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), \(\beta\)-myosin heavy chain (\(\beta\)-MHC), and skeletal muscle (SkM) \(\alpha\)-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, \(\beta\)-MHC, and SkM \(\alpha\)-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 \(\mu\)m), 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 \(\beta\)-MHC and SkM \(\alpha\)-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) 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) MAPK (or ERK) kinases (MEK1 or MEK2) by phosphorylation of a Tyr and a Thr residue in a conserved TEY motif (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), \(\beta\)-myosin heavy chain (\(\beta\)-MHC), and skeletal muscle \(\alpha\)-actin (SkM \(\alpha\)-actin)). In the slightly longer term, expression of constitutive contractile protein genes (e.g. ventricular myosin light chain-2, cardiac muscle \(\alpha\)-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 \(\alpha\),\(\beta\)-adrenergic agonists (22–25)), direct activation of protein kinase C (26–29),
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 insulin-like growth factor-1 (37), and insulin-like growth factor.

**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(N3,E218,D222) (42) 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 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.

**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 ± 62 μm² versus 987 ± 123 μm² 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(ΔN3,E218,D222) + ERK2(wt) (5 μg of each) in addition to pON249 did not significantly increase cell area (1087 ± 107 μm² versus 872 ± 59 μm² 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,E218,D222) replaced MEK1(E217,E221). Again, there was no significant increase in cell area (MEK1(ΔN3,E218,D222) + ERK2(wt), 1147 ± 55 μm²; vector control, 977 ± 64 μm²; 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 α1-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 Kₐ for the binding of phenylephrine to the ventricular α₁-adrenoceptor (about 5 μM in our hands (49)). No stimulation of LUX activity by 0.1 mM phenylephrine was detected using a reporter plasmid that

| MEK1 mutants | Mutation(s) | Consequence | Vector | Reference |
|--------------|-------------|-------------|--------|-----------|
| Rabbit       | Ser221 → Glu | Activating  | pEXV3  | 14, 19    |
| Rabbit       | Ser217 → Glu, Ser221 → Glu | Activating  | pEXV3  | 14, 19    |
| Human        | ΔN3, Ser218 → Glu, Ser222 → Asp | Activating  | pCEP4L | 18        |
| Rabbit       | Ser221 → Ala | Inhibitory  | pEXV3  | 14, 19    |
| ERK2 mutant  | Mouse       |             |        |           |
| Lys52 → Ala  |             | Inhibitory  | pEXV3  | 50        |

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 n 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 MgSO₄, 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 K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], 2 mM MgCl₂ 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.

**Statistical Significance**—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.
MEK1, ERK, and Gene Expression in Cardiomyocytes

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.

Inhibition of the Stimulation of ANF Promoter Activity by Dominant Negative MEK1 and ERK2—MEK1(A221) 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(A221) (plus the ANF reporter plasmid and pON249). With MEK1(A221), ANF promoter activity in the presence of the EC50 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(A221) in conjunction with 5 μg of dominant negative ERK2(A52/A183/F185) (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 Gq-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 an 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 Gq (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 Ser217 and Ser221 and in a LIDS217/MANS221 sequence (14). Mutation of Ser221 to Glu or double mutation of Ser217 and Ser221 to 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(E217/E221) is able to phosphorylate and activate recombinant ERK2 fully in vitro. 2 By combining mutation of the analogous Ser residues in human MEK1 (Ser218 to Glu and Ser221 to 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(E217/E221) and the ability of transfected MEK1(E217/E221) 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

unpaired two-tailed t test versus MEK1(E217/E221) + 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(N3,E218/D222) expression plasmid than with the rabbit MEK1(E217/E221) construct. No stimulation of LUX activity by MEK1(E217/E221) + ERK2(wt) was detected with the promoterless vectors for ANF or TRE/AP-1 (results not shown).

The sensitivity of TRE/AP-1 sites in the TRE2PRL(–36) construct to activation by MEK1 was also examined. Cotransfection of MEK1(E217/E221) 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(N3,E218/D222) + ERK2(wt) increased LUX activity by 682 ± 205-fold (mean ± S.E., n = five separate preparations of cardiomyocytes, p < 0.02 by an
protein phosphatase activity. MEK1(E217/E221) 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(E221) stimulated ANF promoter activity by about 2-fold, and MEK1(E217/E221) 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(E217/E221) cotransfected), activation of ANF promoter activity and, by implication, development of the hypertrophic phenotype. However, the stimulation of ANF promoter activity by phenylephrine is decreased by cotransfection with a plasmid encoding dominant negative MEK1(A221) and inhibition is increased by cotransfecting additionally with dominant negative ERK2(A52/A183/F185). 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, β-MHC, c-fos SRE, and 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). Statistical significance by a paired two-tailed t test as follows: a, p < 0.05; b, p < 0.02; c, p < 0.001 versus transfection with pEXV3 vector; d, p < 0.05 versus transfection with MEK1(wt) + ERK2(wt) expression plasmids.

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 < 0.001 versus transfection with pEXV3 vector; d, p < 0.05 versus transfection with MEK1(wt) + ERK2(wt) expression plasmids.

| Gene          | Species | Nucleotide number | Vector               | Reference |
|---------------|---------|-------------------|----------------------|-----------|
| ANF           | Rat     | −638 to +62       | pSVQALΔ5′            | 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        |

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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(E217/E221) + ERK2(wt). Statistical significance: a, p < 0.05; b, p < 0.02; c, p < 0.001 versus transfections with pEXV3 vector by a paired two-tailed t test.
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 kinase 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 relevant to the development of the hypertrophic phenotype in the ventricular cardiomyocyte.

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