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Biochemistry

Insulin activates a 70-kDa S6 kinase through serine/threonine-specific phosphorylation of the enzyme polypeptide

(growth factor/signal transduction/protein kinase cascade)

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ABSTRACT **The dominant insulin-stimulated ribosomal protein S6 kinase activity was purified to near homogeneity from insulin-treated ^{32}P-labeled rat H4 hepatoma cells and found to copurify with a 70-kDa ^{32}P-labeled polypeptide. The dominant S6 kinase purified from livers of cycloheximide-treated rats is also a 70-kDa polypeptide. Antiserum raised against rat liver S6 kinase specifically immunoprecipitates the purified ^{32}P-labeled H4 hepatoma insulin-stimulated S6 kinase. This antiserum also specifically precipitates insulin-stimulated S6 kinase activity directly from cytosolic extracts of H4 cells. Immune complexes prepared from the cytosol of ^{32}P-labeled H4 cells contain several ^{32}P-labeled polypeptides; only a 70-kDA ^{32}P-labeled peptide, however, is specifically displaced by preadsorption of the antiserum with nonradioactive rat liver S6 kinase. Insulin treatment increases the ^{32}P content of the immunoprecipitated 70-kDa S6 kinase polypeptide 3- to 4-fold over basal levels; ^{32}P-labeled serine, some ^{32}P-labeled threonine, but no ^{32}P-labeled tyrosine are detected after partial acid hydrolysis. Tryptic peptide maps indicate that the insulin-stimulated S6 kinase purified from ^{32}P-labeled H4 cells is phosphorylated at multiple sites distinct from those which participate in autophosphorylation *in vitro*. Autophosphorylation of rat liver S6 kinase *in vitro* does not modify S6 kinase activity. The S6 kinases purified from liver of cycloheximide-treated rat and H4 hepatoma insulin-stimulated enzyme are each completely deactivated by incubation with protein phosphatase type 2A in both autophosphorylating and 40S S6 phosphorylating activities. The phosphatase 2A-deactivated 70-kDa S6 kinase is neither reactivated nor phosphorylated by partially purified insulin-stimulated microtubule-associated protein 2 kinase, in experiments where *Xenopus* S6 kinase II undergoes phosphorylation and partial reactivation. Thus insulin activates the 70-kDa S6 kinase by promoting phosphorylation of specific serine/threonine residues on the enzyme polypeptide, probably through activating an as-yet-unidentified serine/threonine protein kinase distinct from microtubule-associated protein 2 kinase.**

Insulin rapidly stimulates the serine/threonine phosphorylation of an array of proteins in target cells (1). This phenomenon, due to activation of several serine/threonine protein kinases, presumably reflects intermediate signaling reactions in effector pathways for insulin action (2, 3). Although this idea is now widely accepted, evidence supporting a regulatory role for these modifications remains largely indirect in that few examples have been established where insulin-stimulated serine/threonine phosphorylation alters enzyme activity or protein function (4).

An early and well-studied example is insulin-stimulated phosphorylation of 40S ribosomal protein S6 (5, 6); although the functional consequences of S6 phosphorylation remain uncertain, the modification of S6 is clearly catalyzed by one or more insulin-activated "S6" protein kinase (7–9). The major insulin-stimulated S6 kinase purified from *Xenopus* oocytes is a 92-kDa polypeptide, called S6 kinase II (10). This enzyme is regulated through serine/threonine phosphorylation in the oocyte (11); moreover, S6 kinase II, deactivated by protein phosphatase treatment, can be phosphorylated *in vitro* and partially reactivated by another insulin-stimulated serine/threonine protein kinase, called microtubule-associated protein 2 (MAP-2) kinase (12). Although a homolog of S6 kinase II (13) is expressed in avian and mammalian cells (14), most studies examining mitogen-stimulated S6 kinase activity from these sources have detected 70-kDa polypeptides as the major form of stimulated S6 kinase (15–17). We previously purified and characterized an hepatic S6 protein kinase that is the dominant Ca^{2+}/cyclic nucleotide-independent S6 kinase activated in livers of cycloheximide-treated rats (18). This enzyme is a 70-kDa polypeptide unreactive with antisera raised to purified or recombinant *Xenopus* S6 kinase II. The 70-kDa hepatic S6 kinase exhibits chromatographic properties indistinguishable from the dominant S6 kinase activity that appeared during liver regeneration in the rat or in insulin-treated H4 hepatoma cells (19). We present immunochemical and structural evidence that (*i*) the 70-kDa S6 kinase purified from rat liver is closely related, perhaps identical, with the major insulin-stimulated S6 kinase in H4 hepatoma cells and (*ii*) insulin activates the catalytic function of the H4 hepatoma 70-kDa S6 kinase by phosphorylating the enzyme polypeptide at serine/threonine residues.

