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Identification of Mitogen-Responsive Ribosomal Protein S6 Kinase pp90^{rsk}, a Homolog of *Xenopus* S6 Kinase II, in Chicken Embryo Fibroblasts

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Antiserum raised against recombinant *Xenopus* ribosomal protein S6 kinase (*rsk*) was used to identify a 90,000-M_r ribosomal S6 kinase, pp90^{rsk}, in chicken embryo fibroblasts. Adding serum to cells stimulated the phosphorylation of pp90^{rsk} on serine and threonine residues and increased the activity of S6 kinase measured in immune complex assays. *Xenopus* S6 kinase II and chicken embryo fibroblast pp90^{rsk} had nearly identical phosphopeptide maps.

Protein phosphorylation has been implicated as a biochemical mechanism for signal transduction, providing rapid and reversible alterations in protein function. One potential member of a signal-transducing phosphorylation cascade, ribosomal protein S6, is phosphorylated within minutes following treatment of quiescent cells with mitogenic agents such as serum, insulin, and epidermal growth factor (EGF) or with the tumor promoter phorbol-12-myristate-13-acetate (PMA) (5, 18, 20, 24–26). In addition, the phosphorylation of S6 is increased in cells expressing the transforming protein *src* (9). Several laboratories have reported the purification of protein with S6 kinase activity from different tissue sources. These enzymes can be segregated into two groups on the basis of apparent molecular size; enzymes from 90,000 to 92,000 M_r, denoted S6KII, have been thus far isolated only from *Xenopus laevis*, (10, 11) and 65,000 to 70,000-M_r S6 kinase activities have been identified in Swiss 3T3 fibroblasts, chicken embryo fibroblasts (CEF), bovine liver, regenerating rat liver, and chicken embryos (3, 4, 15, 19, 23). Polyclonal antibodies raised against purified *Xenopus* S6KII have been shown to immunoprecipitate an S6 kinase activity from CEF (13); however, this antiserum was unable to detect a specific protein that could have accounted for the activity observed. To date, the relationship between these proteins remains unclear, but the recent cloning of chicken and mouse homologs of the *Xenopus* ribosomal S6KII gene predicts the presence of an 84,000-M_r S6 kinase in both species (1, 16).

The results presented in Fig. 1 suggest the presence of a homolog of *Xenopus* S6KII in CEF. Cell lysates from CEF which had been serum stimulated for 1 h (5) were analyzed by immunoblotting with antiserum 125. The identification and isolation of a cDNA clone representing the *Xenopus* S6KII gene (16), the generation of polyclonal antiserum 125 raised against recombinant *Xenopus rsk* gene product (1, 16), and immunoblot detection of *Xenopus* S6KII (12) have been previously described. To determine whether the 90,000-M_r protein recognized by antiserum 125 was an S6 kinase, immunocomplexes formed between CEF cell lysates and antiserum 125 were assayed for S6 kinase activity. Those assays were compared with assays of S6 kinase activity measured in the same cell lysates without immunoprecipitation. As shown by others and confirmed in this paper (Fig. 2A), serum stimulation of CEF results in an increase in phosphorylation of S6 in 40S ribosomal subunits exogenously added to cell lysates (Fig. 2A, lanes 1 and 2). The same cell lysates were immunoprecipitated with antiserum 125 and then assayed for S6 phosphorylation. The results demonstrated the presence of an immune-specific S6 kinase activity in CEF and that this activity was greater in immunocomplexes formed from serum-stimulated CEF lysates (Fig. 2B). Immunoprecipitation of S6 kinase activity could be blocked by the addition of purified inactive *Xenopus rsk* protein to the antiserum prior to the addition of cell lysate (data not shown). *Xenopus* S6KII has been shown to auto-

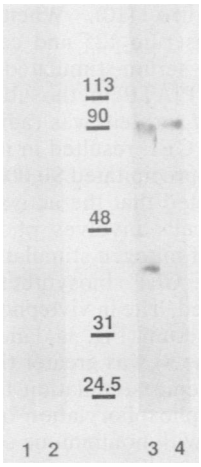

FIG. 1. Immunoblot of *Xenopus* S6KII and CEF cell lysate. Samples of purified *Xenopus* S6KII (lanes 2 and 4) and serum-stimulated CEF cell lysate (lanes 1 and 3) were fractionated on sodium dodecyl sulfate–10% polyacrylamide gels (17) and immunoblotted either with preimmune serum (lanes 1 and 2) or polyclonal antiserum 125 (lanes 3 and 4). Positions of the molecular weight standards (in thousands) are indicated.

