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Mitogen-activated Swiss mouse 3T3 RSK kinases I and II are related to pp44^{mpk} from sea star oocytes and participate in the regulation of pp90^{rsk} activity

(signal transduction/growth control/tyrosine phosphorylation)

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ABSTRACT **Using a recombinant *rsk* gene product as a substrate for *in vitro* kinase assays, we have identified two mitogen-activated Swiss 3T3 RSK protein kinase activities (referred to as RSK kinase I and RSK kinase II, based on their order of elution from phenyl-Sepharose). Polyclonal antisera prepared against maturation-regulated 44-kDa myelin basic protein (MBP) kinase (pp44^{mpk}) purified from sea star oocytes demonstrated immunocrossreactivity with polypeptides of ≈44 kDa in the RSK kinase I preparation and ≈42 kDa in the RSK kinase II preparation, respectively. These polypeptides were also recognized by anti-phosphotyrosine antibodies, and either phosphatase 1B or 2A (tyrosine- and serine/threonine-specific phosphatases, respectively) separately inactivated RSK phosphotransferase activity supporting the notion that tyrosine and serine/threonine phosphorylation are required for activity. *In vitro*, both RSK kinases and MBP kinase phosphorylated recombinant RSK and generated nearly identical two-dimensional tryptic phosphopeptide maps. They also phosphorylated MBP and microtubule-associated protein 2 but not 40S ribosomal protein S6. Furthermore, these protein kinases phosphorylated and partially activated pp90^{rsk} in immune complexes obtained from quiescent cells.**

Several protein serine/threonine (Ser/Thr) kinases have been implicated in the early signal transduction response initiated by mitogens. Two of these, pp70^{S6K} and pp90^{rsk}, were originally identified by their ability to phosphorylate *in vitro* the 40S ribosomal protein S6 (for review, see refs. 1–3). These enzymes are differentially regulated by protein phosphorylation in somatic cells (4–7) and are encoded by distinct genes (8–11). The kinetics of activation of pp90^{rsk} and pp70^{S6K} are consistent with both enzymes participating in the complete phosphorylation of S6, with pp90^{rsk} activated maximally in the first few minutes after mitogenic challenge and pp70^{S6K} 10–20 min later (5–7). There is also evidence that other pp90^{rsk} substrates exist (2, 12) and that a substantial amount of pp90^{rsk} is localized in the nucleus (R.-H. Chen and J.B., unpublished data). Therefore, it is likely that other physiologically significant substrates of pp90^{rsk} exist in both the cytoplasm and nucleus. In addition, the identity of the growth-regulated S6 kinase kinases, upstream in the signaling pathway, is of interest.

In the present study, we sought to identify the murine protein kinases that participate in the phosphorylation/regulation of pp90^{rsk} family members, by using recombinant *rsk* gene product as a substrate for *in vitro* kinase assays. With this assay, a mitogen-regulated RSK kinase activity was previously identified, partially purified, and shown to phosphorylate a subset of the pp90^{rsk} Ser/Thr phosphorylation sites identified in biosynthetically labeled mammalian cells as indicated by comigration of tryptic phosphopeptides separated by two-dimensional thin-layer electrophoresis/chromatography (6, 13). The RSK kinase activity was rapidly stimulated (maximally 1–5 min after growth factor addition), immediately prior to the activation of pp90^{rsk} and was coordinately inactivated (13). The activation/inactivation kinetics of RSK kinase activity also correlated with hyperphosphorylation/dephosphorylation of pp90^{rsk} (7).

Studies with *Xenopus laevis* oocytes in M phase have also pointed to the regulation of the *rsk* gene family of S6 kinases by an unknown meiosis-activated protein Ser/Thr kinase (14). Recent studies have detected the activation of a myelin basic protein (MBP) kinase in maturing *Xenopus* oocytes that slightly precedes stimulation of the S6 kinases (15). The sea star counterpart has been purified to near homogeneity and corresponds to a 44-kDa phosphoprotein (designated pp44^{mpk}, for meiosis-activated protein kinase; ref. 16). Activation of pp44^{mpk} during sea star oocyte maturation near the onset of germinal vescicle breakdown correlates with increased tyrosine and serine phosphorylation of the kinase (17). This observation is reminiscent of the phosphorylation of an insulin-stimulated 42-kDa murine protein Ser/Thr kinase, p42^{map} (for mitogen-activated protein kinase), which also utilizes microtubule-associated protein 2 (MAP-2) and MBP as substrates (18, 19).