MATERIALS AND METHODS

Antiserum to Cycloheximide-Stimulated Rat Liver S6 Kinase. Mice were immunized with rat liver S6 kinase purified through the Mono Q step as described (18), emulsified in Ribi Immunochem adjuvant, and injected i.p.; booster injections were also in Ribi adjuvant. Serum was screened by immunoprecipitation of native rat liver S6 kinase, ^{32}P-labeled in an autophosphorylation reaction by incubation with Mg^{2+} plus [γ-^{32}P]ATP. Approximately 10 ng of the ^{32}P-labeled enzyme was incubated with 1 μl of serum in a total volume of 0.12 ml. Serum from one of three immunized mice was reactive; $\approx$5–10% of ^{32}P-labeled enzyme was recovered in the immune complex, harvested with protein A–Sepharose. Precipitation of the ^{32}P-labeled enzyme was prevented completely by preincubating the antiserum with nonradioactive S6 kinase. Nonimmune mouse serum did not immunoprecipitate the ^{32}P-labeled 70-kDa band. Assay of protein A–Sepharose pellets for S6 kinase activity was positive only for the serum that precipitated labeled 70-kDa polypeptide. The S6 kinase measured in the immunoprecipitate was $\approx$10% that obtained from the same mass of ^{32}P-labeled S6 kinase assayed in free solution. Adding SDS (0.1%) to the immunoprecipitation

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Abbreviation: MAP-2, microtubule-associated protein 2.

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reaction abolished recovery of labeled 70-kDa enzyme polypeptide. Moreover, the sera (at 1:100 dilution) did not immunoblot 10–20 ng of purified S6 kinase transferred to nitrocellulose after SDS/PAGE. Thus, this antiserum only interacts with native nondenatured 70-kDa S6 kinase, the activity of which is expressed in the immunoprecipitate, although in a substantially inhibited way.

^{32}P-Labeling of Cultured Cells. H4 hepatoma cells were grown to confluence in Swim's S77 medium (Sigma)/15% horse serum/5% fetal calf serum. Cells were placed in serum-free medium for 24 hr before hormone treatment. Cells were incubated for 2 hr with 0.55 mCi of $^{32}PO_4$ per 10-cm plate (1 Ci = 37 GBq) in phosphate-free medium before hormone exposure; these conditions provide steady-state labeling of endogenous ^{32}P-labeled polypeptide (19, 20).

Purification of S6 Kinase from ^{32}P-Labeled, Insulin-Treated H4 Cells. ^{32}P-labeled H4 cells (20 plates) and nonradioactive cells (40 plates) were treated with insulin (10^{-7} M, 15–20 min); all subsequent procedures were at 0–4°C. Cells were rinsed with ice-cold phosphate-buffered saline and scraped into 10 mM KP_i, pH 6.5/1 mM EDTA/5 mM EGTA/10 mM $MgCl_2$/2 mM dithiothreitol/1 mM vanadate/50 mM β-glycerophosphate/2 μM leupeptin/2 μM pepstatin/100 nM okadaic acid/1 mM diisopropylfluorophosphate (homogenization buffer). Cells were homogenized in an Ultra Turrax homogenizer (at full speed); the supernatants (100,000 × *g* for 1.5 hr) from nonradioactive and ^{32}P-labeled cells were combined and applied to a DEAE-Sephacel column (1 × 5 cm) equilibrated with homogenization buffer. After gradient elution with NaCl, the S6 kinase activity was further purified by chromatography on SP-Sephadex, heparin-Sepharose, S6 peptide affinity, and Mono Q HR 5/5 column, as described (18).

Immunoprecipitation of S6 Kinase from Crude Extracts. ^{32}P-labeled H4 cells (as above) were solubilized in homogenization buffer (0.5 ml per two plates) containing aprotinin at 100 units/ml and 0.4% Triton X-100. Extracts were centrifuged at 100,000 × *g* for 30 min. Supernatants were incubated with antiserum or normal serum (1:100, final dilution) for 30 min at 5°C; protein A-Sepharose was then added [50–100 μl of a 1:1 slurry in phosphate-buffered saline/2% (vol/vol) Tween 20]. Incubation continued at 5°C with agitation for 3 hr. The immune complexes were sedimented, washed 3–5 times with phosphate-buffered saline/2% Tween 20 and suspended in SDS containing sample buffer for SDS/PAGE. In some experiments, S6 kinase was extracted and immunoprecipitated as above from nonradioactive cells, and the S6 kinase assay was done with the immune complex, using 40S ribosomes as substrate, as described (9, 18).

Tryptic Peptide Mapping of ^{32}P-Labeled S6 Kinase. ^{32}P-labeled S6 kinase eluted from SDS/PAGE was precipitated in chloroform/methanol with added unlabeled carrier (rabbit skeletal muscle phosphorylase b, 25–100 μg), dried, and resuspended in 150 μl of 0.1 M ammonium bicarbonate, pH 8.3. Tosylphenylalanine chloromethyl ketone (TPCK)/trypsin, 1:10 (wt/wt) was added; after incubation at 37°C for 18 hr, further TPCK/trypsin, 1:10 was added, and digestion continued for 8 hr. The digests were dried, resuspended in H_2O, dried again twice, redissolved in a small volume of deionized H_2O, and applied to a cellulose acetate plate. Two-dimensional separation by electrophoresis at pH 3.5 (pyridine/acetic acid/H_2O, 10:100:1890) or at pH 6.5 (pyridine/acetic acid/H_2O, 50:2:450) was followed by chromatography (butanol/acetic acid/pyridine/H_2O, 15:3:10:12). Xylene cyanol FF was used as a marker (0.5 μl of 1 mg/ml of solution).

