MOLECULAR AND CELLULAR BIOLOGY, Apr. 1991, p. 1861–1867
0270-7306/91/041861-07$02.00/0
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Vol. 11, No. 4

Regulation of pp90^{rsk} Phosphorylation and S6 Phosphotransferase Activity in Swiss 3T3 Cells by Growth Factor-, Phorbol Ester-, and Cyclic AMP-Mediated Signal Transduction

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Somatic cell homologs to the *Xenopus laevis* S6 protein kinases (referred to collectively as pp90^{rsk}) have recently been identified and partially characterized. Here we examine alterations in pp90^{rsk} phosphorylation and S6 phosphotransferase activity in response to regulators of multiple signal transduction systems: purified growth factors, phorbol ester, changes in cyclic AMP (cAMP) levels, and sodium vanadate. All reagents tested increased pp90^{rsk} serine and threonine phosphorylation, but only those agents that regulate cell proliferation and sodium vanadate activated its S6 kinase activity. In addition to the cAMP-stimulated phosphorylation of pp90^{rsk}, a simple correlation between the extent of growth-regulated pp90^{rsk} phosphorylation and S6 phosphotransferase activity was not observed. Quantitative phosphorylation of pp90^{rsk} continued to increase after its S6 kinase activity began its return towards basal levels. However, a close correlation between the appearance and disappearance of a slow-mobility form of phosphorylated pp90^{rsk} (by electrophoresis) and pp90^{rsk} activity was observed. In addition, pp90^{rsk} was regulated by both protein kinase C-independent and -dependent signaling mechanisms. The extent of protein kinase C participation, however, varied depending on which growth factor receptor was activated. Furthermore, growth factor-specific differences in the temporal regulation of pp90^{rsk} S6 phosphotransferase activity were also observed. These results support the notion that the complex regulation of the *rsk* gene product constitutes one of the primary responses of animal cells to mitogenic signals.

How protein-tyrosine kinases and other growth-regulating agents modulate cell proliferation remains largely a mystery. In addition to altered tyrosine phosphorylation, consistent changes in protein-serine/threonine phosphorylation are observed when quiescent cells are induced to enter the cell cycle with growth factors or tumor promoters or following expression of active oncogene products. One of the most consistently observed changes associated with the G_0/G_1 transition is the increased phosphorylation of the 40S ribosomal protein S6 at multiple serine residues. Preceding this measured increase in S6 phosphorylation is the stimulation of protein-serine kinase activity capable of phosphorylating S6 in vitro (for a review, see reference 18). Mitogen-stimulated S6 kinases have been purified from chicken embryo fibroblasts (CEF) and Swiss 3T3 cells and exhibit similar biochemical properties, including an estimated molecular size of 65 to 70 kDa (referred to here as pp70-S6K) (5, 16). Similar enzymes have also been purified from developing chicken embryos and bovine, rat, and rabbit liver (5, 13, 19, 21, 32).

S6 phosphorylation at multiple serine residues is also stimulated during the progesterone- or insulin-induced meiotic maturation of *Xenopus laevis* oocytes to mature eggs (10, 15, 22, 27). Activated *Xenopus* S6 protein kinases have been identified that are immunologically related and possess apparent molecular sizes of 90 to 92 kDa (11). One of these has been cloned and sequenced (17). The cDNAs for chicken and murine homologs have also been identified (1). These genes have been given the acronym *rsk* for ribosomal S6 kinase, and the protein products are referred to as pp90^{rsk}. One of the distinguishing features of the predicted protein sequence is the existence of structural information for two distinct and separate protein kinase domains (1, 17). In addition, greater than 80% homology at the protein level has been predicted between the *X. laevis*, chicken, and murine *rsk* gene products, suggesting a strong evolutionary pressure to maintain this unique structure (1). It remains to be determined whether one or both kinase domains are active and if one or both possess S6 phosphotransferase activity.

Although several laboratories have purified biochemically similar, growth-regulated S6 protein kinases from several somatic cell sources, none had identified pp90^{rsk} as an S6 protein kinase, nor has pp70-S6K been identified as an S6 kinase activated during the maturation of *Xenopus* oocytes. Recently, by using antisera prepared against recombinant chicken and *Xenopus rsk* gene products, somatic cell homologs to *Xenopus* S6 protein kinases have been identified in CEF, Swiss 3T3 fibroblasts, and HeLa cells (8, 31). In quiescent cultured cells, serum stimulates a rapid increase in serine and threonine phosphorylation at multiple sites and a concomitant increase in S6 phosphotransferase activity of pp90^{rsk}. Furthermore, these antisera do not immunoprecipitate pp70-S6 kinase purified from chicken embryos or serum-stimulated CEF (8). One possible explanation of why pp90^{rsk} has not been identified previously as a mitogen-stimulated S6 kinase in cultured cells is that serum-stimulated pp90^{rsk} activity is already waning by the time cell lysates are prepared, as described for purification procedures reported previously (5, 8, 16). In addition, we have found that in serum-stimulated (5 min) NIH 3T3 cells overexpressing pp90^{rsk}, greater than 70% of the total S6 phosphotransferase activity in cytoplasmic extracts eluting from DEAE resins is due to pp90^{rsk}. However, measurement of

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S6 phosphotransferase activity by the direct cell lysate assay does not reflect this difference between control NIH 3T3 cells and cells overexpressing $pp90^{rsk}$ (8). The biochemical nature of this apparent inhibition or masking of $pp90^{rsk}$ activity in cell lysates remains to be determined.

