Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7040–7044, September 1990
Cell Biology

Identical M_r 70,000 S6 kinase is activated biphasically by epidermal growth factor: A phosphopeptide that characterizes the late phase

(Swiss 3T3 cells/*in vivo* $^{32}P_i$ labeling/autophosphorylation/phosphorylated amino acids/protein kinase C)

MIRA ŠUŠA AND GEORGE THOMAS*

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

Communicated by Stanley Cohen, July 2, 1990

ABSTRACT **Mitogenic stimulation of quiescent mouse 3T3 cells with epidermal growth factor leads to biphasic S6 kinase activation. The kinases present in both phases of the response have been purified from ^{32}P-labeled cells and shown to contain a phosphoprotein of equivalent M_r 70,000. Chromatographic analysis of the purified S6 kinases on a Mono Q column reveals that (*i*) all ^{32}P-labeled protein coelutes with S6 kinase activity, (*ii*) only those fractions containing S6 kinase autophosphorylate, (*iii*) autophosphorylation is restricted to a single M_r 70,000 protein, and (*iv*) the extent of autophosphorylation directly parallels the degree of S6 kinase activation. Analysis of the two autophosphorylated S6 kinases by two-dimensional tryptic phosphopeptide mapping indicates that they are the same protein. Both *in vivo* ^{32}P-labeled S6 kinases contain phosphoserine and phosphothreonine but no detectable phosphotyrosine. Two-dimensional tryptic peptide maps of the *in vivo* ^{32}P-labeled S6 kinases are essentially identical, except for a single qualitative change in the late-phase S6 kinase.**

When epidermal growth factor (EGF) is added to quiescent Swiss mouse 3T3 fibroblasts, a number of well-defined biochemical events occur that culminate in DNA synthesis and cell division (1). A set of the early events includes the rapid incorporation of 5 mol of phosphate per mol of 40S ribosomal protein S6, a 2- to 3-fold increase in the rate of protein synthesis, and specific alterations in the pattern of translation (2, 3). The activation of these events by EGF occurs in a dose-responsive manner and correlates with the extent of DNA synthesis (2). A number of studies indicate that increased S6 phosphorylation is a necessary prerequisite for the observed changes in the rate and pattern of translation (4, 5).

To understand how this signal is transmitted from the growth factor receptor tyrosine kinase to the 40S ribosome, the phosphorylated residues in S6 have been identified. Most of the phosphate was incorporated into serine residues in a sequential fashion (6) regardless of the mitogen used (7). Initial attempts to detect an EGF-activated S6 kinase failed until phosphatase inhibitors such as β-glycerophosphate were included in the extraction buffer, suggesting that either the kinase or a regulatory subunit of the kinase was controlled by phosphorylation (8). In addition to EGF, other mitogens were shown to stimulate a similar enzyme activity. In all cases, the activity exhibited identical chromatographic properties and behaved as a single entity, indicating that distinct signaling pathways converge on the same S6 kinase (9). In the last few years, this kinase has been shown to be a single polypeptide of M_r 70,000, to phosphorylate the same S6 tryptic peptides as observed *in vivo*, to be activated by serine/threonine phosphorylation, and to be preferentially inactivated by a type 2A phosphatase (10–13). The last two findings suggested that there was at least one step between the EGF receptor tyrosine kinase and the S6 kinase.

Recently, we showed that the S6 kinase was biphasically activated by EGF, suggesting that the response was more complex than originally proposed (14). Each phase of S6 kinase activity behaved identically on a number of chromatographic columns and was inactivated at approximately the same rate by phosphatase 2A. The signaling mechanism controlling the early-phase S6 kinase was not identified, but the late-phase S6 kinase was shown to be under the control of protein kinase C (pkC). These results suggested that EGF triggered two distinct signaling pathways, which converged at different times on the same kinase. However, cDNA homologues to a M_r 92,000 S6 kinase (15, 16), which had been identified only in *Xenopus*, have recently been described in mouse (17, 18). Furthermore, a kinase of similar molecular weight, which phosphorylates S6 *in vitro*, has been reported to be activated within minutes of EGF stimulation of A431 cells (19). These last observations together with our earlier findings (14) have raised questions concerning the identity of the S6 kinase in both phases and the point at which the two signaling pathways converge to induce S6 kinase activation. Here we have addressed these questions and discussed the results in relation to other protein kinases that have been implicated in signal transduction.