RESULTS

Purification of the Insulin-Stimulated S6 Kinase from ^{32}P-Labeled H4 Hepatoma Cells. Insulin treatment activates S6 kinase activity 3- to 10-fold as measured in H4 hepatoma cytosolic extracts. This S6 kinase activity was purified extensively by using the steps described (18) for isolating the major S6 kinase activated from livers of cycloheximide-treated rats. The elution profile of H4 hepatoma S6 kinase activity and ^{32}P-labeled polypeptides from the final Mono Q chromatography are shown in Fig. 1. A single peak of S6 kinase activity coelutes precisely with ^{32}P-labeled 70-kDa polypeptide; the latter exhibits the same mobility in SDS/PAGE as ^{32}P-labeled rat liver S6 kinase, autophosphorylated after purification with Mg^{2+} and [γ-^{32}P]ATP. Moreover, murine antiserum raised against rat liver S6 kinase immunoprecipitates part of H4 hepatoma 70-kDa ^{32}P-labeled polypeptide (Fig. 1 *Bottom*) as well as part of H4 hepatoma S6 kinase activity (data not shown). Two-dimensional peptide maps of tryptic digests, prepared from rat liver and insulin-stimulated H4 hepatoma 70-kDa S6 kinase, each ^{32}P-labeled by autophosphorylation *in vitro* with [γ-^{32}P]ATP, exhibit a similar pattern of ^{32}P-labeled peptides (see Fig. 6). From these findings, we conclude that the 70-kDa ^{32}P-labeled polypeptide is the quantitatively dominant insulin-stimulated H4 hepatoma S6 kinase, which in turn is immunochemically and structurally related (possibly identical) to the 70-kDa rat liver S6 kinase activated by cycloheximide. Electroelution and partial acid hydrolysis of the 70-kDa ^{32}P-labeled polypeptide, purified from insulin-stimulated ^{32}P-labeled H4 cells,

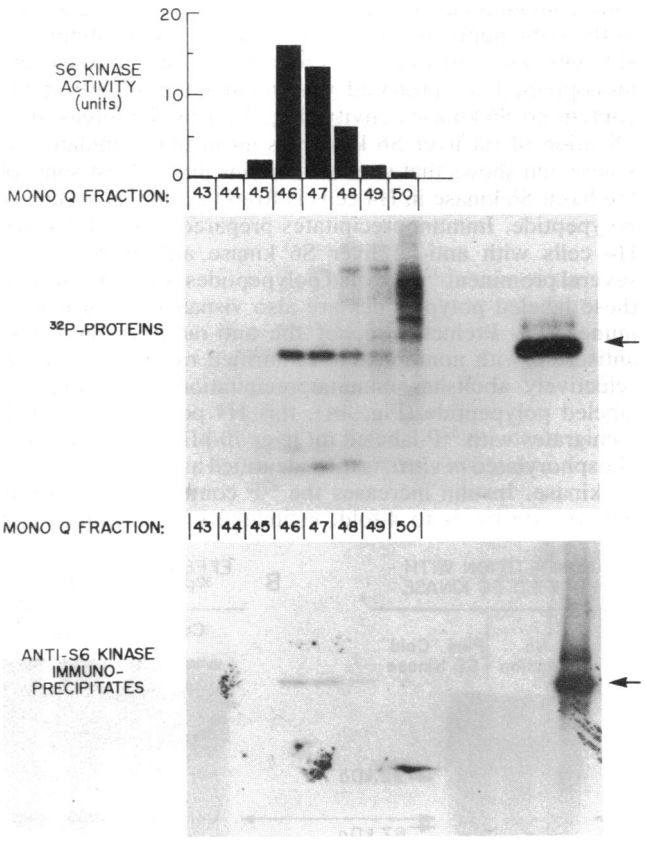

FIG. 1. Purification of the insulin-stimulated S6 kinase from ^{32}P-labeled H4 hepatoma cells. The elution profile of the Mono Q column is shown. (*Top*) S6 kinase activity; this activity represents 16% of the S6 kinase activity in the initial homogenate. (*Middle*) An aliquot of each fraction was subjected to SDS/PAGE; autoradiograph of the gel is shown. (*Bottom*) An aliquot of each fraction was incubated with murine anti-rat liver S6 kinase antiserum. Immune complexes were subjected to SDS/PAGE; an autoradiograph of the gel is shown. An aliquot of rat liver S6 kinase, autophosphorylated *in vitro* with [γ-^{32}P]ATP, was included in the *Middle* and *Bottom* gels and is marked by arrows.

