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Biochemistry

Coinfection of insect cells with recombinant baculovirus expressing pp60$^{v\text{-}src}$ results in the activation of a serine-specific protein kinase pp90^{rsk}

(protein phosphorylation/tyrosine kinases/ribosomal protein S6 kinase)

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ABSTRACT **A recombinant baculovirus was constructed for the production of the serine-specific protein kinase, pp90^{rsk} (where rsk is ribosomal S6 kinase), in insect cells. The *Xenopus* pp90^{rsk} expressed in the infected cells had nearly undetectable enzyme activity in contrast to the same enzyme coproduced with the v-*src* oncogene product pp60$^{v\text{-}src}$. The transforming gene product pp60$^{v\text{-}src}$ very effectively activated pp90^{rsk}, whereas the products of c-*src* and the myristoylation-minus nontransforming virus NY315 were markedly less effective. Only a fraction of the total pp90^{rsk} population was activated, and it could be partially separated from unactivated protein by ion-exchange chromatography. When compared to the unactivated form, the activated enzyme displayed about a 4000-fold increase in the capacity to phosphorylate the ribosomal protein S6. The enhanced enzymatic activity appeared to be due to phosphorylation of pp90^{rsk}.**

A decade has passed since the demonstration that protein kinase oncogene products (1) and normal growth factor protein kinase receptors (2) share the capacity to phosphorylate tyrosine (3). Yet the link between tyrosine phosphorylation and functional modification of proteins has remained largely obscure except for the tyrosine-specific protein kinases, where modification of tyrosine can lead to inhibition or stimulation of enzyme activity (4, 5). The stimulation of tyrosine-specific growth factor receptors at the plasma membrane by mitogens or the activation of membrane-associated oncogene products, such as pp60$^{v\text{-}src}$, often leads to serine- or threonine-specific phosphorylation of major cellular proteins such as the ribosomal protein S6 and histone H1. Thus, signal transduction may involve activation of serine- or threonine-specific protein kinases or inhibition of phosphoprotein phosphatases. The latter enzymes have been reviewed (6).

Several serine/threonine-specific protein kinases have been implicated as potential substrates for tyrosine-specific protein kinases, including microtubule-associated protein (MAP) kinase (7, 8), casein kinase II (9, 10), cdc2/histone H1 kinase (11), the oncogene product Raf-1 (12), and ribosomal S6 kinases (rsks) (13–17). Under appropriate conditions MAP kinase and cdc2/histone H1 kinase are phosphorylated on tyrosine and the former enzyme is activated, whereas the latter is inactivated, by this modification. In each case the enzymes are also phosphorylated on threonine apparently to an extent equal to that of tyrosine. Both sites may need to be modified to achieve a functional change (18, 19). The Raf-1 kinase has been shown to be activated by platelet-derived growth factor receptor phosphorylation *in vitro* (20) and is perhaps a substrate for pp60$^{v\text{-}src}$ *in vivo* (12). Casein kinase II is activated by mitogen stimulation, but there is no evidence that it is phosphorylated directly by growth factor receptors (9, 10). With the exception of genetic evidence on the function of cdc2/histone H1 kinase in the cell cycle of yeast, the significance of any phosphorylation mediated by these serine/threonine-specific kinases remains to be determined. The function of S6 phosphorylation is also not clarified, but its phosphorylation is associated with transition from G_0 to G_1 in the cell cycle (21) and may enhance the protein synthetic capacity of ribosomes.