MATERIALS AND METHODS

Cell Culture, Radioactive Labeling, and Preparation of Extracts. Cells were grown and maintained as described (11). For *in vivo* labeling with $^{32}P_i$, cultures were refed with phosphate-free Dulbecco's modified Eagle's medium containing 6% fetal calf serum, which had been dialyzed against the same medium. The extent of early- and late-phase S6 kinase activation was unchanged after such treatment (unpublished data). On day 7, when cells reached confluence, the volume of the medium was reduced to 10 ml and the cells were labeled overnight with 1 mCi of $^{32}P_i$ (1 Ci = 37 GBq) (Amersham) per 15-cm plate. Cell extracts were prepared as described from unlabeled cultures (14), except that the extraction buffer contained 1 mM instead of 20 mM EGTA, no $MgCl_2$, 10 μM sodium orthovanadate, and 100 μM ammonium molybdate. Radioactively labeled cultures were extracted as described (12).

Purification of S6 Kinase. For each phase of the activation, cells from 50 plates (15 cm) were used to purify the kinase as described (12) with the following modifications: all buffers contained 100 μM sodium orthovanadate and 10 μM ammonium molybdate; the S200 column was run in Mono Q buffer containing no NaF; and the last step of the purification was changed from chromatography on ATP type IV agarose (Pharmacia) to chromatography on a peptide affinity resin (20), which was run in the presence of 1 mM EGTA instead

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.

Abbreviations: EGF, epidermal growth factor; pkC, protein kinase C.

*To whom reprint requests should be addressed.

of EDTA. The peptide used for the affinity column was based on a sequence of a 32-amino acid cyanogen bromide cleavage product of S6, which was previously shown to contain all the sites of phosphorylation (21). This peptide is similar to the one used by Price *et al.* (22). Compared to the ATP column, use of the peptide column resulted in at least a 2-fold greater recovery of kinase activity and a higher purification of the enzyme, as judged by autophosphorylation (unpublished data). Elution of the ^{32}P-labeled proteins from the peptide column was carried out as described (20). The wash steps of 0.2, 0.25, and 0.30 M NaCl removed >99% of the radioactively labeled proteins from the S200 pool, which could easily be monitored by following the Cerenkov radiation. The kinase eluted from the column at ≈0.9 M NaCl and those fractions containing kinase activity were frozen in liquid N_2 and stored at −70°C. In some cases, the kinase pools were concentrated on a 250-μl Mono Q column before further use (see Fig. 3 and ref. 20). Autophosphorylation and S6 kinase assays were carried out essentially as described (12, 14).

Analysis of Phosphopeptides and Phosphorylated Amino Acids. Prior to carrying out either analysis, the S6 kinase pools were precipitated with 10% trichloroacetic acid in the presence of 0.015% sodium deoxycholate and were processed as described (12). The samples were then resuspended in SDS sample buffer, 2.5 μg of bovine serum albumin was added, and the proteins were separated by SDS/PAGE. The bovine serum albumin was localized by nonfixing Cu^{2+} staining (23), the M_r 70,000 region of the gel was cut out, and the protein was recovered by electroelution (24). A typical preparation yielded ≈700 cpm from the early-phase S6 kinase and 500 cpm from the late-phase S6 kinase. It should be noted that the Cerenkov counts detected in the peptide column fractions (see Fig. 3) were substantially higher than those recovered from the gel. This was mostly due to [^{32}P]ATP, which was bound to the kinase and removed only after SDS/PAGE (unpublished data). After electroelution, an additional 7.5 μg of bovine serum albumin was added to the sample, and the mixture was lyophilized to dryness and washed three times with acetone/triethylamine/acetic acid/water (85:5:5:5) to remove any remaining SDS (25). At this point, the sample was divided and used for analysis of either phosphorylated amino acids or phosphopeptides as described (26, 27). For analysis of phosphorylated amino acids, the sample was hydrolyzed for 1 hr and the first and second dimensions of electrophoresis were carried out for 30 and 25 min, respectively. For phosphopeptide analysis, the sample was resuspended in 200 μl of 0.1 M ammonium bicarbonate and digested twice with 15 μg of trypsin (Worthington) for 12 hr with shaking at 37°C. The first dimension of thin-layer electrophoresis was carried out for 45 min for the autophosphorylated kinase and for 60 min for the *in vivo* phosphorylated kinase.

RESULTS

Biphasic Activation of the S6 Kinase. Previously, we purified and analyzed phosphorylated amino acids on the M_r 70,000 kinase from cells stimulated for 1 hr (10, 12). In these studies, no precautions were taken to protect against phosphotyrosine phosphatases. Therefore, any effects on kinase activity due to tyrosine phosphorylation may have been partially or totally abrogated. To protect phosphotyrosine from dephosphorylation, sodium orthovanadate and ammonium molybdate were added to the extraction buffer as potent phosphotyrosine phosphatase inhibitors (28, 29). To determine whether the measured EGF-induced S6 kinase response was increased in cells extracted under the new conditions in comparison to those previously used, the kinetics of kinase activation were monitored after the addition of EGF. The amplitude and the kinetic appearance of each phase were very similar to those recently reported (14), with the early phase peaking at 15 min and the late phase reaching a maximum between 30 and 45 min (Fig. 1). This result suggested that if phosphotyrosine were present in the S6 kinase, it would play little role in activating the enzyme.

