Regulation and Interaction of pp90rsk Isoforms with Mitogen-activated Protein Kinases

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Each of the three known mammalian 90-kDa S6 kinase (pp90rsk) isoforms (RSK1, RSK2, and RSK3) was expressed in transfected cells and further characterized. The kinase activity (immunocomplex toward S6 peptide) of each isoform was activated by in vitro growth factor (epidermal growth factor (EGF)) stimulation; RSK1 was more responsive (10–15-fold) versus RSK2 and RSK3 (2–4-fold). Pretreatment with PD98059 (MEK1 inhibitor) partially (50%) blocked EGF-mediated ERK1 activation and had similar effects on EGF stimulation of each ribosomal S6 kinase (RSK). Cotransfection with dominant-negative MEK1 inhibited activation of each RSK; furthermore, the kinase activity of RSK1, RSK2, and RSK3 was markedly increased by cotransfection with constitutively active MEK1. A specific association between mitogen-activated protein kinases (MAPKs) (ERK1 and ERK2) and RSK isoforms was tested by MAPK immunoblotting after immunoprecipitation of RSKs. ERK1 and ERK2 were present in RSK3 (and to a lesser extent, RSK2) immunoprecipitates, but were absent in RSK1 immunoprecipitates. Both dephosphorylated (from quiescent cells) and phosphorylated (from stimulated cells) MAPKs were associated with RSK2 and RSK3. Deletion mutants of RSK3 were characterized: the C terminus (33 residues) was shown to be required for association with MAPKs. The kinase activity of RSK1 or RSK2 was enhanced by in vitro incubation with ERK1. In contrast, RSK3 activity was not affected by exposure to ERK1. Furthermore, MAPKs in RSK3 immunoprecipitates were phosphorylated by purified MEK1; however, RSK3 kinase activity was unaffected. We conclude that 1) the MEK1-MAPK signaling pathway is both necessary and sufficient for in vivo growth factor-mediated activation of all three RSK isoforms; 2) RSK isoforms differ with respect to growth factor responsiveness and their physical association with MAPK; and 3) formation of the MAPK-RSK complex is mediated by the RSK C terminus.

Although similar signal transduction pathways are activated coincident with the stimulation of cell proliferation, cell differentiation, or metabolic events, it remains unclear what the precise determinants of these discordant biological responses are. Variation in cell context or the amplitude and duration of activation of a given pathway are important parameters (1). The existence of multiple isoforms for each of several classes of protein kinases provides an additional level of specificity. For example, different physiologic processes (mating, cell wall synthesis, osmoregulation) in Saccharomyces cerevisiae are regulated by different mitogen-activated protein kinase (MAPK)1 and MAPK kinase (MEK) homologs (2, 3). Recently, a number of distinct mammalian MEK and MAPK isoforms that participate in growth factor- or stress-activated pathways have also been identified (1, 4, 5).

pp90rsk (ribosomal S6 kinase (RSK)) is an additional important family of signal-transducing Ser/Thr kinases. RSK was initially isolated from Xenopus, where it serves to phosphorylate the 31-kDa protein (S6) that is a component of the 40 S ribosomal subunit (6). Subsequently, an avian RSK and two mouse RSK homologs (rskmo-1 or RSK1 and rskmo-2 or RSK2), a rat RSK1 homolog, and three human RSK isoforms were reported (7–11). The three mammalian RSK isoforms appear to be closely related since they each contain two non-identical complete kinase domains (7, 11). However, RSK3 contains a 33-amino acid N-terminal domain that is enriched in basic residues and bears no homology to other RSKs (11). Recently, we demonstrated that, whereas both N- and C-terminal kinase domains contribute to growth factor-stimulated RSK autophosphorylation, substrate phosphorylation is mediated by the N-terminal kinase domain (12).

In mammalian cells, RSK is activated in response to a broad range of cellular perturbations, including oncogenic transformation (13), stimulation with insulin (13–15) or growth factors and phorbol ester (16), growth hormone (17), changes in cAMP levels (16), heat shock (18), ionizing radiation (19), and T cell receptor activation (20). Since the discovery and characterization of another class of S6 kinase (pp70S6K), it appears that pp70S6K, not pp90rsk, accounts for in vivo phosphorylation of ribosomal S6 in mammalian cells (21–23). In contrast to pp70S6K, the substrate specificity of RSK is relatively broad (23). Furthermore, RSK has been suggested to mediate important physiologic processes, including insulin-stimulated activation of glycogen synthase (via phosphorylation of protein phosphatase 1) (15, 24) and growth factor regulation of cell proliferation (via phosphorylation of nuclear substrates such as c-Jun, c-Fos, and serum response factor) (25).

