Activation of Protein Serine/Threonine Kinases p42, p63, and p87 in Rous Sarcoma Virus-Transformed Cells: Signal Transduction/Transformation-Dependent MBP Kinases

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We have used myelin basic protein immobilized in sodium dodecyl sulfate-polyacrylamide gels to identify protein kinases after gel electrophoresis, followed by protein kinase reactions. This technique has permitted us to detect three protein kinases in serum-deprived cells transformed by p60^{src}. On induction of cellular transformation by a temperature-sensitive v-src, a p87 protein kinase is activated within 30 min and remains activated in fully transformed cells. The p63 protein kinase is not fully activated until 24 h but remains activated in transformed cells. The commonly studied p42^{MBPK} is rapidly activated within 30 min, and its kinase activity decreases significantly by 24 h, when the p63 enzyme is fully activated. The p42^{MBPK}, as well as the p63 and p87 enzymes, are stimulated by transforming p60^{c-src} mutants but not normal c-src or nonmyristylated p60^{c-src}. In addition, the kinase activity of p63 enzyme, but not of p42^{MBPK}, can be induced in okadaic acid-treated chicken embryo fibroblasts, indicating that phosphatase 2A and/or phosphatase 1 may be involved in the regulation of its activity. Additional data indicate that either p42^{MBPK} or p63 activity correlates with the stimulation of the protein kinase p90^{RSK}. Thus, there may be two independent pathways leading to the activation of the RSK gene product.

INTRODUCTION

The relay of extracellular signals to the nucleus involves reversible serine/threonine phosphorylation, as demonstrated by genetic and biochemical studies in yeast and animal cells (Cobb *et al.*, 1991; Crews *et al.*, 1992; Thomas, 1992). The regulation of the relevant protein kinases in these events is critical for the orderly behavior of cells during cell proliferation or differentiation. Recently, the serine/threonine-specific protein kinases that phosphorylate microtubule-associated proteins (MAPs)¹ and myelin basic protein (MBP) *in vitro* have been the

focus of increasing research activity and are the subject of several current reviews (Sturgill and Wu, 1991; Crews *et al.*, 1992; Pelech and Sanghera, 1992; Thomas, 1992).

The MAP/MBP kinases also have been dubbed extracellular-regulated kinases (ERKs) because they are activated by signals, such as nerve growth factor, that result in differentiation, as well as by signals that result in proliferation (Ray and Sturgill, 1987; Shaw *et al.*, 1988; Rossomando *et al.*, 1989; Ahn *et al.*, 1990; Gotoh *et al.*, 1990b; Miyasaka *et al.*, 1990a; Boulton *et al.*, 1991). There are two well-characterized MAP/MBP kinases of *M_r* 42 000 and 44 000 that are activated by phosphorylation on threonine and tyrosine (Ray and Sturgill, 1988) by a *M_r* 45 000 protein kinase related to the yeast protein kinases encoded by the *byr1* and *STE7* genes (Alessandrini *et al.*, 1992; Crews and Erikson, 1992). The p42/44 MBP kinase in turn transmits signals by serine/threonine phosphorylation of downstream substrates (Crews *et al.*, 1992).

¹ Abbreviations used: CEF, chicken embryo fibroblasts; DTT, dithiothreitol; ERK, extracellular-regulated kinases; MAP, microtubule-associated protein; MBP, myelin basic protein; MBPK, myelin basic protein kinase; MEK, MAP kinase extracellular-regulated kinase; SAMK, src-activated myelin basic protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; Tris, tris(hydroxymethyl)aminomethane.

The cDNA cloning of mRNAs encoding the p42/44 MAP/MBP kinases has suggested the existence of a multigene family for these enzymes (Boulton *et al.*, 1991; Rossomando *et al.*, 1991; Gonzalez *et al.*, 1992). Protein kinases with similar sequences also have been identified in yeast and shown to be involved in signal transduction (Courchesne *et al.*, 1989; Elion *et al.*, 1991; Toda *et al.*, 1991). Molecular cloning has uncovered a cDNA, denoted ERK3, that may encode a rat M_r 63 000 MAP/MBP kinase (Boulton *et al.*, 1991), and additional evidence for a distinct human M_r 63 000 MAP/MBP kinase also has been presented recently (Gonzalez *et al.*, 1992). The sequences of these putative protein kinases suggest a distinct form of activation, however, as they lack the phosphorylation site Thr Glu Tyr necessary for the activation of p42/44 forms of MAP/MBP kinases (Gonzalez *et al.*, 1992). Thus, these enzymes are likely to be involved in biochemical events distinct from those of the p42/44 enzymes. Because of their potential for the regulation of important cellular processes, we have studied the activation of protein kinases that phosphorylate MBP in chicken embryo fibroblasts (CEF) transformed by Rous sarcoma virus. This study has detected two new MBP kinase activities of ~ 63 and 87 kD (denoted SAMK, for *src*-activated myelin basic protein kinase) activated as a consequence of $p60^{v-src}$ expression and suggests transformation occurs with only minor involvement of p42/44 MAP/MBP kinases.

