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Inhibition of a Signaling Pathway in Cardiac Muscle Cells by Active Mitogen-activated Protein Kinase Kinase

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Signaling via the Ras pathway involves sequential activation of Ras, Raf-1, mitogen-activated protein kinase kinase (MKK), and the extracellular signal-regulated (ERK) group of mitogen-activated protein (MAP) kinases. Expression from the c-Fos, atrial natriuretic factor (ANF), and myosin light chain-2 (MLC-2) promoters during phenylephrine-induced cardiac muscle cell hypertrophy requires activation of this pathway. Furthermore, constitutively active Ras or Raf-1 can mimic the action of phenylephrine in inducing expression from these promoters. In this study, we tested whether constitutively active MKK, the molecule immediately downstream of Raf, was sufficient to induce expression. Expression of constitutively active MKK induced ERK2 kinase activity and caused expression from the c-Fos promoter, but did not significantly activate expression of reporter genes under the control of either the ANF or MLC-2 promoters. Expression of CL100, a phosphatase that inactivates ERKs, prevented expression from all of the promoters. Taken together, these data suggest that ERK activation is required for expression from the Fos, ANF, and MLC-2 promoters but MKK and ERK activation is sufficient for expression only from the Fos promoter. Constitutively active MKK synergized with phenylephrine to increase expression from a c-Fos- or an AP1-driven reporter. However, active MKK inhibited phenylephrine- and Raf-1–induced expression from the ANF and MLC-2 promoters. A DNA sequence in the MLC-2 promoter that is a target for inhibition by active MKK, but not CL100, was mapped to a previously characterized DNA element (HF1) that is responsible for cardiac specificity. Thus, activation of cardiac gene expression during phenylephrine-induced hypertrophy requires ERK activation but constitutive activation by MKK can inhibit expression by targeting a DNA element that controls the cardiac specificity of gene expression.

INTRODUCTION

Signal transduction pathways that involve activation of Ras and lead to stimulation of the protein kinase cascade involving sequential activation of Raf-1, mitogen-activated protein kinase kinase (MKK),[1] and mitogen-activated protein (MAP) kinase are major regu-

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[1] Abbreviations used: ANF, atrial natriuretic factor; ERK, extracellular signal-regulated protein kinase; MLC-2, myosin light chain-2; MAP kinase, mitogen-activated protein kinase; MKK, MAP kinase kinase, also known as MEK.

lators of cell growth and differentiation (for review see Davis, 1993; Avruch *et al.*, 1994; Hill and Treisman, 1995). For several years, it has been clear that constitutively active Ras and Raf are capable of inducing cell growth whereas recent work from several laboratories shows that constitutively active MKK can induce NIH 3T3 cell growth and transformation (Brunet *et al.*, 1994; Cowley *et al.*, 1994; Mansour *et al.*, 1994), differentiation of PC12 cells (Cowley *et al.*, 1994), and *Xenopus* oocyte maturation (Huang *et al.*, 1995). The fact that active MKK has the same effects as active versions of upstream molecules like Raf-1 or Ras implies that the sequential pathway Ras → Raf-1 → MKK → ERK is sufficient to induce growth or differentiation of mammalian cells. Sequential activation of the same linear cascade appears to function in other signal transduction pathways. For example, genetic evidence in the *Drosophila* sevenless pathway indicates that defects in the growth factor receptor can be compensated for by a gain-of-function mutation in MAP kinase (Brunner *et al.*, 1994). These data provide strong support for the view that sequential activation of this linear signal transduction pathway is both necessary and sufficient to produce complex cellular responses.

Postnatal growth of cardiac muscle occurs by cell hypertrophy rather than cell division, and is characterized by a number of phenotypic changes including activation of immediate early, fetal, and contractile protein genes for example, c-Fos, atrial natriuretic factor (ANF), and myosin light chain-2 (MLC-2). In response to treatment with a variety of different agonists, primary cultures of ventricular myocytes isolated from neonatal rat hearts display many of the features associated with hypertrophy in vivo and provide a useful model to study this nonproliferative growth response (Chien *et al.*, 1991). Previously, we found that introduction of active Ras or active Raf-1 were each necessary and sufficient for expression from the c-Fos, ANF, and MLC-2 promoters in phenylephrine-induced hypertrophy (Thorburn *et al.*, 1993; Thorburn *et al.*, 1994b). Active MAP kinase is also required for induction by phenylephrine (Thorburn *et al.*, 1994a) or Raf-1 (Thorburn *et al.*, 1994b). Phenylephrine and other hypertrophic agonists cause biochemical activation of Ras (Thorburn and Thorburn, 1994), Raf-1 (Thorburn and Thorburn, unpublished data), MKK (Bogoyevitch *et al.*, 1993; Lazou *et al.*, 1994), and ERKs (Bogoyevitch *et al.*, 1993, 1994; Yamazaki *et al.*, 1993; Thorburn *et al.*, 1994a). In addition, dominant-negative Ras or Raf mutants prevent phenylephrine-induced ERK activation (Thorburn, 1994). Taken together, these data suggest that the signal transduction pathway consisting of sequential activation of Ras → Raf-1 → MKK and the ERK family of MAP kinases regulate gene expression during phenylephrine-induced cardiac hypertrophy.

Because MKK1 and MKK2 are the only known activators of the ERKs, and Raf-1 is a major upstream regulator of MKK, we examined the role of MKK in the activation of the c-Fos, ANF, and MLC-2 promoters. Unlike active Ras or Raf-1, constitutively active MKK did not induce significant expression from the ANF or MLC-2 promoters although it was sufficient to induce expression from the Fos promoter. This suggests that other targets downstream of Raf-1, in addition to MKK, must be activated to cause ANF and MLC-2 gene expression during cardiac hypertrophy. This finding provides direct evidence that activation of the simple, sequential kinase cascade is not sufficient to induce all phenotypes associated with activation of Ras and Raf-1. Surprisingly, constitutively active MKK inhibited phenylephrine- and Raf-induced expression from the ANF and MLC-2 promoters. This effect was not indicative of general inhibition of phenylephrine signaling because active MKK synergized with phenylephrine to increase expression from the c-Fos promoter or an AP1-driven plasmid. Inhibition of the MLC-2 promoter by active MKK mapped to a previously identified sequence in the promoter (HF1), which is responsible for cardiac specificity (Zhu *et al.*, 1991).