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; RSK, ribosomal S6 kinase; ERK, extracellular-regulated kinase; EGF, epidermal growth factor; MBP, myelin basic protein; HA, hemagglutinin; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis.
Ser/Thr phosphorylation is required for RSK activation, and RSK is inactivated by incubation with protein phosphatases 1 and 2A (23). Since RSK and specific MAPK isoforms (ERK1-p44MAPK and ERK2-p42MAPK) are coordinately regulated (26), ERK1 and ERK2 have been implicated as upstream activators of RSKs. Furthermore, RSK, ERK1, and ERK2 have been shown to undergo growth factor-stimulated translocation to the cell nucleus (11, 27–29), and there is evidence suggesting a physical association between RSK and ERK1/2 MAPKs both in PC12 cells (30) and in Xenopus oocytes (31). Although both RSK1 (8) and RSK2 (32) were shown to be partially activated in vitro incubation with ERK, we found that RSK3 activity was unaffected by ERK2 under similar in vitro conditions (11). In addition, other investigators have suggested that additional RSK kinases may exist (reviewed in Ref. 25). Therefore, the extent to which ERK1 and ERK2 serve as in vitro upstream RSK activators for any or all RSK isoforms is not well established.

To examine the potential dependence of growth factor-mediated activation of each RSK isoform on the MAPK pathway, we employed both dominant-negative and constitutively activated mutants of MEK1 and a synthetic specific MEK inhibitor. From these studies, we conclude that the MEK-MAPK pathway is not unilaterally dependent on the MEK activation for RSK activation. Various deletion mutants of RSK1, RSK2, and RSK3 were constructed and studied for their ability to associate with MAPKs. The C terminus (33 amino acid residues) of RSK3 was shown to be required for the association with ERK1 and ERK2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ribosomal S6 substrate peptide (RRRLSSLRRA, amino acids 231–239), ERK1/MAPK (isolated from seastar), and a polyclonal RSK2-specific antibody (RSK2-PCT) were from Upstate Biotechnology, Inc. Epidermal growth factor (EGF) and myelin basic protein (MBP) were obtained from Sigma. Recombinant insulin-like growth factor 1 was from Genentech; monoclonal antibody 12CA5 was from Babco (San Rafael, CA); and γ-32P-ATP was from DuPont NEN. The enhanced chemiluminescence immunodetection system (ECL) was purchased from Amersham Corp. The mammalian expression vector pMT2 (33) with a hemagglutinin (HA) epitope sequence inserted (pMT2-HA) and an additional expression vector containing an HA epitope-tagged RSK1 cDNA (pMT2-HA-RSK1) (8) were generously provided by Dr. Joseph Avruch (Massachusetts General Hospital, Boston). MAPK anti--serum was generously provided by Dr. John Blenis (Harvard Medical School, Boston). Purified recombinant constitutively activated MEK enzyme and mouse RSK2 cDNA were kindly supplied by Dr. Raymond Erikson (Harvard University, Cambridge, MA). Two cDNA expression vectors encoding site-directed MEK1 mutants (34) were a gift from Dr. Chris Marshall (Institute of Cancer Research, London). A synthetic inhibitor of MEK (PD98059) (35) was kindly provided by Dr. Alan R. Saltiel (Parke-Davis Pharmaceutical Research, Ann Arbor, MI). A cDNA expression vector (HA-ERK1) encoding epitope-tagged ERK1 was a gift from Dr. Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia).

**Construction of Expression Vectors and Mutagenesis**—For transient expression in COS cells, RSK1 (8), RSK2 (7), and RSK3 (11) cDNAs were subcloned into the mammalian expression vector pMT2-HA. The resultant recombinant proteins contain a 9-amino acid hemagglutinin epitope (YPYDVPDYA) at their N terminus. RSK1 cDNA was introduced into the pMT2-HA vector as described earlier (8); the open reading frame of RSK2 cDNA was subcloned into the XhoI/SmaI sites of pMT2-HA-RSK1 (thereby deleting the coding region of RSK1). The introduction of an XhoI site at the N terminus of RSK2 cDNA was carried out by polymerase chain reaction. RSK3 cDNA was subcloned into pMT2-HA as described previously by Zhao et al. (11).

