MOLECULAR AND CELLULAR BIOLOGY, June 1990, p. 3204–3215
0270-7306/90/063204-12$02.00/0

Vol. 10, No. 6

Identification of *Xenopus* S6 Protein Kinase Homologs (pp90^{rsk}) in Somatic Cells: Phosphorylation and Activation during Initiation of Cell Proliferation

REY-HUEI CHEN AND JOHN BLENIS*

Department of Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611, and Department of Cellular and Molecular Physiology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115

Received 4 December 1989/Accepted 1 March 1990

We have identified human, mouse, and chicken homologs to *Xenopus* S6 protein kinase II (S6KII). In quiescent cells, the apparent molecular mass of the *Xenopus* homologs (referred to as pp90^{rsk}) increased from a range of 81 to 91 to a range of 85 to 92 kilodaltons following serum addition, which is consistent with an increase in protein phosphorylation. Indeed, serum growth factors stimulated pp90^{rsk} phosphorylation at multiple serine and threonine residues. Furthermore, pp90^{rsk} activity was stimulated within seconds of serum addition. Distinct molecular sizes, chromatographic properties, phosphopeptide maps, and kinetics of activation, the lack of immunological cross-reactivity, and analysis of S6 kinase activities in cells that overexpressed pp90^{rsk} suggest that pp90^{rsk} and pp70-S6 protein kinase, a previously identified mitogen- and oncogene-regulated S6 kinase in cultured cells, are distinct and differentially regulated. The notion that both enzymes are regulated by protein phosphorylation was supported by the ability to inactivate their S6 phosphotransferase activities with potato acid phosphatase. These data demonstrate that homologs to the *Xenopus* S6 protein kinases are produced and regulated by protein phosphorylation in somatic cells and that, in addition to a proposed role in *Xenopus* oocyte maturation, these homologs may participate in the initiation of animal cell proliferation.

Several studies have demonstrated that information transmission and processing within cells are manifested at least in part via protein phosphorylation. Indeed, a number of growth factors and oncogene products exhibit or regulate protein-tyrosine or protein-serine/threonine (Ser/Thr) kinase activities. The targets of these enzymes are likely the initial mediators of information that in turn modify a cascade of subsequent signal transducers. With regard to protein phosphorylation, several protein-serine/threonine kinases with the potential to transmit growth-regulating information have been described. These include second messenger-regulated protein kinases such as cyclic AMP-dependent protein kinases, members of the protein kinase C family, Ca^{2+}, calmodulin-dependent protein kinases, and cyclic GMP-dependent protein kinases. In addition, several growth-regulated protein-serine/threonine kinases that do not require known second messengers for activity, including the 65- to 70-kilodalton (kDa) S6 protein kinases (4, 11, 32–34, 42), MAP-2 kinase (35), casein kinase II (24, 38), c-*raf* protein kinase (29, 30), and a 100-kDa epidermal growth factor-regulated protein-Ser/Thr kinase (19), have been identified in animal cells. There is also evidence suggesting that these protein kinases are themselves regulated by protein phosphorylation.

In addition to the protein kinases regulated during the initiation of cell proliferation, several protein-Ser/Thr kinases are regulated during the meiotic maturation of *Xenopus* oocytes (9, 18) and sea star oocytes (28) and during neuronal differentiation of rat PC12 cells (6, 27, 37). One of these, the *Xenopus* 40S ribosomal protein S6 kinase II (S6KII), is a major S6 kinase whose activity is regulated during oocyte maturation (14, 18). However, S6KII has not been previously identified in cultured animal cells. Furthermore, homologs to the 65- to 70-kDa S6 kinases (referred to here as pp70-S6K), major S6 kinases whose activity has been detected in several animal culture systems (7, 21, 31, 41), have not been identified in *Xenopus* oocytes or mature eggs (14, 15, 17).

Xenopus S6KII has been identified as a ~92-kDa phosphoprotein (15). Previous studies suggest that it may participate in the maturation of *Xenopus* oocytes to eggs (18). Its activity is apparently regulated by protein phosphorylation, as evidenced by the absolute requirement for phosphatase inhibitors to maintain activity during lysate preparation and purification (14, 15), the ability to greatly reduce activity with phosphatase 1 or 2A (39), and the partial reactivation of S6 kinase activity by phosphorylation with MAP-2 kinase (39). Two sequences encoding putative S6KII gene products have been isolated from a *Xenopus laevis* cDNA library and partially characterized. One of the unique features of the encoded polypeptide is the apparent existence of two distinct and separate types of ATP-binding and catalytic domains (23). No other protein kinase that possesses this feature has been described to date. In addition, greater than 80% homology at the predicted protein level has been observed between *Xenopus*, chicken, and mouse S6KII homologs, suggesting evolutionary pressure to maintain the unique structure of these potentially critical growth-regulating enzymes (1). The role of these domains in the function of the S6KII polypeptides remains to be determined. It is tempting to speculate that if the separate domains are active, each catalytic domain may exhibit a distinct substrate specificity as well as differential regulation, a key feature for a single protein kinase with the potential to transmit different growth-modulating signals.

Antiserum to purified *Xenopus* S6KII has provided evidence for the existence of a serum- and pp60$^{v\text{-}src}$-regulated S6 kinase activity in cultured chicken embryo fibroblasts

* Corresponding author.

(through the use of an immune-complex protein kinase assay). However, this antiserum is incapable of immunoprecipitating polypeptides from lysates of biosynthetically labeled cells, thus preventing the identification of the enzyme (18). Presumably, this is the result of low antibody affinity. Furthermore, several laboratories have purified a major growth-regulated S6 kinase from cultured chicken and mouse cells and bovine and rat liver that exhibits a molecular size of 65 to 70 kDa (pp70-S6K). This kinase is clearly distinct from purified *Xenopus* S6KII (~92 kDa) and the chicken and mouse homologs, whose predicted molecular sizes are 82 to 84 kDa (1). To resolve this discrepancy and determine if distinct growth-regulated *Xenopus* S6KII homologs indeed exist in a variety of cultured somatic cells, we have generated polyclonal antiserum to purified recombinant chicken and *Xenopus* S6KII polypeptides. This has allowed us to identify an apparent family of immunologically related S6KII polypeptides (referred to collectively as pp90^{rsk}) in cultured chicken, mouse, and human cells and to study their regulation during the G_0-to-G_1 transition of cell growth with a pp90^{rsk}-specific immune-complex protein kinase assay. In addition, we have overexpressed pp90^{rsk} in NIH 3T3 and Swiss 3T3 cells by infection with recombinant retroviruses. These studies, together with a lack of immunological cross-reactivity and distinct chromatographic profiles, demonstrate that pp90^{rsk} and pp70-S6K are distinct mitogen-regulated protein kinases and are not proteolytically related. Results from phosphopeptide analysis suggest that pp90^{rsk} is rapidly phosphorylated at multiple serine and threonine sites. Furthermore, we show that phosphorylation of pp90^{rsk} in cultured cells is required for activity. These data provide evidence for the existence of one or more growth-regulated protein-Ser/Thr kinases that are activated before pp90^{rsk} and in turn activate pp90^{rsk} and probably other targets associated with signal transduction and the control of animal cell proliferation.

MATERIALS AND METHODS

Cell cultures. Chicken embryo fibroblasts (CEF) were prepared as described previously (4) and maintained at 41.5°C in Dulbecco modified Eagle medium (DMEM) supplemented with 10% tryptose phosphate broth, 5% calf serum (GIBCO), and 1% heat-inactivated chicken serum (GIBCO). Swiss 3T3 cells and HeLa cells were passaged in DMEM plus 10% heat-inactivated fetal calf serum (Hyclone); for experiments, these cells were cultured in 5% calf serum instead of 10% fetal calf serum until confluence. NIH 3T3 cells were cultured in DMEM containing 10% calf serum. For all experiments, confluent cells were further cultured in DMEM containing 0.5% calf serum and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.35, for 24 to 48 h prior to serum addition and preparation of cell lysates.

Generation of polyclonal antisera. Polyclonal antisera were prepared against recombinant *Xenopus* S6KII as described previously (23). Antisera to recombinant chicken S6KII were similarly prepared. Briefly, a cDNA fragment encoding amino acids 53 to 752 of chicken S6KII was inserted into the *Eco*RI site of the vector pEV-vrf1, and *Escherichia coli* RR1 was transformed with this construct as described (13). The recombinant chicken S6KII produced in bacteria was isolated from preparative sodium dodecyl sulfate (SDS)-polyacrylamide gels and used to raise antisera in rabbits.

Biosynthetic cell labeling and immunoprecipitation. For labeling with $^{32}P_i$ (ICN Pharmaceuticals Inc.), confluent monolayers were further cultured in DMEM with 0.5% calf serum for 24 to 48 h. The medium was then changed to 4 ml of serum- and phosphate-free medium per 100-mm culture dish for 1 h before being labeled for 2 h with 0.5 to 5 mCi of carrier-free $^{32}P_i$ per dish. For [^{35}S]methionine biosynthetic labeling, the serum-depleted cells were labeled for 6 h with 0.5 to 2 mCi of [^{35}S]methionine (700 to 800 Ci/mmol; Du Pont, NEN Research Products) in 4 ml of methionine-free medium per 100-mm tissue culture dish.

For serum growth factor stimulation, dialyzed fetal calf serum was added to a final concentration of 10% (vol/vol) for a desired time prior to preparation of cell lysates. Biosynthetically labeled cells were then washed with STE (150 mM NaCl, 50 mM Tris chloride, 1 mM EDTA, pH 7.2), scraped in 0.5 ml of lysis buffer (10 mM KPO_4, 1 mM EDTA, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid], 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 40 μg of phenylmethylsulfonyl fluoride per ml [pH 7.2]), Dounce homogenized (25 to 30 strokes with a tight-fitting pestle), and clarified by centrifugation for 5 min at 10,000 × *g*. Cell lysates were always prepared at 4°C. Supernatants were then adjusted to 1% SDS, denatured by heating to 85 to 90°C for 5 min, and diluted with 10 volumes of RIPA buffer (10 mM Tris chloride [pH 7.2], 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM sodium orthovanadate, 40 μg of phenylmethylsulfonyl fluoride per ml). Ten microliters of preimmune serum or antiserum raised against recombinant *Xenopus* or chicken S6KII (antibody excess) was incubated overnight at 4°C with one fourth of the cell lysate from a confluent 100-mm culture dish and then incubated for an additional 20 min with protein A-containing *Staphylococcus aureus*. The immunocomplexes were precipitated by centrifugation at 3,000 × *g* for 3 min and washed twice with RIPA buffer, twice with high-salt buffer (1 M NaCl, 0.1% Nonidet P-40, 10 mM Tris chloride [pH 7.2], 1 mM sodium orthovanadate, 40 μg of phenylmethylsulfonyl fluoride per ml), and once with STE. The immunoprecipitated proteins were solubilized by heating to 85 to 90°C for 3 min in SDS-polyacrylamide gel electrophoresis sample buffer, resolved by SDS–7.5% polyacrylamide gel electrophoresis, and visualized by autoradiography.