At least two distinct kinases can be activated to phosphorylate the 40S ribosomal subunit protein S6 *in vitro*. An S6 kinase of M_r 92,000, denoted S6KII, has been purified from unfertilized *Xenopus* eggs (16, 17). The activated form of this enzyme can be inactivated by dephosphorylation and partially reactivated by MAP kinase *in vitro* (22). Partial peptide sequences were used to identify two *Xenopus* cDNA clones corresponding to mRNA for S6KII, designated α and β, that predict proteins of M_r 83,000 and M_r 71,000, respectively (23). Homologous cDNA clones obtained from chicken and mouse predict proteins that have greater than 85% identity to the *Xenopus* S6KIIα protein (24). Recent evidence indicates that both chicken and mouse cells contain a M_r ≈90,000 S6 kinase (pp90^{rsk}) related to S6KII and that pp90^{rsk} is phosphorylated and activated in mitogen-treated or transformed cells (25, 26). In addition, chicken and mouse cells contain an S6 kinase of M_r ≈ 70,000 that is distinct from pp90^{rsk} (26) and that is also phosphorylated and activated in mitogen-treated cells. When isolated from mitogen-stimulated cells that have been metabolically labeled with inorganic ^{32}P, both enzymes contain phosphoserine and phosphothreonine (13, 25, 27), but no phosphotyrosine is detectable. Phosphotyrosine is found, however, in the M_r ≈90,000 protein isolated from Rous sarcoma virus-transformed cells (25). Since both enzymes are inactivated by dephosphorylation, they are likely to be activated by a serine/threonine protein kinase located on a pathway activated by tyrosine phosphorylation. Thus, only general outlines have emerged concerning the pathways that lead to the phosphorylation of a major cellular phosphoprotein. Reconstruction of the pathways *in vitro* with highly purified components may serve to establish the functional role of each enzyme and the functional significance of each phosphorylation event.

We have constructed recombinant baculoviruses to aid in the study of the activation of pp90^{rsk} *in vitro*. During these studies we noted that pp90^{rsk} produced in these cells had virtually no detectable enzyme activity. However, coproduction of pp90^{rsk} and pp60$^{v\text{-}src}$ in insect cells results in a remarkable degree of pp90^{rsk} activation. This activation is

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Abbreviations: rsk, ribosomal S6 kinase; S6KII, S6 kinase II; MAP, microtubule-associated protein.

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apparently due to phosphorylation of multiple sites, most of which are serine and are silent in the activation event(s).

MATERIALS AND METHODS

Recombinant Baculovirus Construction. Procedures for maintaining cells, purifying viruses, and propagating viruses were performed as suggested by Summers and Smith (28). The transfer vector pVL941 and wild-type baculovirus *Autographa californica* nuclear polyhedrosis virus were the gift of M. Summers (Texas A&M University, College Station, TX). The pAC373 (c-*src*) recombinant baculovirus (29) was the gift of T. Roberts (Dana–Farber Cancer Institute, Boston). The plasmid pSR-XD315(RSV) was the gift of H. Hanafusa (The Rockefeller University, New York).

The full-length coding region of the rsk-α cDNA was isolated by first ligating a synthetic linker molecule (5'-AATTCGGATCCACCATGGCTCTGGGACAACTGGTG-GATCTTTGGC-3') to the *Ava* I–*Eco*RI fragment of pA14-1a (23). After subcloning into the *Eco*RI site of a Bluescript (Stratagene) vector, the full-length cDNA was isolated by partial *Bam*HI digestion. The rsk-β cDNA was isolated after methylation of *Bam*HI sites of pA3-1a (23) and digestion by *Eco*RI and *Pvu* II. *Bam*HI linkers were then ligated to the mixture. The full-length coding region of v-*src* was isolated from a derivative of a plasmid containing v-*src* from the Prague A strain (30). *Bam*HI linkers were ligated to the *Nco* I–*Hin*dIII fragment containing the full-length coding region. The full-length coding region of NY315 was isolated by ligating *Bam*HI linkers to the *Nco* I–*Eco*RI fragment of pSR-XD315 (31). Conditions for various steps in the cloning were as described by the manufacturers (New England Biolabs and BRL) or as previously described (32).

All of the above fragments were resolved by 1% low-melting-point agarose gel electrophoresis. The appropriate fragments were isolated from the gel and ligated directly into the *Bam*HI site of the vector pVL941 (33). Plasmids containing the cDNAs in proper orientation were isolated from transformed bacteria and then used for transfection into Sf9 cells along with purified wild-type baculovirus DNA. Transfections, screening for recombinant viruses, cloning, and propagation of recombinant viruses were performed as described by Summers and Smith (28).

