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The Mitogen-activated Protein Kinase Kinase MEK1 Stimulates a Pattern of Gene Expression Typical of the Hypertrophic Phenotype in Rat Ventricular Cardiomyocytes*

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Adult mammalian ventricular cardiomyocytes are terminally differentiated cells that enlarge adaptively by hypertrophy. In this situation, genes normally expressed in the fetal ventricular cardiomyocyte (*e.g.* atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), and skeletal muscle (SkM) α-actin) are re-expressed, and there is transient expression of immediate early genes (*e.g.* c-*fos*). Using appropriate reporter plasmids, we studied the effects of transfection of the constitutively active or dominant negative mitogen-activated protein kinase kinase MEK1 on ANF, β-MHC, and SkM α-actin promoter activities in cultured ventricular cardiomyocytes. ANF expression was stimulated (maximally 75-fold) by the hypertrophic agonist phenylephrine in a dose-dependent manner (EC_{50}, 10 μM), and this stimulation was inhibited by dominant negative MEK1. Cotransfection of dominant negative MEK1 with a dominant negative mitogen-activated protein kinase (extracellular signal-regulated protein kinase (ERK2)) increased this inhibition. Transfection with constitutively active MEK1 constructs doubled ANF promoter activity. The additional cotransfection of wild-type ERK2 stimulated ANF promoter activity by about 5-fold. Expression of β-MHC and SkM α-actin was also stimulated. Promoter activity regulated by activator protein-1 or c-*fos* serum response element consensus sequences was also increased. We conclude that the MEK1/ERK2 cascade may play a role in regulating gene expression during hypertrophy.

Extracellular signal-regulated protein kinases (ERKs)[1] are members of the mitogen-activated protein kinase (MAPK) family and play an important role in intracellular signaling pathways that lead to the division or differentiation of a number of cell types (reviewed in Refs. 1–7). This is probably attributable to their ability to phosphorylate a variety of transcription factors and other signaling and structural proteins (reviewed in Refs. 5 and 8). Three closely related mammalian ERKs have been identified by molecular cloning (9) with ERK1 and ERK2 being the most widely distributed (10). ERKs are in turn activated by highly specific (11) *M*APK (or *E*RK) *k*inases (MEK1 or MEK2) by phosphorylation of a Tyr and a Thr residue in a conserved TEY motif (reviewed in Refs. 1 and 4). MEKs and ERKs are activated through protein-tyrosine kinase- and G protein (G_q- or G_i-)-coupled receptors (reviewed in Ref. 3). The tyrosine kinase-mediated activation involves Ras and the MEK kinase c-Raf (reviewed in Refs. 12 and 13), which phosphorylates Ser^{217} and Ser^{221} (14) in rabbit MEK1 (or corresponding Ser residues in other MEKs (15–17)). Mutation of these residues to Glu produces a rabbit MEK1 expressing increased constitutive activity (14). Activity can be further increased by deleting additionally an inhibitory domain in the N-terminal region (18). Transfection of such constructs into cultured cells results in differentiation and transformation (18–20).

In adult mammals, the ventricular cardiomyocyte is a terminally differentiated cell that loses its ability to mitose soon after birth. However, in response to the imposition of an increased workload *in vivo*, it adapts hypertrophically to accommodate the increased contractile load (reviewed in Ref. 21). This process contributes substantially to the clinical entity of "cardiac hypertrophy." In both the *in vivo* setting and primary cultures of ventricular myocytes from neonatal rat hearts, a number of characteristic transcriptional modifications distinguish the hypertrophy from normal maturational growth (reviewed in Ref. 21). Following a hypertrophic stimulus, immediate early gene (*e.g.* c-*fos*, c-*jun*, *egr*-1) expression is rapidly and transiently up-regulated. Following this, genes that are only normally expressed in the fetal ventricle are re-expressed (*e.g.* atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), and skeletal muscle α-actin (SkM α-actin)). In the slightly longer term, expression of constitutive contractile protein genes (*e.g.* ventricular myosin light chain-2, cardiac muscle α-actin) is increased. Stimulation of promoter activity for these genes has frequently been used as a marker of the hypertrophic response (reviewed in Ref. 21).

