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MAP kinase- and Rho-dependent signals interact to regulate gene expression but not actin morphology in cardiac muscle cells

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Post-natal growth of cardiac muscle cells occurs by hypertrophy rather than division and is associated with changes in gene expression and muscle fiber morphology. We show here that the protein kinase MEKK1 can induce reporter gene expression from the atrial natriuretic factor (ANF) promoter, a genetic marker that is activated during *in vivo* hypertrophy. MEKK1 induced both stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) activity; however, while the SAPK cascade stimulated ANF expression, activation of the ERK cascade inhibited expression. C3 transferase, a specific inhibitor of the small GTPase Rho, also inhibited both MEKK- and phenylephrine-induced ANF expression, indicating an additional requirement for Rho-dependent signals. Microinjection or transfection of C3 transferase into the same cells did not disrupt actin muscle fiber morphology, indicating that Rho-dependent pathways do not regulate actin morphology in cardiac muscle cells. While active MEKK1 was a potent activator of hypertrophic gene expression, this kinase did not induce actin organization and prevented phenylephrine-induced organization. These data suggest that multiple signals control hypertrophic phenotypes. Positive and negative signals mediated by parallel MAP kinase cascades interact with Rho-dependent pathways to regulate hypertrophic gene expression while other signals induce muscle fiber morphology in cardiac muscle cells.

Keywords: actin morphology/cardiac hypertrophy/MAP kinase/MEKK/Rho

Introduction

Cardiac muscle cells grow by proliferation during fetal development and hypertrophy after birth. Hypertrophic growth of neonatal cardiac muscle cells is associated with distinct changes in the pattern of gene expression and cell morphology. The hypertrophic phenotype can be monitored by analyzing renewed expression of fetal genes such as atrial natriuretic factor (ANF), and cell morphology changes such as the development of highly organized muscle fibers. In neonatal ventricular myocytes, hypertrophy can be induced by different kinds of stimuli including agonists whose receptors couple to heterotrimeric G proteins, growth factors whose receptors are tyrosine kinases, transforming growth factor β, mechanical stretch and phorbol esters that directly activate some isoforms of protein kinase C (Parker *et al.*, 1990; Chien *et al.*, 1991).

We have studied hypertrophic responses induced by phenylephrine, an α_1-adrenergic agonist whose receptor is coupled to a $G_{\alpha q}$-containing heterotrimeric G protein. Ras is biochemically activated by phenylephrine and other hypertrophic stimuli (Sadoshima and Izumo, 1993a,b, 1996; Thorburn, 1994). Ras activation is required for phenylephrine-induced hypertrophy and is sufficient to induce both morphological and genetic markers of hypertrophy (Thorburn *et al.*, 1993). In addition to effects on genes that are induced specifically by hypertrophic stimuli, Ras is also involved in the more general increase in gene expression that is associated with cardiac muscle cell growth (Abdellatif *et al.*, 1994). The Ras effector c-Raf is important in the hypertrophic response since active Raf is sufficient to induce hypertrophic gene expression and Raf activity is increased by hypertrophic stimuli (Thorburn *et al.*, 1994a; Bogoyevitch *et al.*, 1995b). Activation of the Raf–mitogen-activated/extracellular-signal regulated kinase kinase (MEK)–extracellular-signal regulated (ERK) protein kinase cascade also appears to be important in hypertrophy, although in a more complicated manner. Our laboratory and others have shown that interference with ERK activation with catalytically inactive ERK1, the protein phosphatase CL100 or antisense oligonucleotides can inhibit phenylephrine-induced gene expression (Thorburn *et al.*, 1994b, 1995; Glennon *et al.*, 1996). However, conflicting conclusions based on the use of a highly specific MEK inhibitor suggest that the simple conclusion that phenylephrine-induced ERK activation is directly required for expression of genes such as ANF may be incorrect (Post *et al.*, 1996). Furthermore, while active Raf or Ras can induce hypertrophic gene expression, we found that constitutively active MEK was unable to induce hypertrophic gene expression and actually inhibited phenylephrine-induced expression (Thorburn *et al.*, 1995). We have not found that interference with these protein kinases has any effect on the morphological phenotypes associated with the hypertrophic response (Thorburn *et al.*, 1994a,b) although Ras is sufficient to induce these effects. These data suggest that, as in other cells (Joneson *et al.*, 1996), different Ras effector molecules may mediate the different effects associated with Ras-dependent growth stimuli.

While hypertrophic growth is a normal developmental process, cardiac hypertrophy also occurs in adults in response to physiological stress such as ischemia/reperfusion and pressure overload resulting from chronic hypertension. Although initially a compensatory response to the

increased cardiac workload resulting from these stimuli, continued hypertrophy is associated with cardiac dysfunction and eventual heart failure (Levy *et al.*, 1990). *In vivo*, hypertrophic cardiomyopathy may also involve activation of Ras-dependent signaling pathways since transgenic mice that express constitutively active Ras in their left ventricles display many of the features of this syndrome (Hunter *et al.*, 1995). In ventricular myocytes, several hypertrophic stimuli induce activation of the stress-activated protein (SAP) kinases (also called JNKs) (Bogoyevitch *et al.*, 1995a) that function in a parallel mitogen-activated protein (MAP) kinase cascade to the ERKs. It seems likely that activation of these kinases and downstream transcription factors may play a role in the regulation of cardiac hypertrophy as a result of physiological stress. The upstream MAP kinase kinase kinase that plays the equivalent role of Raf in the SAPK pathway is thought to be the kinase MEKK1. MEKK1 was identified initially as a kinase capable of activating MEK, the MAP kinase kinase that activates ERK1 and ERK2 (Lange-Carter *et al.*, 1993). However, MEKK1 is now thought to be primarily responsible for activation of SEK (also known as JNKK) and the SAPK family of MAP kinases (Minden *et al.*, 1994; Yan *et al.*, 1994; Derijard *et al.*, 1995).

Since we had suggested previously that activation of the ERK pathway was important in cardiac hypertrophy but *in vivo* hypertrophy is often a consequence of physiological stress, we were interested in determining whether activation of the SAPK pathway by MEKK could affect hypertrophic phenotypes. We find that MEKK1 will induce hypertrophic gene expression and that this is regulated via both positive and negative signals mediated by the SAPK and ERK protein kinase cascades. In addition, Rho-dependent signals are required for ANF gene expression. The signals that stimulate hypertrophic gene expression are seperable from those that induce muscle fiber formation.

Results

MEKK1 induces ANF gene expression

To test whether MEKK1 could activate hypertrophic gene expression, we performed transient transfection experiments with an ANF-luciferase reporter plasmid and varying amounts of an expression plasmid encoding either a full-length, wild-type MEKK1 molecule or a catalytically inactive mutant (D1369A) (Xu *et al.*, 1996). Figure 1A shows that the wild-type molecule but not the inactive mutant activated the ANF promoter in a dose-dependent manner. These data indicate that overexpression of wild-type MEKK1 is sufficient to induce expression of a gene that is activated during cardiac hypertrophy.

To test the effects of a constitutively active MEKK1 molecule, we co-transfected the reporter plasmid regulated by the ANF promoter with various amounts of an expression plasmid encoding a truncated MEKK1 molecule lacking the putative N-terminal regulatory domain (MEKK1-c) (Xu *et al.*, 1995). As a control, we transfected an AP1-luciferase plasmid since AP1 is a known target for the signaling pathways that are activated by MEKK1. Figure 1B shows that AP1-driven gene expression is stimulated in a dose-dependent manner by MEKK1-c, confirming that the molecule was active and behaving as expected. Increased MEKK1 activity also caused increased activation of AP1 from phenylephrine-stimulated cells. When the ANF promoter was examined (Figure 1C), we found that small amounts of the MEKK1-c expression plasmid were sufficient to induce significant luciferase expression. However, larger amounts of the plasmid inhibited expression irrespective of whether or not the cells were stimulated with the bona fide hypertrophic agonist phenylephrine. Maximal MEKK1-c-induced expression was achieved with 100 ng of expression plasmid per transfection reaction, and this level of gene activation was significantly higher than that observed with phenylephrine alone. This amount of MEKK1-c expression plasmid did not produce maximal AP1-driven expression.

