Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 406–410, January 1988
Biochemistry

Identification and characterization of a mitogen-activated S6 kinase

(Swiss mouse 3T3 cells/orthovanadate/autophosphorylation/protein phosphatase 2A/ribosomal protein S6)

PAUL JENÖ, LISA M. BALLOU, ILSE NOVAK-HOFER*, AND GEORGE THOMAS†

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

Communicated by E. Racker, September 30, 1987 (received for review August 24, 1987)

ABSTRACT **Treatment of Swiss mouse 3T3 cells with epidermal growth factor, orthovanadate, or serum results in the activation of a kinase that phosphorylates protein S6 of the 40S ribosomal subunit *in vitro*. This kinase is eluted as a single peak of activity from either a Mono Q anion-exchange column at 0.34 M NaCl or a Mono S cation-exchange column at 0.20 M NaCl. Treatment of the peak fraction from the Mono S column with phosphatase 2A completely abolishes the activity of the enzyme. The kinase appears to be distinct from protein kinase C, cAMP-dependent protein kinase, and two protease-activated kinases, PAK II and H4P. The kinase has been purified to apparent homogeneity and migrates as a single band at M_r 70,000 in NaDodSO$_4$/polyacrylamide gels. The kinase exhibits the ability to autophosphorylate, and this activity directly parallels S6 phosphorylation activity on the final step of purification. *In vitro*, the kinase incorporates up to 5 mol of phosphate into S6, and the tryptic phosphopeptide maps obtained are equivalent to those from S6 phosphorylated *in vivo*. Most important, treatment of the purified kinase with phosphatase 2A results in complete inactivation of the enzyme, arguing that the activity of the kinase is directly controlled by phosphorylation.**

The activation of protein synthesis and subsequent cell growth in numerous biological systems is preceded by the multiple phosphorylation of S6, a protein of the 40S ribosomal subunit (1–6). In the case of quiescent animal cells stimulated to proliferate by specific mitogens, protein synthesis increases by as much as 3-fold and up to 5 mol of phosphate are incorporated per mol of S6 (7, 8). The function of S6 phosphorylation in this system is not clearly understood; however, a central role in controlling the initiation of protein synthesis has been suggested by several lines of evidence (9–15).

The possibility that protein phosphorylation cascades may be involved in the activation of cell growth has recently focused a great deal of attention on the kinase that phosphorylates S6 (16–24). Earlier we described an S6 kinase activity from Swiss mouse 3T3 cells that was stimulated up to 25-fold by serum, epidermal growth factor (EGF), or sodium orthovanadate (vanadate) (16, 17). The S6 kinase activity in extracts prepared from cells treated with increasing concentrations of EGF was found to closely parallel the dose–response curve observed for S6 phosphorylation in the intact cell (16). In addition, the two-dimensional tryptic phosphopeptide maps derived from S6 phosphorylated *in vitro* and *in vivo* were equivalent (25). Together, these two findings suggested that the S6 kinase activity being followed *in vitro* was responsible for phosphorylating S6 in the intact cell.

To recover full kinase activity from cell extracts, it was crucial to include phosphatase inhibitors such as glycerol 2-phosphate (16, 17). This finding suggested that the kinase might be regulated by phosphorylation and that the kinase inactivator in cell extracts was a phosphatase. Recently, we confirmed the latter suspicion by demonstrating that the major S6 kinase inactivator in cell extracts is a type 2A phosphatase (18). Following a similar approach, a number of laboratories have used glycerol 2-phosphate to extract an apparently equivalent activity from other cell types in which S6 phosphorylation has been induced (19–24). Furthermore, in these studies the S6 kinase activity appeared to behave as a single enzyme.

In the work described here, we employed a number of criteria, including column chromatography and phosphatase sensitivity, to demonstrate that EGF, vanadate, and serum activate the same S6 kinase and that it is distinct from four other kinases known to phosphorylate S6 *in vitro*. Next, we purified this kinase to apparent homogeneity and examined its ability to autophosphorylate and the extent to which it phosphorylates S6. Finally, we tested whether the purified form of the enzyme retains its sensitivity to phosphatase 2A.

MATERIALS AND METHODS

Cell Culture and Extraction. Swiss mouse 3T3 cells were seeded at 1.5×10^6 cells per 850-cm^2 roller bottle (Falcon) in 150 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO) (8). After 8 days when no mitoses were observed, cultures were stimulated for 2 hr at 37°C with 3.8 mM vanadate (BDH) in 10 mM Hepes (Serva) at pH 7.0. The cells then were harvested in extraction buffer containing 0.1 mM phenylmethylsulfonyl fluoride (16) and were homogenized for 10 sec at setting 5 with a tissue homogenizer (Kinematica, Lucerne, Switzerland) and the homogenate was centrifuged at 2°C for 45 min at 450,000 × *g*. The supernatant was frozen and stored in liquid nitrogen.

