Two Distinct Forms of MAPKAP Kinase-2 in Adult Cardiac Ventricular Myocytes†

Dominique Chevalier‡ and Bruce G. Allen*,‡,§

Institut de Cardiologie de Montréal, Centre de Recherche, 5000 rue Bélanger est, Montréal, PQ, Canada, H1T 1C8, and Departments of Medicine and Biochemistry and Groupe de Recherche sur le Système Nerveux Autonome, Université de Montréal, Montréal, PQ, Canada, H3C 3J7

Received December 10, 1999; Revised Manuscript Received March 8, 2000

ABSTRACT: Hsp27 kinase activities were studied in adult rat ventricular myocytes following sequential chromatography on Mono Q and Mono S. A basal level of activity was present following cell isolation. FPLC on Mono Q revealed three peaks of activity, peaks 'a', 'b', and 'c'. A fourth peak, 'd', was detected upon subsequent chromatography of the Mono Q flow-through on Mono S. Immunoblotting revealed that peaks 'a', 'b', and 'c' contained predominantly a 49 kDa form of MAPKAP kinase-2. Peak 'd' contained a 43 kDa form. 'In-gel' kinase assays using hsp27 indicated both forms of MAPKAP kinase-2 were active. No other bands of hsp27 kinase activity were detected. Both forms of hsp27 kinase immunoprecipitated with a MAPKAP kinase-2 antibody and have therefore been named MAPKAP kinase-2α (p49) and MAPKAP kinase-2*β* (p43). MAPKAP kinase-2*β* chromatographed on Superose 12 as a 60.7 kDa monomer whereas the behavior of MAPKAP kinase-2α suggested both a 65.7 kDa monomer and higher molecular mass complexes. Both activities phosphorylated hsp27 on serine residues, and two-dimensional phosphopeptide mapping indicated the same sites were phosphorylated. A tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA), stimulated both MAPKAP kinase-2α and MAPKAP kinase-2*β* activity. Inhibition of MEK activation with PD 98059 or p38α/*β* MAP kinase activity with SB203580 blocked activation by PMA. However, whereas PD 98059 inhibited only the PMA-stimulated activation, SB203580 inhibited both PMA-stimulated and basal hsp27 phosphorylation. These data demonstrate the presence of two forms of MAPKAP kinase-2 in adult ventricular myocytes. Both forms are activated indirectly by the ERK MAP kinase pathway and directly by p38 MAP kinase but independently regulated.

At least four isoforms of p38 MAP kinase[1] have now been characterized including p38α (*1*−*4*), p38β_1/β_2 (*5*, *6*), p38*γ* (*7*, *8*), and p38*δ* (*5*, *9*). Activation of p38 in the heart is thought to play a role in protecting the myocardium. The p38 pathway is activated during myocardial ischemia, and this activation is maintained during reperfusion (*10*, *11*). Similarly, pretreatment with anisomycin, an activator of p38, protects isolated myocytes from ischemic injury whereas inhibition of p38 using SB203580 abolishes the protective effect of preconditioning in isolated hearts (*12*). In cultured neonatal myocytes under conditions of experimental ischemia, two distinct phases of activation of p38 occurred, and inhibition of p38 during the second phase protected these cells from ischemic injury (*13*). This apparent inconsistency may be explained by activation of different isoforms of p38 MAP kinase as p38α and p38*β* are present in the heart. The activities of both p38α and p38*β* were increased in a mouse model of pressure overload and correlated with ventricular hypertrophy (*14*). Transfecting neonatal myocytes with activated mutants of MAP kinase kinase 6b (MKK6b) and MAP kinase kinase 3b (MKK3b), upstream activators of p38α and p38*β*, evoked changes characteristic of the hypertrophic phenotype (e.g., increase in cell size, enhanced sarcomeric organization, and elevated ANF expression) and apoptosis, respectively (*14*). Coexpression of MKK6b with wild-type p38*β* enhanced the hypertrophic response whereas expression of a kinase-dead mutant suppressed this effect. The pro-apoptotic effect of MKK3b is enhanced by wild-type p38α and suppressed by the inactive mutant. In addition, suppression of the endogenous p38*β* activity using the kinase-dead mutant results in increased cell death. Overexpression of constitutively active MAP kinase kinase 6, another selective activator of p38, protects neonatal myocytes against apoptosis (*15*). Thus, in cardiac myocytes it appears that p38*β* induces hypertrophy and protects against apoptosis whereas p38α promotes apoptotic cell death.

The mechanisms whereby p38 exerts its effects upon cardiac myocytes are unknown; however, an understanding of the downstream targets of p38 may yield some clues. The MAP kinase-activated protein kinases (MAPKAP kinases)-

† Supported by grants from the Medical Research Council of Canada (MT-14725), the Quebec Heart and Stroke Foundation, the Fonds de la Recherche en Santé du Québec (FRSQ), and the Montreal Heart Institute Research Center. B.G.A. is currently a Research Scholar of the Heart and Stroke Foundation of Canada.

* Correspondence should be addressed to this author at the Centre de Recherche, Institut de Cardiologie de Montréal, 5000 rue Bélanger est, Montréal, PQ, Canada, H1T 1C8. Tel: (514) 376-3330 (3591). Fax: (514) 376-1355. E-mail: allen@icm.umontreal.ca.

‡ Institut de Cardiologie de Montréal.

§ Université de Montréal.

[1] Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ERK, extracellular signal-related kinase; FPLC, fast protein liquid chromatography; GST, glutathione *S*-transferase; MAP kinase, mitogen-activated protein kinase; MAPKAP kinase-2, MAP kinase-activated protein kinase-2; MBP, myelin basic protein; MEK, MAP/ERK kinase; PKC, protein kinase C; PKI, cyclic AMP-dependent protein kinase inhibitory peptide; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride.

10.1021/bi9928389 CCC: $19.00
Published on Web 04/29/2000

1, -2, and -3 and PRAK/MAPKAP kinase-5 have been shown to be activated by p38 (*4, 16*−*20*). The MAPKAP kinases phosphorylate several proteins including hsp27 (*16*), the cAMP response-element binding protein (CREB) (*17*), and transcription factors ATF-1 (*17*) and serum response factor (SRF) (*21*). Hence, it may be through one or more of these MAPKAP kinase-activated pathways that p38 effects are mediated. The 27 kDa heat shock protein (hsp27), or its murine homologue hsp25, is expressed in many tissues. Hsp27 is phosphorylated in response to different forms of stress (e.g., heat shock, arsenite, osmotic stress, shear stress, and oxidative stress) and cytokines or agonists (e.g., mitogens, serum, interleukin 1, tumor necrosis factor, thrombin, histamine, and phorbol esters) (*2, 22*−*26*). Thus, hsp27 phosphorylation appears to represent a common step in multiple signal transduction pathways. Human hsp27 is phosphorylated at Ser-15, Ser-78, and Ser-82, all located within the kinase recognition motif X-X-Hyd-X-Arg-X-X-Ser-X-X, where Hyd is a bulky hydrophobic residue (*4, 25*). When heat shock, arsenite, or IL-1 stimulation occurs, hsp27 phosphorylation is catalyzed by mitogen-activated protein kinase-activated kinase-2 (MAPKAP kinase-2) in a cascade distinct from that of Erk MAP kinase (*2, 4*). Hsp27 is a cytosolic actin-binding protein that functions to cap the barbed end of filamentous actin (F-actin), thereby inhibiting actin polymerization (*27, 28*). The control of hsp27 actin-binding activity has been linked to its phosphorylation state in that only nonphosphorylated hsp27 binds actin (*29*). Local phosphorylation of hsp27 could thus regulate the polymerization and organization of F-actin by freeing barbed ends on microfilaments for the addition of monomers (*29*). Unphosphorylated hsp27 exists in high molecular mass complexes, and phosphorylation by MAPKAP kinase-2 causes these complexes to dissociate to tetramers (*30, 31*). In addition, phosphorylation of hsp27 decreases its ability to act as a molecular chaperone in vitro (*31*). Hence, phosphorylation of hsp27 is associated with stabilization of the actin cytoskeleton, complex formation, and molecular chaperone activity, and each of these may underlie its cytoprotective effect.

Herein, we show that two distinct forms of hsp27 kinase activity are expressed in adult ventricular cardiac myocytes and are resolved by sequential chromatography on Mono Q and Mono S. Both activities immunoprecipitated with antisera specific for MAPKAP kinase-2 and have therefore been named MAPKAP kinase-2α (Mono Q) and MAPKAP kinase-2β (Mono S). Both forms are present as monomers whereas MAPKAP kinase-2α also forms higher molecular mass complexes. We have demonstrated that both forms are activated directly by SB203580-sensitive forms of p38 MAP kinase and indirectly by the ERK MAP kinase pathway. The differences in activity shown over time in freshly isolated cells indicate that these kinases are regulated independently of one another; however, phosphopeptide mapping demonstrated that they catalyze the phosphorylation of the same sites in hsp27, suggesting the same substrate specificity.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP was from Amersham Pharmacia Biotech. Membrane-grade (reduced) Triton X-100, leupeptin, and PMSF were from Roche Molecular Biochemicals. SDS−polyacrylamide gel electrophoresis reagents, PVDF, and Bradford protein assay reagent were from Bio-Rad. Microcystin LR and phorbol 12-myristate 13-acetate were from Calbiochem. The specific p38 MAP kinase inhibitor SB203580 was a gift from Dr. John C. Lee (SmithKline Beecham, King of Prussia, PA). cAMP-dependent protein kinase inhibitor peptide (PKI, amino acid sequence TTYADFIASGRTGRRNAIHD) was from the University of Calgary Peptide Synthesis Core Facility. Canine hsp27, cloned into the pET24a expression vector (*32*), was a kind gift from Dr. William Gerthoffer, Reno, NV. Anti-ERK1-CT antisera, raised against a synthetic peptide, CGGPFTFDMELDDLPKERLKELIFQETARFQPGAPEAP, which corresponds to residues 333−367 of rat ERK1 MAP kinase and recognizes both ERK1 and ERK2, was a generous gift from Dr. Steven Pelech, Vancouver, BC. Antibodies to MAPKAP kinase-2 (sc-6621) and ERK2 (sc-1647) were from Santa Cruz Biotechnology Inc. HRP-conjugated secondary antibodies were from Jackson Laboratories. Myelin basic protein was purified from bovine brain as described previously (*33*). All other reagents were of analytical grade or best grade available.