Based on the previous identification of SEK as a preferred substrate over MEK for MEKK (Minden *et al.*, 1994; Yan *et al.*, 1994; Cavigelli *et al.*, 1995), a possible explanation for these results is that small amounts of MEKK1-c were preferentially activating the SEK–SAPK pathway and leading to induction of ANF-driven expression. Larger amounts of the MEKK1-c expression plasmid might also activate MEK and ERK, leading to inhibition via the mechanisms through which active MEK inhibits expression in phenylephrine-treated cells (Thorburn *et al.*, 1995). To test whether the two families of MAP kinases were activated differentially by different amounts of MEKK1-c in our experiments, we performed transient transfections with epitope-tagged molecules. Myc-tagged ERK2 and HA-tagged SAPKβ were co-transfected along with increasing amounts of MEKK1-c in the same ratios that were used in the reporter gene expression assays. The tagged molecules were immunoprecipitated and used in *in vitro* kinase assays with myelin basic protein (MBP) or GST–Jun as substrates. Figure 1D shows that both ERK2 and SAPKβ were activated efficiently even by small amounts of MEKK1-c, and there was no obvious preference for SAPK activation over ERK activation in our cells as has been found in other cell types (see, for example, Cavigelli *et al.*, 1995). The amount of MEKK1-c expression plasmid that resulted in maximal ANF-driven expression (100 ng) caused both kinases to be significantly activated.

Effects of active MEK and MEKK on kinase activation and gene expression

Previously, we reported that active MEK was not able to induce ANF-luciferase expression (Thorburn *et al.*, 1995); however, other investigators have reported that similar constitutively active MEK molecules can in fact induce hypertrophic gene expression (Gillespie-Brown *et al.*, 1995). Since we found that active MEKK1-c was an effective activator of the ANF promoter, we designed an experiment to test whether the introduction of active MEK to induce similar amounts of ERK activity resulted in similar amounts of gene expression in cardiac muscle cells.

Figure 2 shows an experiment where various amounts of either an active MEK mutant (ΔN3, S218E, S222D) (Mansour *et al.*, 1994) or the MEKK1-c expression plasmid were transfected into cardiac cells along with tagged Myc-ERK2 and HA-SAPKβ or AP1- or ANF-luciferase reporters. Figure 2A shows IP kinase assays of the Myc-ERK2 and HA-SAPKβ molecules, indicating that both MEKK1-c and MEK could efficiently increase ERK

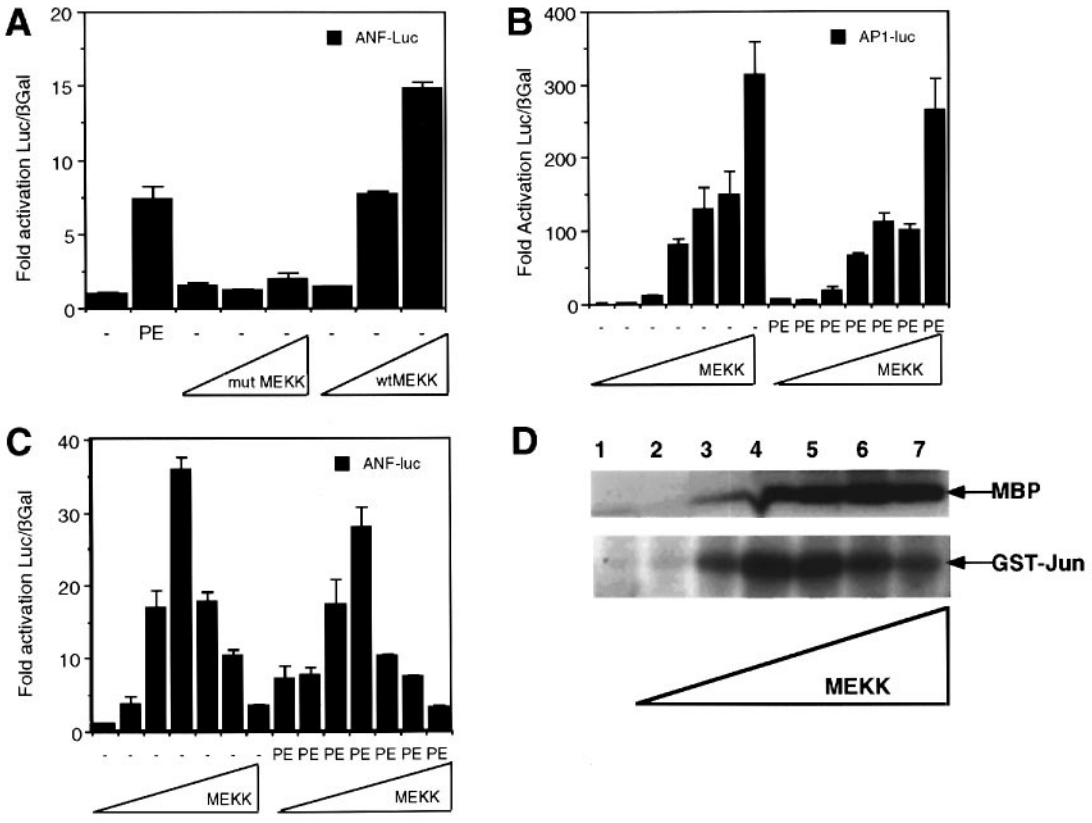

Fig. 1. MEKK induces ANF expression and activation of ERK2 and SAPKβ. (**A**) Empty expression plasmid plus or minus 100 μM phenylephrine (PE) or increasing amounts (10 ng, 100 ng or 3 μg supplemented with empty expression plasmid to make the total 3 μg) of catalytically inactive (mut MEKK) or wild-type MEKK1 (wt MEKK) expression plasmids were transfected along with RSVβGal and ANF-luciferase reporters. (**B**) Increasing amounts of MEKK1-c expression plasmid (1, 10, 100 and 500 ng, 1 and 3 μg, supplemented with empty expression vector to make the total 3 μg) were transfected along with RSVβGal and AP1-luciferase and the cells treated with (PE) or without (–) 100 μM phenylephrine. (**C**) Cells were transfected with the same amounts of MEKK1-c expression vector as in (B) plus ANF-luciferase and RSVβGal. (**D**) Cells were transfected in 6 cm dishes (3× the area used in gene expression experiments) with the same ratios of MEKK1-c expression vector (i.e. 3, 30 and 300 ng, 1.5, 3 or 9 μg plus empty vector as required) plus pEFMycERK2 and pEFHASAPKβ. Kinases were immunoprecipitated and used in kinase assays with MBP and GST–Jun as substrates.

activity while, as expected, only MEKK1-c could elevate SAPK activity. Figure 2B and C indicates that both MEKK1-c and MEK were efficient inducers of AP1-driven gene expression. Conversely (Figure 2D and E), only MEKK1-c was able to induce significant ANF-luciferase expression. As before, increasing the amount of MEKK1-c in the transfection mix beyond 100 ng per reaction led to repression of ANF-driven gene expression. While it is clear that MEKK1-c was a more effective ERK activator than MEK, the amount of ERK activity that is stimulated by the small amounts of MEKK1-c expression plasmid that induce maximal ANF-luciferase expression (100 ng) can be achieved by slightly more MEK expression plasmid (500 ng). Thus ERK activation is not sufficient to explain ANF gene expression.

ERK and SAPK differentially regulate MEKK1-c-induced ANF expression

To test whether activation of the MAP kinases was required for ANF gene expression, we expressed the human phosphatase CL100 which dephosphorylates and thus inactivates MAP kinases (Alessi *et al.*, 1993). The mouse homolog of CL100 has been reported preferentially to dephosphorylate ERKs rather than SAPKs (Sun *et al.*, 1994); however, it is clear that various isoforms of SAPK/JNKs are also inactivated by this phosphatase (Gupta *et al.*, 1996). We found that expression of even small amounts of CL100 in cardiac muscle cells is effective at inhibiting both SAPKβ and ERK2 that had been activated by co-transfection of MEKK1-c (Figure 3A). Figure 3B indicates that expression of CL100 inhibited MEKK1-c-induced ANF-luciferase expression in a dose-dependent manner. Previously, we found that CL100 inhibited phenylephrine-induced ANF-luciferase expression (Thorburn *et al.*, 1995). These data indicate that ERK, SAPK or activity from both kinases are required for ANF expression.

