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S6 kinase in quiescent Swiss mouse 3T3 cells is activated by phosphorylation in response to serum treatment

(phosphatase 2A/phosphorylation cascades/ribosomal protein S6/phospho amino acid analysis)

LISA M. BALLOU, MICHEL SIEGMANN, AND GEORGE THOMAS*

Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland

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ABSTRACT **To investigate the role of phosphorylation in the activation of S6 kinase, the enzyme was isolated from ^{32}P-labeled Swiss mouse 3T3 cells before and after stimulation with serum. The kinase activity was followed through several purification steps, and a radioactive protein of M_r 70,000 was obtained from the stimulated cells. This band was not detected in resting cells. The M_r 70,000 protein exhibited the same size upon NaDodSO$_4$/PAGE as the homogeneous kinase, and it comigrated with the *in vitro* autophosphorylated form of the enzyme. Treatment of the *in vivo*-labeled material with phosphatase 2A led to a loss of kinase activity concomitant with a release of $^{32}P_i$ from the M_r 70,000 protein. The partially dephosphorylated protein migrated faster during PAGE, displaying distinct species of M_r 69,000 and 68,000. Most importantly, phospho amino acid analysis of the labeled S6 kinase showed only phosphoserine and phosphothreonine. These results argue that the S6 kinase is phosphorylated at multiple sites *in vivo* and that it is activated by serine/threonine phosphorylation.**

Cells exposed to mitogens undergo coordinate biochemical changes that culminate in DNA synthesis and cell division. To understand how cell growth is regulated, identification of the mediators that link extracellular signals with intracellular responses is essential. One approach has been to examine growth factor receptors and the events they trigger at the plasma membrane, leading to the discovery that many receptors possess a ligand-activated tyrosine kinase activity. Thus rose the hypothesis that signals were transmitted through phosphorylation cascades (1). However, it has been difficult to identify physiologically relevant substrates for receptor tyrosine kinases and to correlate phosphorylation with any functional change in target proteins (1). Similarly, some receptors were found to act through G proteins and phospholipase C to increase inositol-1,4,5-triphosphate and diacylglycerol, leading to a rise in intracellular Ca^{2+} and activation of protein kinase C (2). Again, how these events couple to the stimulation of cell growth is not clear.

A second approach has been to start with an intracellular process and to delineate the proximal steps leading to the activation of that process. The phosphorylation of 40S ribosomal protein S6 is a useful model for this purpose. Addition of growth factors or expression of oncogenes in quiescent cells leads to the incorporation of up to 5 moles of phosphate into S6 (3, 4). This reaction is catalyzed by a specific S6 kinase that is immediately activated upon treatment with these stimulatory agents (5–19). The phosphates are added to ribosomal protein S6 in a specific order at the carboxyl terminus in a sequence containing 13 amino acid residues (20–22). The later phosphorylation sites are thought to either facilitate or trigger the increased rate of protein synthesis required for cell division (23–25). In earlier studies we found that the mitogen-stimulated S6 kinase in cell extracts rapidly loses activity in the absence of phosphatase inhibitors, suggesting that the inactivator might be a phosphatase (5, 6). This suspicion was confirmed by showing that the major kinase inactivator in these extracts is a type 2A phosphatase (26, 27); this enzyme is distinct from the S6 phosphatase, which is a type 1 enzyme (28). The kinases stimulated by epidermal growth factor, serum, and orthovanadate are all phosphatase-sensitive and behave similarly on cation- and anion-exchange columns (19, 29), arguing that different agents activate the same enzyme. Recently, we purified the kinase from orthovanadate-treated Swiss mouse 3T3 cells (29, 30). This kinase consists of one polypeptide of M_r 70,000, and in its homogeneous form it is still susceptible to phosphatase inactivation (29), implying that the enzyme is activated directly by phosphorylation.

To establish the validity of this hypothesis it is necessary to demonstrate that the kinase becomes phosphorylated in response to mitogenic stimuli and that removal of the phosphate leads to a loss of enzyme activity. To address these questions, the kinase was purified from cells stimulated with serum in the presence of $^{32}P_i$. The effect of phosphatase 2A was examined, and the ^{32}P-labeled phospho amino acid residues were identified. The results not only show that the kinase is directly regulated by phosphorylation–dephosphorylation but also point to the existence of a mitogen-activated kinase cascade.