Purification of Early- and Late-Phase S6 Kinase. To purify early- and late-phase enzymes, it was necessary to minimize cross-contamination of one kinase with the other. Based on the data above, stimulation with EGF for 10 min was chosen for the early-phase S6 kinase activation, a time at which kinase activity was rising, and 60 min was used for the late-phase S6 kinase activation, a time at which the activity in the early phase should have returned to basal levels (Fig. 1 and ref. 14). At each step of purification (see *Materials and Methods*), the enzymes present in both phases eluted in the same position as a single peak of activity. Following this purification protocol, a final recovery of 30% can be obtained (20). To determine the identity of the kinase present in each phase of the activation, the proteins in the peak fraction of both peptide columns were separated on SDS/polyacrylamide gel and analyzed by autoradiography. In both cases, a single ^{32}P-labeled protein band was observed at the identical M_r 70,000 (Fig. 2), suggesting that the two enzymes were equivalent.

S6 Kinase Activity, ^{32}P-Labeled Proteins and Autophosphorylation. To ensure that all ^{32}P-labeled protein eluting with the S6 kinase from the peptide columns represented the M_r 70,000 enzyme, the active S6 kinase pools were chromatographed on a Mono Q column. As shown in Fig. 3, all of the radioactivity from each column was found to coelute with S6 kinase activity. Next, a portion of each fraction was incubated with [γ-^{32}P]ATP, the proteins present were separated by SDS/PAGE, and the products were analyzed by autoradiography. The only protein band visible was again at M_r 70,000. More importantly, this protein was found only in those fractions containing S6 kinase activity and the intensity of autophosphorylation directly paralleled S6 kinase activity (Fig. 3 *Insets*). Thus, in both S6 kinase pools only a single ^{32}P-labeled protein of M_r 70,000 was detected, which had the ability to autophosphorylate.

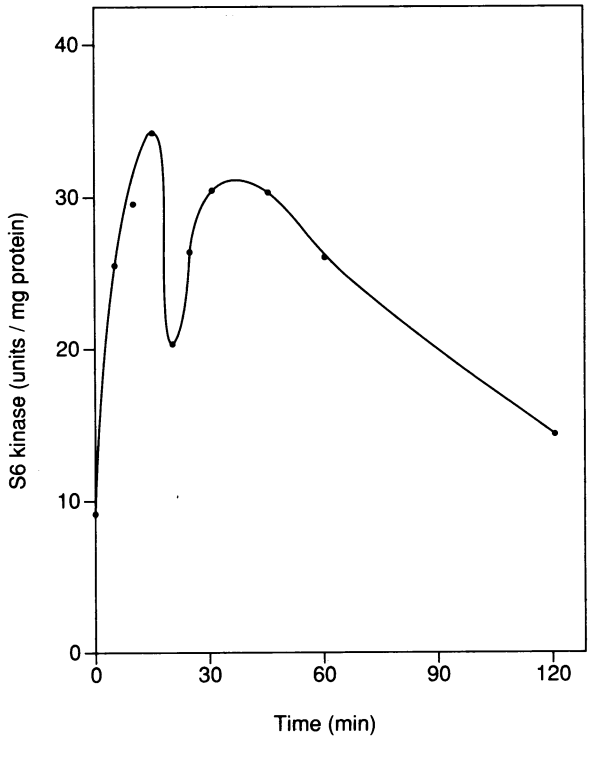

FIG. 1. Biphasic activation of S6 kinase by EGF. Cells were stimulated with EGF for the times indicated, extracted, and assayed.

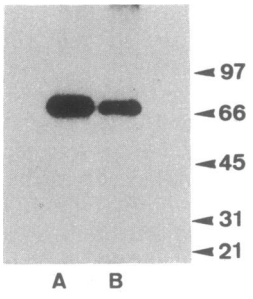

FIG. 2. *In vivo* ^{32}P-labeled early- and late-phase S6 kinase. ^{32}P-labeled cells were stimulated with EGF for 10 min (lane A) or 60 min (lane B). Cells were extracted and S6 kinase was purified. Aliquots corresponding to 150 cpm from the peak peptide column fractions (≈25% of the total kinase purified) were precipitated with trichloroacetic acid and analyzed by SDS/PAGE. Autoradiography was performed for 2 days at −70°C with intensifying screens. Markers on the right indicate molecular weights × 10^{-3}.