In an attempt to inhibit the SAPK pathway more specifically, we also performed experiments with catalytically inactive versions of SEK and SAPKβ. Figure 3C shows that transient transfections with either inactive SEK or an inactive SAPKβ partially inhibit both phenylephrine- and MEKK1-c-induced ANF-luciferase expression. The extent of inhibition of phenylephrine-induced expression was less than that for MEKK1-c-induced expression. We also overexpressed a truncated, dominant-negative Jun molecule (ΔNJun) that lacks the *trans*-activation domain of Jun. This molecule has been shown previously to prevent neuronal cell apoptosis (Ham *et al.*, 1995; Xia *et al.*, 1995), a phenotype that can be induced by MEKK. Expression of ΔNJun also inhibited MEKK1-c-induced

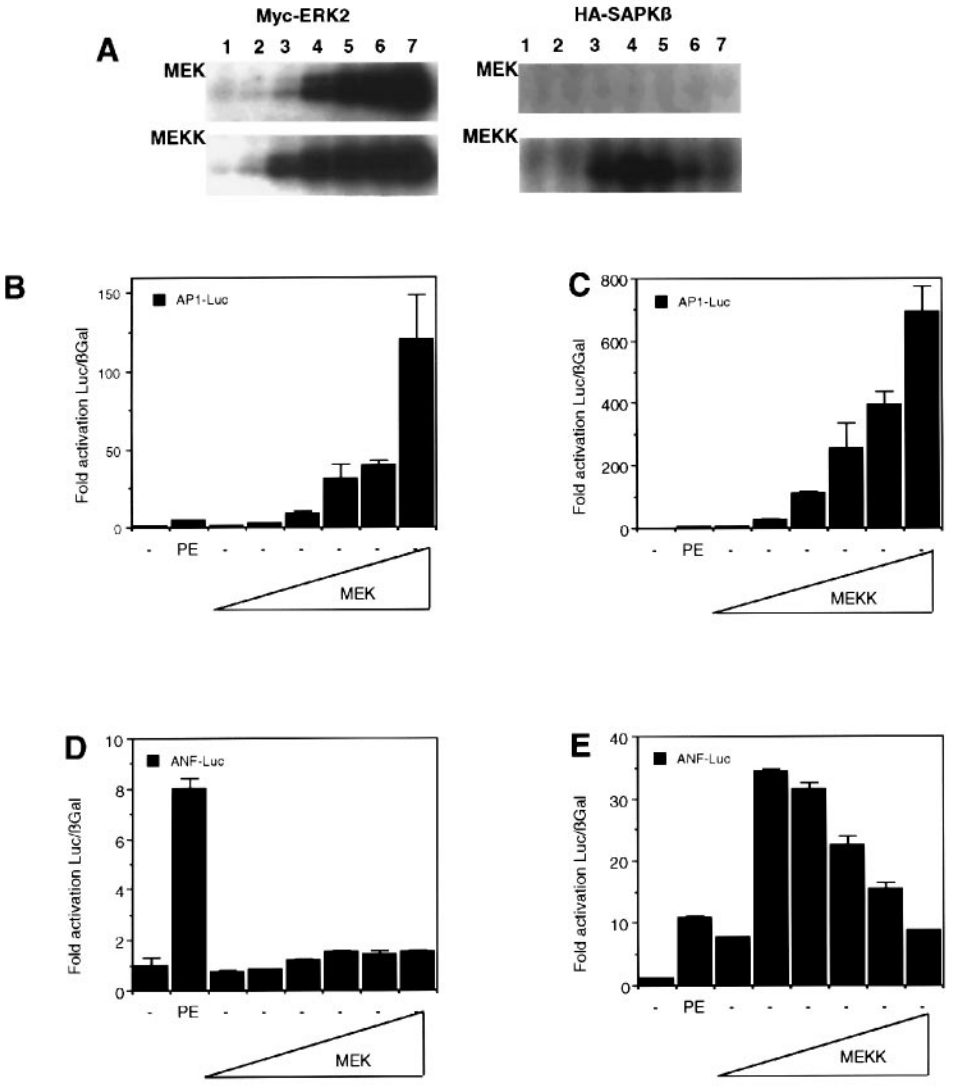

Fig. 2. Effects of MEK and MEKK on MAP kinase activation and gene expression. (**A**) Epitope-tagged kinase assays were performed in 6 cm dishes with Myc-ERK2 and HA-SAPKβ plus increasing amounts (3, 30 and 300 ng, 1.5, 3 and 9 μg) of either MEKK1-c expression plasmid or MEK (ΔN3, S218E, S222D) expression plasmid. (**B**) Increasing amounts of MEK or (**C**) MEKK1-c expression plasmids in the same ratios as in (A) were transfected with AP1-luciferase and RSVβGal. (**D**) The same amounts of the MEK or (**E**) MEKK1-c expression plasmids were transfected with ANF-luciferase plus RSVβGal.

ANF-luciferase expression but had little effect on phenylephrine-induced activation of this promoter. Taken together, these results suggest that SAPK and perhaps AP1 activation is important for ANF expression.

We also performed experiments to inhibit MEK and ERK signaling selectively in the MEKK1-c-transfected cells. In these experiments, we used the MEK-specific inhibitor PD 098059 (Alessi *et al.*, 1995; Dudley *et al.*, 1995). Cells were transfected with the reporter plasmids plus 100 ng of the MEKK1-c expression plasmid and treated with increasing amounts of PD 098059 to inhibit MEK activation (Alessi *et al.*, 1995). Figure 3D shows that treatment with the MEK inhibitor led to increased ANF-luciferase expression in the MEKK1-c-transfected cells. Control kinase assays demonstrated that PD 098059 treatment caused partial but not complete inhibition of MEKK1-c-induced Myc-tagged ERK2 activity without detectably affecting HA-tagged SAPK activity in the same cells (data not shown). Thus while MEKK1 stimulates both MEK–ERK and SEK–SAPK signaling, the two parallel pathways have opposing effects on ANF gene expression, with SAPK-dependent signals leading to stimulation of gene expression while MEK-dependent signals cause inhibition. We cannot discriminate between inhibitory effects on ANF promoter activity that are mediated by MEK itself or by MEK-induced ERK activity.

C3 transferase inhibits phenylephrine- and MEKK1-c-induced ANF expression

Recently, it has become clear that the small G protein Rho can send signals to the nucleus to activate the serum response factor (SRF) and regulate inducible gene expression (Hill *et al.*, 1995). This activity does not appear to involve activation of the currently identified MAP kinases (Hill *et al.*, 1995), although other Rho family GTPases, Rac and Cdc42, can induce SAPK activation (Minden *et al.*, 1995; Coso *et al.*, 1996). To test whether Rho activity was involved in ANF expression, we used the Rho-specific inhibitor C3 transferase.