The precise physiological stimuli that induce cardiac hypertrophy *in vivo* have not been identified, and the cause may be multifactorial. In cultured myocytes, a number of interventions lead to the acquisition of the hypertrophic phenotype. These include sympathoadrenal agonists (especially α_1-adrenergic agonists (22–25)), direct activation of protein kinase C (26–29),

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[1] The abbreviations used are: ERK, extracellular signal-regulated kinase; ANF, atrial natriuretic factor; AP-1, activator protein-1; β-MHC, β-myosin heavy chain; LUX, luciferase; MAPK, mitogen-activated protein kinase; MEK, MAPK (or ERK) kinase; SkM α-actin, skeletal muscle α-actin; SRE, serum response element; TRE, 12-*O*-tetradecanoylphorbol 13-acetate response element; wt, wild type; Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.

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TABLE I
MEK1 and ERK2 expression plasmids

Species	Mutation(s)	Consequence	Vector	Reference
MEK1 mutants				
Rabbit	$Ser^{221} \rightarrow Glu$	Activating	pEXV3	14, 19
Rabbit	$Ser^{217} \rightarrow Glu$, $Ser^{221} \rightarrow Glu$	Activating	pEXV3	14, 19
Human	$\Delta N3$, $Ser^{218} \rightarrow Glu$, $Ser^{222} \rightarrow Asp$	Activating	pCEP4L	18
Rabbit	$Ser^{221} \rightarrow Ala$	Inhibitory	pEXV3	14, 19
ERK2 mutant				
Mouse	$Lys^{52} \rightarrow Ala$, $Thr^{183} \rightarrow Ala$, $Tyr^{185} \rightarrow Phe$	Inhibitory	pEXV3	50

vasoactive peptides (*e.g.* endothelin-1 (30–33) and angiotensin II (34, 35)), growth factors (*e.g.* fibroblast growth factors (36), insulin-like growth factor-1 (37), and insulin-like growth factor-2 (38)), and mechanical stretch (39–41). In the heart, the α_1-adrenergic agonist phenylephrine (42) and many of other hypertrophic agonists activate ERKs (42–47) and, where studied, MEKs (44, 47). On the basis of this correlation, we suggested (42, 44) that activation of the ERK cascade is important in the development of the hypertrophic phenotype. Here, we show directly that specific activation of the ERK cascade using transfected expression plasmids encoding constitutively active MEK mutants leads to stimulation of a variety of promoters of genes known to be up-regulated during the hypertrophic response.

EXPERIMENTAL PROCEDURES

Materials—Sprague-Dawley rats were bred within the National Heart and Lung Institute. Cell culture reagents and other reagents were from Sigma, Life Technologies, or Merck. Details of MEK1 and ERK2 expression plasmids and of firefly luciferase (LUX) reporter plasmids are given in Tables I and II, respectively. Human wild-type (wt) MEK1 and MEK1(ΔN3.E^{218}/D^{222}) were gifts from Dr. N. G. Ahn (Howard Hughes Medical Institute, Dept. of Chemistry and Biochemistry, University of Colorado, Boulder, CO) and Dr. S. J. Mansour (Dept. of Chemistry and Biochemistry and Dept. of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO). The ANF reporter construct was a gift from Dr. K. R. Chien (Dept. of Medicine, University of California San Diego). The β-MHC, SkM α-actin, and c-*fos* serum response element (SRE) reporter constructs were gifts from Dr. M. D. Schneider (Molecular Cardiology Unit, Baylor College of Medicine, Houston, TX). The TRE/AP-1 reporter construct TRE2PRL(−36) was a gift from Dr. J. H. Brown (Dept. of Pharmacology, University of California San Diego) and Dr. M. G. Rosenfeld (Howard Hughes Medical Institute, University of California San Diego). Plasmid pON249 (48) (also from Dr. K. R. Chien), in which β-galactosidase expression is controlled by a constitutive cytomegalovirus promoter, was cotransfected to control for transfection efficiency.