The RSK3 constructs for expression of the isolated N-terminal (RSK3ΔC) and C-terminal (RSK3ΔN) kinase domains were prepared as described previously (12). Removal of amino acids 1–33 (RSK3ΔNT) was done by digesting the XhoI/SmaI fragment encoding amino acids 33–732 of RSK3 into pMT2-HA-RSK1 (thereby deleting the coding region of RSK1). The RSK3ΔCT mutant (lacking amino acids 689–733) was generated by digestion of pMT2-HA-RSK3 with DraI and SacI, filling in of overhangs using Klenow enzyme, and subsequent religation. The chimeric RSK3/RSK1 mutant was made by digestion of pMT2-HA-RSK3 with DraI and SacI, followed by religation together with a DraI/SalI fragment of a small polymerase chain reaction product from the C terminus of RSK1, generated using suitable primers. For expression of RSK3 in stably transfected CHO cells, the Pet/3XHA insert from pMT2-HA-RSK3 was excised and inserted into the expression vector pRCE/CMV (Invitrogen).

**Cell Culture and Protein Expression**—CHO cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C in 5% CO2. For transient expression of RSK1, RSK2, or RSK3 proteins, COS cells (30–50% confluence in 35-mm dishes) were transfected with 1.5 μg of plasmid DNA using 10 μl of Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s protocol. Cells were harvested 72 h post-transfection and solubilized in 500 μl of lysis buffer (1% Nonidet P-40, 0.5% Triton X-100, 10% glycerol). 150 μl NaCl, 1 mM NaVO4, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 50 mM Tris-HCl, pH 7.4). A portion of the lysate (50 μl) was boiled in an equal volume of 2 × Laemmli sample buffer. The remainder was clarified by centrifugation at 20,000 × g for 15 min, and the supernatant was immunoprecipitated as described below. Each expression vector containing different cDNAs with different epitope tags was transfected with 1 μg of RSK cDNA plus 1 μg of MEK1 cDNA. For the experiments using the MEK-specific inhibitor PD98059, transfected COS cells were starved in Dulbecco’s modified Eagle’s medium supplemented with 0.1% bovine serum albumin for 10 h beginning 68 h post-transfection, incubated with PD98059 at a final concentration of 75 μM for 30 min, and subsequently stimulated with EGF for 15 min. The cells were harvested by aspirating the medium, rinsing in ice-cold phosphate-buffered saline, and extracting into ice-cold lysis buffer.

CHO cells were grown in Ham’s F-12 nutrient mixture with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C in 5% CO2. CHO cells were transfected with 10 μg of linearized pBLCMV-RSK3 cDNA according to a standard protocol for calcium phosphate-mediated transfection (36). 24 h following transfection, cells were plated in selection medium that contained 0.8 mg/ml Geneticin (Life Technologies, Inc.). The selected medium was changed every 2 days for 3 weeks, and individual colonies were selected and expanded (38).

**Immunoblotting and Immunoprecipitation**—Immunoprecipitations were performed using clarifying nuclease-digested 12CA5 or RSK2-PCT antibodies (1:250 dilution) and protein A-agarose (1:15 dilution of a 50% suspension in lysis buffer) on a rotating wheel for 3 h or overnight. The agarose beads were pelleted by low speed centrifugation, washed extensively with ice-cold lysis buffer, and then washed twice in kinase assay buffer (10 mM MgCl2, 0.1 mM EGTA, 1 mM dithiothreitol, 30 mM Tris-HCl, pH 7.4). The precipitated proteins were used for subsequent manipulations.

For immunoblotting, proteins were subjected to SDS-PAGE as described previously (37), and then resolved polypeptides were transferred to nitrocellulose membranes using the system of Towbin et al. (38). The membranes were blocked with 10% nonfat dried milk in Towlbin buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) for 2 h at room temperature or overnight at 4 °C and then incubated with antibodies (1:1000 dilution) in 5% nonfat dried milk for 2 h. After removal of unbound antibodies by three washes with 20 min in Towlbin buffer, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (1:1000) for 1 h and washed five times in Towlbin buffer. The targeted proteins were detected using ECL as described by the manufacturer.