MATERIALS AND METHODS

Assays. S6 kinase was assayed according to Olivier *et al.* (28). The catalytic subunit of phosphatase 2A was purified from rabbit skeletal muscle (31) and assayed as described (26).

^{32}P Labeling and Preparation of Extracts. Swiss mouse 3T3 cells were maintained in 15-cm plates and used 8 days after seeding (26). Before labeling, the cells were washed with 10 ml of 150 mM NaCl/20 mM Hepes (Serva, Heidelberg)/pH 7.2, and then with 10 ml of P_i-free Dulbecco's modified Eagle's medium (GIBCO). For serum stimulation, the cells were then incubated for 1 hr in 15 ml of P_i-free medium containing 5 mCi of $^{32}P_i$ (Amersham) and 10% fetal calf serum (GIBCO) that had been dialyzed at 4°C against NaCl/Hepes buffer. The radioactive medium from 10 plates was then removed and pooled, an additional 3 ml of dialyzed serum was added, and the medium was equally distributed onto the next set of 10 plates; this process was repeated for 5 sets of plates. Resting cells were labeled in the same way except that serum was replaced with NaCl/Hepes buffer. After being labeled, the cells were washed twice with 10 ml of ice-cold extraction buffer (15 mM pyrophosphate/1 mM EGTA/1 mM dithiothreitol/1 mM benzamidine/0.1 mM phenylmethylsul-

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*To whom reprint requests should be addressed.

fonyl fluoride, pH 6.5). They were then scraped from the plate, homogenized with 20 strokes of a glass homogenizer, and centrifuged at 12,000 × *g* for 15 min at 2°C. The supernatants were immediately frozen and stored at −70°C.

Purification of S6 Kinase. S6 kinase from serum-stimulated and resting cells was purified an estimated 5000-fold using a modification of the procedure used earlier to obtain the orthovanadate-stimulated enzyme (29, 30). All steps were performed at 4°C, and 0.1 mM phenylmethylsulfonyl fluoride was added after each step. The kinase was stored at −70°C after steps *i*, *iv*, and *v*.

(i) Cation exchange. Extracts from 50 plates of ^{32}P-labeled cells (≈25 ml; ≈25 mg of protein) were thawed and centrifuged for 1 hr at 250,000 × *g*. The supernatants were diluted with 1 vol of 2 mM Na_2HPO_4, pH 6.5, and loaded onto a 1-ml Mono S column (Pharmacia) equilibrated in 50 mM 2-morpholinoethanesulfonic acid/1 mM EGTA/10 mM pyrophosphate/1 mM dithiothreitol/0.1% Triton X-100/1 mM benzamidine, pH 6.5. The column was washed with 5 ml of buffer and then developed with a NaCl gradient (Fig. 1). The flow rate was 0.5 ml/min, and 1-ml fractions were collected. The S6 kinase emerged at ≈180 mM NaCl.

(ii) Anion exchange. Fractions from step *i* (5 ml) were diluted with 20 ml of Mono Q buffer (20 mM triethanolamine/1 mM EGTA/50 mM NaF/1 mM dithiothreitol/1 mM benzamidine/0.1% Triton X-100, pH 7.8), and 20 μM 8-Br-cAMP was added. The enzyme was applied to a 1-ml Mono Q column (Pharmacia), and the column was washed with 5 ml of buffer. The proteins were eluted at 0.6 ml/min with a gradient of NaCl from 0 to 500 mM, and 30 1-ml fractions were collected. The enzyme emerged at ≈300 mM NaCl.

(iii) Gel filtration. Fractions from step *ii* (4 ml) were applied to a column of Sephacryl S-200 (83.5 × 2.6 cm) equilibrated in 10 mM morpholinopropanesulfonic acid (Mops)/0.1 mM EDTA/10 mM NaF/1 mM dithiothreitol/0.1% Triton X-100/1 mM benzamidine, pH 7.4. The flow rate was 26 ml/hr, and fractions of 2.2 ml were collected. The S6 kinase emerged at a position corresponding to M_r 80,000.