We now demonstrate the existence of two growth-regulated, chromatographically distinct RSK protein kinases in Swiss 3T3 fibroblasts. Both RSK kinases I (≈44 kDa) and II (≈42 kDa) were biochemically and immunologically related to sea star pp44^{mpk}, partially activated pp90^{rsk} *in vitro* and were inactivated by protein tyrosine (Tyr) or protein Ser/Thr phosphatases.

MATERIALS AND METHODS

Cell Culture. Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO); experimental cells were grown in DMEM plus 5% calf serum. Confluent cells were further cultured in DMEM containing 0.5% calf serum and 20 mM Hepes (pH 7.35) for 24–48 h prior to mitogen addition.

Protein Kinase Assays. The pp90^{rsk} assays were completed in the immune complex as reported (6). Assays for RSK kinase activity were completed as described (13) except that Nonidet P-40 and Brij-35 (final concentration, 0.05%) were included in the reaction mixture. These assays used recom-

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Abbreviations: re-RSK, recombinant *rsk* gene product; MBP, myelin basic protein; MAP-2, microtubule-associated protein 2; EGF, epidermal growth factor; PKC, protein kinase C.

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binant chicken RSK (re-RSK; amino acids 53–752) as the substrate that was expressed in *Escherichia coli* and purified as described (13). Assays using MBP or MAP-2 as substrates were similarly completed using 2 μg of MBP (Sigma) or MAP-2 (a generous gift from K. S. Kosik, Harvard Medical School) in place of 2 μg of re-RSK polypeptide.

Partial Purification of Growth-Regulated RSK Protein Kinases I and II. The RSK kinases were partially purified with resins similar to those used for MAP-2 kinase purification (20, 21) with the modifications described below. Swiss 3T3 cells (30 × 150 mm tissue culture plates) were grown as described above, stimulated with 10% (vol/vol) calf serum for 5 min, and then lysed in 22 ml of buffer C1 (13) containing pepstatin (10 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonyl fluoride (40 μg/ml) and clarified by centrifugation at 100,000 × *g* for 30 min; the supernatant was loaded over 8 ml of DEAE-Sephacel (Pharmacia) equilibrated in buffer C1. After washing with 2.5 column vol, 30 2-ml fractions were collected (NaCl gradient, 0–300 mM) at a flow rate of 0.2 ml/min. Peak DEAE fractions of RSK kinase activity were pooled and directly applied to a phenyl-Sepharose (Pharmacia) column. After washing with 20 ml of buffer A (22), RSK kinase activity was eluted with a 60-ml gradient as described (21), except that buffer A in the gradient (0–60% ethylene glycol and 250–25 mM NaCl) contained 0.1% Brij-35 and 40 mM β-glycerophosphate in place of 40 mM *p*-nitrophenyl phosphate. Thirty 2-ml gradient fractions followed by 10 wash fractions (60% ethylene glycol/25 mM NaCl) were collected at 0.2 ml/min. The RSK kinase I activity from fractions 21 and 22 was pooled, concentrated against buffer A containing 50% (vol/vol) glycerol, and stored at −20°C. The RSK kinase II (fraction 34) was stored directly at −20°C. Earlier, we had used phenyl-Superose (FPLC) and buffer A without Brij-35 (13). Under these conditions, only a single broad peak of activity was obtained as described by Ray and Sturgill (23). We have also used phenyl-Sepharose as the first column with the conditions described above to verify that both RSK kinase peaks were mitogen-stimulated activities by comparing profiles from quiescent and stimulated cells.

Western Blotting. For Western blotting, proteins were transferred to nitrocellulose (Schleicher & Schuell) from SDS/10% polyacrylamide gels and processed as described (7). Blots were probed with rabbit antiserum raised against a synthetic peptide (GEGA peptide) with the sequence GLAYIGEGAYGMVAC corresponding to protein subdomain I (24) of sea star pp44^{mpk} (1:500), affinity-purified rabbit polyclonal antibodies developed against purified sea star pp44^{mpk} (ref. 17; 1:500), or anti-phosphotyrosine antiserum (ICN; PY20, 1:500) followed by ^{125}I-labeled protein A (ICN).