Figure 4A shows that C3 transferase inhibits phenyl-

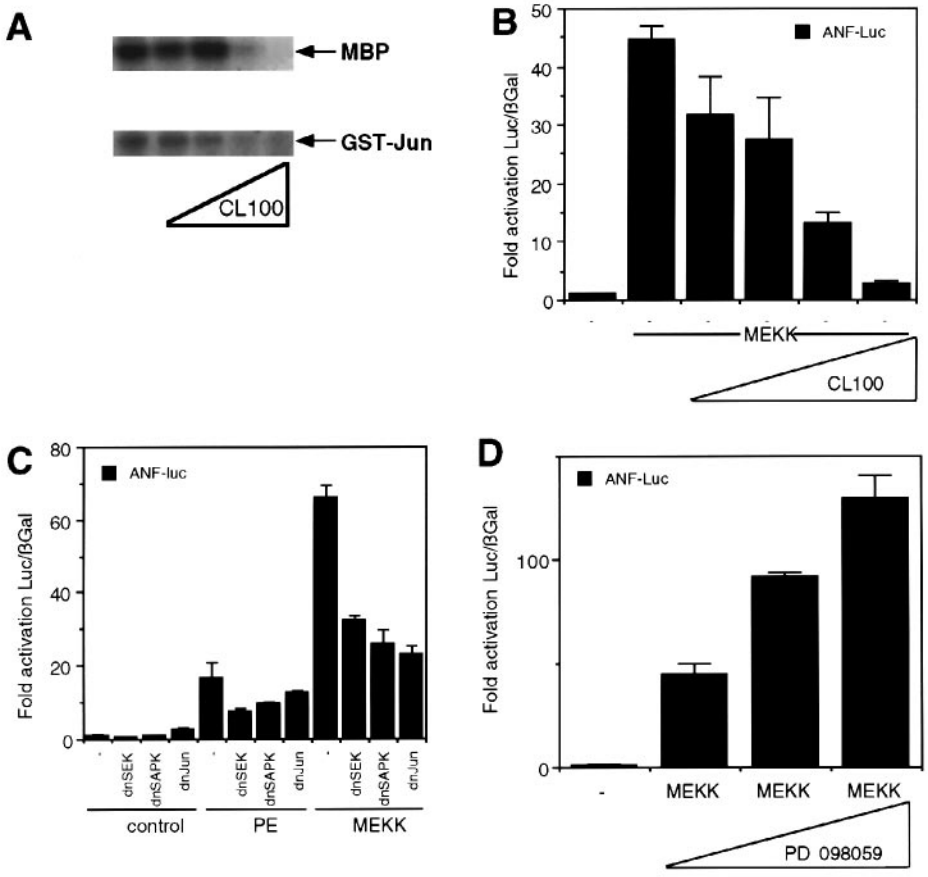

Fig. 3. Different MAP kinase cascades differentially regulate ANF gene expression. (**A**) Increasing amounts of CL100 expression plasmid (0, 3, 30 and 300 ng, 3 μg) were transfected along with tagged ERK2 and SAPKβ plus 300 ng of the MEKK1-c expression plasmid to induce kinase activation and used in immunoprecipitation kinase assays. (**B**) Increasing amounts of CL100 expression plasmid (0, 1, 10 and 100 ng, 1 μg) plus 100 ng of the MEKK1-c expression plasmid and ANF-luciferase and RSVβGal were transfected. (**C**) ANF-luciferase plus RSVβGal plasmids were transfected with empty vector and maintained in serum-free media (–) or treated with 100 μM phenylephrine (PE) or co-transfected with 100 ng of the MEKK1-c expression plasmid (MEKK) along with 3 μg of empty pEF expression plasmid (control), 3 μg of dominant-negative SEK (dnSEK), 3 μg of dominant-negative SAPKβ (dnSAPK) or 3 μg of dominant-negative Jun (dnJun). (**D**) Reporter plasmids were transfected with empty expression vector (–) or 100 ng of the MEKK1-c expression plasmid (MEKK) then treated with increasing amounts of the MEK inhibitor PD 098059 (0, 1, 10 μM) to inhibit MEK activation.

ephrine-induced ANF expression, suggesting that a Rho-dependent signal is required for α-adrenergic agonist-induced gene expression. To test whether Rho was sufficient to induce ANF expression, we performed transfection experiments with a constitutively active molecule (V14Rho). Figure 4B shows that this molecule is able to induce only modest ANF-luciferase activity, suggesting that while Rho may be required for expression, it is not sufficient for efficient expression from this promoter. We did not observe inhibition of phenylephrine-stimulated AP1 activity by C3 transferase (Figure 4C).

To determine whether Rho activity was also required for MEKK1-c-induced gene expression, we performed transfection experiments expressing C3 transferase plus MEKK1-c. Figure 4D shows that C3 transferase partially inhibited MEKK1-c-induced ANF expression. In parallel transfections, this amount of C3 transferase completely abolished phenylephrine-induced ANF expression. As before, C3 transferase did not inhibit AP1 activity (Figure 4E) induced by either phenylephrine or MEKK1-c.

Since we found that both catalytically inactive SAPKβ and C3 transferase partially inhibited MEKK1-c-induced ANF expression, we performed experiments using both inhibitors simultaneously. Figure 4F shows that the two molecules together inhibited expression beyond that observed with either molecule alone. These data indicate that the two inhibitory effects are additive, and suggest that at least two separate signals are required for MEKK1-c-induced ANF expression. One signal is inhibited by inactive SAPKβ and presumably involves SAPK activation, the other signal is inhibited by C3 transferase and is therefore Rho dependent. IP kinase assays indicated that expression of C3 transferase had no significant effect on MEKK1-c-induced ERK2 or SAPKβ activity (data not shown), further supporting the view that MAP kinase-dependent and Rho-dependent signals that regulate ANF expression are separate.

C3 transferase does not inhibit actin organization in cardiac muscle cells

Neonatal cardiac muscle cell hypertrophy is associated with obvious morphological changes. The cells increase in area, become more regularly shaped and develop highly organized muscle fibers as shown by staining to reveal filamentous actin. Figure 5A shows the pattern of actin staining found in unstimulated cells, while Figure 5B

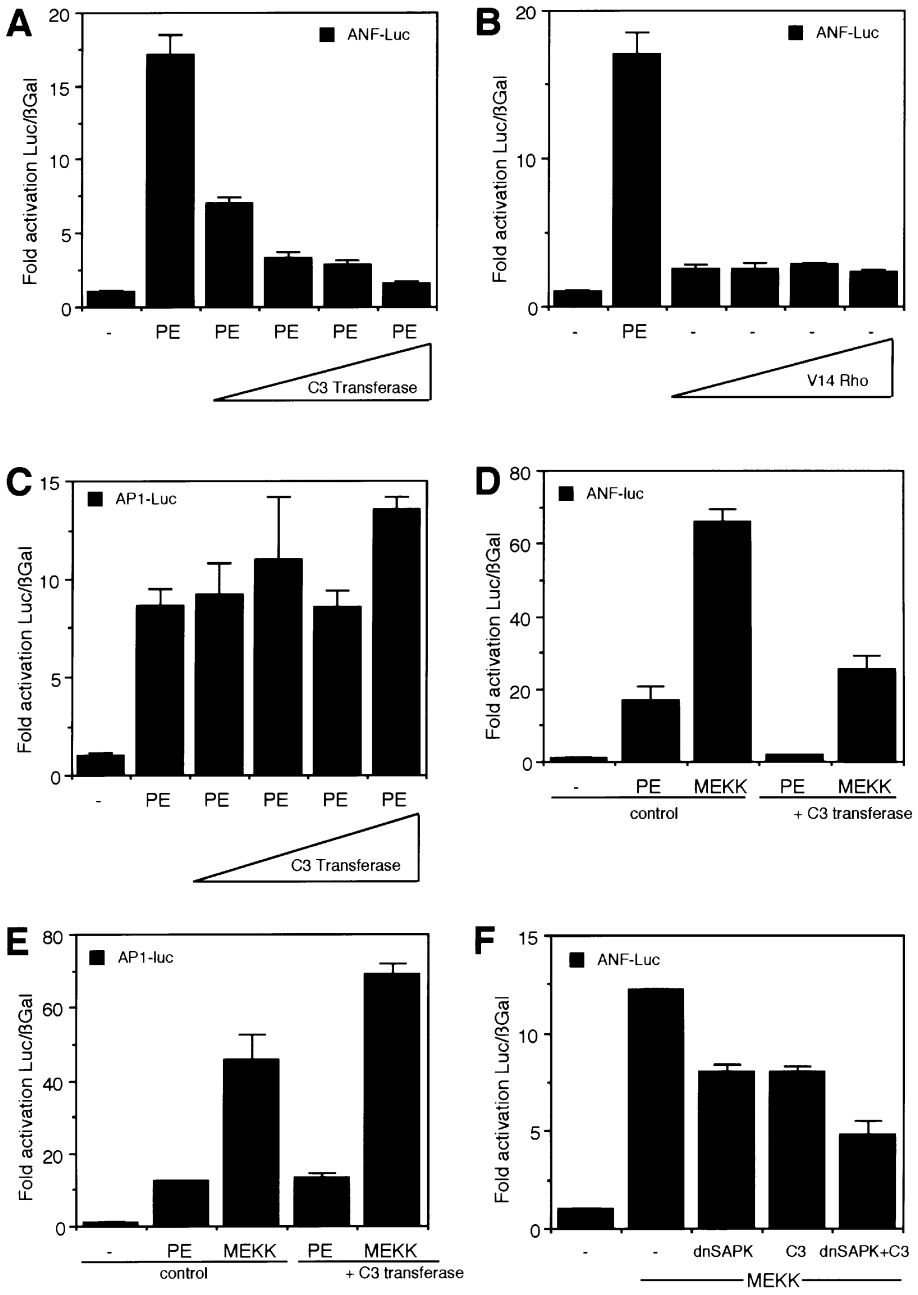

Fig. 4. Rho activity is required for ANF expression. (**A**) Increasing amounts (0, 10 and 100 ng, 1 and 3 μg) of C3 transferase expression plasmid were co-transfected along with ANF-luciferase plus RSVβGal and the cells were treated with 100 μM phenylephrine. (**B**) Empty expression plasmid plus (PE) or minus (–) stimulation or increasing amounts (100 ng, 0.5, 1 and 3 μg) of EFV14Rho were transfected along with ANF-luciferase and RSVβGal. (**C**) Increasing amounts of C3 transferase (as in A) were transfected along with AP1-luciferase and RSVβGal and the cells were treated with 100 μM phenylephrine. (**D**) Cells were transfected with ANF-luciferase plus RSVβGal plus 2.9 μg of empty expression plasmid (control) or the C3 transferase expression plasmid (C3 transferase) and either 100 ng of empty CMV plasmid then treated with phenylephrine (PE) or with 100 ng of the MEKK1-c expression plasmid (MEKK). (**E**) Cells were transfected as in (D) except that AP1-luciferase replaced ANF-luciferase. (**F**) Cells were transfected with 100 ng of the MEKK1-c expression plasmid plus ANF-luciferase, RSVβGal and either 3 μg of empty vector, 1.5 μg of vector plus 1.5 μg of C3 transferase (C3), 1.5 μg of vector plus 1.5 μg of dominant-negative SAPKβ (dnSAPK), or 1.5 μg of C3 transferase plus 1.5 μg of dominant-negative SAPKβ (C3 + dnSAPK).