Isolation of Ventricular Myocytes. Male Sprague−Dawley rats (150−200 g) were injected intraperitoneally with 500 units of heparin and anesthetized with pentobarbital (60 mg/kg). The hearts were then rapidly removed. Calcium-tolerant cardiomyocytes were isolated by Langendorff perfusion as described by Rodrigues and Severson (*34*), resuspended in 10 mL of Joklik MEM buffer supplemented with 25 mM $NaHCO_2$, 1.2 mM $MgSO_4$, 1 mM DL-carnitine, and 500 μM $CaCl_2$ (buffer A), and maintained under 95% O_2/5% CO_2. The preparation provided 8−12 million cells/heart with 80−85% viability, as assessed by counting quiescent cells with rod-shaped morphology.

Stimulation of Myocytes and Preparation of Lysates. Adult ventricular myocytes, in buffer A, were divided into 1 mL aliquots in 1.5 mL microcentrifuge tubes and used immediately after isolation. PMA and inhibitors were added directly to cells in buffer A from 1000-fold stock solutions prepared in either 100% DMSO (PMA, PD 98059) or distilled water (SB203580). Pretreatment with 10 μM SB203580 or 10 μM PD 98059 was for 5 min at room temperature. Stimulation with 10 nM PMA was for 5 min at room temperature. To terminate the incubation, cells were chilled to 0 °C for 1 min, pelleted in a microcentrifuge at 3000 rpm and 5 °C for 5 min, and resuspended in 0.5 mL of ice-cold lysis buffer which comprised 50 mM Tris-HCl (pH 7.5 at 5 °C), 20 mM β-glycerophosphate, 20 mM NaF, 5 mM EDTA, 10 mM EGTA, 1 mM Na_3VO_4, 10 mM benzamidine, 0.5 mM PMSF, 10 μg/mL leupeptin, 5 mM DTT, 1 μM microcystin LR, and 1% (v/v) Triton X-100. Cells were extracted by mixing for 15 min at 5 °C using a clinical rotator, centrifuged at 13000*g* and 5 °C for 15 min, and the soluble fraction was retained. Protein concentrations were determined by the method of Bradford (*35*) using bovine γ-globulin as standard.

Fast Protein Liquid Chromatography of Myocyte Lysates. In preparation for separation by fast protein liquid chromatography (FPLC), lysates were diluted to a protein concentration of 10 mg/mL with lysis buffer, recentrifuged (13000*g*, 10 min, 5 °C), and injected into a 0.5 mL sample loop. The chromatography system was maintained in a chromatography cabinet at 5 °C. Separation was achieved using a Mono Q

HR5/5 column equilibrated with 50 mM Tris-HCl (pH 7.4 at 5 °C), 20 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 5% (v/v) glycerol, 0.03% (v/v) Brij 35, 1 mM benzamidine, 1 μg/mL leupeptin, 1 mM Na_3VO_4, and 0.1% (v/v) β-mercaptoethanol. Following a 5 mL isocratic wash, proteins were eluted using a NaCl gradient (24 mL, 0−0.40 M NaCl; 0.1 mL, 0.40−1.0 M NaCl; 0.9 mL, 1.0 M NaCl) at a flow rate of 0.3 mL/min. Sixty fractions of 0.5 mL were collected. Fractions 1−8 from the Mono Q column, corresponding to the flow-through and wash, were pooled, diluted to 7 mL, and applied to a Mono S HR 5/5 column, using a 10 mL superloop, equilibrated with 20 mM HEPES (pH 7.5 at 5 °C), 20 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 5% (v/v) glycerol, 0.03% (v/v) Brij 35, 1 mM benzamidine, 1 μg/mL leupeptin, 1 mM Na_3VO_4, and 0.1% (v/v) β-mercaptoethanol (buffer B). Following a 5 mL isocratic wash, proteins were eluted using a NaCl gradient (24 mL, 0−0.40 M NaCl; 0.1 mL, 0.40−1.0 M NaCl; 0.9 mL, 1.0 M NaCl) at a flow rate of 0.4 mL/min. Seventy fractions of 0.5 mL were collected.

Assay of ERK Activity. ERK was assayed by its ability to phosphorylate MBP. Each FPLC fraction (20 μL) was assayed for ERK activity. The assay was for 60 min at 30 °C in a final volume of 30 μL in the presence of 50 mM Tris-HCl (pH 7.5 at 30 °C), 13 mM β-glycerophosphate, 0.5 mg/mL MBP, 10 mM $MgCl_2$, 1.3 mM EDTA, 1.3 mM EGTA, 1.0 mM Na_3VO_4, 0.1 mM [γ-^{32}P]ATP (50−100 cpm/pmol), 1 μM PKI, 10 μg/mL leupeptin, and 10 mM DTT. Reactions were initiated by the addition of 10 μL of 3× assay media and terminated by spotting 20 μL onto 1.5 × 1.5 cm squares of P81 phosphocellulose paper which were immediately immersed in 0.5% H_3PO_4. Papers were washed 3 times (5 min each, 500 mL) in 0.5% H_3PO_4 and dried, and ^{32}P incorporation was quantified by Cerenkov counting (*36*).

Assay of Hsp27 Kinase Activity. Hsp27 kinase activities were assayed using recombinant canine hsp27 as substrate (*16*). The assay was for 60 min at 30 °C in a final volume of 30 μL in the presence of 50 mM Tris-HCl (pH 7.5 at 30 °C), 13 mM β-glycerophosphate, 1 μg of hsp27, 10 mM $MgCl_2$, 1.3 mM EDTA, 2 mM EGTA, 1.0 mM Na_3VO_4, 10 μM [γ-^{32}P]ATP (3.3 Ci/mmol), 1 μM PKI, 10 μg/mL leupeptin, and 10 mM DTT. Reactions were initiated by the addition of 10 μL of 3× assay media and terminated by the addition of 10 μL of 4× Laemmli sample buffer. The phosphorylated substrate was separated by electrophoresis on 10−20% SDS−polyacrylamide gradient gels and visualized by autoradiography. The incorporation of [^{32}P]P_i into hsp27 was quantified by phosphorimaging (Bio-Rad GS 525 Molecular Analyzer). For phosphoamino acid analysis and phosphopeptide mapping, 1 μg of hsp27 was phosphorylated for 60 min at 30 °C, in the presence of either MAPKAP kinase-2α or MAPKAP kinase-2β, in 50 mM Tris-HCl (pH 7.5 at 30 °C), 13 mM β-glycerophosphate, 10 mM $MgCl_2$, 1.3 mM EDTA, 2 mM EGTA, 1.0 mM Na_3VO_4, 10 μM [γ-^{32}P]ATP (33 Ci/mmol), 1 μM PKI, 1 μM microcystin LR, 10 μg/mL leupeptin, and 10 mM DTT. Following SDS−PAGE, gels were stained, destained, dried, and autoradiographed. Bands containing hsp27 were cut out of the dried gels and tryptic digests performed in situ using TPCK-trypsin (Worthington) (*37*). Less than 5% of the ^{32}P remained in the gel chips following digestion. Peptides were then dissolved in dH_2O to 2000 cpm/μL. Samples (1 μL) were subjected to two-dimensional thin-layer electrophoresis/chromatography as described previously (*38*). Thin-layer electrophoresis was for 40 min at pH 1.9 and 10 °C. For phosphoamino acid analysis, 5 μL aliquots were diluted in 0.3 mL Reacti-Vials to 50 μL, and an equal volume of concentrated HCl was added. Samples were hydrolyzed for 90 min at 110 °C, dried in a SpeedVac, and redissolved to 2000 cpm/μL in dH_2O containing 0.25 mg/μL phosphoamino acid standards (P-Ser, P-Tyr, P-Thr). A 1.0 μL aliquot was analyzed by thin-layer electrophoresis in dH_2O/acetic acid/pyridine (95:5:0.125) (*39*) at 10 °C and 1200 V for 35 min on Kodak Chromatogram 13255 cellulose sheets without indicator as described previously (*36*).

Electrophoresis and Immunoblotting. Protein samples were subjected to SDS−PAGE at 15 °C, using a discontinuous buffer system (*40*), in 10−20% acrylamide-gradient slab gels (1.5 mm thick) with a 5% acrylamide stacking gel. Gels were stained in 45% (v/v) denatured ethanol, 10% (v/v) acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue R-250 and diffusion-destained in 20% (v/v) denatured ethanol containing 10% (v/v) acetic acid. Destained gels were dried between two sheets of cellophane (BioDesign, Inc.) and exposed to Kodak BioMax MR film for 24 h at −80 °C in cassettes fitted with Kodak TranScreen-HE intensifying screens. Following autoradiography, gels were exposed to Molecular Imaging screens for 48 h, and ^{32}P incorporation was digitized and quantified by phosphorimaging (Bio-Rad GS 525 Molecular Analyzer). For immunoblotting, following SDS−PAGE, samples were transferred at 100 V and 5 °C for 90 min onto PVDF membranes in a buffer comprising 25 mM Tris base, 192 mM glycine, and 5% methanol. Membranes were blocked for 2 h in a solution comprising 5% (w/v) skimmed milk powder (Carnation) in 25 mM Tris (pH 7.5 at 20 °C), 150 mM NaCl (TBS), and 0.05% (v/v) Tween-20 (TBST). Membranes were incubated with primary antibodies, diluted 1:1000 with 2.5% skimmed milk powder in TBS, for 16 h at 5 °C. After washing, membranes were reblocked with TBST containing 2.5% skimmed milk powder, and then incubated in the presence of secondary antibody, horseradish peroxidase-labeled donkey anti-goat immunoglobulin diluted 1:30 000, for 2 h at room temperature. Immune complexes were detected by the ECL Western blotting detection method (Renaissance, NEN Life Sciences) according to the manufacturer's instructions and visualized using Kodak BioMax ML film.

