was still phosphorylated (see retarded band in Fig. 7A, lane 6), indicating that Ser^{112} phosphorylation occurred in the absence of phosphorylation at Ser^{108}. Furthermore, mutation of both Ser^{108} and Ser^{112} sites of MCIP1 abolished the MEKK3-mediated MCIP1 phosphorylation,
suggesting that Ser112 is the priming phosphorylation site for the subsequent phosphorylation of Ser108 of MCIP1. Overall, these results indicate that both Ser108 and Ser112 are phosphorylated in response to MEKK3 activation, and Ser108 phosphorylation is required for phosphorylation at Ser112.

Phosphorylation of MCIP1 at Ser108/Ser112 Is Required for Activation of Calcineurin/NFAT Signaling—To determine whether the phosphorylation of MCIP1 at Ser108 and Ser112 is essential for the activation of calcineurin/NFAT, we cotransfected NFAT-luciferase constructs with active MEKK3 in the presence of different dosages of wild type or mutated MCIP1 (single site or double site mutants S108A, S112A, and S108A/S112A) expression constructs. Mutation of either serine 112 or 108 or both of these two sites resulted in a significantly increased inhibitory effect on MEKK3-mediated NFAT activation compared with wild type MCIP1 (Fig. 7B). Similarly, the inhibitory effect of mutant MCIP1 on Ang II-mediated NFAT activation is significantly higher than that of wild type MCIP1 (Fig. 7C). Thus, these results indicate that S108A and S112A mutants have an inhibitory effect on calcineurin activation. These findings imply that phosphorylation of MCIP1 at both serine sites is essential for MEKK3 or Ang II-mediated NFAT activation, and phosphorylation of MCIP1 can switch its inhibitory effect to a stimulatory effect.

DISCUSSION

The calcium-calmodulin dependent regulation of calcineurin is well recognized. However, the potential importance of protein kinase-mediated regulation of calcineurin is not well known. Here we report the identification of MEKK3, a MAP3K family member as an upstream activator of calcineurin. We have presented multiple lines of evidence that MEKK3 is absolutely required for calcineurin/NFAT activation in response to Ang II, a potent calcineurin/NFAT activator. First, MEKK3-deficient mouse embryo fibroblasts failed to activate calcineurin and NFAT in response to Ang II. Second, Ang II-induced NFAT activation was restored to MEKK3-deficient mouse embryo fibroblasts following the introduction of an MEKK3 expression plasmid. Third, MEKK3 depletion in cardiac myocytes resulted in decreased calcineurin/NFAT activation. Fourth, Ang II-mediated MEKK3 phosphorylation is associated with MCIP1 phosphorylation. Finally, we have demonstrated that MCIP1 phosphorylation is essential for MEKK3- and Ang II-mediated calcineurin/NFAT activation. Thus, MEKK3-induced MCIP1 phosphorylation represents a previously unrecognized signaling pathway regulating calcineurin activation.

MEKK3 Signaling Is Essential for Cardiac Hypertrophy and Heart Development—the MAPK cascades represent a primary mechanism by which cells transduce intracellular signals. Here we demonstrate that MEKK3 signaling controls calcineurin/NFAT activation to regulate the cardiac hypertrophic transcriptional response. Consistent with our in vitro cell culture data, preliminary in vivo studies show that heart-specific expression of active MEKK3 in transgenic mice results in cardiac hypertrophy and sudden death (data not shown). Thus, MEKK3 is sufficient to induce cardiac hypertrophy through calcineurin signaling.

Our studies using MEKK3-deficient MEFs provides genetic evidence, rather than evidence obtained only via overexpression studies in vitro, that MEKK3 plays a crucial role in calcineurin/NFAT activation. Because MEKK3 is a member of the MEKK gene family, its function may be compensated for by other members of the family. However, genetic studies show that only MEKK3 but not MEKK1 or MEKK2 depletion results in embryonic lethality due to cardiovascular and placental defects. Thus, although these MAP3Ks are mediators of signal transduction of the MAPK pathway, they are subject to stimulus and cell type-specific regulation and cannot compensate for each other. Thus, MEKK3, unlike MEKK1 and MEKK2, is likely to be used specifically by the Ang II signaling pathway in NFAT activation.