shows the highly organized pattern that is induced by a 24 h treatment with phenylephrine. Previously we showed that these morphological changes can be induced by Ras (Thorburn *et al.*, 1993) but do not appear to involve ERK or Raf signaling (Thorburn *et al.*, 1994a,b). Rho is known to be a key regulator of actin morphology in other cell types (Ridley and Hall, 1992). Since inhibition of Rho by C3 transferase had a clear effect on hypertrophic gene expression, we also determined whether C3 transferase affected hypertrophic cell morphology. Recombinant C3 transferase protein plus a marker IgG molecule was microinjected into cardiac muscle cells that were then treated with phenylephrine to induce hypertrophy. Surprisingly, injection of C3 transferase plus IgG (Figure 5C) had no effect on actin organization in response to phenylephrine (Figure 5D). As expected, control injections of

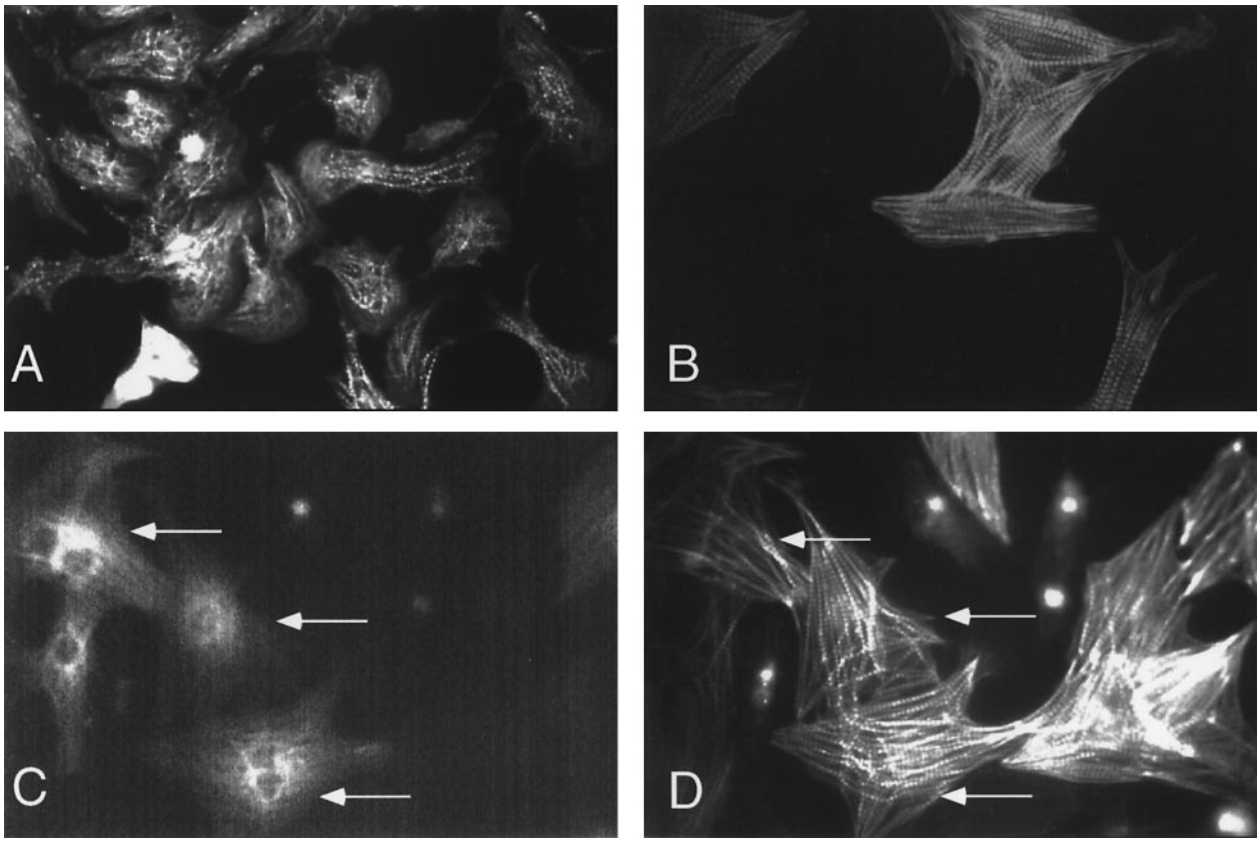

Fig. 5. C3 transferase protein does not inhibit actin organization. (**A**) Phalloidin staining of unstimulated cardiac muscle cells. (**B**) Phalloidin staining of muscle cells treated with 100 μM phenylephrine. (**C**) Cardiac muscle cells injected with rat IgG plus recombinant C3 transferase, stained with FITC-conjugated anti-Rat to identify injected cells. (**D**) The same field of cells as in (C) stained with phalloidin.

IgG alone had no effect on actin morphology (data not shown). As a control to ensure that the C3 protein was active, we performed injection experiments in serum-starved NIH 3T3 cells then treated these cells with 10% fetal calf serum. Injected fibroblasts rounded up within an hour and displayed a significantly altered morphology indicating that the C3 transferase protein was active (data not shown).

One possible explanation for the lack of effect of recombinant C3 protein on the heart cells was that we did not achieve a high enough amount of C3 protein in the cell to produce an inhibitory effect in our relatively long-term assay for actin morphology. We therefore repeated the morphology experiments using microinjected or transfected C3 expression plasmids. Figure 6 shows the results obtained. Cells that contained the C3 expression plasmids were identified by co-injection or co-transfection of a green fluorescent protein (GFP) expression plasmid. Figure 6A shows GFP fluorescence in control cells, indicating that injection of plasmids did not affect phenylephrine-induced morphology (Figure 6B). Figure 6C and D shows cells injected with the C3 expression plasmid plus the GFP plasmid, again indicating that C3 expression did not prevent actin organization. To exclude the possibility that injected plasmids were not as effective as transiently transfected C3 plasmids (which we know to be active in heart cells since they abolish phenylephrine-induced ANF-luciferase expression, Figure 4A), we also monitored actin morphology in transiently transfected cells. Figure 6E shows GFP fluorescence from transiently transfected cells using 3 μg of the expression plasmid in each transfection mix, i.e. the same amount of C3 expression plasmid that led to complete inhibition of ANF-luciferase (Figure 4A). Figure 6F shows the same field stained with phalloidin, indicating again that C3 transferase does not prevent actin organization into muscle fibers. When C3 transferase-containing cells were counted and scored for actin organization into muscle fibers, we found that ~90% of the cells showed clear actin organization after phenylephrine treatment. To exclude the possibility that timing was affecting the outcome of these experiments, we fixed cells either at 24 or 48 h (i.e. when cells were harvested for luciferase assays) after transfection. At both times, the transfected cardiac muscle cells, identified by GFP fluorescence, had highly organized actin and were, in fact, contracting rhythmically before fixation. In further control experiments, we found that injected or transfected C3 transferase expression plasmids prevented luciferase expression from the ANF-luciferase reporter that was monitored by immunofluorescence staining with anti-luciferase antibodies (data not shown). When C3 expression plasmids were injected into NIH 3T3 or REF52 cells, the injected cells displayed marked morphological changes and, after 2–3 h, were rounded, had severely disrupted actin stress fibers and were poorly attached to the dish, further indicating that the expression plasmid was active.