'In-Gel' Kinase Assay. Indicated fractions from Mono Q or Mono S columns were concentrated using UltraFree-MC (Millipore) centrifugal filtration units fitted with 10 kDa nominal molecular weight cutoff membranes. Concentrated samples were resolved on 10−20% acrylamide-gradient gels containing either 0.5 mg/mL MBP (ERK) or 10% mini gels containing 0.5 mg/mL hsp27. After electrophoresis, gels were washed with 20% 2-propanol in 50 mM Tris-HCl (pH 8.0), and then with 50 mM Tris-HCl (pH 8.0) containing 5 mM β-mercaptoethanol (buffer C). Gels were incubated for 60 min in buffer C containing 6 M guanidine hydrochloride, and then washed with, and incubated overnight at 5 °C in, buffer C plus 0.04% Tween-40. Gels were subsequently washed with 50 mM Tris-HCl (pH 8.0) containing 5 mM β-mercaptoethanol, 1 mM EGTA, 10 mM $MgCl_2$, and 0.1 μM PKI. In situ phosphorylation of MBP or hsp27 was performed in 50 mM Tris-HCl (pH 8.0) containing 5 mM

β-mercaptoethanol, 1 mM EGTA, 10 mM $MgCl_2$, 0.1 μM PKI, and 10 μM [γ-^{32}P]ATP (2 μCi/mL) for 3 h at 20 °C. After being washed with a 5% TCA solution containing 10% sodium pyrophosphate, gels were dried and autoradiographed.

Partial Purification of MAPKAP Kinase-2. MAPKAP kinase-2 was partially purified from canine heart as described previously (*41*) with minor modifications. Briefly, canine hearts (90−120 g, Pel Freeze Biologicals) were minced and homogenized using a Waring blender on low for 3 × 30 s in 500 mL of Tris buffer (pH 7.5 at 5 °C) containing 5 mM EGTA, 5 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 1 mM benzamidine, and 0.1 mM PMSF. The resulting homogenate was centrifuged for 35 min at 5000*g*, filtered through cheesecloth, and then centrifuged again for 30 min at 100000*g*. The pH of the supernatant was readjusted to 7.5 with 1.0 M Tris base and then applied to a 2.6 × 7 cm CM-Sephadex G-25 column equilibrated with 20 mM MOPS (pH 7.5 at 5 °C), 1 mM EGTA, 1 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 1 mM benzamidine, and 0.1 mM PMSF (buffer D). The column was washed with equilibration buffer until the A_{280} returned to baseline, and MAPKAP kinase-2 was eluted with 200 mL of buffer D containing 300 mM NaCl. The eluate was adjusted to 45% saturation with solid ammonium sulfate over 30 min, left mixing at 5 °C for 30 min, and then centrifuged for 10 min at 10000*g*. The supernatant was discarded, and the pellet was resuspended in 3 mL of 5 mM MOPS (pH 7.5 at 5 °C), 0.25 mM EGTA, 1.0 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 1 mM benzamidine, and 0.1 mM PMSF (buffer E) and dialyzed for 16 h against 500 mL of buffer with 3 changes. After centrifugation for 60 min at 100000*g*, the supernatant was applied to a 1 × 5 cm DEAE-Sephacel column equilibrated with buffer E and subsequently washed with buffer E. Three fractions of 12 mL each, including the flow-through, were collected. Fractions 1 and 2 were combined, diluted 1:3 with buffer B, and chromatographed on Mono S as described above. The fractions from Mono S were screened for MAPKAP kinase-2 immunoreactivity and the peak fractions pooled and stored at −80 °C.

RESULTS

There are currently five known MAP kinase-activated protein kinases (MAPKAP kinases), and three of these, MAPKAP kinase-2 (*16*), MAPKAP kinase-3 (*42*), and MAPKAP kinase-5/PRAK (*20*), have been shown to phosphorylate hsp27. The activation of the different kinases catalyzing the phosphorylation of hsp27 was studied in adult rat ventricular myocytes. Hsp27 phosphorylating activity was detected in freshly isolated, unstimulated, cardiac myocytes (data not shown). To determine if this represented a short-term activation of hsp27 kinase(s) due to the cell isolation procedure versus a prolonged activation, these activities were determined at various times following cell isolation. Cells were isolated and allowed to recover for 30, 90, or 180 min at room temperature under 95% O_2/5% CO_2, and then lysates were prepared. Cell viability was also determined at each time point. The lysates were resolved by sequential FPLC (see below) on Mono Q and Mono S, and hsp27 phosphorylating activity was assessed. Whereas extracts prepared from unstimulated, snap-frozen whole rat ventricles exhibited no hsp27-phosphorylating activity (data not shown), a basal level of activity was present in myocyte extracts. FPLC on Mono Q revealed a slightly asymmetric peak of hsp27-phosphorylating activity (Figure 1A, peak 'a') eluted at 130 mM NaCl. To establish the stability of this activation over time, myocytes were also incubated at room temperature under 95% O_2/5% CO_2 for 90 or 180 min prior to lysis. These longer incubations revealed that the activation of peak 'a' persisted for up to 3 h and therefore was not a short-term result of the cell isolation procedure. However, at 90 min a much broader peak was observed in comparison with either 30 or 180 min. Peak 'a' was flanked by two additional peaks of activity, eluting at 110 mM (peak 'b') and 145 mM (peak 'c') NaCl. In addition, a low level of activity was detected in Mono Q flow-through and wash fractions. When Mono Q fractions 1−8 were pooled and applied to a Mono S column, a peak of hsp27-phosphorylation activity (peak 'd') was observed eluting at 160 mM NaCl (Figure 1B). The unmasking of the hsp27 kinase activity in the Mono Q flow-through following chromatography on Mono S suggests the presence of an inhibitor of this kinase activity. Whereas the activity eluting from Mono Q as peak 'a' remained unchanged, the activity eluting from Mono S (peak 'd') was detected at 30 min but was absent following 90 or 180 min of incubation.

To determine if MAPKAP kinase-2 copurified with hsp27 kinase activity, samples were resolved on SDS−PAGE, transferred onto PVDF, and probed with specific antisera. Immunoblot analysis revealed a prominent band of 49 kDa in peaks 'a', 'b', and 'c' (Figure 1C). In addition, peak 'a' contained a minor immunoreactive band of 52 kDa. Immunoreactive bands of 43, 49, and 70 kDa were detected in peak 'd'. To determine which of the bands detected by immunoblotting represented active forms of MAPKAP kinase, FPLC fractions were concentrated 10-fold, and in-gel kinase assays were performed with hsp27 as the substrate (Figure 1D). These experiments revealed that peaks 'a', 'b', and 'c' contained a 55 kDa renaturable hsp27 kinase activity whereas in peak 'd' a kinase of 47 kDa was active. The values for molecular mass determined by immunoblotting differ from those determined in the gel kinase assay due to differences in the electrophoretic mobility of the prestained standards on large acrylamide-gradient gels versus isogradic mini gels. The 47 and 55 kDa renaturable kinase activities detected in the in-gel kinase assays correspond to the 43 and 49 kDa immunoreactive bands, respectively. No other bands of hsp27-phosphorylating activity were detected using the in-gel kinase assay system. Similarly, no bands were observed in the in-gel kinase assay if the substrate hsp27 was omitted from the gel (not shown). Occasionally, some of the lower molecular weight form was detected in the Mono Q fractions (e.g., Figure 1D, lane 2), suggesting a weak interaction with Mono Q was possible under the chromatography conditions employed. When the ionic strengths of the lysis buffer and the Mono Q buffer were decreased by using 10 mM MOPS-HCl (pH 7.5) in place of 50 mM Tris-HCl (pH 7.5), greater than 95% of the hsp27 kinase activity was retained on Mono Q (not shown).

To determine the extent to which MAPKAP kinase-2 contributed to the hsp27-phosphorylating activities detected on Mono Q and Mono S, the ability and extent to which these activities were immunoprecipitated by antisera specific to MAPKAP kinase-2 were studied. Peak fractions from both