MATERIALS AND METHODS

Cell Culture, Plasmid, and Virus

Cultures of primary CEF were prepared from *gs*-negative embryos (Spafas, Norwich, CT) and were maintained in Dulbecco's modified Eagle's medium (Flow Laboratories, Rockville, MD) supplemented with 1% chicken serum, 4% calf serum, 50 units/ml penicillin, 5 μ g/ml streptomycin, and 10% tryptose phosphate broth (Bryant and Parsons, 1982) and incubated at 41°C. Cells were routinely subcultured every 2 or 3 d. Transfection of cells with pRL-*src*, which is an infectious nonpermuted molecular clone of PrA RSV, results in the production of virus and a spread within the cell culture (Reynolds *et al.*, 1987). Typically, 1 μ g of pRL DNA was used to transfect one 60-mm dish of CEF 24 h after trypsinization and plating. High titer virus stocks were obtained from cells 10 d after transfection and stored at -70°C . Changes in cell morphology were routinely observed 6–8 d after transfection of pRL-*v-src*, pRL-*c-527F* (Reynolds *et al.*, 1987), pRL-*ts119* (Bryant and Parsons, 1982), or pRL-*dl118C* (Wang and Parsons, unpublished data). Replication of *c-src* and *c-src* mutant 2A-527F, which lack transforming activity, was assessed by resistance to superinfection with PrA RSV (Bryant and Parsons, 1982). The cultures exhibited morphological changes or resistance to superinfection 3–4 d after virus infection with transformation-competent mutants or *c-src* mutants, respectively. Okadaic acid (Sigma Chemical, St. Louis, MO), sodium fluoride (J.T. Baker Chemical, Phillipsburg, NJ) and sodium orthovanadate (Fisher Scientific, Pittsburgh, PA) used here were diluted in cultured medium before treatment of cultured cells.

Preparation of Cytosolic Proteins

Cells were lysed with 30 strokes of a tight-fitting Dounce homogenizer in lysis buffer A (10 mM KH_2PO_4 , 1 mM EDTA, 5 mM ethylene

glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid, 10 mM MgCl_2 , 50 mM β -glycerolphosphate, pH 7.2, 1 mM Na_3VO_4 , 2 mM dithiothreitol [DTT]) supplemented with 1 mM phenylmethylsulfonyl fluoride, 4 mM *p*-nitrophenyl phosphate disodium, and 1 μ M pepstatin. Crude lysates were centrifuged at $150\,000 \times g$ for 30 min to separate cytoplasmic supernatant (S-150) from crude membrane and nuclei pellets. Protein concentration in S-150 was measured using the BCA assay (Pierce, Rockford, IL).

Direct Assays for Phosphorylation of the ERK Gene Product and MBP

The total activity of protein kinases able to phosphorylate the ERK gene product was determined by incubating 5 μ g of cytosolic proteins (S-150) with 0.5 μ g of kinase-negative ERK1 protein containing a substitution of Met-63 for Lys-63 at the ATP-binding site (Alessandrini *et al.*, 1992). The phosphorylation of ERK1/K63M was measured in 20 μ l lysis buffer A supplemented with 50 μ M ATP and 5 μ Ci [γ - ^{32}P]ATP at 30°C for 30 min. The labeled ERK1/K63M proteins were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG), visualized by autoradiography, excised, and counted.

The MBP filter binding kinase assay was carried out by the incubation of 5 μ l fractionated elutes with 5 μ l of 1 mg/ml MBP in 20 μ l kinase reaction buffer (37.5 mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.5, 15 mM MgCl_2 , 0.15 mg/ml bovine serum albumin, 150 μ M ATP, 1 μ Ci [γ - ^{32}P]ATP) at 30°C for 20 min. The kinase reaction was stopped by spotting 25 μ l of the reaction mixture onto P81 ion exchange chromatography filter paper (Whatman International, Hillsboro, Oregon). The filter paper was then washed in 75 mM ice-cold phosphoric acid (10 ml per filter) for 5 min, followed by washing the filter paper at room temperature for 5 min three times. The filter paper was air-dried and scintillation counted.