Transient Transfection of Ventricular Cardiomyocytes in Culture—Myocytes were isolated and cultured by a method based on that of Iwaki *et al.* (24). They were dissociated from the ventricles of 1–2-day-old rat hearts using 0.4 mg/ml collagenase and 0.6 mg/ml pancreatin in 116 mM NaCl, 20 mM Hepes, 0.8 mM Na_2HPO_4, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$ (pH 7.35). The cells were resuspended in maintenance medium (Dulbecco's modified Eagle's medium/medium 199 (4:1 (v/v)) containing 100 units/ml of both penicillin and streptomycin) supplemented with 10% horse serum and 5% fetal calf serum. Cells were preplated for 30 min on uncoated 60-mm culture dishes (Primaria, Falcon) to deplete fibroblasts, and then the myocytes were plated at a final density of 350 cells/mm^2 on 60-mm gelatin-precoated dishes. After 20 h, the medium was changed to maintenance medium (4 ml) containing 4% horse serum.

Myocytes were transfected 24 h after the initial plating by a calcium phosphate precipitation method. Plasmids were diluted in 0.25 M $CaCl_2$, and an equal volume of 50 mM Bes (pH 6.9), 280 mM NaCl, 1.5 mM Na_2HPO_4 was added. After 20 min, cells were transfected with this suspension (1 ml/plate). Myocytes were standardly transfected with 15 μg of LUX reporter plasmid, 4 μg of pON249, and a total of 10 μg of test plasmid(s). In control experiments, test plasmids were replaced by empty vectors. Controls were carried out concurrently for each transfection. In experiments where the dependence of ANF-LUX expression on phenylephrine concentration was studied, test plasmids were omitted.

After transfection for 16–20 h, cells were washed in maintenance medium containing 10% horse serum and then twice with maintenance medium. Cells were incubated for 48 h in maintenance medium, washed twice with ice-cold phosphate-buffered saline, and extracted on ice with 0.1 M potassium phosphate (pH 7.9), 0.5% (v/v) Triton X-100, 1 mM dithiothreitol (0.4 ml) for 15 min. LUX activity was assayed in 0.5 ml of 100 mM Tricine (pH 7.8), 10 mM $MgSO_4$, 2 mM EDTA, 75 μM luciferin, and 5.5 mM ATP. Light emitted was measured using an LKB 1219 RackBeta liquid scintillation counter with the photomultipliers set out of coincidence.

For assessment of transfection efficiency and cell area, cells were washed twice with ice-cold phosphate-buffered saline, fixed with 4% formaldehyde for 10 min, and stained with 0.2 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in phosphate-buffered saline. The number of blue cells in 100 fields was counted for each treatment. Cell area of transfected (blue) cells was estimated using an image grabber and planimetry.

Statistics—Statistical significance was assessed as appropriate by a two-tailed paired or unpaired Student's *t* test with a significant difference taken as being established at $p < 0.05$.