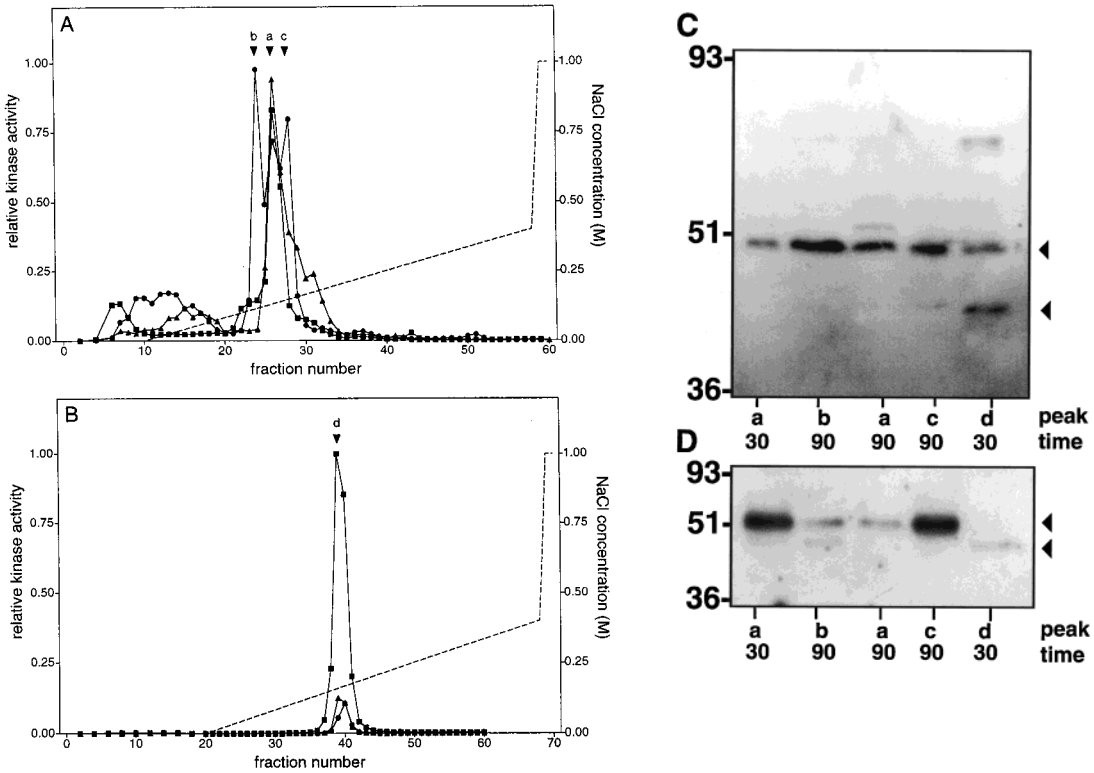

FIGURE 1: MAPKAP kinase-2 activity in unstimulated myocytes. Rat ventricular myocytes were freshly isolated and incubated at room temperature under an atmosphere of 95% O_2/5% CO_2, and then lysates were prepared after 30, 90, or 180 min as described under Experimental Procedures. Five milligrams of lysate was applied to the Mono Q column and chromatographed as described under Experimental Procedures. (A) MAPKAP kinase-2 activity was measured in the indicated Mono Q fractions, using hsp27 as substrate, derived from cells 30 (■), 90 (●), or 180 (▲) min after isolation. The NaCl gradient is shown by the dashed line. (B) Fractions 1−8, corresponding to the flow-through and wash, from the Mono Q column were pooled, diluted to 7 mL with Mono S buffer, applied to a Mono S HR 5/5 column, and chromatographed as described under Experimental Procedures. MAPKAP kinase-2 activity was measured in the indicated Mono S fractions, using hsp27 as substrate, derived from cells 30 (■), 90 (●), or 180 (▲) min after isolation. The NaCl gradient is shown by the dashed line. Hsp27 phosphorylation was quantified by phosphorimaging. Kinase activity was normalized to the highest activity detected in this experiment and expressed as relative kinase activity. The four peaks of hsp27-phosphorylating activity are labeled a−d. (C) and (D) Indicated column fractions were concentrated 10-fold and 20 μL aliquots analyzed by immunoblotting and 'in-gel' kinase assays. (C) Indicated fractions from either the Mono Q or the Mono S columns were resolved on 10−20% acrylamide gradient SDS−PAGE, transferred to PVDF, and probed with MAPKAP kinase-2-specific antisera. (D) Indicated fractions from either the Mono Q or the Mono S columns were resolved on 10% SDS−PAGE mini-gels containing 0.5 mg/mL hsp27, and in situ phosphorylation of hsp27 was assayed as described under Experimental Procedures. Numbers to the left of the panels indicate the positions of the prestained molecular mass marker proteins (in kDa). Letters and numbers below indicate, respectively, the peak and time post-isolation (in min). These results are representative of two (C, D) or three (A, B) independent experiments performed on different myocyte preparations.

Mono Q and Mono S were examined. In both cases, in the presence of the MAPKAP kinase-2-specific sera, the hsp27-phosphorylating activity was recovered in the immune complex whereas in the presence of control preimmune sera the kinase activity remained in the supernatant (Figure 2). Quantification by phosphorimaging revealed that, relative to the control sera, MAPKAP kinase-2 antisera precipitated 94% of the activity in peak 'a' and 96% of the activity in peak 'd'. When purified from rabbit skeletal muscle, MAPKAP kinase-2 was found to exist as two closely related forms of 53 and 60 kDa, respectively (*41*). The two immunoreactive forms of MAPKAP kinase-2 from rat myocytes possessed the same electrophoretic mobility as the two forms present in MAPKAP kinase-2 partially purified from canine heart (Figure 3), and the two activities detected herein were taken to represent these two forms of this enzyme. Thus, we have concluded that both forms of hsp27-phosphorylating activity represent MAPKAP kinase-2 and for the purposes of clarity will refer to p49 as MAPKAP kinase-2α and p43 as MAPKAP kinase-2β.

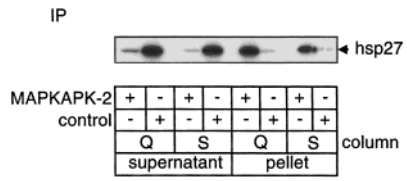

FIGURE 2: Immunoprecipitation of hsp27 kinase activities with MAPKAP kinase-2 antisera. Aliquots (30 μL) of fractions containing the peak of hsp27-phosphorylating activity from the Mono Q and Mono S columns were incubated in the presence of either MAPKAP kinase-2 or preimmune serum precoupled to protein A/G Sepharose at 5 °C for 16 h. A 20 μL aliquot of the supernatant was removed, and the pellets were washed 4 times with 1 mL of 50 mM Tris-HCl (pH 7.4 at 5 °C), 150 mM NaCl, 20 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 5% (v/v) glycerol, 0.03% (v/v) Brij 35, 1 mM benzamidine, 1 μg/mL leupeptin, 1 mM Na_3VO_4, and 0.1% (v/v) β-mercaptoethanol. Hsp27 phosphorylation was subsequently determined as described under Experimental Procedures. This result is representative of two independent experiments.

To determine if the differences in chromatographic properties of MAPKAP kinase-2α and -2β result from the forma-

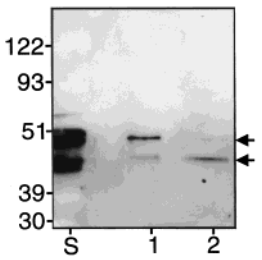

FIGURE 3: MAPKAP kinase-2 immunoreactivity in cardiac myocytes. MAPKAP kinase-2, purified from canine heart as described under Experimental Procedures, and fractions corresponding to the peaks of hsp27 kinase activity from Mono Q and Mono S were resolved on 10−20% acrylamide gradient SDS−PAGE, transferred to PVDF, and probed with MAPKAP kinase-2-specific antisera. The FPLC fractions were concentrated 10-fold. Numbers to the left of the panels indicate the positions of the prestained molecular mass marker proteins (in kDa). Arrows to the right of the panels indicate the positions of the 43 and 49 kDa forms. The numbers below the panel indicate the following samples: S, MAPKAP kinase-2 purified from canine heart; 1, Mono Q peak; 2, Mono S peak. This result is representative of two independent experiments.

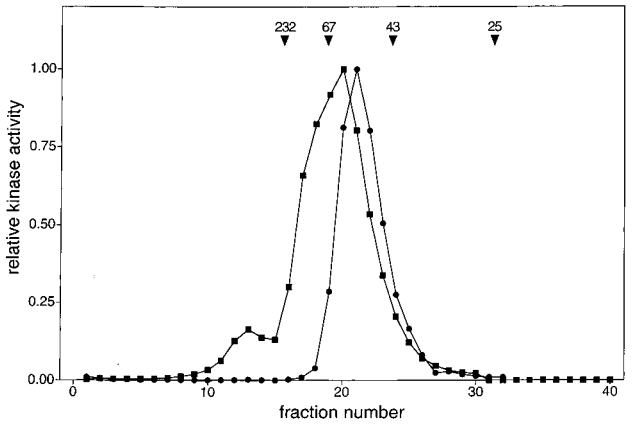

FIGURE 4: Gel filtration of hsp27 kinase activities on Superose 12. Hsp27 kinase activities were resolved by sequential chromatography on Mono Q (■) and Mono S (●). Aliquots (300 μL) from the peak activity fractions were concentrated to 50 μL and applied to a Superose 12 column (1.0 × 30 cm) equilibrated with 50 mM Tris-HCl (pH 7.4 at 5 °C), 150 mM NaCl, 20 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 5% (v/v) glycerol, 0.03% (v/v) Brij 35, 1 mM benzamidine, 1 μg/mL leupeptin, 1 mM Na_3VO_4, and 0.1% (v/v) β-mercaptoethanol. The flow rate was 0.1 mL/min. Between 8.0 mL (V_o) and 16.0 mL, fractions of 0.2 mL were collected and assayed for hsp27 phosphorylation as described under Experimental Procedures. The arrows denote the positions of the marker proteins catalase (232 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa). Peak activities before normalization were 13 200 PD × mm^2 for MAPKAP kinase-2α and 16 600 PD × mm^2 for MAPKAP kinase-2β. This result is representative of two independent experiments.

tion of complexes, myocyte lysates were subjected to sequential chromatography on Mono Q and Mono S, and the fractions corresponding to the peak of hsp27-phosphorylating activity were concentrated 6-fold and resolved on Superose 12 (Figure 4). The apparent molecular masses of MAPKAP kinase-2α and -2β, as determined by gel filtration, were 65 700 and 60 700 Da, respectively. The sequence for MAPKAP kinase-2 predicts a mass of 45 000 Da: hence, both forms were present as monomers. However, whereas MAPKAP kinase-2β eluted as a single symmetrical peak of kinase activity, the elution profile for MAPKAP kinase-2α was asymmetric, suggesting the presence of higher mass species along with the 65 700 Da monomeric form. In addition, a peak of activity eluted with an apparent molecular mass of 240 700 Da. In immune complex assays, each of these activities was precipitated by MAPKAP kinase-2-specific antisera (data not shown). These results suggest that MAPKAP kinase-2α exists both as a monomer and in higher molecular mass complexes, whereas MAPKAP kinase-2β is only present in monomeric form.