(iv) ATP affinity. The Sephacryl S-200 pool (24 ml) was applied to 500 μl of AGATP type 4 resin (Pharmacia) equilibrated in the buffer used in step *iii*. The column was washed with 1 ml of buffer, and the proteins were eluted at 0.2 ml/min with a gradient of ATP from 0 to 30 mM. Twenty 1-ml fractions were collected, and the S6 kinase activity emerged at ≈12 mM ATP.

(v) Concentration. The most active fractions from step *iv* (5 ml) were desalted on 50 ml of Sephadex G-25 medium in 40 mM Tris/1 mM dithiothreitol/0.1 mM EDTA/0.1% Triton X-100/1 mM benzamidine, pH 7.5. The enzyme was then concentrated on 75 μl of Fast Flow Q Sepharose (Pharmacia) and eluted with 225 μl of Mono Q buffer containing 1 M NaCl.

RESULTS

Recovery of a ^{32}P-Labeled M_r 70,000 Protein After Serum Stimulation. Our earlier results suggested that the S6 kinase is activated by phosphorylation (5, 6, 26–29). To test this hypothesis directly, we purified the kinase from ^{32}P-labeled cells before and after stimulation with serum. Because antibodies to immunoprecipitate the protein are not available, the enzyme was isolated by use of a modified version of the procedure first used to purify the activated kinase (refs. 29, 30, and see *Materials and Methods*). An activity profile of the first column showed that the serum-stimulated enzyme was ≈50 times more active than the one from resting cells (Fig. 1). This level of activation was larger than the 10- to 25-fold activation usually obtained with serum (5, 29) and was probably due to a reduction in basal activity following the 1-hr incubation of the resting cells in serum-free medium. Although the S6 kinase activity in resting cells was low, it was

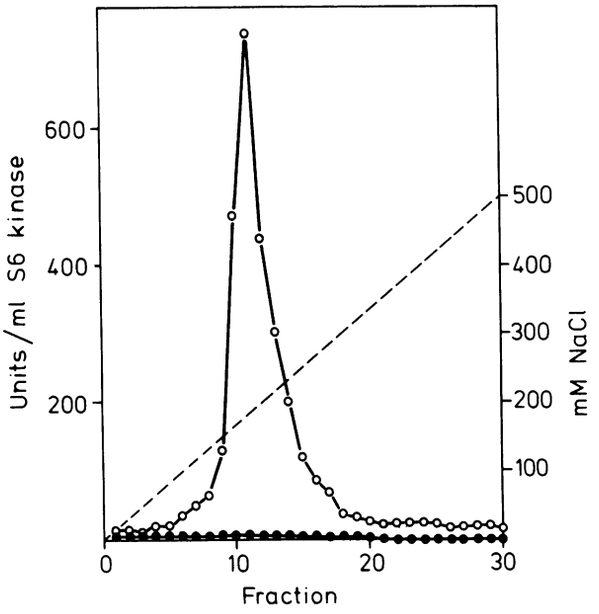

FIG. 1. Elution of S6 kinase from a Mono S column. High-speed supernatants from resting (●) or serum-stimulated (○) cells were subjected to cation-exchange chromatography as described. ---, mM NaCl.

detectable at every purification step, and it coeluted with the activity from stimulated cells. In both cases the enzyme behaved as one entity at each step (data not shown).

To determine whether a radiolabeled protein band in the kinase fractions correlated with enzyme activity, the proteins pooled at each step were examined by autoradiography. In resting cells many ^{32}P-labeled bands were detected early in the purification but virtually all of them were removed after chromatography on Sephacryl S-200 (Fig. 2*A*). Crude fractions from serum-stimulated cells also contained many labeled bands (Fig. 2*B*). After the sizing column, several radioactive proteins were present, including a major species at M_r 70,000. After the ATP-affinity step only two radioactive bands remained—the major protein of M_r 70,000, migrating in a position identical to that previously reported for the S6 kinase (29, 30), and a faint band at M_r 39,000. The M_r 39,000 protein was the only one that could be seen on Coomassie-stained gels (data not shown), and this band can be removed by subsequent steps of purification (29, 30). The final pool of concentrated S6 kinase from serum-stimulated cells contained 5000 cpm of Cerenkov radiation and ≈1000 units of enzyme activity. All subsequent experiments were done with this material.