For protein immunoprecipitation-blocking experiments, the same procedures were performed, except that the antiserum was preincubated with excess recombinant *Xenopus* or chicken S6KII (1 μg) for 30 min at 4°C before immunoprecipitation.

Assays for S6 protein kinase activity. Quiescent cells or cells incubated with serum for various periods were washed in STE, scraped in 0.8 ml lysis buffer, Dounce homogenized (25 to 30 strokes with a tight-fitting pestle), and clarified by centrifugation at 100,000 × *g* for 30 min. Glycerol was then added to a final concentration of 10% (vol/vol), and cell lysates were stored at −5°C. Assays were completed within 2 h of lysate preparation.

Assays for pp70-S6K were performed as previously described (4). Briefly, 10 μl of cell lysate (generally 5 to 10 μg of protein [8]; equal amounts were used within each experiment) were incubated with 33 μg of 40S ribosomal subunit (prepared from rat livers) in a reaction buffer containing 10 mM $MgCl_2$, 100 μM ATP (10 μCi of [γ-^{32}P]ATP), 20 mM Tris chloride (pH 7.5), and 0.05 μg of heat-stable cyclic AMP-dependent protein kinase inhibitor (Sigma Chemical Co.) in a total volume of 50 μl. The reaction mixtures were incubated for 15 min at 30°C, and the reaction was terminated by the addition of 12.5 μl of 5× SDS-polyacrylamide gel elec-

trophoresis sample buffer followed by heating at 90°C for 3 min. S6 was resolved by SDS–12% polyacrylamide gel electrophoresis followed by autoradiography and quantitated by excising the S6 polypeptide from the dried gel and determining associated radioactivity.

Assays for pp90^{rsk} were performed with the immune complex essentially as described previously (18). Cell lysates (equal amounts of protein), prepared as described above, were incubated with antiserum on ice for 1 h and then adsorbed to *S. aureus* for 20 min. The immunocomplexes were washed twice with buffer A (1% Nonidet P-40, 0.5% deoxycholate, 100 mM NaCl, 10 mM Tris chloride [pH 7.2], 1 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 40 μg of phenylmethylsulfonyl fluoride per ml), twice with buffer B (high-salt buffer with 2 mM dithiothreitol), and once with ST (150 mM NaCl, 50 mM Tris chloride [pH 7.2]). Following the final wash, the immunocomplexes were suspended in 20 μl of 1.5× pp90^{rsk} reaction buffer minus ATP and 40S subunits. The reaction was started by the addition of 10 μl of ATP and substrate. The final reaction concentrations were 20 mM HEPES [pH 7.2], 10 mM $MgCl_2$, 50 μM ATP (20 μCi of [γ-^{32}P]ATP), 3 mM β-mercaptoethanol, 0.1 mg of bovine serum albumin per ml, and 20 μg of rat liver 40S ribosomal subunits. The reaction was allowed to proceed for 15 min at 30°C (linear assay conditions) and terminated by the addition of an equal volume of 2× SDS-polyacrylamide gel electrophoresis sample buffer followed by heating at 90°C for 5 min. The *S. aureus* was pelleted, and the supernatant polypeptides were resolved by SDS–12% polyacrylamide gel electrophoresis. To measure pp90^{rsk} autophosphorylating activity, the reaction was performed without added substrate in 1 μM ATP.

Overexpression of pp90^{rsk} in cultured cells. A full-length chicken pp90^{rsk} cDNA (1) was subcloned into the *Eco*RI site of the plasmid pMV-7 (kindly supplied by M. Johnson, Department of Pathology, Northwestern University Medical School). A detailed description of the retroviral expression vector pMV-7 has been given (25). The resulting construct was designated pMV7-chS6KII. Twenty micrograms of CsCl-banded pMV-7 or pMV7-chS6KII plasmid DNA was transfected onto subconfluent ψ_{CRE} cells according to standard protocols for calcium phosphate precipitation. After 48 h, the culture medium was collected, filtered through a 0.2-μm-pore-size filter, and stored at −80°C. Recipient NIH 3T3 or Swiss 3T3 murine fibroblasts (5×10^5 cells per 100-mm dish) were infected with the virus-containing medium in 2 μg of Polybrene per ml for 48 h. The cells were then trypsinized and replated in DMEM containing 200 μg of the active neomycin derivative G418 (Geneticin; GIBCO) per ml. Resistant colonies were cloned by ring isolation after 2 weeks of G418 selection. The cells that stably overexpressed pp90^{rsk} were identified by Northern (RNA) blot analysis and immunoprecipitation of pp90^{rsk} from [^{35}S]methionine-labeled cells.

Potato acid phosphatase treatment. pp70-S6K was purified from CEF as previously described (7). pp90^{rsk} was partially purified under similar conditions through the DEAE chromatography step from Swiss 3T3 cells incubated with serum for 5 min. Purification was monitored by both S6 kinase assays described above. Under these conditions, pp90^{rsk} eluted at approximately 30 to 70 mM NaCl (the major pp90^{rsk} peak), whereas the pp70-S6K eluted at approximately 100 to 180 mM NaCl. For each phosphatase experiment, less than 1 μl of each S6 kinase preparation (pp90^{rsk} and pp70-S6K containing approximately equal S6 phosphotransferase activity) was diluted to a final volume of 4 μl with phosphatase buffer (20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid] [pH 6.8], 20 mM KCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.05% Brij 35), and 1 μl of a 0.375-U/μl stock solution of potato acid phosphatase (Boehringer Mannheim Biochemicals) in storage buffer (phosphatase buffer plus 50% glycerol) was added. Control reactions were performed with 1 μl of heat-denatured (85 to 90°C for 2 min) potato acid phosphatase or, additionally, phosphatase incubations were completed in the presence of phosphatase inhibitors with similar results (not shown). The reaction mixtures were incubated at room temperature for 0 to 30 min and terminated by the addition of an equal volume of cold phosphatase inhibitor buffer (20 mM KPO_4 [pH 7], 100 μM sodium orthovanadate, 20 mM β-glycerophosphate [pH 7.2]). S6 kinase activity after phosphatase treatment was then measured immediately as described above. Under these S6 phosphotransferase conditions, the level of dephosphorylation of S6 was low and accounted for. Although the molar ratio of pp90^{rsk} or pp70-S6K to phosphatase in Fig. 11A is not known, previous reports have shown that the specific activities of the purified enzymes are similar (15, 22). In this experiment, we used similar amounts of enzyme activity.

For dephosphorylation and inactivation of S6 kinase activity associated with the immunocomplex, pp90^{rsk} immunocomplexes from Swiss 3T3 cells were prepared as described above and washed twice with buffer A and twice with phosphatase buffer. Two microliters of heated or unheated potato acid phosphatase shock solution was added to the immune complex resuspended in 50 μl of phosphatase buffer. The phosphatase reaction was allowed to proceed at room temperature for 0 to 30 min and terminated by adding volumes of cold phosphatase inhibitor buffer equal to those of the reaction mixtures. The immunocomplexes were then washed twice with buffer B and once with ST and assayed as described above.

The potato acid phosphatase stock solution was prepared by sedimenting the enzyme out of ammonium sulfate suspension, dissolving it in phosphatase buffer, and dialyzing it against storage buffer. This preparation was stored at −20°C.

Analysis of autophosphorylated peptides generated by limited proteolysis. Autophosphorylation of purified chicken pp70-S6K was completed in the absence of 40S ribosomal subunits in 1 μM ATP. Autophosphorylation of pp90^{rsk} was performed in the immune complexes isolated from serum-stimulated CEF as described above. The reaction mixtures were then resolved by preparative SDS–7.5% polyacrylamide gel electrophoresis, and the labeled proteins were located in the wet gel by autoradiography, excised, and analyzed by limited proteolysis as described by Cleveland et al. (10) with *S. aureus* V8 protease.

Two-dimensional phosphopeptide mapping of biosynthetically labeled pp90^{rsk}. Six 100-mm tissue culture plates of CEF, ten plates of Swiss 3T3 cells, and two plates of HeLa cells were grown to confluence, serum deprived, and labeled with $^{32}P_i$ (4 mCi per plate for CEF and Swiss 3T3 cells and 5 mCi per plate for HeLa cells in 4 ml of phosphate-free DMEM). Half of the plates were incubated with serum for 5 min (Swiss 3T3) or 10 min (CEF and HeLa) before preparation of cell lysates. pp90^{rsk} was then immunoprecipitated, separated by SDS–7.5% polyacrylamide gel electrophoresis, located in the wet gel by autoradiography, excised, eluted, oxidized with performic acid, and subjected to exhaustive digestion with trypsin as previously described (20). Tryptic digests were resolved on cellulose thin-layer chromatography plates by electrophoresis at pH 1.9 (acetic acid–88% formic acid–H_2O [156:50:1794 {vol/vol/vol}]) for 40 min at 1.0

kV in the first dimension followed by ascending chromatography in a buffer composed of *n*-butanol, pyridine, acetic acid, and H_2O (75:50:15:60 [vol/vol]) for 4 h in the second dimension. Labeled tryptic phosphopeptides were detected by autoradiography

Two-dimensional phosphoamino acid analysis. Biosynthetically ^{32}P-labeled pp90^{rsk} was immunoprecipitated from quiescent and serum-stimulated cells (one plate each, labeled with 5 mCi per plate as described above), isolated by elution from preparative SDS–7.5% polyacrylamide gels, and precipitated with trichloroacetic acid (final concentration, 20%) following the addition of carrier bovine serum albumin. pp90^{rsk} was then hydrolyzed in 6 N HCl for 2 h at 100°C. After hydrolysis, 2.5 μg of phosphoserine, 2.5 μg of phosphothreonine, and 5.0 μg of phosphotyrosine were added so that the positions of these phosphoamino acid standards could be monitored following electrophoresis and staining with ninhydrin. The phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis with the first dimension performed in pH 1.9 buffer for 40 min at 1 kV and the second dimension performed in pH 3.5 buffer (pyridine–acetic acid–H_2O [1:10:189 {vol/vol/vol}]) for 20 min at 1.0 kV as previously described (12).