Two-Dimensional Phosphopeptide Mapping of *in Vitro*-Labeled Recombinant RSK. *In vitro*-labeled re-RSK was separated by SDS/7.5% PAGE, located in the wet gel by autoradiography, excised, eluted, oxidized with performic acid and subjected to exhaustive digestion with trypsin (6, 13), and analyzed by two-dimensional thin-layer electrophoresis/chromatography as described (25). Labeled tryptic phosphopeptides were detected by autoradiography.

Activation of pp90^{rsk} by RSK Kinases I and II. Quiescent or 5-min serum-stimulated NIH 3T3 cells overexpressing chicken pp90^{rsk} (6) were lysed and immune complexes were formed as described, except that a chicken-specific RSK N-terminal peptide antiserum was used. After the immune complexes were washed, pellets were resuspended in 20 μl of 10 mM $MgCl_2$/50 μM ATP/20 mM Hepes, pH 7.2/bovine serum albumin (0.1 mg/ml). RSK kinase I or II (5 μl; ≈46.7 fmol·μl^{-1}·min^{-1}) or no enzyme (5 μl; control) was added and incubated at room temperature for 20 min. At the end of this incubation, the immune complexes were washed two times with buffer B and one time with ST buffer and pp90^{rsk} S6 phosphotransferase activity was measured as described (6).

Inactivation of RSK Kinases I and II by Protein Phosphatase 2A or 1B. RSK kinases I and II (equal RSK phosphotransferase activity) were incubated with phosphatase 2A (64 units/ml) and protein tyrosine-specific phosphatase 1B (10 units/ml) separately at 30°C in separate phosphatase buffers [phosphatase 2A buffer, 20 mM Tris·HCl, pH 7.5 (25°C)/5 mM dithiothreitol/5 mM $MgCl_2$; phosphatase 1B buffer, 20 mM Tris·HCl, pH 7.5 (25°C)/5 mM dithiothreitol/2 mM EDTA]. At each time point, aliquots were removed and directly added to RSK kinase reaction mixtures. RSK kinase assays were completed as described above in the presence of phosphatase inhibitors (for phosphatase 2A, 10 mM NaF, 1 mM EDTA, and 0.1 μM okadaic acid; for phosphatase 1B, 500 μM sodium vanadate). Control experiments were completed with RSK kinases I and II incubated with phosphatases in the presence of the appropriate phosphatase inhibitors.

RESULTS

Chromatographic Separation of Two Growth-Regulated RSK Kinase Activities and Their Relationship to Maturation-Activated pp44^{mpk} from Sea Star Oocytes. We have previously

