

# A Role for the p38 Mitogen-activated Protein Kinase Pathway in Myocardial Cell Growth, Sarcomeric Organization, and Cardiac-specific Gene Expression

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**Abstract.** Three hallmark features of the cardiac hypertrophic growth program are increases in cell size, sarcomeric organization, and the induction of certain cardiac-specific genes. All three features of hypertrophy are induced in cultured myocardial cells by  $\alpha_1$ -adrenergic receptor agonists, such as phenylephrine (PE) and other growth factors that activate mitogen-activated protein kinases (MAPKs). In this study the MAPK family members extracellular signal-regulated kinase (ERK), c-jun NH<sub>2</sub>-terminal kinase (JNK), and p38 were activated by transfecting cultured cardiac myocytes with constructs encoding the appropriate kinases possessing gain-of-function mutations. Transfected cells were then analyzed for changes in cell size, sarcomeric organization, and induction of the genes for the

A- and B-type natriuretic peptides (NPs), as well as the  $\alpha$ -skeletal actin ( $\alpha$ -SkA) gene. While activation of JNK and/or ERK with MEKK1<sub>COOH</sub> or Raf-1 BXB, respectively, augmented cell size and effected relatively modest increases in NP and  $\alpha$ -SkA promoter activities, neither upstream kinase conferred sarcomeric organization. However, transfection with MKK6 (Glu), which specifically activated p38, augmented cell size, induced NP and  $\alpha$ -SkA promoter activities by up to 130-fold, and elicited sarcomeric organization in a manner similar to PE. Moreover, all three growth features induced by MKK6 (Glu) or PE were blocked with the p38-specific inhibitor, SB 203580. These results demonstrate novel and potentially central roles for MKK6 and p38 in the regulation of myocardial cell hypertrophy.

CARDIAC myocytes, which are postmitotic, increase in size during postnatal development through a well-studied hypertrophic growth program. Myocardial cell hypertrophic growth is characterized by a number of phenotypic changes, including the activation of several immediate early genes (e.g., c-fos, c-jun, and egr-1), increased expression of genes encoding certain sarcomeric proteins (e.g.,  $\alpha$ -skeletal actin,  $\beta$ -myosin heavy chain, and myosin light chain-2), and the induction of the genes for the A- and B-type cardiac natriuretic peptides (ANP and BNP)<sup>1</sup> (Schneider et al., 1992; Van Bilsen and Chien, 1993; Lembo et al., 1995). Although myocardial mass in the fully developed adult does not generally undergo significant increases in size, in some pathological conditions, such as

overload-induced hypertrophy, adult cardiac myocytes do reenter a hypertrophic growth program very similar to that observed in the developing neonatal heart (Schneider et al., 1992; Van Bilsen and Chien, 1993; Lembo et al., 1995; Van Heugten et al., 1995; Yamazaki et al., 1995).

Cultured neonatal rat ventricular cardiac myocytes have served as a model system for studies aimed at gaining a better understanding of this interesting mechanism of cell growth. Primary myocardial cells respond to a variety of stimuli by undergoing a hypertrophic growth program virtually identical to that observed in the developing neonate and the pathologic adult myocardium (Van Bilsen and Chien, 1993). For example, cultured myocardial cells treated with the  $\alpha_1$ -adrenergic receptor agonist, phenylephrine (PE), various other growth factors, or mechanical loading or electrical pacing of contractions display marked increases in size, enhanced sarcomeric organization, and induction of the cardiac genes associated with the hypertrophic growth program (Simpson, 1983; Komuro et al., 1990; McDonough and Glembotski, 1992; LaMorte et al., 1994; Sadoshima et al., 1995; Bogoyevitch et al., 1995b; Karns et al., 1995; Sprenkle et al., 1995; LaPointe et al., 1996; Thuerlauf and Glembotski, 1997). The convergence

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1. *Abbreviations used in this paper:*  $\alpha$ -SkA,  $\alpha$ -skeletal actin; ANP and BNP, A- and B-type cardiac natriuretic peptides; ATF, activating transcription factor; CMV, cytomegalovirus; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; hsp, heat-shock protein; JNK, NH<sub>2</sub>-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP, MAPK-activated protein kinase; PE, phenylephrine.

of these diverse stimuli on the features that define myocardial cell growth suggests that a select group of intracellular signaling pathways are coordinately activated by these treatments. Accordingly, a better understanding of myocardial cell signaling pathways that contribute to hypertrophic growth is required to fully grasp normal cardiac development as well as counterproductive pathological growth.

Studies of the intracellular signaling mechanisms responsible for myocardial cell growth have focused on the mitogen-activated protein kinases (MAPK), in part because at least one of the MAPKs, extracellular signal-regulated kinase (ERK), has been implicated in mitogenic growth in a variety of cell types (Cobb et al., 1991). Typically, ERK is activated via the well-known sequential pathway sometimes referred to as a MAPK module. MAPK modules consist of three members, a MAPKKK, followed by a MAPKK and then the terminal MAPK, itself. In the case of the ERK pathway, Ras stimulates the MAPKKK, Raf-1, which activates the MAPKK known as MEK (Kyriakis et al., 1992; Davis, 1993).

In cultured cardiac myocytes, PE activates Ras, Raf, MEK, and ERK (Thorburn and Thorburn, 1994; Bogoyevitch et al., 1993a, b; Clerk et al., 1994; Thorburn et al., 1994a), and transfection of constructs encoding active Ras, Raf, or MEK can induce ANP,  $\beta$ -MHC,  $\alpha$ -skeletal actin ( $\alpha$ -SkA) and/or MLC-2 promoter activities (Thorburn et al., 1993, 1994b; Abdellatif et al., 1994; Thuerauf and Glembotski, 1997). Moreover, dominant interfering forms of Ras or Raf can inhibit PE-induced ERK and cardiac gene promoter activity (Thorburn et al., 1993, 1994b; Thorburn, 1994), consistent with a role for ERK in the cardiac growth program. However, the overexpression of active forms of either Ras or ERK does not lead to the sarcomeric organization typical of hypertrophic growth (Thorburn et al., 1994a,b), and inhibiting MEK with PD 098059 does not block PE-induced sarcomeric organization or ANP gene expression (Post et al., 1996). Additionally, while myocardial cell ERK can be activated by some agonists, such as PE, ET, or FGF, each of which can cause hypertrophic growth, it can also be activated by agonists, such as ATP or carbachol, neither of which support myocardial cell growth (Post et al., 1996). These findings suggest that the activation of ERK alone is not sufficient to confer hypertrophic growth and the related gene expression.

Another member of the MAPK family, the stress-kinase c-Jun NH<sub>2</sub>-terminal kinase (JNK) (Davis, 1994; Cano and Mahadevan, 1995; Karin, 1995) has received some attention as a potential mediator of growth in cardiac myocytes. The activation cascade for JNK is somewhat less understood than that for ERK. However, it is believed that in most cells the sequential stimulation of the Ras-like monomeric GTP-binding protein, Rac, perhaps by Ras itself, leads to the activation of MEKK1, a MAPKKK that then activates the MAPKK, MEK4, also called SEK or JNK kinase, culminating in the activation of c-jun kinase (JNK)/MAPK (Lange-Carter et al., 1993; Derijard et al., 1994).

JNK is activated in cultured myocardial cells by the growth promoters PE and ET-1 (Bogoyevitch et al., 1995a), suggesting that it may contribute to the hypertrophic phenotype. Transfection of cultured myocardial cells with a construct encoding active MEKK1 leads to an

approximately twofold increase in myocardial cell size and 5–50-fold enhancement of ANP,  $\beta$ -MHC, and  $\alpha$ -SkA promoter activities (Bogoyevitch et al., 1995a, 1996). However, there have been no reports that JNK activation fosters the sarcomeric organization that is an obligate feature of the hypertrophic phenotype. Thus, it appears that like the ERK pathway, the JNK pathway alone may not be sufficient to support all of the main features associated with the hypertrophic growth program.

The third and most recently characterized member of the MAPK family is the stress-kinase, p38, also known as protein kinase HOG1 (Rouse et al., 1994; Raingeaud et al., 1995). In comparison to the JNK pathway but in contrast to ERK, the p38 pathway is not commonly activated by mitogens but is induced by cell stresses such as endotoxins, osmotic shock, or metabolic inhibitors. The upstream activators of p38 are poorly understood; however, recent studies have resulted in the cloning and characterization of members of the p38 MAPKK module, most notably MKK4, which can activate both JNK and p38 (Lin et al., 1995), and MKK3 (Derijard et al., 1995) and MKK6 (Raingeaud et al., 1996; Han et al., 1996), either of which specifically activate p38. MKK6 is also known as SAPKK3 (Cuenda et al., 1996).

