Cycloheximide injection of rats results in the activation of a protein kinase that phosphorylates 40 S ribosomal protein S6. This Ca\(^{2+}\)/cyclic nucleotide-independent kinase exhibits chromatographic properties that are indistinguishable from the S6 kinase in H4 hepatoma cells whose activity is stimulated by insulin and growth factors and the S6 kinase that is activated during liver regeneration. The enzyme has been purified 50,000-fold to near homogeneity: a critical step in purification employs a peptide affinity column using a synthetic peptide corresponding to the carboxy-terminal 32-amino acid residues of mouse liver S6, which encompasses all S6 phosphorylation sites. The purified enzyme is a 70,000-dalton polypeptide that is reactive with azido-ATP. In addition to 40 S ribosomal S6 and the synthetic peptide, the S6 kinase catalyzes rapid phosphorylation of a number of other protein substrates including histone H2b, glycogen synthase, and ATP citrate lyase; this last protein is phosphorylated by S6 kinase in vitro on the same serine residue that is phosphorylated in response to insulin and epididymal growth factor in intact hepatocytes. Moreover, the S6 kinase catalyzes the phosphorylation of a number of hepatic nonhistone nuclear proteins. This S6 kinase probably underlies the increased hepatic S6 phosphorylation observed after cycloheximide treatment, which in turn corresponds to the mitogen-activated S6 kinase.

S6 is the sole phosphoprotein of the 40 S subunit of the eukaryotic ribosome (1). S6 phosphorylation was first demonstrated by Gressner and Wool (2), who showed that rat liver S6 undergoes a 10-fold increase in \(^{32}\)P content in the course of liver regeneration; a similar increase in hepatic S6 phosphorylation was observed in rats injected with puromycin or cycloheximide (3). In both instances, up to five-charge isomers could be detected on two-dimensional gels of proteins separated from 40 S ribosomal subunits. This is now known to reflect the presence of five or six phosphorylation sites located near the carboxy terminus of the protein (4).

Subsequent studies demonstrated that S6 phosphorylation in cultured cells is augmented within 10 min after exposure to a variety of growth-promoting stimuli including serum, polypeptide growth factors, insulin, and active phorbol esters (5). The ubiquitous appearance of S6 phosphorylation as an early concomitant of the response to growth factors and hormones generated considerable effort toward elucidation of the underlying mechanism. The detection of an activated S6 kinase was first reported by Rosen and co-workers (6). A substantial advance was provided by Novak-Hofer and Thomas (7), who showed that preservation of the activated S6 kinase induced by serum or EGF\(^1\) treatment of 3T3 fibroblasts required homogenization to be carried out in the presence of EGTA and \(\beta\)-glycerophosphate. These homogenization conditions were subsequently demonstrated to permit capture of an S6 kinase activity stimulated by insulin, growth factors, active phorbol esters, and vandase in a wide range of cultured cells (8–11) as well as in progesterone or insulin-treated Xenopus oocytes (12), v-src-transformed chick embryo fibroblasts (13), and regenerating rat liver (14). Extensive purification of several of these activated S6 kinases has been reported. Herein, we report the purification and preliminary characterization of the rat liver S6 kinase that is activated by cycloheximide injection of the animal prior to sacrifice. This enzyme is indistinguishable in its chromatographic properties and requirements for \(\beta\)-glycerophosphate and EGTA from the S6 kinase activated during liver regeneration and by insulin treatment of serum-starved rat hepatoma (H4IIIC) (14) cells. The highly purified rat liver enzyme exhibits a major 70,000-dalton silver-stained polypeptide that comigrates with S6 kinase activity on several chromatographic steps, undergoes \(^{32}\)P-labeling on incubation with [\(\gamma\)\(^{32}\)]ATP, and is the only polypeptide reactive with azido-\([\gamma\)\(^{32}\)]ATP; this polypeptide is the S6 kinase. Although purified as an "S6" kinase, the enzyme exhibits considerable kinase activity toward ATP citrate lyase, glycogen synthase, histone H2b, and a number of unidentified proteins in nuclear extracts. In fact, the enzyme phosphorylates ATP citrate lyase on a single major tryptic peptide at the same serine residue phosphorylated by the cAMP-dependent protein kinase in vitro; this is known to be the site on ATP citrate lyase which undergoes phosphorylation in insulin and EGF-stimulated hepatocytes (15).