Phosphopeptide Maps of Autophosphorylated S6 Kinase. The results in Figs. 2 and 3, together with the finding that the two kinases present in each phase behaved identically through four steps of purification, indicated that they were the same protein. To ensure that this was the case, the two M_r 70,000 autophosphorylated proteins were electroeluted from the gel shown in Fig. 3 and digested with trypsin, and the proteolytic products were analyzed by two-dimensional thin-layer electrophoresis/chromatography. Although the relative intensities of the maps shown in Fig. 4 were different, the identical phosphopeptides were observed, indicating that both kinases represent the same protein. Consistent with this finding, one-dimensional SDS/PAGE analyses of cyanogen bromide peptides of both autophosphorylated kinases were identical (unpublished data).

Analysis of *in Vivo* ^{32}P-labeled S6 Kinase. The results described above support the earlier hypothesis that the two phases of EGF-induced S6 kinase activation were mediated through the same enzyme by two distinct signaling pathways (14). To determine whether these pathways use the same or distinct phosphorylation sites to regulate S6 kinase activity, we first analyzed the phosphorylated amino acids present in each protein. Phosphoserine and to a much lesser extent phosphothreonine were detected in both enzyme preparations, each at approximately the same level (Fig. 5). Hence, the different signaling pathways cannot be distinguished by content of phosphorylated amino acids. The absence of phosphotyrosine was further supported by the observation that alkaline treatment of the gel depicted in Fig. 2 led to a complete removal of phosphate from either protein band and that anti-phosphotyrosine antibodies failed to immunoprecipitate increased S6 kinase activity from EGF-stimulated cell extracts (unpublished data). These results appear to rule out the direct phosphorylation and activation of the S6 kinase by the activated EGF receptor tyrosine kinase.

Tryptic peptides of *in vivo* ^{32}P-labeled S6 kinase from cells treated with EGF for 10 or 60 min were analyzed by two-dimensional thin-layer electrophoresis/chromatography. The maps from the two enzymes showed very similar patterns (Fig. 6), further indicating that the two kinases were identical. However, visual comparison of the maps revealed that the relative amount of phosphate incorporated did differ in some peptides. These differences could not be quantitated because of the low amount of ^{32}P incorporated into the protein. More striking was the appearance of a peptide in the late-phase S6 kinase (arrowhead in Fig. 6*B*) that even after long exposure of the early-phase S6 kinase tryptic maps could not be detected (unpublished data). This was the only qualitative difference detected between the two kinases. In the future it will be of great interest to determine whether this site is directly regulated by pkC.