Autophosphorylation. We showed earlier that the homogeneous kinase displays an apparent M_r of 70,000 on NaDodSO$_4$ gels, it phosphorylates itself *in vitro*, and this phosphorylation has no effect on its activity (29, 30). To test whether the radioactive M_r 70,000 protein could also autophosphorylate, a sample of the kinase was incubated with [γ-^{32}P]ATP and subjected to NaDodSO$_4$/PAGE. The results show that only one protein became autophosphorylated *in vitro* and that it displayed an M_r identical to that of the protein labeled *in vivo* (Fig. 3). Thus, the characteristics of molecular size and autophosphorylation indicated that the ^{32}P-labeled M_r 70,000 protein was the S6 kinase.

Effect of the Catalytic Subunit of Phosphatase 2A. We demonstrated previously that protein phosphatase 2A inactivates the S6 kinase (26–29). Therefore, treatment of the *in vivo*-labeled M_r 70,000 protein with the phosphatase should cause a loss of phosphate corresponding to the loss of enzyme activity. To examine this possibility, the kinase was incubated alone or with the catalytic subunit of phosphatase 2A.

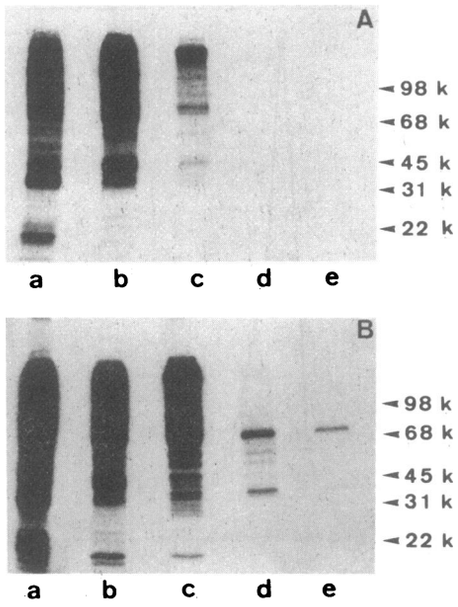

FIG. 2. ^{32}P-labeled proteins in fractions from steps of the kinase purification. Proteins from resting (*A*) and serum-stimulated (*B*) cells (0.75% of each pool) were precipitated with 8.3% (vol/vol) trichloroacetic acid/0.013% deoxycholate. The pellets were washed with 30 μl of −20°C acetone and 30 μl of −20°C ether and then dried under vacuum. The proteins were dissolved in $NaDodSO_4$ sample buffer and subjected to PAGE on 15% gels (26). Autoradiography was performed for 3 days at −70°C using Kodak XAR-5 film and intensifying screens. Lanes: a, high-speed supernatants; b, Mono S column eluate; c, Mono Q column eluate; d, Sephacryl S-200 column eluate; e, AGATP type 4 column eluate. The M_r markers are phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

In control reactions lacking phosphatase, the kinase activity and the phosphorylation state of the M_r 70,000 protein did not change after 15 min at 37°C (Fig. 4*A*). However, with the catalytic subunit of phosphatase 2A a rapid loss of kinase activity occurred, which was paralleled by a loss of ^{32}P from the M_r 70,000 band (Fig. 4*B*). Consequently, the kinase