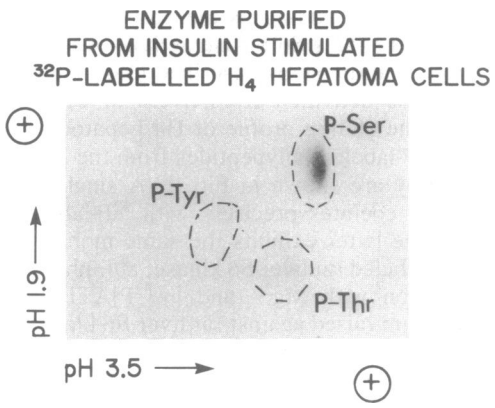

FIG. 2. [^{32}P]Phosphoamino acid analysis of S6 kinase. Insulin-stimulated S6 kinase purified from ^{32}P-labeled H4 cells was subjected to partial acid hydrolysis, followed by two-dimensional, thin-layer electrophoresis (21).

yielded ^{32}P-labeled serine as the predominant phosphoamino acid; a minor component of labeled threonine but no labeled tyrosine was detected (Fig. 2).

Immunoprecipitation of Insulin-Stimulated S6 Kinase from H4 Hepatoma Cytosol. Immunoprecipitates prepared from cytosolic extracts of control- and insulin-stimulated H4 hepatoma cells, using the murine anti-rat liver S6 kinase antiserum, contain S6 kinase activity, which is 3- to 9-fold greater in the immunoprecipitates prepared from insulin-stimulated H4 cells, as compared with control cells. By contrast, immunoprecipitates prepared with nonimmune mouse serum contain no S6 kinase activity (Fig. 3). This reinforces identification of rat liver S6 kinase as an insulin-stimulated S6 kinase and shows that even without insulin, at least some of the basal S6 kinase in H4 cells is from the 70-kDa S6 kinase polypeptide. Immunoprecipitates prepared from ^{32}P-labeled H4 cells with anti-rat liver S6 kinase antiserum contain several prominent ^{32}P-labeled polypeptides (Fig. 4*A*); most of these labeled polypeptides are also visualized with nonimmune sera. Preincubation of the anti-rat liver S6 kinase antiserum with nonradioactive, purified rat liver S6 kinase selectively abolishes immunoprecipitation of 70-kDa ^{32}P-labeled polypeptide (Fig. 4*A*); this H4 polypeptide, which comigrates with ^{32}P-labeled rat liver 70-kDa S6 kinase autophosphorylated *in vitro*, is thus identified as the H4 hepatoma S6 kinase. Insulin increases the ^{32}P content of the 70-kDa polypeptide by 3- to 4-fold, estimated by densitometry of autoradiographs (Fig. 4*B*; ^{32}P content is too low for reliable estimate by liquid scintillation counting).

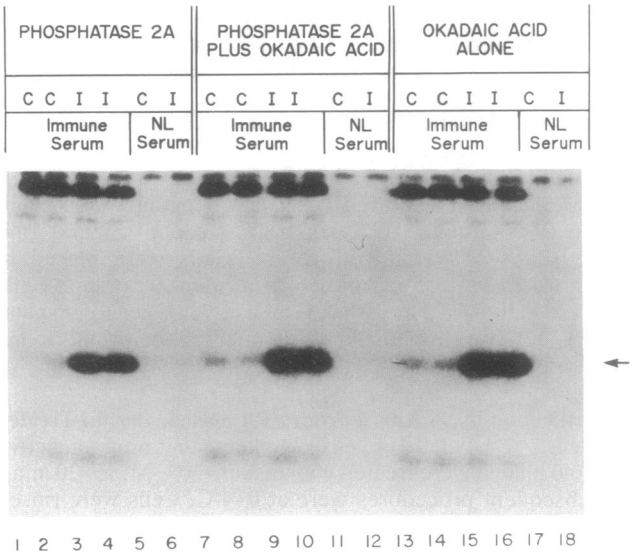

FIG. 3. Immunoprecipitation of S6 kinase activity from H4 cell cytosol. H4 cells were treated with insulin (I) (lanes 3, 4, 6, 9, 10, 12, 15, 16, and 18) (10^{-7} M × 15 min) or diluent (C) (lanes 1, 2, 5, 7, 8, 11, 13, 14, and 17). Cytosolic extracts were prepared and incubated with normal mouse serum (lanes 5, 6, 11, 12, 17, and 18) or mouse anti-rat S6 kinase antiserum (lanes 1–4, 7–10, and 13–16) at a 1:100 dilution. The immune complexes were harvested with protein A-Sepharose, resuspended in 50 mM Mops, pH 7.2/1 mM dithiothreitol/10 mM $MgCl_2$/0.1% Triton X-100/2 mM EGTA (buffer C) and divided into three aliquots. Each aliquot was incubated at 30°C with either phosphatase 2A (1 unit/ml) (lanes 1–6); phosphatase 2A plus okadaic acid (100 nM) (lanes 7–12); or okadaic acid alone (lanes 13–18). After 30 min okadaic acid was added to 100 nM to the samples of lanes 1–6, and S6 kinase activity of the immune complex was assayed by using [γ-^{32}P]ATP and 40S subunits. The reaction was terminated by adding SDS, and the solubilized proteins were subjected to SDS/PAGE. Position of ^{32}P-labeled S6 in the autoradiographed gel is indicated by the arrow. Insulin stimulation of S6 kinase detected in the immune complex assays is 9-fold as determined by densitometry of the 31-kDa band; two other experiments with insulin gave 2.6- and 3.7-fold increases. Phosphatase treatment yields a 50% decrease in the level of insulin-stimulated kinase; basal activity was too low to quantify.