In previous studies, it was shown that a p38-like kinase is induced in isolated perfused rat heart after preconditioning, which is a brief ischemic treatment known to protect cardiac tissue from damage due to subsequent ischemic episodes taking place soon thereafter (Bogoyevitch et al., 1996; Maulik et al., 1996). The possibility that p38 activation could contribute to the protective effects of preconditioning is consistent with findings that p38 can lead to the phosphorylation of heat-shock proteins (Stokoe et al., 1992b), a modification thought to enhance their cell-protective effects. However, the putative role of the p38 pathway in the hypertrophic growth of cardiac myocytes has not been assessed.

The goal of the present study was to compare the effects of activating the p38 pathway with activation of ERK and JNK on the three major features of the myocardial cell hypertrophic growth program—cell size, sarcomeric organization, and activation of cardiac gene expression. It was found that only p38 activation conferred all three features of myocardial cell growth in a manner similar to that observed using PE. These results suggest that p38 plays a central role in mediating the cardiac hypertrophic growth pathway, a concept that could also be applicable to other terminally differentiated cell types.

## Materials and Methods

### Cell Culture

Primary ventricular myocytes were prepared from 1–4-d-old neonatal rats as previously described (Sprenkle et al., 1995; Thuerauf and Glembotski, 1997). After the enzymatic dissociation of ventricular tissue, the cells were plated onto uncoated plastic dishes in DME/F-12 (1:1)/10% FBS for 1 h during which time most of the fibroblasts adhered to the dish. The recovered cells were then transfected (see below), plated on fibronectin-coated plastic dishes or glass slides ( $3 \times 10^5$  cells/mm<sup>2</sup>), and then maintained for about 18 h in DME/F-12 (1:1)/10% FBS. The cultures were then washed briefly with medium, refed with serum-free DME/F-12 (1:1), maintained for 48 h, and then either extracted for reporter enzyme assay (see below)

or fixed and then analyzed by immunocytofluorescence or staining with fluorescent phalloidin.

## Transfections

After preplating (see above), myocardial cells were resuspended at a density of 30 million cells/ml minimal medium (DMEM:F12 [GIBCO BRL, Gaithersburg, MD] containing 1  $\mu$ g/ml BSA) and transfections were carried out as described previously (Sprenkle et al., 1995; Thuerauf and Glembofski, 1997). Briefly, for each transfection, 300  $\mu$ l, or 9 million cells, were mixed with 15–30  $\mu$ g of either ANP-3003GL (Sprenkle et al., 1995), BNP-2501GL (Thuerauf and Glembofski, 1997),  $\alpha$ -SkA-394GL (MacLellan et al., 1994), or pG5E1bLuc (Raingeaud et al., 1996); 3–9  $\mu$ g of cytomegalovirus- $\beta$ -galactosidase (used as a normalization reporter except where the test construct is known to activate CMV promoter activity [Gillespie-Brown et al., 1995; Paradis et al., 1996; Post et al., 1996]); and in some experiments, 15–45  $\mu$ g of an activated Ras, Rac, Raf-1, JNKKK, MKK6, activating transcription factor-2 (ATF2)/Gal4, or MEF2C/Gal4 expression construct (see below). The levels of plasmid used in each culture within an experiment were equalized using empty vector DNA, such as pCEP. Each 300- $\mu$ l aliquot was then electroporated in a Bio-Rad (Hercules, CA) Gene Pulser at 700 V, 25  $\mu$ F, 100  $\Omega$  in a 0.2-cm-gap cuvette, a protocol that allows for the selective transfection of only cardiac myocytes (Sprenkle et al., 1995). This procedure results in an  $\sim$ 30% viability (Sprenkle et al., 1995); Accordingly, the 3 million viable cells were plated into fibronectin-coated 35-mm wells, at  $1 \times 10^6$  cells/well, into 24-mm wells at  $0.5 \times 10^6$  cells/well, or into four-chamber Lab Tek chamber slides at  $0.3 \times 10^6$  cells/2 cm<sup>2</sup> well.

## Reporter Enzyme Assays

Transfected cells were maintained in DMEM:F12 supplemented with 10% FBS for  $\sim$ 16 h after electroporation. The cells were then washed thoroughly and the medium was replaced with minimal medium. Unless otherwise stated, 24 h later, the medium was again replaced with minimal medium  $\pm$  50  $\mu$ M phenylephrine with 1  $\mu$ M propranolol added to block  $\beta$ -adrenergic receptors. Luciferase and  $\beta$ -galactosidase assays were performed as described (Sprenkle et al., 1995). Luciferase activity was measured for 30 s on a Bio Orbit 1251 Luminometer (Pharmacia Biotech, Inc., Piscataway, NJ). Data are expressed as "Relative Luciferase (Rel Luc)" = arbitrary integrated luciferase units/ $\beta$ -galactosidase units, representative of at least three independent experiments performed with two different plasmid preparations, and represent the mean and SEM of triplicate 35- or 24-mm wells.

## Test Expression Constructs

To assess the effects of various signaling proteins, the following constructs were used: pDCR H-Ras<sup>V12</sup> (codes for activated Ha-Ras; from D. Barsagi, State University of New York at Stony Brook, NY), pDCR Rac<sup>V12</sup> (codes for activated Rac; from M. Cobb and J. Frost, University of Texas Southwestern Medical Center, Dallas, TX), RSV-Raf-1 BXB (codes for activated Raf-1 kinase; from U. Rapp, University of Wurzburg, Wurzburg, Germany), pCMV5 MEKK<sub>COOH</sub> (codes for activated MEKK-1; from G. Johnson, University of Colorado, Denver, CO), pcDNA3 MKK6 (Glu) (codes for activated MKK6, or p38/MAPKK; from R. Davis, University of Massachusetts, Worcester MA), ATF2/Gal4 (codes for the ATF2 transcriptional activation domain fused to the Gal4 DNA-binding domain; from R. Davis), MEF2C/Gal4 (codes for MEF2C fused to the Gal4 DNA-binding domain; from J. Han, The Scripps Research Institute, La Jolla, CA), MEF2C-S/Gal4 (codes for mutant [Ser to Ala 387] MEF2C fused to the Gal4 DNA-binding domain; from J. Han), pG5E1bLuc (codes for 5X Gal4 sites cloned upstream of a prolactin promoter driving luciferase expression; from R. Davis). Preliminary experiments using different concentrations of each construct verified that optimal doses were chosen.

## Immunocytofluorescence: Morphometric Analyses

To study the effects of activating the MAPK pathways on cell size and sarcomeric organization, myocardial cells were cotransfected with a test expression construct or an empty vector control and CMV- $\beta$ -galactosidase. Double-staining experiments demonstrated a cotransfection efficiency of  $\sim$ 85%. After treatment for 48 h with or without PE, cultures were fixed, as described (McDonough and Glembofski, 1992). Transfected cardiac myocytes were identified by immunostaining for  $\beta$ -galactosidase using a Texas red-conjugated anti-mouse IgG. Since the  $\beta$ -galactosidase is cytosolic, staining was uniform throughout the myocardial cells, facilitating the

determination of cell area (see below). The same samples were also stained with BODIPY-conjugated phalloidin, an actin-specific stain, to evaluate sarcomeric organization, as described (McDonough and Glembofski, 1992).

To study the effects of the test expression constructs on endogenous cardiac gene expression, myocardial cells were cotransfected with a test expression construct or an empty vector control and ANP-3003GL. After fixation, the cells were immunostained for luciferase using a rabbit anti-luciferase antiserum, and they were immunostained for ANP using a mouse monoclonal antibody to rat ANP (Glembofski et al., 1987) and visualized using differential fluorescence. Generally, positive ANP staining was visualized using a Texas red-conjugated anti-mouse IgG, and positive luciferase staining was visualized using an FITC-conjugated anti-rabbit IgG.

## Morphometric Analyses

**Cell Size.** Transfected myocytes that immunostained positively for  $\beta$ -galactosidase were microscopically visualized under fluorescent illumination and photographed. The photographic images were then digitally acquired using a scanner (model ES-1200C; Epson America, Inc., Torrance, CA) attached to a Apple Power Mac 8500 (Cupertino, CA). The area in pixels of each digitized image was determined using NIH Image software and compared to a standard image possessing an area of 1  $\mu$ m<sup>2</sup>. This enabled the designation of area, in square micrometers, to each cell image; between 20 and 50 images of different cells derived from each treatment were analyzed. The values reported are the mean areas, in square micrometers  $\pm$  standard error.