**Protein Phosphatase 2A, ERK1/MAPK, and MEK Treatment**—The immunocomplexes were incubated with protein phosphatase 2A as described earlier (11) and terminated by three washes with 1 ml of ice-cold kinase assay buffer. The samples with and without the protein phosphatase 2A treatment were resuspended in kinase assay buffer with 0.5 mM ATP. 40 μg of ERK1/MAPK was added, and the incubation (total volume of 20 μl) was then allowed to proceed at 30 °C for 15 min. The reactions were terminated by chilling on ice and washing three times with ice-cold kinase assay buffer. The prepared immunocomplexes were assayed for kinase activity using 5′ peptide as substrate as described below. Treatment of immunocomplexes with purified active MEK enzyme was performed in a total volume of 15 μl of kinase assay buffer containing 10 μl of 5 mg/ml bovine serum albumin, 0.5 mM dithiothreitol, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM MgCl2, and 0.5 mM dithiothreitol. The enzyme reaction was allowed to proceed for 15 min at 37 °C, and the reaction was terminated by chilling on ice and washing three times with ice-cold kinase assay buffer. The prepared immunocomplexes were assayed for kinase activity using 5′ peptide as substrate as described below.
buffer including 0.5 mM ATP at 30 °C for 15 min and terminated by adding 3 × Laemmli sample buffer.

**Protein Kinase Assay**—Kinase activity was measured by resuspending the final immunocomplexes in 15 μl of kinase assay buffer supplemented with 100 μM [γ-32P]ATP (5 μCi). MBP was used at a final concentration of 0.5 μg/μl, and the final concentration of S6 peptide was 250 μM. The reaction mixture was incubated at 30 °C for 10 min and then stopped by pipetting the mixture onto pieces of phosphocellulose P-81 paper (Whatman), followed by five washes in 150 mM phosphoric acid or by adding 3 × Laemmli sample buffer directly and subsequent separation by SDS-PAGE. The incorporation of 32P into S6 peptide adsorbed on the P-81 paper was determined by Cerenkov counting.

### RESULTS

**Expression of RSK1, RSK2, and RSK3 in COS Cells**—The coding regions of RSK1, RSK2, and RSK3 were subcloned into a eukaryotic expression vector (pMT2) for transient expression in COS cells. This expression vector encodes 13 additional in-frame amino acids immediately after the N-terminal initiator methionine and includes a 9-residue epitope (YPYDVPDYA) from influenza HA that is reactive with the 12CA5 monoclonal antibody (39). The resultant constructs were transfected into COS cells, and the expressed recombinant proteins were isolated 72 h following transfection. As shown in Fig. 1A, the overexpressed RSK1, RSK2, and RSK3 proteins were readily detected in lysates derived from transfected cells by immunoblotting with the 12CA5 antibody. Although all three isoforms could be overexpressed at a similar level in COS cells (immunoblotting using total cell lysates), the solubility of each recombinant protein in the applied lysate buffer was quite different. A substantial fraction of RSK1 and a large majority of RSK2 were recovered in the clarified lysate. In contrast, a smaller fraction (~5%) of the total expressed RSK3 protein was soluble; thus, the majority of RSK3 was present in the pellet after centrifugation of the total lysate. However, the soluble fraction of RSK3 protein was identical in molecular size to in vivo translated HA-RSK3 protein and was detected by antibodies directed against the N (12CA5) and C (using a specific anti-peptide antibody) termini of the protein (data not shown). We subsequently isolated solubilized RSK isoforms by immunoprecipitation from clarified lysates using the anti-HA epitope antibody (12CA5). Fig. 1B shows that all three RSK isoforms (with or without EGF stimulation) could be isolated by immunoprecipitation from transfected COS cells. When aliquots of these immunocomplexes were used to measure their kinase activity toward S6 peptide, we found that all three HA-RSK proteins could phosphorylate the S6 peptide substrate and that kinase activity was augmented by in vitro EGF stimulation. However, RSK1 was much more responsive (10–15-fold) compared with RSK2 and RSK3 (2–4-fold). The degree to which EGF stimulation resulted in an electrophoretic mobility shift (detected by immunoblotting) was more pronounced in the case of RSK1, although the mobility of RSK2 and RSK3 proteins was also affected by EGF (Fig. 1B). Similar effects of EGF on RSK2 (endogenous) and RSK3 (low level stable expression) in CHO cells were also observed (data not shown).