DEAE chromatography of pp70-S6K and pp90^{rsk} from NIH 3T3 cells overexpressing pp90^{rsk}. Five 150-mm plates of NIH 3T3 cells overexpressing pp90^{rsk} or NIH 3T3 cells infected with the control pMV7 retrovirus minus the chS6KII insert were grown in DMEM containing 5% calf serum to confluence. The medium was then changed to DMEM plus 0.5% calf serum and 20 mM HEPES, pH 7.35, for 48 h. The serum-depleted cells were stimulated with 10% (vol/vol) fetal calf serum for 5 min before preparation of cell lysates. The cells were lysed in 5 ml of buffer C1 (10 mM potassium phosphate [pH 7.1], 1 mM EDTA, 5 mM EGTA, 5 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.05% Brij 35, 10% [vol/vol] glycerol), clarified by centrifugation at 100,000 × *g* for 30 min, and fractionated with approximately equal amounts of protein, measured as described previously (8), over 8 ml of DEAE-Sephacel (Pharmacia Biotechnology, Inc.). Forty 2-ml fractions were collected (0 to 300 mM NaCl gradient). pp90^{rsk} activity from 200-μl samples was measured in the immune complexes as described above. Assays for S6 phosphotransferase activity were performed by the direct assay under the same reaction conditions as described for pp90^{rsk} assays, except that 25 ng of heat-stable cyclic AMP-dependent protein kinase inhibitor was added to the 25-μl reaction mixture, which included 5 μl of the appropriate fraction.

RESULTS

Identification of pp90^{rsk} in chicken embryo fibroblasts, Swiss 3T3 fibroblasts, and HeLa cells. Antisera to recombinant *Xenopus* and chicken S6KII were prepared as described in Materials and Methods. As shown in Fig. 1A through C, both antisera were capable of immunoprecipitating [^{35}S]methionine-labeled, immune-specific polypeptides from quiescent CEF, Swiss 3T3, and HeLa cell lysates (molecular masses, ~81 to 91 kDa) that were not detected with preimmune serum (compare lane 1 with lane 3 and lane 9 with lane 11). These molecular sizes are consistent with the predicted molecular weights of pp90^{rsk} on the basis of sequence information (1, 23) and predict the existence of a family of immunologically related S6KII protein kinases and/or modified forms of S6KII polypeptides. After the addition of serum growth factors to quiescent cells, these immune-specific polypeptides migrated with an apparently greater molecular size, as detected by SDS-polyacrylamide gel electrophoresis (molecular size, ~85 to 92 kDa; compare lane 3 with lane 4 and lane 11 with lane 12). Shown in Fig. 1D is a direct comparison of the immunoprecipitated pp90^{rsk} polypeptides from quiescent and serum-stimulated CEF, Swiss 3T3 cells, and HeLa cells illustrating the decreasing molecular sizes of the major [^{35}S]methionine-labeled pp90^{rsk} polypeptides in these species. In serum-stimulated CEF (Fig. 1A), a closely migrating doublet was consistently observed at ~90 to 92 kDa with several antisera prepared against recombinant chicken S6KII and with anti-*Xenopus* S6KII antiserum. Anti-*Xenopus* S6KII antiserum (α-XeS6KII) also recognized an immune-specific polypeptide with a molecular mass of ~53 kDa. The immunoprecipitation of this polypeptide was blocked by recombinant *Xenopus* S6KII but only partially blocked with recombinant chicken S6KII (Fig. 1A, lanes 11 to 16). In serum-stimulated mouse Swiss 3T3 cells (Fig. 1B), a closely migrating doublet runs at ~88 to 90 kDa, with a less abundant polypeptide at ~85 kDa. Anti-chicken S6KII no. 6 antiserum (α-chS6KII, the anti-chicken S6KII antiserum referred to throughout this report) also immunoprecipitated a ~73-kDa polypeptide from Swiss 3T3 cells which was not observed in chicken cells, and the detection of this protein was also blocked with both recombinant proteins. In serum-stimulated HeLa cells (Fig. 1C), as in Swiss 3T3 cells, the ~85- to 90-kDa polypeptides were specifically immunoprecipitated with both antisera; however, in HeLa cells the ~85-kDa polypeptide was much more abundant than observed in Swiss 3T3 cells. Also, in HeLa cells, α-chS6KII recognized a ~73-kDa polypeptide that was blocked with both recombinant proteins, and the α-XeS6KII antiserum recognized a ~110-kDa polypeptide that was blocked with recombinant *Xenopus* S6KII but only partially blocked by recombinant chicken S6KII. We have prepared three additional α-chS6KII antisera (nos. 2, 3, and 5), all of which specifically immunoprecipitate the ~81- to 92-kDa pp90^{rsk} polypeptides from quiescent and serum-stimulated cells. The immunoprecipitation of these proteins was also specifically blocked with the appropriate recombinant S6KII polypeptides. No other immune-specific polypeptides were recognized by more than one of these polyclonal antibodies. In addition, only the ~81- to 92-kDa pp90^{rsk} polypeptides were detected in cells of all three species used in this study. The additional immune-specific polypeptides may represent related proteins or proteins with related antigenic sequences recognized by specific antisera (for example, protein kinases with kinase domains related to one of the two distinct kinase domains found in pp90^{rsk}). It is unlikely that they represent part of a pp90^{rsk} protein complex, since cell lysates were heated to 85°C for 5 min in 1% SDS before immunoprecipitation of 10-fold-diluted lysates. Finally, none of the four polyclonal antisera prepared against recombinant chicken S6KII recognizes autophosphorylated pp70-S6K purified from serum-stimulated CEF or developing chicken embryos (data not shown).

Serum-stimulated phosphorylation of pp90^{rsk}. The change in the electrophoretic mobility of pp90^{rsk} (to slower-migrating species) following growth factor addition is consistent with protein phosphorylation. To demonstrate that growth-modulated phosphorylation of pp90^{rsk} in cultured cells does occur, serum-depleted, quiescent cells were labeled with $H_3{}^{32}PO_4$, and then serum was added for 5 min (Swiss 3T3) or 10 min (CEF and HeLa). Phosphorylation of the ~85- to 92-kDa polypeptides, immunoprecipitated with either α-chS6KII (Fig. 2A) or α-XeS6KII (Fig. 2B), in-

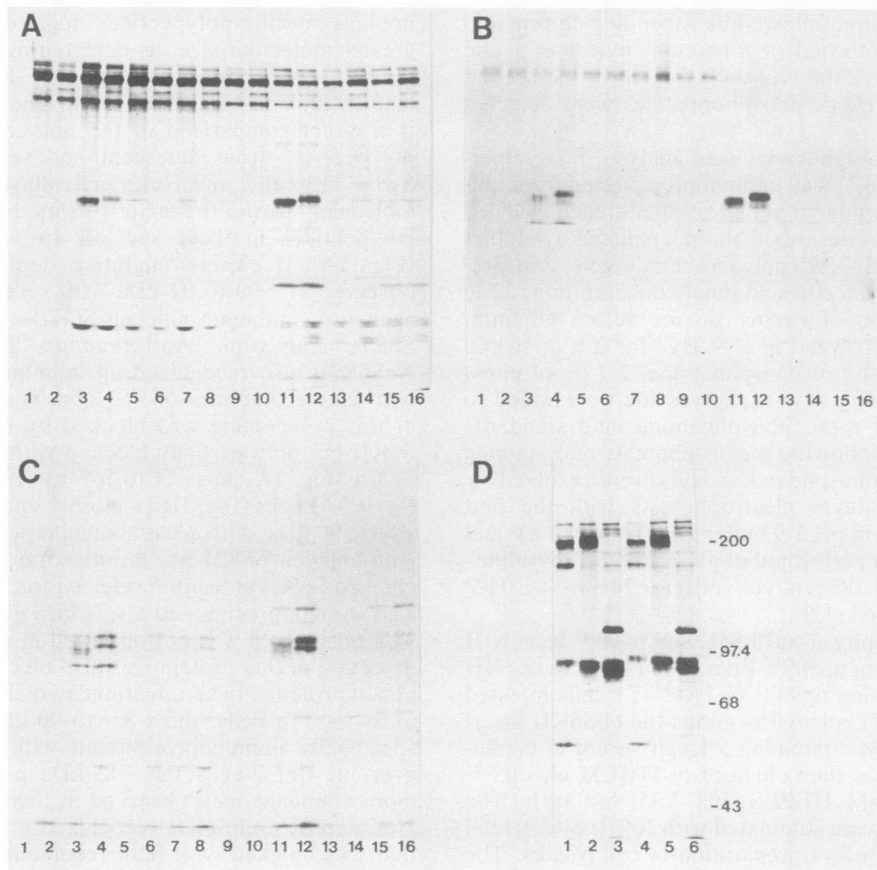

FIG. 1. Identification of pp90^{rsk} in CEF, Swiss 3T3 cells, and HeLa cells. Confluent monolayers of CEF (A), Swiss 3T3 cells (B), and HeLa cells (C) were cultured in 5 ml of DMEM plus 0.5% calf serum for 24 h, followed by labeling with [^{35}S]methionine (1 mCi per plate for CEF, 2 mCi per plate for Swiss 3T3 cells, and 0.5 mCi per plate for HeLa cells) in methionine- and serum-free DMEM for 6 h. Cell lysates were then prepared from quiescent cells (odd-numbered lanes) and cells stimulated with 10% dialyzed fetal calf serum (even-numbered lanes) for 5 min (Swiss 3T3 cells) or 10 min (CEF and HeLa cells). Immunoprecipitations were performed with polyclonal antiserum raised against recombinant chicken S6KII (α-chS6KII; panels A through C, lanes 3 through 8) or *Xenopus* S6KII (α-XeS6KII; panels A through C, lanes 11 through 16). Lanes 1, 2, 9, and 10 represent immunoprecipitations with the appropriate preimmune sera. For immunoprecipitation-blocking experiments, the antisera were preincubated with 1 μg of gel-purified recombinant chicken S6KII (lanes 5, 6, 15, and 16) or *Xenopus* S6KII (lanes 7, 8, 13, and 14). Panel D shows direct size comparisons (electrophoretic mobility in SDS-polyacrylamide gels) of pp90^{rsk} immunoprecipitated with α-XeS6KII antiserum from quiescent (lanes 1 to 3) and serum-stimulated (lanes 4 to 6) CEF (lanes 1 and 4), Swiss 3T3 cells (lanes 2 and 5), and HeLa cells (lanes 3 and 6). The migration of molecular weight standards (in thousands) is indicated.

creased severalfold following serum addition. In the experiment shown in Fig. 2C, pp90^{rsk} was immunoprecipitated with α-XeS6KII from HeLa cells labeled with [^{35}S]methionine or $H_3{}^{32}PO_4$ and run together on an SDS-polyacrylamide gel. As shown, the [^{35}S]methionine-labeled pp90^{rsk} and ^{32}P-labeled pp90^{rsk} phosphoproteins comigrated, supporting their identity. No other immune-specific phosphoproteins were immunoprecipitated by these antisera.