**Sarcomeric Organization.** Transfected myocytes were identified by positive  $\beta$ -galactosidase immunostaining and observed using a Texas red-compatible filter. The cells were then viewed after phalloidin staining using an FITC-compatible filter and scored positively for sarcomeric organization if the myofilament alignment resembled that in cells treated with PE. Approximately 50–100 cells from each of three cultures per treatment were assessed. Generally, treating CMV- $\beta$ -galactosidase/pCEP-transfected cells with PE (positive control) or without (negative control) resulted in  $\sim$ 25 and 2% of the  $\beta$ -galactosidase-positive cells scoring for sarcomeric organization, respectively. In experimental cultures transfected with CMV- $\beta$ -galactosidase and a test construct, the number of cells scoring positive for sarcomeres was normalized to (divided by) the number of cells scoring positive for sarcomeres obtained with PE, which gave maximal values, and the results are displayed as percentages of maximal values.

## MAPK Assays

To test the effects of treatments on MAPK activity levels, hemagglutinin (HA)-tagged forms of ERK1 (pCEP4 HA-wt-ERK1 from M. Cobb), JNK (SRa-HA-JNK from G. Johnson) or p38 (pCEP4 HA-wt-p38Hog1 from M. Cobb) were cotransfected with the test constructs. After the appropriate times, cultures were extracted in a buffer containing 10 mM Tris, pH 7.6, 1% Triton X-100, 0.05 M NaCl, 5 mM EDTA, 2 mM *o*-vanadate, and 20  $\mu$ g/ml aprotinin. After brief centrifugation, extracts were incubated for 2 h at 4°C with HA monoclonal antibody (12CA5; Boehringer-Mannheim Corp., Indianapolis, IN), bound to protein A-Sepharose (Pharmacia Biotech, Inc.) and immune-complex kinase assays were carried out using the appropriate substrates, as described (Derijard et al., 1994; Post et al., 1996). Briefly, reactions were initiated by the addition of 1  $\mu$ g of the appropriate substrate, MBP for ERK, GST-c-Jun for JNK, Phas-I for p38, and 6  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (5,000 Ci/mmol) in a final volume of 30  $\mu$ l of kinase buffer (20 mM Hepes, pH 7.4, 20 mM MgCl<sub>2</sub>, 20 mM  $\beta$ -glycerophosphate, 2 mM DTT, 20  $\mu$ M ATP). After 30 min at 25°C, the reactions were terminated by the addition of Laemmli sample buffer, and the phosphorylation level of substrate proteins was evaluated by SDS-PAGE followed by autoradiography and phosphorimage analyses.

In each experiment, two identically treated cultures ( $1.5 \times 10^6$  cells/35-mm dish) were used for each treatment, and after densitometric analyses of the exposed phosphorimage plates, values for each treatment were averaged.

## Results

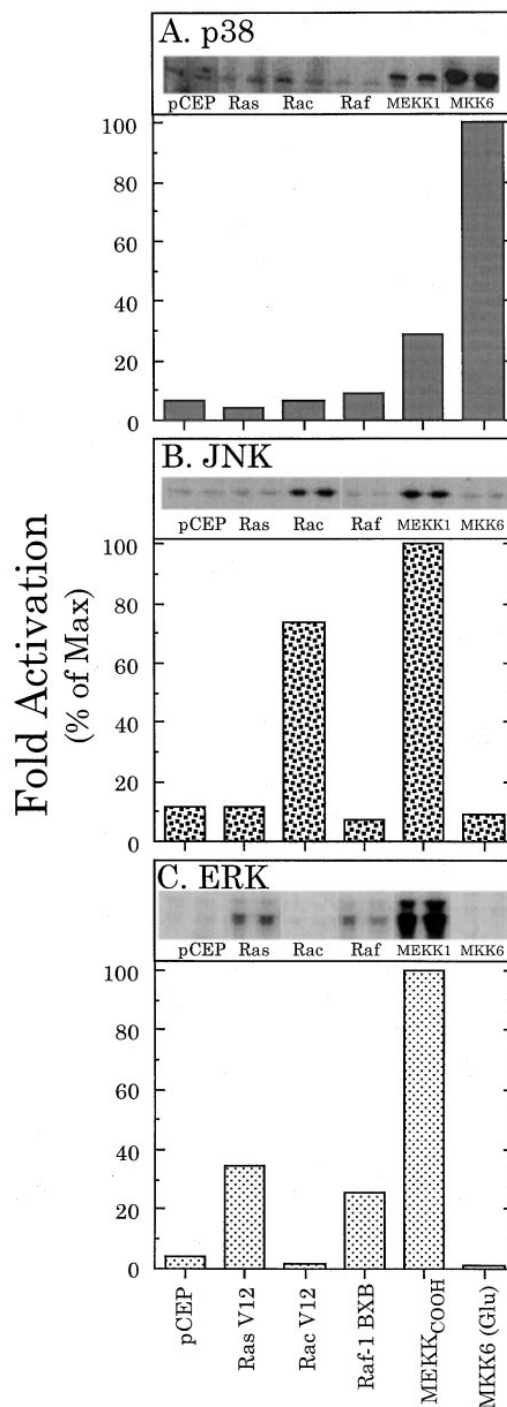
### *MKK6 (Glu) Selectively Activates p38 in Myocardial Cells and Strongly Stimulates Cardiac Gene Expression*

To characterize the effects of overexpressing various sig-

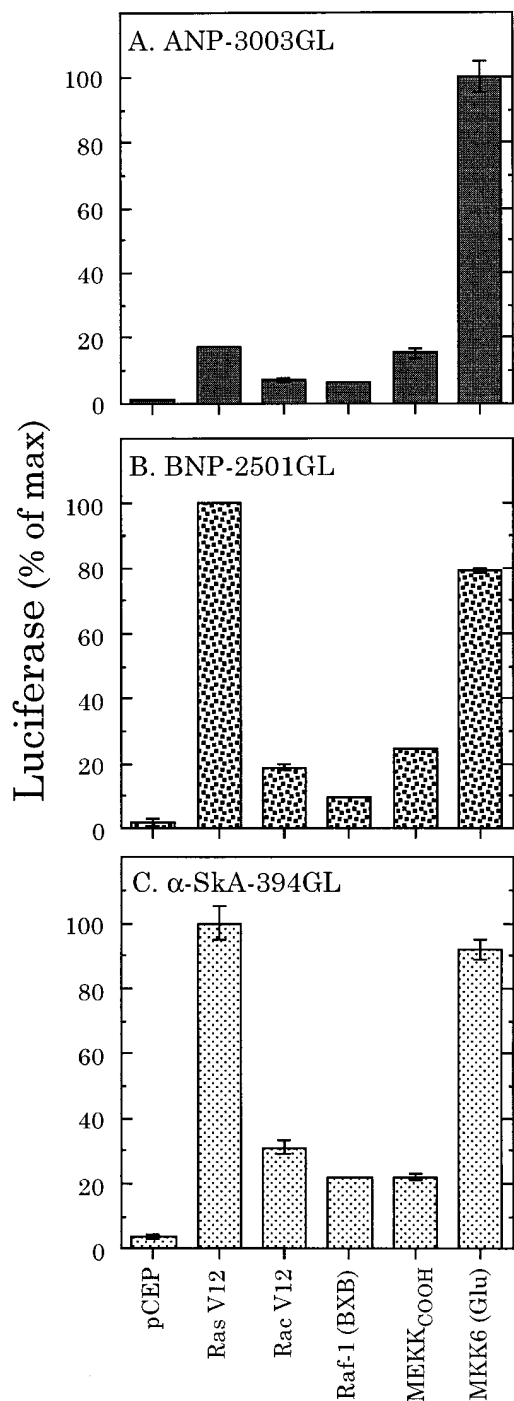
naling proteins on each of the three MAPK family members in the cardiac context, myocardial cells were cotransfected with constructs encoding gain-of-function forms of Ras, Rac, Raf-1 kinase, JNKK kinase, or p38 kinase, and constructs encoding HA-tagged p38, JNK, or ERK. In the cardiac myocytes, Ras V12 served as a poor activator of either p38 or JNK, but as expected, it was a strong activator of ERK (Fig. 1). Rac V12 had no effect on p38 or ERK in the cardiac cells; however, it strongly activated JNK, consistent with its hypothesized ability to serve as an upstream activator of MEKK1 (Lange-Carter et al., 1993; Derijard et al., 1994). Raf BXB, which encodes an active form of Raf-1 kinase (Bruder et al., 1992; Kolch et al., 1993), served primarily as an ERK activator, while MEKK<sub>COOH</sub>, a truncated active form of MEKK1 (Lange-Carter et al., 1993), mildly activated p38 by about fourfold, moderately activated JNK by 8–10-fold, as expected, but more strongly stimulated ERK by about 25-fold (Fig. 1). The ability of MEKK<sub>COOH</sub> to activate both JNK and ERK is consistent with results in other cell types (Minden et al., 1994). Importantly, however, MKK6 (Glu), an activated form of the p38 kinase, MKK6 (Raingeaud et al., 1996), potently activated p38 in the cardiac myocytes by about 16-fold, with no effect on either ERK or JNK (Fig. 1). A kinase-dead form of MKK6, MKK6 (K82A) (Raingeaud et al., 1996), did not activate p38 in the cardiac cells (not shown). These results verify the utility of constructs encoding activated forms of Raf, MEKK1, and MKK6 as stimulators of the MAPK pathways, and in particular, they clearly show that MKK6 serves as a very selective p38 activator in cardiac myocytes.