Enzyme Assays. S6 kinase activity was measured using 40S ribosomal subunits prepared from rat liver (26). Reaction mixtures contained, in a final volume of 10 μl, 50 mM 4-morpholinepropanesulfonic acid (Mops; pH 7.0), 1 mM dithiothreitol, 10 mM $MgCl_2$, 30 μM [γ-^{32}P]ATP (10–30 cpm/fmol, Amersham), 10 mM *p*-nitrophenyl phosphate, 17 μg of 40S subunits, 10 units of the heat-stable inhibitor of cAMP-dependent protein kinase (PKI), and 5 μl of enzyme fractions that had been diluted 1:10 in Mono S buffer (see legend to Fig. 3). After incubation at 37°C for 30 min, reactions were terminated by addition of 3 μl of electrophoresis sample buffer and analyzed by NaDodSO$_4$/PAGE followed by autoradiography as described (18). The assay conditions for protein kinase C were identical to those above, except that 5 mM $CaCl_2$ and 0.8 μg of L-α-phosphatidyl-L-serine (Sigma) per assay were included. A

Abbreviations: EGF, epidermal growth factor; vanadate, sodium orthovanadate; PKI, heat-stable inhibitor of cAMP-dependent protein kinase; $2A_c$, catalytic subunit of phosphatase 2A.

*Present address: Laboratory of Biochemistry-Endocrinology, Department of Research, University Clinic Medical School, CH-4031 Basel, Switzerland.
†To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

unit of kinase activity equals 1 pmol of phosphate incorporated into S6 per minute. Autophosphorylation of the S6 kinase was carried out in a final volume of 10 μl in Mono S buffer (Fig. 3) containing 1 μM [γ-^{32}P]ATP (300–1000 cpm/fmol) and 2 μl of the indicated fractions at 37°C for 30 min. The reaction was stopped and the gels were prepared for autoradiography as described above.

Purification of the S6 Kinase. The kinase was purified by sequential chromatography on Fast Flow S Sepharose, Mono Q, Sephacryl S200, ATP-substituted agarose, hydroxylapatite, and Mono S. The detailed purification and characterization of the kinase will appear elsewhere. The overall recovery was ≈0.2% with a 3000-fold purification and a final specific activity of 0.6 μmol per min per mg of protein at 100 μM ATP and 20 mM $MgCl_2$. The low recovery was due to freeze–thawing of the sample between chromatographic steps. The enzyme was stored at −20°C in Mono S buffer (Fig. 3) containing 50% (vol/vol) ethylene glycol.

Two-Dimensional PAGE and Phosphopeptide Analysis. *In vitro* phosphorylation of S6 was carried out as described in the legend to Fig. 4. For labeling with ^{32}P, ribosomes were incubated with 10 μCi (370 kBq) of [γ-^{32}P]ATP under these conditions. The 40S ribosomal proteins were isolated and S6 was either analyzed by two-dimensional PAGE (27) or digested with trypsin for analysis of phosphopeptides as described (28, 29).

RESULTS

Activation of a Common S6 Kinase. Unfractionated extracts prepared from 3T3 cells stimulated with EGF, vanadate, or serum contain higher levels of S6 kinase activity than extracts from quiescent cells (16, 17). To test whether these mitogens stimulated a common activity and whether this activity represented one or more kinases, extracts from quiescent cells and from cells treated for 30 min with one of the three agents were analyzed by Mono Q (Pharmacia) anion-exchange chromatography. Resting cells yielded two minor peaks of activity that were eluted at 0.23 M and 0.34 M NaCl (Fig. 1 *A–C*). Following mitogen stimulation, only the activity in the latter peak increased. In each case >90% of the activity applied to the column was recovered in this peak. A similar analysis on a Mono S cation-exchange column also revealed a single major peak of S6 kinase activity, eluted at 0.20 M NaCl (data not shown). Earlier we showed that partially purified preparations of the vanadate-stimulated enzyme can be inactivated by protein phosphatases (18). To determine whether each of these kinases shared this property, the peak fractions from the Mono S columns were incubated with purified phosphatase 2A catalytic subunit ($2A_c$). In each case this treatment led to almost complete inactivation of the kinase within 15 min (Table 1). Based on this evidence it appears that a single S6 kinase is activated by EGF, vanadate, and serum.