Multiple phosphorylations of pp90^{rsk} in cultured cells occurred in response to serum. Phosphoamino acid analysis of pp90^{rsk} demonstrated that the serum-stimulated increase in phosphorylation occurred at both serine and threonine residues (Fig. 3). The two-dimensional tryptic phosphopeptide maps (Fig. 4) obtained from chicken, mouse, and human pp90^{rsk} are closely related, consistent with the high degree of homology predicted by protein sequence information from chicken and mouse cDNA (1). A composite summary of the phosphopeptide maps is illustrated in Fig. 4G. Addition of serum to quiescent cells resulted in a decrease in the levels of some phosphopeptides (e.g., phosphopeptide 4; this decrease may also be due to increased phosphorylation at other sites within the same phosphopeptide which result in a change in mobility) and dramatic increases in several phosphopeptides (phosphopeptides 5 to 9), whereas little change was observed in others (phosphopeptides 1 to 3). We have analyzed mixes from quiescent and serum-stimulated pp90^{rsk} tryptic phosphopeptides and found the migration of the similarly numbered phosphopeptides (1 to 3) to be identical within each animal species analyzed.

Stimulation of pp90^{rsk} immune-complex kinase activity. An S6KII immune-complex protein kinase assay was used to specifically study the regulation of pp90^{rsk} activity (see reference 18 and Materials and Methods). Addition of serum to quiescent CEF, Swiss 3T3 cells, or HeLa cells resulted in a 10- to 50-fold increase in pp90^{rsk} S6 phosphotransferase activity. Furthermore, as shown in Fig. 5A through C, immunoprecipitation of pp90^{rsk} activity was blocked by preincubation of α-chS6KII with recombinant chicken S6KII (CEF, Swiss 3T3 cells, and HeLa cells [lanes 3 and 4]) or *Xenopus* S6KII (CEF and Swiss 3T3 cells [lanes 5 and 6]). The partial blocking of HeLa pp90^{rsk} immune-complex activity by recombinant *Xenopus* S6KII (Fig. 5C, lanes 5 and

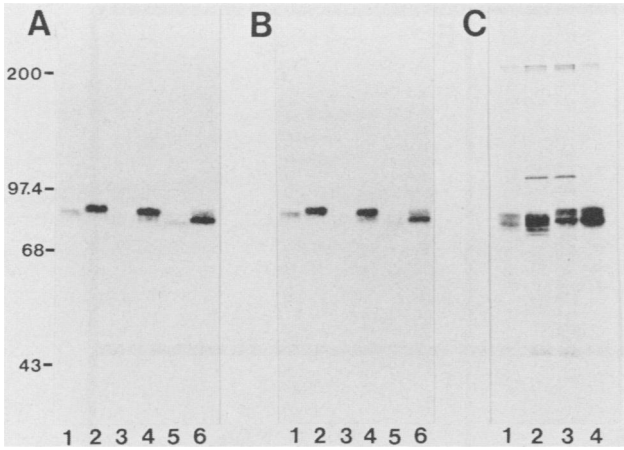

FIG. 2. Serum-stimulated phosphorylation of pp90^{rsk} in CEF, Swiss 3T3 cells, and HeLa cells. Immunoprecipitation of biosynthetically $^{32}P_i$-labeled pp90^{rsk} was performed with α-chS6KII antiserum (A) and α-XeS6KII antiserum (B) from CEF (lanes 1 and 2), Swiss 3T3 cells (lanes 3 and 4), and HeLa cells (lanes 5 and 6). Briefly, confluent cell monolayers were cultured in DMEM containing 0.5% calf serum for 24 h and then in 4 ml of serum- and phosphate-free DMEM for 1 h, followed by the addition of inorganic $^{32}P_i$ for an additional 2 h (1 mCi per plate for CEF, 2 mCi per plate for Swiss 3T3 cells, and 0.5 mCi per plate for HeLa cells). Cell lysates were then prepared from quiescent cells (odd-numbered lanes) and cells stimulated with 10% dialyzed fetal calf serum (even-numbered lanes) for 10 min (CEF and HeLa cells) or 5 min (Swiss 3T3 cells). Immunoprecipitations were then completed as described in Materials and Methods. The migration of molecular weight standards (in thousands) is indicated in panel A. Panel C shows a direct comparison of [^{35}S]methionine- and $^{32}P_i$-labeled pp90^{rsk} immunoprecipitated from HeLa cells with α-XeS6KII antiserum and analyzed by SDS-polyacrylamide gel electrophoresis. HeLa cells were cultured and labeled with [^{35}S]methionine (lanes 2 and 3) or $^{32}P_i$ (lanes 1 and 4) as described above and in the legend to Fig. 1. Lanes: 1 and 2, pp90^{rsk} from quiescent cells; 3 and 4, pp90^{rsk} from cells incubated for 10 min with serum.

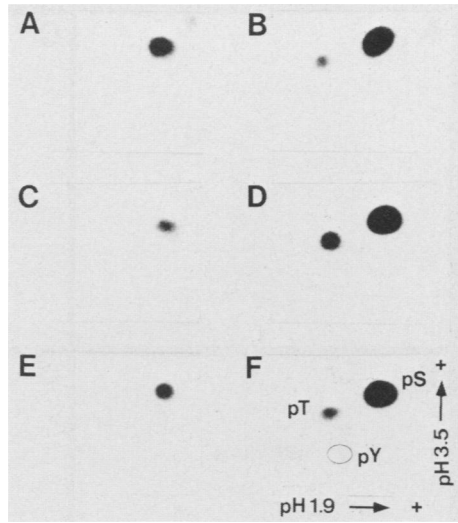

FIG. 3. Phosphoamino acid analysis of pp90^{rsk} from quiescent and serum-stimulated CEF, Swiss 3T3 cells, and HeLa cells. Biosynthetically ^{32}P-labeled pp90^{rsk} was isolated by immunoprecipitation with α-XeS6KII antiserum from quiescent (A, C, and E) or serum-stimulated (B, D, and F) cells (CEF [A and B], Swiss 3T3 cells [C and D], and HeLa cells [E and F]) and further processed for phosphoamino acid analysis as described in Materials and Methods. The directions of electrophoresis are indicated in panel F. pS, Phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

6) was consistent with the partial blocking of [^{35}S]methionine- or ^{32}P-labeled HeLa pp90^{rsk} immunoprecipitated by α-chS6KII (Fig. 1C, lanes 7 and 8, and data not shown). Finally, in addition to α-chS6KII, growth-regulated immune-complex pp90^{rsk} activity was detected with all other antisera prepared against chicken or *Xenopus* recombinant S6KII.

In addition to S6 kinase activity, we were able to detect pp90^{rsk} autophosphorylating activity in the immune complex. As shown in Fig. 5D with quiescent and serum-stimulated Swiss 3T3 cells (lanes 1 and 2), this activity was also growth factor dependent. We have consistently observed that a majority of the autophosphorylated pp90^{rsk} comigrates on SDS-polyacrylamide gels with the 90-kDa polypeptide of the in vivo ^{32}P-labeled 88- and 90-kDa doublet from serum-stimulate Swiss 3T3 cells (Fig. 5D; compare lanes 2 and 3). This result provides preliminary support for the premise that the most-highly phosphorylated pp90^{rsk} polypeptide (hyperphosphorylated pp90^{rsk}) is the active S6 kinase and autophosphorylating activity. Additional experiments to address this possibility are in progress.

pp90^{rsk} and pp70-S6 kinase are distinct growth factor-regulated protein-serine/threonine kinases. We have established that *Xenopus* S6KII homologs indeed exist in somatic cells as growth factor-activated protein kinases (pp90^{rsk}). However, in contrast to the S6 kinase purified from *Xenopus* eggs (S6KII), the only growth-regulated S6 kinase activity identified to date in cultured animal cells is that of pp70-S6 kinase. To further address the question of the relationship of these enzymes, we overexpressed pp90^{rsk} in NIH and Swiss 3T3 cells. In Fig. 6 are the results obtained with a cloned NIH 3T3 pp90^{rsk} overexpressor cell line; the results with Swiss 3T3 pp90^{rsk} overexpressors were the same. As shown, serum-stimulated (10 min) pp90^{rsk} activity in the pp90^{rsk} overexpressor cell line (as measured by the immune-complex assay) was increased severalfold relative to that in serum-stimulated 3T3 cells infected with the control retroviral vector (Fig. 6B; compare lanes 4 and 2). However, the parallel measurement of S6 kinase activity by the cell lysate assay revealed relatively little change in total S6 phosphotransferase activity (Fig. 6A; compare lanes 2 and 4). In a similar experiment (5-min serum stimulation), DEAE chromatography (Fig. 7) demonstrated that pp90^{rsk} and pp70-S6K are biochemically distinct and furthermore showed that in the overexpressor cell line (Fig. 7B), pp90^{rsk} can contribute greater than 70% of the total S6 phosphotransferase activity measured under our assay conditions. However, supporting the results shown in Fig. 6, no significant difference in S6 kinase activity eluting at 100 to 180 mM NaCl (pp70-S6K) was detected. Interestingly, two peaks of pp90^{rsk} activity were detected by the immune-complex assay and appeared to be overproduced, differentially modified products of the single introduced gene (compare Fig. 7A and B). Thus, even in cells that overexpress pp90^{rsk}, changes in cell lysate S6 phosphotransferase activity appear to reflect changes in pp70-S6 kinase, not pp90^{rsk}, activity. Finally, phosphopeptides generated by partial V8 proteolysis of autophosphorylated pp90^{rsk} and pp70-S6K and separated by polyacrylamide gel electrophoresis are clearly unrelated (Fig. 8). These results further demonstrate that pp70-S6K is not a proteolytic product of pp90^{rsk} and together provide substantial evidence that these growth-regulated protein-Ser/Thr kinases are biochemically distinct.

