MOLECULAR AND CELLULAR BIOLOGY, Mar. 1996, p. 1212–1219 Vol. 16, No. 3
0270-7306/96/$04.00+0

Evidence for Two Catalytically Active Kinase Domains in pp90^{rsk}

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Received 20 July 1995/Returned for modification 15 September 1995/Accepted 16 December 1995

Mitogen-activated protein kinase and one of its targets, pp90^{rsk} (ribosomal S6 kinase [RSK]), represent two serine/threonine kinases in the Ras-activated signalling cascade that are capable of directly regulating gene expression. pp90^{rsk} has been shown to have two highly conserved and distinct catalytic domains. However, whether both domains are active and which domain is responsible for its various identified phosphotransferase activities have not been determined. Here we demonstrate that the N-terminal domain is responsible for its phosphotransferase activity towards a variety of substrates which contain an RXXS motif at the site of in vitro phosphorylation, including serum response factor, c-Fos, Nur77, and the 40S ribosomal protein S6. We also provide evidence that the C-terminal domain is catalytically active and can be further activated by mitogen-activated protein kinase phosphorylation.

Two families of growth factor-regulated S6 serine/threonine kinases have been identified and characterized. One of these includes several enzymes ranging in molecular size from 85 to 92 kDa which are referred to collectively as ribosomal S6 kinase (RSK) (3). The other family of S6 kinases has two members (with molecular masses of ~70 and ~85 kDa, referred to collectively as pp70^{S6k}) that are expressed from a single gene which is alternately spliced (16). Although both families of S6 kinases are growth regulated and phosphorylate S6 in vitro, several lines of evidence indicate that pp70^{S6k} is the S6 kinase responsible for regulating S6 phosphorylation in somatic cells (2, 4, 10). Several candidate RSK substrates that are phosphorylated by RSK in vitro at sites that are phosphorylated in vivo have been identified. Furthermore, the kinetics of their phosphorylation in vivo correlates with the activation of RSK, as does their cellular distribution. These include the transcription factors c-Fos (6), serum response factor (SRF) (24), and Nur77 (13) and the serine/threonine kinase glycogen synthase kinase 3-β (28, 31). However, direct evidence that these are targets of RSK in the cell is lacking.

One interesting feature of RSK is that its amino acid sequence predicts the presence of two unrelated protein serine/threonine kinase domains that are likely not the result of gene duplication (1, 20). The C-terminal catalytic domain shares significant homology (30% identity) with the catalytic domain of phosphorylase *b* kinase. The N-terminal catalytic domain resembles the catalytic domains of cyclic AMP (cAMP)-dependent kinase, protein kinase C, and pp70^{S6k}. Because of the homology to pp70^{S6k} and because these three related kinases are also capable of phosphorylating S6 in vitro, it has been hypothesized that the N-terminal catalytic domain is responsible for the S6 phosphotransferase activity of RSK. However, whether this domain phosphorylates S6, c-Fos, SRF, or Nur77 has not yet been demonstrated. In spite of the homology of the RSK C-terminal catalytic domain to phosphorylase *b* kinase, RSK has not been shown to phosphorylate phosphorylase *b* in vitro (14) and there is no evidence that the C-terminal kinase domain is active.

RSK phosphotransferase activity towards its known in vitro substrates is regulated by serine/threonine phosphorylation. Evidence in support of this conclusion includes (i) a correlation between mitogen-stimulated phosphorylation and activation (7, 15, 32), (ii) loss of activity following phosphatase treatment (7, 15), and (iii) partial activation following phosphorylation by either of two *erk*-encoded mitogen-activated protein kinases (MAPKs), p42^{MAPK} and p44^{MAPK} (11, 29). In addition to being coordinately regulated with RSK, MAPK has been shown to directly phosphorylate or regulate the phosphorylation of RSK at several serine and threonine residues in vitro at sites phosphorylated in vivo (9). Indeed, one potential MAPK phosphorylation site has been identified in a murine RSK homolog, rsk^{mo}-2, as a threonine in subdomain VIII of the C-terminal catalytic domain (30). An analogous phosphorylation site has also been identified as a conserved regulatory phosphorylation site in several protein kinases such as cAMP-dependent protein kinase, cyclin-dependent kinases, and MAPK (33). Additionally, MAPK and RSK have been shown to physically interact (19, 26) and we have mapped the interaction site of MAPK to the C terminus of RSK (17). Together, this information suggests that MAPK might regulate the activity of the C-terminal catalytic domain of RSK.

To better understand how MAPK regulates RSK activity, we have constructed two mutants of RSK, one which inactivates the N-terminal catalytic domain and the other which inactivates the C-terminal catalytic domain. We have also expressed both halves of RSK separately in *Escherichia coli* to examine their activities. We provide evidence that the N-terminal domain is responsible for phosphorylation of several in vitro targets of RSK, including S6, c-Fos, SRF, and Nur77. We also provide evidence that the C-terminal domain is active and can be further activated in vitro by MAPK phosphorylation. These results suggest the possibility that the N-terminal catalytic domain of RSK is regulated upon activation of the C-terminal catalytic domain following its phosphorylation by MAPK.

MATERIALS AND METHODS

Materials. Tissue culture media and sera were purchased from GIBCO (Grand Island, N.Y.). Enhanced chemiluminescence reagents were obtained from Amersham (Amersham, England). [γ-^{32}P]ATP was procured from New England Nuclear (Boston, Mass.). Recombinant proteins c-Fos, Nur77, and SRF were gifts from T. Curran, L. F. Lau, and M. Greenberg, respectively. The recombinant plasmid pGEX2T-S6 was a gift from C. Kuo and G. Crabtree. The retroviral expression vector pMV7 was a gift from M. Johnson.