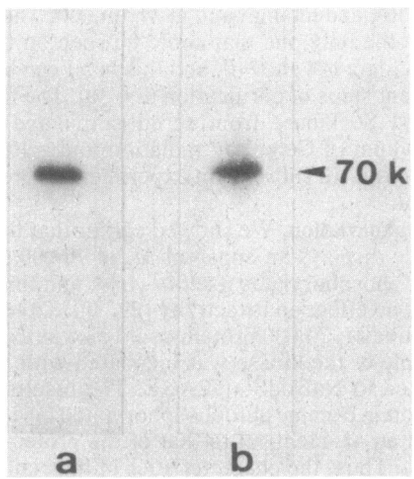

FIG. 3. Comigration of the ^{32}P-labeled M_r 70,000 protein with autophosphorylated S6 kinase. Lane a, 5 μl of concentrated S6 kinase from ^{32}P-labeled stimulated cells were subjected to $NaDodSO_4$/PAGE followed by autoradiography for 2 days (see legend of Fig. 2). Lane b, 1 μl of kinase was mixed with 9 μl of buffer A (50 mM Mops/1 mM dithiothreitol/10 mM $MgCl_2$/0.1% Triton X-100, pH 7.2), containing 1 μM [γ-^{32}P]ATP (1000 cpm/fmol, Amersham). After 30 min at 37°C, the autophosphorylated kinase was subjected to PAGE followed by autoradiography for 1 hr.

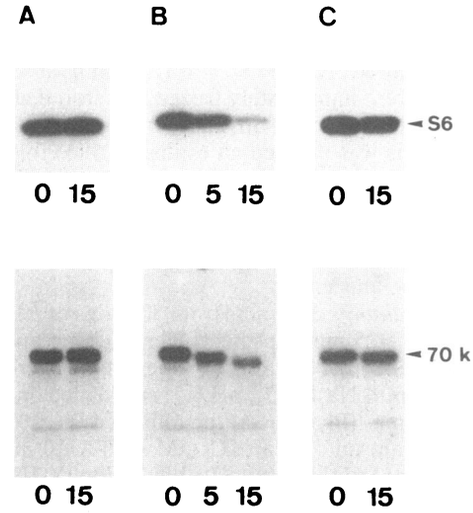

FIG. 4. Loss of S6 kinase activity and loss of ^{32}P from the M_r 70,000 protein. Concentrated S6 kinase from ^{32}P-labeled stimulated cells was dialyzed against buffer A for 2 hr at 4°C. Upper panels represent *in vitro* phosphorylated S6, whereas lower panels represent *in vivo* phosphorylated S6 kinase. (*A*) Control reactions containing 5 μl of kinase and 5 μl of buffer A were incubated at 37°C for the indicated times in min. One μl was removed and assayed immediately for S6 kinase activity, and the remaining 9 μl were subjected directly to $NaDodSO_4$/PAGE. (*B*) The same as *A* except that the reaction also contained the catalytic subunit of phosphatase 2A at 2.5 units/ml. (*C*) The same as *B* except that 10 mM *p*-nitrophenyl phosphate was included. Autoradiography for S6 was for 30 min, and for the M_r 70,000 kinase, autoradiography was for 2 weeks.

migrated faster in the gels, generating two new species of M_r 69,000 and 68,000. In contrast to the kinase, the M_r 39,000 contaminant was unaffected by the phosphatase.

Others have shown that the extent of phosphorylation can affect the mobility of specific proteins during $NaDodSO_4$/PAGE (32–34). To ensure that the appearance of the lower-M_r bands in Fig. 4*B* was due to dephosphorylation, the kinase and phosphatase were incubated together with *p*-nitrophenyl phosphate, a phosphatase inhibitor. This compound largely prevented the shift in M_r, loss of ^{32}P, and loss of kinase activity (Fig. 4*C*), supporting the argument that all three events are due to dephosphorylation of the kinase. This conclusion was further tested by monitoring the release of $^{32}P_i$ from the kinase by one-dimensional thin-layer electrophoresis during the reaction shown in Fig. 4*B*. This analysis showed a time-dependent release of free phosphate from the kinase (Fig. 5), confirming that dephosphorylation was occurring.

Phospho Amino Acid Analyses. To identify the phosphorylated residues in the *in vivo*-labeled kinase, an acid-hydrolyzed sample was analyzed by two-dimensional thin-layer electrophoresis. The majority of the ^{32}P was found in phosphoserine, with a substantial amount also present in phosphothreonine (Fig. 6*A*). No phosphotyrosine was detected; however, this absence could be due to the phosphatase inhibitors used or the time of stimulation. Similar results were obtained with the *in vitro*-labeled autophosphorylated kinase, except that there was relatively less phosphothreonine (Fig. 6*B*). It should also be noted that the sample derived from *in vivo*-labeled material contained several phosphopeptides migrating near the origin that were not seen in the autophosphorylated sample. Taken together, these results indicate that the kinase is activated by phosphorylation of serine or threonine residues. Thus, if the S6 kinase participates in a phosphorylation cascade initiated by a growth factor receptor, there must be at least one additional serine/threonine kinase coupling these two enzymes.