pp90^{rsk} and pp70-S6K exhibit distinct activation and inac-

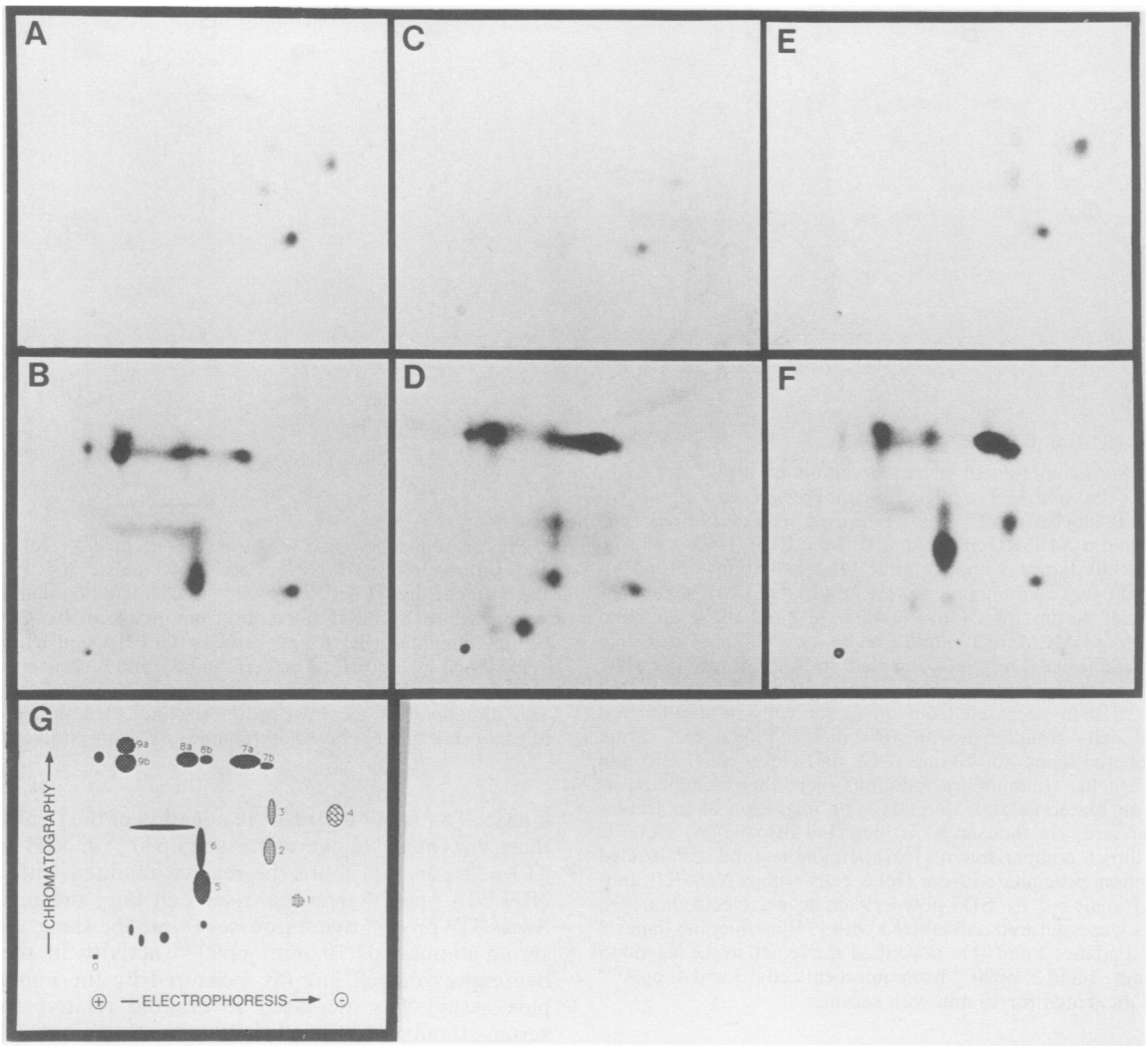

FIG. 4. Tryptic peptide mapping of biosynthetically ^{32}P-labeled pp90^{rsk} from CEF, Swiss 3T3 cells, and HeLa cells. pp90^{rsk} was isolated by immunoprecipitation with α-XeS6KII antiserum from cells biosynthetically labeled with [^{32}P]orthophosphate and further processed for extensive tryptic digestion. (A and B) CEF; (C and D) Swiss 3T3 cells; (E and F) HeLa cells. Panels A, C, and E were from quiescent cells, and panels B, D, and F were from serum-stimulated cells. The positions of the major phosphopeptides are numbered and indicated in panel G. The position of the origin (o) and the directions of first-dimensional electrophoresis and second-dimensional chromatography are also indicated in panel G.

tivation kinetics. pp90^{rsk} was activated within seconds of serum addition to quiescent CEF and Swiss 3T3 and HeLa cells (Fig. 9A through C). In Swiss 3T3 cells, a fourfold activation of pp90^{rsk} activity was measured by 15 s, reaching maximal activity (45-fold) by 2 min (Fig. 9B, panel a). By 10 min, the activity was already beginning to decline, whereas pp70-S6K activity (Figure 9B, panel b) was just reaching maximal activation (5.4-fold). Similar results were obtained with CEF (Fig. 9A). Interestingly, in HeLa cells pp70-S6K activity (Fig. 9C, panel b) was not significantly stimulated (generally less than 1.5-fold) by serum after addition to serum-depleted (24 to 48 h) confluent cells, whereas pp90^{rsk} activity (Figure 9C, panel a) was maximally activated (8.3-fold) by 5 to 10 min.

In addition to examining the initial rate of activation, a longer kinetic analysis was performed to compare the kinetics of inactivation and determine how long into G_1 these enzymes remained activated. Immune-complex pp90^{rsk} activity was maximal at 5 to 10 min after serum addition to quiescent CEF, Swiss 3T3 cells, or HeLa cells but had nearly returned to basal levels by 4 h (Fig. 10). At 30 min to 2 h after serum addition, the time previously used to provide a source of growth factor-stimulated cells for purification of pp70-S6K (7, 21), less than 50% of the maximal pp90^{rsk} activity was measured. This may account in part for the reason why pp90^{rsk} activity has not been previously identified following chromatography of lysates from stimulated cells or is only seen as a minor peak of S6 kinase activity eluting at approximately 100 mM NaCl during anion-exchange Mono Q chromatography (4). In contrast to pp90^{rsk} activity, pp70-S6K activity was nearly maximal in CEF and Swiss 3T3 cells from 10 min to 2 h and was greater than 50% of the maximal level at 4 h following serum addition (Fig. 10).

Regulation of pp90^{rsk} and pp70-S6 protein kinase by protein phosphorylation. Previous studies have shown that serum-stimulated pp70-S6K is phosphorylated on serine and threonine residues and that the enzyme can be inactivated by

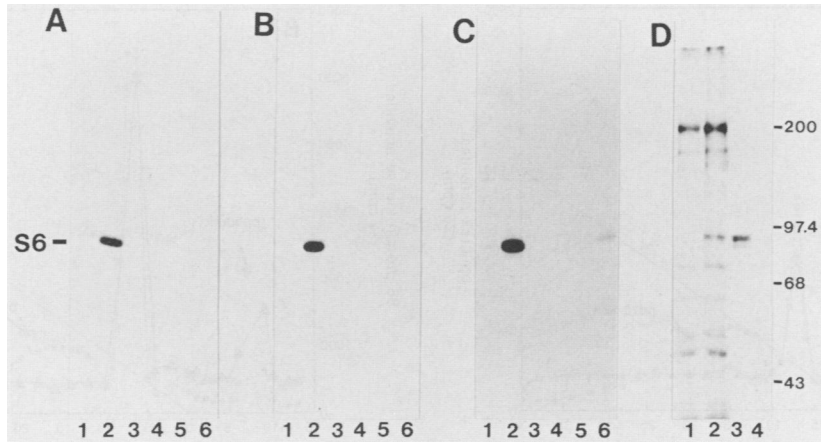

FIG. 5. Serum stimulation of pp90^{rsk} immunocomplex kinase activity and its specific blocking with recombinant chicken and *Xenopus* S6KII. Confluent CEF (A), Swiss 3T3 cells (B), and HeLa cells (C) were cultured in DMEM containing 0.5% serum for 48 h. Cell lysates were then prepared from these quiescent cells with or without incubation in 10% fetal calf serum before cell lysate preparation (odd-numbered lanes, quiescent cells; even-numbered lanes, serum-stimulated cells) (incubation, 10 min for CEF and HeLa cells and 5 min for Swiss 3T3 cells). Lysates were then assayed for pp90^{rsk} immunocomplex protein kinase activity with α-chS6KII antiserum as described in Materials and Methods (lanes 1 and 2). For immunocomplex kinase-blocking experiments, the antiserum was preincubated with 2 μg of recombinant chicken S6KII (lanes 3 and 4) or 5 μg of recombinant *Xenopus* S6KII (lanes 5 and 6). Panel D illustrates results obtained when comparing the electrophoretic mobility of pp90^{rsk} labeled in vitro in an immunocomplex autophosphorylating reaction (lanes 1 and 2) with that of biosynthetically ^{32}P-labeled pp90^{rsk} immunoprecipitated from Swiss 3T3 cells (lanes 3 and 4). pp90^{rsk} autophosphorylation and immunoprecipitation of ^{32}P-labeled pp90^{rsk} were performed as described in Materials and Methods and the legend to Fig. 2. Lanes: 1 and 4, results obtained from quiescent cells; 2 and 3, results obtained from cells incubated with fetal calf serum for 5 min.