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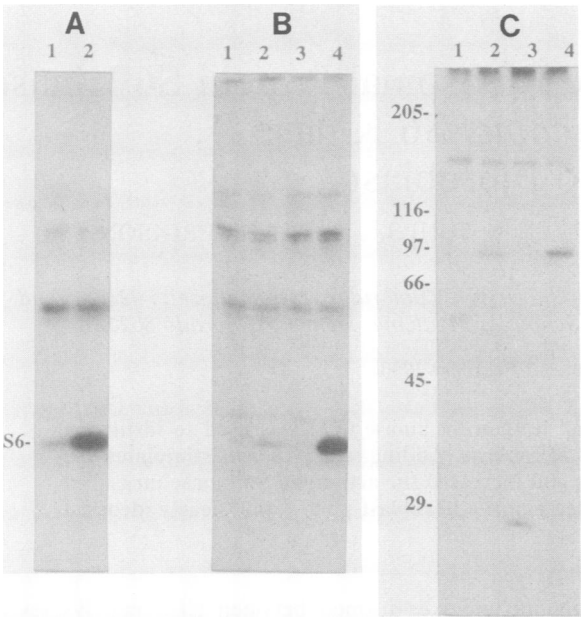

FIG. 2. Identification of a serum-stimulated S6 kinase recognized by antiserum 125. Cell lysates were prepared from serum-starved CEF and from serum-starved CEF incubated for 1 h with 10% calf serum. (A) Equal amounts of control CEF (lane 1) and serum-stimulated CEF (lane 2) lysates were incubated for 15 min at 30°C in the presence of 20 mM Tris (pH 7.6), 10 mM $MgCl_2$, 50 μM ATP (1 mCi/5 mM), and 20 μg of 40S ribosomal subunits prior to resolution on 10% polyacrylamide gels and autoradiography. (B) Immunocomplex kinase assays were performed with control CEF (lanes 1 and 2) and serum-stimulated CEF (lanes 3 and 4) lysates and with preimmune serum (lane 1 and 3) and antiserum 125 (lanes 2 and 4). The position of S6 is indicated. (C) Autophosphorylation of serum-stimulated pp90 in an immunocomplex. Control and serum-stimulated CEF lysates were immunoprecipitated and assayed for kinase activity in the absence of exogenous substrate as described for panel A but incubated 30 min at 30°C with 5 μM ATP (2 mCi/5 mM). Lanes are as described for panel B. The migration of molecular weight standards (in thousands) is indicated.