MEKK1-c does not induce morphological phenotypes associated with hypertrophy

We also tested whether active MEKK was able to induce the morphological phenotypes associated with cardiac

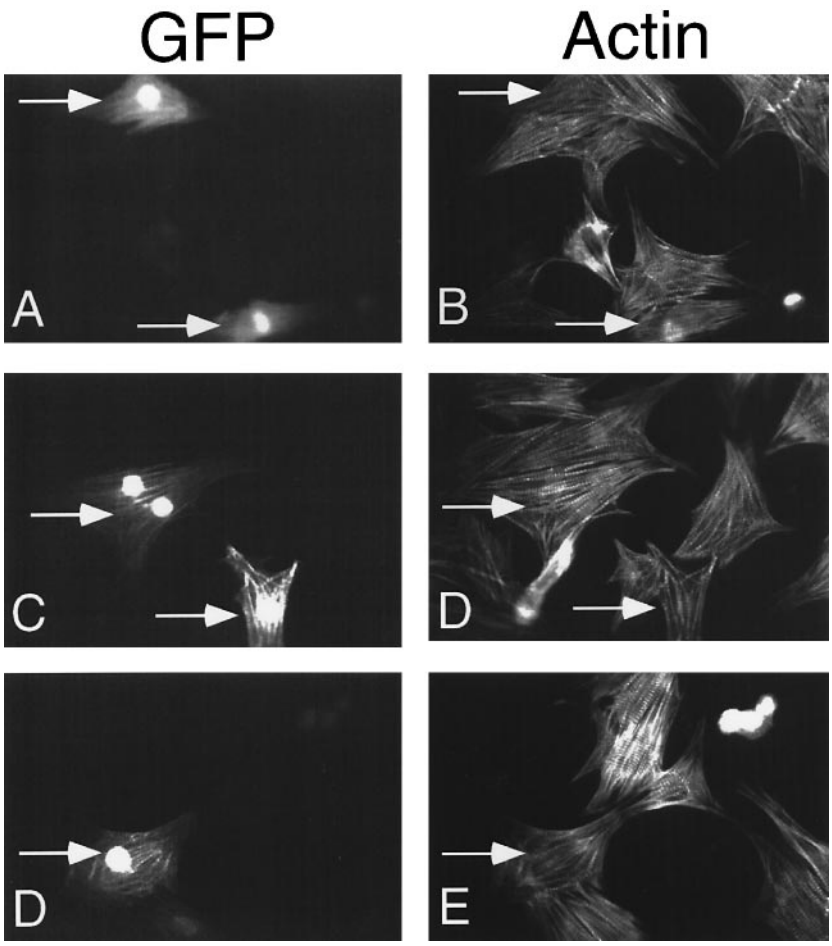

Fig. 6. C3 transferase expression plasmids do not prevent actin organization. (**A**) GFP fluorescence in control cells injected with 0.5 mg/ml GFP expression vector and treated with phenylephrine. (**B**) Same field as (A) stained with rhodamine–phalloidin. (**C**) GFP fluorescence from cells injected with 0.5 mg/ml GFP expression plasmid plus 0.5 mg/ml C3 transferase expression plasmid. (**D**) Same field as (C) stained with phalloidin. (**E**) GFP fluorescence from cells transfected with 1 μg of GFP expression plasmid plus 3 μg of C3 transferase expression plasmid. (**F**) Same field as (E) stained with phalloidin.

hypertrophy. For these experiments, the MEKK1-c expression plasmid was transfected into cells along with the GFP expression plasmid. Cells were monitored for GFP fluorescence to identify transfected cells and stained for actin organization. Figure 7A and B shows control cells that were transfected with the GFP expression plasmid and empty vector, indicating that transfection alone does not induce muscle organization. Figure 7C and D shows cells expressing small amounts of MEKK1-c (100 ng of expression plasmid per transfection mix, the amount that caused maximal ANF-luciferase expression); these cells did not display morphological phenotypes associated with hypertrophy. Figure 7E and F shows cells that were transfected with 100 ng of the MEKK1-c expression plasmid then treated with the MEK inhibitor PD 098059; these cells also did not show any organization into muscle fibers. Thus the conditions that lead to maximal MEKK1-driven ANF-luciferase expression were not sufficient to induce the morphological phenotypes associated with hypertrophy.

Figure 8 shows the results obtained when MEKK1-c was expressed in phenylephrine-treated cells. Surprisingly, expression of MEKK1-c inhibited actin organization into muscle fibers in response to phenylephrine treatment (Figure 8C and D), although surrounding untransfected muscle cells, e.g. the cell between the two transfected cells on the right hand side of the field, had organized muscle fibers. Control cells (Figure 8A and B) showed no disruption. Similar inhibition was observed whether small (100 ng) or large amounts (3 μg) of the MEKK1-c expression plasmid were used in the transfection mix or whether cells were injected or transfected. Unlike the C3 transferase-containing cells, MEKK1-c-containing cells when identified by GFP fluorescence before fixation did not contract even when the dish was treated with phenylephrine and neighboring cells were capable of contraction, further indicating that expression of MEKK1-c prevents functional muscle fiber formation.

Discussion

In this study, we have shown that MEKK1 can cause expression of a marker gene, ANF, that is induced by hypertrophic stimuli in cardiac muscle cells. In our cells, MEKK1 appears to be almost equally good at activating the ERK and SAPK pathways, unlike the situation in other cell types where MEKK1 preferentially activates the SAPK pathway (Minden *et al.*, 1994; Yan *et al.*, 1994). Thus there may be cell type-specific differences in the effects of introduction of these various upstream kinases

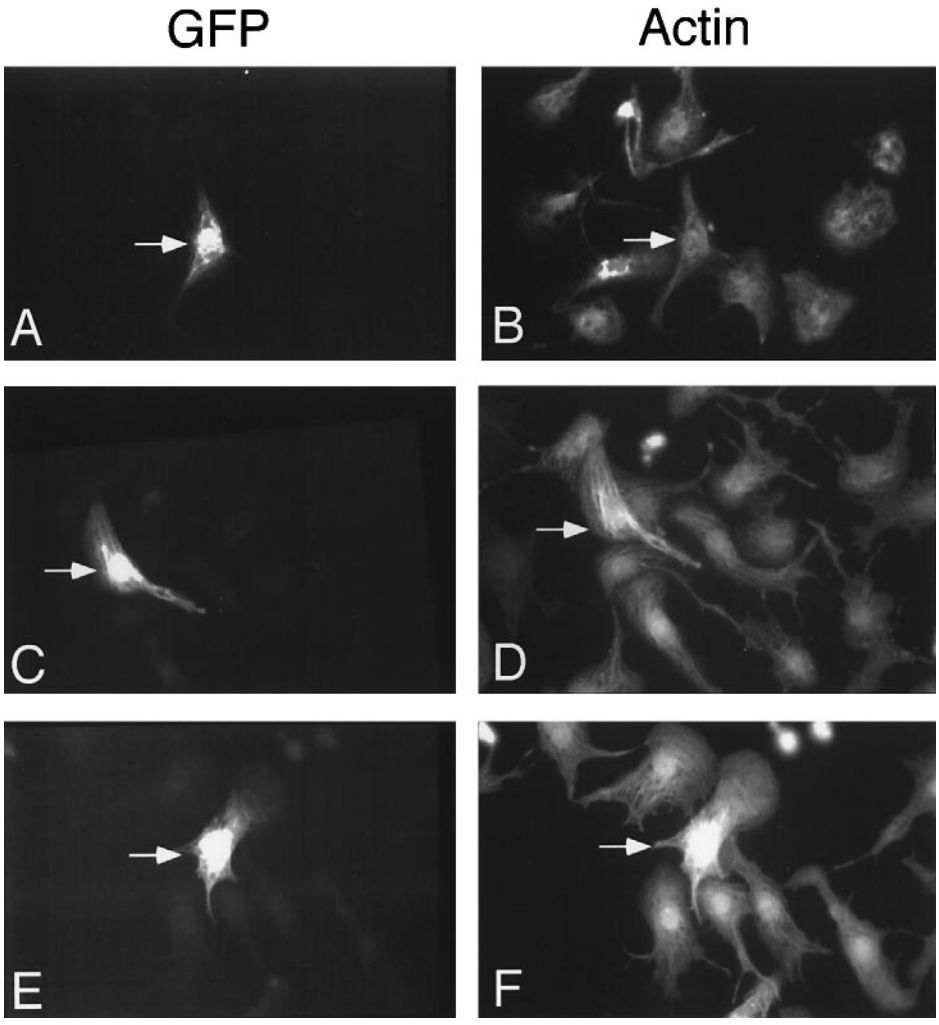

Fig. 7. MEKK1 does not induce actin organization. (**A**) and (**B**) Cells transfected with 1 μg of GFP expression plasmid and 3 μg of empty vector. (**C**) and (**D**) Cells transfected with 1 μg of GFP expression plasmid, 100 ng of MEKK1-c expression plasmid and 2.9 μg of empty vector. (**E**) and (**F**) Cells transfected as in (C) and (D) but treated with 10 μM PD 098059 to inhibit MEK activity.

and it may not be possible to extrapolate from one cell type to another.