MATERIALS AND METHODS

Cell Culture and Transfections

All experiments were performed by calcium phosphate-mediated transient transfection of primary neonatal rat ventricular myocytes. Myocytes were prepared, cultured, and transfected as previously described (Thorburn and Thorburn, 1994; Thorburn *et al.*, 1994a,b). Transfections were performed in triplicate in 10-cm or 3.5-cm dishes, using the amounts of plasmid DNA noted in each figure legend. The MKK molecules were expressed from the CMV-driven plasmid pMCL, which is based on the plasmid pCEP4 (Invitrogen, La Jolla, CA). The estrogen-regulated Raf-1 expression vector (pCEP4ΔRaf-1:ER) expresses the kinase domain of Raf-1 fused to the steroid-binding domain of the human estrogen receptor. The Raf-1 protein kinase activity of this molecule is dependent on the addition of exogenous estradiol to the culture medium (Samuels *et al.*, 1993). The c-Fos-, ANF-, and MLC-2-luciferase plasmids contained 0.75 kb, 3 kb, and 2.7 kb of promoter sequences, respectively. Experiments were also performed using a 250-bp MLC-2 promoter fragment. The AP1-luciferase plasmid contains two copies of a consensus AP1 binding site upstream of a basal promoter whereas the HF1-luciferase molecule consisted of three copies of the 28-bp HF1 sequence (Zhu *et al.*, 1991) upstream of the basal promoter in the plasmid pGL3 Promoter (Promega, Madison, WI). The control, SV40-driven β-galactosidase expression plasmid used to normalize for transfection efficiencies was pSVβGal (Promega). After washing to remove the calcium phosphate precipitate, the cells were maintained in serum-free media (supplemented as required with phenylephrine, PMA, or β-estradiol) for 48 h before harvesting for kinase assays or β-galactosidase and luciferase assays. Kinase assays of HA-tagged ERK2 (42-kDa MAP kinase) were performed after precipitation with the 12CA5 monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) as previously described (Faure *et al.*, 1994; Thorburn, 1994; Thorburn *et al.*, 1994b). Reporter enzyme assays were performed with reagents from Promega as described by the manufacturer. Variation in transfections efficiency was controlled by dividing the luciferase activity for each dish by the corresponding β-galactosi-

dase activity. The data shown in each figure are from a single representative transfection experiment, in which values represent the mean ± SE of enzyme activities from separate dishes. Each experiment was performed at least three times with different preparations of cells and plasmids.

RESULTS

Expression of Mutant MKK Molecules in Cardiac Myocytes

To test whether expression of active MKK in cardiac muscle cells mimicked the effects of phenylephrine or expression of active Ras or Raf-1, we performed experiments with a number of MKK mutants. Two constitutively active mutants were used. MKK (S218E/S222D) is activated by the introduction of negatively charged residues at the positions that are phosphorylated when MKK is activated by Raf-1; MKK (ΔN3/S218E/S222D) has an additional deletion of an inhibitory domain. These mutants constitutively display 80× and 410× wild-type activity, respectively (Mansour *et al.*, 1994). In addition, we used the wild-type molecule and a catalytically inactive molecule MKK (K97 M), which has the essential lysine at the catalytic site mutated to a methionine residue. Figure 1 shows that expression of the two constitutively active mutants increased co-transfected, HA-tagged ERK2 activity while expression of the wild-type kinase and MKK (K97 M) did not. The extent of ERK2 activation by active MKK was considerably greater than that observed with transient (10 min) phenylephrine treatment of wild-type–transfected cells. This high level of ERK2 activity was similar to that previously observed when cells were transfected with constitutively active Ras (Thorburn, 1994) or ΔRaf-1:ER, an estrogen-regulatable Raf-1 molecule that had been continually activated by estradiol treatment for 2 days (Thorburn, unpublished data). These data indicate that expression of the activated forms of MKK results in high levels of ERK activity in transfected cardiac myocytes.

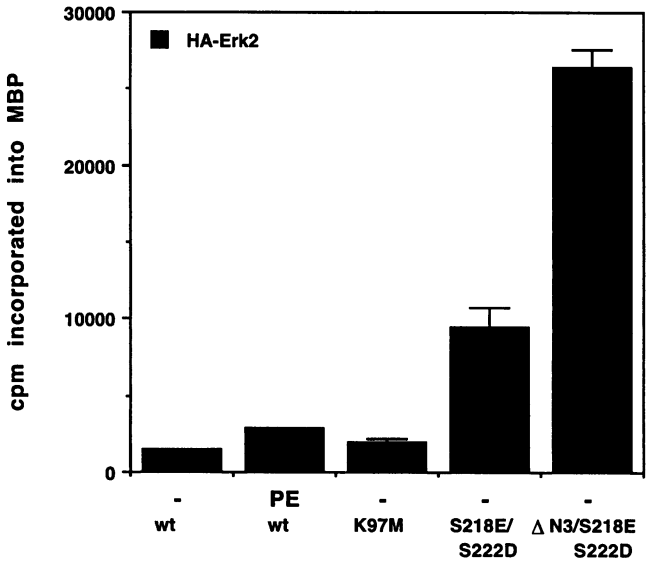

Figure 1. Expression of active MKK in cardiac myocytes induces ERK activation. Cells in 10-cm dishes were transfected with 8 μg of an expression vector (pCEP4L ERK2) for HA-tagged ERK2 and 24 μg of either wild-type, kinase-deficient (K97 M), or two constitutively active (S218E/S222D and ΔN3/S218E/S222D) forms of MKK. Epitope-tagged EEK2 was purified by immunoprecipitation and used in an in vitro kinase assay with myelin basic protein (MBP) as substrate.

Constitutively Active MKK Causes Expression from the c-Fos or AP-1-driven Promoters, but not from the ANF or MLC-2 Promoters

Cardiac muscle cell hypertrophy is associated with increased expression of various genes (Chien *et al.*, 1991). Three classes of genes are activated: immediate early, fetal, and contractile protein genes. We therefore examined expression from the Fos, ANF, and MLC-2 promoters as examples from each of these classes. Active Ras or Raf-1 can induce expression from these promoters (Thorburn *et al.*, 1993; Thorburn *et al.*, 1994b). To determine whether constitutively active MKK could likewise induce expression from these promoters, transient transfections were performed with the various MKK expression vectors co-transfected with an SV40 promoter-driven β-galactosidase plasmid and Fos-, AP1-, ANF-, or MLC-2-luciferase. Constitutively active MKK induced expression from the AP1-driven plasmid and from the Fos promoter (Figure 2, A and B). The more active MKK mutant caused higher levels of expression than the less active mutant. This was expected because the more active molecule also produced higher levels of ERK2 activation, and both AP1 and the Fos promoter responded to ERK activation. When expression of co-transfected ANF- and MLC-2-driven luciferase reporters was analyzed, constitutively active MKK did not induce significant expression from these promoters, although in cells co-transfected with wild-type MKK, phenylephrine induced expression as expected (Figure 2, C and D). We occasionally noted a modest (1.5- to 2-fold) activation of the ANF promoter with the less active mutant. No activation was ever observed with the more active MKK mutant and no activation of the MLC-2 promoter was observed with either MKK mutant. Thus, active MKK, although sufficient to induce expression from the Fos and AP1-driven promoters, is not sufficient to induce expression from either the ANF or MLC-2 promoters. These data indicate that not all phenotypes associated with active Raf-1 are necessarily also associated with active MKK.