Role of 70-kDa S6 Kinase Phosphorylation in Enzyme Activity. Incubation of the purified rat liver S6 kinase with

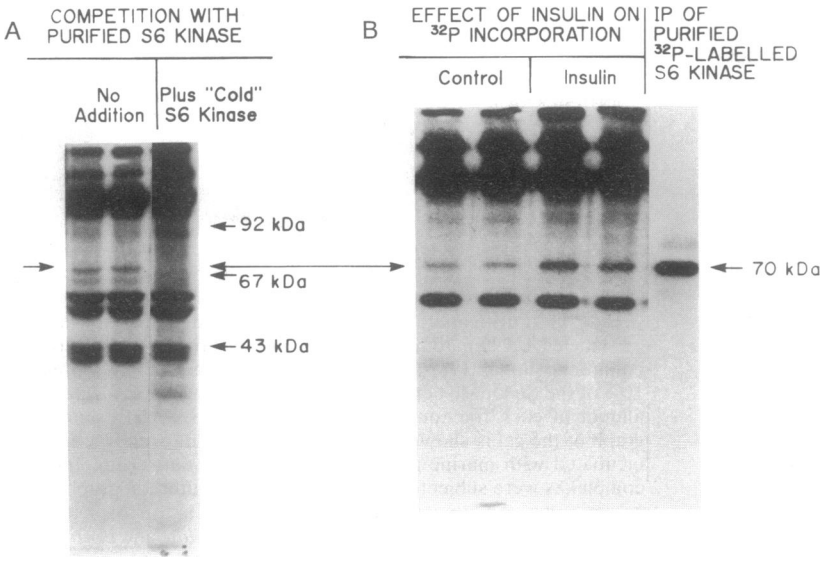

FIG. 4. Immunoprecipitation of ^{32}P-labeled S6 kinase from cytosol of control- and insulin-stimulated ^{32}P-labeled H4 cells. (*A*) The two lanes at left exhibit the ^{32}P-labeled polypeptides immunoprecipitated from cytosol of insulin-stimulated cells by anti-rat liver S6 kinase antiserum. The right lane demonstrates the effect of preincubating antiserum with nonradioactive rat liver S6 kinase. Notice the selective inhibition of immunoprecipitation of the 70-kDa H4 ^{32}P-labeled polypeptide. (*B*) ^{32}P-labeled H4 cells were exposed to insulin (10^{-7} M × 15 min) or diluent, and a cytosolic extract was prepared as described. Immunoprecipitation was done by adding mouse anti-rat liver S6 kinase antiserum at 1:100 dilution. The immune complexes were solubilized into SDS and subjected to SDS/PAGE. An aliquot of ^{32}P-autophosphorylated rat liver S6 kinase is shown in the far right lane. Densitometry indicated that the ^{32}P-labeled 70-kDa band contained 3 times more ^{32}P after insulin treatment; two additional experiments yielded 2.9- and 4.3-fold stimulation.

highly purified serine/threonine/specific protein phosphatases markedly inhibits S6 kinase activity. Incubation with phosphatase 2A can abolish completely both the 40S phosphorylating (Fig. 5) and autophosphorylating activities of the S6 kinase (data not shown). This activity loss is solely due to the phosphatase action, as kinase inactivation is completely prevented by okadaic acid, a potent, selective inhibitor of phosphatase 1 and 2A. Phosphatase 1 can also inactivate rat liver S6 kinase in a reaction prevented by highly purified protein phosphatase inhibitor 2. Compared at equal phosphorylase A phosphatase units, phosphatase 1 is only 5% as potent as phosphatase 2 in inactivating S6 kinase (Fig. 5*B*). The S6 kinase purified from insulin-stimulated H4 hepatoma cells is also deactivated by protein phosphatase 2A, although 4- to 5-fold higher concentrations of phosphatase activity are necessary than for deactivating rat liver S6 kinase purified from cycloheximide-treated rats (Fig. 5*C*). Finally, treatment of the immunoprecipitates from control- and insulin-treated H4 hepatoma cells with phosphatase 2A significantly reduces S6 kinase activity in both, in an okadaic-sensitive manner (Fig. 3).