The abilities of the various expression constructs to activate three cardiac genes (ANP, BNP, and  $\alpha$ -SkA) that serve as hallmarks of the hypertrophic growth program were tested using ANP-3003GL, BNP-2501GL, or  $\alpha$ -SkA-394GL. These reporter constructs possess 3,003, 2,501, or 394 bp of the ANP, BNP, or  $\alpha$ -SkA 5'-flanking sequences, respectively. As expected from previous studies (Thorburn et al., 1993; MacLellan et al., 1994; Thuerlauf and Glembotski, 1997), Ras V12 served as a strong activator of both natriuretic peptide (NP) promoters, fostering up to 50-fold activation of luciferase expression (Fig. 2). The Rac V12 construct also activated these promoters, but less strongly than Ras,  $\sim$ 10-fold; this may reflect the differential efficacies of ERK and JNK as inducers of the cardiac genes studied. Although Raf BXB and MEKK<sub>COOH</sub> stimulated NP and  $\alpha$ -SkA promoter activities by up to 20-fold, most notable were the effects of the p38-activating construct, MKK6 (Glu), which stimulated up to 130-fold (Fig. 2). These findings suggest that while each of the MAPK pathways can stimulate cardiac natriuretic peptide and  $\alpha$ -SkA gene expression, the p38 pathway as stimulated with MKK6 (Glu) confers the strongest induction of the three genes studied.

**Figure 1.** Activation of p38, JNK, and ERK MAP kinases in myocardial cells. Myocardial cells were cotransfected with an expression construct encoding activated Ras (Ras V12), Rac (Rac V12), Raf (Raf-1 BXB), JNK kinase (MEKK<sub>COOH</sub>), p38 kinase (MKK6 [Glu]), or an empty vector control (pCEP) and either HA-p38, HA-JNK, or HA-ERK. After a 48-h incubation in se-



rum-free media, the cultures ( $\sim 3 \times 10^6$  cells each) were extracted and incubated with an HA monoclonal antibody, and the appropriate kinase assay was carried out on the resulting immune complex, as described in the Materials and Methods. After exposing the resulting SDS gel to a phosphorimager plate, each phosphorylated substrate band was digitized and printed (see the inset of each panel). The relative density of each band was determined using Molecular Dynamics Image Quant software (Sunnyvale, CA). Each treatment was carried out on two identical cultures, and the average of the band density for each treatment was then normalized to the maximal value obtained in each experiment. Shown is the percentage of the maximum; the average variation between duplicate samples was 10% or less. This is representative of three identical experiments that produced similar results.



**Figure 2.** Effects of Ras, Rac, and MAP kinase pathway expression constructs on cardiac-specific promoter activities in myocardial cells. Myocardial cells were cotransfected with an expression construct encoding activated Ras (Ras V12), Rac (Rac V12), Raf (Raf-1 BxB), JNK kinase (MEKK<sub>COOH</sub>), p38 kinase (MKK6 [Glu]), or an empty vector control (pCEP) and either an ANP (ANP-3003GL), BNP (BNP-2501GL), or  $\alpha$ -SkA ( $\alpha$ -SkA-394GL) promoter/luciferase reporter construct. These reporter constructs contain either the full-length, 3,003 bp of rat ANP 5'-flanking sequence, the full-length, 2,501 bp of rat BNP 5'-flanking sequence, or -394 bp of the rat  $\alpha$ -SkA 5'-flanking sequence driving the expression of a luciferase reporter. All cultures were also transfected with CMV- $\beta$ -galactosidase. After a 48-h incubation in serum-free media, the cultures were extracted, and luciferase and

### **MKK6 (Glu) Increases Cardiac Myocyte Size and Sarcomeric Organization**

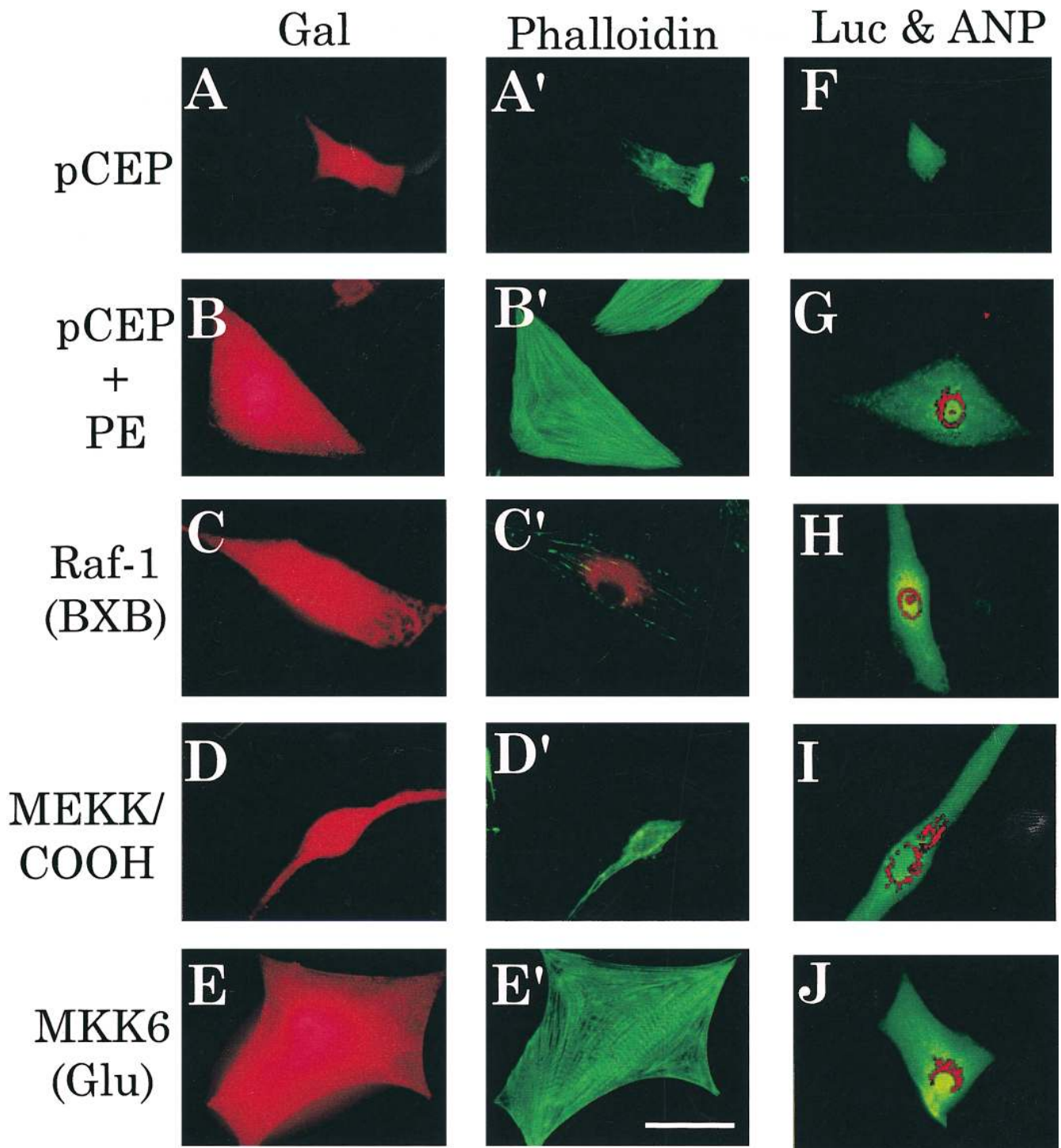
Further studies were undertaken to compare the effects of Raf BxB, MEKK<sub>COOH</sub>, and MKK6 (Glu) with the gold standard, PE, on other features of the program, such as cell size and sarcomeric organization. Compared to cells maintained in control media (Fig. 3, A and A'), the PE-treated cells were much larger (Fig. 3 B), displaying an approximately two- to three-fold increase in area (Fig. 4 A), and they possessed a high degree of sarcomeric organization (Fig. 3 B'). In general, the PE-treated cultures possessed about 10-fold more myocytes displaying organized sarcomeres than the control cultures (Fig. 4 B). PE-treated cultures also displayed significantly increased levels of endogenous ANP expression, observed as the prototypical perinuclear staining found often in hypertrophic cardiac myocytes (Fig. 3, F [control] vs. G [PE-treated]). Interestingly, cultures transfected with Raf BxB or MEKK<sub>COOH</sub> displayed increases in size (Figs. 3, C [BxB] and D [MEKK<sub>COOH</sub>], and 4 A), and while the usual shape of the Raf BxB-treated cells was similar to PE-treated cells, the MEKK<sub>COOH</sub>-treated cells were almost always very long and thin. Moreover, while either Raf BxB (Fig. 3 H [BxB]) or MEKK<sub>COOH</sub> (Fig. 3 I [MEKK-1]) fostered the induction of endogenous ANP expression, neither construct supported sarcomeric organization (Fig. 3 C' [BxB] and 3 D' [MEKK-1]; also see Fig. 4 B). These results suggested that neither ERK (Raf BxB) alone nor ERK and JNK (MEKK<sub>COOH</sub>) were sufficient to confer all the features of the hypertrophic phenotype.