**EXPERIMENTAL PROCEDURES**

S6 Kinase Purification—Fifty rat livers, removed 1 h after an intraperitoneal injection of cycloheximide (50 mg/kg of body weight) were homogenized in buffer A (10 mM KPi, pH 6.5, 1 mM EGTA, 5 mM EGTA, 10 mM MgCl\(_2\), 1 mM DTT, 1 mM vanadate, 50 mM \(\beta\)-glycerophosphate, 2 \(\mu\)M leupeptin, 2 \(\mu\)M pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM diisopropyl fluorophosphate) at a ratio of 3 ml/g, wet weight, of liver. The suspension was centrifuged for 2 h at 35,000 rpm in a Beckman Ti-45 rotor (100,000 \(\times G\) for 2 h); the supernatant was removed, filtered through glass wool, and added to 300 ml of settled DEAE-Sephadex (Pharmacia LKB Biotechnology Inc.) equilibrated in buffer A. The slurry was stirred for 1 h and washed in a Buchner funnel with ~2 liters of buffer A, transferred to 400 ml of settled DEAE- Sephadex (Pharmacia LKB Biotechnology Inc.) equilibrated in buffer A. The slurry was stirred for 1 h and washed in a Buchner funnel with ~2 liters of buffer A, transferred to

\(^{1}\)The abbreviations used are: EGF, epidermal growth factor; MOPS, 3\((N\text{-morpholino})propanesulfonic\) acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kinase A, cAMP-dependent protein kinase; kinase C, protein kinase C; DTT, dithiothreitol; EGTA, [\(\text{ethylenebis(oxethylenetrilithilo)\text{etracetic acid}}\); HPLC, high pressure liquid chromatography; NEM, N-ethylmaleimide.
a column (5 × 60 cm), washed with 0.3 liter of buffer A + 0.05 M NaCl, and then eluted with a 3.5-liter gradient of NaCl (0.05-0.8 M) in buffer A. The dominant peak of S6 kinase activity eluted between 0.2 and 0.4 M NaCl. A trailing shoulder or second peak, variable in amount, was usually seen eluting around 0.45-0.5 M NaCl. The first peak was pooled (1400 ml), brought to 0.05% Brij 35, dialyzed twice against 2 liters of buffer B (10 mM KP, pH 6.5, 1 mM EDTA, 5 mM EGTA, 5 mM MgCl₂, 0.1 mM vanadate, 0.1% β-glycerol phosphate, 0.05% Brij 35, 2 mM β-mercaptoethanol, and 10% glycerol) to a conductivity of <3 mmho, and mixed with 200–250 ml of SP-Sepharose C-50 (Pharmacia) equilibrated with buffer B. After 30 min with occasional agitation, the slurry was transferred into a column (5 × 30 cm), washed with 2 bed volumes of buffer B, and eluted with a 1,500-ml gradient of NaCl (to 0.5 M) in buffer B. S6 kinase activity emerged as a broad bifid peak, the earlier and usually larger peak centered around 0.18-0.21 M, the latter peak around 0.26-0.3 M NaCl. The entire bifid peak was pooled, dialyzed against 4 liters of buffer C (20 mM Tris-HCl, pH 6.5, 1 mM EGTA, 2 mM EDTA, 2 mM DTT, 0.1% Triton X-100, 10 mM β-glycerol phosphate, and 10% glycerol) to a conductivity of <2 mmho, and applied at 1.0 ml/min to a 25-cm (1.5 × 7 cm) column of heparin-Sepharose (Pharmacia). The column was washed with 50 ml of buffer C and eluted with a ~200-ml gradient of NaCl (to 1 M) in buffer C. S6 kinase eluted as a double peak between 0.37 and 0.6 M NaCl. The eluate was dialyzed versus buffer C and applied at 0.2 ml/min to a 1 × 7-cm S6 peptide affinity column equilibrated in buffer C. This column was prepared by reacting approximately 70 mg of S6 peptide with ~8 ml of Affi-Gel 10 (Bio-Rad) for 12 h at 5 °C. Excess sites were blocked with Tris-HCl, and the resin was washed with 0.5 M NaCl followed by buffer C. The sequence of this peptide, Lys-Glu-Ala-Lys-Glu-Ala-Lys-Arg-Arg-Asp-Thr-Ser-Thr-Ile-Ala-Lys-Arg-Arg-Leu-Ser-Leu-Arg-Ala-Ser-Thr-Gln-Lys-Glu-Leu-Ser-Lys-Glu-Lys-Arg-Arg-Leu-Ser-Leu-Arg-Ala-Thr-Ser-Lys-Gly-Gly-Gly-Ser-Gln-Lys, corresponds to the carboxy-terminal amino acids of rat liver ribosomal protein S6 (16). The column was washed with 45 ml of buffer C and developed at 0.5 ml/min with a 180-ml gradient of NaCl (to 0.5 M) in buffer C. S6 kinase activity eluted as a single peak between 0.17 and 0.31 M NaCl. The first Mono Q eluate was applied to a 42-cm column of Sephacryl S-300 equilibrated with buffer C (0.05 M NaCl) and eluted with a 180-ml gradient of NaCl (to 1 M) in buffer C. S6 kinase activity eluted as a single peak between 0.17 and 0.31 M NaCl. 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The eluate was dialyzed versus 3 liters of buffer C and applied at 0.8 ml/min to a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer C. The column was washed with buffer C, and S6 kinase activity was eluted with a 70-ml gradient of NaCl (to 0.5 M) in buffer C (collecting 1.5-ml fractions). S6 kinase activity always elutes in four or five consecutive fractions as a double peak between 0.22 and 0.28 M (Fig. 1). Concentration prior to gel filtration was achieved by adsorption/elution from the Mono Q column. The first Mono Q eluate was diluted by the addition of 3 volumes of buffer C, reequilibrated to the Mono Q HR 5/5 column (reequilibrated with buffer C), and eluted with a 4-ml gradient of NaCl (0.5 M) in buffer C. S6 kinase emerged as a total volume of 1.0-1.2 ml with ~50% recovery. The second Mono Q eluate was applied to a 1 × 42-cm column of Sephacryl S-300 (Pharmacia) equilibrated in buffer C plus 0.05 M NaCl. Elution was carried out at 0.3 ml/min, and S6 kinase activity emerged as a single symmetric peak just ahead of bovine serum albumin (Fig. 2). A representative purification is quantified in Table I, and the gel electrophoretic analysis of the last two steps is shown in Fig. 3.