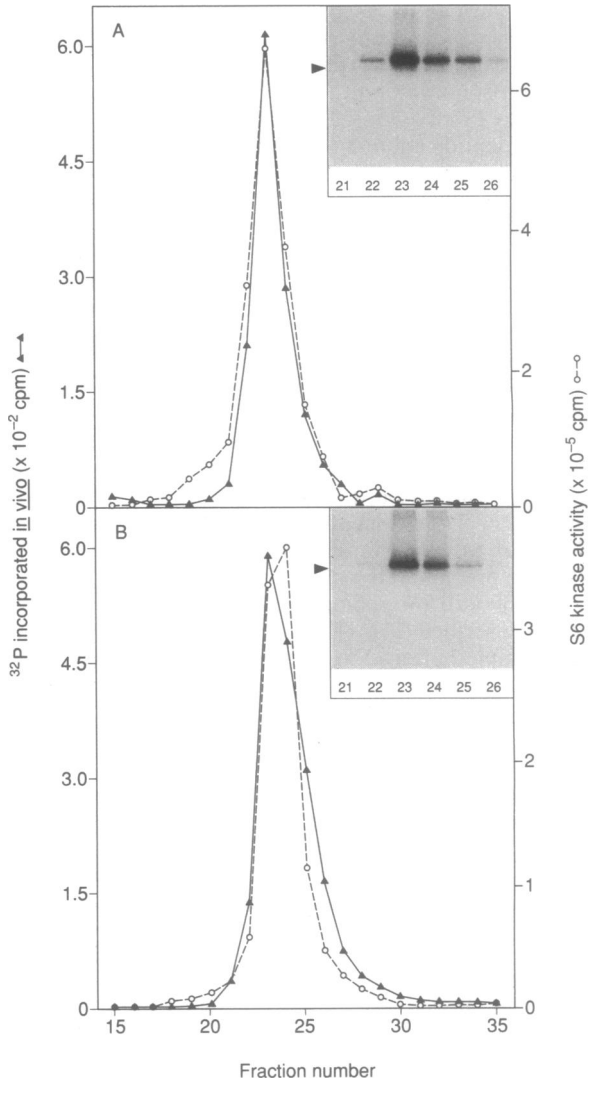

FIG. 3. Coelution of *in vivo* ^{32}P-labeled M_r 70,000 protein, S6 kinase activity, and M_r 70,000 autophosphorylating activity. Pools from the peptide column were applied to a 250-μl Mono Q column (Pharmacia) and eluted with a 0–0.5 M gradient of NaCl. Fractions were analyzed for Cerenkov counts (▲) or S6 kinase activity (○), and 0.6% of each fraction was used for autophosphorylation (*Insets*). Arrowheads indicate the position of bovine serum albumin (M_r 66,000). (*A*) Early-phase S6 kinase. (*B*) Late-phase S6 kinase. Autophosphorylated M_r 70,000 band in fractions 22–26 contained 18,140 cpm (*A*) and 7730 cpm (*B*).

DISCUSSION

Here we show that both phases of EGF-induced S6 kinase activation are mediated by the apparent same M_r 70,000 enzyme (Figs. 2–4 and 6). This enzyme was initially identified as a specific soluble kinase activity that was responsible for serum- and EGF-induced S6 phosphorylation (8) and, after its purification, was termed the "mitogen-activated S6 kinase" (10). A number of S6 kinases with similar molecular weights have now been identified from many different sources, including chicken, mouse, rat, rabbit, and cow (13, 22, 30–33). In contrast, a distinct M_r 92,000 enzyme, termed S6 kinase II, has been described only in *Xenopus* (15, 16). However, recent identification of cDNA homologues to *Xenopus* S6 kinase II in chicken and mouse (18) indicates that the existence of the M_r 92,000 enzyme is widespread. Based on

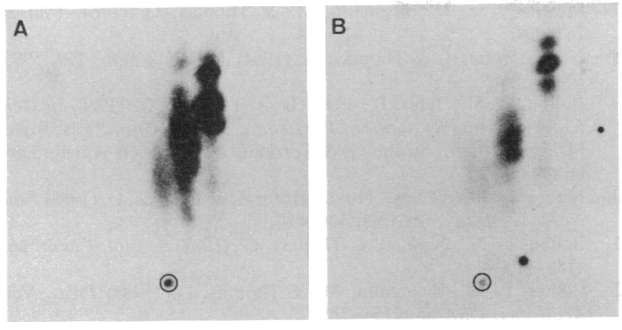

FIG. 4. Tryptic phosphopeptide analysis of autophosphorylated early- and late-phase S6 kinase. The autophosphorylated S6 kinase (≈0.8% of the total enzyme purified) was electroeluted from the SDS gel and digested with trypsin. Phosphopeptides were separated by thin-layer electrophoresis at pH 1.9 for 45 min in the first dimension (left to right) and by thin-layer chromatography in the second dimension (bottom to top). Phosphopeptides were visualized by autoradiography. Circles indicate the origin. (*A*) Early-phase S6 kinase (≈600 cpm loaded; exposure, 1 week at −70°C). (*B*) Late-phase S6 kinase (≈100 cpm loaded; exposure, 3 weeks at −70°C).

the recent cDNA cloning of the M_r 70,000 kinase (46), it is clear that this enzyme is not a degradation product of the *Xenopus* enzyme. These results raise the question of why the *Xenopus* enzyme has not been detected in other systems in which S6 phosphorylation is known to increase. A number of possibilities could explain this, including stability of the kinase, its kinetics of activation, and its ability to phosphorylate S6 in comparison to the M_r 70,000 kinase. Although preliminary data indicate that some of these possibilities may play a role (L. M. Ballou, H. Luther, and G.T., unpublished data), more thorough studies will be required to evaluate the relative importance of the M_r 92,000 enzyme in mediating *in vivo* S6 phosphorylation.

Previously we demonstrated that activation of the early- and late-phase S6 kinase was controlled by a pkC-independent and -dependent signaling pathway, respectively (14). These pathways could not be distinguished by tryptic phosphopeptide maps of autophosphorylated kinase or by their content of phosphorylated amino acids (Figs. 4 and 5). However, tryptic maps of the *in vivo* labeled protein kinases revealed some quantitative differences between individual peptides and a single unique peptide in the late-phase S6 kinase (Fig. 6). It was not possible to determine whether any