In this report we show that different mitogenic signals originating from a variety of diverse signal transducers converge rapidly, leading to the serine/threonine phosphorylation of $pp90^{rsk}$ and activation of its S6 phosphotransferase activity in Swiss 3T3 fibroblasts. The pp70-S6 protein kinase is also activated by these agents but with slower kinetics. All reagents tested generated an oscillatory regulation of $pp90^{rsk}$ and pp70-S6 phosphotransferase activities. In addition, we observed the contribution of protein kinase C-dependent and -independent signals in the G_0/G_1 regulation of $pp90^{rsk}$ to be mitogen dependent. In contrast, regulation of pp70-S6K activity by all growth factors was in general less dependent upon protein kinase C-mediated signal transduction. The role of protein phosphorylation in the regulation of $pp90^{rsk}$ activity is supported by the observed inactivation of the mitogen-stimulated enzyme with protein phosphatase. However, a simple correlation between the amount of $pp90^{rsk}$ phosphorylation and S6 phosphotransferase activity was not observed, whereas a correlation between the appearance of a slower-mobility form of $pp90^{rsk}$ on sodium dodecyl sulfate (SDS)-polyacrylamide gels and activity was observed. This unique pattern of growth-regulated phosphorylation may be particularly significant in view of the unusual structure (two distinct kinase domains) of $pp90^{rsk}$. These data are consistent with the regulation of $pp90^{rsk}$ by a complex series of converging phosphorylation and dephosphorylation events.

MATERIALS AND METHODS

Cell culture and mitogen stimulation. Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (GIBCO); cells for experiments were grown in DMEM plus 5% calf serum. 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 mitogen addition. The following mitogens were used in these experiments: epidermal growth factor (EGF; 25 ng/ml; IMCERA Products), recombinant human basic fibroblast growth factor (FGF; 10 ng/ml; kindly provided by Bradley B. Olwin, University of Wisconsin, Madison), recombinant human platelet derived-growth factor-β (PDGF; 5 ng/ml; Amgen), phorbol myristate acetate (PMA; 100 ng/ml; Sigma), sodium orthovanadate (500 μM; Fisher), the cyclic AMP (cAMP) analog N^6-2'-*O*-dibutyryladenosine 3',5'-cyclic monophosphate (dibutyryl-cAMP; 0.5 mM in dimethyl sulfoxide [DMSO]; Sigma), and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM in DMSO; Sigma). Mitogens were added for the times indicated in the figure legends prior to preparation of cell lysates.

Assays for S6 protein kinase activity. Cell-free lysates from quiescent cells or cells incubated with various mitogens for the times indicated in the figure legends were prepared and assays for pp70-S6K were completed as described previously (3). Assays for $pp90^{rsk}$ were completed with the immune complex as described before (12).

Biosynthetic cell labeling, immunoprecipitation, and Western immunoblot analysis. Serum-depleted confluent cells were further cultured in 4 ml of serum- and phosphate-free medium per 100-mm culture dish for 1 h, followed by labeling for 2 h with 2 mCi of carrier-free $^{32}P_i$ (ICN) per dish.

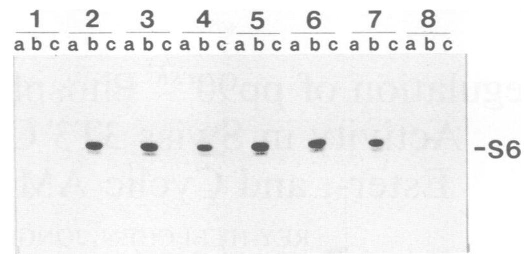

FIG. 1. Activation of $pp90^{rsk}$ activity by various mitogens. Cell lysates were prepared from quiescent Swiss 3T3 cells or cells incubated with various mitogens for 5 min and assayed for $pp90^{rsk}$ activity by the immune complex protein kinase assay. Panel 1, Quiescent cells; panel 2, fetal calf serum (10%, 10.2-fold activation); panel 3, EGF (25 ng/ml, 11.6-fold); panel 4, recombinant human basic FGF (10 ng/ml, 7.2-fold); panel 5, recombinant human PDGF (5 ng/ml, 12.6-fold); panel 6, PMA (100 ng/ml, 11.3-fold); panel 7, sodium orthovanadate (500 μM, 7.0-fold); panel 8, dibutyryl-cAMP (0.5 mM, 1.7-fold). Lanes: a, immunoprecipitations with preimmune serum; b, immunoprecipitations with antiserum raised against recombinant chicken $pp90^{rsk}$; c, immunoprecipitations with antiserum preincubated with 2 μg of recombinant chicken $pp90^{rsk}$. The position of S6 is indicated.