Fig. 1. Mono Q chromatography. The peak S6 kinase activity from the peptide affinity column was dialyzed versus buffer C and applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 10 ml of buffer C. The S6 kinase was eluted with a linear NaCl gradient of NaCl to 0.5 M (70 ml) at 0.5 ml/min with collection of 1.5-ml fractions.
The dried gel was exposed to discharge the 32P-histidine phosphoenzyme intermediate. The mixture was then desalted over a column equilibrated with 10 mM NaPi, pH 7.5, 1 mM EGTA, 4 mM sodium citrate, 10 mM DTT, and 0.3 mM DTT.  The void volume was pooled, and a portion of the 32P-lyase (-1 mg) was subjected to trypsin digestion and subjected to automated Edman degradation with carrier protein so as to identify the cycle at which 32P was released.

Azido-ATP Labeling—S6 kinase in buffer C was extensively dialyzed against buffer C minus DTT. After cooling to 0–4 °C on ice and the addition of MgCl2 to 3 mM, ~55 pmol of B-azidoadenosine 5'-triphosphate [γ-32P] (5.7 Ci/mmol) (ICN) was added to ~20 mg of enzyme in a 96-well plate on ice, in a final volume of 60 μl, either in the presence or absence of unlabeled ATP (0.4 mM). The samples were then irradiated for 30 s with an Am Mineralight at a distance of 1 cm. The reactions were stopped by the addition of SDS quench, subjected to SDS-PAGE on a 10–15% gradient gel, and fixation, the silver stain; the lower panels (b and d) are autoradiographs.