Preparation of Cell-Free Lysates. Sf9 cells were seeded at 2×10^6 cells per 60-mm dish and allowed to attach to the dish overnight. Infections were done by adding virus to a multiplicity of infection of 10. At appropriate times after infection, cells were rinsed gently with lysis buffer A [10 mM potassium phosphate, pH 7.05/1 mM EDTA/5 mM EGTA/10 mM $MgCl_2$/50 mM β-glycerophosphate/1 mM Na_3VO_4/2 mM dithiothreitol/phenylmethylsulfonyl fluoride (40 μg/ml)], then scraped in 0.5–1.0 ml of lysis buffer. Cell membranes were disrupted by 25 strokes in a Dounce homogenizer. The lysate was clarified by centrifugation at 100,000 $\times$ *g* for 30 min at 4°C. For radiolabeled proteins, cells were starved in methionine-free medium for 20 min and then labeled in methionine-free medium containing [^{35}S]methionine at 100 μCi/ml (Tran^{35}S-label, ICN; 1 Ci = 37 GBq) for 4 hr.

Immunoprecipitation, Immunoblot Analysis, and Kinase Assay. Lysates containing 100–150 μg of total protein, as determined by the method of Bradford (34), were incubated with 5 μl of rabbit serum 125, raised against pp90^{rsk} antigen produced in bacteria (24), and 100–150 μl of buffer (100 mM NaCl/10 mM Tris·HCl, pH 6.5/1 mM EDTA/1% Nonidet P-40/0.5% sodium deoxycholate). Immunocomplexes were adsorbed to protein-A-containing *Staphylococcus aureus*. The staphylococcus immunocomplexes were washed several times with buffer and then equilibrated to 150 mM NaCl/10 mM Tris·HCl, pH 7.2/2 mM dithiothreitol prior to kinase assay or washed several times in buffer (10 mM Tris·HCl, pH 7.2/150 mM NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% SDS) before being solubilized by boiling 2 min in electrophoresis sample buffer (35). For kinase assay, the bacteria immunocomplexes were resuspended in a final volume of 30 μl of reaction buffer, at final concentrations of 20 mM Tris·HCl, pH 7.25/10 mM $MgCl_2$/5 μM ATP (10 μCi of [γ-^{32}P]ATP). Incubation was carried out at 30°C for 30 min. The reaction was stopped by adding 30 μl of 2$\times$ electrophoresis sample buffer (35) and boiling for 2 min. S6 phosphorylations using the bacteria immunocomplexes were also done in a total volume of 30 μl with 50 μM ATP (5 μCi of [γ-^{32}P]ATP) and 40S ribosomal subunits (0.1–0.25 mg/ml) prepared from *Xenopus laevis* ovaries as described (17). The reaction was incubated at 30° for 15 min. Lysates were also used directly for S6 phosphorylation under the same reaction conditions as above. Proteins were resolved by 10% polyacrylamide/SDS gel electrophoresis as described by Laemmli (36). Phosphatase-treated rsk or fractions from the S-Sepharose column were resolved by SDS/PAGE and transferred electrophoretically to a 0.45-μm (pore size) nitrocellulose filter. The filter was blocked in 10% (vol/vol) calf serum/150 mM NaCl/50 mM Tris·HCl, pH 7.5 at 25°C for 1 hr. The filter was then incubated with a 1:200 dilution of antibody in blocking solution overnight at 4°C. Subsequent steps were as described in the Vectastain ABC kit (Vector Laboratories).

Partial Purification of rsk Proteins. For purification of activated pp90^{rsk}, insect cells were doubly infected with *rsk-α* and v-*src* baculoviruses. Cells were harvested 28–32 hr after infection, washed with ice-cold buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.2) and then homogenized in lysis buffer A as described above. Leupeptin and pepstatin A were also added to the lysis buffer, both at 10 μg/ml, final concentration. The lysate was clarified by 100,000 $\times$ *g* centrifugation for 30 min at 4°C. The supernatant was applied to a 5-ml S-Sepharose fast flow (Pharmacia) column equilibrated in buffer B [10 mM potassium phosphate, pH 7.2/1 mM EDTA/5 mM EGTA/0.1 mM Na_3VO_4/10 mM β-glycerophosphate/0.05% Brij 35 (Pierce)/5 mM $MgCl_2$/2 mM dithiothreitol/10% (vol/vol) glycerol]. The bound proteins were eluted in a 60-ml gradient of 0–500 mM NaCl in buffer B. Fractions were assayed for S6 kinase activity. The peak of activity was pooled and applied to a 4-ml hydroxylapatite (Bio-Gel HT, Bio-Rad) column equilibrated in buffer B. Proteins were eluted in a 40-ml gradient of 10–400 mM potassium phosphate (pH 7.2) in buffer B. Fractions were assayed for S6 kinase activity. Fractions with peak activity were pooled, dialyzed against 100 vol of buffer B for 3 hr at 4°C, and then applied to a 2-ml heparin-agarose (Sigma) column. Bound proteins were eluted with a 40-ml gradient of 0–500 mM NaCl in buffer B. Fractions were assayed for S6 kinase activity. Peak activity was pooled and dialyzed against buffer B with 50% glycerol overnight at 4°C and then stored at −20°C. The *rsk* gene product was also purified from lysates of Sf9 cells infected with recombinant *rsk-α* baculovirus and harvested 48 hr after infection. Lysates were prepared and applied to an S-Sepharose column as above. Fractions containing *rsk-α* gene product as determined by Coomassie staining were pooled and applied to a hydroxylapatite column. Approximately 50–70% of the rsk protein flows through the column. The flow-through fractions containing rsk were pooled and dialyzed against buffer B containing 50% glycerol.