Other Kinases. A number of kinases have been reported to phosphorylate S6 *in vitro*, including the cAMP-dependent protein kinase (30–32), two protease-activated kinases termed PAK II and H4P (33, 34), and protein kinase C (35, 36). The S6 kinase does not appear to be protein kinase C, since it was eluted from a Mono Q column at a higher salt concentration (0.34 M versus 0.16 M NaCl) and was not affected by phospholipid and Ca^{2+} (Fig. 1*D*). To test for the presence of cAMP-dependent protein kinase, the enzyme was assayed in the presence of PKI. The inhibitor had no effect on the S6 kinase activity, whereas it completely abolished the activity of cAMP-dependent protein kinase (Table 2). Finally, limited proteolysis of the enzyme with trypsin led to an almost complete loss of S6 kinase activity, though it did activate a kinase present in unfractionated cell extracts (Table 2). Thus, the S6 kinase described here appears to be distinct from these four kinases.

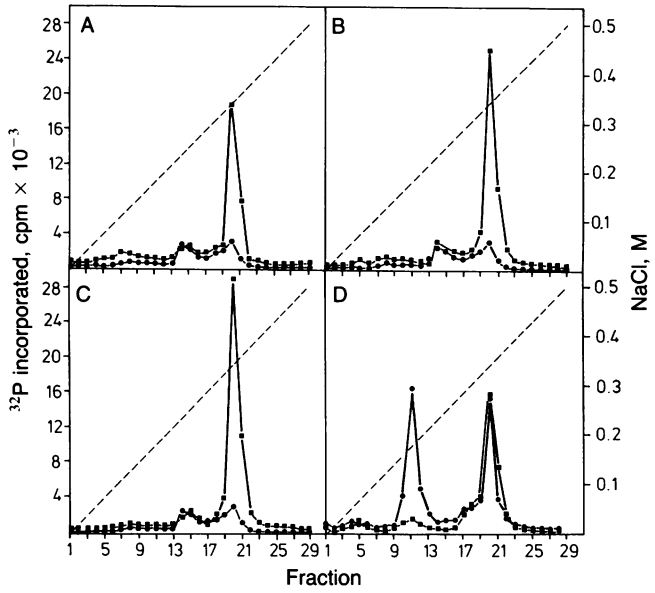

FIG. 1. Mono Q anion-exchange chromatography of S6 kinase. Unfractionated extract (5 ml) from ≈2.5 × 10^7 cells was applied to a 1.0-ml Mono Q column (Pharmacia) at a flow rate of 1 ml/min. After the column was washed with 3 ml of buffer B (20 mM Tris·HCl, pH 7.5/0.5 mM EGTA/2 mM EDTA/2 mM dithiothreitol/0.1% Triton X-100), bound material was eluted with a 30-ml linear gradient from 0 to 0.5 M NaCl (---) in buffer B. Fractions (1 ml) were collected and assayed for S6 kinase activity. Cells were stimulated for 30 min with 5 nM EGF (*A* and *D*), 1 mM vanadate (*B*), or 10% serum (*C*). In *D*, Triton X-100 was omitted from buffer B. This did not alter the elution position of the S6 kinase but did lead to a slight reduction in recovered kinase activity. ■ in *A–D*, mitogen-stimulated cells; ● in *A–C*, untreated quiescent cells; ● in *D*, EGF-stimulated cells, kinase assayed in the presence of Ca^{2+} and phospholipid.

Purification of the S6 Kinase. For purification of the kinase, vanadate was chosen for three reasons as the agent to activate the enzyme *in vivo*. First, as indicated above, the kinase stimulated by vanadate appears to be the one stimulated by EGF and serum. Second, in contrast to EGF, activation of the kinase by vanadate is persistent rather than transient (17). Third, the extent of activation at higher concentrations of vanadate is equivalent to that achieved

Table 1. Inactivation of partially purified S6 kinase by phosphatase $2A_c$

Mitogen	S6 kinase activity, milliunits	
	0 min	15 min
None	1.6	0.3
EGF	8.0	0.6
Vanadate	5.7	0.1
Serum	11.8	0

Extracts from cells treated with mitogens as described in the legend of Fig. 1 were subjected to chromatography on a Mono S column. Fractions from Mono S rather than Mono Q were chosen because they contain no endogenous phosphatase activity (18). The peak fractions of S6 kinase activity were diluted 1:10 with buffer A (50 mM Mops, pH 7.2/1 mM dithiothreitol/10 mM $MgCl_2$/0.1% Triton X-100), and a 2.5-μl aliquot was mixed with an equal volume of buffer A with 36 milliunits of rabbit skeletal muscle phosphatase $2A_c$. After a 0- or 15-min preincubation at 37°C the samples were assayed for S6 kinase activity. In control experiments lacking phosphatase, the kinases lost only about 10% of their activity (data not shown).