We found that small amounts of constitutively active MEKK1 were strong activators of ANF-luciferase expression while larger amounts of MEKK1-c led to inhibition both in the absence and presence of phenylephrine. The experiments shown in Figure 2 indicate that activation of either high or low levels of ERK activity are not sufficient to explain ANF induction. Expression of catalytically inactive SEK or SAPK mutants inhibits MEKK1-c-induced ANF expression while inhibition of MEK by treatment with PD 098059 caused further stimulation of ANF promoter-driven gene expression. Thus the level of MEKK1-induced ANF-luciferase expression is the product of positive signals mediated via the SEK–SAPK pathway and inhibitory signals mediated via MEK. Since MEKK1-c appears to induce hypertrophic gene expression in part via the SAPKs, these results may be relevant for the understanding of hypertrophic gene expression during clinically important cellular stresses, such as cardiac ischemia or pressure overload, that are likely to cause SAPK activation. In this respect, it was reported recently that ischemia/reperfusion stimulates SAPK activation in heart muscle. These authors also showed that active MEKK could induce ANF expression (Bogoyevitch *et al.*, 1996). SAPKs are also activated by ischemia/reperfusion of kidney cells (Pombo *et al.*, 1994).

The experiments showing that transient transfection of C3 transferase expression plasmids inhibits both phenylephrine- and MEKK1-c-induced ANF expression indicate that hypertrophic gene expression also requires Rho-dependent signals. Interestingly, while relatively large amounts of C3 transferase (3 μg per transfection) completely abolished phenylephrine-induced ANF expression, this amount of the inhibitor only partially inhibited MEKK1-c-induced expression. The simplest explanation of these results is that at least two separate signals regulate ANF expression. One signal is dependent upon SAPK activation while the other signal is Rho-dependent. While both signals seem to have a role to play, the relative importance of these signals may be different for phenylephrine- and MEKK1-induced ANF expression. This idea is supported by the observation that inactive SEK or SAPK is a fairly effective inhibitor of MEKK-induced ANF expression but a poor inhibitor of phenylphrine-induced expression, while C3 transferase is a more effective inhibitor of phenylephrine-induced than MEKK-induced expression. We have not been able to demonstrate

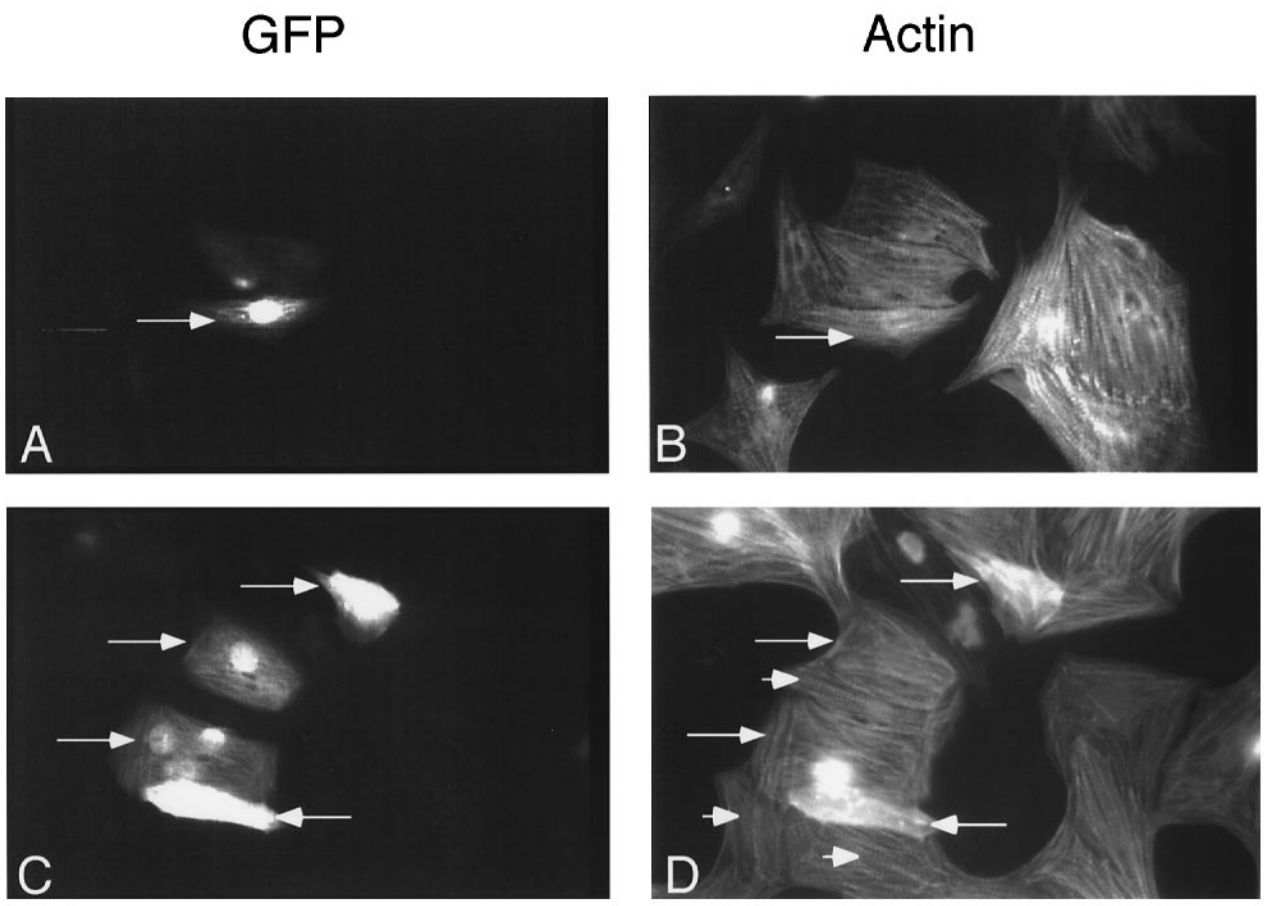

Fig. 8. MEKK1 disrupts phenylephrine-induced muscle fiber organization. (**A**) and (**B**) Control cells transfected with 1 μg of GFP expression plasmid plus 3 μg of empty vector. (**C**) and (**D**) Cells transfected with 1 μg of GFP expression plasmid, 100 ng of MEKK1-c expression plasmid and 2.9 μg of empty vector. The cells were stimulated with phenylephrine to induce organization after transfection. The larger arrows indicate cells that were transfected and show disrupted muscle fibers, while the smaller arrows indicate neighboring, untransfected cells that have normal muscle fiber organization.

inhibition of phenylephrine-induced ANF expression by catalytically inactive MEKK1; this result is consistent with the idea that there may be functional differences between the mechanism of activation of this promoter by these two stimuli. A likely Rho-dependent target for ANF gene expression is the SRF whose activity is known to be dependent upon Rho in fibroblasts (Hill *et al.*, 1995). An SRF-like activity is important for both basal and phenylephrine-stimulated ANF expression in cardiac muscle cells (Sprenkle *et al.*, 1995), and SRF has been implicated in hypertrophic stimulation of the skeletal α-actin gene (MacLellan *et al.*, 1994; Karns *et al.*, 1995).