**Cotransfection of Individual RSK Isoforms with Different MEK1 Mutants**—MEKs (also known as MAPK kinases) are dual specificity kinases that exhibit a high degree of specificity for phosphorylation of MAPKs on threonine and tyrosine residues. MEK1 and MEK2 are currently the only known kinases that activate ERK1 and ERK2 (40–42). The MEK1 enzyme has been shown to be activated through phosphorylation on 2 serine residues within the catalytic domain: Ser-217 and Ser-221 (43, 44). By mutating these sites, dominant-negative and constitutively activated mutants of MEK1 have been generated (34, 45), and these mutants have been successfully used to study cellular proliferation (45), cell differentiation, and transformation (34). We used both dominant-negative (A217) and constitutively active (E217/E221) MEK1 mutants (34) that were cotransfected with vectors containing individual RSK isoforms in COS cells. As shown in Fig. 2, the EGF-stimulated (and to some extent, basal) activity of each RSK isoform toward S6 peptide was attenuated by cotransfection with dominant-negative MEK1. In the case of RSK1, activation by EGF was reduced from 5.7 to 2.9-fold; for RSK2, activation was reduced from 2.6 to 2.1-fold; and for RSK3, activation was reduced from 3.5 to 2.2-fold. In addition, coexpression of constitutively active MEK1 markedly augmented the activity of all three RSK isoforms in an EGF-independent fashion. These results suggest that activation of MEK1 is both necessary and sufficient for activation of each RSK isoform.

**Inhibitory Effect of PD98059 on Activation of Individual Recombinant RSK Isoforms in COS Cells**—A synthetic inhibitor of the mitogen-activated protein kinase cascade (PD98059) was recently described by Dudley et al. (35). PD98059 was shown to prevent activation of MEK and subsequently to in-
ERK2. To do so, several deletion mutants were constructed with these mutants were subjected to immunoprecipitation (Fig. 5). The cell lysates prepared from COS cells transfected with the 12CA5 antibody and subsequent immunoblotting with anti-MAPK antiserum. As shown in Fig. 3, growth factor-mediated activation of all three RSK isoforms was substantially reduced by prior incubation with PD98059. This result, together with that from cotransfection with MEK1 mutants, clearly demonstrates that activation of each RSK isoform is related to the MEK1 signaling pathway.

Association of ERK1 and ERK2 with RSK2 and RSK3, but Not With RSK1—Based on co-immunoprecipitation, RSK was reported to associate with ERK1 (26) and ERK2 (31). To investigate the association of individual RSK isoforms with ERK1 and ERK2, COS cells were transfected with HA-RSK1, HA-RSK2, or HA-RSK3 cDNAs, followed by immunoprecipitation of the corresponding epitope-tagged RSK proteins using the 12CA5 antibody and subsequent immunoblotting with anti-MAPK antiserum. As shown in Fig. 4, RSK2 and RSK3 immunocomplexes contained both ERK1 and ERK2. In contrast, ERK1 and ERK2 were not present in RSK1 immunocomplexes despite the fact that greater amounts of RSK1 than RSK3 were present. Importantly, in vitro EGF stimulation had no effect on the extent to which ERK1 and ERK2 associated with either RSK2 or RSK3. Indeed, EGF mediated phosphorylation (activation) of ERK1 and ERK2 as evidenced by a characteristic up-shift in electrophoretic mobility (Fig. 4) (46). Using stably transfected CHO cells that express epitope-tagged RSK3, we were also able to detect the presence of MAPK (ERK2 > ERK1) in RSK3 immunoprecipitates (data not shown).

