Ras Regulates NFAT3 Activity in Cardiac Myocytes*

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Multiple distinct signal transduction pathways have been implicated in the development of cardiac myocyte hypertrophy. These hypertrophic pathways include those regulated by the Ras superfamily of small GTPases and a separate calcineurin-regulated pathway that culminates in the activation of the transcription factor NFAT3. In this report, we demonstrate a functional interaction between Ras-regulated and calcineurin-regulated pathways. In particular, expression in neonatal myocytes of a constitutively active form of Ras (V12ras), but not activating mutants of Rac1, RhoA, or Cdc42, results in an increase in NFAT activity. Similarly, expression of an activated Ras, but not other small GTPases, results in the nuclear translocation of an NFAT3 fusion protein. Expression of a dominant negative ras gene product blocks phenylephrine-stimulated NFAT transcriptional activity and the ligand-stimulated NFAT3 nuclear localization. Ras proteins appear to function upstream of calcineurin, because cyclosporin A blocks the ability of V12ras to stimulate NFAT-dependent transcription and nuclear localization. Similarly, expression of a dominant negative ras gene inhibits phenylephrine-stimulated calcineurin activity. Pharmacological inhibition of MEK1 or expression of a dominant negative form of c-Raf or ERK2 inhibits phenylephrine-stimulated NFAT3 activation. Conversely, NFAT activity was stimulated by expression of constitutively active forms of c-Raf or MEK1. Taken together, these results imply that, in cardiac myocytes, a Ras-regulated pathway involving stimulation of mitogen-activated protein kinase regulates NFAT3 activity.

Due to their inability to divide, terminally differentiated cardiac myocytes respond to a diverse range of stimuli, including hypertension, myocardial infarction, or inborn mutations in sarcomeric gene products by undergoing hypertrophy. Initially, this increase in myocyte size leads to an increase in contractile power allowing the heart to meet the increased demands induced by these precipitating genetic or acquired conditions. However, with time this initially beneficial response turns maladaptive. Indeed, the presence of cardiac hypertrophy car-

1 The abbreviations used are: ANF, atrial natriuretic factor; BNP, B-type natriuretic peptide; IL-2, interleukin-2; JNK, c-Jun N-terminal kinase; HA-JNK, epitope-tagged form of JNK; MAPK, mitogen-activated protein kinase ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol 3-kinase; bp, base pair(s); GFP, green fluorescence protein.

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mune cells have demonstrated that T cell activation results in an increase in intracellular calcium leading to the subsequent activation of the cytoplasmic phosphatase calcineurin. Once activated, calcineurin dephosphorylates NFAT resulting in nuclear translocation of NFAT. Once in the nucleus, NFAT binds DNA in a sequence-specific fashion to activate expression of a host of gene products involved in T cell activation. The best studied example of NFAT-mediated gene expression involves interleukin-2 (IL-2) production following T cell activation.

The importance of the pathway described above is evident by the discovery that the immunosuppressant cyclosporin A functions by inhibiting calcineurin and, hence, subsequent NFAT activation and T cell responsiveness. The relevance of this pathway to cardiac hypertrophy is underscored by the observation that cardiac-targeted transgenic animals expressing constitutively activated forms of either calcineurin or NFAT3 produced ventricular hypertrophy (16). Similarly, pharmacological inhibition of calcineurin by the administration of cyclosporin A inhibited some (16, 18, 19) but not necessarily all (19–21) forms of genetic or acquired ventricular hypertrophy.

Given the expanding list of pathways implicated in the hypertrophic signaling cascade, it seems possible that some degree of crosstalk or interactions might exist between these seemingly diverse members. In this report, we have specifically attempted to understand whether there is any potential interaction between hypertrophic pathways regulated by the various small GTPases and the more recently described NFAT-dependent pathway.