RESULTS

Transfection Efficiency and Cell Size—Transfection efficiency, determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining, was 2%. For a given experiment, the number of blue cells per 100 fields did not change significantly with any treatment. Promoter activities have not therefore been corrected for β-galactosidase activity and are presented in terms of light emitted relative to a vector control. After exposure to 0.1 mM phenylephrine for 48 h, the area of the cardiomyocytes transfected with pON249 was 1433 ± 122 μm^2 *versus* 987 ± 123 μm^2 for similarly transfected control cells cultured in serum-free medium (mean ± S.E., n = nine randomly selected cells for each condition, $p < 0.025$ by an unpaired two-tailed *t* test). In the following experiments, the areas of 50 randomly selected transfected cells were measured. Details of expression plasmids are given in Table I. Transfection of cardiomyocytes with MEK1(E^{217}/E^{221}) + ERK2(wt) (5 μg of each) in addition to pON249 did not significantly increase cell area (1087 ± 107 μm^2 *versus* 872 ± 59 μm^2 for cells transfected with 10 μg of vector, mean ± S.E., $p = 0.078$ by an unpaired two-tailed *t* test). In an analogous experiment, MEK1(ΔN3.E^{218}/D^{222}) replaced MEK1(E^{217}/E^{221}). Again, there was no significant increase in cell area (MEK1(ΔN3.E^{218}/D^{222}) + ERK2(wt), 1147 ± 55 μm^2; vector control, 977 ± 64 μm^2; mean ± S.E., $p = 0.068$ by an unpaired two-tailed *t* test).

Stimulation of ANF Promoter Activity by Phenylephrine—We confirmed earlier observations by others (25) that the α_1-adrenergic agonist phenylephrine stimulated ANF promoter activity (Fig. 1). Stimulation was readily detectable even at low (<0.5 μM) concentrations of phenylephrine, with light emitted being typically 1 million to 2 million counts/4 s at maximally effective concentrations of agonist. Stimulation was maximally about 75-fold and was half-maximal at about 10 μM phenylephrine (Fig. 1). The latter agrees well with the K_D for the binding of phenylephrine to the ventricular α_1-adrenoreceptor (about 5 μM in our hands (49)). No stimulation of LUX activity by 0.1 mM phenylephrine was detected using a reporter plasmid that

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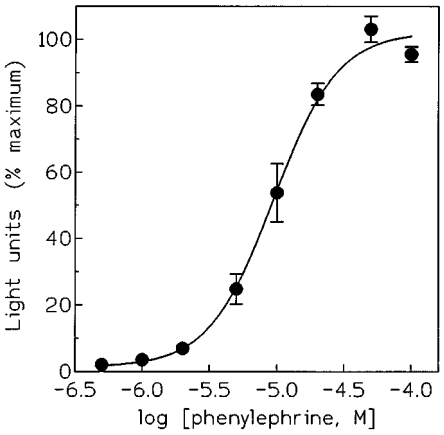

FIG. 1. **Dependence of ANF-LUX expression on phenylephrine concentration.** Following transfection of cardiomyocytes with the ANF reporter plasmid (15 μg/plate) and pON249 (4 μg/plate) for 16–20 h, the cells were exposed to phenylephrine for 48 h, and expression of LUX activity was measured as described under "Experimental Procedures." For each of the six separate experiments (minimum of three at any given phenylephrine concentration), data were fitted to sigmoid curves using the GraphPad (San Diego) Inplot 4 program. Data were normalized taking the derived maximum luciferase activity as 100%, averaged, and replotted. Data are means ± S.E.

lacked the ANF promoter sequence.

Stimulation of Promoter Activity by MEK1—Details of expression plasmids and LUX reporter constructs are given in Tables I and II, respectively. ANF promoter activity was not stimulated significantly by transfection with ERK2(wt) or with MEK1(wt) alone but was stimulated about 2-fold by transfection with a combination of these plasmids (Fig. 2). Transfection with MEK1(E^{221}) or MEK1(E^{217}/E^{221}) also doubled ANF promoter activity, but only the result with MEK1(E^{221}) was statistically significant (Fig. 2). The greatest stimulation (4.5–5-fold) of ANF promoter activity was observed when MEK1(E^{221}) or MEK1(E^{217}/E^{221}) was cotransfected with ERK2(wt) (Fig. 2). This stimulation was significantly greater than in transfections with MEK1(wt) + ERK2(wt) (Fig. 2).