Phosphatase Assays. Phosphatase 2A was a gift of H. C. Li (Mount Sinai School of Medicine, New York). Okadaic acid was a gift of Hirota Fujiki (National Cancer Institute, Tokyo). Partially purified activated pp90^{rsk} was treated with 0.1 unit of phosphatase 2A in the presence or absence of 10 nM okadaic acid. Reaction mixtures (20–30 μl) contained 20 mM Tris·HCl (pH 7.25), 10 mM $MgCl_2$, and bovine serum albumin (0.05 mg/ml). Samples were incubated at 30°C for 30 min and then either reactions were stopped with 0.25 vol of 5$\times$ sample buffer and resolved directly by gel electrophoresis for immu-

noblotting or 40S ribosomes, ATP, and [γ-^{32}P]ATP were added with subsequent incubation at 30°C for 15 min prior to adding sample buffer and resolving by SDS/PAGE.

RESULTS

The need for sufficient pp90^{rsk} protein for studies on its activation prompted us to use the baculovirus expression vector system to produce recombinant proteins. Recombinant baculoviruses expressing *Xenopus* rsk-α protein, which corresponds to pp90^{rsk} and rsk-β protein, which is truncated at the COOH terminus by some 104 amino acids compared to rsk-α, were used to infect Sf9 cells. As Fig. 1*A* shows, virus-infected cells, when labeled with [^{35}S]methionine 36–40 hr after infection, had reduced synthesis of most cellular proteins (lanes 2–4) when compared to uninfected cells. Infections by the recombinant rsk-α or -β-producing viruses resulted in unique bands at M_r 83,000 and M_r 71,000 (lanes 2 and 3, respectively) when compared to the wild-type infected cells (lane 4). Immunoprecipitation demonstrated that these proteins were recognized by anti-rsk serum (Fig. 1*B*, lanes 2 and 3). These molecular weights are similar to those of the products of *in vitro* transcription and translation of the rsk-α and rsk-β cDNAs (24). Although not readily apparent on the autoradiograph, a doublet was consistently seen in Coomassie blue-stained gels of immunoprecipitates of both the α and β proteins. The appearance of the doublet is reproducible and has also been observed in preparations of the enzyme purified from unfertilized *Xenopus* eggs (17).