Since MAPKs were more readily detected in RSK3 than in RSK2 immunoprecipitates, we elected to map the domain in RSK3 that was responsible for the association with ERK1 and ERK2. To do so, several deletion mutants were constructed with these mutants were subjected to immunoprecipitation with the 12CA5 antibody. The resulting immunocomplexes were resolved by SDS-PAGE, followed by immunoblotting with the 12CA5 antibody. Another aliquot was used to measure ERK1 kinase activity using MBP as substrate. The incorporation of 32P into MBP by HA-ERK1 was assayed by separation of the mixture on 15% SDS-polyacrylamide gel and subsequent autoradiography. Kinase activity was measured using S6 peptide as substrate.
In Vitro Regulation of RSKs by ERK1/MAPK and MEK—
Mammalian RSK1 and RSK2 have been reported to be phos-
phorylated and partially activated in vitro by incubation with
ERK2 (8, 32). In contrast, we recently reported that the kinase
activity of RSK3 isolated from transfected COS cells was un-
affected by in vitro treatment with ERK2 (11). In the present
study, we compared the ability of all three RSK isoforms to be
activated in vitro by MAPK under the same experimental
conditions. As shown in Fig. 6, when recombinant RSK1, RSK2,
and RSK3 were immunoprecipitated from briefly starved
transfected COS cells and incubated with ERK1, the kinase
activity of RSK1 or RSK2 was increased 2–3-fold, whereas
RSK3 activity was unaffected. Alternatively, a similar experi-
ment was conducted using CHO cells that were stably trans-
fected with RSK3 cDNA (CHO-RSK3). CHO-RSK3 cells ex-
pressed much lower levels of RSK3 than transiently trans-
fected COS cells, as judged by immunoblotting of total cell
lysates (data not shown). Since (untransfected) CHO cells also
express substantial levels of RSK2 (data not shown), we were
able to compare the activity of recombinant RSK3 (isolated
using 12CA5) versus endogenous RSK2 (immunoprecipitated
with RSK2-PCT) to be activated in vitro by ERK1. In separate
experiments using COS cells transfected with RSK1, RSK2, or
RSK3, we demonstrated that the RSK2-PCT antibody recog-
nizes only RSK2 (data not shown). Unfortunately, antibodies
that we raised against RSK3 (11) fail to immunoprecipitate this
isoform. As shown in Fig. 6, ERK1 incubation increased the
activity of endogenous RSK2 derived from CHO cells, but had
no effect on RSK3, which was also recovered from CHO cells.
In additional experiments, immunoprecipitates containing RSK1
(from COS cells), RSK2 (from COS or CHO cells), or RSK3
(from COS or CHO cells) were incubated with protein phos-
phatase 2A, followed by removal of phosphatase and exposure to
ERK1 (Fig. 6). RSK1 and RSK2 were completely inactivated by
protein phosphatase 2A and partially (5–25%) reactivated by
ERK1. In contrast, RSK3 could only be partially inactivated by
protein phosphatase 2A (70%); subsequent ERK1 treatment
failed to promote RSK3 reactivation. Thus, both RSK1 and
RSK2 were activated by MAPK as previously reported (21, 44),
whereas RSK3 activity was unaffected by in vitro exposure to
ERK1, similar to what we previously observed using ERK2
(11).

RSK3 was shown to be associated with ERK1 and ERK2 in
vitro (Fig. 4), and EGF-mediated activation of RSK3 was also
shown to require the MEK1 signaling pathway (Figs. 2 and 3).
Since constitutively active MEK1 was sufficient to promote in
vitro activation of RSK3, we assessed whether in vitro incu-
bation of activated MEK1 with the ERK1/2-RSK3 complex would
augment RSK3 activity. Although both ERK1 and ERK2 in the

FIG. 6. Influence of in vitro ERK1/MAPK incubation on RSK
activity. Immunocomplexes were prepared from COS cells transfected
with epitope-tagged RSKs using antibody 12CA5 (for HA-RSK1, HA-
RSK2, or HA-RSK3), from CHO cells with RSK2-specific antibodies (for
endogenous RSK2), or from stably transfected CHO cells that express
HA-RSK3 with the 12CA5 antibody. Aliquots of these immunocom-
plexes were then incubated with or without ERK1/MAPK. Alterna-
tively, immunoprecipitated RSKs were first incubated with protein
phosphatase 2A (PP2A), followed by incubation with or without ERK1/
MAPK. After these incubations, kinase activity toward S6 peptide was
assayed. Results are expressed as a percentage of values obtained
without treatment with ERK1 or protein phosphatase 2A (referred to as
basal kinase activity and set at 100%). Background values obtained
using cells transfected with vector alone were <5% of basal kinase
activity. Similar results were obtained in three independent
experiments.
RSK3 immunocomplex were readily phosphorylated by MEK1. RSK3 activity was unaffected (data not shown). These results suggest that ERK1 and ERK2 that are physically associated with RSK3 are accessible and can be activated by MEK. However, once activated, RSK3-associated ERK1 and ERK2 are unable to augment the kinase activity of RSK3.