Activation of other promoters (see Table II) up-regulated during the hypertrophic response was also examined (Fig. 3). Under optimal conditions for ANF promoter stimulation (*i.e.* transfection with ERK2(wt) + MEK1(E^{217}/E^{221})), the β-MHC promoter activity was stimulated 15-fold, c-*fos* SRE activity was stimulated 10-fold, and SkM α-actin activity was stimulated 5-fold. Despite the higher constitutive specific activity of human MEK1(ΔN3.E^{218}/D^{222}) over rabbit MEK1(E^{217}/E^{221}), transfection of cardiomyocytes with ERK2(wt) + MEK1(ΔN3.E^{218}/D^{222}) did not significantly increase the activities of the ANF, β-MHC, and SkM α-actin promoters or of the c-*fos* SRE over ERK2(wt) + MEK1(E^{217}/E^{221}) (results not shown). However, the reasons for this could have been trivial (*e.g.* from differences in levels of expression of MEK1). Levels of expression of the constructs (even epitope-tagged constructs) cannot be easily assessed in cultured cardiomyocytes because of the low transfection efficiency.

The sensitivity of TRE/AP-1 sites in the TRE2PRL(−36) construct to activation by MEK1 was also examined. Cotransfection of MEK1(E^{217}/E^{221}) with ERK2(wt) stimulated LUX activity significantly by 24.0 ± 7.3-fold (mean ± S.E.), whereas cotransfection of rabbit MEK1(wt) and ERK2(wt) resulted in a statistically insignificant 3.0 ± 1.0-fold (mean ± S.E.) increase (Fig. 3). Human MEK1(wt) + ERK2(wt) also increased LUX activity 5.0 ± 1.4-fold (mean ± S.E., n = five separate preparations of cardiomyocytes). MEK1(ΔN3.E^{218}/D^{222}) + ERK2(wt) increased LUX activity by 682 ± 205-fold (mean ± S.E., n = five separate preparations of cardiomyocytes, $p < 0.02$ by an unpaired two-tailed t test *versus* MEK1(E^{217}/E^{221}) + ERK2(wt)). Activation by the wild-type MEK1 constructs alone in pEXV3 or pCEP4L did not differ. The AP-1-regulated promoter was the only one that showed significantly greater activation with the human MEK1(ΔN3.E^{218}/D^{222}) expression plasmid than with the rabbit MEK1(E^{217}/E^{221}) construct. No stimulation of LUX activity by MEK1(E^{217}/E^{221}) + ERK2(wt) was detected with the promoterless vectors for ANF or TRE/AP-1 (results not shown).

Inhibition of the Stimulation of ANF Promoter Activity by Dominant Negative MEK1 and ERK2—MEK1(A^{221}) acts in a dominant negative manner in NIH 3T3 cells to block stimulation of DNA synthesis by serum (19). Cardiomyocytes were transfected with 5 μg of MEK1(wt) or MEK1(A^{221}) (plus the ANF reporter plasmid and pON249). With MEK1(A^{221}), ANF promoter activity in the presence of the EC_{50} concentration of phenylephrine (10 μM) was 70 ± 1% of that in the presence of MEK1(wt) (mean ± S.E., n = five separate preparations of cardiomyocytes, $p < 0.001$ by a paired two-tailed t test). When cells were cotransfected with 5 μg of MEK1(A^{221}) in conjunction with 5 μg of dominant negative ERK2($A^{52}/A^{183}/F^{185}$) (50), ANF promoter activity decreased to 54 ± 6% of that in the presence of ERK2(wt) + MEK1(wt) (mean ± S.E., n = five separate preparations of cardiomyocytes, $p < 0.001$ by a paired two-tailed t test).