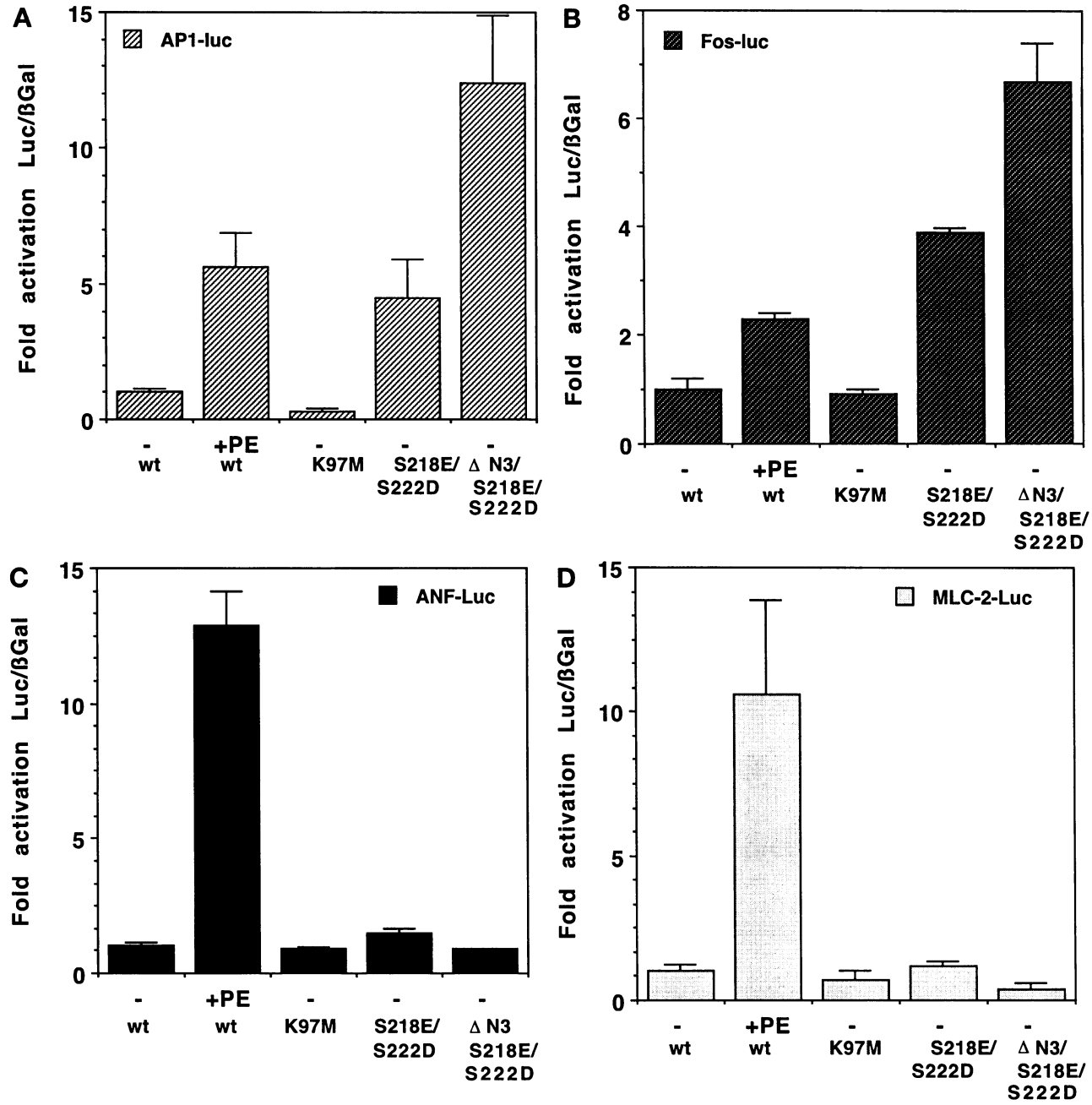

Figure 2. Active MKK induces AP1- and Fos-luciferase but not ANF- or MLC-2-luciferase. (A) Cells were transfected in 3.5-cm dishes with 3 μg of the various MKK expression plasmids, 0.7 μg of pSVβGal, plus 1 μg of a luciferase plasmid driven by two AP1 sites. Cells transfected with wild-type MKK were treated with or without 100 μM phenylephrine (PE) as a positive control for activation of the promoters. The activity of the two reporter plasmids was determined by measuring β-galactosidase and luciferase activities. (B–D) Transfections were performed as in panel A except that 3 μg of Fos-luciferase, ANF-luciferase, or MLC-2-luciferase, respectively, was used in place of the AP1 luciferase.

The MAP Kinase Phosphatase CL100 Inhibits Expression from the Fos, ANF, and MLC-2 Promoters

Previously, we found that catalytically inactive ERK1 and purine analogues that inhibit ERK activation prevented phenylephrine-induced activation of Fos-, ANF- and MLC-2-luciferase expression. These data were interpreted as meaning that ERK activity was required for expression from these promoters during the hypertrophic response (Thorburn *et al.*, 1994a). The

data in Figure 2 indicate that the ANF and MLC-2 promoters respond differently from the Fos promoter and AP1-driven genes to expression of the ERK activator MKK. We therefore wished to use a different approach to confirm the requirement for activity of the ERKs for expression from the cardiac-specific promoters. CL100 is a dual specificity phosphatase that dephosphorylates and thus inactivates the MAP kinases ERK1 and ERK2 (Alessi *et al.*, 1993). CL100 is the human homologue of a mouse phosphatase MKP1 (Sun *et al.*, 1993), and constitutive expression of this molecule prevents Ras- and serum-induced gene expression and cell growth (Sun *et al.*, 1994; Brondello *et al.*, 1995).

To determine whether CL100 inhibited Fos-, AP1-, ANF-, or MLC-2-luciferase expression, transient transfections were performed with either an empty expression plasmid or the CL100 expression plasmid. As shown in Figure 3, CL100 expression significantly reduced expression from all the promoters tested. These data confirm that expression from all the promoters, including ANF and MLC-2, is inhibited when ERK activity is blocked, although it remains possible that other MAP kinase family members that are also inhibited by CL100 may play a role in activation of these promoters. Combined with the data from the experiments shown in Figure 2 and our previous studies (Thorburn *et al.*, 1994a), we suggest that ERK activation is required for expression from the Fos, ANF, and MLC-2 promoters, and that ERK activation by MKK is sufficient to induce expression from the Fos promoter but not the ANF or MLC-2 promoters.