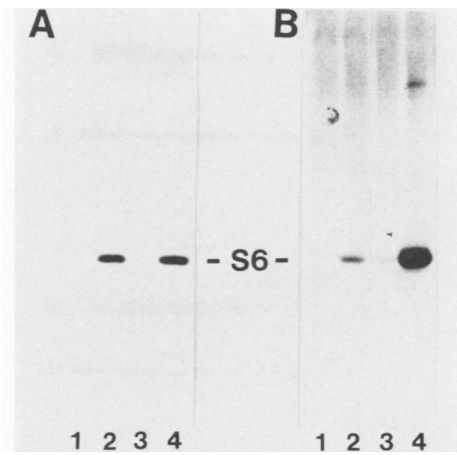

FIG. 6. Characterization of pp70-S6K activity and pp90^{rsk} immunocomplex activity in cells that overexpress pp90^{rsk}. NIH 3T3 cells were infected with pMV7-chS6KII recombinant retrovirus and G418-resistant clones selected as described in Materials and Methods. Resistant clones overexpressing pp90^{rsk} were identified by Northern analysis and immunoprecipitation of [^{35}S]methionine-labeled pp90^{rsk}. One such clone was used in this experiment. Cell lysates were prepared from quiescent (lane 1) and serum-stimulated (lane 2; stimulation with 10% [vol/vol] serum for 10 min) NIH 3T3 cells infected with a control pMV7 retrovirus not containing the pp90^{rsk} sequence and from quiescent (lane 3) and serum-stimulated (lane 4; stimulation with 10% [vol/vol] serum for 10 min) NIH 3T3 cells that overexpressed pp90^{rsk}. Assays for pp70-S6 protein kinase activity (A) and pp90^{rsk} immunocomplex protein kinase activity (B) were performed as described in Materials and Methods. The in vitro incorporation of ^{32}P into 40S ribosomal protein S6 was as follows: (A) 60 (lane 1), 474 (lane 2), 41 (lane 3), and 610 cpm (lane 4); (B) 93 (lane 1), 504 (lane 2), 169 (lane 3), and 6,250 cpm (lane 4).

protein phosphatase 2A (2, 3). *Xenopus* egg S6KII can be inactivated with protein phosphatase 1 or 2A (39). In this report, we have demonstrated that pp90^{rsk} is phosphorylated at multiple sites on serine and threonine residues following the addition of serum growth factors to quiescent cells (Fig. 2 to 4), suggesting the involvement of protein phosphorylation in its regulation. The somatic-cell pp90^{rsk}, partially purified from Swiss 3T3 cells (as described in Materials and Methods) or isolated in the immune complex, can be inactivated by treatment with potato acid phosphatase (Fig. 11). Potato acid phosphatase also inactivated pp70-S6K. In addition, the autophosphorylating activity of pp90^{rsk} was abolished by potato acid phosphatase (Fig. 11B, insert).

DISCUSSION

In this report we demonstrate with antiserum prepared against recombinant *Xenopus* or chicken S6KII that: (i) multiple species and/or multiple phosphorylated forms of immunologically related S6KII polypeptides (pp90^{rsk}) exist in CEF, Swiss 3T3 cells, and HeLa cells (Fig. 1 and 2); (ii) increased phosphorylation of pp90^{rsk} is observed following the addition of serum growth factors to quiescent cells (Fig. 2); (iii) serine and threonine phosphorylation occur at multiple sites (Fig. 3 and 4); (iv) pp90^{rsk} phosphorylation appears to regulate enzyme activity, as S6 phosphotransferase and autophosphorylating activities are greatly reduced following treatment with potato acid phosphatase (Fig. 11); (v) the activation and inactivation kinetics (regulation) of pp90^{rsk} and pp70-S6K are clearly distinct (Fig. 9 and 10); and (vi) analysis of the chromatographic behavior of pp90^{rsk} and pp70-S6K, phosphopeptides of the autophosphorylated enzymes, and regulation of pp90^{rsk} and pp70-S6K activities in 3T3 pp90^{rsk} overexpressors and in HeLa cells further supports the notion that these growth-regulated enzymes are distinct and also suggests that pp90^{rsk} phosphotransferase activity is not readily detectable in the direct cell lysate S6 phosphotransferase assays used (Fig. 6, 7C, 8, and 9).

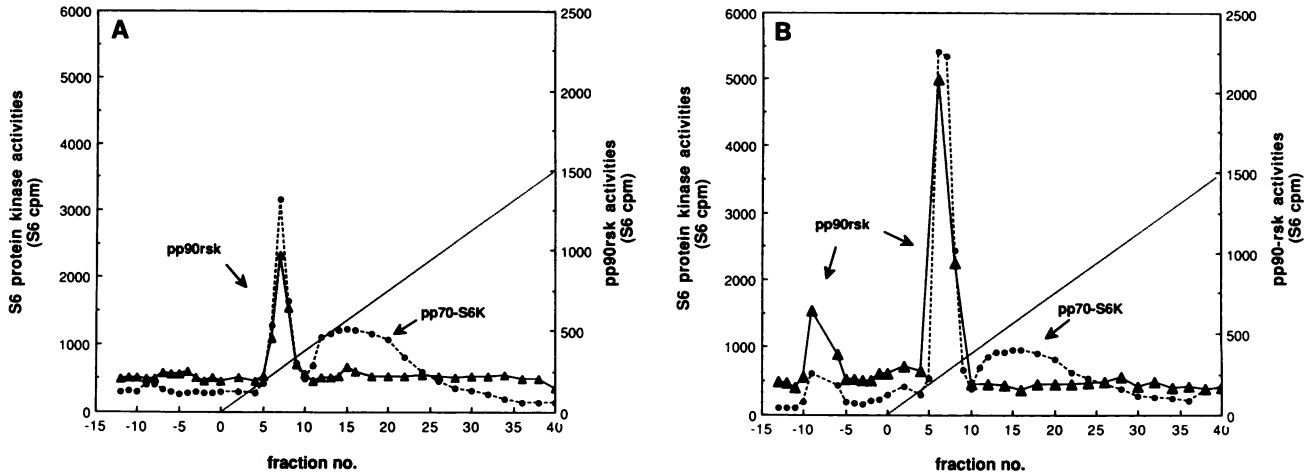

FIG. 7. Analysis of pp70-S6K and pp90^{rsk} activities in NIH 3T3 pp90^{rsk} overexpressors by DEAE chromatography. Quiescent NIH 3T3 cells infected with the control pMV7 retrovirus (A) or NIH 3T3 cells overexpressing pp90^{rsk} (B) were stimulated with 10% (vol/vol) serum for 5 min. Cell lysates were then prepared and fractionated by DEAE chromatography. S6 phosphotransferase activity and pp90^{rsk} activity were measured by the direct cell lysate assay and immune-complex protein kinase assay, respectively. The flowthrough and wash fractions were collected before the start of the gradient and are designated as negative numbers. The gradient (0 to 300 mM NaCl) is also indicated (· · · · · ·).

Indeed, pp90^{rsk} appears to be one of the most rapidly activated nonreceptor, growth factor-regulated protein kinases described to date. It remains to be determined whether the somatic-cell pp90^{rsk} is also activated during the G_2-to-M transition in the cell cycle, as is observed for S6KII during the meiotic maturation of *Xenopus* oocytes. Regulation of pp90^{rsk} activity following addition of serum exhibits a biphasic nature with a rapidly stimulated, transient early peak (0 to 10 min) followed by a less pronounced, more slowly inactivated second peak (Fig. 10). The molecular basis for this differential regulation is at present not known. Serum-stimulated pp70-S6K activity exhibits a monophasic activation curve. This latter observation is different from that described by Susa et al. (40) for serum-stimulated S6 kinase activity. However, we have observed multiple activation peaks for pp90^{rsk} and pp70-S6K with purified growth factors (unpublished data). It has been proposed that two kinetically distinct peaks of S6 kinase activity are regulated separately by protein kinase C-independent and -dependent pathways (40). An alternative explanation for the two peaks (as mea-

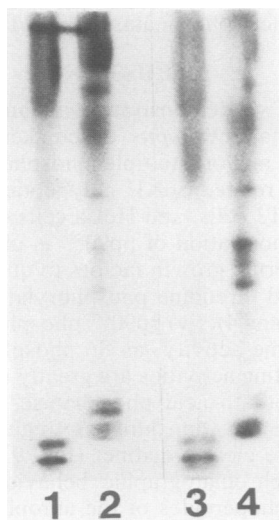

FIG. 8. Analysis of V8 phosphopeptides of autophosphorylated pp70-S6K and pp90^{rsk} by limited proteolysis. Purified chicken pp70-S6K (lanes 1 and 3) and pp90^{rsk} immunoprecipitated from serum-stimulated (5 min) CEF (lanes 2 and 4) were autophosphorylated in vitro and phosphopeptides were generated with 5 ng (lanes 1 and 2) or 50 ng (lanes 3 and 4) of *S. aureus* V8 protease.

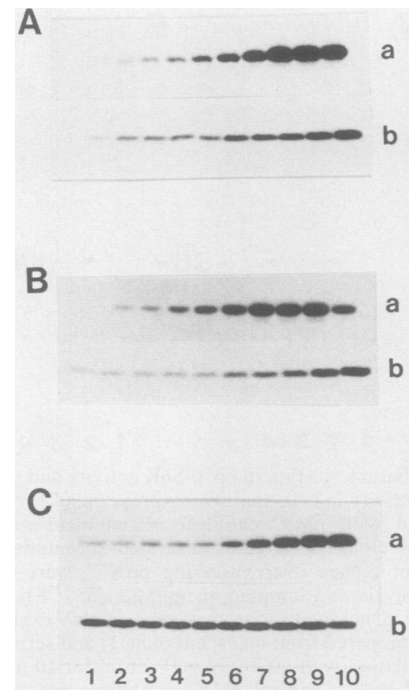

FIG. 9. Rapid-activation kinetics of pp90^{rsk} immunocomplex kinase and pp70-S6 kinase activities in CEF, Swiss 3T3 cells, and HeLa cells. Quiescent cells were stimulated with 10% fetal calf serum for various periods (lanes 1 through 10, no stimulation and 15, 30, 45, 60, 90, 120, 180, 300, and 600 s of stimulation, respectively). Cell lysates were then prepared and assayed for pp90^{rsk} immunocomplex kinase activity (panels a) and pp70-S6 kinase activity (panels b). (A) CEF; (B) Swiss 3T3 cells; (C) HeLa cells.