DISCUSSION

One pathway that stimulates promoter activities of genes induced during hypertrophy involves the binding of agonists to G_q-coupled receptors, thereby stimulating phospholipase Cβ-mediated hydrolysis of membrane phosphatidylinositols (30, 49, 51–55). The ensuing increase in *sn*-1,2-diacylglycerol concentrations activates the appropriately sensitive isoforms of protein kinase C (56–60), and this leads indirectly to a increase in promoter activities (reviewed in Ref. 21). Equally, direct activation of protein kinase C induces the hypertrophic phenotype (26–29, 57, 61, 62) as does transfection of myocytes with constitutively active G_q (63). Hypertrophic agonism and protein kinase C activation also stimulate the MEK/ERK cascade (42–46), which is strongly implicated in the regulation of cell growth and differentiation (reviewed in Refs. 2–5 and 7). We have proposed that activation of MEK and ERK is an important aspect of the hypertrophic response (42, 44).

Activation of rabbit MEK1 involves phosphorylation of Ser^{217} and/or Ser^{221} in a $LIDS^{217}MANS^{221}$ sequence (14). Mutation of $Ser^{221} \rightarrow$ Glu or double mutation of Ser^{217} and $Ser^{221} \rightarrow$ Glu produces MEK1 species, which are 30–40-fold more active than the unphosphorylated enzyme (14). However, *in vitro*, these mutated species express only about 0.5% of the activity of recombinant wild-type MEK1 that had been phosphorylated by c-Raf (14). Despite this relatively low level of activity, recombinant MEK1(E^{217}/E^{221}) is able to phosphorylate and activate recombinant ERK2 fully *in vitro*.[2] By combining mutation of the analogous Ser residues in human MEK1 ($Ser^{218} \rightarrow$ Glu and $Ser^{222} \rightarrow$ Asp) with deletion of an N-terminal 20-amino acid predicted α-helix, Mansour *et al.* (18) produced a MEK1 that was 400 times more active than the unphosphorylated wild-type MEK1. As discussed in Ref. 19, the apparent discrepancy between the low level of constitutive activity of recombinant MEK1(E^{217}/E^{221}) and the ability of transfected MEK1(E^{217}/E^{221}) to activate ERK can be rationalized as follows. First, a small activation of MEK may be sufficient to activate ERK fully (64). Second, following exposure of cells to suitable agonists, endogenous MEK activity is stimulated but rapidly returns to basal values (47), presumably because of

[2] P. H. Sugden, unpublished observations.

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TABLE II
Luciferase reporter constructs

The SRE consensus sequence is $CC(A/T)_6GG$. The TRE/AP-1 reporter contained two AP-1 (TGAGTCA) consensus sequences in a 34-nucleotide sequence from the promoter region of the α-chorionic gonadotrophin gene contained within two *Xho*I sites.

Gene	Species	Nucleotide number	Vector	Reference
ANF	Rat	−638 to +62	pSV0ALΔ5′	25
β-MHC	Rat	−667 to +38	pXP1	75
SkM α-actin	Chicken	−394 to +24	pXP1	70, 75, 76
c-*fos* SRE	Mouse	−318 to −291 centred on the SRE (nucleotide −309 to −300), −56 to +109 (neutral)	pXP2	76, 77
TRE/AP-1	Rat	−36 to +34 (minimal prolactin promoter) preceded by two AP-1 sequences	pSV2ALΔ5′	78