In-Gel MBP Kinase Assay

For preparation of the MBP immobilized SDS-PAG, 0.4 mg/ml of MBP was added into SDS-PAG solution mixture before polymerization. Cell extract S-150 containing 20 μ g of protein was resolved in the MBP-SDS-PAG, followed by rinsing the gel with 20% isopropanol in buffer B (100 mM Tris-HCl, pH 8, 5 mM 2-mercaptoethanol). The gel was denatured in buffer B containing 6 M guanidine-HCl, followed by renaturation with buffer B containing 0.04% Tween 40 at 4°C for 16 h (Kameshita and Fujisawa, 1989). The renatured MBP-SDS-PAG was soaked in kinase buffer (20 mM Tris, pH 7.2, 10 mM MgCl_2 , 15 mM β -glycerolphosphate, 0.3 mM Na_3VO_4) at 22°C for 30 min followed by a kinase reaction that was carried out by incubating the gel with kinase buffer supplemented with 50 μ Ci of [γ - ^{32}P]ATP and 50 μ M ATP at 22°C for 30 min. Then the gel was washed five times with 500 ml of 1% pyrophosphate in 5% trichloroacetic acid. Radio-labeled proteins were detected by autoradiography.

Immune Complex Protein Kinase Assay and Immunoblots

Immunoprecipitation of $p90^{\text{RSK}}$ was carried out by incubating 150 μ g of cytosolic protein (S-150) with 1 μ l of RSK-specific polyclonal antibodies (serum 125) at 0°C for 1 h. Immune complexes were adsorbed to 10 μ l of formalin-fixed *Staphylococcus aureus* at 0°C for 30 min and washed three times with STE buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM Na_2EDTA) supplemented with 0.1 mM DTT and 0.1% NP-40, and once with ST buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl) supplemented with 1 mM DTT. Immune complexes of $p90^{\text{RSK}}$ were suspended in 20 μ l of kinase buffer (20 mM Tris-HCl, pH 7.2, 10 mM MgCl_2 , 50 μ M γ -ATP, 5 μ Ci [γ - ^{32}P]ATP) and incubated at 30°C for 15 min using 0.25 μ g of ribosomal 40S protein as substrate. 40S subunits were prepared as described previously (Erikson *et al.*, 1991). The products of the reaction were electrophoresed in a 10%

SDS-PAG, visualized by autoradiography, and the S6 protein was excised and counted.

The proteins were resolved by electrophoresis in a 10% SDS-PAG and transferred to a nitrocellulose sheet (pore size 0.4 μ m). To detect ERK proteins, the nitrocellulose sheet was blocked by incubation in ST buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl) supplemented with 3% nonfat milk at room temperature for 30 min. ERK proteins were detected by incubation of the sheet with ERK1/ERK2 protein-specific rabbit polyclonal antibodies no. 691 (Santa Cruz Biotechnology, Santa Cruz, California) at room temperature for 2 h, followed by rinsing the sheet three times with ST buffer. Rabbit antibodies were then detected by incubation of the sheet with 1 μ Ci/ml [125 I] Protein A (Dupont, New England Nuclear Research Products, Wilmington, Delaware) at room temperature for 2 h, followed by rinsing the sheet as described above.

RESULTS

Identification of MBP Kinases in *src*-Transformed CEF

Both the *ERK1* and *ERK2* gene products, p44^{ERK1} and p42^{ERK2}, have been shown to be activated during mitogenic stimulation of quiescent cells (Ahn *et al.*, 1990; Ferrell and Martin, 1990; Chung *et al.*, 1991; Posada *et al.*, 1991) and to phosphorylate MBP as well as MAP in vitro (Chung *et al.*, 1991). We used MBP immobilized in SDS-PAG to identify the MBP kinases (MBPKs) with the in-gel MBP kinase assay described by Kameshita and Fujisawa (1989). Serum-stimulation of quiescent CEF showed induction of MBP kinase activity of a 42-kDa protein, p42^{MBPK} (Figure 1, lane 2). In agreement with the earlier reports (Gotoh *et al.*, 1990b; Miyasaka *et al.*, 1990b; L'Allemain *et al.*, 1991; Posada *et al.*, 1991), p42^{MBPK} was activated transiently during the G₀/G₁ transition in CEF (Figure 1, lane 2) and activated constitutively in p60^{v-src} transformed cells (Figure 1, lane 3). Although we were not able to detect the kinase activity of a p44^{MBPK} in chicken cells by the in-gel MBP kinase assay, murine p44^{ERK1} has been detected with this technique (Kameshita and Fujisawa, 1989). It is possible chicken cells have a very low level of this ERK gene product. In addition to p42^{MBPK}, two soluble proteins of 63 and 87 kDa consistently displayed high MBP kinase activity in *v-src* transformed CEF (Figure 1, lane 3) and were designated p63^{SAMK} and p87^{SAMK}. In other experiments, prolonged treatment of quiescent cells with serum up to 30 h has not revealed activation of either p63^{SAMK} or p87^{SAMK}. It should be noted that we have failed to detect either protein kinase C or activated p90^{RSK} with the in-gel assay, and thus these enzymes cannot account for the enzyme activity observed.