When myocardial cells were transfected with MKK6 (Glu), they were on average four times larger than control cells (Figs. 3, E and 4 A), and notably, they displayed sarcomeric organization that was visually similar to that observed upon PE treatment (Figs. 3 E' and 4 B). Consistent with the high degree of sarcomeric ordering was the finding that cells transfected with MKK6 (Glu) displayed spontaneous contractile activity. Moreover, like PE-treated cells, MKK6 (Glu)-transfected myocardial cells possessed significantly elevated levels of endogenous ANP (Fig. 3 J), consistent with the ability of MKK6 (Glu) to strongly activate NP promoter activities (Fig. 2). Thus, it was apparent that MKK6 (Glu), a selective p38 activator in the cardiac myocytes, was able to mimic the three hallmark features of the hypertrophic growth program.

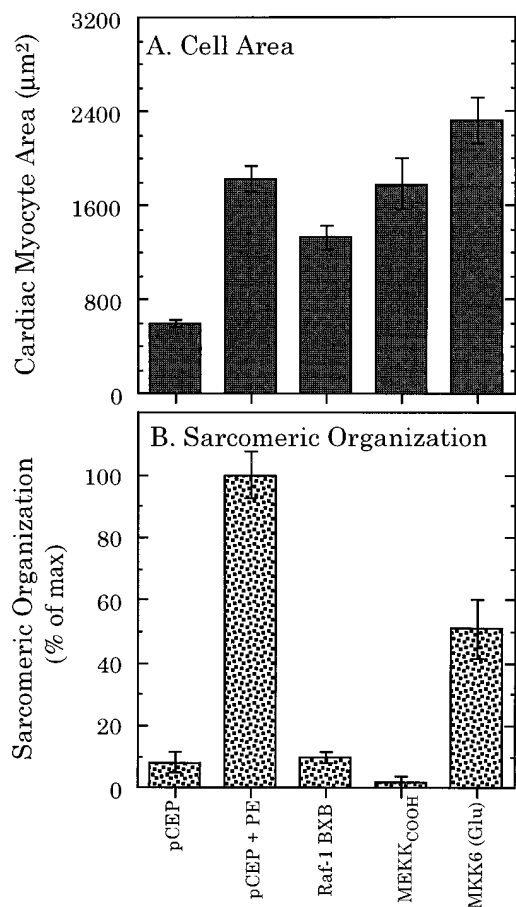
### **The Effects of PE and MKK6 Are Sensitive to a p38 Inhibitor**

To demonstrate that the effects of MKK6 (Glu) and PE on myocardial cell growth and gene expression involved p38, cultures were treated with the highly specific p38 inhibitor,

$\beta$ -galactosidase enzyme activities were assessed, as described in the Materials and Methods. Values for luciferase enzyme units obtained with each treatment were normalized to the maxima. Values are means  $\pm$  SE,  $n = 3$  cultures. In this experiment, luciferase values were not normalized to  $\beta$ -galactosidase since one of the constructs, MEKK<sub>COOH</sub>, is a strong inducer of CMV-driven reporter expression, and it is believed that such normalization can be misleading, as previously reported (Gillespie-Brown et al., 1995; Paradis et al., 1996; Post et al., 1996).

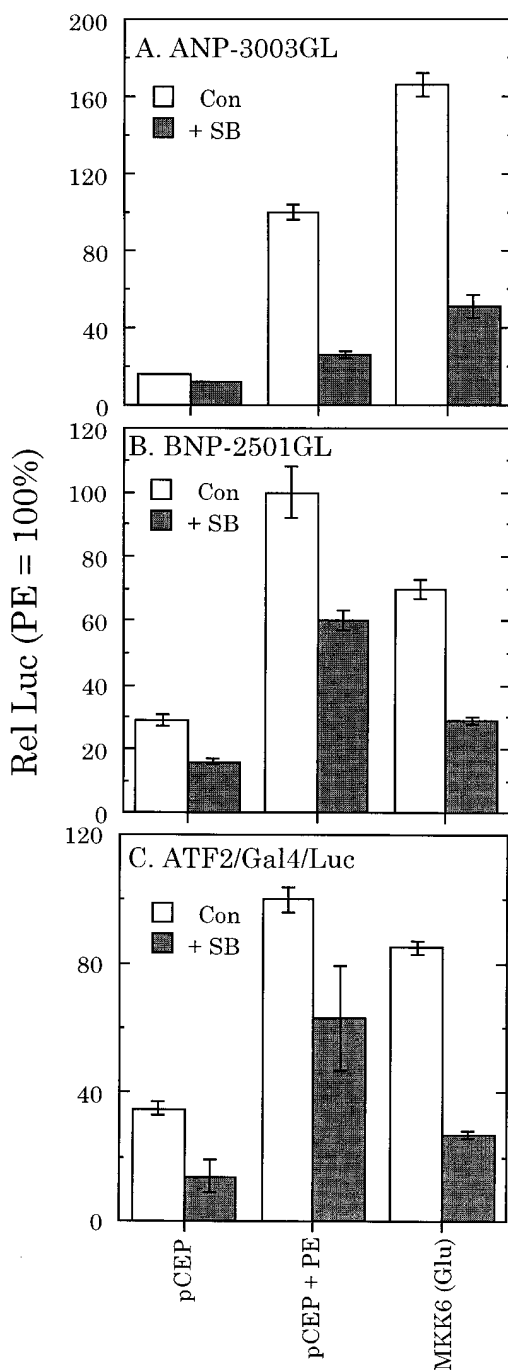


**Figure 3.** Fluorescent microscopic analyses of the effects of Raf-1 BXB, MEKK<sub>COOH</sub>, or MKK6 (Glu) expression constructs on size, sarcomeric organization, and endogenous cardiac-specific gene expression in myocardial cells. Myocardial cells were cotransfected with Raf (Raf-1 BXB), JNKK kinase (MEKK<sub>COOH</sub>), p38 kinase (MKK6 [Glu]), or an empty vector control (pCEP) and CMV- $\beta$ -galactosidase (A-E) or ANP-3003GL (F-J), as described in the legend for Fig. 1. After 48 h of incubation in either serum-free control media or in the same media containing 10  $\mu$ M of the  $\alpha_1$ -adrenergic receptor agonist, phenylephrine (PE) + 1  $\mu$ M propranolol (the latter to block potential binding to  $\beta$ -adrenergic receptors), cultures were fixed in paraformaldehyde. (A-E)  $\beta$ -galactosidase expression (Gal), used to identify transfected cells, was visualized with a Texas red-conjugated second antibody and photographed using a rhodamine-compatible filter. (A'-E') Actin organization in the same  $\beta$ -galactosidase-positive cells shown in A-E was assessed by staining them with BODIPY-conjugated phalloidin (Phalloidin) and photographing them using an FITC-compatible filter. (F-J) In a separate experiment, luciferase expression (Luc), used to identify transfected cells, was visualized with an FITC-conjugated second antibody and photographed using an FITC-compatible filter. The same cells were also assessed for endogenous ANP expression (ANP), viewed with a Texas red-conjugated second antibody, and photographed using a rhodamine-compatible filter. The digitized photographic images of luciferase- and ANP-positive cells were overlaid using Adobe Photoshop (San Jose, CA), and the resulting montage was prepared in Claris MacDraw Pro. Bar, 50  $\mu$ M.