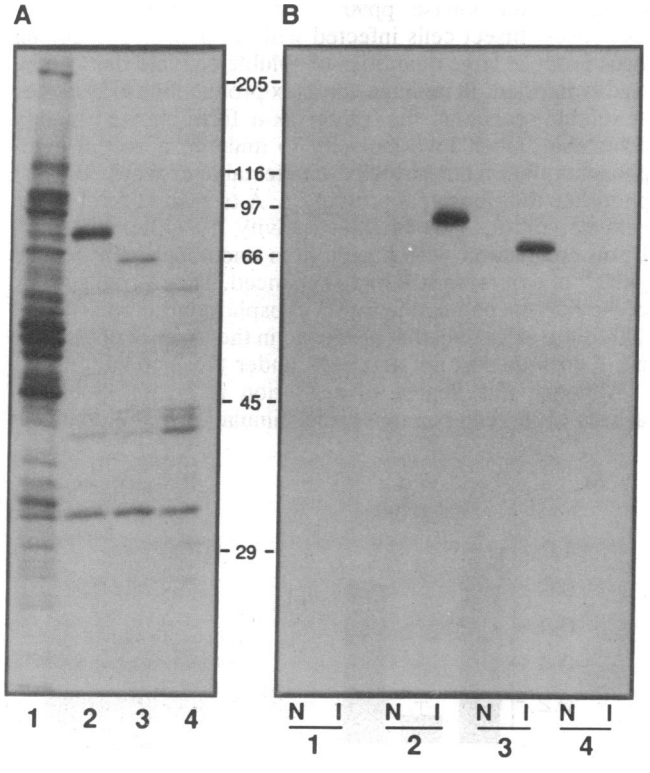

FIG. 1. [^{35}S]Methionine labeling and immunoprecipitation of rsk products from insect cells. Recombinant baculoviruses encoding rsk-α or rsk-β were used to infect Sf9 cells. Labeled lysates were resolved directly by SDS/PAGE or immunoprecipitated with either preimmune or immune serum and then resolved. (*A*) Direct analysis of labeled protein from uninfected (lane 1), *rsk-α*-infected (lane 2), *rsk-β*-infected (lane 3), or wild-type virus-infected (lane 4) Sf9 cells. (*B*) Immunoprecipitation of labeled protein from uninfected (lanes 1), *rsk-α*-infected (lanes 2), *rsk-β*-infected (lanes 3), or wild-type virus-infected (lanes 4) Sf9 cells. N, preimmune serum; I, anti-rsk serum. Molecular weight standards ($\times 10^{-3}$) are indicated.

The functional activity of the kinases produced in Sf9 cells was noted initially by their ability to autophosphorylate. The signal seen by labeling with [γ-^{32}P]ATP was much less intense than expected, since sufficient quantities of rsk proteins are produced by the cells to result in detectable Coomassie stainable protein upon immunoprecipitation. The phosphorylation signal seen in rsk-β was less than that seen in rsk-α, perhaps because of a loss of an autophosphorylation site in the truncated protein or lower specific activity of the truncated form. Attempts to show specific phosphorylation of the S6 protein in 40S ribosomes were unsuccessful.

We attempted to mimic the pathway of activation reported for Rous sarcoma virus-transformed chicken embryo fibroblasts by simultaneously infecting Sf9 cells with recombinant baculoviruses containing the cDNA for pp60$^{v\text{-}src}$ and either *rsk-α* or *rsk-β*. Autophosphorylation of immunoprecipitates of cell lysates from uninfected or infected Sf9 cells is shown in Fig. 2*A*. The background activity in uninfected and wild-type infected cells was quite low (lanes 1 and 7). The rsk-β protein (lanes 5 and 6) showed significantly less activity than rsk-α with or without v-*src* expression. The activity is apparent only on prolonged exposure of the autoradiograph. Coproduction of v-*src* and rsk-α resulted in greatly enhanced autophosphorylation of rsk-α compared to rsk-α alone (lane 4) and resulted in heterogeneous migration of rsk-α. The most slowly migrating form of phosphorylated rsk-α had an approximate M_r 90,000, similar to the molecular weight of purified *Xenopus* S6KII. Coinfection with v-*src* and *rsk-β* (lane 5) did not result in a detectable change in the migration of rsk-β. When immunoprecipitates of these lysates were used to phosphorylate 40S ribosomes (Fig. 2*B*), only the doubly infected v-*src* and *rsk-α* sample (lane 3) showed significant phosphorylation of the S6 protein. Prolonged exposure of the gel revealed no phosphorylation of S6 above background in the remaining lanes, suggesting at least a 100-fold increase in the activity of rsk-α as the result of pp60$^{v\text{-}src}$ expression.

To determine whether other *src* gene products would have a similar effect, baculoviruses expressing pp60$^{c\text{-}src}$ or pp60^{NY315} were coproduced with rsk-α in Sf9 cells (Fig. 2*C*). Again, immunoprecipitates of lysates from double-infected cells were used to phosphorylate 40S ribosomes. Lane 1 shows the activity in v-*src*- and *rsk-α*-infected cells. The activity in c-*src*- and *rsk-α*-infected cells (lane 2) shows significantly less phosphorylation of S6 but was above the level of activity in cells infected with *rsk-α* alone (data not shown). The activity in NY315- and *rsk-α*-infected cells (lane 3) was intermediate but reproducibly lower than in v-*src* and *rsk-α* cells. The protein levels of the various *src* gene products and pp90^{rsk} were equivalent upon immunoblot analysis (data not shown). More rsk protein was found in the higher molecular weight forms in the v-*src* and NY315 than in the c-*src* double-infected cells.