Generation of polyclonal antiserum. Peptide-specific rabbit polyclonal antiserum was prepared against amino acids 32 to 51 (NPPCKAKSDITWVEKD LVDS) of the avian RSK predicted protein sequence, which is absent from mammalian sequences (1). Rabbit polyclonal antiserum against amino acids 733

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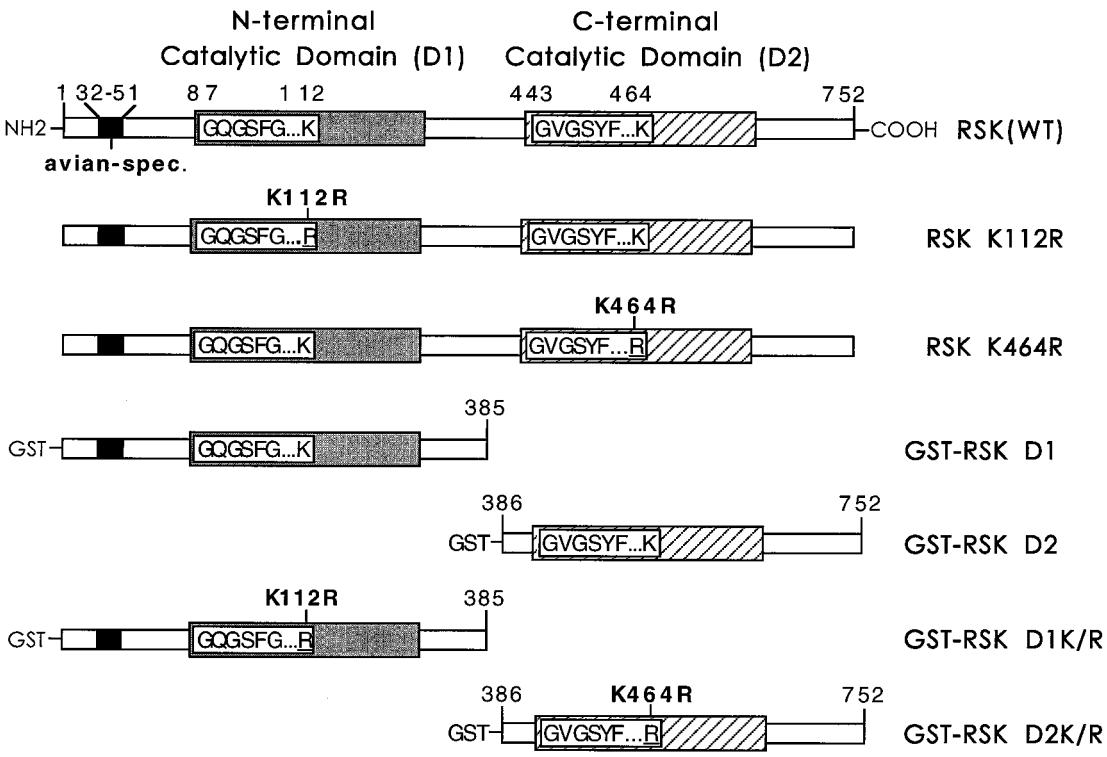

FIG. 1. Schematic diagram of pp90^{rsk} cDNA. All mutant and GST fusion protein constructions employed the avian pp90^{rsk} cDNA containing a unique sequence (black box) against which avian-specific antiserum was generated. The catalytic domains (gray and striped boxes) are represented by the GXXG. . .K motif found in conserved region II of the catalytic domain of protein kinases. Catalytically inactive mutants were generated by changing lysine 112 to arginine in the N-terminal catalytic domain or the corresponding lysine 464 to arginine in the C-terminal catalytic domain. The N-termini of pp90^{rsk} constructs D1 and D2 (amino acids 1 to 385 and 386 to 752, respectively) were tagged with GST.

to 752 (PIKSSILAQRRVKKLPSTTL) of the avian RSK predicted protein sequence, which is 80% identical to the murine C-terminal RSK sequence (1), was also raised. Antiserum recognizing the C terminus of MAPK has been previously described (8). A synthetic C-terminal MAPK- or ERK-activating kinase (MEK) peptide made according to predicted amino acids 375 to 393 (CSTIGLNQPS TPTHAASI) of the murine MEK1 sequence (12) was used to raise antiserum in rabbits.

Generation of pp90^{rsk} mutants. The pp90^{rsk} mutants were generated by the method of Kunkel et al. (22). The template was chicken *rsk* cDNA (1) cloned into M13mp18. The oligonucleotide sequences used for mutagenesis are available upon request and will not be described here. The wild-type and mutant *rsk* cDNAs were individually subcloned into the *Eco*RI site of the pMT2 plasmid. All point mutations were confirmed by sequencing upon each round of subcloning.

Expression and purification of recombinant proteins from bacteria. Glutathione *S*-transferase (GST) fusion proteins containing either the N-terminal catalytic domain (D1, amino acids 1 to 385) or the C-terminal catalytic domain (D2, amino acids 386 to 752) of avian pp90^{rsk} or full-length rat ERK1 were constructed with PCR-generated fragments subcloned into the pGEX2T plasmid vector (Pharmacia). The oligonucleotide sequences used for PCR are available upon request and will not be described here. Recombinants were transformed into *E. coli* DH5α, and fresh bacterial cultures were grown at 37°C to an optical density (A_{600}) of 2.0. Expression of recombinant proteins was induced with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at 28°C for 6 h. The bacteria were harvested and lysed by sonication in buffer G (phosphate-buffered saline [PBS]–50 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride). Postsonication, Triton X-100 was added to a 1% (vol/vol) final concentration and lysates were clarified by centrifugation at 12,000 × *g*. Soluble GST fusion proteins were adsorbed to glutathione-Sepharose beads (GIBCO), washed, and eluted with 20 mM reduced glutathione (Sigma)–50 mM Tris (pH 8.0). The protein concentrations of the fusion proteins (and other recombinant proteins used as substrates for in vitro kinase assays) were determined by Coomassie brilliant blue R-250 (Sigma) staining with bovine serum albumin (BSA) as a standard. All recombinant proteins were stable with respect to degradation under all assay conditions as determined by Coomassie staining.

Transient expression of pp90^{rsk}. BHK cells were maintained in Dulbecco's modified Eagle medium supplemented with 5% (vol/vol) calf serum and 20 U of penicillin–20 μg of streptomycin per ml. For transient expression of pp90^{rsk} constructs, a confluent 10-cm-diameter tissue culture dish was subcultured 1:40 approximately 16 h prior to transfection. BHK cells were used for these experiments because of the high transfection efficiency achieved. BHK cells were transfected with pMT2-based plasmids encoding wild-type or mutant RSK (5 or 10 μg per 10-cm-diameter dish) by the calcium phosphate method (25). The medium was replaced with Dulbecco's modified Eagle medium containing 0.5% (vol/vol) calf serum and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4) 16 h posttransfection, and serum stimulation (final concentration, 10% [vol/vol]; 7.5 min) was carried out 24 h thereafter.