**Fig. 3. Gel electrophoresis of purified S6 kinase.** An aliquot from each of the peak fractions of S6 kinase activity corresponding to the Mono Q chromatography shown in Fig. 1 (a and b) and the subsequent Sephacryl S-300 chromatography shown in Fig. 2 (c and d) were autophosphorylated with magnesium and [γ-32P]ATP (see under "Experimental Procedures") and subjected to SDS-PAGE. The upper panels (a and c) show the silver stain; the lower panels (b and d) are autoradiographs.

**TABLE I**  
Purification of S6 kinase from cycloheximide-stimulated rats

| Protein | Activity | Volume | Purification | Recovery | Specific activity |
|---------|----------|--------|--------------|----------|------------------|
| Extract, control | 266 | 341 | 100 | -fold | % | pmol/ min/mg |
| Extract, cycloheximide Rx | 10,065 | 92,590 | 1,880 | 1 | 100 | 9.20 |
| DEAE-Sephadex | 426.4 | 126,140 | 680 | 32 | 136 | 294 |
| SP-Sephadex | 56.2 | 102,240 | 56.2 | 198 | 110 | 1,819 |
| Heparin-Sephadex | 19.35 | 46,368 | 92 | 260 | 50 | 2,386 |
| Peptide affinity | 0.285 | 63,912 | 103 | 24,437 | 69 | 224,820 |
| Mono Q | 0.001 | 29,280 | 12 | 52,228 | 32 | 480,500 |
| Sephacryl S-300 | 0.014 | 1,156 | 6.5 | 47,795 | 6.6 | 439,714 |

**RESULTS**

S6 kinase is purified from the livers of cycloheximider-treated rats in a six-step procedure, ~50,000-fold, with 5–10% recovery (Table I). The DEAE column (step 1) consistently shows, in addition to the major peak of S6 kinase, a second peak that elutes later and varies in amount from a trailing
shoulder to a distinct peak containing 30–50% as much S6 kinase activity as the dominant earlier eluting peak. This second peak, which is also seen occasionally in extracts from insulin-treated H4 cells and regenerating liver, has not been further characterized; only the consistently observed, early eluting, major DEAE peak was taken for further purification. At the SP-Sephadex (step 2), heparin-Sepharose (step 3), and Mono Q (step 5, Fig. 1) steps, the S6 kinase activity is usually recovered as two overlapping but distinguishable peaks. The molecular basis for the appearance of these two peaks is not known. On elution from the Mono Q column (step 5), each fraction in this dual peak of S6 kinase activity exhibits a dominant 70-kDa silver-stained polypeptide that usually appears as a doublet, and a considerable number of minor polypeptide bands (Fig. 3). Incubation of each fraction containing S6 kinase activity with magnesium and [γ-32P]ATP reveals that the 70/68 kDa polypeptide doublet undergoes phosphorylation as do minor polypeptides of 66 and 58 kDa (Fig. 3); the latter polypeptides exhibit progressively lower 32P incorporation, although still, however, overlapping both peaks S6 kinase of activity. In addition, some isolates exhibit a 95-kDa 32P-labeled polypeptide in the Mono Q fractions corresponding to the earlier half of the double peak of S6 kinase; these preparations also occasionally show traces of a 32P-labeled 93-kDa polypeptide, eluting slightly later than the 95 kDa but still within the earlier peak. In this (first) peak of S6 kinase, the 95/93-kDa 32P-polypeptide exhibits perhaps 10% of the silver stain intensity and 32P content as the 70/68-kDa array. Incubation of the pooled Mono Q peak of S6 kinase activity with azido-[32P]ATP is associated with labeling of only the 70/68-kDa polypeptide; the 95-kDa species, when present, is not reactive with azido-ATP (Fig. 4). If the leading fraction of S6 kinase activity observed on Mono Q, which contains only the 70- and 95-kDa 32P-peptides, is subjected to gel filtration (step 6), only a single peak of S6 kinase activity is observed; the 70- and 95-kDa polypeptides and enzyme activity all coelute at an apparent molecular mass of 70 kDa (Fig. 2); the 70-kDa polypeptide visualized on silver stain (Fig. 3c) corresponds to the major 32P-labeled polypeptide (Fig. 3d). The 32P-labeled 95-kDa polypeptide is seen as a minor band on autoradiography.