**DISCUSSION**

Several lines of investigation have suggested that RSK is phosphorylated and activated by ERK1/2 MAPK isoforms (14, 21, 47, 48). In addition to these earlier observations, Sutherland et al. (32) showed that RSK2 purified from rabbit skeletal muscle was partially reactivated by *in vitro* incubation with ERK2. They also identified two potential MAPK phosphorylation sites within a TPCYTA motif in subdomain VIII of the C-terminal kinase domain. MAPKs are known to be proline-directed (49), and only the first Thr in a synthetic peptide was shown to be phosphorylated by ERK2; thus, this residue was suggested to be the major MAPK phosphorylation site (32).

Furthermore, Grove et al. (8) showed that peptide maps of recombinant RSK1 isolated from 32P-labeled cells were similar to the pattern of *in vitro* RSK1 phosphorylation by ERK2. On the other hand, other evidence suggests that additional kinases may participate in the *in vivo* activation of RSK or that ERK1/2 MAPKs may not be upstream of RSK in a protein kinase cascade. These findings can be summarized as follows. 1) A growth factor-activated kinase(s) other than MAPK reportedly phosphorylates RSK *in vitro* (27). 2) In certain cell contexts, the kinetics of RSK phosphorylation and ERK1/2 activation may be somewhat discordant (16). 3) In contrast to the potent activation of MAPK by MEK, *in vitro* activation of RSK by ERK1 and ERK2 is only partial (8, 14, 47). 4) Induced expression of a Cdc2-like kinase in PC12 cells results in activation of RSK (50). 5) We recently showed that recombinant RSK3 from COS cells could not be reactivated *in vitro* by ERK2/MAPK (11). In addition, EGF-stimulated *in vivo* activation of RSK3 was preserved with mutation of the putative MAPK phosphorylation site (Thr-570), which is also present in this RSK isoform (12).

In this study, we sought to determine whether ERK1/2 MAPKs mediate *in vivo* activation of RSK and whether potential differences in the regulation of individual RSK isoforms by MAPK might exist. It is important to note that serum deprivation and EGF stimulation had different effects on the activity of RSK1 *versus* RSK2 and RSK3 when studies of transfected COS cells were performed. The activity of RSK1 paralleled that seen with ERK1: serum starvation markedly diminished activity, and subsequent EGF stimulation caused a >5-fold increase (compare Figs. 1B and 3A). In contrast, RSK2 and RSK3 retained substantial activity in the face of serum deprivation and were only stimulated 2–4-fold. Coexpression of dominant-negative MEK1 (34) attenuated the EGF-stimulated activity of all three RSK isoforms (Fig. 2). In addition, treatment with the synthetic MEK inhibitor PD98059 (35) substantially reduced EGF-mediated activation of RSK1, RSK2, and RSK3. It is important to note that the effects of PD98059 are specific for the MEK-ERK1/2 pathway since a number of other protein kinases including phosphatidylinositol 3-kinase and alternative MAPKs (SAPK/JNK and p38) are reportedly unaffected by the inhibitor (35). Using both approaches, we noted a small amount of residual EGF-stimulated RSK activation; this was likely due to the fact that inhibition of ERK activation was incomplete (see Fig. 3A). Different mechanisms of MEK inhibition can also be implicated since dominant-negative MEK1 tended to reduce basal RSK activity whereas PD98059 did not. This is consistent with previous results that suggest that PD98059 is an allosterically reversible inhibitor of MEK activation that does not appear to inhibit pre-activated MEK (35, 51).

Importantly, we also demonstrated that coexpression of constitutively active MEK1 caused a marked increase in the activity of each RSK isoform that was independent of serum starvation or EGF stimulation (Fig. 2). Therefore, our results indicate that MEK is necessary for *in vivo* growth factor-mediated activation of all three RSKs and that MEK activation is also sufficient to stimulate *in vivo* activation of the RSKs. Since ERK1 and ERK2 are the only known substrates for MEK, these findings suggest that activation of ERK1 and ERK2 is also both necessary and sufficient for activation of RSKs.