**EXPERIMENTAL PROCEDURES**

**Myocyte Preparation**—Neonatal ventricular myocytes were prepared from hearts of 2-day-old Harlan Sprague-Dawley rats. Excised hearts were treated overnight with cold trypsin digestion in calcium and magnesium free Hepes-buffered salt solution followed by collagenase digestion in Leibovitz’s L-15 media (Worthington Biochemical, NJ). Myocytes were subsequently isolated by 40 trituration strokes, and debris was removed by passage through a 70-μm filter. The cells were pelleted by low speed centrifugation and subject to two 30-min rounds of preplating to allow for the purification of myocytes from contaminating fibroblasts. Previous immunohistochemical staining has verified that this procedure results in a population of cells that is over 90% myocytes (12). Cells were plated on laminin-coated 6-well dishes at a density of 3 × 10^5 cells/cm² in plating media that consisted of Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1, v/v) with 15 mM Hepes, pH 7.5, 2 mg/ml bovine serum albumin, 10 mg/liter insulin, 5.5 mg/liter transferrin, 5 μg/liter selenium, 2 mg/liter ethanolamine, 10,000 units of penicillin, and 10,000 μg/ml streptomycin supplemented with 5% horse serum (Life Technologies, MD). Cells were transfected 24 h after plating. Eighteen hours after transfection, the medium was changed to serum free plating medium, and phenylephrine (Sigma Chemical Co., St. Louis, MO), cyclosporin A (Sigma), or PD98059 (Calbiochem, La Jolla, CA) was added for the remaining 48 h prior to harvest.

**Plasmids and Transfection**—Plasmids encoding activated forms of Ras (V12ras), Rac1 (V12rac), RhoA (V14rhoA), Cdc42H (V12cdc42H), c-Raf (Raf BXB), or MEK1 (MEK-E) or plasmids encoding dominant negative forms of Ras (N17ras), Rac1 (N17rac), RhoA (N19rhoA), c-Raf (Raf301), and ERK2 (ERK185) have been described previously (22–24). All plasmids were generous gifts of S. Gutkind (National Institutes of Health). The Ras effector mutant (V12/C40), which exclusively activates phosphatidylinositol 3-kinase (PI3K) but not c-Raf or other Ras effector molecules, has also been described previously (25). For analysis of NFAT activity, a luciferase reporter containing three tandem repeats of the NFAT3-binding sites derived from the BNP promoter was routinely used. To confirm the effects seen with V12ras alone. Amount of total transfected empty vector or small GTPase was maintained at 6 μg. C, effects of V12ras or V14rhoA on NFAT activity using a reporter plasmid containing two tandem repeats of the NFAT3-binding sites derived from the BNP promoter.

**FIG. 1.** V12ras expression activates NFAT activity. A, cardiac myocytes were transfected with plasmids encoding constitutively active forms of Ras, Rac1, RhoA, or Cdc42 along with a NFAT-dependent reporter gene derived from the IL-2 promoter. All activity was normalized to an internal renilla transfection standard and is expressed as -fold change in NFAT activity compared with an empty vector control (−). B, addition of activated forms of Rac1 or RhoA does not potentiate the effects seen with V12ras alone. Amount of total transfected empty vector or small GTPase was maintained at 6 μg. C, effects of V12ras or V14rhoA on NFAT activity using a reporter plasmid containing two tandem repeats of the NFAT3-binding sites derived from the BNP promoter.

For luciferase assays, except where indicated, myocytes were transfected using 4 μg of the indicated small GTPase or corresponding empty vector and 1 μg of the indicated NFAT reporter construct and 0.1 μg of the pPRL-TK renilla transfection control plasmid. Cells were harvested 66 h after transfection, and the ratio of luciferase to renilla activity was measured using the dual luciferase assay in accordance with the man-
manufacturer’s recommendation (Promega, WI). Where indicated, cells were treated with either phenylephrine, cyclosporin A, wortmannin, or the MEK1 inhibitor PD98059 (20 μM). All experiments were performed at least three times, and one representative experiment performed in triplicate is routinely shown. Statistical significance (*) is determined by a paired t test with a value of \( p < 0.05 \) considered significant.

**NFAT3 Localization**—Following transfection with GFP-NFAT3 fusion protein construct (2.5 μg) and a construct encoding the indicated small GTPase, dominant negative c-Raf, ERK2, or empty vector control (2.5 μg), cells were visualized by a Nikon TE300 fluorescence microscope. For quantification purposes, cells were considered positive if the nuclear fluorescence was the sole or predominant localization of GFP-NFAT3. Values represent the mean percentage ± S.D. of cells demonstrating nuclear predominant fluorescence obtained from three wells of a single representative experiment (~200 cells).

**Kinase and Phosphatase Assays**—For analysis of c-Jun N-terminal (JNK) kinase activity, myocytes were transiently transfected with mammalian expression vectors encoding an epitope-tagged form of JNK (HA-JNK) with or without the indicated small GTPase. Twenty-four hours after transfection, cells were washed three times and maintained in serum-free medium for an additional 12 h. Unstimulated cells or cells stimulated for 30 min with phenylephrine (50 μM) were subsequently harvested in lysis buffer as described previously (26). Transfected HA-JNK was immunoprecipitated from 30 μg of protein lysate using an anti-HA mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The activity of the immune complex was assayed using 2 μg of GST-ATF-2 as a substrate (26). The reactions were terminated with SDS-polyacrylamide gel electrophoresis sample buffer, and samples were then subjected to electrophoresis on 12% polyacrylamide gels followed by autoradiography.