Thus, several lines of evidence indicate that the 70-kDa polypeptide is the S6 kinase, particularly the absolute coincidence of this polypeptide with the S6 kinase activity on both the Mono Q and gel filtration columns and its reactivity with azido-ATP. Moreover, the rate of 32P incorporation from [γ-32P]ATP into the 70-kDa polypeptide is independent of enzyme dilution over a 10-fold range (not shown), suggesting that phosphorylation occurs via an intramolecular mechanism. The minor 95-kDa polypeptide is variably present, lacking entirely in some isolates; it is not labeled with azido-ATP, and on partial digestion with S. aureus V8 protease, it exhibits an array of 32P-peptide entirely distinct from those generated from the 32P-labeled 70-kDa polypeptide analyzed in parallel (not shown). The 95-kDa polypeptide appears thus to be a minor contaminant that can act as a substrate for the S6 kinase in vitro and which is structurally and functionally unrelated to the 70-kDa polypeptide.

**Regulatory Properties**—ATP is the preferred nucleotide triphosphate for the S6 kinase (Km ATP = 143 μM, Fig. 5); kinase activity is unaffected by excess concentrations of a variety of nucleotide triphosphates other than ATP (Table II). Addition of AMP does significantly reduce S6 kinase activity. Optimal activity is attained with magnesium at 1–10 mM. Considerable activity is also observed with manganese as the sole divalent cation at manganese concentrations near that of ATP; by contrast, excess free manganese is strongly inhibitory (Fig. 6). S6 kinase is unaffected by cyclic nucleotides, calcium, calmodulin, phospholipids, and diglycerides (Table II), distinguishing it from known ligand-regulated kinases. Heparin and polylysine are mildly inhibitory, whereas protamine is a potent inhibitor; polyglutamic acid at comparable concentrations is without effect. The S6 kinase activity is also inhibited by increasing ionic strength and is particularly sensitive to inhibition by NaF (Fig. 7). The requirement for EGTA is no longer evident in the purified enzyme; dilution of enzyme into EGTA-free buffers is associated with a slight increase in activity (not shown). We have not ascertained the step at which the requirement for EGTA EDTA is no longer observed. S6 kinase activity is profoundly inhibited by several transition metals (Table II). The presence of —SH groups critical to activity is indicated by the potent inhibition of the enzyme by low concentrations of NEM (see Fig. 10B). S6 kinase activity exhibits a broad pH optimum between 6.5 and 7.8 (not shown).

**Substrate Specificity**—The purified S6 kinase phosphorylates a peptide that corresponds to the carboxyl-terminal 32

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**Fig. 4. Azido [γ-32P]ATP labeling of S6 kinase.** S6 kinase (~20 ng) was photoaffinity labeled with 8-azido-[γ-32P]adenosine triphosphate at 0 °C in the absence (lane 3) or presence (lane 4) of 0.4 mM unlabeled ATP, according to “Experimental Procedures.” The same preparation autophosphorylated by [γ-32P]adenosine triphosphate at 30 °C is shown in lanes 1 and 2.

**Fig. 5. Kinetic parameters of S6 kinase.** Activities of S6 kinase were measured as described under “Experimental Procedures,” utilizing the synthetic S6 peptide varying (A) S6 peptide concentration and (B) ATP concentration.
TABLE II
Modifiers of S6 kinase activity
S6 kinase activity was assayed in the presence of the indicated concentration of modifier by the S6 peptide method. Assays also contained 40 mM MOPS, pH 7.4, 8 mM MgCl₂, 1 mM DTT, 5 μM heat-stable inhibitor of kinase A peptide, and 100 μM [γ-32P]ATP. For effect of phospholipids, phosphatidylserine was 400 pg/ml, diolein concentration of modifier by the S6 peptide method. Assays also was 40 pg/ml, and Ca²⁺ was 0.25 mM, final concentration.