Constitutively Active MKK Inhibits Phenylephrine-induced ANF and MLC-2 Promoter Activity

Because MKK activity is not sufficient to induce expression from the ANF or MLC-2 promoters, we tested whether active MKK was capable of increasing expression induced by phenylephrine. We found (Figure 4A) that rather than increasing activation, active MKK actually inhibited phenylephrine-induced activation of both promoters. The more active mutant MKK (ΔN3/S218E/S222D) was a better inhibitor than the less active mutant MKK (S218E/S222D), suggesting that inhibition was dependent on the level of kinase activity. This was tested by transfecting cells with increasing amounts of the MKK (ΔN3/S218E/S222D) mutant. Figure 4B shows that the inhibitory effect of active MKK was dose-dependent, indicating that inhibition is correlated with the amount of active MKK transfected. As expected, increasing the amount of active MKK did not induce activation in the absence of phenylephrine treatment. The inhibitory effect was not due to general inhibition of gene expression or to general inhibition of phenylephrine signaling because active MKK increased both phenylephrine- and phorbol ester-induced activation of an AP1-driven reporter plasmid (Figure 4C). Phenylephrine increased MKK (ΔN3/S218E/S222D)–induced AP1 activity beyond that observed with either stimulus alone in a dose-dependent manner (Figure 4D). Similarly, Fos-lucif-

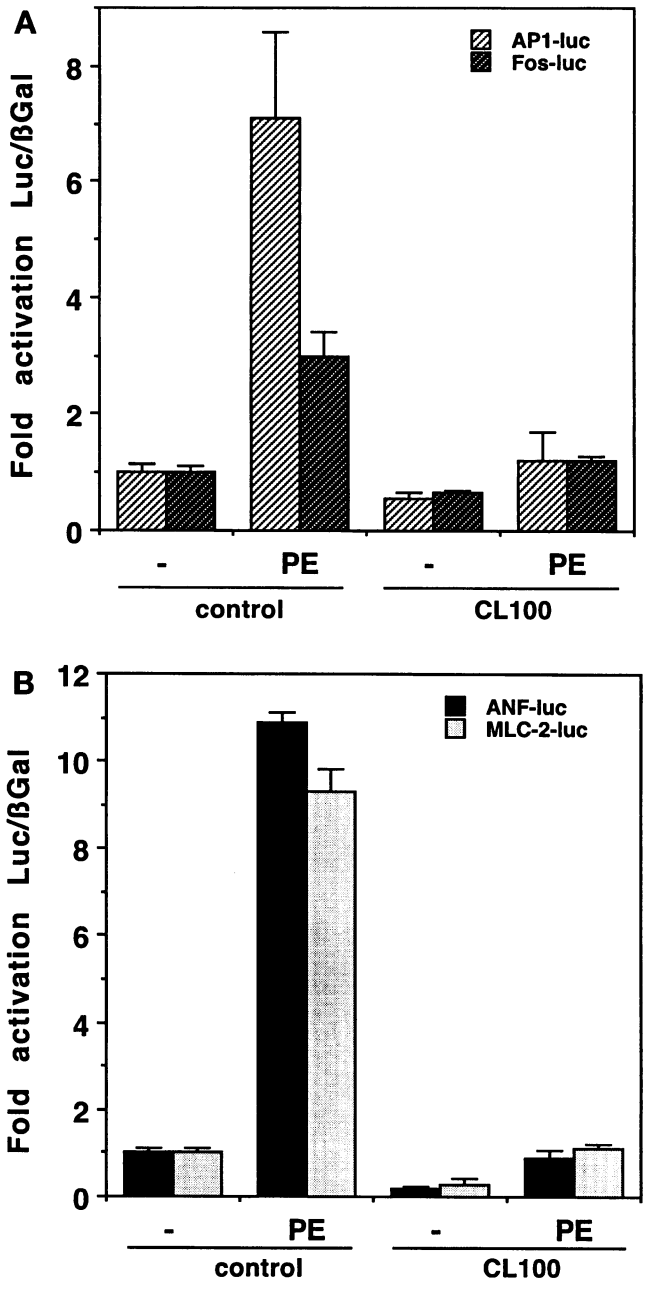

Figure 3. CL100 blocks activation of AP1-, Fos-, ANF-, and MLC-2-luciferase. (A) Transfections were performed with 1 μg of AP1-luciferase or Fos-luciferase and 0.7 μg of pSVβGal plus 3 μg of either empty expression plasmid or a CL100 expression plasmid. Cells were treated with or without phenylephrine. (B) Transfections were performed as in panel A except that 1 μg of ANF-luciferase or MLC-2-luciferase was used in place of AP1- or Fos-luciferase.