In addition to the essential role of MEKK3 in calcineurin activation in cardiac hypertrophy, as we have shown here, MEKK3 is also essential for early embryonic cardiovascular development (41). We show here that lack of MEKK3 in mouse embryo fibroblasts results not only in failure to induce calcineurin and NFAT activation in response to Ang II stimulation but also in decreased basal (unstimulated) levels of both calcineurin and NFAT activity. Our results suggest that the cardiac defect in MEKK3-deficient mouse embryos may be secondary to calcineurin/NFAT deficiency, since targeted disruption of NFATc3 and NFATc4 genes also leads to embryonic lethality at embryonic day 10.5 and with similar cardiac defects seen in MEKK3-deficient embryos (thin ventricles, pericardial effusion, and a reduction in ventricular myocyte proliferation) (43). Thus, the NFAT deficiency resulting from MEKK3 deficiency may contribute to the cardiac defects seen in MEKK3-deficient mouse embryos. In addition, MEKK3 also functions upstream of another transcription factor, MEF2C, which is important in early cardiovascular development (42). Mice deficient in MEF2C show early cardiovascular defects, similar to those observed in MEKK3-deficient mice (41). Thus, the similar cardiovascular phenotype seen in MEKK3, NFAT, and MEF2C null mice imply that cardiovascular defects in MEKK3-deficient mice may be due to defective NFAT and/or MEF2C activity. Taken together, our current findings, along with other early studies, imply that MEKK3 plays an essential role in cardiac hypertrophy and cardiovascular development by controlling the functions of two key cardiac transcription factors: NFAT and MEF2C.

MEKK3 Functions Downstream of the Angiotensin Receptor to Mediate MCIP1 Phosphorylation—the MAPKs play an essential role in regulating many critical cellular processes including growth, differentiation, apoptosis, and stress-related responses. In this respect, a large diversity of extracellular stimuli activate MAPKs, including growth factors, hormones, cytokines, antigens, and many physical chemical stimuli such as oxidative stress and heat shock. These diverse extracellular stimuli initiate signals via receptors or “sensors,” which activate intracellular protein kinase cascades. The downstream signaling pathways of MAP3Ks have been intensively studied and include activation of multiple kinases, c-Jun N-terminal kinase, p38, extracellular signal-regulated kinase, and BMK1. Our studies demonstrate that active MEKK3 is required for calcineurin/NFAT activation. Thus, it is important to determine the signaling cascade downstream of MEKK3 that mediates calcineurin/NFAT activation.

The upstream signaling pathways that regulate and activate MAP3Ks remain largely unknown. It has been reported that MEKK3 physically associates with interleukin-1-associated protein to regulate downstream gene expression (44). Other studies have shown that MEKK3 functions downstream of RIP and TRAF2 in tumor necrosis factor receptor-mediated signaling (42, 44). Our studies are the first to show that Ang II induces phosphorylation of MEKK3 and that MEKK3 functions downstream of the AT1 receptor to mediate calcineurin/NFAT activation. Calcium-dependent activation of calcineurin is well recognized in cardiac hypertrophy. Our studies indicate that MEKK3 signaling is also essential for calcineurin activation in response to Ang II stimulation. Whether the MEKK3 pathway and the calcium-dependent pathway regulate calcineurin signaling in a parallel fashion or in an upstream and downstream fashion in response to Ang II stimulation has not been addressed. Ang II-mediated calcineurin/NFAT activation is in part mediated through Gαq-coupled signaling. Recent studies indicate that WNK1 acts upstream of MEKK3 to activate BMK1 (45). Therefore,
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FIGURE 8. Proposed model of Ang II-induced calcineurin/NFAT activation by MEKK3 signaling. Using cardiac hypertrophy as a model system, we have shown that Ang II induces calcineurin/NFAT activation through phosphorylation of MEKK3. We propose that phosphorylated MEKK3 acts upstream in a signaling cascade leading to MCIP1 phosphorylation. The phosphorylation of MCIP1 switches its inhibitory effect to stimulatory effect on calcineurin/NFAT signaling. The kinase that directly mediates MCIP1 phosphorylation has not been identified.