The above findings are supported by data that were recently reported by Sale et al. (52). These investigators found that insulin-mediated RSK activation (immunocomplex kinase assay using "anti-p90<sup>pp60</sup>" antibody) was diminished in 3T3-L1 cells where antisense oligonucleotides had been used to suppress ERK1 and ERK2 expression.

RSK protein is reportedly co-immunoprecipitated from PC12 cells with ERK1 (30) and from *Xenopus* oocytes with ERK2 (31). In the latter case, the association of RSK with ERK2 was evident with cells in G<sub>2</sub> phase (when MAPK is inactive), but was not detected in M phase oocytes with active ERK2. It was therefore suggested that MAPK-RSK exists as an inactive heterodimer that dissociates upon concomitant activation of both enzymes (31). To correlate MAPK dependence of RSK activation with the potential for an *in vivo* physical association between RSKs and ERK1/2, immunoprecipitates from COS cells expressing individual RSK isoforms were immunoblotted with anti-ERK1/2 antibodies. Unfortunately, we could not utilize the opposite approach (MAPK immunoprecipitation followed by RSK immunoblotting) since the anti-ERK1/2 antisera was not useful for immunoprecipitation of MAPK under nonenriching conditions. Despite the fact that MAPK was required for EGF-mediated activation of all three RSK isoforms, ERK1 and ERK2 were detected only in RSK2 and RSK3 immunoprecipitates. It is distinctly possible that MAPKs directly interact with RSK1 in a transient fashion, which was not strong enough to be detected using the co-immunoprecipitation approach. This may be particularly important in light of the fact that RSK1 was the most responsive to growth factor stimulation (which was sensitive to MEK1 inhibition).

In contrast to the results reported by Hsiao et al. (31) using *Xenopus* oocytes, we also demonstrated that both inactive (dephosphorylated) and active (phosphorylated) ERK1 and ERK2 were associated with RSK2 or RSK3 to a similar extent (see Fig. 4). Additionally, we used a mutant form of RSK3 (ablation of both ATP-binding sites) (12) to show that ERK1 and ERK2 were also readily immunoprecipitated by kinase-deficient RSK3 (data not shown). Thus, the physical association between RSKs and MAPKs in mammalian cells appears to be independent of the state of activation of both ERK1 and ERK2, and of RSK.

ERK1/2 abundance was greatest in RSK3 immunocomplexes, despite lower amounts of this isoform in soluble cell protein lysates. We therefore used RSK3 deletion mutants to map the protein domain responsible for the physical association with MAPKs. ERK1/2 association with RSK3 was preserved with selective deletion of either kinase domain or with deletion of the RSK3-specific C-terminal extension region. In contrast, expressed proteins with a deletion of the C terminus (44 residues) or replacement of the last 33 amino acids with the RSK1 C terminus lost the ability to associate with ERK1 and ERK2. This region of RSK3 is more homologous to RSK2 (70%) than to RSK1 (57%), suggesting that an amino acid motif that is common to RSK2 and RSK3 accounts for their ability to form a more stable complex with MAPKs. It is also interesting to note that the C-terminal truncation mutant of RSK3...
(RSK3ΔC) displayed a ~60% reduction in basal kinase activity
and was minimally EGF-responsive (~1.3-fold) (data not
shown). In contrast, the chimeric protein in which the RSK3 C
terminus was replaced with residues from RSK1 displayed the
normal EGF stimulation characteristics of RSK3 (data not
shown). These results are consistent with a model in which
both RSK1 and RSK3 physically interact with ERK1 and ERK2
through their respective C termini (with different kinetics);
this interaction may also be required for growth factor-mediated
RSK activation.

As discussed above, we previously showed that RSK3 activity
was unaffected by in vitro incubation with ERK2 (11). This
result contrasts with the ability of RSK3 to strongly associate
with ERK1 and ERK2 and its dependence on MEK1 for in vivo
activation by EGF. Therefore, we sought to directly compare
the potential in vitro regulation of all three RSKs by MAPK.
With recombinant RSK1 and either recombinant or endoge-
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