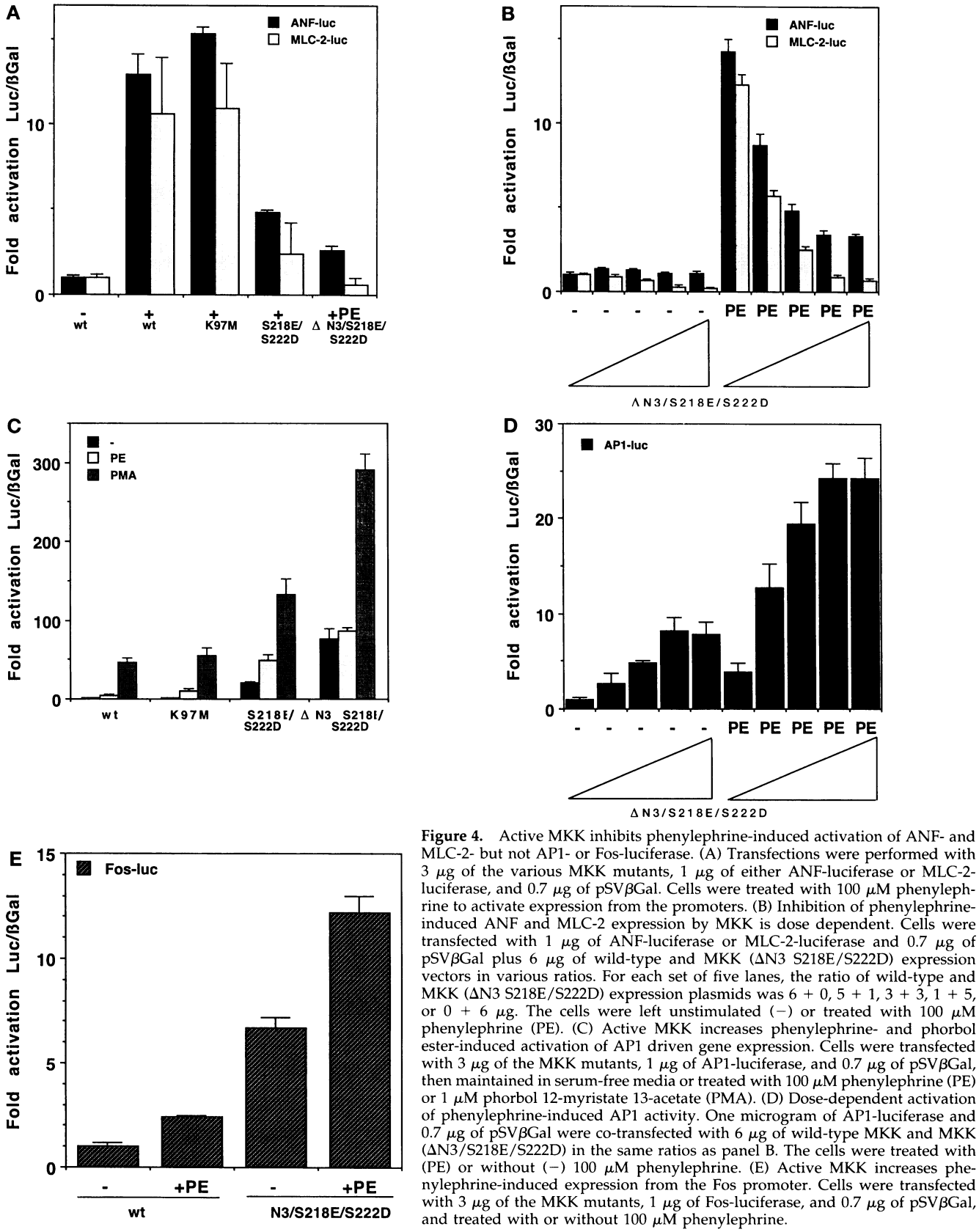

Figure 4. Active MKK inhibits phenylephrine-induced activation of ANF- and MLC-2- but not AP1- or Fos-luciferase. (A) Transfections were performed with 3 μg of the various MKK mutants, 1 μg of either ANF-luciferase or MLC-2-luciferase, and 0.7 μg of pSVβGal. Cells were treated with 100 μM phenylephrine to activate expression from the promoters. (B) Inhibition of phenylephrine-induced ANF and MLC-2 expression by MKK is dose dependent. Cells were transfected with 1 μg of ANF-luciferase or MLC-2-luciferase and 0.7 μg of pSVβGal plus 6 μg of wild-type and MKK (ΔN3 S218E/S222D) expression vectors in various ratios. For each set of five lanes, the ratio of wild-type and MKK (ΔN3 S218E/S222D) expression plasmids was 6 + 0, 5 + 1, 3 + 3, 1 + 5, or 0 + 6 μg. The cells were left unstimulated (−) or treated with 100 μM phenylephrine (PE). (C) Active MKK increases phenylephrine- and phorbol ester-induced activation of AP1 driven gene expression. Cells were transfected with 3 μg of the MKK mutants, 1 μg of AP1-luciferase, and 0.7 μg of pSVβGal, then maintained in serum-free media or treated with 100 μM phenylephrine (PE) or 1 μM phorbol 12-myristate 13-acetate (PMA). (D) Dose-dependent activation of phenylephrine-induced AP1 activity. One microgram of AP1-luciferase and 0.7 μg of pSVβGal were co-transfected with 6 μg of wild-type MKK and MKK (ΔN3/S218E/S222D) in the same ratios as panel B. The cells were treated with (PE) or without (−) 100 μM phenylephrine. (E) Active MKK increases phenylephrine-induced expression from the Fos promoter. Cells were transfected with 3 μg of the MKK mutants, 1 μg of Fos-luciferase, and 0.7 μg of pSVβGal, and treated with or without 100 μM phenylephrine.

erase was also further activated when phenylephrine-treated cells were transfected with constitutively active MKK (ΔN3/S218E/S222D) (Figure 4E).

Constitutively Active MKK Inhibits Raf-1–induced ANF and MLC-2 Promoter Activity

Previously, we found that activation of an estrogen-regulated Raf-1 molecule was sufficient to induce expression from the ANF and MLC-2 promoters (Thorburn *et al.*, 1994b). To test whether active MKK inhibited this Raf-mediated effect, we performed an experiment where cells were transfected with the ΔRaf-1:ER expression plasmid along with wild-type MKK or MKK (ΔN3/S218E/S222D) and ANF-luciferase or MLC-2-luciferase. Raf-1 activity was induced, in the absence of other stimulation, by adding estradiol to the media. Active MKK inhibited ΔRaf-1:ER–induced activation of ANF and MLC-2 (Figure 5A) but did not inhibit AP1-driven expression (Figure 5B). Unlike the case of active MKK plus phenylephrine (Figure 4D), stimulation with both active MKK and activated ΔRaf-1:ER did not further increase AP1-driven expression beyond that observed with active MKK alone (Figure 5B).

Inhibition by Active MKK of the MLC-2 Promoter Maps to a Sequence Responsible for Cardiac-specific Expression

We wished to identify sequences in the promoters of the cardiac-specific genes that were responsible for inhibition by active MKK. To do this, we focused on the MLC-2 promoter as this has been the subject of considerable analysis in transient transfection studies and transgenic mice. The experiments described above were all performed with a large fragment of the MLC-2 promoter encompassing 2.7 kb of upstream sequence. However, cardiac specificity can be demonstrated for a much smaller promoter fragment containing only 250 bp of upstream sequence (Henderson *et al.*, 1989; Lee *et al.*, 1992). Initially, we tested whether the lack of induction by active MKK and inhibition of phenylephrine-induced stimulation that we observed for the long promoter was also true for this smaller fragment. As shown in Figure 6A, the 250-bp promoter responded in the same way as the longer promoter to co-transfection with the mutant kinase.

The 250-bp MLC-2 promoter has been studied in considerable detail. Several sequence elements that are important in conferring cardiac specificity and induction have been identified (Zhu *et al.*, 1991, 1993; Navankasattusas *et al.*, 1992, 1994; Lee *et al.*, 1994). These include a negative regulatory sequence (HF3) that seems to prevent noncardiac expression (Lee *et al.*, 1994), an E box (MLE) that binds the helix-loop-helix transcription factor USF (Navankasattusas *et al.*, 1994), and a sequence (HF1) that is sufficient and required for