For the experiment shown in Fig. 4, mitogens were added for the last 5 min of this period. For the experiment shown in Fig. 5B, the labeling time for all time points was 2 h, with serum or sodium vanadate added for the indicated times. Cells were then washed with STE (150 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, pH 7.2), scraped into 0.5 ml of lysis buffer, Dounce homogenized, and clarified by centrifugation for 5 min at 10,000 × *g*. 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-Cl [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). One-fourth of the cell lysates from a confluent 100-mm culture dish was used for immunoprecipitation analysis, and the rest was used for phosphoamino acid analysis of immunoprecipitated $pp90^{rsk}$. The lysates were incubated with antiserum overnight at 4°C and then incubated with *Staphylococcus aureus* for another 20 min. The immunocomplexes were then washed twice with RIPA, twice with high-salt buffer, and once with STE (8). The immunoprecipitated proteins were then solubilized by heating at 90°C for 3 min in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, resolved by SDS–7.5% polyacrylamide gel electrophoresis, and visualized by autoradiography.

For Western blot analysis, immunoprecipitated *rsk* polypeptides were transferred to nitrocellulose (Schleicher & Schuell; 0.45 μm pore size), incubated with recombinant *rsk* polyclonal antibody (1:250 dilution), and detected with ^{125}I-labeled protein A (ICN).

RESULTS

Growth factor-stimulated activation of $pp90^{rsk}$ and pp70-S6 kinase activities. We have recently shown that antibodies prepared against recombinant chicken *rsk* S6 protein kinase specifically immunoprecipitate $pp90^{rsk}$ from Swiss 3T3 cells and that S6 phosphotransferase activity can be monitored through the use of an immunocomplex $pp90^{rsk}$ assay (8). Using this assay and a direct cell-free lysate S6 phosphotransferase assay (3), we analyzed the effect of a variety of mitogenic agents on the activation state of both $pp90^{rsk}$ and