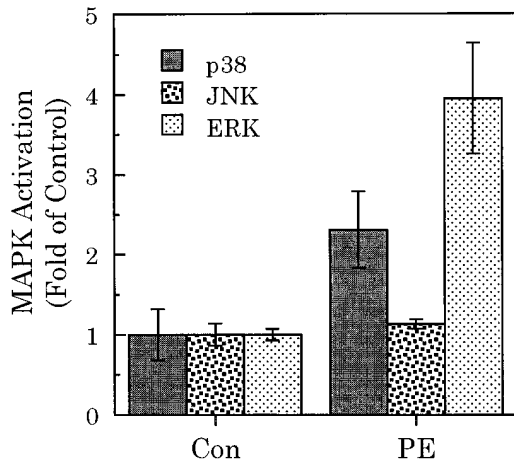


**Figure 4.** Morphometric analyses of the effects of Raf-1 BXB, MEKK<sub>COOH</sub>, or MKK6 (Glu) expression constructs on size and sarcomeric organization in myocardial cells. (A) Photographic images of myocardial cells transfected and treated as described in the legend to Fig. 3, A–E, were digitized and the areas (μm<sup>2</sup>) of ~20–50 cells from each treatment were determined using NIH Image software, as described in the Materials and Methods. Shown are the mean area values for each treatment ± SE (*n* = 3 cultures). (B) The myofilament structure in myocardial cells transfected and treated as described in the legend to Fig. 3, A'–E', was evaluated using BODIPY-phalloidin to stain actin. Upon visually inspecting 50–100 transfected (i.e., β-galactosidase-positive) cells per treatment using a fluorescence microscope, cells were scored for possessing organized sarcomeres (i.e., appearing similar to cells shown in Fig. 3, B' or E'). The number of cells transfected with test construct that scored positive for sarcomeric organization was normalized to (divided by) the maximal values for sarcomeric organization, which were obtained by PE treating cells that had been transfected with the empty vector control; the results are shown as a percentage of these maximal values. Generally, ~25% of the cells transfected with the empty vector control and then treated with PE displayed highly organized sarcomeres. Shown are the mean area values for each treatment ± SE (*n* = 3 cultures).

SB 203580 (Young et al., 1993). At 20 μM, SB 203580 has been shown to block p38/MAPK, while concentrations as high as 100 μM have been shown to have no effect on 20 other protein kinases tested, including ERK and JNK (Cuenda et al., 1995). In the present study, SB 203580 (20 μM) blocked PE and MKK6 (Glu)-inducible NP promoter activity by between 40 and 70% (Fig. 5, A and B)



**Figure 5.** Effects of SB 203580 on ANP-, BNP-, or ATF2-dependent luciferase production in myocardial cells. Myocardial cells were cotransfected with MKK6 (Glu) or an empty vector control (pCEP and pCEP + PE) and either ANP-3003GL, BNP-2501GL, or pG5E1bLuc reporter constructs. In C, cells were also transfected with ATF2/GAL4 (codes for the ATF2 transcriptional activation domain fused to the Gal4 DNA-binding domain). All cells were also transfected with CMV-β-galactosidase for normalization purposes. Cultures were then maintained for 48 h with or without SB 203580 (20 μM) or with or without DMSO (vehicle control) and with or without PE (10 μM) + propranolol (1 μM), as shown and then extracted and assayed for luciferase and β-galactosidase reporter activities, as described in Materials and Methods. Luciferase enzyme units were normalized to β-galactosidase, and the values obtained with each treatment were normalized to pCEP + PE. Values are means ± SE, *n* = 3 cultures.



**Figure 6.** Effects of PE on the activities of p38, JNK, and ERK in myocardial cells. Myocardial cells were treated for 30 min with or without PE (10  $\mu$ M) + propranolol (1  $\mu$ M) and then extracted and subjected to SDS-PAGE followed by Western analyses using antibodies (New England Biolabs, Inc., Beverly, MA) that detect p38, JNK, or ERK only when activated by dual phosphorylation on Thr180 and Tyr182 (following the manufacturer's protocols). Developed blots were then analyzed using a Molecular Dynamics PhosphorImager. Each bar represents the mean blot intensity of three identically treated cultures  $\pm$  SE.

and decreased MKK6 (Glu)-activated  $\alpha$ -SkA by >90% (not shown). Additionally, myocardial cell p38 was shown to be activated by PE, as was ERK; however, JNK was not stimulated under these conditions (Fig. 6). Taken together, these results confirmed a central role for p38 in MKK6 (Glu) induction of cardiac gene expression and strongly suggest that the ability of PE to induce NP expression is at least partly due to the MKK6/p38 pathway.

The stress-activated MAPK pathways, especially p38, are well-known stimulators of ATF2 (Gupta et al., 1995; Raingeaud et al., 1996). ATF2 can dimerize with other ATF family members (e.g., cAMP response element-binding protein [CREB] or ATF-1), Rb, NF- $\kappa$ B, or *c-jun* and enhance transcription through cAMP response elements, AP-1 sites, or NF- $\kappa$ B sites. Accordingly, the abilities of MKK6 (Glu) or PE to activate ATF2-dependent transcription in cardiac myocytes were tested. Myocardial cells were cotransfected with a reporter plasmid possessing GAL4 DNA-binding sites cloned upstream of luciferase, a construct encoding a fusion protein composed of the ATF2 transactivation domain and the GAL4 DNA-binding domain, and either MKK6 (Glu) or pCEP. Transfection with MKK6 (Glu) or treatment pCEP-transfected cells with PE enhanced ATF2-mediated luciferase production (Fig. 5 C). Moreover, SB 203580 served as a potent inhibitor of MKK6 (Glu)-activated ATF2, consistent with a major requirement for p38. Interestingly, PE-activated ATF2 was only partially blocked by SB 203580 (Fig. 5 C), as was PE-activated BNP transcription (Fig. 5 B), suggesting that the ability of this agonist to activate cardiac gene expression is partly dependent on p38, or a very similar kinase.

The SB 203580 compound also had potent inhibitory effects on myocardial cell size and sarcomeric organization

induced by PE or MKK6 (Glu) (Fig. 7). PE- or MKK6 (Glu)-mediated sarcomeric organization and increases in cell size were reduced by >90% by SB 203580 (Fig. 8). These results are consistent with a central role for a p38-like pathway in myocardial cell growth conferred by PE or by MKK6 (Glu).