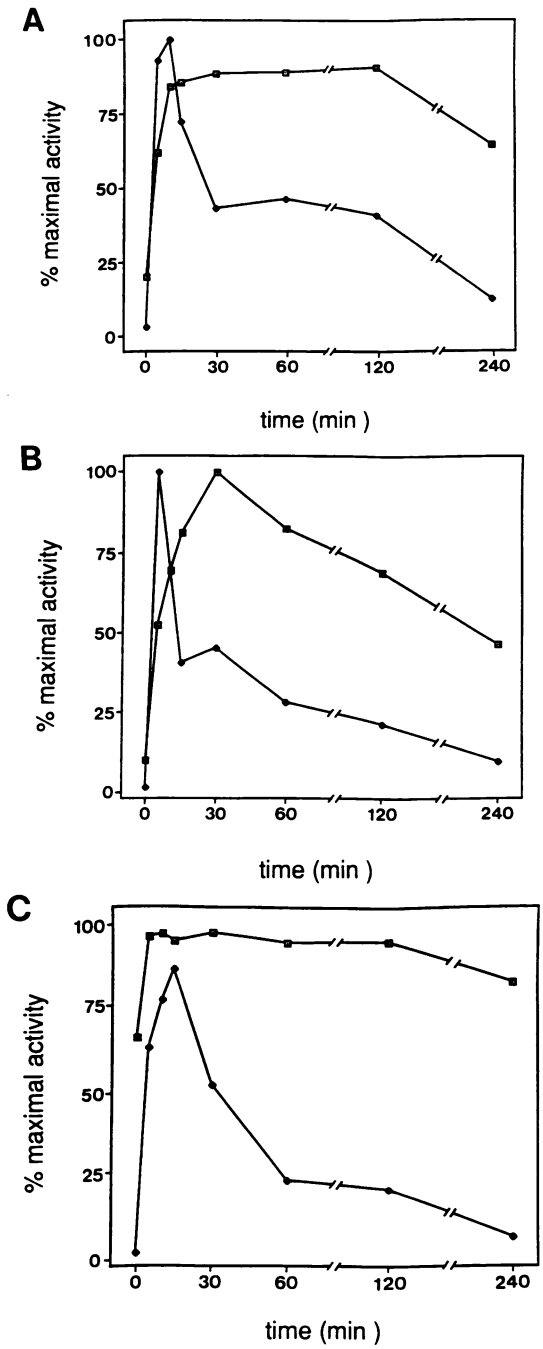

FIG. 10. Physiological inactivation of pp70-S6K and pp90^{rsk} activities in early G_1 after addition of serum to CEF, Swiss 3T3 cells, and HeLa cells. Cell lysates from quiescent cells incubated with 10% fetal calf serum for the indicated times were prepared and assayed for pp70-S6 kinase (□) and pp90^{rsk} immunocomplex kinase (◆) activities. The maximum activity in each time course was designated as 100% activity. The graphs are the averages of the results from two or three separate experiments. (A) CEF (n = 3); (B) Swiss 3T3 cells (n = 3); (C) HeLa cells (n = 2). The maximum fold increases measured in these kinetic studies for pp70-S6K and pp90^{rsk} activities, respectively, were as follows: CEF, 3.8 and 13; Swiss 3T3 cells, 8.5 and 28.6; and HeLa cells, 1.5 and 23.5.

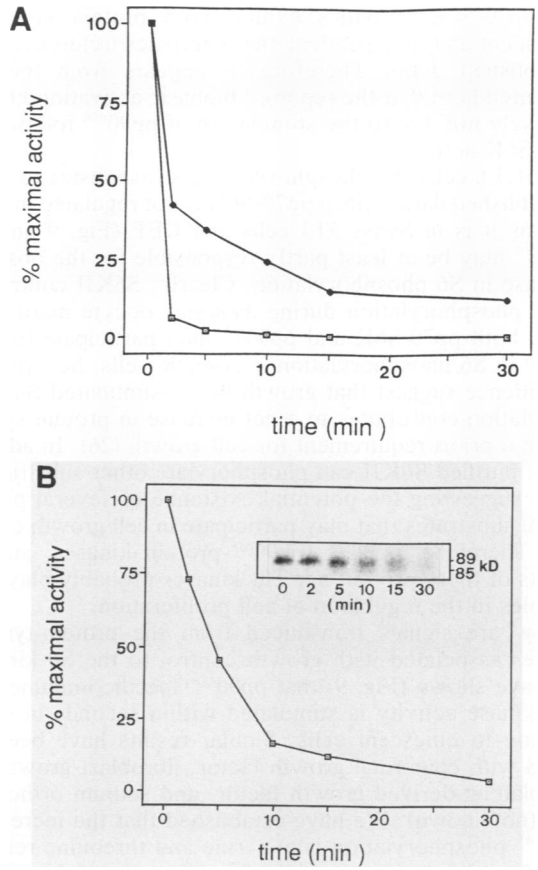

FIG. 11. Inactivation of pp70-S6K and pp90^{rsk} activities by potato acid phosphatase. (A) Kinetics of inactivation of pp70-S6K and pp90^{rsk}. Purified pp70-S6K (□) and partially purified pp90^{rsk} (◆) were incubated with potato acid phosphatase (in this experiment, we used approximately equal S6 phosphotransferase activities) for the indicated times (0, 2, 5, 10, 15, and 30 min). The residual S6 kinase activity following phosphatase treatment was calculated as the percentage of the control kinase activity observed for the same enzyme incubated with heat-inactivated potato acid phosphatase for the same period. Similar results were obtained when the control was incubation of the protein kinases with potato acid phosphatase plus phosphatase inhibitors. (B) Kinetics of inactivation of pp90^{rsk} immunocomplex kinase activity. pp90^{rsk} immunocomplexes prepared from quiescent Swiss 3T3 cells stimulated with 10% fetal calf serum for 5 min were incubated with potato acid phosphatase for the indicated times as described in Materials and Methods. The immunocomplexes were then washed several times and assayed for pp90^{rsk} and autophorphorylating activities. The residual pp90^{rsk} immunocomplex kinase activity following potato acid phosphatase treatment was calculated as the percentage of the control kinase activity observed for the immunocomplex incubated with heat-inactivated potato acid phosphatase. The insert is an autoradiogram showing the kinetics of inactivation of pp90^{rsk} autophosphorylating activity following potato acid phosphatase treatment. The apparent size of pp90^{rsk} is indicated in kilodaltons (kD).

sured by the direct assay), in light of the rapid stimulation of pp90^{rsk} activity described here, is that under the lysate preparation and assay conditions used by these authors, they may be detecting pp90^{rsk} activity and therefore the first peak is due to pp90^{rsk} followed by the slower activation of pp70-S6 kinase activity. However, in addition to demonstrating the small effect that changes in pp90^{rsk} activity have on our direct cell lysate assay (Fig. 6, 7, 9, and 10), we have found that at both early and late stages of activation, pp90^{rsk}

and pp70-S6K activities exhibit both protein kinase C-dependent and -independent characteristics (reference 5 and unpublished data). Therefore, it appears from the data presented here that the reported biphasic activation kinetics are likely not due to the stimulation of pp90^{rsk} followed by pp70-S6K activities.

In HeLa cells, S6 phosphorylation is responsive to serum (unpublished data). Since pp70-S6K is not regulated in HeLa cells as it is in Swiss 3T3 cells and CEF (Fig. 9 and 10), pp90^{rsk} may be at least partly responsible for the observed increase in S6 phosphorylation. Clearly, S6KII contributes to S6 phosphorylation during *Xenopus* oocyte maturation. Thus, both pp70-S6K and pp90^{rsk} may participate to some extent in S6 phosphorylation in somatic cells. Several lines of evidence suggest that growth factor-stimulated S6 phosphorylation contributes to a net increase in protein synthesis, an a priori requirement for cell growth (26). In addition to S6, purified S6KII can phosphorylate other substrates in vitro, suggesting the potential existence of several physiological substrates that may participate in cell growth control (16). Therefore, pp90^{rsk}, pp90^{rsk}-protein kinases, and the targets of these protein-Ser/Thr kinases probably play critical roles in the regulation of cell proliferation.

How are signals transduced from the protein-tyrosine kinases associated with growth control to the S6 kinases? We have shown (Fig. 9) that pp90^{rsk}-specific immune-complex kinase activity is stimulated within seconds of serum addition to quiescent cells. Similar results have been obtained with epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, and sodium orthovanadate (not shown). We have established that the increase in pp90^{rsk} phosphorylation is at serine and threonine residues (Fig. 3) and occurs at multiple sites (Fig. 4) and that pp90^{rsk} can be inactivated by dephosphorylation with potato acid phosphatase (Fig. 11). These results, in addition to different activation and inactivation kinetics (Fig. 9 and 10), suggest the potential existence of distinct, mitogen-stimulated S6 kinase-kinases that may be involved in the regulation of pp70-S6K and pp90^{rsk} activities. It is believed that this protein-Ser/Thr kinase(s) are in turn modulated by protein phosphorylation. Because of the multiple phosphorylation and rapid stimulation of pp90^{rsk} activity, we anticipate that one or more putative pp90^{rsk}-protein kinases will be found to be regulated directly by protein-tyrosine kinases.