For assessment of calcineurin activity, cells were infected with a recombinant adenovirus encoding N17ras (Ad.N17ras) or a control adenovirus lacking a transgene (Ad.d3312). Both viruses have been described previously (27), and the infection was performed at a multiplicity of infection of 25. Twenty-four hours after infection, the viral particles were removed and the media was replaced with serum free media with or without phenylephrine. Forty eight hours later, cardiomyocytes were harvested in lysis buffer (50 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 1 mM EDTA, 1 mM CaCl2) supplemented with protease inhibitor mixture (Roche Molecular Biochemicals), and clarified by centrifugation. The Quantizyme assay system AK-804 was performed by using 1 μg of protein lysate according to the manufacturer’s procedure (BIOMOL, Plymouth Meeting, PA). Calcineurin phosphatase activity was measured spectrophotometrically by detecting free-phosphate released from the calcineurin-specific RII phosphopeptide as described previously (28). Results are from one of three similar experiments each performed in triplicate.

**RESULTS**

To understand the relationship between the Ras family of GTPases and the regulation of NFAT3, we transfected cultures of neonatal rat myocytes with plasmids encoding constitutively active forms of Ras, Rac1, RhoA, and Cdc42 along with an NFAT-dependent reporter plasmid. As demonstrated in Fig. 1A, expression of an activated ras gene (V12ras) but not similar
forms of Rac1, Cdc42, or RhoA resulted in an increase in NFAT activity. In six separate experiments, each performed in triplicate, expression of V12ras resulted in anywhere from a 2- to 6-fold increase in NFAT activity. This degree of increased NFAT activation seen with V12ras was comparable to what we observed with phenylephrine stimulation (see Fig. 3). Because separately, activated forms of Rac1 and RhoA were incapable of activating NFAT, we next tested whether these small GTPases could synergize with V12ras. As seen in Fig. 1, addition of activated forms of RhoA or Rac1 to V12ras tended to inhibit activation rather than potentiate V12ras-stimulated reporter gene activity. The basis for this inhibition is unknown but may reflect competition for shared downstream molecules.

The NFAT reporter used in Fig. 1 (A and B) represents a sequence derived initially from the promoter of IL-2, a known NFAT-responsive gene in T cells. The predominant form of NFAT in cardiac myocytes is NFAT3. Recent evidence suggests that BNP expression in myocytes is regulated by NFAT3, and an authentic consensus binding site has been found in the BNP promoter (16). To confirm the effects of V12ras using an authentic NFAT3 binding site, we constructed a reporter plasmid containing two tandem NFAT3 sites, derived from the BNP promoter, upstream of luciferase. As demonstrated in Fig. 1C, expression of V12ras stimulated NFAT activity using this reporter. Again activation of NFAT activity was specific for Ras and not shared by other small GTPases such as V12rc. Using this reporter, the level of activation observed with V12ras expression, as well as with phenylephrine stimulation (data not shown), tended to be slightly less robust than with the standard NFAT reporter. These differences may relate to the spacing of the tandem NFAT3 sites or due to the fact that NFAT proteins predominantly bind in a cooperative fashion with other transcription factors (17).

Activation of NFAT involves the dephosphorylation of the transcription factor by calcineurin, resulting in its translocation from the cytosol to the nucleus. To confirm the effects of V12ras on NFAT activity in neonatal myocytes, we constructed a GFP-NFAT3 fusion construct. Transfection of this construct into myocytes revealed that, under basal conditions, the fusion protein was either distributed in the cytosol with nuclear exclusion of GFP or a combination of both cytosol and nucleus (Fig. 2, A and B). A nuclear-predominant fluorescence was routinely observed in less than 5% of control cells. As demonstrated in Fig. 2C, expression of V12ras resulted in a shift in GFP-NFAT3 localization with a significant number of cells now displaying nuclear predominant fluorescence. Quantification of these observations confirmed that activated Ras expression, but not activated forms of other small GTPases, resulted in a 5- to 10-fold increase in the number of cells expressing a nuclear predominant form of the GFP-NFAT3 fusion protein (Fig. 2D).