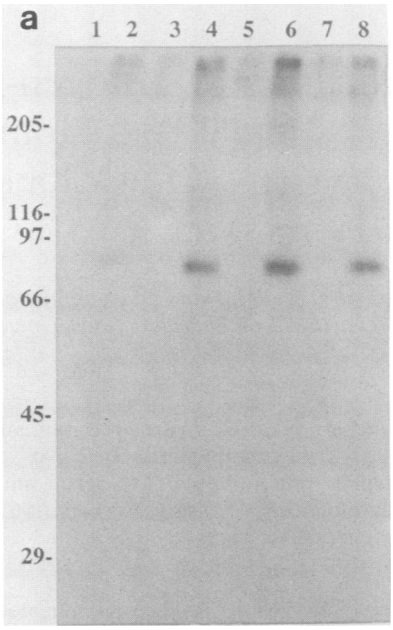

FIG. 3. Immunoprecipitation and phosphoamino acid analysis of pp90 S6 kinase from biosynthetically ^{32}P-labeled CEF. (a) CEF were starved for phosphate and serum for 18 h prior to incubation for 1 h with $H_3{}^{32}PO_4$ alone (lanes 1 and 2), $H_3{}^{32}PO_4$ and dialyzed 10% calf serum (lanes 3 and 4), 100 ng of PMA per ml (lanes 5 and 6), or EGF plus insulin (lanes 7 and 8). One hour after ^{32}P addition, the cells were harvested, solubilized by boiling in 1% sodium dodecyl sulfate, and immunoprecipitated with control serum (lanes 1, 3, 5, 7, and 9) or antiserum 125 (lanes 2, 4, 6, and 8). Immunoprecipitates were resolved on sodium dodecyl sulfate–10% polyacrylamide gels and subjected to autoradiography for 72 h. The migration of molecular weight standards (in thousands) is indicated. (b) CEF were biosynthetically ^{32}P labeled, as described for panel a, and incubated for 1 h with 10% dialyzed calf serum, 100 ng of PMA per ml, or EGF plus insulin. Immunoprecipitated pp90 was processed for phosphoamino acid analysis as previously described (8). The positions of phosphoamino acid standards, as visualized by ninhydrin, and the origin are indicated. NY72-4-infected CEF were maintained at 41°C or shifted to 35°C for 24 h prior to labeling. Autoradiograms of the phosphoamino acids resolved by two-dimensional thin-layer electrophoresis (8) are as follows: panel A, control; panel B, 10% dialyzed calf serum; panel C, 100 ng of PMA per ml; panel D, EGF plus 1.6 μM insulin; panel E, NY72-4 grown at 41°C; panel F, NY72-4 grown at 35°C.

phosphorylate in vitro (10). When immunocomplexes formed between antiserum 125 and cell lysates of control (Fig. 2C, lane 2) or serum-stimulated (lane 4) CEF were incubated with [γ-^{32}P]ATP in the absence of exogenous substrate, a 90,000-M_r protein was radiolabeled. Moreover, serum stimulation of CEF resulted in increased phosphorylation of the immunoprecipitated 90,000-M_r protein (pp90).

It has been suggested that the activation of S6 kinase in mitogen-stimulated cells involves phosphorylation of the enzyme. The effect of mitogen stimulation on the phosphorylation of pp90 in CEF biosynthetically labeled with $H_3{}^{32}PO_4$ was examined. The in vivo phosphorylation of pp90 after treatment with serum (Fig. 3a, lane 4), PMA (lane 6), or EGF plus insulin (lane 8) was greater than that of untreated cells (lane 2). Mitogenic stimulation had no effect on the pattern or extent of phosphorylation observed for samples immunoprecipitated with nonimmune serum (Fig. 3A, lanes 1, 3, 5, and 7). In parallel experiments, immunocomplex phosphorylation of pp90 and S6 was elevated similarly by these mitogens. Moreover, increased phosphorylation of pp90 and S6 was detected in immunoprecipitates of extracts from CEF infected with the temperature-sensitive Rous sarcoma virus mutant, NY72-4, when cells were grown at the permissive temperature (35°C) for $pp60^{src}$ activation (Table 1). The extent of phosphorylation, quantitated and normalized for protein concentration, is presented in Table 1.

The same biosynthetically labeled CEF lysates were used to identify the specific amino acids phosphorylated in pp90 in response to mitogen treatment. Phosphoserine was the predominate phosphoamino acid in pp90 immunoprecipitated from quiescent cells treated for 1 h with serum (Fig. 3b, panel B), PMA (panel C), and EGF plus insulin (panel D). Stimulation with each of these mitogens also resulted in the phosphorylation of pp90 on threonine. It should also be noted that serine and threonine were the only phosphorylated amino acids detected in cells after only 10 min of stimulation with serum (data not shown). In extracts from CEF infected with NY72-4 and grown at 41°C, serine and threonine were the only phosphorylated amino acids detectable in pp90 (Fig. 3b, panel E). In addition to phosphoserine