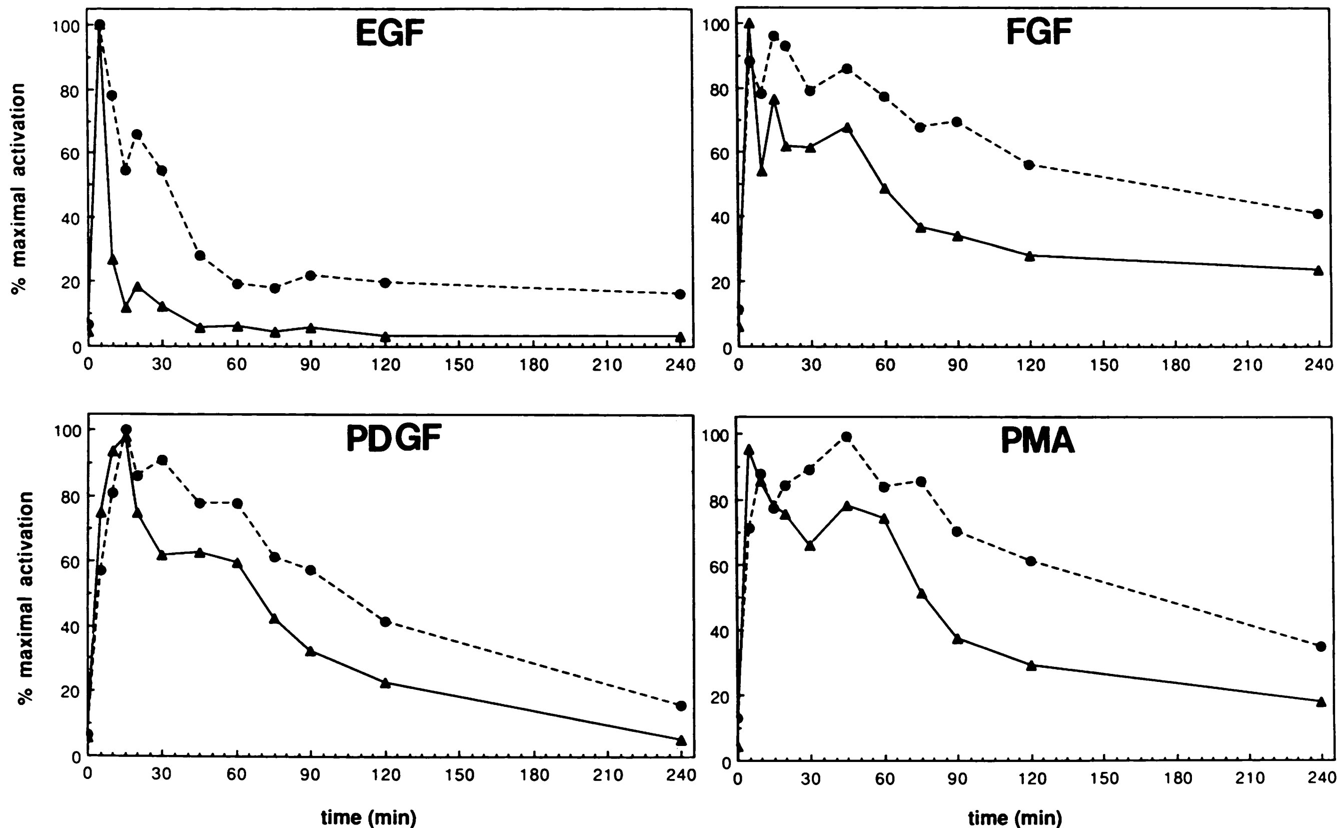

FIG. 2. Activation and inactivation of pp90^{rsk} and pp70-S6K activities during the G_0/G_1 cell cycle transition. Cell lysates were prepared from quiescent Swiss 3T3 cells incubated with various mitogens (EGF, FGF, PDGF, and PMA) for 5, 10, 15, 20, 30, 45, 60, 75, 90, 120, and 240 min and assayed for pp90^{rsk} (▲) and pp70-S6K (●) activities. Maximal activity during this time course is designated as 100%. These values represent the average of two experiments for each mitogen.

pp70-S6 kinase (Fig. 1 and 2). Both enzyme activities were stimulated severalfold within 5 min after addition of serum (10% [vol/vol]), EGF (25 ng/ml), FGF (10 ng/ml), PDGF (5 ng/ml), PMA (100 ng/ml), and sodium orthovanadate (Na_3VO_4, 500 μM). Only a relatively small effect on pp90^{rsk} S6 phosphotransferase activity was seen after intracellular concentrations of cAMP were raised by incubation with 0.5 mM dibutyryl-cAMP and 0.5 mM IBMX; similar results were seen with 0.5 mM 8-bromo-cAMP and 20 μM forskolin. Immunoprecipitation of pp90^{rsk} activity was blocked by preincubation of immune sera with recombinant chicken *rsk* gene product (Fig. 1, lanes c), and immunoprecipitable S6 phosphotransferase activity was not detected with preimmune serum (Fig. 1, lanes a).

Maximal stimulation of pp90^{rsk} immunocomplex activity was observed by 5 to 10 min, and this was followed by a return to basal levels by 2 to 4 h (Fig. 2). Maximal stimulation of pp70-S6K activity lagged just behind that of pp90^{rsk}. Furthermore, pp70-S6 protein kinase remained partially activated after the observed decline in pp90^{rsk} activity. Interestingly, we have consistently observed, with every reagent tested, a reproducible, mitogen-stimulated oscillation of both S6 protein kinase activities. In general, the initial stimulated pulse of pp90^{rsk} activity was the greatest (5 to 10 min), followed by two or three rapidly diminishing additional pulses.

pp90^{rsk} and pp70-S6 kinase are differentially regulated by protein kinase C-dependent and -independent signalling pathways. Our results clearly indicate that activation of protein kinase C leads to the stimulation of pp90^{rsk} activity. To determine whether protein kinase C is required for activation of pp90^{rsk} by growth factors or sodium vanadate, immunocomplex pp90^{rsk} activity was measured in cells depleted of protein kinase C as a result of prolonged treatment with phorbol ester. In these experiments we also wanted to determine whether protein kinase C was required at different stages of the observed oscillating activation process. Recent studies have suggested that regulation of pp70-S6 protein kinase activity in Swiss 3T3 fibroblasts can be divided into two distinct phases; the early phase does not require protein kinase C, whereas the later phase is completely dependent on it (29).

Based on our detailed kinetic analysis of the regulation of both pp90^{rsk} and pp70-S6 protein kinase activities following growth factor addition, we analyzed the regulation of both enzymes at several distinct points of the activation curve in normal Swiss 3T3 cells and PMA-treated cells. In contrast to the recent study mentioned above (29), we have been unable to consistently demonstrate a significant dependence on protein kinase C for pp70-S6K activation at the later portion of the activation curve for all factors tested (Fig. 3), supporting earlier findings (4, 24). However, regulation of pp90^{rsk} activity by PDGF or sodium vanadate did appear to be significantly attenuated at later points of the G_0/G_1 activation process, whereas stimulation of pp90^{rsk} activity by EGF and FGF was not significantly and reproducibly altered by

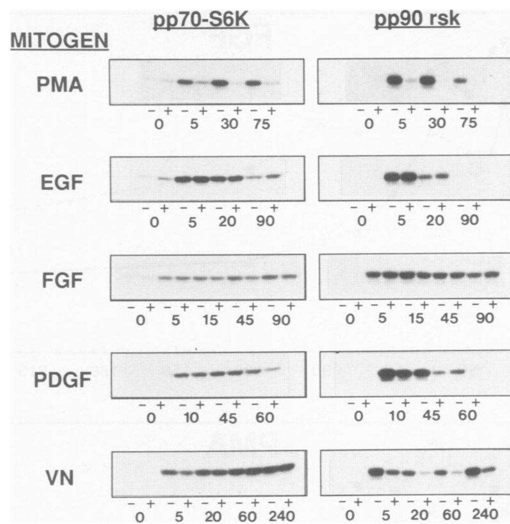

FIG. 3. Regulation of pp90^{rsk} and pp70-S6K activities by protein kinase C-dependent and -independent pathways. Quiescent Swiss 3T3 cells were untreated (−) or incubated with PMA (100 ng/ml) (+) for 20 h prior to the addition of various mitogens for the indicated times (minutes). The pp70-S6K and pp90^{rsk} activities were then measured. VN, Sodium orthovanadate.

protein kinase C downregulation. At present it is difficult to interpret the partial stimulation or attenuation of kinase activity in protein kinase C-downregulated cells after growth factor or vanadate addition; however, it is clear that the agents tested can modulate pp70-S6 kinase and pp90^{rsk} kinase activities throughout the activation process by both protein kinase C-independent and -dependent mechanisms.