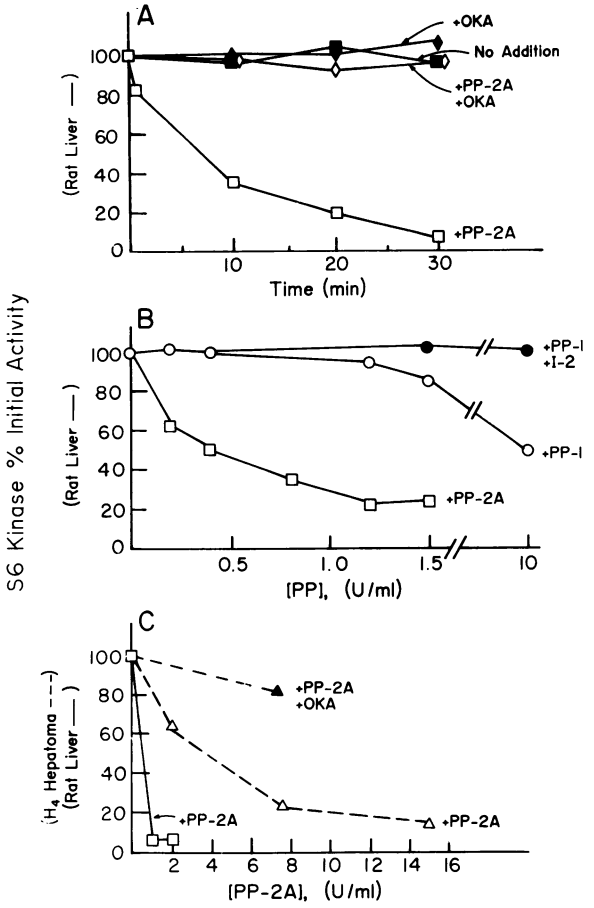

FIG. 5. Deactivation of purified S6 kinase by phosphatase treatment. (*A*) Time course of S6 kinase deactivation by phosphatase 2A (PP-2A): S6 kinase from rat liver (20 units/ml) was incubated at 30°C with phosphatase 2A (1 unit/ml) (□) in buffer C (see Fig. 3). At time points indicated, okadaic acid (OKA) was added to 200 nM, and aliquots were taken for S6 kinase assay. Controls included S6 kinase plus phosphatase 2A plus okadaic acid (200 nM) (◇), S6 kinase alone (■), and S6 kinase plus okadaic acid (◆). (*B*) Concentration dependence of S6 kinase deactivation by phosphatase 1 versus phosphatase 2A. S6 kinase from rat liver (25 units/ml) was incubated in buffer C at 30°C with various concentrations of phosphatase 2A (□), phosphatase 1 (○), and phosphatase 1 plus inhibitor 2 (20 μg/ml) (●). For phosphatase 1, deactivation was terminated by adding inhibitor 2 to a final concentration of 16 μg/ml, whereas phosphatase 2A action was terminated by adding *p*-nitrophenyl phosphate (final concentration, 20 mM). (*C*) Concentration dependence of phosphatase 2A deactivation of insulin-stimulated H4 hepatoma S6 kinase (△, ▲) versus rat liver S6 kinase: each S6 kinase was incubated at 10 units/ml for 30 min at 30°C with phosphatase 2A as indicated. Phosphatase action was terminated by adding okadaic acid to 0.1 μM. S6 kinase assays were done by using S6 peptide (18) as substrate.

These findings indicate that the catalytic activity of both the rat liver 70-kDa and the insulin-stimulated H4 hepatoma 70-kDa S6 kinases depends on one or more Ser(*P*)/Thr(*P*) residues. Inasmuch as insulin stimulates serine/threonine phosphorylation of the 70-kDa S6 kinase polypeptide, the insulin-induced activation is probably caused by the insulin-stimulated serine/threonine phosphorylation. More rigorous proof will require identification of the specific residues phosphorylated in response to insulin and the demonstration that phosphorylation of one or more of these residues stimulates enzyme activity.

Several lines of evidence indicate that increased phosphorylation of enzyme activity seen after insulin treatment is not from an insulin-stimulated autophosphorylation of S6 kinase: autophosphorylation *in vitro* of the enzyme purified in an "activated" state from livers of cycloheximide-treated rats, under conditions that incorporate ≈0.3 mol of phosphorus per subunit, does not further activate kinase activity toward 40S subunits (data not shown). S6 kinase inactivated with phosphatase 2A cannot be reactivated by incubation with Mg^{2+} and ATP; in fact, as indicated above, prior phosphatase treatment of S6 kinase reduces autophosphorylation comparably with 40S S6 phosphorylation. These observations indicate that the autophosphorylating activity of S6 kinase seen after purification only reflects the extent of prior activation and does not itself further stimulate catalytic activity of the kinase; presumably autophosphorylation (at least *in vitro*) does not involve the serine residues responsible for enzyme activation. This conclusion is supported by examining tryptic digests of H4 hepatoma S6 kinase, autophosphorylated with Mg^{2+} plus [γ-^{32}P]ATP *in vitro* after purification, as compared