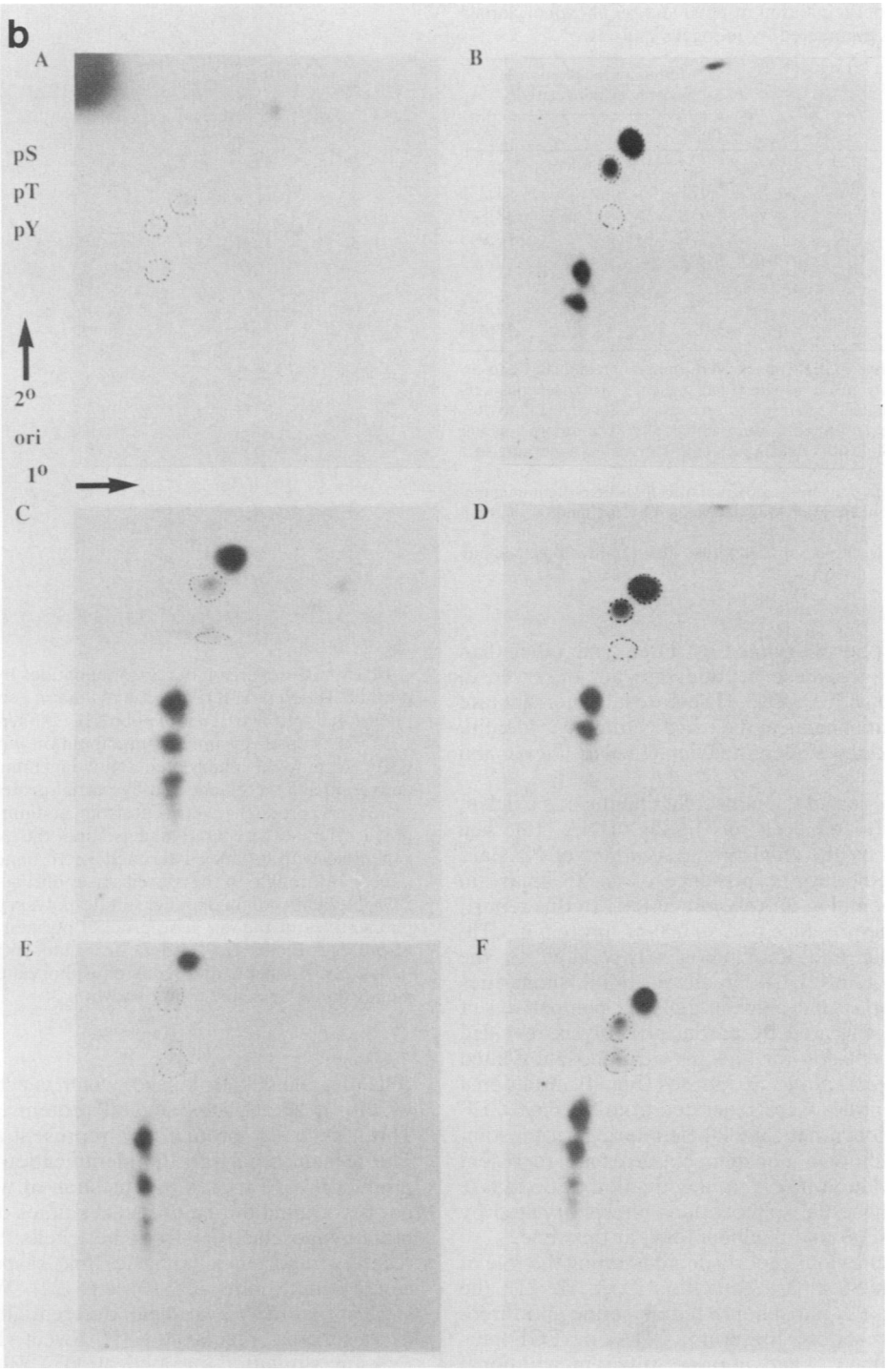

FIG. 3—*Continued.*

and phosphothreonine, phosphotyrosine could be detected in immunoprecipitated pp90 (Fig. 3b, panel F) when the temperature of the NY72-4-infected cells was shifted to 35°C.