Following sequential chromatography on Mono Q and Mono S of lysates from arsenite-stimulated PC12 cells or heat-shocked HeLa cells, MAPKAP kinase-2 activity was detected, using the substrate peptide KKLNRTLSV, in the flow-through fractions but was not retained by Mono Q (*4*). Using intact canine hsp27, we have always observed MAPKAP kinase-2α activity in cardiac myocytes. Similarly, a MAPKAP kinase-2 activity was detected using hsp27 in Mono Q fractions from neutrophils following stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (*43*) and MRC-5 fibroblasts stimulated with interleukin-1 or tumor necrosis factor (*24*). The consensus phosphorylation sequence for MAPKAP kinase-2 is X-X-Hyd-X-Arg-X-X-Ser-X-X, where Hyd is a bulky hydrophobic residue (Phe, Leu, Ile) (*44*). Small changes in this consensus sequence, such as changing serine to threonine, are not well tolerated by MAPKAP kinase-2. Hsp27 contains three serine residues in sequences fitting this consensus sequence that are phosphorylated in vivo in response to growth factors and heat shock: Ser-15, Ser-78, and Ser-82 of human hsp27 (*16*), corresponding to Ser-15, Ser-82, and Ser-86 of canine hsp27 (*45*). Ser-82 in the human sequence, corresponding to Ser-86 in the canine sequence, was preferentially phosphorylated in vitro by MAPKAP kinase-2 purified from rabbit skeletal muscle (*16*). One possible explanation for not detecting MAPKAP kinase-2α activity is that the two forms of MAPKAP kinase-2 have different preferences with respect to the sites phosphorylated in hsp27 and the peptide is only suitable as a substrate for MAPKAP kinase-2β. To test this possibility, hsp27 was phosphorylated by either MAPKAP kinase-2α or MAPKAP kinase-2β and subjected to both phosphoamino acid analysis and phosphopeptide mapping. Phosphoamino acid analysis revealed that both forms of MAPKAP kinase-2 phosphorylated hsp27 solely on serine (Figure 5). This is consistent with the findings of Stokoe et al. (*44*) where replacement of serine with threonine in the substrate peptide KKLNRTLSV resulted in a 33-fold decrease in phosphorylation by MAPAKP kinase-2. Two-dimensional phosphopeptide mapping confirmed that MAPKAP kinase-2α and MAPKAP kinase-2β phosphorylated hsp27 at the same sites. The tryptic phosphopeptide maps of hsp27 phosphorylated by MAPKAP kinase-2α (Figure 6A), MAPKAP kinase-2β (Figure 6B), and a mixture of the two (Figure 6C) were identical. In each case, three phosphopeptides were detected: two intense and one weak. These results indicate that the two forms of MAPKAP kinase-2 do not differ in selectivity of phosphorylation sites on hsp27. The lack of MAPKAP kinase-2 activity retained on Mono Q reported by Rouse et al. (*4*) may be a result of cell type, as the regulation of both JNK and MAP kinase kinase have been shown to differ depending upon the nature of the cellular stress or the cell type employed (*46*).

Hsp27 becomes phosphorylated in many cells in response to stimuli such as tumor necrosis factor (*25*, *47*−*49*),

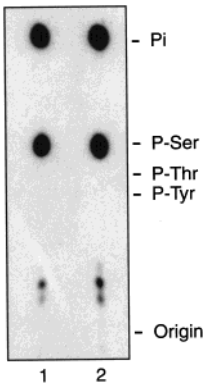

FIGURE 5: Phosphoamino acid analysis of hsp27 phosphorylated by MAPKAP kinase-2α and MAPKAP kinase-2β. [^{32}P]Hsp27 phosphorylated by MAPKAP kinase-2α (lane 1) or MAPKAP kinase-2β (lane 2) was separated by SDS−PAGE, identified by autoradiography, and excised from the gel as described under Experimental Procedures. Following in situ tryptic digestion in gel chips and acid hydrolysis, phosphoamino acids were separated and identified by thin-layer electrophoresis. This result is representative of two independent experiments.

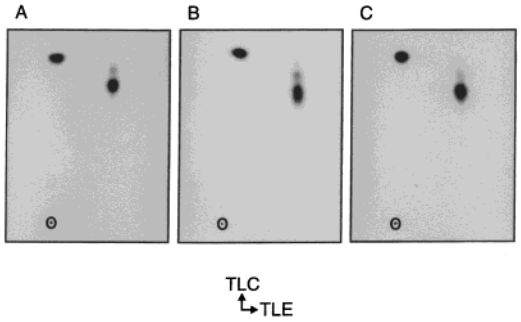

FIGURE 6: Phosphopeptide mapping of hsp27 phosphorylated by MAPKAP kinase-2α and MAPKAP kinase-2β. Hsp27 was phosphorylated by incubation with MAPKAP kinase-2α or MAPKAP kinase-2β for 60 min. After separation by SDS−PAGE, hsp27 bands were identified by autoradiography and excised from the gel. Following tryptic digestion, peptides were separated by two-dimensional thin layer electrophoresis/chromatography, and phosphopeptides were identified by autoradiography. (A) MAPKAP kinase-2α; (B) MAPKAP kinase-2β; (C) MAPKAP kinase-2α + MAPKAP kinase-2β. The 'O' on each panel indicates the original sample loading site. The cathode (−) and anode (+) are on the left and right sides, respectively, of each panel. This result is representative of two independent experiments.

interleukin-1 (*48*, *50*), platelet-derived growth factor (*50*), fMLP (*43*), fibroblast growth factor (*25*, *50*), arsenite (*25*), serum (*25*), thrombin (*25*), tumor-promoting phorbol esters (*25*, *49*, *51*, *52*), fluid shear stress (*23*), and heat shock (*25*, *53*−*55*). Several of the stimuli that induce the phosphorylation of hsp27 are known to activate ERK, and ERK has been shown to phosphorylate and activate MAPKAP kinase-2 in vitro (*41*). To determine if the ERK MAP kinase cascade could directly activate either MAPKAP kinase-2α or MAPKAP kinase-2β in vivo in cardiac myocytes, myocytes were treated with phorbol 12-myristate 13-acetate (PMA, 10 nM) for 5 min prior to lysis. Tumor-promoting phorbol esters, including PMA, represent the most potent known activators of the ERK MAP kinase pathway in cardiac myocytes. First, experiments were performed to ensure that the ERK MAP kinase cascade remained intact in these cells following isolation and was responding under the experimental conditions employed. FPLC of the myocyte lysates on Mono Q revealed two major peaks of PMA-stimulated MBP kinase

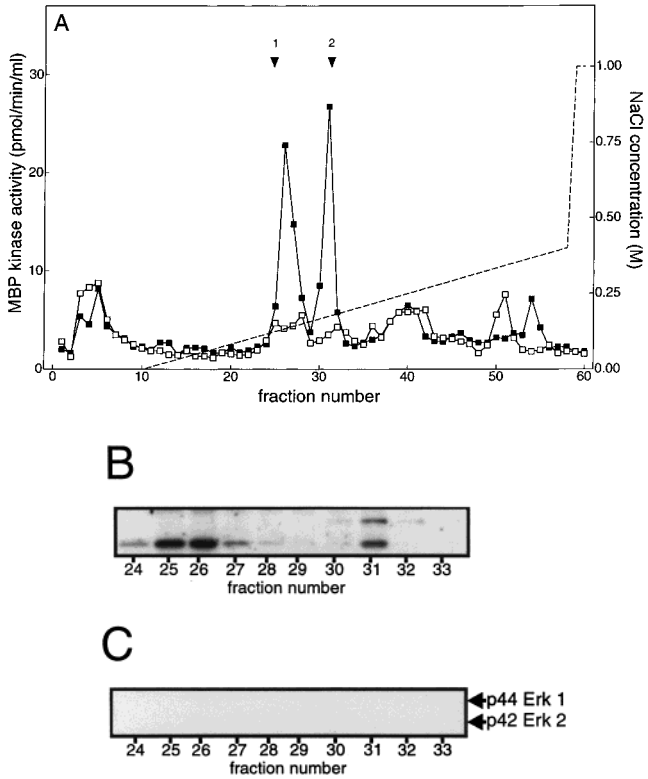

FIGURE 7: Activation of ERK MAP kinases by PMA. Rat ventricular myocytes were isolated, and the preparation was equally divided into 4 tubes, and then immediately treated as follows: control (A, □); treated with 10 nM PMA for 5 min (A, ■); pretreated for 5 min with 10 μM PD 98059 followed by 10 nM PMA for 5 min (A, ▼); pretreated for 5 min with 10 μM SB203580 followed by 10 nM PMA for 5 min (A, ▲). Following treatment, the cells were transferred onto ice and lysates prepared. A 5 mg sample of each lysate was applied to a Mono Q HR 5/5 column and chromatographed as described under Experimental Procedures. (A) Column fractions were assayed for myelin basic protein (MBP) phosphorylating activities. The two peaks of activity are labeled 1 and 2. The NaCl gradient is shown by the dashed line. (B and C) Selected column fractions (Mono Q fractions 24 through 33) were concentrated 20-fold and resolved on 10−20% acrylamide-gradient SDS−PAGE gels containing 0.5 mg/mL myelin basic protein, and in situ phosphorylation of myelin basic protein was assayed as described under Experimental Procedures. (B) PMA-treated cells; (C) control cells. Numbers below the panels indicate the column fractions from panel A. This result is representative of six independent experiments.

activity (Figure 7A, peaks 1 and 2) eluting at 140 and 190 mM NaCl. Four criteria were used to identify the peaks of MBP kinase activity as the ERK MAP kinases. First, the Mono Q elution profile is similar to that previously described for ERK in cells exposed to PMA. Second, immunoblot analysis of Mono Q fractions using antisera specific to both ERK1 and ERK2 revealed the presence of immunoreactive bands at 44 kDa (ERK1) in both peaks 1 and 2 whereas the 42 kDa band (ERK2) was detected only in peak 1 (not shown). These results were confirmed using ERK2-specific antibody (Santa Cruz cs-1647). Third, when Mono Q fractions were concentrated and resolved on 10−20% acrylamide-gradient SDS−PAGE gels containing MBP and MBP phosphorylation was determined in situ in the gels, peak 1 MBP kinase activity migrated at 42 kDa and peak 2 MBP kinase activity migrated at 44 kDa (Figure 7B). Peak 2 also contained an unidentified 41 kDa MBP kinase activity. No phosphorylation was observed in the in-gel assays when