Table 2. Effect of PKI and trypsin on the EGF-activated S6 kinase

Sample	S6 kinase, milliunits/ml
cAMP-dependent protein kinase	38.0
cAMP-dependent protein kinase + PKI	0.4
EGF-activated S6 kinase	15.0
EGF-activated S6 kinase + PKI	15.8
Unfractionated cell extract	9.3
Unfractionated cell extract + trypsin	19.7
EGF-activated S6 kinase + trypsin	1.6

To examine the effect of PKI, 2 μl of the EGF-activated S6 kinase (Fig. 1A, fraction 20) or the catalytic subunit of cAMP-dependent protein kinase (20 μg/ml; Sigma) were preincubated in the presence or absence of 5 units of PKI for 10 min at 22°C in a final volume of 10 μl containing 20 mM Mops (pH 7.0). A 2-μl aliquot from each sample was then diluted 1:10 with 50 mM Mops, pH 7.0/1 mM dithiothreitol/10 mM $MgCl_2$/10 mM *p*-nitrophenyl phosphate and assayed essentially as described in *Materials and Methods*. To examine the effect of trypsin, 48 μl of EGF-activated S6 kinase or the same amount of unfractionated cell extract from EGF-stimulated cells was incubated in the presence or absence of 2 μl of trypsin (0.1 mg/ml; Sigma) for 5 min at 37°C. The reaction was terminated by adding 1 μl of soybean trypsin inhibitor (12 mg/ml; Sigma). A 2-μl aliquot from each sample was then diluted 1:50 and assayed as described above.

with serum. Thus, vanadate offered an efficient and inexpensive method for obtaining reasonable amounts of activated kinase from large numbers of 3T3 cells. The specific activity of the S6 kinase in extracts from vanadate-treated cells varied between 100 and 200 pmol per min per mg of protein. The enzyme was purified by the procedure outlined in *Materials and Methods*, and the polypeptide compositions of the fractions obtained from the final cation-exchange column were analyzed by $NaDodSO_4$/15% PAGE followed by silver staining. The fraction containing the peak activity showed a single band with an apparent molecular weight of 70,000 under reducing conditions (Fig. 2). This band migrated slightly faster when dithiothreitol was omitted from the sample buffer or when examined on 10% acrylamide gels, yielding a polypeptide band at M_r 65,000 (data not shown). The former finding indicates the existence of intramolecular disulfide bonds, which apparently must be reduced to obtain a sharp protein band. From 1 g of cell extract protein, approximately 500 ng of pure S6 kinase could be obtained.

Autophosphorylation. In general, most protein kinases have the ability to catalyze an autophosphorylation reaction. To test whether the M_r 70,000 protein would also autophosphorylate, fractions from the final purification step (Fig. 3) were incubated with [γ-^{32}P]ATP in the absence of 40S subunits. The only phosphorylated band detected was in fractions 16–19 (Fig. 3 *Inset*), at a molecular weight identical to that of the kinase. This activity directly overlapped with the S6 phosphorylation activity (Fig. 3), further supporting the notion that the M_r 70,000 protein is the kinase. The physiological relevance of this autophosphorylation is unclear, since less than 0.1 mol of phosphate was incorporated per mol of enzyme. However, autophosphorylation of the M_r 70,000 protein has proved to be a much more sensitive method for detecting the enzyme than silver staining.

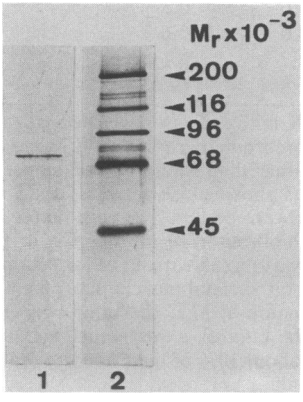

FIG. 2. $NaDodSO_4$/PAGE of purified S6 kinase. Lane 1: a 10-μl aliquot (≈10 ng of protein) from the peak fraction of the cation-exchange column (see Fig. 3) was reduced with 100 mM dithiothreitol and subjected to electrophoresis in a 15% polyacrylamide gel; the gel was stained with silver as previously described (37). Lane 2: 100 ng of each molecular weight marker (myosin heavy chain, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin).