Stable expression of pp90^{rsk}. A cDNA encoding the open reading frame of mutant avian *rsk* K464R (K-to-R mutation at position 464) was cloned into the *Eco*RI site of pMV7, a retroviral expression vector previously described (21). Recombinant retroviral plasmid (10 μg) was transfected into a packaging cell line, Ψ_{CRE} cells, as detailed above. These transfected cells were cultivated in the presence of active G418 (400 μg/ml; GIBCO) for 2 weeks, conditions under which parental Ψ_{CRE} cells all perish. Pools of neomycin-resistant Ψ_{CRE} cells were tested for chicken pp90^{rsk} expression by immunoblot analysis as detailed below. Conditioned medium was harvested from pooled chicken pp90^{rsk}-expressing Ψ_{CRE} cells after 24 h in the absence of G418. This virus-containing medium was filtered through a 0.2-μm-pore-size filter and added 1:1 to normal medium with 2 μg of Polybrene (Sigma) per ml on a subconfluent 10-cm-diameter tissue culture dish of NIH 3T3 cells. After 48 h, these cells were washed once with PBS and then cultivated in the presence of G418 for 2 weeks. Neomycin-resistant clones were isolated and further screened by immunoblot analysis for chicken pp90^{rsk} expression.

Immunoblot analyses. Total cell lysate was prepared as previously described (7), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. The membranes were first blocked in PBS with 0.2% Tween 20–2% BSA and then incubated with the avian-specific RSK antiserum (1:1,000 in blocking buffer) (see Fig. 2) and with the C-terminal RSK antiserum (1:1,000) and C-terminal MAPK antiserum (1:2,000) together (see Fig. 10). Secondary antibody, anti-rabbit immunoglobulin G coupled to peroxidase, was detected by enhanced chemiluminescence (enhanced chemiluminescence reagents; Amersham).

Immune-complex kinase assays. Cell lysates were prepared and immune-complex kinase assays for pp90^{rsk} were performed as previously described (7), with 1 μg of recombinant GST-S6, c-Fos, SRF, or Nur77 as substrate. The reaction was in a total volume of 30 μl and incubated at 30°C for 15 min. ^{32}P incorporation was quantitated by the Molecular Dynamics PhosphorImager and

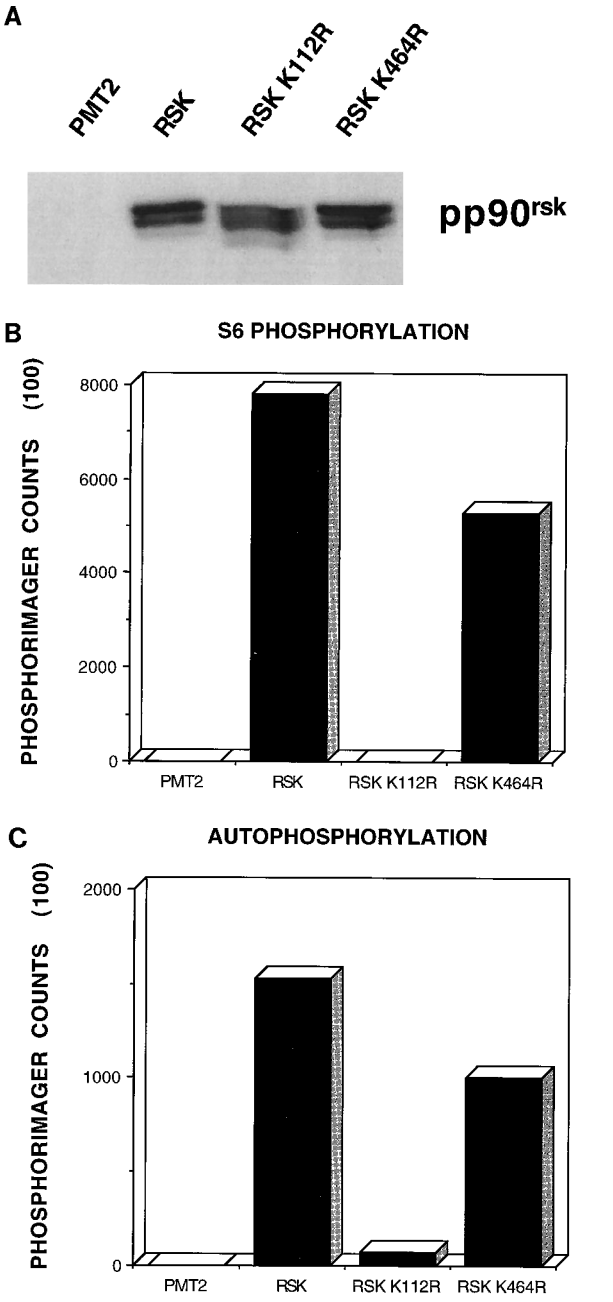

FIG. 2. Transient expression of pp90^{rsk} in BHK cells. (A) Western blot (immunoblot) of total cell lysate with the avian-specific pp90^{rsk} antibody. Full-length pp90^{rsk} enzyme was expressed as wild-type protein (RSK) or with a single-point mutation (K112R or K464R) and isolated from serum-stimulated cells. PMT2 represents the transient transfection of the vector plasmid alone. (B) S6 kinase activities of transiently expressed wild-type and K-to-R mutants of pp90^{rsk}. Lysates from BHK cells transiently transfected with vector alone (pMT2), RSK, K112R, or K464R were immunoprecipitated with avian-specific pp90^{rsk} antiserum and by immune-complex kinase assays performed with S6 as substrate. (C) Autophosphorylation activities of wild-type pp90^{rsk}, and K112R and K464R point mutants. Assays were performed as above in the absence of any exogenous substrate. The results depicted in panels B and C are representative of four sets of experiments.

ImageQuant software (see Fig. 2, 3, 4, and 10) and by scintillation counting of the appropriate excised band (see Fig. 6, 7, and 8).

Preparation of activated GST-ERK1*. Swiss 3T3 cells were serum starved for 48 h and then stimulated for 5 min with 10% (vol/vol) calf serum and 1 nM calyculin A. Cell-free lysates were immunoprecipitated with antiserum against MEK1 under conditions as described above and by immune-complex kinase assays performed in the presence of unlabelled ATP for 2 h at 30°C with GST-ERK1 as the substrate. The MEK immune complex was cleared from the reaction mixture by centrifugation, and the supernatant containing the activated GST-ERK1 was dialyzed against 50 mM Tris (pH 8.0)–100 mM NaCl–50% (vol/vol) glycerol.