To address the structural relatedness of CEF pp90 and *Xenopus* S6KII, we compared the phosphopeptides of these proteins generated by partial proteolysis by elastase (7). The samples compared in Fig. 4 are autophosphorylated, purified *Xenopus* S6KII (lanes 1, 4, and 7); immunoprecipitated, biosynthetically labeled pp90 from serum-stimulated CEF (lanes 2, 5, and 8); and immunoprecipitated, autophosphorylated pp90 from serum-stimulated CEF (lanes 3, 6, and 9). Both concentrations of elastase generated comigrating phosphopeptides with nearly identical patterns for autophosphorylated, purified *Xenopus* S6KII (Fig. 4, lanes 4 and 7) and autophosphorylated, immunoprecipitated CEF pp90 (lanes 6 and 9). The additional labeled peptides of pp90 from biosynthetically labeled CEF (Fig. 4, lanes 5 and 8) suggest the

TABLE 1. Immunoprecipitation of pp90 and S6 phosphorylating activities stimulated by mitogens and v-*src*[a]

Stimulant	Immunoprecipitation of phosphorylating activity[b]	
	pp90	S6
Mitogen		
None	421	3,232
Serum	1,918	8,847
PMA	1,778	10,945
EGF plus insulin	1,343	9,022
NY72-4 (41°C)[c]	591	3,675
NY72-4 (35°C)[c]	3,025	17,498

[a] Control and stimulated CEF lysates were immunoprecipitated and assayed for kinase activity in the absence and presence of exogeneous substrates. Immunoprecipitates were resolved on a sodium dodecyl sulfate–10% polyacrylamide gel and subjected to autoradiography. The autoradiograms were scanned with a Shimadzu dual wavelength chromatoscanner and data recorder.

[b] Arbitrary absorbance units per microgram of cell lysate protein immunoprecipitated. Protein concentration was determined by the method of Bradford (6).

[c] Temperature-sensitive Rous sarcoma virus mutant. Growth temperature is given in parentheses.

existence of sites phosphorylated by an enzyme other than S6 kinase itself, since these peptides are not observed in autophosphorylated CEF pp90. These results corroborate the previous immunochemical data, indicating near identity between the S6 kinases identified in *Xenopus laevis* and CEF.

We previously reported the molecular cloning of a chicken cDNA (1) related to the *Xenopus* S6KII cDNA (16). The proteins predicted by the chicken and *Xenopus* cDNAs are 86% identical, predicting the presence of an S6 kinase in chicken that is a homolog of *Xenopus* S6KII. In this report, we demonstrate the presence of a 90,000-M_r protein in CEF that is functionally and immunologically related to the 92,000-M_r *Xenopus* S6KII. As predicted by the similarities between the *Xenopus* and chicken cDNAs, comparison of phosphopeptides generated by partial proteolysis revealed nearly identical peptides for the in vitro-phosphorylated pp90 CEF and pp92 *Xenopus* S6 kinases (Fig. 4). Additional unique phosphopeptides were generated from the pp90 CEF protein that was biosynthetically labeled during serum stimulation. These additional phosphopeptides may represent sites of phosphorylation involved in activation of S6 kinase and presumably those phosphopeptides phosphorylated by an S6 kinase kinase(s) that is unidentified at this time.

Consistent with previous reports demonstrating the role of phosphorylation in S6 kinase activation (2, 3, 12, 21), the pp90 CEF S6 kinase is phosphorylated on serine and threonine residues in response to serum, PMA, or EGF plus insulin (Fig. 3). pp60^{src} activation resulted in additional phosphorylation of pp90 on tyrosine. While direct activation of S6 kinase by pp60$^{v\text{-}src}$could account for this event, the functional significance of tyrosine phosphorylation is unclear because of the absence of phosphotyrosine in pp90 at 10 min and 1 h after stimulation with other mitogens (Fig. 3).