Rho-dependent pathways regulate actin morphology by inducing the formation of actin stress fibers and focal adhesions in fibroblasts (Ridley and Hall, 1992, 1994; Nobes and Hall, 1995). We therefore suspected that interference with Rho-dependent signaling might prevent the organization of filamentous actin into muscle fibers. Since C3 transferase had no effect on phenylephrine-induced actin organization while it did inhibit gene expression, we conclude that Rho-dependent signals do not regulate actin organization into muscle fibers. These data do not exclude a possible role for Rho in muscle organization that is induced by other stimuli. We also performed experiments to test whether dominant-negative Ras, Rac, Cdc42 or Rho could inhibit muscle fiber formation. We found that neither dominant-negative Rho nor Rac had any effect on muscle organization; however, dominant-negative Ras-transfected or injected cells sometimes showed disorganized muscle fibers as did occasional dominant-negative Cdc42-transfected cells. However, when we counted the transfected cells and scored them as organized or disorganized, the number of organized cells after transfection with these mutants was not statistically different from controls (data not shown). This result suggests that there may be a requirement for Ras- and also perhaps Cdc42-dependent signals in muscle organization but that it will be difficult to characterize further the mechanisms responsible using these approaches. Active Ras can induce hypertrophic morphology (Thorburn *et al.*, 1993); however, we have been unable to demonstrate induction of actin organization in cells injected or transfected with activated mutants of Cdc42, Rac or Rho.

MEKK1-c was an effective inhibitor of actin organization. In contrast to the experiments with the dominant-negative G proteins, almost every MEKK1-c-transfected cell had disrupted muscle fibers. This result shows that it is possible to disrupt muscle fiber formation in the transfection assays, indicating that the failure to do so with a molecule such as C3 transferase is not simply because it is impossible to inhibit organization in response to a strong hypertrophic stimulus such as phenylephrine. Active

MEKK1-c was not sufficient to induce hypertrophic morphology in the absence of other stimulation, although it was a very effective activator of hypertrophic gene expression. These data suggest that MEKK1 is not a physiologically relevant inducer of hypertrophic morphology. Since the catalytic domain of MEKK1 can interact directly with the effector domain of GTP-bound Ras (Russell *et al.*, 1995), one hypothesis to explain the inhibitory effect of MEKK1-c on phenylephrine-induced actin organization is that this molecule might bind to the Ras effector domain and prevent interaction with a different Ras effector molecule that regulates actin organization in heart muscle cells.

In conclusion, the data presented here indicate that multiple signaling pathways regulate cardiac gene expression during hypertrophic growth. Activation of gene expression occurs as a result of signals that are dependent upon SEK and SAPK activity, while a second positive signal requires Rho activity. In addition to these positive signals, signaling via MEK leads to inhibition of the ANF promoter. Thus two parallel MAP kinase cascades have opposite effects on a growth-regulated promoter. In other situations, ERK and SAPK pathways can have similar effects on gene expression, for example by activating ternary complex factors such as Elk1 (Gille *et al.*, 1995; Whitmarsh *et al.*, 1995). Differential effects of these two MAP kinase cascades can account for survival versus apoptosis of PC12 cells (Xia *et al.*, 1995), but in this case it is not clear what the target genes responsible for this differential effect might be. Since the ANF promoter is regulated both positively and negatively by these pathways, this experimental system may provide a useful opportunity to characterize mechanisms of differential effects of parallel MAP kinase cascades. We currently are analyzing the ANF promoter to identify further the DNA elements and transcription factors that might mediate these differential effects. The regulation of actin into muscle fibers in response to hypertrophic stimuli does not involve these signaling pathways, although it can be inhibited by active MEKK1. Future studies using MEKK1-mediated inhibition of actin organization should provide a way to characterize further the molecular mechanisms that regulate muscle organization.

Materials and methods

Cell culture

Primary ventricular cardiac myocytes were isolated from 1-day-old rats by collagenase digestion and maintained in Dulbecco's modified Eagle's medium (DMEM)/medium 199 (4/1) supplemented with penicillin and streptomyocin. For transfection experiments, cells were plated at a density of 2.5×10^5 per 3.5 cm dish (for gene expression assays) or 7.5×10^5 per 6 cm dish (for epitope-tagged kinase assays). For morphology experiments, cells were plated on chamber slides at the same density.

Plasmids

Expression plasmids for full-length wild-type or catalytically inactive (D1369A) MEKK1 and truncated MEKK1-c consisting of only the catalytic domain were constructed in cytomegalovirus (CMV)-driven plasmids (Xu *et al.*, 1995, 1996). The expression plasmid for Myc-tagged ERK2 was constructed by subcloning the ERK2 cDNA into an elongation factor 1α (EF)-driven plasmid downstream of a Myc tag (provided by Richard Treisman). EF-driven C3 transferase and the bacterial expression vector for GST–C3 transferase plasmids were provided by Art Alberts and Richard Treisman. HA-tagged SAPKβ was obtained in the expression plasmid pMT2 from Jim Woodgett and subcloned into an EF-driven plasmid into which the HA tag sequence had been inserted. The truncated Jun expression plasmid was constructed by PCR amplification of the sequences from amino acid 169 to the stop codon and inserted into the EF-Myc plasmid. The ANF-luciferase plasmid (provided by Ken Chien) was driven by a 3003 bp promoter sequence, while the AP1-luciferase plasmid was regulated by a duplicated AP1-binding sequence upstream of a basal promoter. Catalytically inactive SAPKβ was made by site-directed mutagenesis of the wild-type sequence to substitute the catalytic lysine in the ATP-binding site with an alanine residue and was provided by Jeff Frost. The inactive SEK_{AL} cDNA was obtained from Jim Woodgett and subcloned into the EF vector. The CMV-driven ΔN3, S218E, S222D MEK construct (Mansour *et al.*, 1994) was provided by Natalie Ahn. CL100 in the expression vector pSG5 was provided by Steve Keyse. The V14Rho expression plasmid was constructed by inserting the V14Rho cDNA (provided by Alan Hall) into the EF-Myc vector.

Gene expression and kinase assays

Transient transfections were performed in triplicate by calcium phosphate-mediated transfection as previously described (Thorburn *et al.*, 1993, 1994a,b, 1995) using the amounts of plasmid DNAs noted in the figure legends for each experiment. Where required, the total amount of plasmid in the transfection mix was kept constant by adding the relevant empty expression vectors. For luciferase assays, ANF-luciferase or AP1-luciferase reporters were co-transfected with a Rous sarcoma virus (RSV)–β-galactosidase reporter plasmid (provided by Michael Kapiloff) that was used to normalize transfection efficiencies. Luciferase and β-galactosidase assays were performed with reagents from Promega (Madison, WI) or Tropix (Bedford, MA) as described by the manufacturer. Transfection efficiencies were normalized by dividing the luciferase activity from each dish by the β-galactosidase activity from the same dish. Data in each experiment are presented as the mean ± SD of triplicates from a representative experiment. All experiments were performed at least three times with different preparations of plasmids and primary cells, producing qualitatively similar results.

Epitope-tagged kinase assays were performed as before (Thorburn *et al.*, 1994a, 1995). In all cases, both Myc-tagged ERK2 and HA-tagged SAPKβ were co-transfected with expression vectors for the various kinases or CL100 to be tested, then the cell lysate was split for separate immunoprecipitations using the 9E10 (Myc) or 12CA5 (HA) monoclonal antibodies and finally used in kinase assays with MBP (Sigma, St Louis, MO) or GST–Jun (1–169) as substrates respectively.

Cell morphology assays

Cells for morphological analysis were plated on chamberslides. Microinjection experiments were performed as previously described in the presence of 20 mM 2,3 butanedione monoxime (Sigma) to prevent excessive contraction as a result of calcium influx (Thorburn *et al.*, 1993). Recombinant C3 protein was produced as a GST fusion protein, cleaved with thrombin and injected at a needle concentration of 1 mg/ml along with 4 mg/ml rat IgG to identify injected cells; control injections contained rat IgG alone. One hour after injection, phenylephrine was added to the media. Cells were fixed 24 h later and stained with FITC-conjugated anti-Rat to identify injected cells and rhodamine–phalloidin (Sigma) to show filamentous actin. For studies with expression plasmids, injections were performed with a GFP expression plasmid pGreenLantern (BRL, Gaithersburg, MD) to identify cells. The C3 transferase expression plasmid or MEKK1-c expression plasmid and the GFP plasmid were injected at a concentration of 0.5 mg/ml each in the needle. Cells were maintained in serum-free media for 1 h to allow expression (Alberts *et al.*, 1993), then treated with phenylephrine for 24 h before fixation and staining with rhodamine–phalloidin. Injected cells were identified by virtue of their GFP fluorescence. Transient transfections were also performed with these plasmids. For transient transfection experiments, cells were fixed at both 24 and 48 h after phenylephrine treatment with identical results. Control injections in NIH 3T3 and REF52 cells were performed in cells that had been starved in 0.1% serum-containing media for 24 h prior to injection, then treated with 10% fetal bovine serum after injection.

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