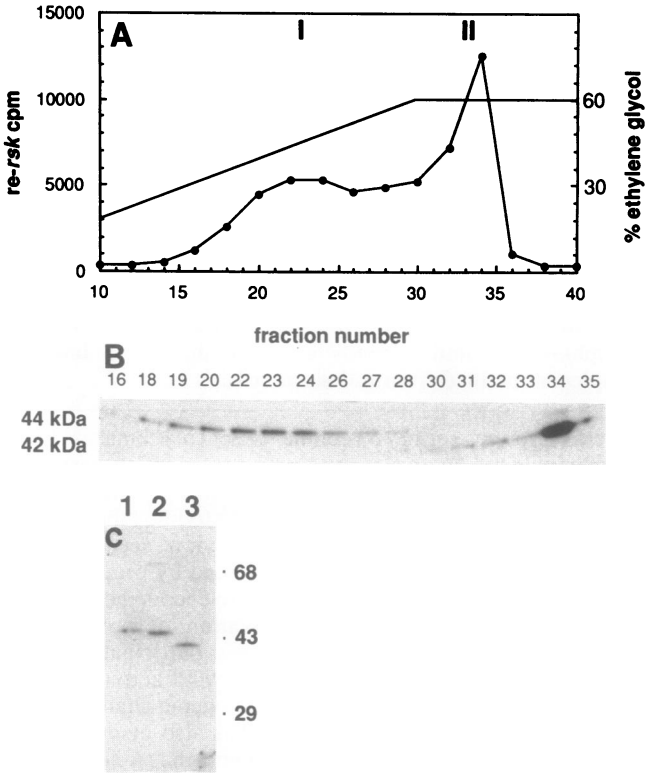

FIG. 1. Detection of two RSK kinase activities in serum-stimulated Swiss 3T3 cells. Following DEAE-Sephacel chromatography, RSK kinase activity was further fractionated by phenyl-Sepharose column chromatography. (*A*) Based on order of elution, the two peaks of RSK phosphotransferase activity are referred to as RSK kinase I and RSK kinase II. (*B*) Fifty-microliter aliquots of the indicated fractions were further characterized by Western blot analysis using anti-pp44^{mpk} polyclonal antibodies (1:500 dilution of each) and ^{125}I-labeled protein A. Two cross-reacting polypeptides migrating at ≈44 and ≈42 kDa were detected and these coeluted with peaks I and II, respectively. (*C*) Approximately equal RSK phosphotransferase activity of purified pp44^{mpk} (MBP kinase) (lane 1), RSK kinase I (lane 2), and RSK kinase II (lane 3) was size fractionated on a SDS/10% polyacrylamide gel and transferred to nitrocellulose paper. This blot was probed with pp44^{mpk} antiserum (1:500 dilution). With this analysis, the sizes of pp44^{mpk}, RSK kinase I, and RSK kinase II were calculated to be ≈45, 44.2, and 41.7 kDa, respectively.

demonstrated the existence of a broad peak of mitogen-stimulated RSK kinase activity eluting at ≈110 mM NaCl from DEAE resin (13). Similar results have also been described for MAP-2 kinase activity (from insulin-stimulated 3T3 L1 cells; ref. 21) and MBP kinase activity (from maturation-induced sea star oocytes; ref. 26). In both cases, these enzymes subsequently eluted as single peaks from hydrophobic interaction resins. Using different buffer conditions, we have observed that RSK kinase activity from serum-stimulated Swiss 3T3 cells yielded two peaks of mitogen-stimulated activity eluting from phenyl-Sepharose whether used as the first column (not shown) or the second column (following DEAE) of a purification scheme (Fig. 1*A*). Initial experiments suggested that they were closely related activities with properties similar to that described for purified pp44^{mpk}. Using antiserum prepared against purified pp44^{mpk} and Western blot analysis, we identified cross-reacting polypeptides of ≈44 and ≈42 kDa that coeluted with RSK kinase peaks I and II, respectively (Fig. 1*B*). A direct comparison by SDS/PAGE and Western blot analysis using anti-pp44^{mpk} polyclonal antiserum, for pp44^{mpk} (≈45 kDa), RSK kinase I, and RSK kinase II is shown in Fig. 1*C*. In this experiment, approximately equal recombinant RSK phosphotransferase activity was loaded on each lane. Continued characterization of these protein kinases revealed that in addition to phosphorylating recombinant RSK *in vitro*, MBP and MAP-2 were also substrates, whereas the 40S ribosomal protein S6 was not (data not shown). The two-dimensional separation of tryptic phosphopeptides of recombinant RSK phosphorylated *in vitro* by RSK kinase I or II revealed comigrating phosphopeptides, and sea star pp44^{mpk} exhibited nearly identical specificity (Fig. 2). The numbered phosphopeptides correspond to *in vivo* labeled tryptic phosphopeptides shown previously (6, 13).

RSK Kinases I and II Activate pp90^{rsk} Activity *in Vitro*. pp90^{rsk} and RSK kinase activities are coordinately regulated by a variety of mitogens and by pp60$^{v\text{-}src}$ (13). Supporting the notion that these protein kinases are participants of a growth-modulated protein phosphorylation cascade, both RSK kinases I and II can partially activate pp90^{rsk} *in vitro*. In the experiment presented in Fig. 3, pp90^{rsk} (isolated from quiescent cells) in the immune complex was incubated in kinase reaction buffer (lane 1) or with RSK kinase I (lane 2) or RSK kinase II (lane 3) under phosphorylation conditions. After the immune complexes were washed to remove the RSK kinases,