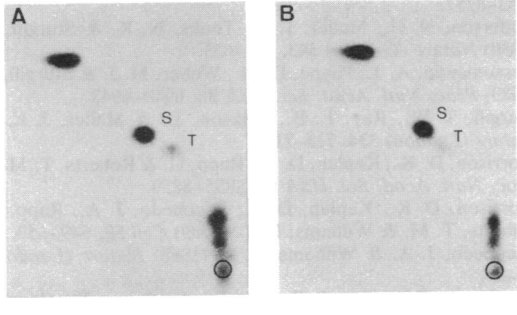

FIG. 5. Phosphoamino acid analysis of *in vivo* ^{32}P-labeled early- and late-phase S6 kinase. The S6 kinase hydrolyzates were separated by thin-layer electrophoresis at pH 1.9 in the first dimension (bottom to top) and at pH 3.5 in the second dimension (right to left). Phosphorylated amino acids present were visualized by autoradiography and identified with unlabeled markers, which were stained with ninhydrin. Circles indicate the origin; S, phosphoserine; T, phosphothreonine. Products directly above the origin represent incompletely cleaved peptides and the one in the upper left represents free inorganic phosphate. (*A*) Early-phase S6 kinase. (*B*) Late-phase S6 kinase. Each sample contained ≈50 cpm and exposure time was 10 days at −70°C.

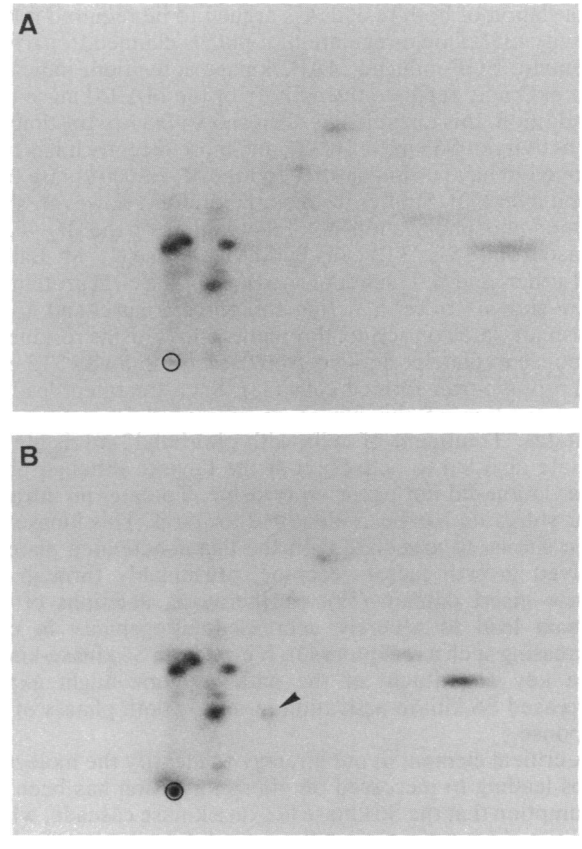

FIG. 6. Tryptic phosphopeptide analysis of *in vivo* ^{32}P-labeled early- and late-phase S6 kinase. Approximately 25% of the purified S6 kinase from either phase of activation (see Fig. 2) was digested with trypsin. Tryptic phosphopeptides were separated by electrophoresis at pH 1.9 for 60 min in the first dimension (left to right) and by chromatography in the second dimension (bottom to top) and were visualized by autoradiography. Circles indicate the origin and the arrowhead indicates a peptide unique to the late-phase S6 kinase. (*A*) Early-phase S6 kinase. (*B*) Late-phase S6 kinase. Both samples contained ≈100 cpm and exposure time was 3 weeks at −70°C.

of the *in vivo* labeled peptides were equivalent to those observed *in vitro* because of different trypsin cleavage conditions, electrophoretic times, and limited amounts of *in vivo* labeled kinase. Although the *in vivo* peptide maps indicated that both signaling pathways are converging on the same kinase, the sites of activation may in part be distinct. This difference may be linked to the approximate 1.5-fold increased ability of the early- versus the late-phase S6 kinase to autophosphorylate. It is difficult to speculate on the significance of these differences without more knowledge of the amount of kinase present in each phase, phosphorylation sites with respect to number, their location in the kinase, and those that affect activity. It would be of interest to analyze the late-phase S6 kinase from cells stimulated with phorbol 12-myristate 13-acetate or a more potent mitogen of Swiss 3T3 cells that operates principally through pkC, such as platelet-derived growth factor or bombesin. If higher phosphorylation of the unique S6 kinase peptide were observed, the direct role of pkC could be addressed *in vitro*. Preliminary experiments show that the S6 kinase is a substrate for pkC, but as yet that modification has had no substantial consequence on S6 kinase activity (unpublished data).