The enzyme activities demonstrated in Figure 1 were further analyzed by the chromatographic fractionations commonly used for the ERK gene products p42 and p44. Cell lysates were fractionated on DEAE-Sephacel columns, and the MBP kinase activity detected by conventional as well as in-gel assays was determined. These MBP kinase-containing fractions were applied to a phenyl-sepharose column and eluted with an ethylene

Figure 1. Identification of activated MBPKs in CEF by the in-gel MBP kinase assay. Cell extracts were prepared from quiescent CEF (lane 1), quiescent CEF after stimulation with 20% serum for 10 min (lane 2), and *v-src* transformed CEF that were maintained in serum-free medium for 36 h (lane 3). Cell extracts containing 20 μ g of cytosolic proteins (S-150) were analyzed by the in-gel MBP kinase assay. The regions that show phosphorylation of MBP indicate the activity of MBPKs. Bars indicate p42^{MBPK} (p42^{ERK2}), p63^{SAMK}, and p87^{SAMK}, which were consistently activated in *v-src* transformed CEF.

glycol gradient (Figure 2A). We show here that p63^{SAMK} and p42^{MBPK} were detected by the in-gel assay (Figure 2B) and that the p42 enzyme but not the p63 enzyme is recognized by a broadly cross-reactive MBP kinase antibody. Thus, biochemical and immunological data establish with near certainty that the MBP kinase of 42 kDa detected here is encoded by a chicken ERK gene. The p87 activity is not efficiently recovered from these columns.

To confirm that we had detected serine/threonine kinases, cytosolic proteins were isolated from transformed CEF and resolved in a conventional SDS-PAG. After processing for the in-gel kinase assay, gel slices containing p42^{MBPK}, p63^{SAMK}, or p87^{SAMK} were incubated with MBP and [γ - 32 P]ATP as described in MATERIALS AND METHODS. MBP phosphorylated by p42^{MBPK}, p63^{SAMK}, and p87^{SAMK} yielded both phosphoserine and phosphothreonine residues. No phosphotyrosine was detected in MBP phosphorylated by any protein kinase eluted from these gels.

Activation of MBP Kinases on Cellular Transformation Induced by a *ts v-src* Mutant

To investigate the correlation between the activation of kinases that can phosphorylate the ERK gene product (MEK, for MAP kinase extracellular-regulated kinase kinase) (Alessandrini *et al.*, 1992; Crews and Erikson, 1992), p42^{MBPK}, p63^{SAMK}, p87^{SAMK}, and p90^{RSK} on cellular transformation by p60^{v-src}, cells were infected with a *v-src* mutant *ts119* (Bryant and Parsons, 1982) and cultivated at the nonpermissive temperature (41°C). The cultures were then incubated in serum-free medium at 41°C for 36, 35.5, 35, 24, 12, or 0 h, followed by a shift from 41°C to the permissive temperature (37°C) for 0, 0.5, 1, 12, 24, or 36 h, respectively. Thus, all cells were cultured in serum-free medium for the same interval. As transformation proceeded, the cell cultures showed a progression of changes from the spindle normal morphology to a typically rounded refractile transformed morphology. Figure 3B shows the total activity of MEKs,

Figure 2. Chromatographic analysis of MBP kinase activity in *v-src* transformed CEF. Transformed cell lysates (20 mg of S-150) were fractionated on a DEAE-Sephacel column, and fractions containing MBP kinase activity were applied to a Phenyl-Sepharose fast performing liquid chromatography (FPLC) column as described by Ray and Sturgill (1988). Proteins were eluted with a linear gradient of ethylene glycol (0–60%) and 3-ml fractions were collected for assay. (A) MBP kinase activity was measured by the MBP filter binding assay described in MATERIALS AND METHODS. (B) *src*-transformed cell lysate (S) containing 5 μ g of cytosolic proteins (S-150) and 30 μ l from fractions 16, 19, 21, 23, and 25 was analyzed to determine the activities of p63^{SAMK} and p42^{MBPK} by the in-gel MBP kinase assay. (C) Immunoblot analysis of p42^{MBPK} in 25 μ g of *src*-transformed cell lysate (S-150) (S) or 150 μ l from fractions 16, 19, 21, 23, and 25 using ERK1 and ERK2 antibody, as described in MATERIALS AND METHODS.