Two-dimensional phosphopeptide mapping. Purified recombinant GST-RSK D2 or the GST-RSK D2 K-to-R mutant (GST-RSK D2 K/R) (2 μg) was phosphorylated in vitro by MEK-activated GST-ERK1 (0.5 μg) or allowed to autophosphorylate in the presence of [γ-^{32}P]ATP for 20 min under the assay conditions described in the legend to Fig. 5. Reaction products were resolved by SDS-PAGE, and phosphorylated recombinant RSK proteins were identified by autoradiography. The phosphorylated proteins were eluted from the wet gel and processed for tryptic phosphopeptide analysis as previously described (7).

RESULTS

Mutant constructions. In order to address the function of the N-terminal and C-terminal catalytic domains of RSK, we generated point mutants by changing each of the required lysines in the ATP-binding domains to arginine in the context of the full-length avian protein for expression in mammalian cells (Fig. 1). The N-terminal mutation is a lysine 112-to-arginine mutation. The C-terminal mutation is a lysine 464-to-arginine mutation. The avian sequence has greater than 79% identity with *Xenopus*, murine, and human sequences (1, 20, 23). Previous experiments have shown that the avian, murine, and human RSK proteins are regulated in a similar fashion (7). In addition, the avian protein has a unique sequence (amino acids 32 to 51) against which we have generated polyclonal antiserum, allowing us to differentiate between endogenous and transfected RSK. We have also expressed each half of RSK, with or without the K-to-R mutation, as GST fusion proteins in *E. coli*. These various mutant constructions are illustrated in Fig. 1.

The RSK N-terminal catalytic domain phosphorylates S6, c-Fos, SRF, and Nur77. BHK cells were transiently transfected with the various RSK point mutant constructs and assayed for RSK phosphotransferase activity (Fig. 2). Serum stimulation was used to ensure that the RSK enzyme was activated. Expression of the wild-type, K112R, and K464R alleles was detected by immunoblot analysis (Fig. 2A). Lysates containing approximately equivalent amounts of the exogenous RSK protein were immunoprecipitated with avian-specific RSK antiserum and subjected to immune-complex kinase assays with S6 as a substrate (Fig. 2B). These data demonstrate that the transiently expressed wild-type protein is active and phosphorylates S6, as does the K464R mutant. The K112R mutant was incapable of phosphorylating S6. These results demonstrate that the N-terminal catalytic domain is responsible for S6 phosphotransferase activity in vitro.

RSK has been shown to phosphorylate several transcription factors in vitro, including c-Fos, SRF, and Nur77 (6, 13, 24). We tested whether phosphorylation of these transcription factors was mediated by the N- or C-terminal catalytic domain. The results shown in Fig. 3 demonstrate that the N-terminal catalytic domain of RSK is responsible for phosphorylation of all three transcription factors in vitro.

We also examined the ability of the exogenous wild-type and mutant RSK proteins to autophosphorylate in vitro (Fig. 2C). The wild-type and K464R alleles exhibited readily detectable autophosphorylating activity. However, the K112R mutant displayed greatly reduced, though reproducible, autophosphorylating activity. This result suggests that the autophosphorylation of RSK is predominantly mediated by its N-terminal kinase domain.

In order to determine if the N-terminal kinase domain activity observed with RSK K464R was growth regulated, we stably expressed this mutant in NIH 3T3 cells by using the

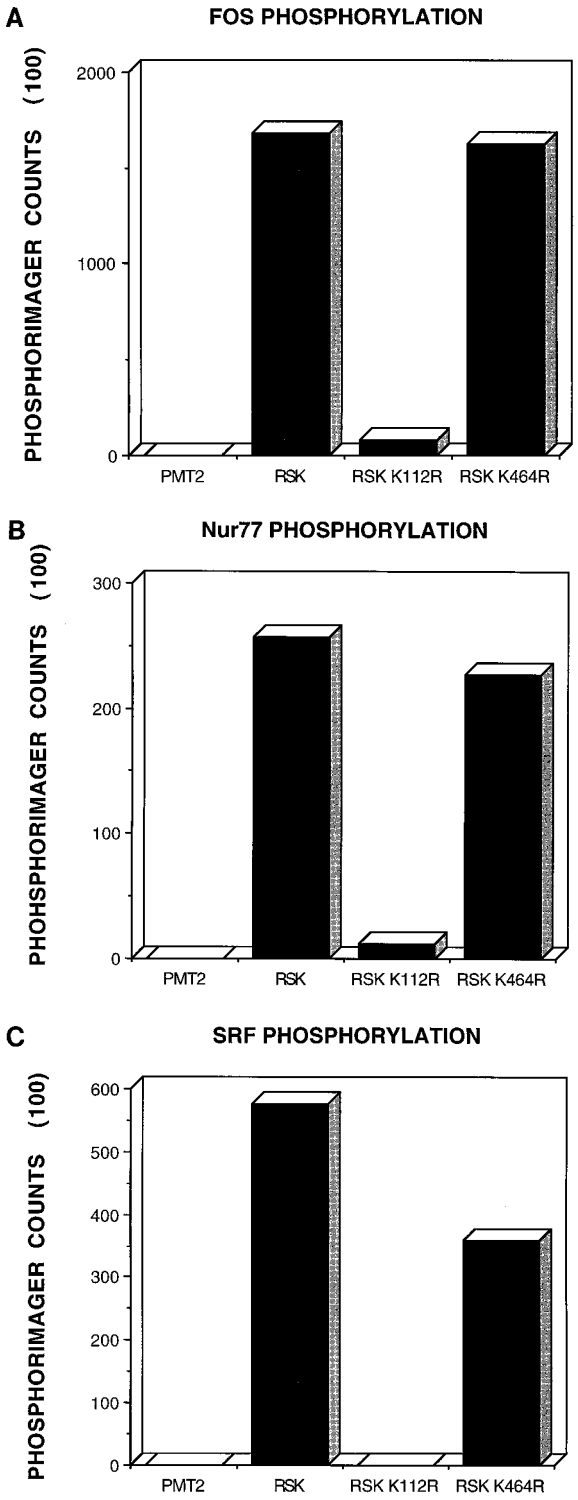

FIG. 3. Kinase activities of wild-type and K-to-R mutants of pp90^{rsk} towards c-Fos, Nur77, and SRF. Immune-complex kinase assays were performed as described in the legend to Fig. 2B, with recombinant c-Fos (A), Nur77 (B), or SRF (C) as substrates for the transiently expressed wild-type RSK, K112R, and K464R. The results depicted are representative of two sets of experiments.