It is also possible that the point of integration of both pathways resides in a kinase upstream of the S6 kinase. The two obvious candidates for such an enzyme are the MAP2 kinase and the product of protooncogene c-*raf*. MAP2 kinase is phosphorylated on tyrosine and threonine (34) and phos-

phorylation of both residues is argued to be required for the activity (35). Down-regulation of pkC is claimed to partially attenuate EGF-induced MAP2 kinase activation, indicating that pkC may regulate the activity of the MAP2 kinase (36). In addition, this enzyme has been shown *in vitro* to stimulate the activity of S6 kinase II (37), and more recently it has been claimed to have a similar effect on the M_r 70,000 kinase from rabbit liver (32). Studies from our laboratory, however, show neither phosphorylation nor reactivation of the M_r 70,000 kinase from Swiss 3T3 cells by MAP2 kinase (L. M. Ballou, H. Luther, and G.T., unpublished data). The c-raf protein has been shown to be a serine/threonine kinase and to be phosphorylated on serine, threonine, and tyrosine residues in response to platelet-derived growth factor in Swiss 3T3 cells and in v-src-transformed cells (38). Such treatment leads to the activation of the kinase as measured in immunoprecipitates. Treatment of cells with phorbol 12-myristate 13-acetate also led to activation of the kinase, although phosphorylation did not occur on tyrosine. To date, no intracellular substrate has been identified for c-raf. This kinase has been shown to associate with the ligand-activated platelet-derived growth factor receptor, presumably through the kinase insert domain (39). Furthermore, deletions of this domain lead to severely decreased mitogenicity in cells expressing such a receptor (40). If c-raf is an S6 kinase-kinase or a key constituent of the pathway, one might expect decreased S6 kinase activation in one or both phases of the response.

A critical element in our strategy to identify the molecular steps leading to increased S6 phosphorylation has been the assumption that the S6 kinase lies on a kinase cascade, which is initiated by activation of the growth factor receptor tyrosine kinase. This view has recently been challenged by the use of anti-insulin receptor antibodies, which are claimed to mimic many of insulin's actions, although they induced only very weak receptor autophosphorylation and no activation of the receptor kinase as measured against exogenous substrates (41, 42). Among the events activated is the S6 kinase and S6 phosphorylation. Instead of these events being triggered by the tyrosine kinase activity of the receptor, it is argued that they may be activated by a conformational change in the receptor, which is induced by antibody binding. This conformational change may lead to noncovalent interactions of the receptor with guanine nucleotide binding regulatory proteins, $p21^{c\text{-}ras}$ GTPase-activating protein, phospholipases, phosphatidylinositol 3-kinase, ion channel proteins, or other protein kinases and in this way trigger their activation. There is evidence for other protein kinases being activated during the early mitogenic response (43–45), some of which may be implicated in the activation of the S6 kinase. Future studies are necessary to elucidate the role of these kinases in regulating S6 phosphorylation.

We would like to thank M. Siegmann and Dr. S. Ferrari for their assistance in carrying out some of these studies, as well as Drs. L. M. Ballou, K. Balmer, B. A. Hemmings, and S. Morley for their critical review of the manuscript. We are grateful to C. Wiedmer for her patience in typing the manuscript and to T. Landolt for maintenance of cells.