Although the v-*src* and *rsk-α* doubly infected cells had S6 kinase activity, it appeared that most of the rsk-α protein produced in these cells comigrated with the inactive protein produced in single infected cells, as judged by Coomassie blue staining. A purification scheme was devised to separate the active protein from unactivated forms (Fig. 3). All forms of protein produced by recombinant virus bound to S-Sepharose fast flow and were eluted with a salt gradient. Representative fractions from the column were resolved by SDS/PAGE and immunoblotted with anti-rsk serum. As seen in Fig. 3*A*, the slower migrating forms (fraction 27) were eluted earlier than those forms that migrate at the predicted molecular weight (fraction 41). Fig. 3*B* demonstrates that the majority of the S6 kinase activity resided in the early fractions containing the slowest migrating forms of pp90^{rsk}, suggesting that only the most slowly migrating form of the kinase makes a significant contribution to S6 phosphorylation.

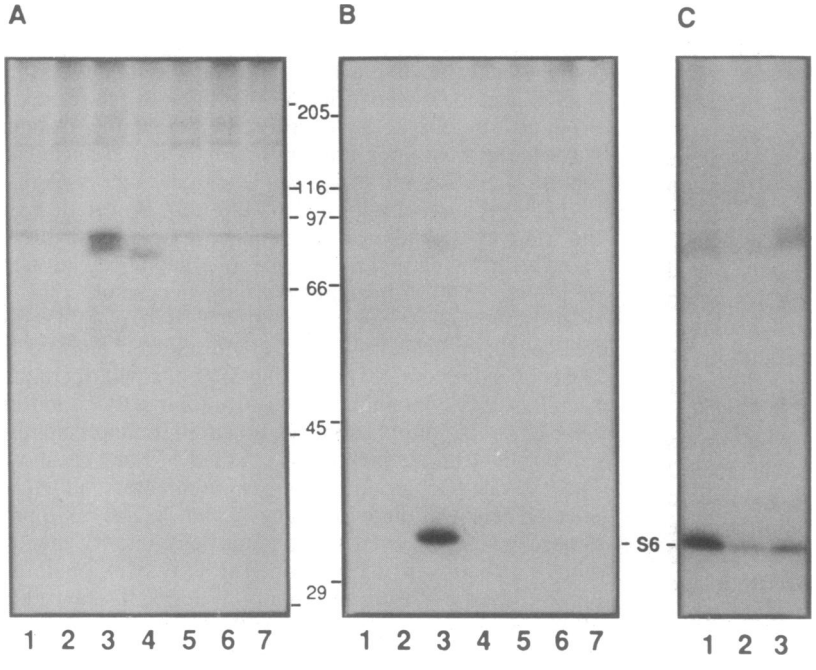

FIG. 2. Activation of *rsk*. Lysates from Sf9 cells 28 hr after infection were immunoprecipitated, washed, and allowed to autophosphorylate or phosphorylate 40S ribosomes in the presence of [γ-^{32}P]ATP. (*A*) Autophosphorylation in immunoprecipitates from uninfected (lane 1), v-*src*-infected (lane 2), v-*src*- plus *rsk*-α-infected (lane 3), *rsk*-α-infected (lane 4), v-*src*- plus *rsk*-β-infected (lane 5), *rsk*-β-infected (lane 6), or wild-type virus-infected (lane 7) Sf9 cells. (*B*) Phosphorylation of 40S ribosomal subunits by immunoprecipitates. Lanes are identical to those in *A*. (*C*) Phosphorylation of 40S ribosomal subunits of immunoprecipitates from lysates of Sf9 cells, which were v-*src*- plus *rsk*-α-infected (lane 1), c-*src*- plus *rsk*-α-infected (lane 2), or NY315- plus *rsk*-α-infected (lane 3). Molecular weight standards ($\times 10^{-3}$) and S6 migration are indicated.