using kinase-negative recombinant ERK1 as a substrate in quiescent cells (lane 1) and in cells infected with *ts v-src* (lanes 2–7). On induction of cellular transformation by *ts v-src*, MEKs were activated within 30 min (Figure 3A, lane 3), followed by a decrease in activity by 24 h (Figure 3A, lane 6). In parallel with MEK activation by *v-src*, p42^{MBPK} was rapidly activated within 30 min, followed by a distinct decrease within 24 h (Figure 3B). The kinase activity of p42^{MBPK} appeared to correlate closely with the activity of MEK. The p63^{SAMK} was not fully activated until 24 h, when the kinase activity of p42^{MBPK} had fallen significantly (Figure 3B, lanes

6 and 7). The p87^{SAMK} was activated within 30 min and remained activated in transformed cells. The kinase activity of p90^{RSK} was rapidly induced within 30 min and remained activated in transformed cells (Figure 3C). Thus, under these conditions, activation of pp90^{RSK} (Figure 3C) showed kinetics distinct from those of p42^{MBPK} (Figure 3B) or MEK(s) (Figure 3A).

Figure 3. Activation of MEKs, p42^{ERK2}, p63^{SAMK}, p87^{SAMK}, and pp90^{RSK} in CEF on transformation induced by *ts v-src*. CEF were infected with a *v-src* mutant 119V and maintained at the nonpermissive temperature (41°C). Cultures were incubated in serum-free medium at 41°C for 36, 35.5, 35, 24, 12, or 0 h, followed by a shift from 41°C to the permissive temperature (37°C) for 0, 0.5, 1, 12, 24, or 36 h, respectively. (A) Quiescent CEF (lane 1); *ts v-src*-infected CEF after temperature-shift for 0 h (lane 2); 1 h (lane 4); 12 h (lane 5); 24 h (lane 6); 36 h (lane 7). (B) Cytosolic cell lysates (S-150) were prepared from quiescent CEF (lane 1) and *ts v-src*-infected cells after being shifted from 41 to 37°C for 0, 0.5, 1, 12, 24, or 36 h (lanes 2–7). Activity of MEKs in 5 μ g of cytosolic proteins (S-150) was determined by direct kinase assay with bacteria-produced recombinant ERK1 protein, K63M (substitution mutation of Met-63 for Lys-63) as substrate (see MATERIALS AND METHODS). (C) Cell extracts containing 20 μ g of cytosolic proteins (S-150) were analyzed by the in-gel MBP kinase assay to determine the activities of p42^{ERK2}, p63^{SAMK}, and p87^{SAMK}. (D) The kinase activity of pp90^{RSK} isolated from cytosolic cell lysates was determined by an immune complex kinase assay using 40S ribosomal subunits as substrate (see MATERIALS AND METHODS Erikson *et al.*, 1991). Arrows indicate the substrates used in kinase reaction. Bars indicate p42^{ERK2}, p63^{SAMK}, and p87^{SAMK}.

Correlation Between Cellular Transformation Induced by *c-src* Mutants and the Activation of MEKs, $p90^{RSK}$, and of MBP Kinases $p42$, $p63$, and $p87$

To further investigate the $p60^{src}$ -stimulated signaling pathways, we determined the correlation between activation of MEKs, $p42^{MBPK}$, $p63^{SAMK}$, $p87^{SAMK}$, and $p90^{RSK}$ and cell transformation by *c-src* mutants that yield distinct phenotypes. The *c-src* gene encodes a nononcogenic protein, $p60^{c-src}$ (Iba *et al.*, 1984; Parker *et al.*, 1984; Johnson *et al.*, 1985). CEF that overexpress $p60^{c-src}$ do not show a significant alteration in cell morphology. Substitution of Phe-527 for Tyr-527 converts $p60^{c-src}$ into a kinase-active transforming protein $p60^{c-527F}$ (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Reynolds *et al.*, 1987). Cells transformed by $p60^{c-527F}$ exhibit a typical transformed morphology. Additional mutation of Gly-2 to Ala-2 in $p60^{c-527F}$ results in a nontransforming, nonmyristylated, kinase-active *c-src* variant of $p60^{2A-527F}$ (Reynolds *et al.*, 1989). Sequence alterations within the SH-3 region of the *c-src* protein result in partial activation of the $p60^{c-src}$ transforming potential (Potts *et al.*, 1988). Cells transformed by $p60^{dl118C}$, which has a deletion of residues 118–122 within the SH-3 region of $p60^{c-src}$, show an elongated fusiform morphology.