Phosphorylation of pp90^{rsk} in Swiss 3T3 cells. The mitogens examined above were further analyzed for their ability to stimulate the phosphorylation of pp90^{rsk} (Fig. 4). Quiescent, ^{32}P-labeled confluent 3T3 monolayers were incubated with each factor for 5 min prior to cell lysis and immunoprecipitation. These results demonstrate a rapid increase in pp90^{rsk} phosphorylation, as indicated by both the altered mobility (higher apparent molecular weight on SDS-polyacrylamide gels, which is consistent with an increase in protein phosphorylation) and the overall increase in incorporation of ^{32}P into pp90^{rsk} following growth factor addition. Phosphoamino acid analysis revealed a large increase in serine phosphorylation, a smaller increase in threonine phosphorylation, and no apparent changes in tyrosine phosphorylation. Like the serum-stimulated enzyme (8), pp90^{rsk} activated in quiescent cells incubated with EGF, FGF, PDGF, PMA, or sodium vanadate (Fig. 1) was inactivated following incubation with potato acid phosphatase (not shown). Since pp90^{rsk} is phosphorylated at multiple serine and threonine sites, as determined by two-dimensional phosphopeptide mapping (8, 11), it remains to be determined whether dephosphorylation of one or more sites is responsible for the inactivation of S6 phosphotransferase activity.

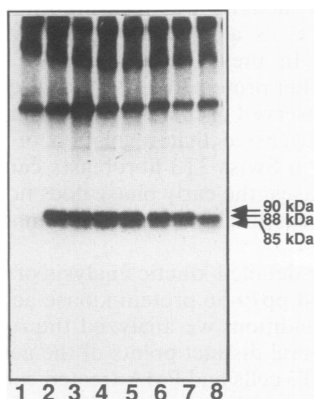

FIG. 4. Phosphorylation of pp90^{rsk} in response to various mitogens. Quiescent Swiss 3T3 cells were labeled with $^{32}P_i$ and incubated with various mitogens as described in the legend to Fig. 1 for 5 min. pp90^{rsk} was then immunoprecipitated from these cells and analyzed by SDS–7.5% polyacrylamide gel electrophoresis. Lane 1, Quiescent cells; lane 2, serum (10%); lane 3, EGF (25 ng/ml); lane 4, FGF (10 ng/ml); lane 5, PDGF (5 ng/ml); lane 6, PMA (100 ng/ml); lane 7, sodium vanadate (500 μM); lane 8, dibutyryl-cAMP and IBMX (0.5 mM each). The molecular masses of pp90^{rsk} are indicated.

An increase in cellular levels of cAMP also stimulated the phosphorylation of pp90^{rsk}. However, the change in mobility was not as great as seen with serum, EGF, FGF, PDGF, PMA, or sodium vanadate. These results suggest that activation of cAMP-dependent protein kinase (PKA) results in phosphorylation of pp90^{rsk} but with an apparent lower stoichiometry and/or at different sites. This phosphorylation does not result in significant activation of pp90^{rsk} S6 phosphotransferase activity (Fig. 1, lane 8b), and this correlates with the inability of cAMP alone to act as a mitogen in these cells.

Differential phosphorylation and modulation of pp90^{rsk} activity. In Swiss 3T3 cells, pp90^{rsk} is rapidly phosphorylated at serine and threonine residues in response to serum (8), a variety of growth factors, and sodium vanadate (Fig. 2). Interestingly, quantitative phosphorylation of immunoprecipitated pp90^{rsk} in response to serum growth factors (Fig. 5B) continued to increase after its S6 phosphotransferase activity began to decline (Fig. 5A). This increase however, occurred in the faster-migrating (on SDS-polyacrylamide gels) species, whereas the slower-migrating phosphoprotein (apparent greater molecular size) disappeared. Thus, the appearance and disappearance of the slower-migrating species correlate with the regulation of pp90^{rsk} S6 kinase activity. This correlation is also evident when examining pp90^{rsk} phosphorylation and S6 phosphotransferase activity following addition of sodium vanadate to quiescent Swiss 3T3 cells. With sodium vanadate, pp90^{rsk} S6 kinase activities at 1 to 4 h after treatment were significantly enhanced (Fig. 5A) compared with the results obtained with growth factors or PMA (Fig. 2). Indeed, sodium vanadate-stimulated pp90^{rsk} exhibited a reproducible activation (0 to 10 min), inactivation (20 min), and reactivation of its S6 phosphotransferase activity in Swiss 3T3 cells. The slower-migrating *rsk* phosphoprotein also appeared (5 min), disappeared (20 min), and reappeared (1 to 4 h) in parallel with pp90^{rsk} S6 kinase activity. During this time, the phosphorylation of the faster-migrating species (~88 kDa) continued to increase, as seen following serum addition. The appearance and disappearance of a slow-mobility form of pp90^{rsk} as detected by Western analysis (Fig. 5C, indicated with arrow) further support the observations with ^{32}P-labeled pp90^{rsk} (Fig. 5B) and regulation of S6 phosphotransferase activity (Fig. 5A). A correlation between S6 kinase activity and hyperphosphorylation has also recently been observed in insect cells coinfected with recombinant baculovirus expressing pp60$^{v\text{-}src}$ and *Xenopus* pp90^{rsk} (33).