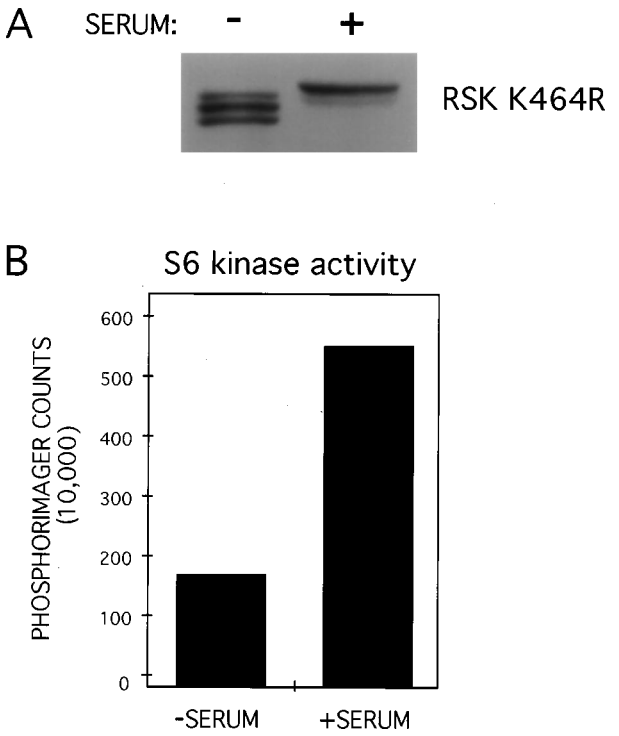

FIG. 4. RSK K464R exhibits mitogen-activated phosphotransferase activity towards S6. The mutant avian RSK K464R was stably overexpressed in NIH 3T3 cells. These cells were starved in 0.5% (vol/vol) calf serum for 24 h (−) and stimulated with 10% (vol/vol) calf serum for 10 min prior to harvesting (+). Whole-cell lysates were subjected to immunoblot analysis (A) with the avian-specific RSK antiserum and by immune-complex assays with S6 as substrate as described in Materials and Methods. Results depicted in panel B are representative of two sets of experiments.

pMV7 retroviral expression system (21). We have previously stably expressed wild-type avian RSK in NIH 3T3 cells by using this system and found the ectopically expressed enzyme to be mitogen regulated in a similar fashion to the endogenous kinase (7). As shown in Fig. 4A, the ectopically expressed RSK K464R protein exhibited a decreased electrophoretic mobility upon serum stimulation, consistent with RSK protein hyperphosphorylation. Consistent with its hyperphosphorylation, RSK K464R S6 phosphotransferase activity was stimulated upon serum addition (Fig. 4B). We have been unable to stably express RSK K112R in NIH 3T3 cells by using the same retroviral system. Interestingly, we have also observed reduced expression of RSK K112R compared with that of RSK K464R in the transient-expression experiments in BHK cells, suggesting a potential negative growth effect of the N-terminal kinase inactive protein. Experiments are now under way to determine if RSK K112R indeed inhibits growth.

The isolated C-terminal catalytic domain of RSK exhibits phosphotransferase activity. The experiments depicted in Fig. 2 indicated that the C-terminal catalytic domain of RSK possessed a small but reproducible amount of autophosphorylation activity. To explore this possibility further, GST fusions of the isolated N- and C-terminal catalytic domains of RSK (D1 and D2, respectively) were expressed as soluble proteins in *E. coli* and purified from a glutathione affinity resin. As shown in Fig. 5, purified GST-RSK D2 wild type (WT) was capable of autophosphorylating. The ability of GST-RSK D2 to autophosphorylate was dependent upon an active catalytic domain since GST-RSK D2 K/R no longer autophosphorylated. Under these

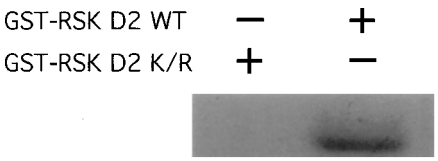

FIG. 5. The C-terminal kinase domain of pp90^{rsk} exhibits autophosphorylation activity. Purified recombinant GST-RSK D2 WT or GST-RSK D2 K/R (2 μg) was incubated in kinase buffer (10 mM $MgCl_2$–20 mM HEPES [pH 7.2]–30 mM β-mercaptoethanol–1 mg of BSA per ml) with 50 μM [γ-^{32}P]ATP (30 cpm/fmol) in a total volume of 30 μl for 20 min at 30°C.

conditions, GST-RSK D1 did not autophosphorylate and GST-RSK D2 did not phosphorylate GST protein when added to the kinase reaction (data not shown). One possible explanation for the lack of detectable autophosphorylating activity with GST-RSK D1 is that the recombinant protein may not be in a fully native conformation.

In order to determine if the kinase activity observed with GST-RSK D2 alone was due to an intramolecular or intermolecular mechanism, experiments were performed to examine the effects of limiting dilution on GST-RSK D2 phosphorylation. First, as shown in Fig. 6, we examined the rate of GST-RSK D2 autophosphorylation and compared it with that of GST-ERK1 under conditions similar to those used to define autophosphorylation of GST-ERK2 (27, 35). We found the initial rates of autophosphorylation of GST-RSK D2 and GST-ERK1 to be similar. Further characterization revealed that GST-RSK D2 phosphorylation was not affected by limiting dilution (Fig. 7A) and that the initial rate of this autokinase activity was also concentration independent (Fig. 7B). Combined, these results are consistent with the conclusion that soluble GST-RSK D2 is capable of autophosphorylation via an intramolecular mechanism. We have been unable to detect phosphotransferase activity towards several exogenous substrates with GST-RSK D2 or GST-RSK D1 in vitro.

The isolated RSK C-terminal kinase domain autophosphorylation activity can be further stimulated by MAPK. Current

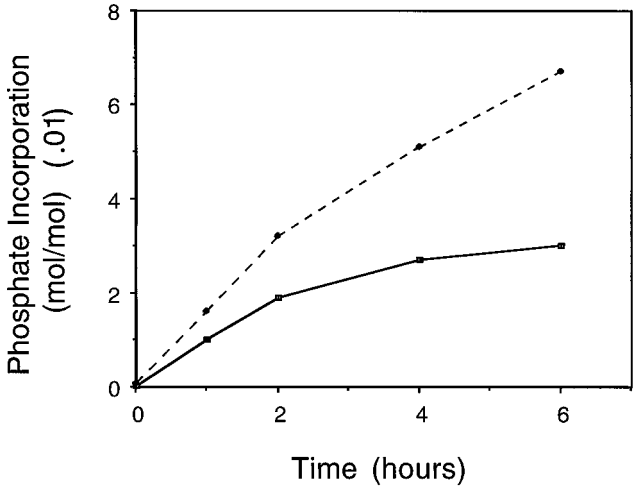

FIG. 6. Autophosphorylation time course for GST-ERK1 and GST-RSK D2. Purified recombinant ERK1 (4 μg; broken line) or RSK D2 (4 μg; solid line) was incubated in a total volume of 80 μl with kinase buffer and 50 μM [γ-^{32}P]ATP (44 cpm/fmol) at 30°C. At the indicated times, aliquots (20 μl) of the kinase reaction mixture were removed and the reaction was terminated with sample buffer. After SDS-PAGE, the ERK1 and RSK D2 bands were excised and the ^{32}P incorporation was determined by scintillation counting. These results are representative of four sets of experiments.