We next sought to understand the role of Ras proteins in physiological activation of NFAT in cardiac myocytes. To begin to address this, we transfected myocytes with dominant negative forms of either Ras, Rac1, or RhoA proteins and stimulated cells with phenylephrine. As previously noted, phenylephrine led to an ~3-fold increase in NFAT activity (Fig. 3). Expression of N17ras had no effect on phenylephrine-stimulated NFAT activity. The functional effects of N17ras expression in cardiac myocytes was confirmed by analyzing phenylephrine-stimulated c-jun N-terminal kinase (JNK) activity. As seen in Fig. 3B, N17ras expression inhibited the activation of JNK by phenylephrine under conditions in which it had no effect on phenylephrine-stimulated NFAT activity. Similarly, in a separate series of three experiments performed in triplicate, expression of N19RhoA did not effect phenylephrine-stimulated NFAT activity (control = 2.2 ± 0.8-fold and N19RhoA = 2.8 ± 1.2-fold, p = 0.57). In contrast, expression of N17ras reduced phenylephrine-stimulated NFAT reporter activity (Fig. 3A). Similarly, treatment of cells with phenylephrine led to an increased nuclear localization of the GFP-NFAT3 fusion protein (Fig. 4A). The ability of phenylephrine to stimulate this nuclear translocation was significantly inhibited by N17ras expression (Fig. 4B).

We next sought to analyze at what level Ras intersects with the NFAT3 pathway. To understand whether this occurs upstream or downstream of calcineurin, we transfected myocytes with V12ras and subsequently treated cells with the calcineurin inhibitor cyclosporin A. As demonstrated in Fig. 5A, the addition of cyclosporin A inhibited in a concentration-dependent fashion the ability of V12ras to stimulate NFAT activity. Similarly, treatment of cells with cyclosporin A blocked V12ras-stimulated NFAT nuclear localization (Fig. 5B). These results argue that the effects of Ras are most likely upstream of calcineurin activation. To further pursue this notion, we infected cells with a recombinant adenovirus encoding N17ras or with a control adenovirus lacking a transgene. As demonstrated in Fig. 6, in control infected cells stimulation of myocytes with phenylephrine led to an increase in calcineurin activity consistent with previous results (28). In cells expressing N17ras, although basal calcineurin activity was not significantly effected, the rise in calcineurin activity seen following ligand stimulation was inhibited.

Ras proteins activate a number of downstream effector pathways. Perhaps the best characterized pathway involves the activation of MAPK that proceeds through c-Raf and MEK1 activation (29). To test whether this pathway was involved in NFAT activation in cardiac myocytes, we treated cells with the specific MEK1 inhibitor PD98059. As seen in Fig. 7A, treat-
ment of cells with this compound inhibited NFAT transcriptional activity induced by either V12ras expression or by phenylephrine stimulation. Similarly, PD98059 treatment inhibited nuclear translocation of GFP-NFAT3 induced by either V12ras expression or phenylephrine stimulation (Fig. 7B).

Consistent with the results obtained by pharmacological inhibition, as demonstrated in Fig. 8, expression of a dominant negative form of c-Raf or ERK2 prevented phenylephrine-stimulated NFAT3 nuclear localization. To further address whether the activation of the MAPK pathway was sufficient to activate NFAT, we expressed constitutively activated forms of c-Raf or MEK1. As seen in Fig. 9, these constructs activated NFAT activity to a similar degree as V12ras. In addition, the ability of both the activated c-Raf and MEK1 constructs to stimulate NFAT activity was inhibited by cyclosporin treatment.

Although these results suggest a significant role for a pathway involving Ras-Raf-MEK1 and MAPK, it does not exclude a role for other Ras-regulated pathways. One downstream effector of Ras implicated in cardiac hypertrophy is PI3K (30). To ascertain whether this Ras-regulated pathway participated in V12ras-stimulated NFAT activation, we asked whether the ability of V12ras to stimulate NFAT activity could be inhibited by the PI3K inhibitor wortmannin. As demonstrated in Fig. 10A, treatment of V12ras-expressing cells with wortmannin produced a modest but concentration-dependent inhibition of NFAT activity. Similarly, expression of the Ras effector mutant V12/C40 capable of specifically activating PI3K but not other Ras targets such as c-Raf produced a small increase in NFAT activity (Fig. 10B). These results suggest that, although activation of Raf-MEK1-MAPK-dependent pathways is necessary and sufficient for NFAT activation in cardiac myocytes, other Ras effector pathways may also participate.
DISCUSSION