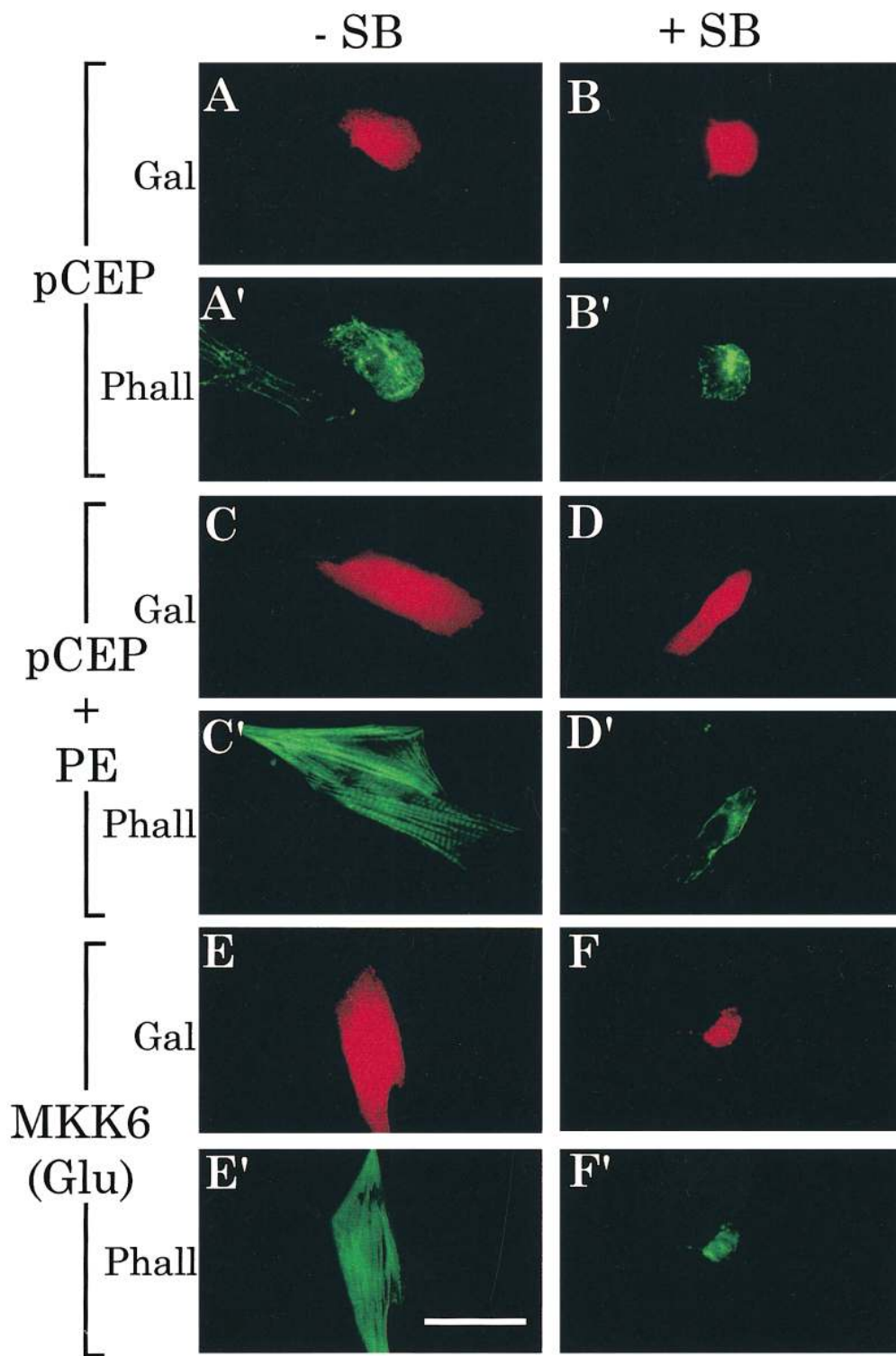
Recent studies have established that the transcription factor, MEF2C, serves as a specific substrate for p38, such that phosphorylation on serine 387 confers MEF2C-mediated transcriptional activation in RAW 264.1 cells (Han et al., 1997). Accordingly, the abilities of PE or MKK6 (Glu) to activate MEF2C-dependent transcription in cardiac myocytes were tested. Myocardial cells were cotransfected with a reporter plasmid possessing GAL4 DNA-binding sites cloned upstream of luciferase and a construct encoding a fusion protein comprised of the MEF2C transactivation domain and the GAL4 DNA-binding domain. Using this system, the activation of MEF2C after phosphorylation on serine 387 can be studied in the cardiac myocytes without interference from any endogenous MEF2 family members. Treatment with PE or transfection with MKK6 (Glu)-enhanced MEF2C-mediated luciferase production (Fig. 9, *MEF2C/Gal4*). When cells were transfected with an altered MEF2C/Gal4 chimera, in which serine 387 was mutated to alanine, neither PE nor MKK6 (Glu) conferred luciferase induction (Fig. 9, *MEF2C-S/Gal4*). These results indicate that like MKK6 (Glu), PE can activate MEF2C in cardiac myocytes, an event that requires phosphorylation at serine 387 by p38. This result further supports the notion that in part, PE enhances myocardial cell growth, sarcomeric organization, and the related gene expression through a pathway involving p38 or a very similar kinase.

## Discussion

Under certain conditions, cardiac myocytes undergo non-mitotic, hypertrophic growth that is typified by dramatic increases in cell size, high degrees of sarcomeric organization, and enhanced expression of certain cardiac-specific genes. Several results from this study indicate that MKK6-activated p38 is sufficient to confer the three main features of this unique hypertrophic growth program. First, MKK6 (Glu), which amongst the MAPKs induces only p38 (Fig. 1 and Raingeaud et al., 1996), conferred sarcomeric organization, increased cell size, and increased cardiac gene expression in a manner similar to the well-characterized  $\alpha_1$ -adrenergic receptor agonist, PE. Second, when induced by MKK6, all three features of the growth program could be blocked by the p38-specific inhibitor, SB 203580. Interestingly, PE-enhanced sarcomeric organization, cell size, and, to some extent, cardiac gene induction were also blocked by SB 203580, indicating that the p38 pathway probably plays an important role in  $\alpha_1$ -adrenergic receptor signaling in myocardial cells. However, since the effects of PE on BNP transcription and ATF2-enhanced transcription were only partially blocked by SB 203580, it appears that while p38 may be central to some features of the hypertrophic response, it may play only a partial role in mediating other aspects of the growth program.

To our knowledge, this is the first report to document that the activation of the p38/MAPK pathway can mimic



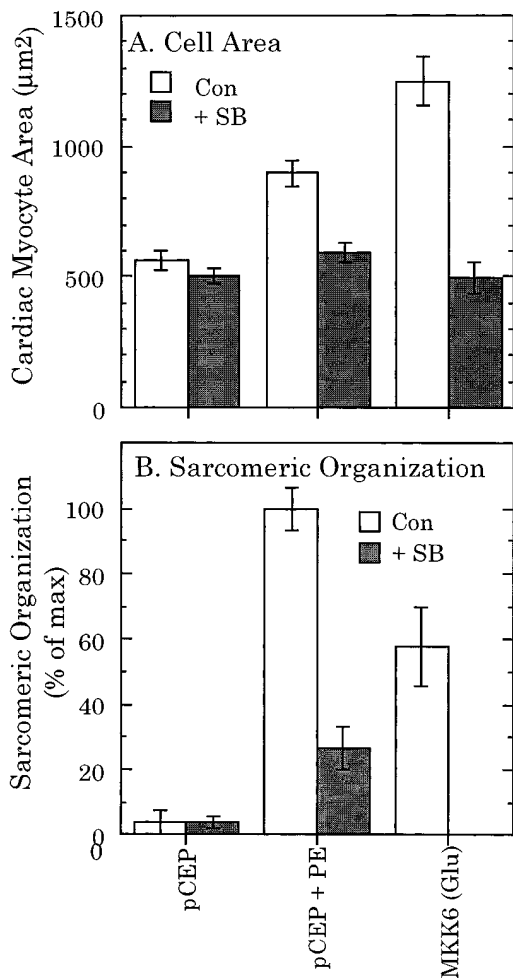


*Figure 7.* Fluorescent microscopic analyses of the effects of SB 203580 on size and sarcomeric organization in myocardial cells. Myocardial cells were cotransfected with MKK6 (Glu) or an empty vector control (pCEP and pCEP + PE) and CMV- $\beta$ -galactosidase and then plated on glass slides, as described in the legend for Fig. 3. After maintenance for 48 h in serum-free control medium with or without SB 203580 (20  $\mu$ M) or DMSO (vehicle control) and with or without PE (10  $\mu$ M) + propranolol (1  $\mu$ M), as shown, cultures were fixed in paraformaldehyde and immunostained for  $\beta$ -galactosidase expression (*Gal*) (A–F), and the same cultures were stained for actin with BODIPY-phalloidin (*Phall*) (A'–F'). Bar, 50  $\mu$ m.

the morphological changes and gene inductive effects of growth factor treatment in any cell type. Thus, while p38/MAPK is known as being a stress-activated kinase, it can apparently contribute to cell growth in a manner that may represent a compensatory response to stress. Although such a role for p38 contrasts with earlier findings that p38 induces apoptosis (Xia et al., 1995), it is consistent with recent studies indicating that this stress kinase can promote

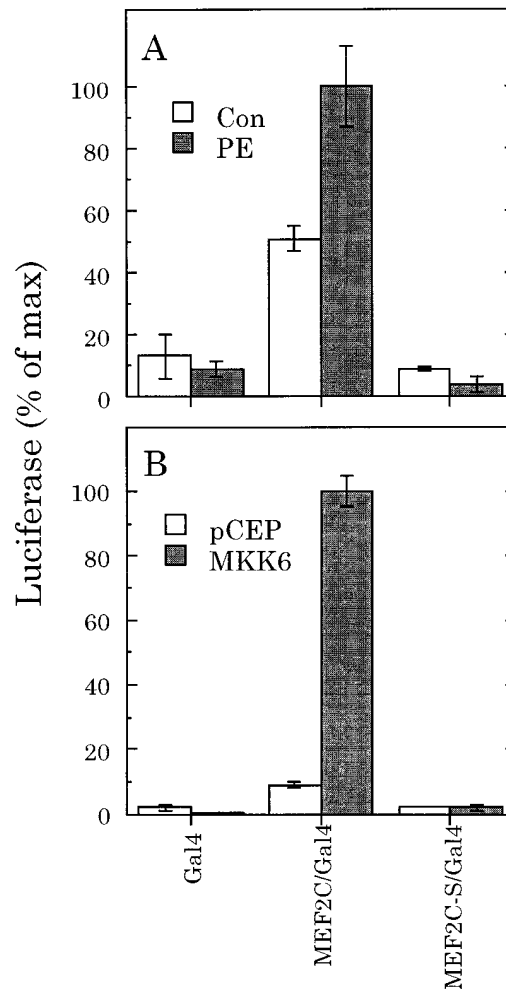
survival in certain cell types (Juo et al., 1997); in this respect, p38 appears to function in a cell-specific manner.