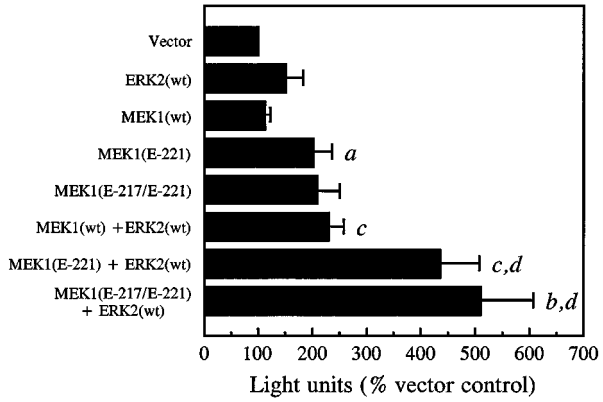

FIG. 2. **Stimulation of ANF-LUX expression by MEK1 and ERK2 expression plasmids.** Cardiomyocytes were transfected with the ANF reporter plasmid (15 μg/plate), pON249 (4 μg/plate), and MEK1 and ERK2 expression plasmids (total of 10 μg/plate, 5 μg of each or 5 μg of empty vector when appropriate) for 16–20 h as described under "Experimental Procedures." Cells were extracted after a further 48 h in maintenance medium. Results (mean ± S.E., n = five separate experiments) are expressed relative to control transfections with the pEXV3 vector (10 μg). Statistical significance by a paired two-tailed t test as follows: *a*, $p < 0.05$; *b*, $p < 0.02$; *c*, $p < 0.01$ *versus* transfection with pEXV3 vector; *d*, $p < 0.05$ *versus* transfection with MEK(wt) + ERK(wt) expression plasmids.

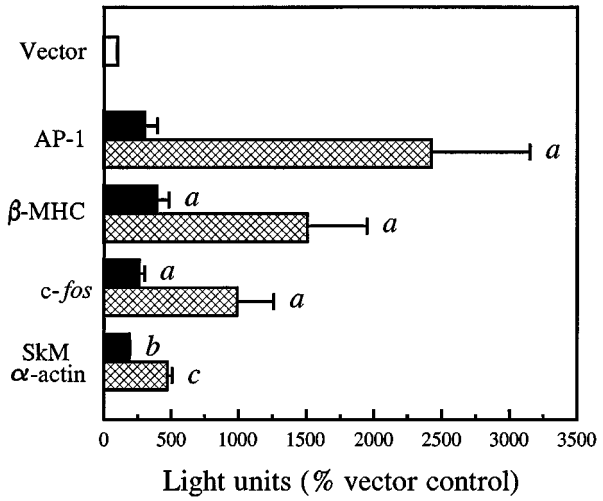

FIG. 3. **Stimulation of AP-1-regulated, β-MHC, c-*fos* SRE, and SkM α-actin promoters by MEK1 and ERK2 expression plasmids.** Cardiomyocytes were transfected with reporter plasmids for TRE/AP-1, β-MHC, c-*fos* SRE, or SkM α-actin (15 μg/plate) and additionally with pON249 (4 μg/plate), and MEK1 and ERK2 expression plasmids (5 μg of each/plate) for 16–20 h as described under "Experimental Procedures." Cells were extracted after a further 48 h in maintenance medium. Results (mean ± S.E., n = five separate experiments) are expressed relative to control transfections with the pEXV3 vector (10 μg). The *open bar* shows the vector control (100%), the *solid bars* represent transfections with MEK1(wt) + ERK2(wt), whereas the *cross-hatched bars* represent transfections with $MEK1(E^{217}/E^{221})$ + ERK2(wt). Statistical significance: *a*, $p < 0.05$; *b*, $p < 0.05$; *c*, $p < 0.001$ *versus* transfections with pEXV3 vector by a paired two-tailed t test.

protein phosphatase activity. $MEK1(E^{217}/E^{221})$ is not subject to such regulation.

To examine whether MEK (and hence its sole substrate, ERK (11)) plays any role in the regulation of expression of hypertrophic marker genes, we transfected cardiomyocytes with plasmids encoding constitutively active MEK1. Transfection with $MEK1(E^{221})$ stimulated ANF promoter activity by about 2-fold, and $MEK1(E^{217}/E^{221})$ showed the same trend (Fig. 2). Stimulation was increased to approximately 5-fold when ERK2(wt) was additionally cotransfected (Fig. 2). Thus, ERK may be limiting in the cardiomyocyte. Under optimal conditions (ERK2(wt) and $MEK1(E^{217}/E^{221})$ cotransfected), activities of promoters for β-MHC and SkM α-actin and the c-*fos* SRE were stimulated by up to 15-fold (Fig. 3). Cotransfection of $MEK1(\Delta N3.E^{218}/D^{222})$ (18) with ERK2(wt) did not increase promoter activation further (results not shown).