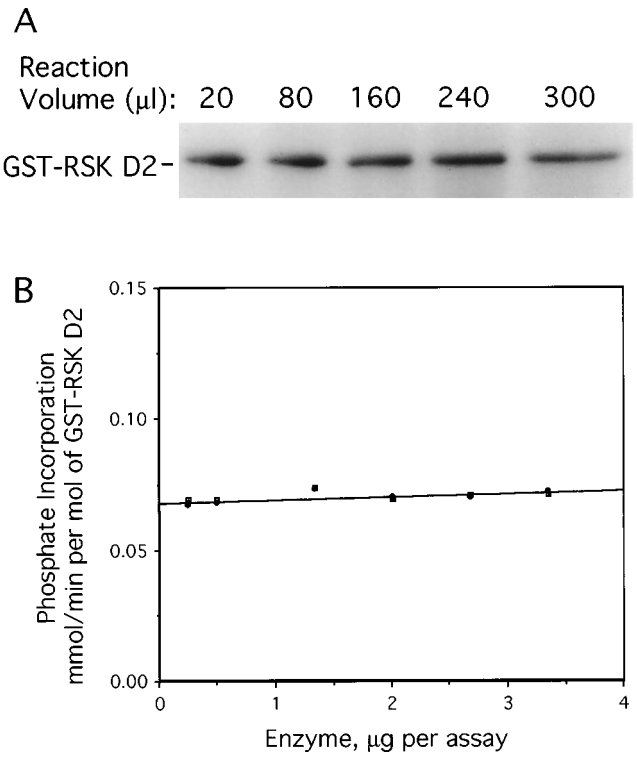

FIG. 7. (A) Effect of limiting dilution on the autophosphorylation of GST-RSK D2. Purified recombinant GST-RSK D2 (2 μg) was autophosphorylated for 30 min under kinase assay conditions as described in the legend to Fig. 5 in total reaction volumes ranging from 20 to 300 μl. ^{32}P incorporation was equivalent for all lanes as quantitated by a Molecular Dynamics PhosphorImager and ImageQuant software. (B) Effect of enzyme concentration on the initial rate of GST-RSK D2 autophosphorylation. Samples of GST-RSK D2 (0.2 to 3.4 μg) were autophosphorylated in 30 μl of kinase buffer and 50 μM [γ-^{32}P]ATP (30 cpm/fmol) for 10 min at 30°C. After SDS-PAGE, the rate of autophosphorylation for GST-RSK D2 was determined by liquid scintillation counting of the excised RSK band.

evidence indicates that a threonine in subdomain VIII of the C-terminal catalytic domain of rsk^{mo}-2 is the site targeted by MAPK (30). This result and the fact that this conserved phosphorylation site is regulatory in many other protein kinases predict that MAPK regulates the C-terminal catalytic domain of RSK. To investigate this possibility, we asked if MAPK phosphorylation stimulated the observed autophosphorylating activity of GST-RSK D2. For these experiments, soluble GST-MAPK (ERK1) was purified from *E. coli* and activated in vitro by MEK. As shown in Fig. 8, phosphorylation of RSK D2 by MEK-activated MAPK (GST-ERK1*) appeared to further stimulate its autophosphorylating activity severalfold, indicating that MAPK can directly modulate the activity of the C-terminal kinase domain of RSK. GST-ERK1* failed to phosphorylate GST-RSK D1 WT or K/R (data not shown).

The phosphorylation of GST-RSK D2 was further characterized by two-dimensional tryptic phosphopeptide mapping as shown in Fig. 9. The map of GST-RSK D2 that was allowed to autophosphorylate (equivalent to lane 1 in Fig. 8) revealed a single tryptic phosphopeptide, indicated with an asterisk (Fig. 9A). In vitro phosphorylation of GST-RSK D2 K/R by GST-ERK1* (Fig. 9B) yielded a map containing a single major phosphopeptide (open arrow) and several minor phosphopeptides. Analysis of GST-RSK D2 WT that was phosphorylated by GST-ERK1* in vitro resulted in a more complex tryptic phosphopeptide map (Fig. 9C). Spots seen in Fig. 9C included

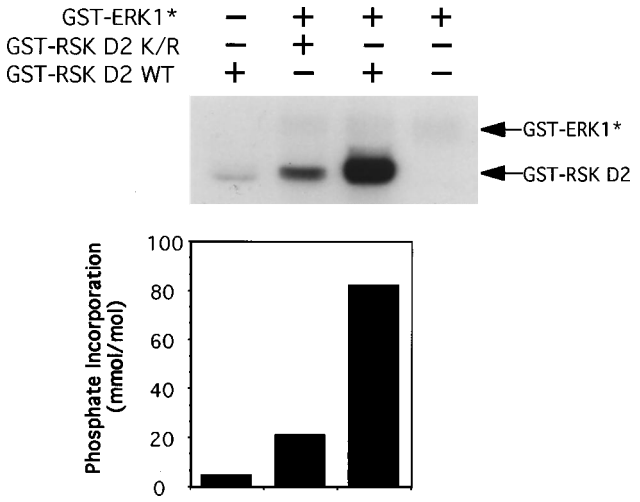

FIG. 8. RSK C-terminal kinase domain autophosphorylation activity can be further stimulated by MAPK. GST-RSK D2 or GST-RSK D2 K/R (2 μg) was incubated with MEK1-activated MAPK (GST-ERK1*; 0.5 μg) and subjected to an in vitro kinase assay for 20 min as described in the legend to Fig. 5. ^{32}P incorporated into GST-RSK D2 (WT or K/R) was determined by scintillation counting of the excised RSK band. The results depicted by the bar graph are representative of four sets of experiments.

the tryptic phosphopeptide obtained upon autophosphorylation of GST-RSK D2 (asterisk), the spots seen as a result of GST-ERK1* phosphorylation of GST-RSK D2 K/R (open arrow, for example), and the appearance of several new tryptic phosphopeptides (solid arrows). These novel tryptic phosphopeptides appear to be the result of new sites targeted by intramolecular autophosphorylation of the activated RSK D2. Mixing experiments confirmed that these peptides were indeed novel and that the peptides labelled with the asterisks were identical (data not shown).