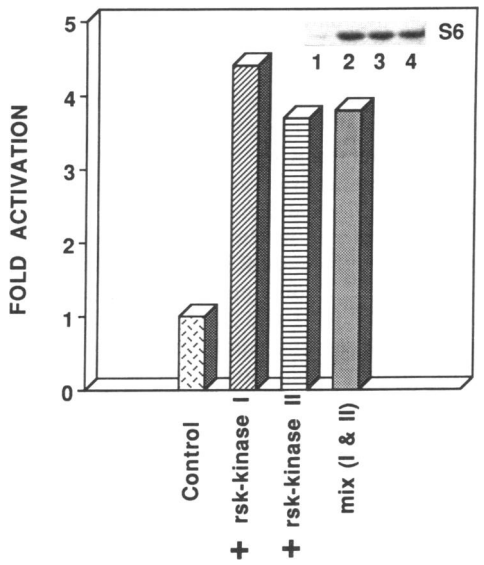

FIG. 3. Activation of pp90^{rsk} from quiescent cells *in vitro* with RSK kinase I or II. Quiescent NIH 3T3 cells overexpressing chicken pp90^{rsk} (6) were lysed and immune complexes were formed with a chicken-specific RSK N terminus peptide antiserum. Under these conditions, immune complex S6 phosphotransferase activity was low. (*Inset*) The pp90^{rsk} immune complexes were incubated in RSK kinase reaction buffer (lane 1) or in reaction buffer with RSK kinase I (lane 2) or RSK kinase II (lane 3, using approximately equal RSK phosphotransferase activity as in lane 2), or in an equal mixture of the two RSK kinases (lane 4). Bar graph presents the extent of activation of pp90^{rsk} in the immune complex by the RSK kinases.

40S ribosomal subunits were added in the presence of [γ-^{32}P]ATP to analyze pp90^{rsk} S6 phosphotransferase activity. Both RSK kinases activated pp90^{rsk} ≈4-fold in this experiment. A mixture of the two (lane 4), with a combined activity equal to that used in lane 2 or 3, yielded similar results. In a separate experiment, pp44^{mpk} also activated pp90^{rsk} S6 phosphotransferase activity severalfold (data not shown). The extent of activation of pp90^{rsk} observed *in vitro* over several experiments was 10–25% of the activation obtained when the quiescent cells used in these experiments were first incubated with epidermal growth factor (EGF) or serum prior to formation of the immune complex and measurement of pp90^{rsk} activity. Activation of pp90^{rsk} was not