The content of phosphotyrosine in cellular proteins and the level of $p60^{src}$ in these cells was determined with phosphotyrosine antibodies and $p60^{src}$ -specific monoclonal antibody by immunoblot analysis. Cells that expressed *c-src* mutants *dl118C*, *c-527F*, or *2A-527F* exhibited significant increases in the overall level of phosphotyrosine content compared with cells overexpressing wt *c-src* protein, although the $p60^{src}$ levels were nearly the same. Cells that expressed $p60^{2A-527F}$ had an elevated phosphotyrosine content, a normal morphology, and no increased activity of MEKs (Figure 4A, lane 5), $p42^{MBPK}$, $p63^{SAMK}$, $p87^{SAMK}$ (Figure 4B, lane 5). The activity of $p90^{RSK}$ (Figure 4C, lane 5) increased two- to threefold under these conditions. In contrast, we observed a correlation of cellular transformation induced by $p60^{dl118C}$, as well as by $p60^{c-527F}$ (Figure 4A, lanes 3 and 4), and the activation of MEKs (Figure 4A, lanes 3 and 4), $p42^{MBPK}$, $p63^{SAMK}$, $p87^{SAMK}$ (Figure 4B, lanes 3 and 4), and $p90^{RSK}$ (Figure 4C, lanes 3 and 4). The kinase activity of $p63^{SAMK}$ was induced to a greater extent in the *c-527F*-transformed cells (Figure 4B, lane 4) than in *dl118C*-transformed fusiform cells (Figure 4B, lane 3). Thus, the extent of morphological transformation appeared to correlate closely with increased $p63^{SAMK}$ activity.

Activation of MEKs, $p42^{MBPK}$, $p63^{SAMK}$, $p87^{SAMK}$, or $p90^{RSK}$ by Treatment with Phosphatase Inhibitors

Several lines of evidence show that serine/threonine-specific phosphatase 2A dephosphorylates and inacti-

Figure 4. Activation of MEKs, $p42^{ERK2}$, $p63^{SAMK}$, $p87^{SAMK}$, and $p90^{RSK}$ on transformation induced by *c-src* mutants. (A) Activity of MEKs in 5 μg of cytosolic proteins (S-150) was determined by direct kinase assay with bacteria-produced recombinant ERK1 protein (K63M) as substrate. (B) Cell lysates containing 20 μg of cytosolic proteins were analyzed by the in-gel MBP kinase assay to determine the activities of $p42^{ERK2}$, $p63^{SAMK}$, and $p87^{SAMK}$. (C) The kinase activity of $p90^{RSK}$ was determined by the immune complex kinase assay using 40S ribosomal subunits as substrate. Normal CEF (lane 1); CEF expressing wt *c-src* (lane 2), *dl118C* (lane 3), *c-527F* (lane 4), and *2A-527F* (lane 5). Arrows indicate the substrates used in kinase reaction. Bars indicate $p42^{ERK2}$, $p63^{SAMK}$, and $p87^{SAMK}$.

vates $p44/42^{ERK1/ERK2}$ as well as $p90^{RSK}$ in vitro (Sturgill *et al.*, 1988). Okadaic acid and sodium fluoride are inhibitors of serine/threonine phosphatases 1 and 2A (Cohen and Cohen, 1989; Cohen *et al.*, 1990). Okadaic acid has been reported to activate $p42$ and $p44$ MBP kinase as well as to induce a pseudomitotic phase in the treated murine cells (Gotoh *et al.*, 1990a; Kipreos and Wang, 1990; Yamashita *et al.*, 1990). Treatment of serum-deprived CEF with okadaic acid resulted in the activation of $p63^{SAMK}$ and $p87^{SAMK}$ (Figure 5B, lane 2) and profoundly elevated the activity of $p90^{RSK}$ (Figure 5C, lane 2) but did not activate MEKs (Figure 5A, lane 2) or $p42^{MBPK}$ (Figure 5B, lane 2). The MBPK activity detected immediately below $p63^{SAMK}$ was not observed consistently (Figure 6). Serum stimulation of cells pretreated with phosphatase inhibitors did not appear to influence significantly the activities of $p63^{SAMK}$ or $p87^{SAMK}$ but did elevate the levels of MEKs and $p42^{MBPK}$ (Figure 5, lanes 2–4 vs. 6–8). Okadaic acid repeatedly elevated $p90^{RSK}$ activities to a greater extent than did serum during the course of these studies (Figure 5C, lane 2 vs. 5).