S6 Phosphorylation and Tryptic Peptide Mapping. Following stimulation of quiescent cells with various mitogens, S6 becomes multiply phosphorylated. The extent of phosphorylation can be determined by the altered mobility of the increasingly phosphorylated derivatives on two-dimensional polyacrylamide gels. Analysis of 40S ribosomes from fasted rat liver showed S6 mainly in the dephosphorylated state, with a small amount of the monophosphorylated derivative S6a (Fig. 4A). After incubation with the mitogen-activated kinase, S6 was present as the highly phosphorylated derivatives S6c–S6e (Fig. 4B). Furthermore, in the presence of [γ-^{32}P]ATP the only phosphorylated protein detected was S6 (Fig. 4C). To examine the specificity of this reaction, ^{32}P-labeled S6 was treated with trypsin and analyzed by two-dimensional thin-layer electrophoresis. The pattern obtained was almost identical to that previously reported for highly phosphorylated S6 derived from serum-stimulated 3T3 cells (Fig. 4D) (27). Peptides 3–10 were clearly visible, whereas peptides 1, 2, and 11 were only weakly labeled (32). Thus, the purified enzyme appears to phosphorylate S6 to the same extent and with the same specificity as observed *in vivo*.

Effect of Phosphatase 2A. The data in Table 1 and our earlier studies (18) suggested that the mitogen-stimulated S6 kinase might be either directly or indirectly regulated by phosphorylation. As can be seen in Fig. 5, incubation of the purified kinase with phosphatase $2A_c$ led to a time-dependent and complete loss of activity that was prevented in the presence of *p*-nitrophenyl phosphate, an efficient inhibitor of the phosphatase. Thus, the activity of the S6 kinase appears to be directly controlled by phosphorylation.

DISCUSSION

To understand the regulation of S6 phosphorylation during the mitogenic response will require the identification of the kinases and phosphatases involved in controlling this event. Previous physiological and biochemical studies from this laboratory argued that the mitogen-stimulated activity followed in cell extracts was responsible for phosphorylating S6 in the intact cell (16, 17, 25). Further, it was implied that EGF, vanadate, and serum were stimulating the same activity. Here we present data that support this hypothesis and further argue that the kinase in all three cases is a single entity. A number of groups have recently described activation of a similar enzyme in other systems (19, 20, 22–24). The findings to date suggest that all of these enzymes might be identical; however, final proof awaits further studies.

The results in Fig. 1 and Table 2 argue that the enzyme described here is distinct from the cAMP-dependent protein kinase, protein kinase C, and the protease-activated kinases PAK II (33) and H4P (34). For the latter three enzymes this conclusion is supported by several additional lines of evidence. First, desensitization of protein kinase C by phorbol esters does not block subsequent activation of the S6 kinase by other mitogens or by oncogenes (19, 23, 38). Furthermore, in Swiss 3T3 cells, unlike other cell types, EGF does not appear to be linked to phospholipid turnover, diacylglycerol production, or protein kinase C activation (39–41), even though it is a potent activator of the kinase in Swiss 3T3 cells. With regard to PAK II and H4P, the proforms of these two kinases have molecular weights similar to that of the S6 kinase but require protease treatment to become activated.