S6KII purified from unfertilized *Xenopus* eggs can be inactivated by treatment with phosphatase 2A (22), and the activity can be partially restored by phosphorylation with MAP kinase. Fig. 4*A* shows the loss of the capacity to phosphorylate S6 after the partially purified kinase is treated with phosphatase 2A. Treatment with phosphatase 2A eliminated 90% of the phosphorylation of S6. Approximately 90% of the protein kinase activity remained after phosphatase treatment in the presence of 10 nM okadaic acid, a potent 2A inhibitor (lane 3) (37). These experiments also showed (Fig. 4*B*) that phosphatase treatment of the active kinase resulted in a shift in migration detected by immunoblotting. The untreated kinase (lane 1) migrated with the uppermost band at M_r ≈90,000. Treatment with phosphatase 2A (lane 2) resulted in loss of the uppermost band. Phosphatase treatment of the kinase in the presence of 10 nM okadaic acid (lane 3) resulted in no significant change in migration.

The best estimate of the degree of activation is presented in Table 1. The relative amounts of kinase, both unactivated and activated, were quantitated by immunoblotting. The same partially purified preparations were used to phosphorylate the S6 protein in 40S ribosomes. The phosphorylated S6 protein was excised from gels, and incorporated ^{32}P was quantitated. As shown in Table 1, an average value for activation was 4000-fold.

DISCUSSION

We describe here the activation of a highly conserved serine-specific protein kinase, pp90^{rsk}, by using recombinant baculoviruses. Insect cells infected with a *rsk*-α or β-encoding virus produce large quantities of soluble enzyme that can be readily purified. In immune complex protein kinase assays or in soluble reactions, the intact rsk-α form showed readily detectable, albeit low, capacity to undergo apparent autophosphorylation but virtually undetectable capacity to phosphorylate the ribosomal protein S6. A truncated form of the enzyme, rsk-β, showed little activity by either criterion. Upon coinfection with baculovirus encoding pp60$^{v\text{-}src}$, the pp90^{rsk} activity was strikingly enhanced. The overall activity of the enzyme population for S6 phosphorylation was at least 100-fold greater than that produced in the absence of *src*. The rsk-β enzyme was not activated under these conditions.

Although this degree of activation is greater than that usually observed upon mitogenic stimulation of cultured cells

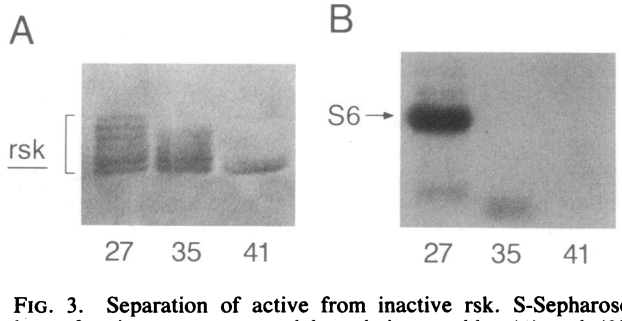

FIG. 3. Separation of active from inactive rsk. S-Sepharose column fractions were assayed by rsk immunoblot (*A*) and 40S phosphorylation activity (*B*). Insect cells were infected with *rsk*-α and v-*src* baculoviruses. The proteins from a cell lysate were eluted from a 5-ml S-Sepharose column. Fractions (1 ml) were collected and representative fractions were analyzed. (*A*) Twenty microliters of representative fractions was resolved by SDS/PAGE on 8% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with rsk antiserum. (*B*) Ten microliters was used for assaying direct 40S phosphorylation. The S6 band and rsk forms are indicated.

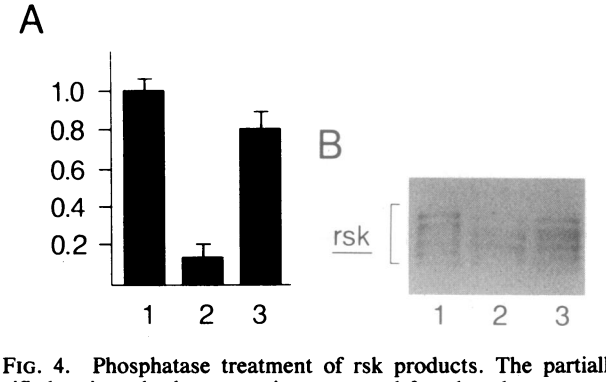

FIG. 4. Phosphatase treatment of rsk products. The partially purified activated rsk preparation was used for phosphatase treatment. (*A*) The relative decrease in S6 kinase activity in rsk preparations treated with phosphatase 2A is shown. Data (mean ± SD) for at least four determinations were normalized to rsk/okadaic acid/boiled phosphatase (bar 1). Rsk/phosphatase 2A (bar 2), and rsk/phosphatase 2A/okadaic acid (bar 3) are shown. (*B*) Immunoblot with rsk antiserum shows preparations that were untreated (lane 1), treated with phosphatase 2A (lane 2), or treated with phosphatase 2A plus okadaic acid (lane 3). The rsk protein is indicated.