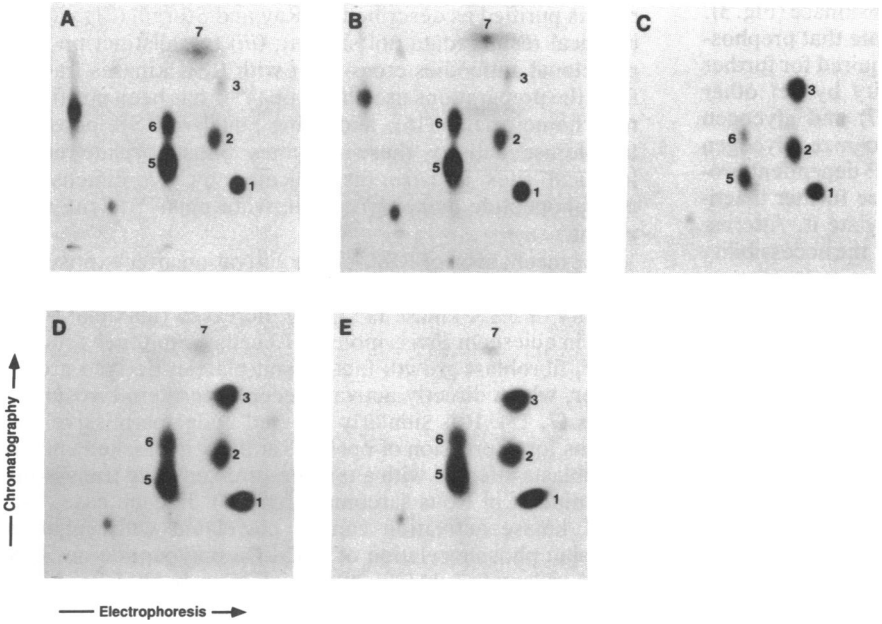

FIG. 2. Tryptic phosphopeptide mapping of recombinant RSK phosphorylated *in vitro* by RSK kinases I and II and pp44^{mpk}. Two-dimensional tryptic phosphopeptide mapping was completed as described (*Materials and Methods*; refs. 6 and 13). Approximately equal cpm for each sample was spotted. (*A*) Tryptic peptide map of re-RSK phosphorylated *in vitro* by RSK kinase I. (*B*) Tryptic peptide map of re-RSK phosphorylated *in vitro* by RSK kinase II. (*C*) Tryptic peptide map of re-RSK phosphorylated *in vitro* by pp44^{mpk}. (*D*) Equal mixture of *A* and *C*. (*E*) Equal mixture of *B* and *C*. The numbering system correlates with that described for RSK labeled *in situ* (6) or *in vitro* (13).

obtained when incubation with RSK kinase I or II was completed in the presence of a nonhydrolyzable ATP analog, AMP-PNP.

RSK Kinases I and II Are Tyrosine Phosphorylated and Inactivated by Protein Tyr and Protein Ser/Thr Phosphatases. To determine whether RSK kinases I and II are tyrosine phosphorylated, we transferred our partially purified preparations to nitrocellulose and probed these Western blots with anti-phosphotyrosine antibodies (PY20). Shown in Fig. 4 are RSK kinases I and II (equal RSK phosphotransferase activity) probed with a second $pp44^{mpk}$ antibody (anti-GEGA peptide antibody, lanes 1 and 2) and a duplicate blot probed with anti-phosphotyrosine antibody (lanes 3 and 4). The inability to directly immunoprecipitate ^{32}P-labeled phosphoproteins from biosynthetically labeled cells with these antisera prevented us from completing a direct phospho amino acid analysis. However, the notion that RSK kinases I and II are tyrosine phosphorylated and regulated by phosphorylation was supported by the ability to inactivate RSK phosphotransferase activity of both enzymes with phosphotyrosine-specific phosphatase 1B (Fig. 5*A*). Both RSK kinases were also inactivated with the phosphoserine/threonine-specific phosphatase 2A (Fig. 5*B*).

DISCUSSION

re-RSK polypeptide has been successfully used as a substrate for *in vitro* assays to probe for the existence of growth-modulated RSK kinase activities (13). Using this assay we now provide evidence for the existence of two chromatographically distinct, serum-stimulated RSK protein kinases, referred to here as RSK kinase I (pp44) and RSK kinase II (pp42) based on their order of elution from phenyl-Sepharose. We also show that both RSK protein kinases are related to the meiotically regulated sea star oocyte $pp44^{mpk}$ by several criteria. They exhibit similar sizes based on SDS/PAGE and the cross-reactivity of each RSK kinase with polyclonal antibodies raised against purified $pp44^{mpk}$ (17) and a synthetic GEGA peptide (Figs. 1 and 4). Like $pp44^{mpk}$ (S.L.P., unpublished data), the RSK kinases also immunoreacted with anti-phosphotyrosine monoclonal antibody (Fig. 4), and they were inactivated by pretreatment with protein Tyr phosphatase 1B (Fig. 5*A*) or protein Ser/Thr phosphatase 2A (Fig. 5*B*). These kinases phosphorylated re-RSK polypeptide *in vitro*, generating comigrating tryptic phosphopeptides (Fig. 3), as well as phosphorylating MAP-2 and MBP. Each elicited a partial activation of $pp90^{rsk}$ from quiescent NIH 3T3 cells that overexpressed the chicken form of this S6 kinase (Fig. 3). The partial reactivation observed may indicate that prephosphorylation of $pp90^{rsk}$ by these kinases is required for further activation of its S6 phosphorylating activity by yet other kinases. For example, casein kinase I (27) and glycogen synthase kinase 3 (28) preferentially recognize glycogen synthase that is prephosphorylated by cAMP-dependent protein kinase and casein kinase II and produce further inactivation of the enzyme when they phosphorylate it. Alternatively, the partial activation may also reflect the accessibility