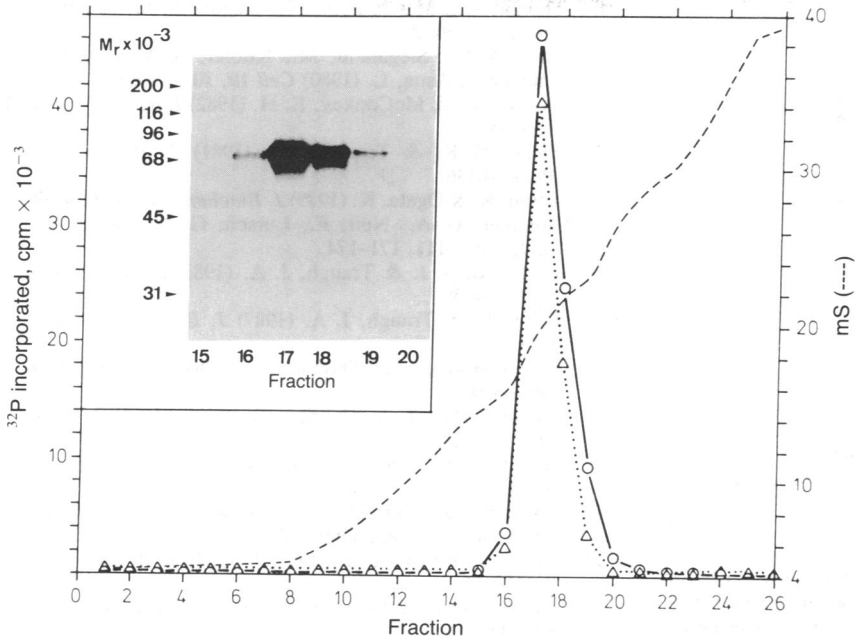

FIG. 3. Cation-exchange chromatography of S6 kinase. For the final purification step, enzyme pooled from the hydroxylapatite column was chromatographed on a 450-μl Mono S column equilibrated in 25 mM phosphate buffer, pH 6.5/1 mM EGTA/1 mM EDTA/1 mM glycerol 2-phosphate/1 mM benzamidine/2 mM dithiothreitol/20% ethylene glycol/0.1% Triton X-100/0.05% PEG 6000 (Mono S buffer). Proteins were eluted at a flow rate of 100 μl/min with a linear gradient up to 0.5 M NaCl. Fractions (100 μl) were collected and tested for enzymatic activity in the presence (○) or absence (△) of 40S subunits. The broken line indicates the conductivity of each fraction. (*Inset*) Autoradiogram of $NaDodSO_4$/PAGE analysis of fractions 15–20 assayed in the absence of 40S subunits. Molecular weight markers were identical to those used in Fig. 2.

When the S6 kinase is treated with protease it becomes inactivated. Although we cannot rule out the possibility that we have isolated a proteolytic fragment of either PAK II, H4P, or protein kinase C, this seems unlikely because the protease-treated forms of these enzymes are substantially smaller (M_r 45,000–55,000) than the S6 kinase reported here.

Two additional S6 kinases have been purified to apparent homogeneity: a M_r 92,000 protein isolated from unfertilized *Xenopus laevis* eggs (21, 42) and a M_r 67,000 kinase purified from bovine liver (43). The enzyme from eggs had a specific activity of 41 nmol per min per mg and *in vitro* it incorporated 4–5 mol of phosphate into S6. The phosphopeptide maps generated under these conditions were identical to those observed *in vivo*, and the enzyme was highly specific for S6. The enzyme from bovine liver was purified more than 50,000-fold and had a specific activity of 22 nmol per min per mg. *In vitro* it appeared to phosphorylate S6 to a higher degree than either protein kinase C or the cAMP-dependent protein kinase, but neither the stoichiometry of phosphorylation nor phosphopeptide maps were reported. The apparent molecular weight was similar to that described here for the S6 kinase; however, the specific activities (22 versus 600 nmol per min per mg) are quite different. The relationship between these two enzymes and the S6 kinase described here is unknown. One of the distinctive features of the 3T3 cell enzyme is its extreme sensitivity to phosphatases. Thus, it will be important to determine whether any of the other kinases that phosphorylate S6 also share this property (19–24, 42, 43).

The amplification of intracellular signals through phosphorylation cascades has been an attractive model both in the field of mitogenesis and in other systems. However, there