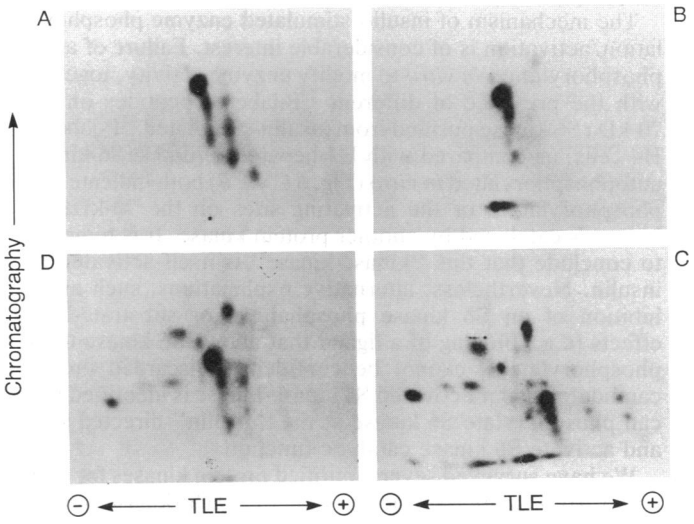

FIG. 6. ^{32}P-labeled peptide maps of S6 kinase after complete tryptic digestion. (*A*) Rat liver 70-kDa S6 kinase autophosphorylated *in vitro* with Mg^{2+} plus [γ-^{32}P]ATP. (*B*) H4 hepatoma S6 kinase (see Fig. 1) purified from ^{32}P-labeled insulin-stimulated cells and autophosphorylated *in vitro* with [γ-^{32}P]ATP; overall ^{32}P incorporation *in vitro* exceeded endogenous ^{32}P by 400-fold. (*C*) Insulin-stimulated 70-kDa S6 kinase purified from ^{32}P-labeled H4 hepatoma cells. (*D*) Mixture of equal cpm of ^{32}P from digests in *B* and *C*. Samples were subjected to thin-layer electrophoresis (TLE) at pH 3.5, followed by TLC. The origin and location of xylene cyanol marker are indicated by dashed circles on the autoradiographs.

with digests prepared from the 70-kDa S6 kinase ^{32}P-labeled *in situ* and purified from insulin-stimulated ^{32}P-labeled H4 hepatoma cells. The ^{32}P-labeled peptide maps show the labeled peptides in the digest of insulin-stimulated labeled enzyme isolated from labeled cells that are not detected in the enzyme autophosphorylated *in vitro* with [γ-^{32}P]ATP after purification (Fig. 6). These tryptic ^{32}P-labeled peptides in the insulin-stimulated enzyme probably contain the "activating" phosphorylation sites.

An obvious candidate for the protein kinase mediating insulin activation of the 70-kDa S6 kinase is the insulin-stimulated MAP-2 kinase, based on the ability of the latter enzyme to phosphorylate and partially reactivate *Xenopus* S6 kinase II (12). We, therefore, examined the ability of MAP-2 kinase (prepared by J. Kyriakis in this laboratory or supplied by T. Sturgill, Univ. of Virginia) to phosphorylate and reactivate rat liver 70-kDa S6 kinase, in comparison with *Xenopus* S6 kinase II (a gift from J. Maller and E. Erikson, Univ. of Colorado), after both S6 kinases have been deactivated by phosphatase 2A. Although reactivation of *Xenopus* S6 kinase II by MAP-2 kinase was readily confirmed, rat liver 70-kDa S6 kinase was not a substrate for the insulin-stimulated MAP-2 kinase.

DISCUSSION

These results demonstrate that a major insulin-stimulated S6 kinase in H4 hepatoma cells is immunochemically and structurally related (possibly identical) to the 70-kDa rat liver S6 protein kinase, initially purified from livers of cycloheximide-treated rats. This enzyme depends completely on Ser(*P*)/Thr(*P*) residues for its catalytic activity, as witnessed by complete deactivation of both H4 hepatoma and rat liver S6 kinases by treatment with phosphatase 2A. Moreover, the ability of insulin to stimulate serine/threonine-specific phosphorylation of the enzyme polypeptide, concomitant with activation of its catalytic function, strongly supports the conclusion that insulin-stimulated phosphorylation underlies enzyme activation.

The mechanism of insulin-stimulated enzyme phosphorylation/activation is of considerable interest. Failure of autophosphorylation *in vitro* to modify enzyme activity, together with the presence of different ^{32}P-labeled peptides on the 70-kDa S6 kinase purified from insulin-stimulated ^{32}P-labeled H4 cells, as compared with H4 hepatoma 70-kDa S6 kinase autophosphorylated *in vitro* (Fig. 6 *C* vs. *B*) both indicate that phosphorylation of the activating sites on the 70-kDa S6 kinase is catalyzed by another protein kinase. It is tempting to conclude that this "kinase-kinase" is itself activated by insulin. Nevertheless, alternative explanations, such as inhibition of an S6 kinase phosphatase, or substrate-level effects (e.g., binding of a ligand that allows S6 kinase to be phosphorylated), cannot be confidently discarded until a candidate insulin-activated S6 kinase-kinase is identified that can phosphorylate S6 kinase at the "insulin"-directed sites and activate S6 kinase catalytic function.