...It is possible that MEKK3 may function downstream of the AT1 receptor via activated WNK1 signaling to mediate calcineurin/NFAT activation. Recent studies report that dimerization through the catalytic domain is essential for MEKK2 activation (46). Thus, dimerization of MEKK3 mediated by AT1 receptor activation may be another possible mechanism for MEKK3 activation. The signaling pathways linking AT1 receptor activation to MEKK3 phosphorylation are the subject of ongoing investigations. It is also important to examine whether MEKK3 is essential for calcineurin/NFAT activation in response to other hypertrophic stimuli. The results obtained from these experiments will shed light regarding the role of MEKK3-mediated calcineurin/NFAT signaling in cardiac hypertrophy.

MEKK3-induced MCIP1 Phosphorylation Enhances Calcineurin Signaling—Our studies indicate that overexpression of MCIP1 can inhibit MEKK3-induced NFAT activation. This result is consistent with a number of previously published observations regarding the inhibitory effects of MCIP1 on calcineurin activity in genetically modified mice. For example, mice with a targeted disruption of the MCIP1 gene show an exaggerated hypertrophic response when crossed to β-MHC-CnA transgenic mice. These findings support the hypothesis that MCIP1 acts as a suppressor of calcineurin signaling (47). Targeted disruption of the MCIP1 gene (termed calspressin I by Ryem et al. (29)) results in enhanced calcineurin activity in lymphocytes and the activation of a number of immune response genes regulated by NFAT. Transgenic mouse studies by Hill et al. (33) showed that over expression of MCIP1 in the heart suppresses hypertrophy in response to activated calcineurin as well as to isoproterenol infusion, aortic banding, and voluntary wheel running. These in vivo transgenic findings are analogous to our in vitro observations that overexpression of MCIP1 in cardiac myocytes prevented MEKK3-mediated calcineurin activation and the cardiac hypertrophic transcriptional response.

Our studies provide evidence that the inhibitory effects of MCIP1 on calcineurin activity are relieved by an inducible protein kinase. Our results support a model in which hypertrophic stimulation (e.g. Ang II) results in calcineurin/NFAT activation via MEKK3-induced MCIP1 phosphorylation (Fig. 8). Our findings are in good agreement with a recent report showing that phosphorylation of RCN1, a yeast ortholog of MCIP1, activates calcineurin signaling (36). However, at this moment, the direct kinases that function downstream of MEKK3 to mediate MCIP1 phosphorylation and how the phosphorylation of MCIP1 switches its inhibitory effect to a stimulatory effect on calcineurin signaling are still unknown. Using the NFAT-luciferase cotransfection bioassay, we have screened a series of constitutively active protein kinases (including phosphatidylinositol 3-kinase, mTOR, AMPK, TAK1, MEK5, MEK6, MEK7, c-Jun N-terminal kinase, and p38). Among these kinases, phosphatidylinositol 3-kinase, mTOR, AMPK, MEK6, p38, and c-Jun N-terminal kinase failed to activate the NFAT-luciferase reporter. In addition to constitutively active MEKK3, only constitutively active TAK1 and MEK5 activated the NFAT-luciferase reporter, suggesting that TAK1 and/or MEK5 may function in parallel or in series with MEKK3 to induce NFAT activation.

In addition, sequence analysis of MCIP1 by the Scansite motif search program predicts that glycoprotein synthase kinase 3β and a MAPK, but not MEKK3 itself, mediate MCIP1 phosphorylation at Ser110 and Ser112 in the SP domain. MEK5 and BMK1 are protein kinases that function downstream of MEKK3 and may be the potential kinases downstream of MEKK3 to mediate MCIP1 phosphorylation (48). In addition, glycoprotein synthase kinase 3β has been previously shown in vitro to directly phosphorylate MCIP1 (35). Hilioti et al. (36) showed that phosphorylation of RCN1 by Mck1, a member of the glycoprotein synthase kinase 3 family of protein kinases, stimulates calcineurin signaling. Thus, it is also possible that glycoprotein synthase kinase 3β functions downstream of MEKK3 to mediate MCIP1 phosphorylation. Future investigation to identify the kinase(s) that phosphorylate MCIP1 will greatly advance our current understanding of the role of protein kinase-mediated regulation of calcineurin signaling in cardiac hypertrophy. These studies will reveal potential attractive therapeutic targets to inhibit cardiac hypertrophy and even heart failure at the early stage.

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