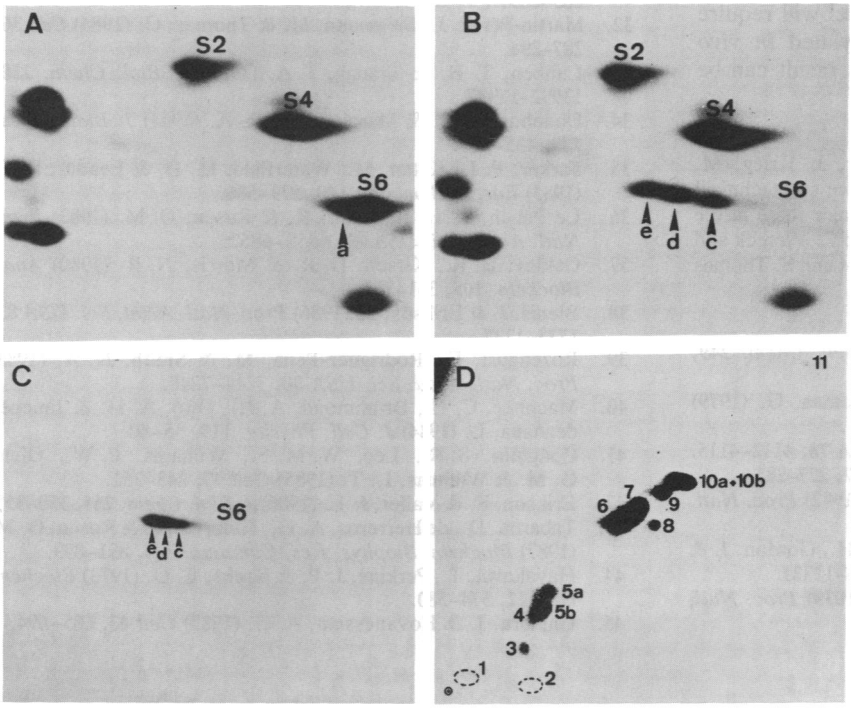

FIG. 4. Two-dimensional PAGE and tryptic phosphopeptide analysis of S6. Rat liver 40S ribosomes (300 μg) were phosphorylated essentially as described by Martin-Pérez and Thomas (29) in the absence (*A*) or presence (*B* and *C*) of the S6 kinase (45 units) and analyzed by two-dimensional PAGE. (*D*) Two-dimensional tryptic phosphopeptide map of S6 derived from a portion of the ^{32}P-labeled S6 shown in *C*. *A* and *B* show Coomassie blue-stained gels; *C* and *D* show autoradiograms.

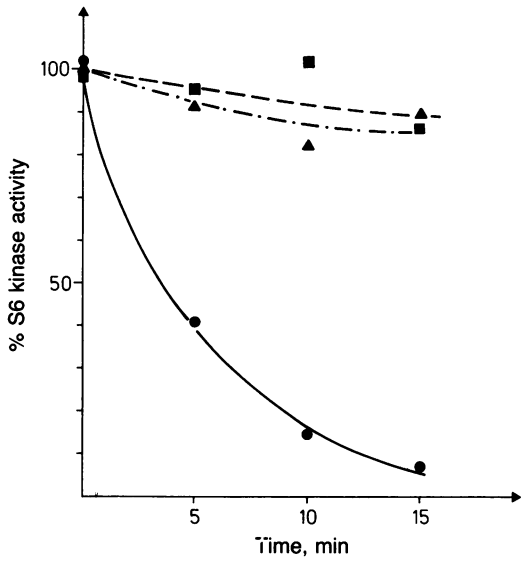

FIG. 5. Phosphatase 2A inactivation of the S6 kinase. Pure S6 kinase (33 milliunits) was incubated alone (▲–·–▲), in the presence of 12 milliunits of phosphatase $2A_c$ (●—●), or in the presence of phosphatase $2A_c$ plus 10 mM *p*-nitrophenyl phosphate (■–■) for the indicated times at 37°C. The samples were then immediately assayed for S6 kinase activity.

are only a few known examples of kinases that are activated by other kinases (44, 45). Previous studies in this laboratory, employing phosphatase inhibitors (16, 17, 25) or purified phosphatases (18), led to the suggestion that either the kinase or a regulatory component of the kinase was controlled by a phosphorylation–dephosphorylation mechanism. The sensitivity of the homogeneous form of the enzyme to phosphatase $2A_c$ argues that it is direct phosphorylation of the kinase that controls its activity. Such a mechanism would be expected to respond rapidly to physiological signals and to be readily reversible. Two additional observations support this view. First, pretreatment of cells with cycloheximide does not appear to alter the kinase response (19). Second, following desensitization of the kinase by EGF, the enzyme can be rapidly reactivated by vanadate (17). Rigorous proof of such a model will require that the kinase be shown to be phosphorylated *in vivo* following mitogenic stimulation and that this result can be reproduced *in vitro*.

We thank J. Knesel, T. Landolt, H. Luther, J. Krieg, M. Siegmann, A. Olivier, and A. Ziegler for their advice and technical assistance. We also thank N. Jäggi, who was a major force in the purification of the kinase. Finally, we acknowledge C. Viereck and S. Kozma for their critical reading of the manuscript and S. Thomas for typing the manuscript.