As stated previously, several groups have reported purification of homogeneous S6 kinase activities. With the exception of 92,000-M_r *Xenopus* S6KII (10, 11), S6 kinases purified from several tissues and species ranged in apparent molecular weight from 65,000 (in chicken embryos [4]) to 70,000 (in Swiss mouse 3T3 cells). Giugni and colleagues have recently reported the partial purification from A431

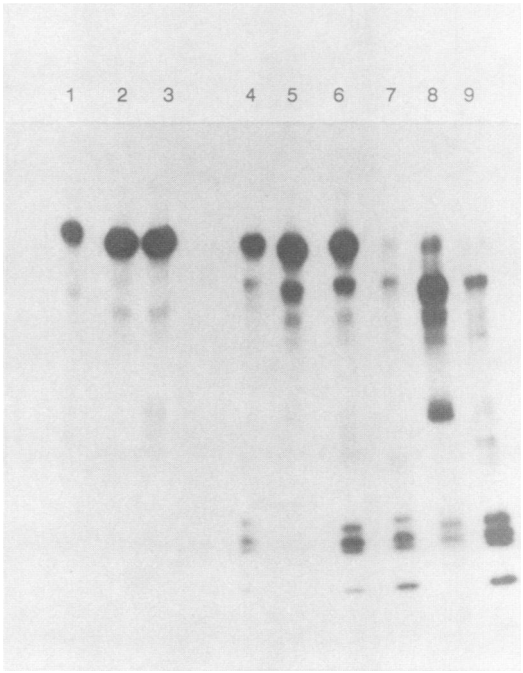

FIG. 4. Comparison of phosphopeptides by partial proteolysis of purified *Xenopus* S6KII and the avian *rsk* gene product. Autophosphorylated purified *Xenopus* S6KII, biosynthetically ^{32}P-labeled CEF *rsk* isolated by immunoprecipitation with antiserum 125, and CEF *rsk* autophosphorylated following immunoprecipitation with antiserum 125 were analyzed by partial proteolysis. The phosphorylated *rsk* products were cut from sodium dodecyl sulfate-10% polyacrylamide preparative gels, and the phosphopeptides were generated with 0 (lanes 1 through 3), 10 (lanes 4 though 6), or 100 (lanes 7 through 9) ng of elastase during resolution on 11 to 20%-gradient sodium dodecyl sulfate-polyacrylamide gels (7). Phosphopeptides of the *rsk* gene product purified from *X. laevis* unfertilized eggs (lanes 1, 4, and 7), from biosynthetically labeled CEF (lanes 2, 5, and 8), and from phosphorylated CEF immunocomplexes (lanes 3, 6, and 9) are shown.

cells of a 100,000-M_r kinase, which is activated by EGF and which phosphorylates several proteins, including S6 (14). This 100,000-M_r protein may represent a *rsk* gene product. The present report on the identification of a 90,000-M_r *rsk* product in CEF raises the question of why this protein has not been found during previous studies on purification of S6 protein kinase activity from these cells. Susa and colleagues recently reported a biphasic time response by S6 kinase activity in extracts of 3T3 cells (22). While these authors suggest that there is a single, differentially activated enzyme in these cells, recent data (L. Sweet, D. Alcorta, and R. Erikson, submitted for publication; J. Blenis, personal communication) suggest that there are two distinct enzymes in both CEF and 3T3 fibroblasts and that pp90^{rsk} is maximally activated within minutes after mitogenic stimulation. Previous reports on the purification of S6 kinase utilized cells stimulated for 1 h, perhaps inducing a systematic bias that could have resulted in discarding pp90^{rsk} during purification in favor of the other major contributor to S6 phosphorylation, 65,000-M_r protein. The data reported here show that pp90^{rsk} has the potential to contribute to the phosphorylation of S6 following the mitogenic stimulation of cells. Its role in S6 phosphorylation and the phosphorylation of other substrates remains to be determined.

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