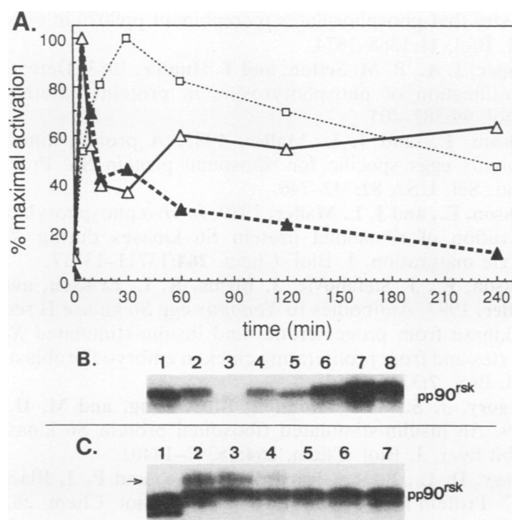

FIG. 5. Correlation between hyperphosphorylation and regulation of pp90^{rsk} S6 phosphotransferase activity. Quiescent Swiss 3T3 cells were incubated with serum or sodium orthovanadate for 5 min to 4 h. (A) Serum-stimulated pp70-S6K (□), serum-stimulated pp90^{rsk} (▲), and vanadate-stimulated pp90^{rsk} (△) activities were measured as described in the text. Maximal activity during these time courses is designated as 100%. (B) The autoradiogram represents biosynthetically ^{32}P-labeled pp90^{rsk} immunoprecipitated from quiescent cells stimulated with serum or vanadate. Lane 1, serum, 2 h; lane 2, serum, 5 min; lane 3, quiescent control; lane 4, vanadate, 5 min; lane 5, vanadate, 20 min; lane 6, vanadate, 1 h; lane 7, vanadate, 2 h; lane 8, vanadate, 4 h. (C) In a separate experiment, quiescent 3T3 cells were incubated with serum for various times, immune complexes were formed, and pp90^{rsk} polypeptides were analyzed by SDS-PAGE followed by transfer to nitrocellulose and Western analysis. Lane 1, Quiescent cells; lanes 2 to 7, serum added for 2, 5, 15, 30, 60, and 120 min, respectively. The arrow indicates the appearance of the slow-mobility form of pp90^{rsk} that correlates with the activation of its S6 phosphotransferase activity.

DISCUSSION

In this study we have examined the regulation of somatic cell pp90^{rsk} by a variety of mitogenic agents. Addition of FGF, EGF, PDGF, PMA, cAMP-elevating reagents, or Na_3VO_4 to quiescent Swiss 3T3 cells results in increased serine and threonine phosphorylation of pp90^{rsk}. All but increases in cAMP levels significantly stimulated pp90^{rsk} S6 phosphotransferase activity as detected in a specific immune-complex protein kinase assay. Although pp90^{rsk} activated by serum, purified growth factors, and phorbol ester is inactivated by protein phosphatase (8; data not shown), a simple increase in phosphorylation alone may not account for the stimulation of pp90^{rsk} S6 kinase activity, as we present evidence for distinct posttranslational pp90^{rsk} phosphorylation events. It appears that phosphorylation of pp90^{rsk} by a coordinately regulated *rsk* kinase (resulting in a slower-migrating pp90^{rsk} phosphoprotein) is required for activation. In the accompanying article (8a), we present evidence for the existence of a distinct, coordinately regulated protein kinase that phosphorylates a recombinant *rsk* gene product in vitro. Its relationship to other potential pp90^{rsk} cofactor-independent protein kinases is discussed.

In addition, our data provide evidence for the existence of a second growth-regulated protein kinase that is responsible for the continued phosphorylation of pp90^{rsk} and perhaps its inactivation. Alternatively, a site-specific, mitogen-regulated protein phosphatase may be activated, and this may account for the partial dephosphorylation and inactivation of pp90^{rsk} activity. Under these conditions, the inactivation of a single putative *rsk* kinase activity might not be necessary for the inactivation of pp90^{rsk}, and it could continue phosphorylating pp90^{rsk} at the sites not involved in regulating S6 phosphotransferase activity. These and other possibilities are currently being examined, as well as the possibility that this second phosphorylation event could play a role in the regulation of the second ATP-binding and catalytic domain of pp90^{rsk}.