We have surveyed several purified protein kinases for their ability to phosphorylate/activate the 70-kDa S6 kinase, with or without prior deactivation by phosphatase 2A. Negative results were obtained with kinase A catalytic subunit, casein kinase II, glycogen synthase kinase 3 (with and without prior incubation of the 70-kDa S6 kinases with casein kinase II and Mg^{2+} and ATP) and several preparations of insulin-stimulated MAP-2 kinase. In addition, insulin treatment of H4 cells does not alter the ^{32}P content of c-raf protein as visualized in immunoprecipitates, suggesting that c-raf protein does not mediate the insulin activation of S6 kinase (J.R.G., J.A., and U. Rapp, unpublished work).

We have analyzed cDNA of rat origin corresponding to the 70-kDa S6 kinase and an 85-kDa homolog of *Xenopus* S6 kinase α (22). The deduced amino acid sequences of the 70- and 85-kDa enzymes, although 56% identical in their catalytic domains, differ extensively outside of the catalytic domain. The inability of the 70-kDa rat liver S6 kinase to be phosphorylated by the insulin-stimulated MAP-2 kinase further distinguishes the 70-kDa enzyme from the 85-kDa *Xenopus* S6 kinase II and its homologs. It appears likely that these two types of S6 protein kinases, 85-kDa versus 70-kDa, although both hormonally regulated by phosphorylation of the enzyme polypeptide, are independently regulated.

The multiple S6 kinases detected thus far appear to reflect the existence of a family of enzymes related by structural and regulatory features as well as substrate specificity. These enzymes function as intermediate signaling molecules in hormone action, even though their precise role in the program remains uncertain. Identification of the kinases that regulate the catalytic function of the 70-kDa rat liver S6 kinase described herein may clarify the nature of the targets of the activated insulin receptor.

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1. Avruch, J., Nemenoff, R. A., Pierce, M. W., Kwok, Y. C. & Blackshear, P. J. (1985) in *Molecular Basis of Insulin Action*, ed. Czech, M. P. (Plenum, New York), pp. 263–296.
2. Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P. & Lewis, R. E. (1988) *J. Biol. Chem.* **263,** 11017–11020.
3. Avruch, J., Tornqvist, H. E., Gunsalus, J. R., Yurkow, E. J., Kyriakis, J. M. & Price, D. J. (1990) in *Handbook of Experimental Pharmacology*, eds. Cuatrecasas, P. & Jacobs, S. (Springer, Berlin), Vol. 92, pp. 313–366.
4. Degerman, E., Smith, C. J., Tornqvist, H., Vasta, V., Belfrage, P., Manganiello, V. C. & Belfrage, P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 533–537.
5. Thomas, G., Siegmann, M. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3952–3956.
6. Smith, C. J., Wejksnora, P. J., Warner, J. R., Rubin, C. S. & Rosen, O. M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2725–2729.
7. Novak-Hofer, I. & Thomas, G. (1984) *J. Biol. Chem.* **259,** 5995–6000.
8. Tabarini, D., Heinrich, J. & Rosen, O. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4369–4373.
9. Nemenoff, R. A., Gunsalus, J. R. & Avruch, J. (1986) *Arch. Biochem. Biophys.* **245,** 196–203.
10. Erikson, E. & Maller, J. L. (1986) *J. Biol. Chem.* **261,** 350–355.
11. Erikson, E. & Maller, J. L. (1989) *J. Biol. Chem.* **264,** 13711–13717.
12. Sturgill, T. W., Ray, L. B., Erikson, E. & Maller, J. L. (1988) *Nature (London)* **334,** 715–718.
13. Jones, S. W., Erikson, E., Blenis, J., Maller, J. L. & Erikson, R. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3377–3381.
14. Alcorta, D. A., Crews, C. M., Sweet, L. J., Bankston, L., Jones, S. W. & Erikson, R. L. (1989) *Mol. Cell. Biol.* **9,** 3850–3859.
15. Blenis, J., Kuo, C. J. & Erikson, R. L. (1987) *J. Biol. Chem.* **262,** 14373–14376.
16. Jeno, P., Ballou, L. M., Novak-Hofer, I. & Thomas, G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 406–410.
17. Ballou, L. M., Siegmann, M. & Thomas, G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7154–7158.
18. Price, D. J., Nemenoff, R. A. & Avruch, J. (1989) *J. Biol. Chem.* **264,** 13825–13833.
19. Nemenoff, R. A., Price, D. J., Mendelsohn, M. J., Carter, E. A. & Avruch, J. (1988) *J. Biol. Chem.* **263,** 19455–19460.
20. Tornqvist, H. E., Gunsalus, J. R., Nemenoff, R. A., Frackelton, A. R., Pierce, M. W. & Avruch, J. (1988) *J. Biol. Chem.* **263,** 350–359.
21. Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W. & Osathanondh, R. (1982) *J. Biol. Chem.* **257,** 15162–15166.
22. Banerjee, P., Ahmad, M., Grove, J. R., Koslosky, C., Price, D. J. & Avruch, J. (1990) *Proc. Natl. Acad. Sci. USA*, in press.