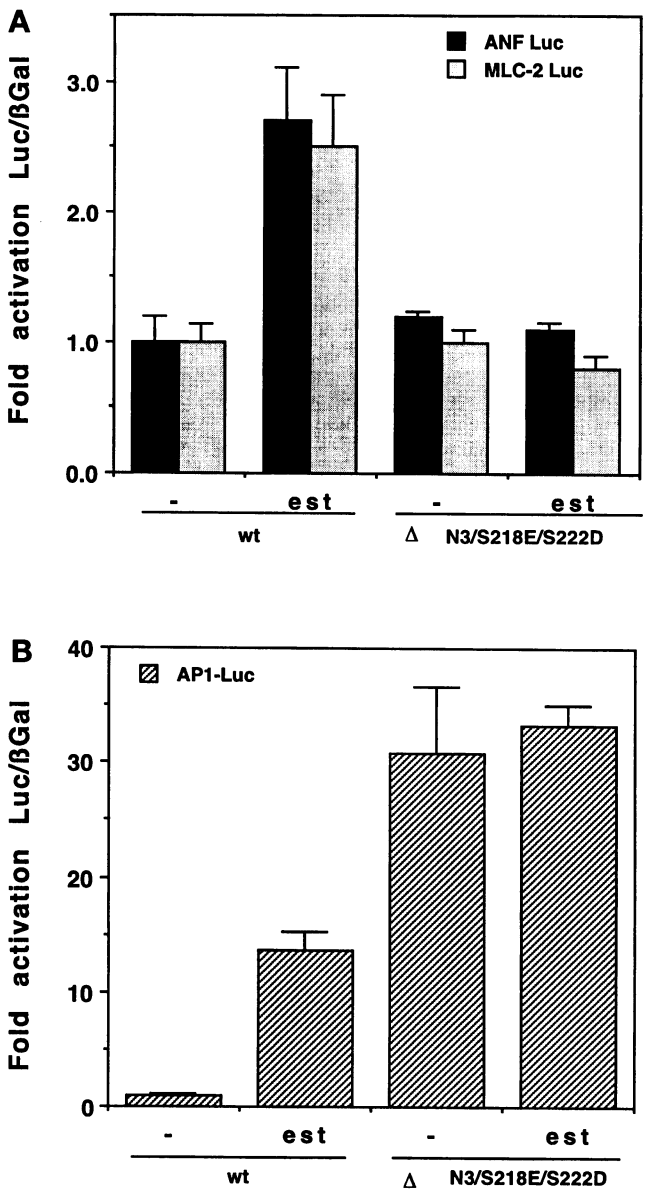

Figure 5. Active MKK inhibits Raf-induced expression of ANF and MLC-2. (A) Cells were transfected with 3 μg of an estradiol-inducible Raf-1 molecule (pCEP4 ΔRaf-1:ER), 3 μg of wild type or MKK (ΔN3 S218E/S222D), 1 μg of ANF-luciferase or MLC-2 luciferase, and 0.7 μg of pSVβGal. Raf-1 activity was induced by adding 1 μM β-estradiol to the media. (B) Active MKK does not inhibit Raf-1–induced AP1 activity. Cells were transfected with 3 μg of pCEP4 ΔRaf-1:ER, 1 μg of AP1-luciferase, 0.7 μg of pSVβGal, and 3 μg of either wild-type MKK or MKK (ΔN3/S218E/S222D).

cardiac-specific expression (Zhu *et al.*, 1991; Lee *et al.*, 1994). The HF1 sequence consists of at least two protein-binding sites, HF1a and HF1b. It is thought that this sequence confers cardiac specificity by interaction of a tissue-restricted transcription factor at one site and a ubiquitous factor at the other (Zhu *et al.*, 1991,

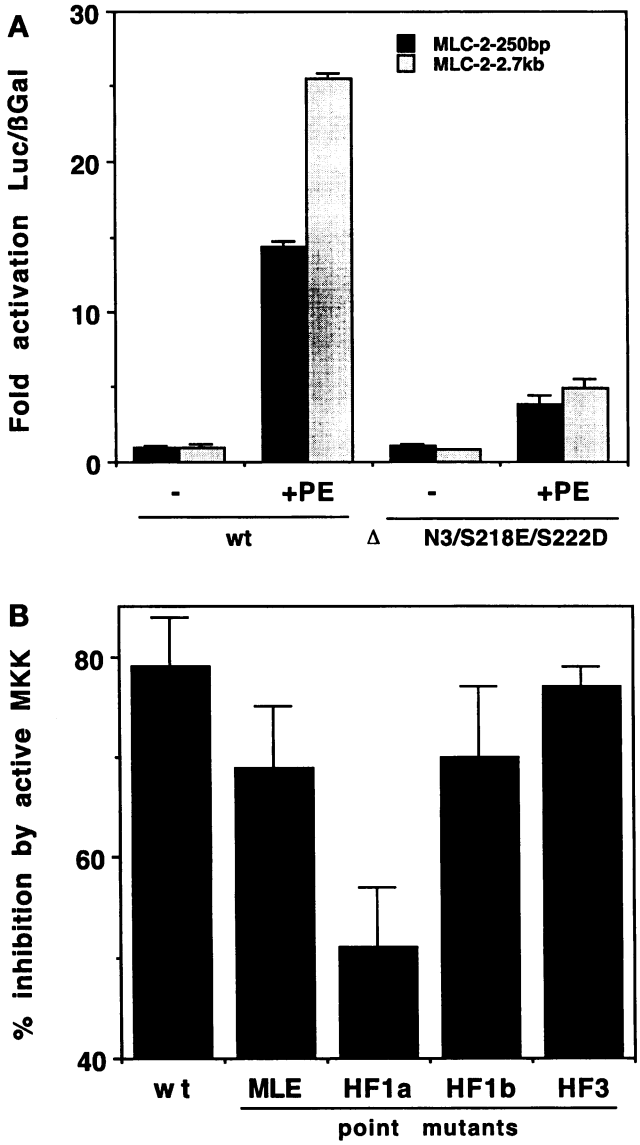

Figure 6. Inhibition of a 250-bp MLC-2-luciferase plasmid by active MKK. (A) Cells were transfected with 1 μg of the 2.7-kb MLC-2-luciferase or a 250-bp MLC-2-luciferase plasmid plus 0.7 μg of pSVβgal and 3 μg of either wild-type MKK or MKK (ΔN3/S218E/S222D). The cells were stimulated with or without 100 μM phenylephrine to induce expression. (B) Transfections were performed with 1 μg of wild-type 250-bp MLC-2-luciferase or point mutants that inactivate the previously characterized HF3, MLE, HF1a, or HF1b DNA elements (Lee *et al.*, 1994) along with 0.7 μg of pSVβGal and 3 μg of either wild-type MKK or MKK (ΔN3/S218E/S222D). The cells were stimulated with 100 μM phenylephrine and the percent inhibition by MKK (ΔN3/S218E/S222D) compared with phenylephrine-treated wild-type transfected cells was calculated for each mutant and the wild-type promoter.

1993; Navankasattusas *et al.*, 1992; Zhou and Chien, 1995).

To screen for possible target sequences that could mediate inhibition of the MLC-2 promoter by active MKK, we performed transient transfection experiments with mutated versions of the 250-bp promoter containing inactivating point mutations in each of these various DNA elements (Zhu *et al.*, 1991; Navankasattusas *et al.*, 1992). Cells were transfected with the wild-type or mutant promoters along with constitutively active MKK (ΔN3/S218E/S222D) or the wild-type MKK expression construct and stimulated with phenylephrine. The percent of inhibition by active MKK compared with the wild-type molecule was calculated for each mutant. Figure 6B shows the results obtained. Constitutively active MKK inhibited phenylephrine-induced expression of all the promoters but was less effective at inhibiting expression of one of the HF1 mutants (HF1a) than the others. This result suggested that the HF1 sequence might be the target for inhibition by active MKK. However, this experiment must be interpreted cautiously. Mutation of the HF1 sequence results in considerably reduced expression from this promoter (Zhu *et al.*, 1991, 1993; Navankasattusas *et al.*, 1992). Thus it was possible that we observed less difference between the wild-type MKK- and MKK (ΔN3/S218E/S222D)-transfected cells with the HF1a mutant simply because this molecule was much less active to begin with.