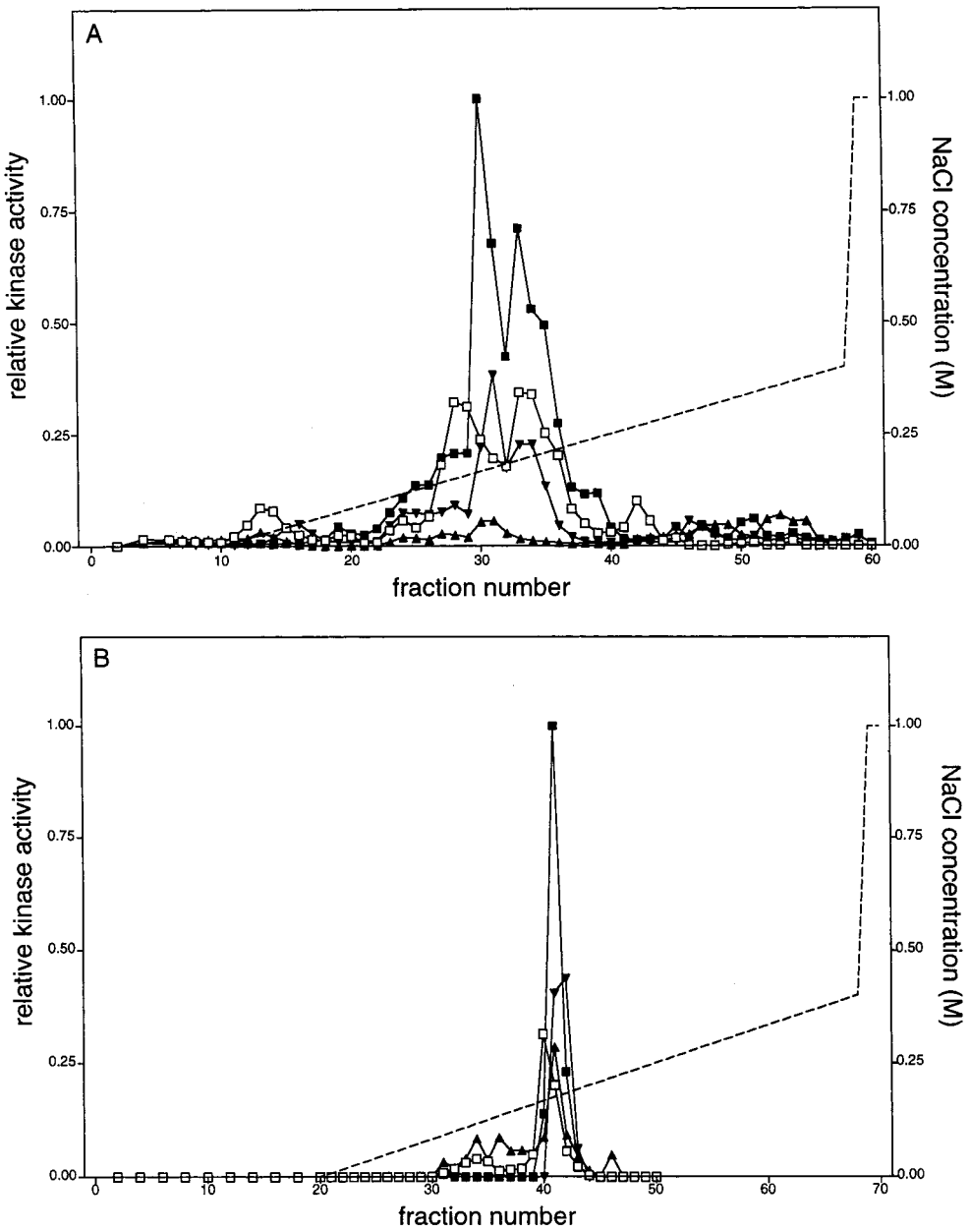

FIGURE 8: Effect of PD 98059 or SB203580 upon PMA-stimulated hsp27 kinase activity. Rat ventricular myocytes were isolated and immediately treated as described below. Following treatment, the cells were transferred onto ice and lysates prepared. A 5 mg sample of each lysate was applied to a Mono Q HR 5/5 column and chromatographed as described under Experimental Procedures. (A) MAPKAP kinase-2 activity was measured in the indicated Mono Q fractions, using hsp27 as substrate. Treatments were as follows: control (□); 10 nM PMA for 5 min (■); pretreated for 5 min with 10 μM PD 98059 followed by 10 nM PMA for 5 min (▼); pretreated for 5 min with 10 μM SB203580 followed by 10 nM PMA for 5 min (▲). (B) Fractions 1−8, corresponding to the flow-through and wash, from the Mono Q column were pooled, applied to a Mono S HR 5/5 column, and chromatographed as described under Experimental Procedures. MAPKAP kinase-2 activity was measured in the indicated Mono S fractions, using hsp27 as substrate. The NaCl gradient is shown by the dashed line. Hsp27 phosphorylation was quantified by phosphorimaging. Kinase activity was normalized to the highest activity detected in this experiment and expressed as relative kinase activity. These results are representative of three independent experiments performed on different myocyte preparations

Mono Q fractions from unstimulated cell lysates were examined (Figure 7C). Similarly, no activity was detected when MBP was omitted from the gel. Finally, both of these peaks were absent if cells were pretreated with an inhibitor of MEK activation, PD 98059 (10 μM, 5 min). Thus, the ERK cascade was intact in these cells, and the conditions employed were appropriate for activation of this pathway.

In light of results in Figure 1 suggesting differences in the activation or inactivation of MAPKAP kinase-2α and MAPKAP kinase-2β, we determined if any of the hsp27 kinase activities were downstream of the ERK pathway. Myocytes were stimulated with PMA (10 nM, 5 min), and hsp27 phosphorylation was examined following sequential chromatography on Mono Q and Mono S (Figure 8A,B). PMA increased hsp27 kinase activity by 2−3-fold over the basal activity. To determine the extent to which hsp27 phosphorylation is regulated by p38 in vivo, cells were pretreated with a specific inhibitor for the α and β forms of p38 MAP kinase, SB203580 (10 μM, 5 min). Blocking of p38α/β for 5 min inhibited hsp27 phosphorylation in both the Mono Q and Mono S fractions in both the presence (Figure 8A,B) and absence (Figure 9A,B) of PMA. Whereas

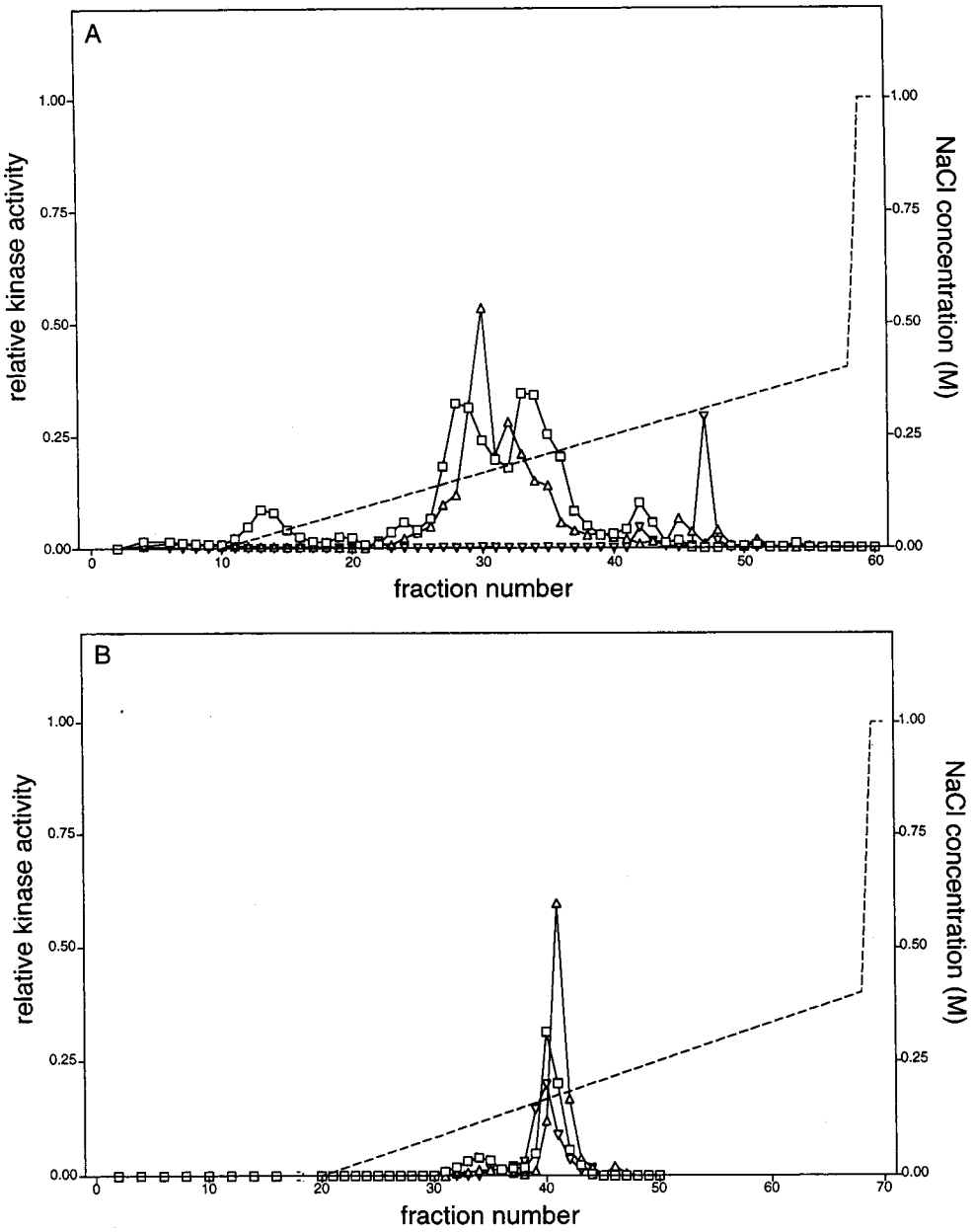

FIGURE 9: Effect of PD 98059 or SB203580 upon basal hsp27 kinase activity. Rat ventricular myocytes were isolated and immediately treated as described below. Following treatment, the cells were transferred onto ice and lysates prepared. A 5 mg sample of each lysate was applied to a Mono Q HR 5/5 column and chromatographed as described under Experimental Procedures. (A) MAPKAP kinase-2 activity was measured in the indicated Mono Q fractions, using hsp27 as substrate. Treatments were as follows: control (□); treated for 5 min with 10 μM PD 98059 (△); treated for 5 min with 10 μM SB203580 (▽). The NaCl gradient is shown by the dashed line. (B) Fractions 1−8, corresponding to the flow-through and wash, from the Mono Q column were pooled, applied to a Mono S HR 5/5 column, and chromatographed as described under Experimental Procedures. MAPKAP kinase-2 activity was measured in the indicated Mono S fractions, using hsp27 as substrate. The NaCl gradient is shown by the dashed line. Hsp27 phosphorylation was quantified by phosphorimaging. These results are representative of three independent experiments performed on different myocyte preparations.

blocking MEK activation with PD 98059 was able to block the PMA-mediated activation of MAPKAP kinase-2, this compound did not inhibit the basal hsp27 phosphorylation. The fact that the basal activity was inhibited by SB203580 suggests that MAPKAP kinase-2 is under strong phosphatase control and that the MAPKAP kinase-2 activity measured in nonstimulated cells is a result of tonic activation of the entire p38 pathway. Taken together, these data indicate that p38α and/or p38β are in vivo regulators of hsp27-phosphorylating activities in adult ventricular myocytes whereas any effects of the ERK pathway are indirect, involving members of the ERK cascade acting upon upstream components of the p38 cascade.