On the basis of our findings and the discussion above, there are at least two potential candidates for a pp90^{rsk}-protein kinase that have been previously described. The MAP-2 kinase has been shown to be rapidly activated by insulin in 3T3-L1 cells and phosphorylated on threonine and tyrosine residues and can be inactivated with phosphatase 2A (36). Previous studies have shown that phosphatase 2A-inactivated *Xenopus* S6KII can be partially reactivated by the insulin-stimulated MAP-2 kinase via phosphorylation at threonine residues (39). This would not account, however, for the increased pp90^{rsk} serine phosphorylation following growth factor stimulation and may be responsible for the partial reactivation observed. Another protein-serine/threonine kinase with the potential to be a pp90^{rsk}-protein kinase is the product of the proto-oncogene c-*raf* (Raf-1). Raf-1 is phosphorylated in response to a variety of growth factors or oncogene products. In addition to increased serine and threonine phosphorylation, an increase in tyrosine phosphorylation has been observed in response to platelet-derived growth factor or activation of pp60$^{v\text{-}src}$, the oncogenic protein-tyrosine kinase of Rous sarcoma virus (29, 30). It will be of interest to determine if the regulation of MAP-2 or Raf-1 kinases is coordinated with the activation of pp90^{rsk} that we have described here (Fig. 9 and 10), and if either or both can reactivate dephosphorylated pp90^{rsk}. Independent purification of the putative pp90^{rsk}-protein kinase(s) will be required to resolve questions of its identity.

ACKNOWLEDGMENTS

We express our gratitude to R. L. Erikson, L. J. Sweet, and D. A. Alcorta for kindly providing chicken S6KII cDNA and for discussions of unpublished data and to M. Johnson for advice and vectors necessary to construct the pMV7-chS6KII recombinant retrovirus. We also thank H. Green, M. J. Birnbaum, L. F. Lau, R. Costa, M. Johnson, and L. J. Sweet for helpful comments on the manuscript.

This research was supported by Public Health Service grant RO1-CA46595 from the National Institutes of Health and a grant from the Leukemia Research Foundation.

LITERATURE CITED

1. **Alcorta, D. A., C. M. Crews, L. J. Sweet, L. Bankston, S. W. Jones, and R. L. Erikson.** 1989. Sequence and expression of chicken and mouse *rsk*: homologs of *Xenopus laevis* ribosomal S6 kinase. Mol. Cell. Biol. **9**:3850–3859.
2. **Ballou, L. M., P. Jeno, and G. Thomas.** 1988. Protein phosphatase 2A inactivates the mitogen-stimulated S6 kinase from Swiss mouse 3T3 cells. J. Biol. Chem. **263**:1188–1194.
3. **Ballou, L. M., M. Siegmann, and G. Thomas.** 1988. S6 kinase in quiescent Swiss mouse 3T3 cells is activated by phosphorylation in response to serum treatment. Proc. Natl. Acad. Sci. USA **85**:7154–7158.
4. **Blenis, J., and R. L. Erikson.** 1985. Regulation of a ribosomal protein S6 kinase activity by the Rous sarcoma virus transforming protein, serum or phorbol ester. Proc. Natl. Acad. Sci. USA **82**:7621–7625.
5. **Blenis, J., and R. L. Erikson.** 1986. Stimulation of ribosomal protein S6 kinase activity by pp60$^{v\text{-}src}$ or by serum: dissociation from phorbol ester-stimulated activity. Proc. Natl. Acad. Sci. USA **83**:1733–1737.
6. **Blenis, J., and R. L. Erikson.** 1986. Regulation of protein kinase activities in PC12 pheochromocytoma cells. EMBO J. **5**:3441–3447.
7. **Blenis, J., C. J. Kuo, and R. L. Erikson.** 1987. Identification of a ribosomal protein S6 kinase regulated by transformation and growth promoting stimuli. J. Biol. Chem. **262**:14373–14376.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
9. **Cicirelli, M. F., S. L. Pelech, and E. G. Krebs.** 1988. Activation of multiple protein kinases during the burst in protein phosphorylation that precedes the first meiotic cell division in *Xenopus* oocytes. J. Biol. Chem. **263**:2009–2019.
10. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulphate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
11. **Cobb, M.** 1986. An insulin-stimulated ribosomal S6 protein kinase in 3T3-L1 cells. J. Biol. Chem. **261**:12994–12999.
12. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–405.
13. **Crowl, R., C. Seamans, P. Lomedico, and S. McAndrew.** 1985. Versatile expression vectors for high-level synthesis of cloned gene products in *Escherichia coli*. Gene **38**:31–38.
14. **Erikson, E., and J. L. Maller.** 1985. A protein kinase from *Xenopus* eggs specific for ribosomal protein S6. Proc. Natl. Acad. Sci. USA **82**:742–746.
15. **Erikson, E., and J. L. Maller.** 1986. Purification and characterization of a protein kinase from *Xenopus* eggs highly specific for ribosomal protein S6. J. Biol. Chem. **261**:350–355.
16. **Erikson, E., and J. L. Maller.** 1988. Substrate specificity of ribosomal protein S6 kinase II from Xenopus eggs. Sec. Mess. Phosphoprot. **2**:135–143.
17. **Erikson, E., and J. L. Maller.** 1989. *In vivo* phosphorylation and

activation of ribosomal protein S6 kinases during *Xenopus* oocyte maturation. J. Biol. Chem. **264**:13711–13717.

18. **Erikson, E., J. Stefanovic, J. Blenis, R. L. Erikson, and J. L. Maller.** 1987. Antibodies to *Xenopus* egg S6 kinase II recognize S6 kinase from progesterone- and insulin-stimulated *Xenopus* oocytes and from proliferating chicken embryo fibroblasts. Mol. Cell. Biol. **7**:3147–3155.
19. **Giugni, T. D., K. Chen, and S. Cohen.** 1988. Activation of a cytosolic serine protein kinase by epidermal growth factor. J. Biol. Chem. **263**:18988–18995.
20. **Gould, K. L., and T. Hunter.** 1988. Platelet-derived growth factor induces multisite phosphorylation of pp60$^{c\text{-}src}$ and increases its protein-tyrosine kinase activity. Mol. Cell. Biol. **8**:3345–3356.
21. **Jeno, P., L. M. Ballou, I. Novak-Hofer, and G. Thomas.** 1988. Identification and characterization of a mitogen-activated S6 kinase. Proc. Natl. Acad. Sci. USA **85**:406–410.
22. **Jeno, P., N. Jaggi, H. Luther, M. Siegmann, and G. Thomas.** 1989. Purification and characterization of a 40S ribosomal protein S6 kinase from vanadate-stimulated Swiss 3T3 cells. J. Biol. Chem. **264**:1293–1297.
23. **Jones, S. W., E. Erikson, J. Blenis, J. L. Maller, and R. L. Erikson.** 1988. A *Xenopus* ribosomal protein S6 kinase has two apparent kinase domains that are each similar to distinct protein kinases. Proc. Natl. Acad. Sci. USA **85**:3377–3381.
24. **Karland, J. K., and M. P. Czech.** 1988. Insulin-like growth factor I and insulin rapidly increase casein kinase II activity in Balb/c 3T3 fibroblasts. J. Biol. Chem. **263**:15872–15875.
25. **Kirschmeier, P., J. Housey, M. Johnson, A. Perkins, and I. B. Weinstein.** 1988. Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. DNA **7**:219–225.
26. **Kozma, S. C., S. Ferrari, and G. Thomas.** 1989. Unmasking a growth factor/oncogene-activated S6 phosphorylation cascade. Cell. Signaling **1**:219–226.
27. **Matsuda, Y., and G. Guroff.** 1987. Purification and mechanism of activation of a nerve growth factor-sensitive S6 kinase from PC12 cells. J. Biol. Chem. **262**:2832–2844.
28. **Meijer, L., S. L. Pelech, and E. G. Krebs.** 1987. Differential regulation of histone H1 and ribosomal S6 kinases during sea star oocyte maturation. Biochemistry **26**:7968–7974.
29. **Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF B-receptor. Cell **58**:649–657.
30. **Morrison, D. K., D. R. Kaplan, U. Rapp, and T. M. Roberts.** 1988. Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. USA **85**:8855–8859.
31. **Nemenoff, R. A., D. J. Price, M. J. Mendelsohn, E. A. Carter, and J. Avruch.** 1988. An S6 kinase activated during liver regeneration is related to the insulin-stimulated S6 kinase in H4 hepatoma cells. J. Biol. Chem. **263**:19455–19460.
32. **Novak-Hofer, I., and G. Thomas.** 1984. An activated S6 kinase in extracts from serum- and epidermal growth factor-stimulated Swiss 3T3 cells. J. Biol. Chem. **259**:5995–6000.
33. **Novak-Hofer, I., and G. Thomas.** 1985. Epidermal growth factor-mediated activation of an S6 kinase in Swiss mouse 3T3 cells. J. Biol. Chem. **260**:10314–10319.
34. **Pelech, S. L., B. B. Olwin, and E. G. Krebs.** 1986. Fibroblast growth factor treatment of Swiss 3T3 cells activates a subunit S6 kinase that phosphorylates a synthetic peptide substrate. Proc. Natl. Acad. Sci. USA **83**:5968–5972.
35. **Ray, L. B., and T. W. Sturgill.** 1987. Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 *in vitro*. Proc. Natl. Acad. Sci. USA **84**:1502–1506.
36. **Ray, L. B., and T. W. Sturgill.** 1988. Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine *in vivo*. Proc. Natl. Acad. Sci. USA **85**:3753–3757.
37. **Rowland, E. A., T. H. Muller, M. Goldstein, and L. A. Greene.** 1987. Cell-free detection and characterization of a novel nerve growth factor-activated protein kinase in PC12 cells. J. Biol. Chem. **262**:7504–7513.
38. **Sommercorn, J., J. A. Mulligan, F. L. Lozeman, and E. G. Krebs.** 1987. Activation of casein kinase II in response to insulin and to epidermal growth factor. Proc. Natl. Acad. Sci. USA **84**:8834–8838.
39. **Sturgill, T. W., L. B. Ray, E. Erikson, and J. L. Maller.** 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature (London) **334**:715–718.
40. **Susa, M., A. R. Olivier, D. Fabbro, and G. Thomas.** 1989. EGF induces biphasic S6 kinase activation: late phase is protein kinase C-dependent and contributes to mitogenicity. Cell **57**: 817–824.
41. **Tabarini, D., A. Garcia de Herreros, J. Heinrich, and O. M. Rosen.** 1987. Purification of a bovine liver S6 kinase. Biochem. Biophys. Res. Commun. **144**:891–899.
42. **Tabarini, D., J. Heinrich, and O. M. Rosen.** 1985. Activation of S6 kinase activity in 3T3-L1 cells by insulin and phorbol ester. Proc. Natl. Acad. Sci. USA **82**:4369–4373.