Table 1. Activation of rsk

Sample	Relative amount of rsk	S6, cpm incorporated	Relative fold activation
Activated	0.1	2.1×10^4	3.8×10^3
Activated	0.01	2.2×10^3	4.0×10^3
Unactivated	10	5.5×10^2	1

Partially purified preparations of activated and unactivated rsk were used for analysis. The relative amount of rsk in each preparation was estimated by immunoblotting and Coomassie blue staining. Phosphorylation of S6 protein was performed under conditions of enzyme and substrate concentrations where assays were linear. Three to six determinations were made at each concentration. Radiolabeled phosphate incorporated into S6 was quantitated by liquid scintillation spectrometry. Total incorporation minus phosphorylation of S6 without added kinase is indicated. Relative activation was determined by dividing cpm by relative amount of rsk present and then normalizing to a value of unactivated rsk. With a specific activity of 2.8×10^4 cpm/pmol of ATP, the activated rsk incorporated phosphate into S6 at a rate of 52 pmol per min per ml. The estimated concentration of rsk in this preparation was 10 μg/ml; therefore, the rate is 52 pmol per min per mg.

(35), it does not reflect the total potential range or degree of change in activity of an individual molecule. Upon purification of activated pp90^{rsk} from infected cells, only a small fraction of the population appeared to contribute to the enhanced enzyme activity. Partial resolution of these various forms can be achieved (Fig. 3), and the increased S6 protein kinase activity is associated with a form of pp90^{rsk} that migrates very slowly during gel electrophoresis. The increased activity and change in mobility are apparently due to phosphorylation, since both can be reversed by phosphatase treatment.

These data support previous reports that assert that S6 protein kinases are activated by phosphorylation and inactivated by dephosphorylation (22, 38) but do not address other molecular questions concerning the number of steps involved. In the present study, the steps in the pathway to pp90^{rsk} activation are unknown. It is clear that only a fraction of the enzyme is activated, perhaps because there are insufficient levels of an intervening kinase(s), such as MAP kinase, or because such a kinase is partially inactivated in virus-infected cells. We have not addressed the issue of tyrosine phosphorylation of rsk in the present studies. In coinfected cells, pp90^{rsk} is extensively phosphorylated on tyrosine (data not shown); however, since only a small fraction of the total population of enzymes contributes to S6 phosphorylation, the modifications of a homogeneous form of the activated enzyme need to be determined. A great deal of phosphorylation of pp90^{rsk} seems to occur without significant increase in enzyme activity (for example, see fraction 35, Fig. 3*A*). Thus, these modifications are silent until all modifications have occurred. These studies also do not address the possibility that two or more enzymes are required to activate pp90^{rsk}. Finally, in regard to the significance of tyrosine phosphorylation, it should be noted that both pp90^{rsk} and the $M_r \approx 70{,}000$ S6 kinase show only serine and threonine phosphorylation when isolated from cells after mitogen stimulation (13, 25, 27).

We also note here that both pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ encoded by NY315, a nontransforming Rous sarcoma virus mutant that fails to associate with the plasma membrane but retains protein kinase activity, were less efficient activators of pp90^{rsk}. Both of these enzymes are produced in quantities and with protein kinase activities similar to pp60$^{v\text{-}src}$; thus their failure to produce effects equal to v-*src* may be attributable to such factors as substrate recognition or subcellular localization. In this regard, others (29) studied pp60$^{c\text{-}src}$ expression in infected insect cells and found pp60$^{c\text{-}src}$ enzyme activity was constitutively high, apparently because tyrosine-527 of the c-*src* product is not phosphorylated.

The studies reported here provide relatively large quantities of enzymes for potential *in vitro* reconstruction of pp90^{rsk} activation. In addition, they provide insight into the role of protein phosphorylation as it relates to enzyme activation. The results raise a concern about attribution of functional significance after the mere detection of modification, since it may not alter enzyme activity.

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