To further investigate whether the HF1 sequence was indeed a target for MKK-induced inhibition of expression, we constructed a luciferase reporter driven by three copies of the 28-bp HF1 sequence (Zhu *et al.*, 1991) upstream of the basal SV40 promoter in the plasmid pGL3 promoter. Transient transfections with this plasmid or pGL3 promoter and either empty expression vector, the ERK phosphatase, CL100, or constitutively active MKK (ΔN3/S218E/S222D) were performed. Figure 7 shows the result. As expected, the HF1 sequence was sufficient to allow expression in cardiac muscle cells as indicated by comparing expression from the pGL3 promoter plasmid and the HF1-containing plasmid. The HF1 sequence did not confer significant inducibility by phenylephrine. It should be noted that the luciferase activity is normalized to the β-galactosidase activity from the pSVβGal plasmid. As expected given the general stimulation of expression and protein synthesis associated with the hypertrophic response, we obtained about a twofold activation of β-galactosidase activity upon phenylephrine treatment. Thus, phenylephrine treatment of the HF1-luciferase–transfected cells did result in about twofold activation of luciferase expression; however, unlike the case with the full promoter, this mirrors the general effect on gene expression and protein synthesis associated with hypertrophy. When the HF1-driven plasmid was co-transfected with the CL100 molecule there was no inhibition of expression compared with the control transfections. This suggests that the HF1 sequence does not require ERK activity for expression and thus does not explain the effect of ERK inhibition

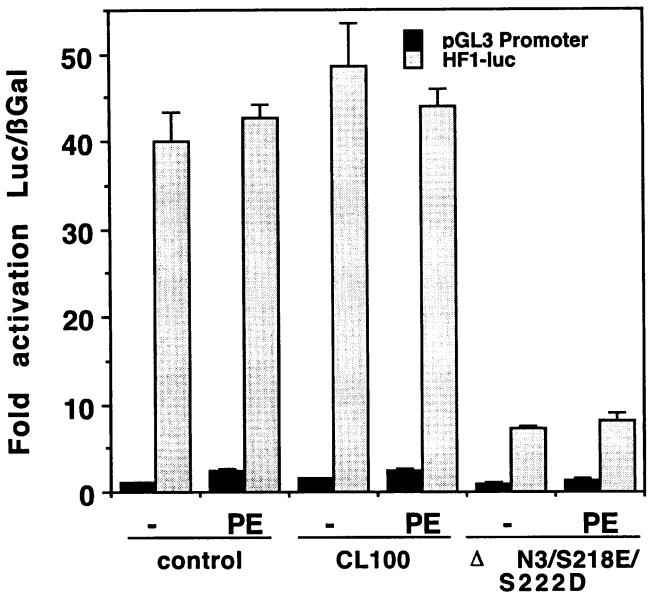

Figure 7. A luciferase plasmid driven by the HF1 sequence is inhibited by MKK (ΔN3/S218E/S222D). (A) 1 μg of pGL3 promoter or HF1-luciferase was transfected along with 0.7 μg of pSVβGal and 3 μg of empty expression vector, CL100 expression plasmid, or MKK (ΔN3/S218E/S222D). Cells were stimulated with or without phenylephrine.

on the full promoter. Notably, MKK (ΔN3/S218E/S222D) severely inhibited HF1-driven expression both with and without phenylephrine treatment, indicating that the HF1 sequence is a target for inhibition by active MKK.

DISCUSSION

We have found that constitutively active MKK1 is capable of inducing ERK2 activation and c-Fos or AP1-driven gene expression in primary neonatal cardiac myocytes. However, unlike active Ras (Thorburn *et al.*, 1993) or Raf-1 (Thorburn *et al.*, 1994b), active MKK1 is not sufficient to induce transcription from the ANF or MLC-2 promoters, two cardiac-specific promoters that are activated during ventricular myocyte hypertrophy. MKK1 and MKK2 (also known as MEK1 and MEK2) both activate ERK1 and ERK2. However, the two MKK isoforms are not identical at least in terms of their ability to interact with active Ras and Raf. Activated Ras and Raf preferentially bind to and activate MKK1 rather then MKK2 (Jelenik *et al.*, 1994). We have previously demonstrated Ras and Raf dependence of the signal transduction pathway leading to hypertrophic gene expression and so our studies with MKK1 are focused on the isoform that is more likely to be physiologically relevant. However, MKK2 may play a different role in these cells.

Expression of dominant-negative ERK1 or the ERK phosphatase CL100, prevents phenylephrine-induced expression from the ANF or MLC-2 promoters (Figure 3; Thorburn *et al.*, 1994a). Dominant-negative ERK1 also prevents Raf-1–induced expression (Thorburn *et al.*, 1994b), suggesting that the Raf-1 → MKK → ERK pathway must be active for expression to occur. MKP-1, the mouse homologue of CL100, has been reported to be highly specific for the ERKs and not to significantly dephosphorylate JNK-1 (Sun *et al.*, 1994). However, other members of the MAP kinase family could also be inactivated when CL100 is expressed in our cells and we cannot exclude the possibility that other MAP kinases are required for activation of these genes. Cells transfected with constitutively active MKK showed high levels of ERK activity. Therefore, we conclude that although ERK activity may be necessary for phenylephrine and Raf-induced expression from the ANF and MLC-2 promoters, it is not sufficient to induce expression from these promoters. Activation of the Raf → MKK → ERK pathway is, however, sufficient to explain expression from the Fos promoter, an earlier gene expression event associated with the hypertrophic response.

The simplest explanation of these data is that at least two signals emanate from Raf-1 and are required for expression from the ANF and MLC-2 promoters. The first signal is the well characterized activation of MKK and the ERKs. We do not know the identity of the second positive signal. Apart from MKK, the only potential target for Raf-1 suggested so far is IκB, the regulator of the NFκB transcription factor (Li and Sedivy, 1993). However, preliminary experiments indicate that a reporter gene driven by two copies of an NFκB-binding sequence upstream of a basal SV40 promoter is not induced by Raf or phenylephrine in primary cardiac myocytes. This is not consistent with IκB being a second downstream target for Raf-1 in the induction of the ANF and MLC-2 promoters. The requirement for two signals from Raf-1 contrasts with activation of AP1-driven genes and the Fos promoter, growth and transformation of NIH 3T3 cells, and differentiation of PC12 cells, where activation of MKK and therefore ERKs can mimic the effects observed with active Raf-1 or Ras (Brunet *et al.*, 1994; Cowley *et al.*, 1994; Mansour *et al.*, 1994). This experimental system may therefore provide a useful means for identifying other physiologically important Raf-1 targets.