Activation could also be mediated by second messenger-regulated protein kinases such as the cAMP-dependent protein kinases and members of the protein kinase C family. Raising cAMP levels within cells results in increased pp90^{rsk} phosphorylation. However, the cAMP-mediated increase in pp90^{rsk} phosphorylation appears to occur to a lower stoichiometry than mitogen-stimulated pp90^{rsk} phosphorylation, as judged by its faster migration on SDS gels (Fig. 4). Furthermore, an increase in cellular cAMP levels does not result in significant activation of pp90^{rsk} activity (Fig. 1). At present, the physiological significance of the cAMP-mediated phosphorylation is unknown. It is also not known whether pp90^{rsk} is directly phosphorylated by cAMP-dependent protein kinase within the cell; however, PKA poorly phosphorylates recombinant *rsk* gene product in vitro (8a). It is worth noting that activated PKA in *Xenopus* oocytes prevents progesterone-induced maturation and S6 phosphorylation, whereas the activation of PKA in the experiments described here does not prevent the mitogen-stimulated activation of pp90^{rsk} (not shown). These results may indicate species differences in the regulation of the *rsk* gene product or differences in the regulation of pp90^{rsk} during meiosis versus mitosis.

Protein kinase C clearly participates in the activation of pp90^{rsk} after addition of PMA to quiescent cells (Fig. 1 to 3). Regulation of pp90^{rsk} activity by FGF and EGF can proceed via protein kinase C-independent mechanisms. Our results indicate that signal transduction by the PDGF receptor and sodium vanadate, with regard to the activation of pp90^{rsk}, exhibits a greater dependence upon an active protein kinase C (Fig. 3). Interestingly, growth factors, phorbol ester, and sodium vanadate generate a reproducible series of recurring bursts (cycling) of both pp90^{rsk} and pp70-S6K activities during the G_0/G_1 transition. The significance of the oscillating pp70-S6K and pp90^{rsk} activities is unclear at this time. A similar observation has been described for the regulation of c-*src* protein-tyrosine kinase activity following the addition of EGF to cultured cells (20). The oscillatory behavior of pp90^{rsk} is also reminiscent of the oscillation of Ca^{2+} fluxes in fibroblasts in response to mitogenic signals (for reviews, see references 2 and 26). Since Ca^{2+} ionophores can activate pp70-S6K (6, 23) and pp90^{rsk} (unpublished) in cultured cells, future studies will examine the ability of Ca^{2+}- and calmodulin-dependent protein kinases to reactivate dephosphorylated pp90^{rsk} in vitro.

Biphasic stimulation of EGF-regulated pp70-S6 kinase activity as measured in cell-free lysates has also been reported (29). The first peak is protein kinase C-independent, whereas stimulation of the second peak of S6 kinase activity is dependent upon protein kinase C and is required for mitogenesis. Sweet et al. (30) have suggested that the first peak is due to pp90^{rsk} and the second to pp70-S6K. As shown in Fig. 2, we observed both protein kinases to exhibit reproducible fluctuations in activity in response to PMA, EGF, FGF, and PDGF. However, we have been unable to

reproducibly demonstrate an absolute requirement for protein kinase C for the primary, secondary, or tertiary stimulations of pp70-S6K activity (Fig. 3). Thus, we are also presently unable to unambiguously determine the requirement or the need for multiple stimulations of pp70-S6 protein kinase activity for mitogenesis, as suggested by Susa et al. (29). Additionally, PMA rapidly activates protein kinase C when added to cultured fibroblasts (14, 25), and we have observed similar kinetics of PMA- and growth factor-stimulated activation of both S6 kinases in Swiss 3T3 cells (Fig. 2), in contrast to the much slower activation of S6 kinase activity by phorbol ester described recently (29). It is possible that these conflicting observations are due to 3T3 clonal cell line differences and/or differences in experimental cell culture techniques. Protein kinase C downregulation does result in partial attenuation of sodium vanadate- and PDGF-activated stimulation of $pp90^{rsk}$ activity, whereas activation of $pp90^{rsk}$ by EGF or FGF results in small changes in activity. Further characterization of these observations is necessary. However, it is clear that different growth factor receptors rapidly generate independently and differentially regulated signals that rapidly converge upon the activation of $pp90^{rsk}$. Future experiments will be aimed at purifying the S6 kinase-protein kinases and identifying additional downstream targets of the pp70-S6 kinase- and $pp90^{rsk}$-mediated signalling processes.

ACKNOWLEDGMENTS

This research was supported by United States Public Health Service research grant CA-46595 from the National Institutes of Health and grant JFRA-257 from the American Cancer Society.

We thank Yang Lu for technical assistance, Bradley B. Olwin (University of Wisconsin, Madison) for his gift of recombinant human basic fibroblast growth factor, and M. J. Birnbaum and S. L. Pelech for helpful comments on the manuscript.