FIG. 5. Release of P_i from the M_r 70,000 protein. Dialyzed S6 kinase was incubated with the catalytic subunit of phosphatase 2A for the times indicated (see legend of Fig. 4*B*). The 10-μl samples were then heated at 95°C for 5 min, dried under vacuum, and dissolved in pH 3.5 electrophoresis buffer (35). One-dimensional thin-layer electrophoresis was done as described (35), and the plate was exposed to film for 2 weeks.

DISCUSSION

Our previous results showing that the S6 kinase is sensitive to protein phosphatases (26–29) gave rise to a simple model for regulation of the enzyme: In resting cells most of the kinase is in an inactive and unphosphorylated state, and upon treatment with mitogens the enzyme becomes phosphorylated and activated. We show here that extracts from serum-stimulated cells, in contrast to resting cells, contain a phosphorylated protein that follows S6 kinase activity through several purification steps. All properties of the protein examined here indicate this molecule to be equivalent to the S6 kinase. The idea that the kinase is activated by phosphorylation is supported by the fact that the protein was unlabeled in resting cells (Fig. 2). In part, this could be explained by the slower uptake of $^{32}P_i$ from the medium by quiescent cells (36). However, because the total radioactivity incorporated into resting-cell protein was only 5- to 10-times lower than in stimulated cells (data not shown), this effect cannot solely account for the complete absence of ^{32}P in the M_r 70,000 protein. Further support for direct involvement of phosphorylation in activating the kinase comes from the fact that treatment with phosphatase 2A leads to the release of $^{32}P_i$ from the protein and a parallel loss of kinase activity (Fig. 4). Interestingly, not all phosphoproteins are dephosphorylated by this phosphatase; for instance, the M_r 39,000 contaminant in the kinase preparation was unaffected by the enzyme. Final proof of this model would be an increase in the absolute amount of phosphate incorporated into the protein upon mitogenic stimulation, especially because basal S6 kinase activity in resting cells is also abolished by phosphatase 2A (29). Presently, this experiment is difficult to do because of the apparently low amount of the enzyme (29, 30).

Upon phosphatase treatment, the M_r 70,000 protein migrated faster in $NaDodSO_4$ gels. An apparent decrease in molecular weight has also been reported for unphosphorylated species of glycogen synthase kinase III (32), the double-stranded RNA-dependent kinase that phosphorylates eukaryotic initiation factor 2 (33), and protein kinase C (34). Two observations suggest that the lower-M_r species of S6 kinase are generated by dephosphorylation and not proteolysis. First, $^{32}P_i$ is released during the reaction, indicating dephosphorylation of the protein by the phosphatase (Fig. 5), and second, the appearance of the lower-M_r bands is blocked by a phosphatase inhibitor (Fig. 4). These two additional radioactive bands suggest the presence of at least three phosphates in the enzyme. These phosphates may represent the activating phosphorylation site(s), autophosphorylation sites that became labeled *in vivo*, and "silent" phosphorylation sites that do not affect enzyme activity. To date, we have found no effect of autophosphorylation on the activity of the kinase (ref. 29 and data not shown).

The relationship between the sites phosphorylated *in vivo* and those obtained by *in vitro* autophosphorylation needs to be determined. The pattern of acid-hydrolyzed phosphopeptides visible near the origins in Fig. 6 suggests that some of the sites will be distinct and some may represent the activating phosphorylation site.