The mechanism by which MKK6-mediated p38 activation could lead to myocardial cell hypertrophic growth remains to be elucidated; however, recent work has revealed several downstream p38 targets that could be involved. For example, p38 phosphorylates and activates several transcription factors, such as ATF2 and Elk-1 (Gupta et



**Figure 8.** Morphometric analyses of the effects of SB 203580 on size and sarcomeric organization in myocardial cells. (A) Photographic images of  $\beta$ -galactosidase-positive myocardial cells transfected and treated as described in the legend to Fig. 3 were digitized, and the areas were determined as described in the legend to Fig. 4 A. Shown are the mean area values for each treatment  $\pm$  SE ( $n = 3$  cultures). The SB 203580 vehicle, DMSO, was included in all controls and had a slight inhibitory effect itself on the ability of PE and the test constructs to increase cell area. (B) The myofilament structure was evaluated using BODIPY-phalloidin to stain actin, as described in the legend to Fig. 4 B. Shown are the mean area values for each treatment  $\pm$  SE ( $n = 3$  cultures).

al., 1995; Livingstone et al., 1995; Raingeaud et al., 1995, 1996), which could augment the expression of cardiac-specific genes induced during hypertrophy. Many of these inducible genes are known to possess relevant *cis*-acting sequences, including serum response elements, cAMP response elements, AP-1 sites, and NF- $\kappa$ B sites. Most recently, it has been demonstrated that the muscle cell-enriched transcription factor, MEF2C, a MADS box protein known to bind to A/T-rich regions of muscle-specific genes and known to be required for proper growth and development of cardiac muscle (Edmondson et al., 1994; Olson and Srivastava, 1996), serves as a substrate for p38 but not ERK or JNK (Han et al., 1997). In that report, the p38-specific phosphorylation was shown to lead to the activation of MEF2C as a transcription factor. Both the rat ANP



**Figure 9.** Effects of PE or MKK6 (Glu) on MEF2C-dependent luciferase production in myocardial cells. Myocardial cells were transfected with pG5E1bLuc and either Gal4 (codes for Gal4 DNA-binding domain only), MEF2C/Gal4 (codes for MEF2C fused to the Gal4 DNA-binding domain), or MEF2C-S/Gal4 (codes for mutant [Ser to Ala 387] MEF2C fused to the Gal4 DNA-binding domain). All cells were also transfected with CMV- $\beta$ -galactosidase for normalization purposes. (A) Cultures were maintained with or without PE (10  $\mu$ M) + propranolol (1  $\mu$ M), as shown, and then extracted and assayed for luciferase and  $\beta$ -galactosidase reporter activities, as described in Materials and Methods. (B) Cultures were also transfected with the control vector, pCEP, or with the test construct, MKK6 (Glu), maintained in control media for 24 h, and then extracted and assayed for luciferase and  $\beta$ -galactosidase reporter activities, as described in Materials and Methods. Luciferase enzyme units were normalized to  $\beta$ -galactosidase, and the values obtained with each treatment were normalized to MEF2C/Gal 4 + PE (A) or MEF2C/Gal 4 + MKK6 (Glu) (B). Values are means  $\pm$  SE,  $n = 3$  cultures.

and BNP 5'-flanking regions, as well as regulatory regions of other genes induced during the hypertrophic growth program (e.g.,  $\alpha$ -skeletal actin and  $\beta$ -myosin heavy chain genes), contain A/T-rich regions that are required for transcriptional induction and could bind MEF2C or related proteins (MacLellan et al., 1994; Thuerlauf et al., 1994; Karns et al., 1995; Sprenkle et al., 1995). Thus, it is possible that p38 could phosphorylate and activate transcription

factors that augment the expression of genes that participate in the cardiac growth program.

Alternatively, p38 may activate other downstream kinases that serve as the final steps in the signaling program. For example, it is well known that p38 can phosphorylate and activate MAP kinase-activated protein kinases (MAPKAPs)-1, -2, and -3 (MAPKAP-3 a.k.a. 3pK) (Stokoe et al., 1992a; Young et al., 1993; Rouse et al., 1994; English et al., 1995; Ludwig et al., 1996; McLaughlin et al., 1996; Sithanandam et al., 1996; Tan et al., 1996). In response to p38 activation by growth factors, MAPKAPs have been shown to phosphorylate and activate selected transcription factors, such as CREB and ATF-1, usually at protein kinase A/CaMK consensus sequences (Tan et al., 1996). Thus, it is possible that via the MAPKAPs, myocardial cell p38 stimulation could culminate with the activation of transcription factors often thought of as being downstream of non-MAPKs, e.g., protein kinase A or CaMK.

In addition to altering the function of transcription factors, p38-mediated MAPKAP/3pK activation could modulate other pathways that might favor cell survival, and in cardiac myocytes, these pathways could contribute to the development of myocardial cell-specific features, such as myofilament organization. For example, MAPKAP-2 has been shown to be activated during ischemic preconditioning of isolated rat hearts (Bogoyevitch et al., 1996; Maulik et al., 1996). Such preconditioning is known to serve as a myocardial stress adaptation, resulting in enhanced protection from ischemia-induced myocardial cell death (Murry et al., 1986; Parratt, 1994; Cumming et al., 1996; Gottlieb et al., 1996). In part, it is believed that this cardioprotection is derived from the induction and activation of heat-shock proteins (hsp's) 27 and 70 (Marber et al., 1993; Mestril et al., 1994; Parratt, 1994), both of which are known to protect cells from apoptosis (Mehlen et al., 1996; Samali and Cotter, 1996; Sharma et al., 1996). Interestingly, MAPKAP-2 and -3 have been shown to phosphorylate hsp 27, a modification known to enhance its protective properties (Ahlers et al., 1994; Huot et al., 1995). Moreover, after phosphorylation induced by either heat-shock or mitogen stimulation, hsp 27 has been shown to bind to and stabilize actin filaments in mouse fibroblasts (Lavoie et al., 1993). Such hsp 27-mediated filament stabilization in cardiac myocytes could be a major contributor to the striking sarcomeric organization observed upon MKK6-mediated p38 activation. Intracellular signaling pathways leading to hsp activation and/or phosphorylation in cardiac myocytes could conceivably extend back to  $\alpha_1$ -adrenergic receptors. Indeed, adrenergic receptor stimulation has been shown to activate hsp's in a variety of cell types; most notable is the finding that  $\alpha_1$ -adrenergic agonists activate hsp 70 in rat aortic cells (Chin et al., 1996) and in rat cardiac cells (Meng et al., 1996).

In summary, our results demonstrate a role for MKK6 and p38 in myocardial cell hypertrophic growth and gene expression. This is consistent with the view that the hypertrophic growth program represents a compensatory response of the myocardium to stress. In a physiological context, the mediators of the hypertrophic response are often hemodynamic stresses, such as increases in blood pressure or volume. Accordingly, increases in myocardial cell size and contractile function afforded by such growth could be

viewed as cellular adaptations designed to counteract a physiological stress. Indeed, induction of the cardiac natriuretic peptide genes, which encode hormones that decrease blood pressure and volume, represent an endocrine compensatory response by the myocardium. The findings that the p38 pathway can mediate all three primary features of the myocardial cell growth program represent a major advancement in our understanding of the signals that regulate this important induction process. Future studies aimed at determining how other signaling pathways, perhaps even the other MAPK pathways, complement the p38 pathway and how p38 itself contributes to myocyte growth, sarcomeric organization, and cardiac gene induction, should reveal new roles for this interesting stress-kinase pathway in the heart.

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