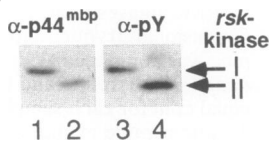

FIG. 4. RSK kinases I and II are recognized by anti-phosphotyrosine antibodies. Approximately equal RSK phosphotransferase activities of RSK kinase I (lanes 1 and 3) and RSK kinase II (lanes 2 and 4) were separated by SDS/10% PAGE, transferred to nitrocellulose, and probed with anti-$pp44^{mpk}$ GEGA peptide antiserum (lanes 1 and 2; 1:500 dilution) or anti-phosphotyrosine antiserum (lanes 3 and 4; PY20 at 1:500 dilution).

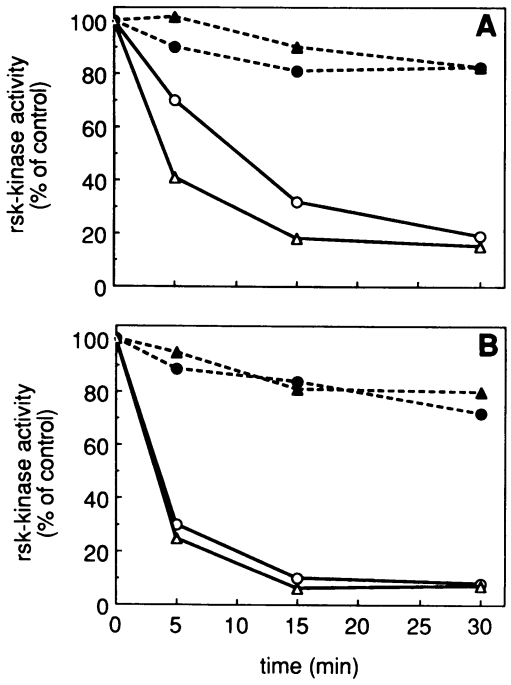

FIG. 5. Inactivation of RSK kinases I and II with protein-tyrosine phosphatase 1B or protein Ser/Thr phosphatase 2A. RSK kinase I or II was incubated for various times with phosphatase 1B (*A*) or phosphatase 2A (*B*) in the absence or presence of the appropriate phosphatase inhibitors prior to measurement of RSK phosphotransferase activity. ○, RSK kinase I incubated with 1B (*A*) or 2A (*B*) without phosphatase inhibitors; △, RSK kinase II incubated as described above without phosphatase inhibitors; ●, RSK kinase I incubated as described above in the presence of phosphatase inhibitors; ▲, RSK kinase II incubated as described above in the presence of phosphatase inhibitors. After phosphatase incubation, all RSK phosphotransferase assays were completed in the presence of equivalent concentrations of phosphatase inhibitors. 100% equals the activity at zero time (control). The extent of inhibition is described as % of control.

of $pp90^{rsk}$ in the immune complex. It is unlikely that the observed activation is due to another contaminating kinase(s) for several reasons: (*i*) a contaminating ($pp90^{rsk}$ activating) kinase is not likely to copurify with the two RSK kinases that were separated based on significant differences in hydrophobicity (by phenyl-Sepharose); (*ii*) other RSK kinase preparations purified as described by Ray and Sturgill (21) yielded identical results (data not shown); (*iii*) two distinct $pp44^{mpk}$ polyclonal antibodies cross-react with RSK kinases I and II from the preparations used here, $pp44^{mpk}$ has been purified to near homogeneity (16), and using equal re-RSK phosphotransferase activity these enzymes phosphorylate nearly identical sites *in vitro* (as indicated by two-dimensional phosphopeptide mapping) and activate $pp90^{rsk}$ to the same extent.