Because ERK activation is required for phenylephrine- or Raf-1–induced expression from the ANF and MLC-2 promoters (Figure 3; Thorburn *et al.*, 1994a,b), it was surprising to find that constitutively active MKK actually inhibited phenylephrine- and Raf-1–induced expression from the ANF and MLC-2 promoters (Figures 4 and 5). The dose dependence of this inhibition suggests that it is dependent upon the amount of MKK or ERK activity in the cells. Because

active MKK synergized with phenylephrine to superactivate AP1-driven expression and expression of Fos-luciferase, inhibition appears to be specific to the cardiac promoters. The increased AP1 activity observed with both active MKK and phenylephrine (Figure 4D) may indicate that phenylephrine induces other signals that increase AP1 activity in addition to ERK activation. Such a signal could be activation of the JNK kinase. This kinase is thought to be primarily responsible for c-Jun activation and can be a target for active Ras through MEK kinase (Lange-Carter and Johnson, 1994; Minden *et al.*, 1994a,b).

MKK-induced inhibition of gene expression could be mediated by a variety of methods. Phosphorylation of the Ras guanine nucleotide exchanger Sos by ERKs has been suggested as a feedback inhibition mechanism (Cherniack *et al.*, 1994) and, in support of this view, Sos phosphorylation is correlated with disassembly of the Grb2-Ras complex after insulin stimulation (Cherniack *et al.*, 1995). However, no direct evidence that this is a mechanism to stop signaling exists as yet. Similarly, estrogen-induced ΔRaf-1:ER prevents NIH 3T3 cell proliferation induced by tyrosine kinase-linked growth factors. This inhibition is correlated with phosphorylation of Sos and the activation of a vanadate-sensitive phosphatase that prevents ERK activation (Samuels and McMahon, 1994). Feedback inhibition of a molecule like Sos that functions early in the signaling pathway would be expected to switch off the entire pathway. In our experiments, signaling to the Fos promoter or the AP1 transcription factor by phenylephrine is further stimulated by active MKK, suggesting that the basic kinase cascade is intact and functional. Because we obtain high levels of ERK2 activity in the MKK (ΔN3/S218E/S222D)–transfected cells, the effects that we observe are not likely to result from ERK inhibition by phosphatases. The mechanism of inhibition in our experiments therefore seems to be quite different from that occurring during ΔRaf-1:ER–mediated inhibition of 3T3 cell growth (Samuels and McMahon, 1994).

Raf-1–induced expression of ANF and MLC-2 is inhibited by active MKK, indicating that the inhibited step must be at or downstream of Raf-1 (Figure 5A). However, Raf-1–induced activation of these promoters is also inhibited by expression of dominant-negative ERK1 (Thorburn *et al.*, 1994b). It appears that Raf-1 signaling to the ERKs through MKK is required for expression from these promoters but that constitutive activation of MKK, and thus ERKs, inhibits expression. An explanation for these observations is provided by the data showing that the HF1 sequence that is responsible for the cardiac specificity of MLC-2 expression (Zhu *et al.*, 1991; Lee *et al.*, 1994) is a target for inhibition by constitutively active MKK but not CL100 (Figure 7). Thus, the positive requirement for ERK activation and the inhibitory effect of constitutive MKK activity are mediated through different DNA elements in the MLC-2 promoter.

The HF1 sequence has been analyzed in detail. At least two protein binding sites are important. The HF1a site has been reported to bind two ubiquitous proteins, USF (Navankasattusas *et al.*, 1994) and the Y-box protein EFI_A/YB-1 (Zhou and Chien, 1995). The HF1b site can bind MEF2/RSRF proteins (Zhu *et al.*, 1991; Navankasattusas *et al.*, 1992) but also a novel, tissue-restricted zinc finger protein (Zhu *et al.*, 1993). It is thought that gene expression from this DNA element is regulated by controlling the binding of these various proteins. In particular, binding of USF to the HF1a sequence may inhibit expression (Navankasattusas *et al.*, 1994) whereas YB-1 binding may stimulate expression (Zhou and Chien, 1995). It will be interesting to determine whether activation of MKK and ERK activity affects the biological functions of these or other transcription factors at this site.

In conclusion, our data suggest that the regulation of some phenotypes that are under the control of the Ras pathway is more complicated than the simple sequential linear kinase cascade that can activate Fos gene expression, NIH 3T3 cell growth, or PC12 cell differentiation (Figure 8). We think that activation of the

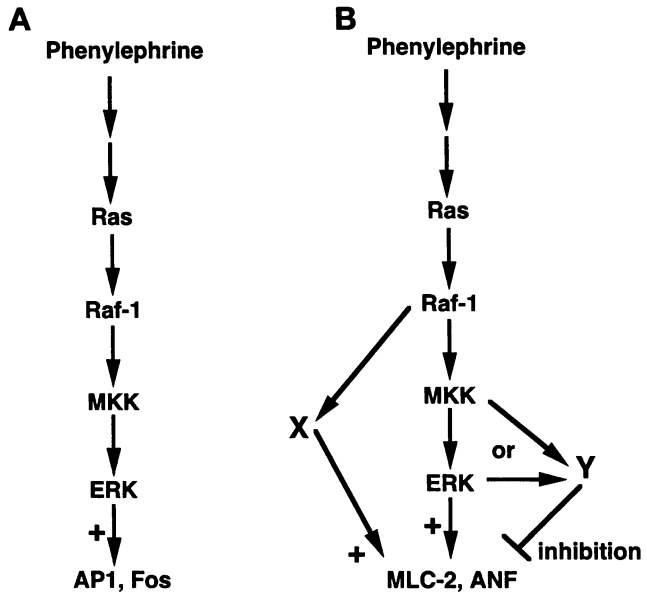

Figure 8. Models for the activation of AP1- or Fos-driven gene expression (A) and ANF- or MLC-2-driven gene expression (B) by activation of the Ras pathway after stimulation of ventricular cardiac myocytes with phenylephrine. AP1 and the Fos promoter are positive targets for the simple sequential kinase cascade, while ANF- and MLC-2-driven expression requires more complex signaling. Raf-1 signaling to both the ERKs and another positive factor (X) are required for expression. In addition, constitutive activation of MKK or ERKs inhibits expression through another factor (Y) by preventing activation of a DNA element responsible for cardiac-specific expression.

ANF and MLC-2 promoters during hypertrophic stimulation requires both activation of the ERKs and also some other positive signal downstream of Raf-1. In addition, expression can be inhibited by repressing a transcription activation element that confers cardiac-specificity. Analysis of the MLC-2 promoter suggests that these disparate effects appear to be mediated through discreet DNA elements. The linear kinase cascade is sufficient to explain early genetic events associated with the hypertrophic response, like expression of Fos. However, increased ANF or MLC-2 expression, which are later events, are more complicated. Further study of the regulation of these promoters during cardiac myocyte growth may provide a useful experimental system to study signal transduction mechanisms whose regulation is more complicated than the pathways that have been the focus of analysis so far.

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