REFERENCES

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* ribosomal S6 kinase. Mol. Cell. Biol. **9**:3850–3859.
2. **Berridge, M. J., and R. F. Irvine.** 1989. Inositol phosphates and cell signalling. Nature (London) **341**:197–205.
3. **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.
4. **Blenis, J., and R. L. Erikson.** 1986. Stimulation of ribosomal protein S6 kinase activity by $pp60^{v-src}$ or by serum: dissociation from phorbol ester-stimulated activity. Proc. Natl. Acad. Sci. USA **83**:1733–1737.
5. **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.
6. **Blenis, J., C. J. Kuo, D. A. Alcorta, and R. L. Erikson.** 1987. Role of second messengers in regulating the growth-associated S6 protein kinase activity, p. 181–192. *In* M. C. Cabot, W. L. McKeehan, and G. Sato, (ed.), Mechanisms of signal transduction by hormones and growth factors. Alan R. Liss, Inc., New York.
7. **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.
8. **Chen, R.-H., and J. Blenis.** 1990. Identification of *Xenopus* S6 protein kinase homologs ($pp90^{rsk}$) in somatic cells: phosphorylation and activation during the initiation of cell proliferation. Mol. Cell. Biol. **10**:3204–3215.

8a. **Chung, J., R.-H. Chen, and J. Blenis.** 1991. Coordinate regulation of $pp90^{rsk}$ and a distinct protein-serine/threonine kinase activity that phosphorylates recombinant $pp90^{rsk}$ in vitro. Mol. Cell. Biol. **11**:1868–1874.

9. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–405.
10. **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.
11. **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.
12. **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.
13. **Gregory, J. S., T. G. Boulton, B.-C. Sang, and M. H. Cobb.** 1989. An insulin-stimulated ribosomal protein S6 kinase from rabbit liver. J. Biol. Chem. **264**:18397–18401.
14. **Halsey, D. L., P. R. Girard, J. F. Kuo, and P. J. Blackshear.** 1987. Protein kinase C in fibroblasts. J. Biol. Chem. **262**:2234–2243.
15. **Hanocq-Quertier, J., and E. Baltus.** 1981. Phosphorylation of ribosomal proteins during maturation in *Xenopus laevis* oocytes. Eur. J. Biochem. **120**:351–355.
16. **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.
17. **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.
18. **Kozma, S. C., S. Ferrari, and G. Thomas.** 1989. Unmasking a growth factor/oncogene-activated S6 phosphorylation cascade. Cell. Signaling **1**:219–226.
19. **Kozma, S. C., H. A. Lane, S. Ferrari, H. Luther, M. Siegmann, and G. Thomas.** 1989. A stimulated S6 kinase from rat liver: identity with the mitogen activated S6 kinase of 3T3 cells. EMBO J. **8**:4125–4132.
20. **Luttrell, D. K., L. M. Luttrell, and S. J. Parsons.** 1988. Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblasts that overexpress $pp60^{c-src}$. Mol. Cell. Biol. **8**:497–501.
21. **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.
22. **Nielsen, P. J., G. Thomas, and J. L. Maller.** 1982. Increased phosphorylation of ribosomal protein S6 during meiotic maturation of *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA **79**:2937–2941.
23. **Novak-Hofer, I., W. Kung, and U. Eppenberger.** 1988. Role of extracellular electrolytes in the activation of ribosomal protein S6 kinase by epidermal growth factor, insulin-like growth factor 1, and insulin in Zr-75-1 cells. J. Cell Biol. **106**:395–401.
24. **Pelech, S. L., and E. G. Krebs.** 1987. Mitogen-activated S6 kinase is stimulated via protein kinase C-dependent and -independent pathways in Swiss 3T3 cells. J. Biol. Chem. **262**:11598–11606.
25. **Pelech, S. L., K. E. Meier, and E. G. Krebs.** 1986. Rapid microassay for protein kinase C translocation in Swiss 3T3 cells. Biochemistry **25**:8348–8353.
26. **Rink, T. J., and T. J. Hallam.** 1989. Calcium signalling in non-excitable cells: notes on oscillations and store refilling. Cell Calcium **10**:385–395.
27. **Stith, B. J., and J. L. Maller.** 1984. The effect of insulin on intracellular pH and ribosomal protein S6 phosphorylation in oocytes of *Xenopus laevis*. Dev. Biol. **102**:79–89.
28. **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.
29. **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.

30. **Sweet, L. J., D. A. Alcorta, and R. L. Erikson.** 1990. Two distinct enzymes contribute to biphasic S6 phosphorylation in serum-stimulated chicken embryo fibroblasts. Mol. Cell. Biol. **10:**2787–2792.
31. **Sweet, L. J., D. A. Alcorta, S. W. Jones, E. Erikson, and R. L. Erikson.** 1990. Identification of mitogen-responsive ribosomal protein S6 kinase pp90^{rsk}, a homolog of *Xenopus* S6 kinase II, in chicken embryo fibroblasts. Mol. Cell. Biol. **10:**2413–2417.
32. **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.
33. **Vik, T. A., L. J. Sweet, and R. L. Erikson.** 1990. Coinfection of insect cells with recombinant baculovirus expressing pp60$^{v\text{-}src}$ results in the activation of a serine-specific protein kinase pp90^{rsk}. Proc. Natl. Acad. Sci. USA **87:**2685–2689.