DISCUSSION

In this report we demonstrate the presence of two distinct forms of hsp27 kinase activity in adult rat cardiac myocytes. Sequential chromatography on Mono Q and Mono S resulted in complete separation of these two activities. The different hsp27 kinase activities could result from one of the following: (1) the expression of different kinases; (2) the same kinase as part of different complexes; (3) posttranslational modification; or (4) alternative splicing. There are currently five known kinases that have been shown to catalyze the phosphorylation of hsp27: the cyclic AMP-dependent protein kinase (PKA) (*56*), protein kinase C (PKC) (*56*, *57*),

MAPKAP kinase-2 (*16*), MAPKAP kinase-3 (*42*), and MAPKAP kinase-5/PRAK (*20, 58*). Phosphorylation by PKC is lipid-dependent, and the experiments conducted herein were performed without lipid and in the presence of EGTA plus the inhibitory peptide of PKA (PKI, 1 μM); hence, these kinases would not be active. All hsp27 phosphorylation was sensitive to inhibition of p38 MAP kinase with SB203580. Furthermore, hsp27 kinase activities on both Mono Q and Mono S were immunoprecipitated by an antibody to human MAPKAP kinase-2. In addition, MAPKAP kinase-3 requires a higher concentration of NaCl for elution from Mono S than MAPKAP kinase-2 (*42*), making MAPKAP kinase-3 an unlikely candidate for the hsp27 kinase activity retained on Mono Q. Hence, it is unlikely that MAPKAP kinase-3 or MAPKAP kinase-5 underlies either of the peaks of hsp27 kinase activity detected in myocytes. We have therefore referred to the activity retained on Mono Q as MAPKAP kinase-2α and that on Mono S as MAPKAP kinase-2β. Chromatographically different forms of MAPKAP kinase-2 activity may result from its incorporation into complexes. This has been ruled out as both MAPKAP kinase-2α and MAPKAP kinase-2β activities behaved as monomers during gel filtration. Murine cells contain 45 and 55 kDa forms of MAPKAP kinase-2 (*59*), and this kinase was purified from rabbit skeletal muscle as two forms having molecular masses of 53 000 and 60 000 Da. These two forms did not result from proteolysis during purification as their relative abundance remained unchanged (*41*). Amino acid sequencing of six tryptic peptides from each protein revealed identical structures (*41*), establishing that these are closely related isoforms of MAPKAP kinase-2 or derived from the same gene. Using northern blot analysis, Stokoe et al. (*44*) detected only a single 3.3 kb transcript for MAPKAP kinase-2 in six human tissues, including skeletal muscle, whereas Zu et al. detected mRNA of both 3.3 and 4.8 kb (*60*). MAPKAP kinase-2α and MAPKAP kinase-2β differed in their apparent molecular masses on immunoblots, in gel kinase assays, and following gel filtration, suggesting the difference is one of posttranslational modification rather than the formation of complexes. The two forms do not arise from differences in phosphorylation as the 53 and 60 kDa forms persisted following dephosphorylation with PP2A and rephosphorylation with ERK (*41*). In contrast, the differences between peaks 'a−c' could represent the incorporation of MAPKAP kinase-2α into complexes which, in turn, modify its chromatographic behavior on Mono Q. However, as peak 'a' does not diminish in the presence of peaks 'b' and 'c', this is not just a shift, but the activation of additional pools of MAPKAP kinase-2. Alternatively, 'a', 'b', and 'c' may differ due to phosphorylation at sites other than the activation sites. As the activation of MAPKAP kinase-2 is dependent upon its phosphorylation, the differences in time-courses of activation/inactivation of the individual forms likely reflect changes in the phosphorylation state of the various forms of this enzyme. This, in turn, results from differences in interactions with activating kinases or inactivating phosphatase activities. Thus, the two forms of hsp27 kinase activity detected in ventricular myocytes, referred to herein as MAPKAP kinase-2α and MAPKAP kinase-2β, represent two distinct forms of MAPKAP kinase-2.

MAPKAP kinase-2 was originally identified as a protein kinase that is only active after phosphorylation by ERK (*41*). Subsequent work showed it to be activated in vivo by p38 MAP kinase (*1*−*4*). Tumor-promoting phorbol esters, potent activators of the ERK MAP kinase cascade, have been shown to induce phosphorylation of hsp27 in vivo (*25, 49, 51, 52, 60, 61*). However, others have reported a lack of effect of phorbol esters upon hsp27 phosphorylation (*24*). These differences may be cell-type-related as cell-type differences have been associated with the regulation of transfected c-Jun N-terminal kinases (JNK) and MAP kinase kinase isoforms (*46*) and with the responses of the ERK pathway to PMA (*62*). As the ERKs are not able to phosphorylate hsp27 directly, this activation must involve the action of a member of the ERK pathway upon MAPKAP kinase-2 or higher in the p38 pathway. In the present study, whereas inhibition of MEK activation with PD98059 blocked only PMA-induced activation of hsp27 kinase, all hsp27 kinase activity was sensitive to inhibition by SB203580. Hence, MAPKAP kinase-2 lies downstream of p38, and the point of interaction between the ERK pathway and p38 pathway is at or upstream of p38. An alternative explanation for these results is that MAPKAP kinase-2 requires phosphorylation by both ERK and p38 for activation. However, phosphorylation by either ERK (*4, 41*) or p38 (*63*) is sufficient for full activation in vitro. In addition, PD 98059, which blocks the activation of MEK, inhibited the PMA-stimulated but not the basal activity. These findings are not consistent with a requirement for phosphorylation by both Erk and p38 MAP kinases. Purified MAPKAP kinase-2 is inactivated upon incubation with PP2A (*41*), and the rapid effect of SB203580 we observed upon basal MAPKAP kinase-2 activity indicates rapid regulation of this enzyme by an associated phosphatase activity. Thus, in ventricular myocytes, both MAPKAP kinase-2α and MAPKAP kinase-2β are directly activated by an SB203580-sensitive form of p38 MAP kinase and indirectly by the ERK MAP kinase pathway.

In vivo, MAPKAP kinase-2 has been shown to phosphorylate hsp27 (*16*), the cAMP response-element binding protein (CREB) (*17*), and transcription factors ATF-1 (*17*) and serum response factor (SRF) (*21*). A bipartite nuclear localization sequence is present near the C-terminus of MAPKAP kinase-2 (*44*) and in Swiss 3T3 cells transfected with a green-fluorescent protein−MAPKAP kinase-2 fusion protein (GFP−MK2), the fusion protein localized predominantly in the nucleus (*64*). Exposure of cells to stressful conditions, including anisomycin, H_2O_2, or arsenite, which activate the p38 pathway, resulted in translocation of GFP−MK2 from the nucleus into the cytoplasm. Translocation was complete by 40 min of stimulation and blocked by SB203580. Similarly, phosphorylation of CREB and ATF-1 in response to fibroblast growth factor (FGF) peaked after 15 min of stimulation and then declined (*17*). Hence, upon activation of the p38 MAP kinase pathway, MAPKAP kinase-2 undergoes a p38 MAP kinase-dependent relocation to the cytosol. Nuclear export of MAPKAP kinase-2 could represent down-regulation of kinase activity in the nucleus following a brief pulse of nuclear phosphorylation, or, alternatively, the localization of MAPKAP kinase-2 away from its cytosolic substrates may serve to enhance the specificity of p38 MAP kinase-mediated responses. Hsp27 is a cytosolic actin-binding protein that functions to cap the barbed end of filamentous actin (F-actin), thereby inhibiting actin polymerization (*27, 28*). The control of hsp27 actin-

binding activity (*29*), but not its subcellular localization (*61*), has been linked to its phosphorylation state in that only nonphosphorylated hsp27 can bind actin (*29*). Hence, phosphorylation of hsp27 is associated with stabilization of the actin cytoskeleton, and this may be the mechanism underlying its cytoprotective effect. As mentioned above, hsp27 is phosphorylated in vivo by several kinases, including MAPKAP kinase-2, and dephosphorylated by protein phosphatase 2A (*65*). Activation of MAPKAP kinase-2 is via p38 MAP kinase, and several isoforms of this kinase have now been characterized including p38α (*1*−*4*), p38β_1/β_2 (*5, 6*), p38γ (*7, 8*), and p38δ (*5, 9*). The isoforms α and β are both expressed in heart tissue, and in cultured neonatal cardiac myocytes p38α appears to promote apoptosis (*13, 14*) whereas p38β promotes hypertrophy (*14*). Both of these forms of p38 activate MAPKAP kinase-2 (*6, 9*). Hence, our present findings extend the results previously obtained using neonatal myocytes. MAPKAP kinase-2α and -2β may function as members of divergent or distinct p38 pathways, coupling different p38 isoforms to unique downstream effector molecules and thereby mediating the different physiological effects attributed to this pathway.

In summary, we have demonstrated the presence of two distinct forms of MAPKAP kinase-2 activity, MAPKAP kinase-2α and MAPKAP kinase-2β, in ventricular myocytes and shown complete separation of these two activities by sequential chromatography on Mono Q and Mono S. Both forms are present as monomers whereas MAPKAP kinase-2α also forms higher molecular mass complexes. We have demonstrated that both forms are activated directly by SB203580-sensitive forms of p38 MAP kinase and indirectly by the ERK MAP kinase pathway. The differences in activity shown over time in freshly isolated cells indicate that these kinases are regulated independently of one another; however, phosphopeptide mapping demonstrated that they catalyze the phosphorylation of the same sites in hsp27, suggesting the same substrate specificity.