The stimulation of ANF promoter activity by $MEK1(E^{221})$ or $MEK1(E^{217}/E^{221})$, even with cotransfection of ERK2(wt) (Fig. 2), was much less than the 75-fold stimulation seen with maximally effective concentrations of phenylephrine (Fig. 1). In contrast, the agonist-stimulated and the $MEK1(E^{217}/E^{221})$-stimulated responses were similar in magnitude in the PC12 cell line (19). The relatively low constitutive activities of $MEK1(E^{221})$ and $MEK1(E^{217}/E^{221})$ may be more important in the primary cultures of cardiomyocytes than in established cell lines (19). The time courses of protein expression from the constructs may differ between cell types. Alternatively, the MEK/ERK cascade may not be of prime importance in the regulation of ANF promoter activity and, by implication, development of the hypertrophic phenotype. However, the stimulation of ANF promoter activity by phenylephrine is decreased by transfection with a plasmid encoding dominant negative $MEK1(A^{221})$ and inhibition is increased by cotransfecting additionally with dominant negative $ERK2(A^{52}/A^{183}/F^{185})$. Thorburn *et al.* have also recently shown that transfection of dominant negative ERK1 (as well as chemical inhibition of ERK) blocked activation of the ANF, MLC-2 and c-*fos* promoters by phenylephrine (45).

The detailed mechanisms involved in the activation of promoters for hypertrophic marker genes are unclear. For the ANF gene, the region principally responsible for phenylephrine-inducible expression (base pairs -323 to -638) contains TRE/AP-1, AP-2, CRE, Egr-1 and SRE/CArG consensus sequences (25). Cotransfection of $MEK1(E^{217}/E^{221})$ or $MEK1(\Delta N3.E^{218}/D^{222})$ with ERK2(wt) stimulates at TRE/AP-1-regulated expression by 24- or 680-fold, respectively. This is the only example we found of $MEK1(E^{217}/E^{221})$ and $MEK1(\Delta N3.E^{218}/D^{222})$ differing significantly. Activation at TRE/AP-1 sites involves the binding of heterodimers of members of the Fos and Jun families (AP-1 complexes), transactivation being stimulated by the phosphorylation of Jun (reviewed in Ref. 65). Although ERK can phosphorylate the N-terminal transactivation domain of c-Jun (66), a separate

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Jun N-terminal kinase (also known as stress-activated protein kinase) family has been identified (reviewed in Refs. 67 and 68). However, c-Jun N-terminal kinase/stress-activated protein kinases are not activated by MEK1 (69). Furthermore, Fig. 3 shows that MEK1 + ERK2 is capable of activation at AP-1 sites, presumably by phosphorylation of the AP-1 complex. An alternative pathway for activation of the ANF and SkM α-actin promoters is through their SRE/CArG sequences (25, 70). This sequence is present in the native c-*fos* promoter (reviewed in Ref. 71) and in the c-*fos* construct used here. Phosphorylation of the transcription factor Elk-1 (p62-TCF) by ERK increases its transactivating activity at SRE/CArG (72). Alternatively, other transcription factors (*e.g.* Egr-1, expression of which is coregulated with c-*fos* (73)) may be involved in the mediation of the hypertrophic response (24, 25, 74).

Our overall conclusions are that activation of the MEK/ERK cascade can stimulate ANF-, β-MHC-, c-*fos* SRE-, SkM α-actin-, and TRE/AP-1-regulated promoter activity. This may be of relevance to the development of the hypertrophic phenotype in the ventricular cardiomyocyte.

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