Cells that were treated with sodium fluoride showed significant elevation of MEK, $p42^{MBPK}$, $p87^{SAMK}$, and $p90^{RSK}$ activity (Figure 5, A–C, lane 3) but not $p63^{SAMK}$ (Figure 5B, lane 3) activity. Serum stimulation of cells

Figure 5. Activation of MEKs, p42^{ERK2}, p63^{SAMK}, p87^{SAMK}, and p90^{RSK} by treatment of CEF with phosphatase inhibitors okadaic acid, sodium fluoride, or sodium vanadate. Cytosolic cell lysates that were analyzed in the following assays were prepared from quiescent CEF (lane 1) and cells that were individually treated with 200 nM okadaic acid, 25 mM sodium fluoride, and 4 mM sodium vanadate for 1 h (lanes 2–4 and 6–8) followed by serum stimulation for 10 min (lanes 5–8). (A) Activity of MEKs in 5 μ g of cytosolic proteins was determined by a direct assay using bacteria-produced recombinant ERK1 protein (K63M) as substrate. The extent of phosphorylation of recombinant ERK1 protein was determined by excision of the ERK1 protein bands from gels and scintillation counting. The value for the kinase activity of MEKs isolated from quiescent cells has been set to 1 and the relative kinase activities of MEKs assayed from phosphatase inhibitor-treated cells were determined. (B) Cell lysates containing 20 μ g of cytosolic proteins were analyzed by the in-gel MBP kinase assay to determine the kinase activities of p42^{ERK2}, p63^{SAMK}, and p87^{SAMK}. Bars indicate p42^{ERK2}, p63^{SAMK}, and p87^{SAMK}. (C) The kinase activity of p90^{RSK} was determined by the immune complex kinase assay using 40S subunits

pretreated with sodium fluoride resulted in additional increases of MEK (Figure 5A, lane 7) and p42^{MBPK} (Figure 5B, lane 7) but not p90^{RSK} (Figure 5C, lane 7) activity. Treatment of cells with sodium vanadate also resulted in elevated levels of MEK (Figure 5A, lane 4), p87^{SAMK}, p42^{MBPK} (Figure 5B, lane 4), and p90^{RSK} activity (Figure 5C, lane 4) but not p63^{SAMK} (Figure 5B, lane 4) activity. Although comparable levels of p42^{MBPK} activity were induced by sodium vanadate (Figure 5B, lane 4) and sodium fluoride (Figure 5B, lane 3), the level of p90^{RSK} activity was distinctly lower in sodium vanadate-treated cells (Figure 5C, lane 4) than in sodium fluoride-treated cells (Figure 5C, lane 3). Serum stimulation of cells pretreated with sodium vanadate did not result in an additional increase in MEK (Figure 5A, lane 8), p42^{MBPK} (Figure 5B, lane 8), or p90^{RSK} (Figure 5C, lane 8) activity. Nor did we detect additional p90^{RSK} activity on serum stimulation of quiescent cells pretreated with okadaic acid or sodium fluoride (Figure 5C). These results taken together indicate that the increased kinase activity of p90^{RSK} is correlated with activation of either p63^{SAMK} or p42^{MBPK}.

Autophosphorylation and Exogenous Phosphorylation Activities of p42^{MBPK}, p63^{SAMK}, and p87^{SAMK}

Quiescent CEF were treated with serum, okadaic acid, sodium fluoride, or sodium vanadate. Cell extracts (20 μ g of S-150) were analyzed in both MBP-immobilized (Figure 6A) and conventional SDS-PAGE (Figure 6B) by the in-gel kinase assay under identical conditions. Figure 6A shows the kinase activity in phosphorylation of MBP, and Figure 6B shows the kinase activity detected in the absence of substrate. Only p87^{SAMK} exhibited any significant activity in autophosphorylation. Both p42^{MBPK} and p63^{SAMK} showed a very low level of apparent autophosphorylation activity.