The mechanism of RSK kinase activation after exposure of cultured cells to diverse stimuli is particularly intriguing. The activity of RSK kinase is rapidly increased (maximal in 1–5 min) in quiescent Swiss mouse 3T3 cells by mitogens such as EGF, fibroblast growth factor, and platelet-derived growth factor, which directly activate receptor/protein-tyrosine kinases (7, 13). It is similarly affected under permissive conditions for expression of $pp60^{v\text{-}src}$ activity in chicken embryo fibroblasts infected with a temperature-sensitive transformation mutant of Rous sarcoma virus (13). In each case, such RSK kinase activation can be correlated with enhanced tyrosine phosphorylation of a 42-kDa polypeptide on SDS/polyacrylamide gels (20, 29), which is probably RSK kinase II. Tumor-promoting phorbol esters that directly activate the

protein (Ser/Thr) kinase C (PKC) family also lead to rapid tyrosine phosphorylation of the 42-kDa phosphoprotein (29–32) and activation of RSK kinase activity in these cells (13). Furthermore, other factors that do not seem to work by directly activating protein-tyrosine kinases but that regulate such diverse biological processes as the regulation of catecholamine release from chromaffin cells also appear to stimulate tyrosine phosphorylation of this 42-kDa polypeptide (33). Interestingly, EGF or fibroblast growth factor but not platelet-derived growth factor produces maximal stimulation of RSK kinase activity when PKC has been down-regulated by prior treatment of the cells with phorbol esters (7, 13), indicating a differential requirement of PKC for signal transduction by different receptor tyrosine kinases and suggesting that PKC is not essential for signaling the activation of the RSK kinases. The ability of protein Ser/Thr phosphatases and protein-tyrosine phosphatases to independently inactivate the RSK kinases points to these enzymes as integrators of diverse signal transduction pathways. Whether different agonists produce the selective activation of one RSK kinase over the other remains to be established. However, it appears that insulin treatment of murine 3T3 L1 cells may result in primarily $pp42^{map}$ stimulation (21, 34).

Recent studies indicate that two ≈42-kDa polypeptides on SDS/polyacrylamide gels that become tyrosine phosphorylated during *Xenopus* oocyte maturation (J. Sanghera, S.L.P., J. Posada, and J. Cooper, unpublished data) as well as a 44- to 45-kDa phosphoprotein and $pp42^{map}$ in EGF-treated 3T3 (J. Sanghera, S.L.P., T. Rossomando, M. Weber, and T. Sturgill, unpublished data) are immunologically related to sea star $pp44^{mpk}$. Another MAP-2 kinase (referred to as *erk-1* encoded) has recently been purified and a partial cDNA was obtained (35). Microprotein sequencing of sea star $pp44^{mpk}$ peptides has revealed ≈70% identity with the predicted rat brain *erk-1*-encoded polypeptides (S.L.P. and R. Aebersold, unpublished data). Finally, two FPLC Mono Q resolved EGF-stimulated MBP kinase activities from Swiss 3T3 cells, identified as E3 and E4, upon gel filtration exhibited molecular masses of ≈30 and ≈50 kDa, respectively. These preparations could stimulate an S6 peptide kinase activity (B1) *in vitro* that behaved as an ≈110-kDa protein on Superose (36). The relationship of these activities to the ≈42-kDa RSK kinase II, the ≈44-kDa RSK kinase I, and $pp90^{rsk}$ is not clear.

Thus, it appears that RSK kinases I and II, $pp44^{mpk}$, $pp42^{map}$, and *erk-1*-encoded polypeptides are members of a family of growth-regulated protein Ser/Thr kinases that receive signals from protein-tyrosine and protein Ser/Thr kinases and transmit information downstream via a protein phosphorylation cascade to additional targets such as $pp90^{rsk}$, which in turn transmits information to the 40S ribosomal protein S6 and likely other cytoplasmic and nuclear (R.-H. Chen and J.B., unpublished data) substrates. Clearly, the continued characterization of the $pp90^{rsk}$ family, the RSK kinases, and the future identification of the RSK kinase protein-tyrosine and protein Ser/Thr kinases that participate in the regulation of this signal transduction pathway, as well as the identification and characterization of additional physiological substrates of the protein kinases in this signaling system, will improve our understanding of the processes regulating normal and neoplastic cell growth.

While this manuscript was in review polyclonal antibodies were generated to the predicted N terminus and C terminus of the erk-1 sequence (34). These antisera recognize RSK kinases I and II in the preparations used here, and in the immune complex assay both phosphorylate re-RSK. Furthermore, both enzymes are immunoprecipitated from quiescent cells and upon serum stimulation as Tyr/Thr-phosphorylated, activated protein Ser/Thr kinases (J.C. and J.B., unpublished data).

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