ACKNOWLEDGMENT

We thank Dr. Terry Hebert for critical reading of the manuscript and Dr. Angela Clerk for thoughtful discussion.

REFERENCES

1. Han, J., Lee, J.-D., Bibbs, L., and Ulevitch, R. J. (1994) *Science 265*, 808−811.
2. Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994) *Cell 78*, 1039−1049.
3. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fischer, S. M., Livi, G. P., White, J. R., Adams, J. L., and Young, P. R. (1994) *Nature 372*, 739−746.
4. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) *Cell 78*, 1027−1037.
5. Jiang, Y., Gram, H., Zhao, M., New, L., Gu, J., Feng, L., Di Padova, F., Ulevitch, R. J., and Han, J. (1997) *J. Biol. Chem. 272*, 30122−30128.
6. Kumar, S., McDonnell, P. C., Gum, R. J., Hand, A. T., Lee, J. C., and Young, P. R. (1997) *Biochem. Biophys. Res. Commun. 235*, 533−538.
7. Li, Z., Jian, Y., Ulevitch, R. J., and Han, J. (1996) *Biochem. Biophys. Res. Commun. 228*, 334−340.
8. Mertens, S., Craxton, S. M., and Goedert, M. (1996) *FEBS Lett. 383*, 273−276.
9. Goedert, M., Cuenda, A., Craxton, M., Jakes, R., and Cohen, P. (1997) *EMBO J. 16*, 3563−3571.
10. Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., Marshall, C. J., and Sugden, P. H. (1996) *Circ. Res. 79*, 162−173.
11. Yin, T., Sandhu, G., Wolfgang, C. D., Burrier, A., Webb, R. L., Rigel, D. F., Hai, T., and Whelan, J. (1997) *J. Biol. Chem. 272*, 19943−19950.
12. Weinbrenner, C., Liu, G.-S., Cohen, M. V., and Downey, J. M. (1997) *J. Mol. Cell. Cardiol. 29*, 2383−2391.
13. Mackay, K., and Mochly-Rosen, D. (1999) *J. Biol. Chem. 274*, 6272−6279.
14. Wang, Y., Huang, S., Sah, V. P., Ross, J., Jr., Brown, J. H., Han, J., and Chien, K. R. (1998) *J. Biol. Chem. 273*, 2161−2168.
15. Zechner, D., Craig, R., Hanford, D. S., McDonough, P. M., Sabbadini, R. A., and Glembotski, C. C. (1998) *J. Biol. Chem. 273*, 8932−8239.
16. Stokoe, D., Engel, K., Campbell, D. G., Cohen, P., and Gaestel, M. (1992) *FEBS Lett. 313*, 307−313.
17. Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P., and Comb, M. J. (1996) *EMBO J. 15*, 4629−4642.
18. Sithanandam, G., Latif, F., Duh, F. M., Bernal, R., Smola, U., Li, H., Kuzmin, I., Wixler, V., Geil, L., Shrestha, S., Lloyd, P. A., Bader, S., Sekido, Y., Tartof, K. D., Kashuba, V. I., Zabarovsky, E. R., Dean, M., Klein, G., Lerman, M. I., Minna, J. D., Rapp, U. R., and Allikmets, R. (1996) *Mol. Cell. Biol. 16*, 868−876.
19. McLaughlin, M. M., Kumar, S., McDonnell, P. C., Van Horn, S., Lee, J. C., Livi, G. P., and Young, P. R. (1996) *J. Biol. Chem. 271*, 8488−8492.
20. New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L. J., Kato, Y., Parry, G. C. N., and Han, J. (1998) *EMBO J. 17*, 3372−3384.
21. Heidenreich, O., Neininger, A., Schratt, G., Zinck, R., Cahill, M. A., Engel, K., Kotlyarov, A., Kraft, R., Kostka, S., Gaestel, M., and Nordheim, A. (1999) *J. Biol. Chem. 274*, 14434−14443.
22. Mehlen, P., Kretz-Remy, C., Briolay, J., Fostan, P., Mirault, M., and Arrigo, A. (1995) *Biochem. J. 312*, 367−375.
23. Li, S., Piotrowicz, R. S., Levin, E. G., Shyy, Y. J., and Chien, S. (1996) *Am. J. Physiol. 271*, C994−C1000.
24. Guesdon, F., Freshney, N., Waller, R. J., Rawlinson, L., and Saklatvala, J. (1993) *J. Biol. Chem. 268*, 4236−4243.
25. Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A., and Anderson, C. W. (1992) *J. Biol. Chem. 267*, 794−803.
26. Levin, E. G., and Santell, L. (1991) *J. Biol. Chem. 266*, 174−181.
27. Miron, T., Wilchek, M., and Geiger, B. (1988) *Eur. J. Biochem. 178*, 543−553.
28. Miron, T., Vancompernolle, K., Vandekerckhove, J., Wilchek, M., and Geiger, B. (1991) *J. Cell Biol. 114*, 255−261.
29. Lavoie, J. N., Hickey, E., Weber, L. A., and Landry, J. (1993) *J. Biol. Chem. 268*, 24210−24214.
30. Clerk, A., Michael, A., and Sugden, P. H. (1998) *Biochem. J. 333*, 581−589.
31. Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A., Buchner, J., and Gaestel, M. (1999) *J. Biol. Chem. 274*, 18947−18956.
32. Larsen, J. K., Gerthoffer, W. T., Hickey, E., and Weber, L. A. (1995) *Gene 161*, 305−306.
33. Chevalier, D., and Allen, B. G. (2000) *Protein Expression Purif. 18*, 229−234.
34. Rodrigues, B., and Severson, D. L. (1997) in *Biochemical Techniques in the Heart* (McNeill, J. H., Ed.) pp 101−115, CRC Press, New York.
35. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
36. Allen, B. G., Andrea, J. E., and Walsh, M. P. (1994) *J. Biol. Chem. 269*, 29288−292948.

37. Weber, L. P., Van Lierop, J. E., and Walsh, M. P. (1999) *J. Physiol. 516*, 805−824.
38. Boyle, W. J., Van der Geer, P., and Hunter, T. (1991) *Methods Enzymol. 201*, 110−149.
39. Allen, B. G., and Katz, S. (1991) *Biochemistry 30*, 4334−4343.
40. Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.
41. Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C., and Cohen, P. (1992) *EMBO J. 11*, 3985−3994.
42. Clifton, A. D., Young, P. R., and Cohen, P. (1996) *FEBS Lett. 302*, 209−214.
43. Krump, E., Sanghera, J. S., Pelech, S. L., Furuya, W., and Grinstein, S. (1997) *J. Biol. Chem. 272*, 937−944.
44. Stokoe, D., Caudwell, B., Cohen, P. T. W., and Cohen, P. (1993) *Biochem. J. 296*, 843−849.
45. Larsen, J. K., Yamboliev, I. A., Weber, L. A., and Gerthoffer, W. T. (1997) *Am. J. Physiol. 273*, L930−L940.
46. Butterfield, L., Zentrich, E., Beekman, A., and Heasley, L. E. (1999) *Biochem. J. 338*, 681−686.
47. Hepburn, A., Demolle, D., Boeynaems, J. M., Fiers, W., and Dumont, J. (1988) *FEBS Lett. 227*, 175−178.
48. Kaur, P., and Saklatvala, J. (1988) *FEBS Lett. 241*, 6−10.
49. Arrigo, A. P. (1990) *Mol. Cell. Biol. 10*, 1276−1280.
50. Saklatvala, J., Kaur, P., and Guesdon, F. (1991) *Biochem. J. 277*, 635−642.
51. Welsh, W. J. (1985) *J. Biol. Chem. 260*, 3058−3062.
52. Regazzi, R., Eppemberger, U., and Fabbro, D. (1988) *Biochem. Biophys. Res. Commun. 152*, 62−68.
53. Arrigo, A. P., and Welch, W. J. (1987) *J. Biol. Chem. 262*, 15359−15369.
54. Chretien, P., and Landry, J. (1988) *J. Cell. Physiol. 137*, 157−166.
55. Oesterreich, S., Benndorf, R., and Bielka, H. (1990) *Biomed. Biochim. Acta 49*, 219−226.
56. Gaestel, M., Schröder, W., Benndorf, R., Lippmann, C., Buchner, K., Hucho, F., Erdmann, V. A., and Bielka, H. (1991) *J. Biol. Chem. 266*, 14721−14724.
57. Maizels, E. T., Peters, C. A., Kline, M., Cutler, R. E., Jr., Shanmugam, M., and Hunzicker-Dunn, M. (1998) *Biochem, J. 332*, 703−712.
58. Ni, H., Wang, X. S., Diener, K., and Yao, Z. (1998) *Biochem. Biophys. Res. Commun. 243*, 492−496.
59. Cano, E., Doza, Y. N., Ben-Levy, R., Cohen, P., and Mahadevan, L. C. (1996) *Oncogene 12*, 805−812.
60. Zu, Y. L., Ai, Y., Gilchrist, A., Maulik, N., Watras, J., Sha'afi, R. I., Das, D. K., and Huang, C. K. (1997) *J. Mol. Cell Cardiol. 29*, 2159−2168.
61. Piotrowicz, R. S., and Levin, E. G. (1997) *J. Biol. Chem. 272*, 25920−25927.
62. Rossinni, G. P., Pinna, C., and Malaguti, C. (1999) *Biochem. Pharmacol. 58*, 279−284.
63. Ben-Levy, R., Leighton, I. A., Doza, Y. N., Attwood, P., Morrice, N., Marshall, C. J., and Cohen, P. (1995) *EMBO J. 14*, 5920−5930.
64. Engel, K., Kotlyarov, A., and Gaestel, M. (1998) *EMBO J. 17*, 3363−3371.
65. Cairns, J., Qin, S., Philp, R., Tan, Y. H., and Guy, G. R. (1994) *J. Biol. Chem. 269*, 9176−9183.

BI9928389