DISCUSSION

In this communication we have identified two MBP kinase activities, p63 and p87, that are constitutively elevated in cells transformed by p60^{v-src} or p60^{c-src} transforming mutants. These MBP kinase activities are not elevated by serum-stimulation of normal cells, however. The activity of the more commonly studied p42^{MBPK}, which is serum stimulated, is only modestly elevated in fully transformed cells. We also noted that the *src*-

as substrate. The extent of phosphorylation of S6 was determined by excision of the S6 bands from gels and scintillation counting. The value for the activity of p90^{RSK} isolated from quiescent cells has been set to 1 and the relative kinase activities of p90^{RSK} that were immunoprecipitated from phosphatase inhibitor-treated cells were determined.

Figure 6. Autophosphorylation and MBP phosphorylation by $p42^{\text{ERK2}}$, $p63^{\text{SAMK}}$, and $p87^{\text{SAMK}}$. Quiescent CEF (lane 1) were stimulated with 20% serum for 10 min (lane 2) or treated with 200 nM okadaic acid (OA), 25 mM sodium fluoride (NaF), or 4 mM sodium vanadate (Na_3VO_4) for 1 h (lanes 3–5). Cytosolic cell lysates were electrophoresed in both MBP-immobilized and conventional SDS-polyacrylamide gels followed by the process of the in-gel kinase assay. A shows kinase activity in phosphorylation of MBP. B shows kinase activity in apparent autophosphorylation.

activated serine/threonine kinases, $p63^{\text{SAMK}}$ and $p87^{\text{SAMK}}$, but not the $p42^{\text{MBPK}}$, are stimulated by treatment of cells with 200 nM okadaic acid. Although the in-gel assay used here (Kameshita and Fujisawa, 1989; Leever and Marshall, 1992) previously has been used to detect $p42^{\text{MBPK}}$ and $p44^{\text{MBPK}}$ in rodent cells that had been stimulated with serum or treated with 1 μM okadaic acid, no p63 or p87 MBP kinase activity was reported (Gotoh *et al.*, 1990a). In contrast to the earlier reports, in these studies we have failed to activate $p42^{\text{MBPK}}$ with okadaic acid. There is no obvious explanation for these discrepancies other than the use of CEF or the specific conditions for the experiments used in this study. We are in the process of exploring the effects of varying okadaic acid concentrations and time of treatment of CEF. The expression of p63 and p87 in rodent cells under a variety of conditions is under study as well. The data shown here on the treatment of cells with protein phosphatase inhibitors sodium fluoride and sodium vanadate dissociate activation of $p42^{\text{MBPK}}$ from activation of $p63^{\text{SAMK}}$ and provide additional evidence that these enzymes operate in distinct signal transduc-

tion pathways. These data also show that $p90^{\text{RSK}}$ is activated in concert with either $p42^{\text{MBPK}}$ or $p63^{\text{SAMK}}$.

Both $p63^{\text{SAMK}}$ and $p87^{\text{SAMK}}$ have a potentially important role in cellular transformation, as they appear to mark a signal transduction pathway that is distinct from the normal response to the growth factors found in serum. Thus, the additional characterization of these enzymes and their substrates is of importance. To this end, we are in the process of purifying the activated p63 and p87 kilodalton kinases described here. The previously described ERK3 gene may encode p63, as it allegedly produces an MBPK related to $p42^{\text{MBPK}}$ and $p44^{\text{MBPK}}$. No obvious candidate gene has been reported to date that may encode the p87 protein kinase, however. MAP (MBP) kinases have previously been reported to be constitutively activated in *src* or G_{12} oncogene-transformed rat fibroblasts (Gupta *et al.*, 1992). In that report, conventional soluble assays on cell and column extracts did not reveal the molecular nature of the enzymes responsible for the activity, however. Thus, it will be of value to determine the response of $p63^{\text{SAMK}}$ and $p87^{\text{SAMK}}$ in cells transformed by other oncogenes.

These studies raise new questions concerning the activation of $p90^{\text{RSK}}$. It has been assumed, based on earlier reports, that $p90^{\text{RSK}}$ is activated as the result of serine/threonine phosphorylation by $p42/44^{\text{MBPK}}$ (Sturgill *et al.*, 1988; Chung *et al.*, 1991). In this report, we show, however, that $p90^{\text{RSK}}$ is activated by okadaic acid with no evident increase in $p42^{\text{MBPK}}$, whereas $p63^{\text{SAMK}}$ and $p87^{\text{SAMK}}$ show significant stimulation. Although $p90^{\text{RSK}}$ could be in part activated because of the inhibition of phosphatase 1 and 2A, it would be surprising if such a mechanism could account for the full stimulation detected here. We shall attempt to determine whether purified $p63^{\text{SAMK}}$ or $p87^{\text{SAMK}}$ is able to phosphorylate and contribute to the activation of $p90^{\text{RSK}}$.

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