1. Carpenter, G. & Cohen, S. (1979) *Annu. Rev. Biochem.* **48,** 193–216.
2. Thomas, G., Martin-Pérez, J., Siegmann, M. & Otto, A. M. (1982) *Cell* **30,** 235–242.
3. Thomas, G., Thomas, G. & Luther, H. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5712–5716.
4. Kozma, S. C., Ferrari, S. & Thomas, G. (1989) *Cell. Signalling* **1,** 219–225.
5. Hershey, J. W. B. (1989) *J. Biol. Chem.* **264,** 20823–20826.
6. Martin-Pérez, J. & Thomas, G. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 926–930.
7. Martin-Pérez, J., Siegmann, M. & Thomas, G. (1984) *Cell* **36,** 287–294.
8. Novak-Hofer, I. & Thomas, G. (1984) *J. Biol. Chem.* **259,** 5995–6000.
9. Ballou, L. M., Jenö, P., Friis, B. & Thomas, G. (1988) in *Gene Expression and Regulation: The Legacy of Luigi Gorini*, eds. Bissel, M. J., Dehò, G., Sironi, G. & Torriani, A. (Elsevier, Amsterdam), pp. 333–342.
10. Jenö, P., Ballou, L. M., Novak-Hofer, I. & Thomas, G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 406–410.
11. Ballou, L. M., Jenö, P. & Thomas, G. (1988) *J. Biol. Chem.* **263,** 1188–1194.
12. Ballou, L. M., Siegmann, M. & Thomas, G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7154–7158.
13. Jenö, P., Jäggi, N., Luther, H., Siegmann, M. & Thomas, G. (1989) *J. Biol. Chem.* **264,** 1293–1297.
14. Šuša, M., Olivier, A. R., Fabbro, D. & Thomas, G. (1989) *Cell* **57,** 817–824.
15. Erikson, E. & Maller, J. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 742–746.
16. Erikson, E. & Maller, J. L. (1986) *J. Biol. Chem.* **261,** 350–355.
17. Jones, S. W., Erikson, E., Blenis, J., Maller, J. L. & Erikson, R. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3377–3381.
18. Alcorta, D. A., Crews, C. M., Sweet, L. J., Bankston, L., Jones, S. W. & Erikson, R. L. (1989) *Mol. Cell. Biol.* **9,** 3850–3859.
19. Giugni, T. D., Chen, K. & Cohen, S. (1988) *J. Biol. Chem.* **263,** 18988–18995.
20. Lane, H. A. & Thomas, G. (1990) *Methods Enzymol.*, in press.
21. Krieg, J., Hofsteenge, J. & Thomas, G. (1988) *J. Biol. Chem.* **263,** 11473–11477.
22. Price, D. J., Nemenoff, R. A. & Avruch, J. (1989) *J. Biol. Chem.* **264,** 13825–13833.
23. Lee, C., Levin, A. & Branton, D. (1987) *Anal. Biochem.* **166,** 308–312.
24. Martin-Pérez, J. & Thomas, G. (1987) *Methods Enzymol.* **146,** 369–375.
25. Konigsberg, W. H. & Henderson, L. (1983) *Methods Enzymol.* **91,** 254–259.
26. Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) *Methods Enzymol.* **99,** 387–402.
27. Gould, K. L. & Hunter, T. (1988) *Mol. Cell. Biol.* **8,** 3345–3356.
28. Swarup, G., Cohen, S. & Garbers, D. L. (1982) *Biochem. Biophys. Res. Commun.* **107,** 1104–1109.
29. Tonks, N. K., Diltz, C. D. & Fisher, E. H. (1988) *J. Biol. Chem.* **263,** 6731–6737.
30. Tabarini, D., Garcia de Herreros, A., Heinrich, J. & Rosen, O. M. (1987) *Biochem. Biophys. Res. Commun.* **144,** 891–899.
31. Blenis, J., Kuo, C. J. & Erikson, R. L. (1987) *J. Biol. Chem.* **262,** 14373–14376.
32. Gregory, J. S., Boulton, T. G., Sang, B.-C. & Cobb, M. H. (1989) *J. Biol. Chem.* **264,** 18397–18401.
33. Kozma, S. C., Lane, H. A., Ferrari, S., Luther, H., Siegmann, M. & Thomas, G. (1989) *EMBO J.* **8,** 4125–4132.
34. Ray, L. B. & Sturgill, T. W. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3753–3757.
35. Anderson, N. G., Maller, J. L., Tonks, N. K. & Sturgill, T. W. (1990) *Nature (London)* **343,** 651–653.
36. Rossomando, A. J., Payne, D. M., Weber, M. J. & Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6940–6943.
37. Sturgill, T. W., Ray, L. B., Erikson, E. & Maller, J. L. (1988) *Nature (London)* **334,** 715–718.
38. Morrison, D. K., Kaplan, D. R., Rapp, U. & Roberts, T. M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8855–8859.
39. Morrison, D. K., Kaplan, D. R., Escobedo, J. A., Rapp, U. R., Roberts, T. M. & Williams, L. T. (1989) *Cell* **58,** 649–657.
40. Escobedo, J. A. & Williams, L. T. (1988) *Nature (London)* **335,** 85–87.
41. Ponzio, G., Contreres, J.-O., Debant, A., Baron, V., Gautier, N., Dolais-Kitabgi, J. & Rossi, B. (1988) *EMBO J.* **7,** 4111–4117.
42. Sung, C. K., Maddux, B. A., Hawley, D. M. & Goldfine, I. D. (1989) *J. Biol. Chem.* **264,** 18951–18959.
43. Palfrey, H. C., Nairn, A. C., Muldoon, L. L. & Villereal, M. L. (1987) *J. Biol. Chem.* **262,** 9785–9792.
44. Sommercorn, J., Mulligan, J. A., Lozeman, F. J. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8834–8838.
45. Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P. & Lewis, R. E. (1988) *J. Biol. Chem.* **263,** 11017–11020.
46. Kozma, S.- C., Ferrari, S., Bassand, P., Siegmann, M., Totty, N. & Thomas, G. (1990) *Proc. Natl. Acad. Sci USA*, in press.