To unambiguously assess the ability of GST-ERK1* to stimulate the autophosphorylating activity of the C-terminal kinase domain of RSK, we incubated GST-RSK D2 with GST-ERK1* or GST-ERK1 K/R (catalytically inactive MAPK) under kinase assay conditions in the presence of unlabelled ATP. The enzymes were then separated by Mono Q anion exchange chromatography (Fig. 10A and B) and the ability of the purified GST-RSK D2 to autophosphorylate was examined. As shown in Fig. 10C and D, the ability of the GST-RSK D2 incubated with GST-ERK1* to autophosphorylate was activated sevenfold over that of the GST-RSK D2 incubated with GST-ERK1 K/R under conditions with equal amounts of the purified RSK proteins. These results support the data in Fig. 8 and 9 which indicate that MAPK phosphorylation of the C-terminal RSK catalytic domain activates its phosphotransferase activity.

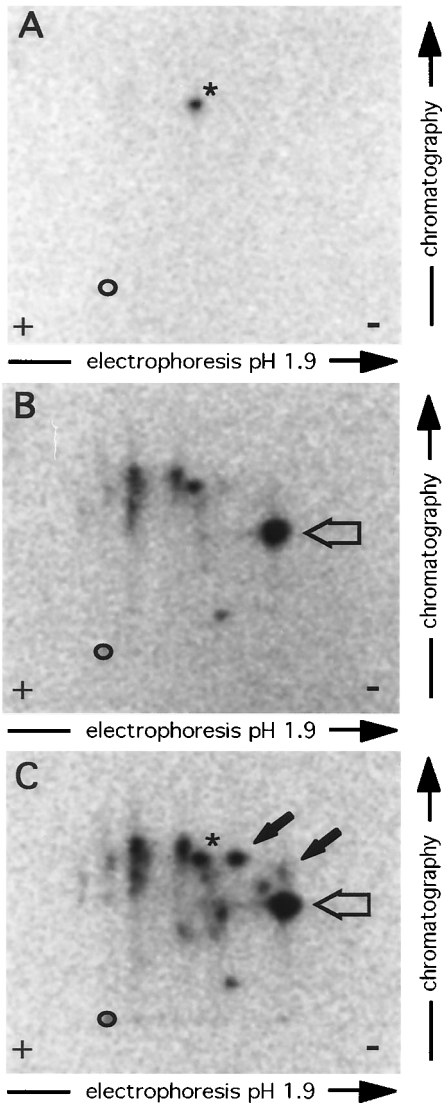

FIG. 9. Two-dimensional tryptic phosphopeptide analysis of RSK D2 phosphorylation. GST-RSK D2 was phosphorylated under the conditions described in the legend to Fig. 8. Phosphorylated products were eluted from the SDS-polyacrylamide gel and processed as described in Materials and Methods. (A) Tryptic phosphopeptide map of GST-RSK D2 allowed to autophosphorylate; (B) tryptic phosphopeptide map of GST-RSK D2 K/R phosphorylated in vitro by GST-ERK1* (MEK-activated MAPK); (C) tryptic phosphopeptide map of GST-RSK D2 WT phosphorylated in vitro by GST-ERK1*. Asterisks denote the migrations of the single phosphopeptides detected under autophosphorylation conditions used for GST-RSK D2. Open arrows indicate major phosphopeptides seen upon GST-ERK1* phosphorylation of GST-RSK D2 K/R. Filled arrows denote the migration of several new tryptic phosphopeptides phosphorylated as a result of the activation of RSK D2 by MEK-activated MAPK. The open circle in each panel marks the origin.

DISCUSSION

In this report we have begun to address the question of how MAPK regulates the activity of one of its downstream targets, pp90^{rsk}. This is of interest for many reasons. First, RSK has two distinct, highly conserved protein kinase domains (20) that possibly provide a mechanism for receiving upstream signals, amplifying them, and transmitting them along distinct signalling paths. Second, RSK, along with MAPK, transmits growth factor-regulated signals to the nucleus for the modulation of immediate-early gene expression (8). Indeed, RSK has been shown to phosphorylate several transcription factors in vitro at sites phosphorylated in vivo, and the activity of RSK parallels these phosphorylation events in many instances (3). Additionally, RSK can regulate yet another protein kinase in this cascade, glycogen synthase kinase 3-β, which has been proposed to regulate the phosphorylation and function of several targets, such as c-Jun (5, 28). Third, understanding the regulation of MAPK and RSK may be important not only in early growth factor-regulated signalling events but also for regulation of the G_2-to-M transition, particularly during *Xenopus* oocyte maturation (15, 34).