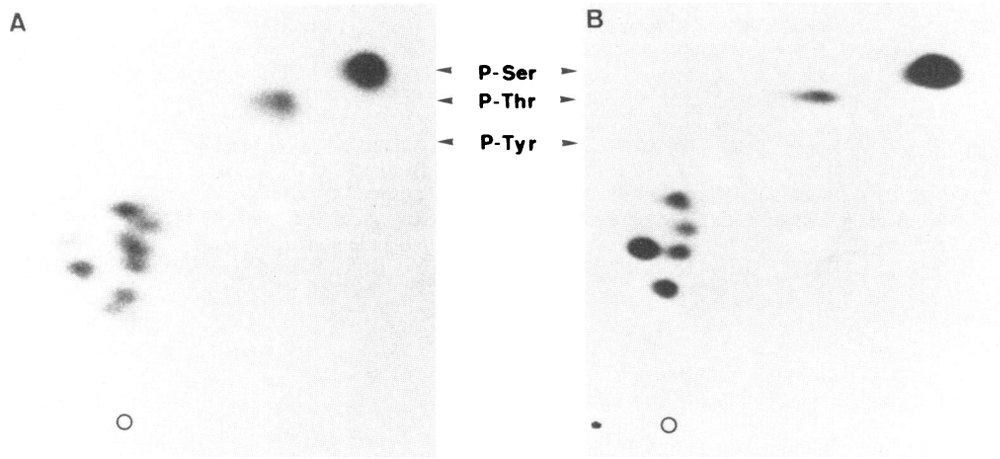

FIG. 6. Phospho amino acid analyses. Twenty μl of concentrated kinase from ^{32}P-labeled stimulated cells (*A*) and autophosphorylated S6 kinase (*B*) (see legend of Fig. 3) were subjected to $NaDodSO_4$/PAGE (26). The radioactive M_r 70,000 proteins were located by autoradiography, excised from the gel, and electroeluted overnight in 100 mM $(NH_4)_2HCO_3$/0.1% $NaDodSO_4$ using 18 μg of ovalbumin as a carrier. The samples were evaporated to dryness in a Speedvac vacuum and the pellets were washed with 200 μl of 90% (vol/vol) acetone. After drying under vacuum, the proteins were hydrolyzed with HCl for 1 hr (35). The samples were dried again and then subjected to two-dimensional thin-layer electrophoresis (35); the first dimension was from left to right, and the second dimension was from bottom to top. Autoradiography was for 2 weeks. ○, Origin.

The mechanism of S6 kinase activation has for several years been a matter for speculation. The enzyme can be stimulated *in vivo* by growth factors that act through receptor tyrosine kinases (5–8, 10, 12, 18), by expression of $pp60^{v\text{-}src}$ (13, 15), by microinjection of active insulin receptors (18), and by orthovanadate (6), an inhibitor of phosphotyrosine-protein phosphatases (37). In addition, high concentrations of phosphotyrosine protect the enzyme in crude extracts (6). From these results the S6 kinase has been suggested to be activated by tyrosine phosphorylation (6, 8, 14, 16, 17, 38). However, we show here that only phosphoserine and phosphothreonine are detectable in the activated enzyme (Fig. 6). This agrees with our previous finding that the kinase is sensitive to phosphatases 1 and 2A (26, 27), which are relatively specific for phosphoseryl and phosphothreonyl residues (39). Alternatively, S6 kinase might be activated by protein kinase C because the former enzyme becomes stimulated in cells treated with phorbol esters or hormones that increase diacylglycerol levels (7, 9, 10, 13–15). However, down-regulation of protein kinase C by prolonged treatment with phorbol esters does not completely abolish S6 kinase activation induced by a second mitogen (9, 10, 13, 15), indicating that protein kinase C-independent pathways also play a role in S6 kinase activation. In support of this conclusion, we were unable to reactivate phosphatase-treated S6 kinase with purified protein kinase C (Mira Susa, L.M.B. and G.T., unpublished data).

The S6 kinase kinase may be an enzyme distinct from those already known to play a role in signal transduction, and its activity might be modulated by tyrosine phosphorylation. Preliminary reports have described two kinases that may fit these criteria: one phosphorylates microtubule-associated protein 2 (40) and the other can be assayed using histone as a substrate (41). Identification of the S6 kinase kinase would be a valuable link in tracing the signal transduction pathways leading to S6 phosphorylation and cell growth.

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