Our results demonstrate an essential role for Ras proteins in the regulation of NFAT3 activity in cardiac myocytes. In particular, we demonstrate that activated Ras stimulates NFAT activity and nuclear translocation and a dominant negative form of Ras blocks phenylephrine-stimulated NFAT activation. The ability of V12ras or phenylephrine to stimulate NFAT activity is significantly inhibited by treatment with the pharmacological MEK1 inhibitor PD98059, suggesting that activation of MAPK is necessary for NFAT activation. This is also supported by the inhibition of phenylephrine-stimulated NFAT3 translocation seen with expression of a dominant negative form of c-Raf and ERK2. Our results also place the Ras/Raf/MEK1/MAPK pathway upstream of calcineurin, because cyclosporin blocks the ability of constitutively active mutants in this pathway to activate NFAT. In addition, expression of N17ras inhibits the rise in calcineurin activity seen following phenylephrine stimulation.

Previous studies in immune cells has also implicated Ras proteins in the activation of NFAT. Expression of V12ras can mimic in part T cell receptor activation, although expression of activated Ras is not by itself sufficient to activate NFAT (31). Coexpression of an activated form of calcineurin or treatment with calcium ionophores appears however to synergize with a Ras signal to produce full NFAT activation (31–34). In both T cells and other immune cells a role for Rac proteins has also been demonstrated with experiments demonstrating that a dominant negative form of Rac can inhibit NFAT activation (35, 36). The activation of NFAT3 in cardiac myocytes therefore appears to differ in significant fashion from immune cells. In particular, our studies suggest that V12ras is sufficient alone to activate NFAT3. In addition, we observed no effects of N17rac or other small GTPases on phenylephrine-stimulated NFAT activity. The level of activation seen with V12ras was comparable to what was observed with phenylephrine stimulation, and thus there is no evidence that an additional calcium-dependent stimulus was needed. These latter differences may relate to the large differences in calcium handling between immune cell and contractile cardiac myocytes.

The elucidation of multiple pathways that contribute to cardiac hypertrophy represent an important advance in our understanding of this condition. The development of therapeutic options to treat hypertrophy will undoubtedly be dependent on a deeper understanding of the inter-relations and hierarchy of

**FIG. 8.** Expression of a dominant negative c-Raf or ERK2 inhibits NFAT3 nuclear localization. The percentage of cells demonstrating nuclear predominant staining was assessed following phenylephrine stimulation in the presence or absence of a vector control or a dominant negative form of c-Raf or ERK2.

**FIG. 9.** Expression of an activated form of c-Raf (Raf-BXB) or MEK1 (MEK-E) activates NFAT in a calcineurin-dependent fashion. The level of NFAT transcriptional activity was determined in myocytes transfected either with empty vector or with activated forms of c-Raf or MEK1 and then treated with or without cyclosporin (CSA;10 ng/ml).

**FIG. 10.** A role for PI3K in Ras activation of NFAT. A, effects of increasing concentrations (nanomolar) of the PI3K inhibitor wortmannin on V12ras-stimulated NFAT activity. B, comparison of the NFAT stimulatory activity of V12ras with the effector mutant V12/C40 Ras that activates only PI3K-dependent pathways.
the various pathways. The results presented here place Ras and subsequent MAPK activation upstream of calcineurin and NFAT activation in cardiac myocytes. This underscores a central role for Ras in the development of hypertrophy. Our preliminary evidence suggests that an MAPK-dependent pathway represents the major pathway through which Ras proteins activate NFAT. Nonetheless, other Ras effectors may also contribute as we do observe a small but noticeable contribution from PI3K-dependent pathways in the activation of NFAT (Fig. 10).

Although this study increases our understanding of the molecular mechanisms underlying cardiac hypertrophy, many questions remain. For instance, it is unclear to what degree the activation of NFAT by Ras proteins contributes to the ability of Ras proteins to induce hypertrophy in vivo (6). Similarly, the mechanism through which MAPK activation leads to NFAT activation is unknown. Recent evidence suggests that a Raf/MEK1/MAPK-dependent pathway is involved in the regulation of intracellular calcium transients in cardiac myocytes (37). As such, these results are consistent with what is described here and suggest MAPK is upstream of calcineurin and may regulate the phosphatase by altering intracellular calcium levels.

The ability of Ras proteins to produce hypertrophy in vitro and in vivo (11, 12, 15) but their apparent inability, as demonstrated here, to regulate NFAT activity suggests that not all described pathways leading to hypertrophy overlap. The activation of c-jun N-terminal kinase (JNK) and the p38 MAPK regulators of NFAT activity suggests that not all Ras effectors may also contribute as we do observe a small but noticeable contribution from PI3K-dependent pathways in the activation of NFAT (Fig. 10).

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