Although RSK was first cloned and sequenced several years

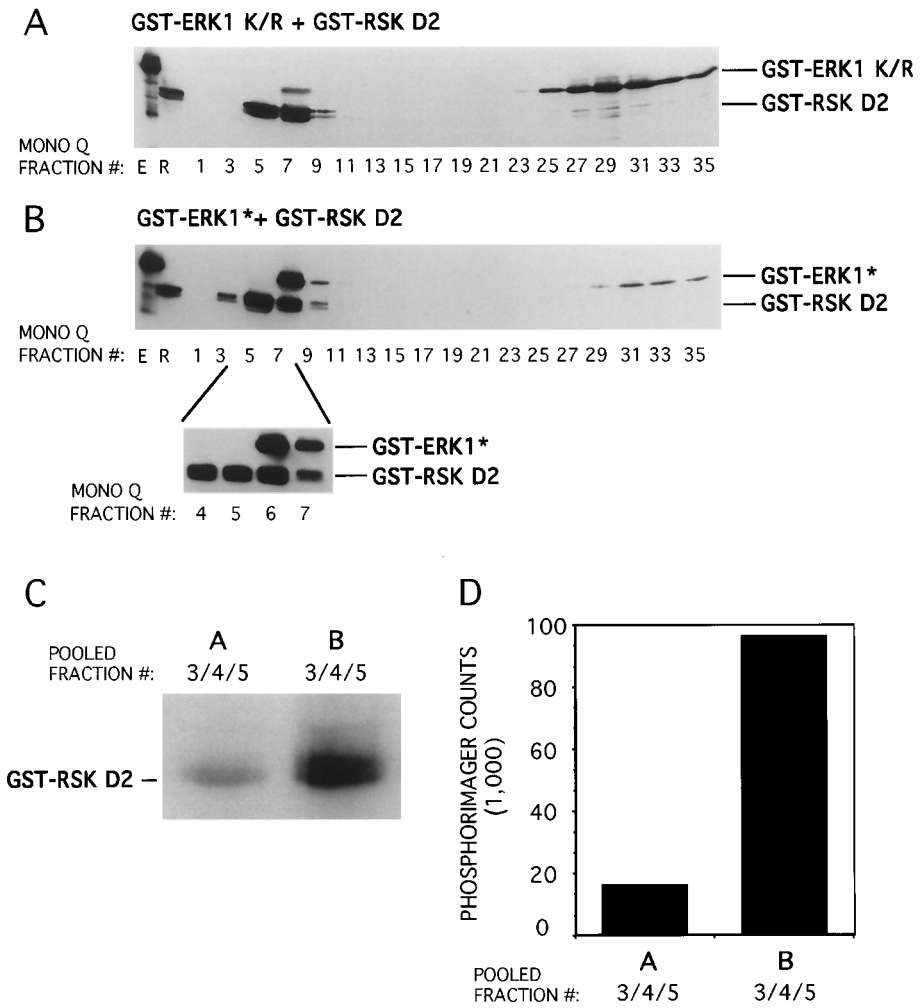

FIG. 10. Activation of RSK D2 autophosphorylation by MAPK (ERK1). Large-scale in vitro kinase assays (total reaction volume, 600 μl) with GST-RSK D2 (120 μg) and either GST-ERK1 K/R (inactive MAPK; 15 μg) or GST-ERK1* (MEK-activated MAPK; 15 μg) were performed in kinase buffer with 50 μM unlabelled ATP for 20 min at 30°C. The reaction mixtures were then fractionated by anion exchange chromatography with the Pharmacia fast-performance liquid chromatography system with Mono Q columns. Mono Q column buffer was 20 mM Tris (pH 7.2)–2 mM EGTA–25 mM β-glycerophosphate–2 mM dithiothreitol–1 mM sodium orthovanadate. The columns were eluted with a 0 to 500 mM linear gradient of NaCl, beginning at fraction 10, and 0.5-ml fractions were collected. Aliquots (30 μl) of alternate fractions were used for immunoblot analysis with antiserum recognizing the RSK C terminus and antiserum against MAPK (controls for immunoblot analysis in panels A and B, E [GST-ERK1 K/R] and R [GST-RSK D2]). Fractions 4 and 5 did not contain MAPK, as shown in the lower half of panel B, an overexposed immunoblot of 10-μl aliquots of fractions 4 to 7 from the column in panel B. This was also true for fractions 4 and 5 from the column in panel A (data not shown). Fractions 3 to 5 of each column were pooled, and the GST-RSK D2 was adsorbed to glutathione-Sepharose beads. The beads and bound protein were washed in kinase buffer. Equal amounts of GST-RSK D2 bound to beads (as determined by Coomassie blue staining) were incubated in kinase buffer and 50 μM [γ-^{32}P]ATP (11 cpm/fmol) in a total reaction volume of 80 μl for 30 min at 30°C (C and D). ^{32}P incorporation was quantitated by a Molecular Dynamics PhosphorImager and ImageQuant software.

ago (1, 20), it had not been shown which catalytic domain possessed S6 phosphotransferase activity. Here we demonstrate that the N-terminal catalytic domain is responsible for its S6, c-Fos, SRF, and Nur77 phosphotransferase activities. The N-terminal catalytic domain is also responsible for a majority of the autophosphorylation in the context of the full-length molecule, as judged by the results shown in Fig. 2. On the basis of the data presented in Fig. 5 to 7, however, it appears that the C-terminal catalytic domain is also capable of autophosphorylating. It is possible that both domains are necessary for full autophosphorylation activity. Thus far we have been unable to show that the C-terminal catalytic domain can phosphorylate several other standard in vitro substrates. We are presently testing additional proteins as candidate targets for the C-terminal kinase domain.

An equally important question that we have begun to address is whether the C-terminal catalytic domain is regulated. On the basis of the above-described results, this has become a very interesting question since (i) MAPK has recently been shown to interact with RSK (19, 26) and we have mapped the region of this interaction within the carboxy terminus of RSK (unpublished results) and (ii) MAPK has been shown to phosphorylate rsk^{mo}-2 at a threonine (30) in kinase subdomain VIII, which is predicted to be a regulatory phosphorylation site on the basis of its conservation and regulatory role in several other protein kinases. This phosphorylation site should be regulatory for the C-terminal and not the N-terminal catalytic domain. Indeed we provide evidence that the C-terminal kinase domain is active with regard to its ability to autophosphorylate in vitro and that MAPK can phosphorylate and further stimulate the C-terminal domain autophosphorylating activity.

Although the N-terminal catalytic domain is clearly growth

factor activated, the mechanism of its activation remains unclear. One possibility is that MAPK phosphorylates the C-terminal kinase domain of avian RSK, making it catalytically active towards the N-terminal domain. Activation could thus occur via an intramolecular or intermolecular process in which the C-terminal kinase domain phosphorylates and activates the N-terminal domain. RSK isolated from quiescent cells and phosphorylated in vitro by MAPK has been shown to have increased levels of autophosphorylating activity (18). To date, we have been unable to demonstrate phosphotransferase activity of the C-terminal domain of RSK, either in the context of the full-length K112R mutant or the MAPK-activated GST-RSK D2, towards the N-terminal domain or any other exogenous substrate tested in vitro. Additionally, Erikson and Maller (14) have been unable to detect any phosphorylase *b* phosphotransferase activity from RSK as one might predict on the basis of sequence homology between the C-terminal kinase domain and phosphorylase *b* kinase. Furthermore, RSK K464R S6 phosphotransferase activity is still responsive to growth factors. However, in the latter case we cannot rule out the possibility that the endogenous RSK is phosphorylating the ectopically expressed RSK in an intermolecular reaction. The continued determination of the mechanism of RSK activation and the identification and characterization of its targets are important in improving our understanding of Ras-regulated cytoplasmic-to-nuclear signal transduction, the regulation of gene expression, and control of the cell cycle.

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