1. Gressner, A. M. & Wool, I. G. (1974) *J. Biol. Chem.* **249**, 6917–6925.
2. Haselbacher, G. K., Humbel, R. E. & Thomas, G. (1979) *FEBS Lett.* **100**, 185–190.
3. Decker, S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4112–4115.
4. Ballinger, D. & Hunt, T. (1981) *Dev. Biol.* **87**, 277–285.
5. Nielsen, P. J., Thomas, G. & Maller, J. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2937–2941.
6. Nielsen, P. J., Manchester, K. L., Towbin, H., Gordon, J. & Thomas, G. (1982) *J. Biol. Chem.* **257**, 12316–12321.
7. Thomas, G., Siegmann, M. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3952–3956.
8. Thomas, G., Martin-Pérez, J., Siegmann, M. & Otto, A. M. (1982) *Cell* **30**, 235–242.
9. Thomas, G., Siegmann, M., Kubler, A. M., Gordon, J. & Jimenez de Asua, L. (1980) *Cell* **19**, 1015–1023.
10. Duncan, R. & McConkey, E. H. (1982) *Eur. J. Biochem.* **123**, 535–538.
11. Tolan, D. R. & Traut, R. R. (1981) *J. Biol. Chem.* **256**, 10129–10136.
12. Terao, K. & Ogata, K. (1979) *J. Biochem. (Tokyo)* **86**, 597–603.
13. Bommer, U.-A., Noll, F., Lutsch, G. & Bielka, H. (1980) *FEBS Lett.* **111**, 171–174.
14. Burkhard, S. J. & Traugh, J. A. (1983) *J. Biol. Chem.* **258**, 14003–14008.
15. Palen, E. & Traugh, J. A. (1987) *J. Biol. Chem.* **262**, 3518–3523.
16. Novak-Hofer, I. & Thomas, G. (1984) *J. Biol. Chem.* **259**, 5995–6000.
17. Novak-Hofer, I. & Thomas, G. (1985) *J. Biol. Chem.* **260**, 10314–10319.
18. Ballou, L. M., Jenö, P. & Thomas, G. (1987) *J. Biol. Chem.*, in press.
19. Tabarini, D., Heinrich, J. & Rosen, O. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4369–4373.
20. Blenis, J. & Erikson, R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7621–7625.
21. Erikson, E. & Maller, J. L. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 742–746.
22. Nemenoff, R. A., Gunsalus, J. R. & Avruch, J. (1986) *Arch. Biochem. Biophys.* **245**, 196–203.
23. Pelech, S. L., Olwin, B. B. & Krebs, E. G. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5968–5972.
24. Matsuda, Y. & Guroff, G. (1987) *J. Biol. Chem.* **262**, 2832–2844.
25. Novak-Hofer, I., Martin-Pérez, J. & Thomas, G. (1986) in *Hormones and Cell Regulation*, Colloque Institut National de la Santé et de la Recherche, ed. Nunez, J. (Libbey, London), Vol. 139, pp. 181–197.
26. Thomas, G., Gordon, J. & Rogg, H. (1978) *J. Biol. Chem.* **253**, 1101–1105.
27. Siegmann, M. & Thomas, G. (1987) *Methods Enzymol.* **146**, 362–369.
28. Martin-Pérez, J. & Thomas, G. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 926–930.
29. Martin-Pérez, J. & Thomas, G. (1987) *Methods Enzymol.* **146**, 369–376.
30. Del Grande, R. W. & Traugh, J. A. (1982) *Eur. J. Biochem.* **123**, 421–428.
31. Wettenhall, R. E. H. & Cohen, P. (1982) *FEBS Lett.* **140**, 263–269.
32. Martin-Pérez, J., Siegmann, M. & Thomas, G. (1984) *Cell* **36**, 287–294.
33. Lubben, T. H. & Traugh, J. A. (1983) *J. Biol. Chem.* **258**, 13992–13997.
34. Donahue, M. J. & Masaracchia, R. A. (1984) *J. Biol. Chem.* **259**, 435–440.
35. Parker, P. J., Katan, M., Waterfield, M. D. & Leader, D. P. (1985) *Eur. J. Biochem.* **148**, 579–586.
36. Le Peuch, C. J., Ballester, R. & Rosen, O. M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6858–6862.
37. Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363.
38. Blenis, J. & Erikson, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1733–1737.
39. Rozengurt, E., Rodriguez-Pena, M. & Smith, K. A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7244–7248.
40. Macphee, C. H., Drummond, A. H., Otto, A. M. & Jimenez de Asua, L. (1984) *J. Cell. Physiol.* **119**, 35–40.
41. Coughlin, S. R., Lee, W. M. F., Williams, P. W., Giels, G. M. & Williams, L. T. (1985) *Cell* **43**, 243–251.
42. Erikson, E. & Maller, J. L. (1986) *J. Biol. Chem.* **261**, 350–355.
43. Tabarini, D., de Herreros, A. G., Heinrich, J. & Rosen, O. M. (1987) *Biochem. Biophys. Res. Commun.* **144**, 891–899.
44. Hayakawa, T., Perkins, J. P. & Krebs, E. G. (1973) *Biochemistry* **12**, 574–580.
45. Galabru, J. & Hovanessian, A. G. (1985) *Cell* **43**, 685–694.