| Modifier, concentration | Activity % |
|-------------------------|------------|
| No addition             | 100        |
| cAMP, 10 μM             | 93.6       |
| cGMP, 10 μM             | 104.3      |
| Polylysine, 50 μg/ml    | 48.0       |
| Heparin, 50 μg/ml       | 62.2       |
| Protamine, 25 μg/ml     | 4.8        |
| Polyglutamic acid, 50 μg/ml | 102.1 |
| Diolein/phosphatidylserine | 99.7 |
| Diolein/phosphatidylserine/Ca⁴⁺ | 96.2 |
| CaCl₂, 0.25 mM          | 95.6       |
| Ca⁴⁺/calmodulin, 10 μg/ml | 96.5 |
| MnCl₂, 10 mM            | 6.8        |
| CuSO₄, 10 mM            | 0.6        |
| CoCl₂, 10 mM            | 0          |
| Ammonium molybdate, 0.5 mM | 107 |
| ZnSO₄, 10 mM            | 0.1        |
| DTT, 10 mM              | 116        |
| UTP, 1.67 mM            | 96.6       |
| CTP, 1.67 mM            | 85.7       |
| ATP, 1.67 mM            | 103.6      |
| AMP, 1.67 mM            | 11.4       |
| NADH, 1.07 mM           | 33.3       |

![Fig. 6. Mg²⁺/Mn²⁺ concentration dependence of S6 kinase.](image)

S6 kinase was assayed using the S6 peptide (see under “Experimental Procedures”) with increasing concentrations of MgCl₂ (ocz) or MnCl₂ (A) at 0.1 mM [γ-32P]ATP. The reaction contained 0.06 mM EGTA.

![Fig. 7. Inhibition of S6 kinase by NaF and NaCl.](image)

S6 kinase (20 units/ml) was assayed by standard S6 peptide assay in the presence of increasing levels of NaF (ocz) or NaCl (ocz).

TABLE III
Relative rate of substrate phosphorylation
S6 kinase, kinase C, and kinase A were normalized by their ability to phosphorylate S6 peptide. The actual amount of protein kinase in each assay tube was: kinase A catalytic subunit (Sigma P2645), 1.3 ng; kinase C (Lipidex 1002), 4.3 ng; S6 kinase (Mono Q step, Table I), 6.1 ng. All assays contained 60 mM MOPS, pH 7.4, 10 mM MgCl₂, 1 mM DTT. For assay of kinase C, reactions also contained 400 μg/ml phosphatidylserine and 40 μg/ml dodecyl. Concentrations of individual protein substrates are indicated in far left column. After incubation of reactions for 10 min at 30 °C, reactions were terminated by addition of SDS quench, and samples were run on 7 or 15% SDS-polyacrylamide gels. Appropriate bands of the dried gels were cut, and [32P] was determined by liquid scintillation counting. MAP-2, microtubule-associated protein-2.

| Substrate Protein kinase activity | Kinase A S6 kinase Kinase C |
|---------------------------------|---------------------------|
| Kinase A concentration (pmol/min) | 750 | 753 | 753 |
| Kinase C concentration (pmol/min) | 81.60 | 753 | 753 |
| 40 S ribosomes (pmol/min) | 250.80 | 80.10 |
| ATP citrate lyase (pmol/min) | 56.8 | 0.25 |
| Glycogen synthase (pmol/min) | 122.60 |
| Acetyl-CoA carboxylase (pmol/min) | 60.25 |
| Histone H2b (pmol/min) | 410.20 | 81.60 |
| Histone H1 (pmol/min) | 82.70 | 4.54 |
| MAP-2 (pmol/min) | 3.48 | 0.69 |
| Phosphatase (pmol/min) | 2.9 | 0 |
with the tryptic peptide that bears the major site of phosphorylation by kinase A shown previously to have the sequence Thr-Ala-Ser-(Pro)-Phe-Ser-Glu-Ser-Lys (Fig. 8). Moreover, when the \(^{32}\)P-labeled tryptic peptide isolated from lyase phosphorylated by either kinase A or the S6 kinase is subjected to automated Edman degradation, a burst of \(^{32}\)P is released only at the third cycle. Thus, S6 kinase phosphorylates the same serine residue phosphorylated in vitro by kinase A; this serine is also the residue phosphorylated in intact liver cells stimulated by either insulin or glucagon (15, 23). S6 kinase phosphorylates skeletal muscle glycogen synthase at a considerable rate, ~50% that observed for 40 S subunits, and comparable to the rate of glycogen synthase phosphorylation catalyzed by the cAMP-dependent protein kinase. We have not determined the site of phosphorylation or the effect on glycogen synthase activity. S6 kinase phosphorylates histone H2b and microtubule-associated protein-2, ~20-25% as rapidly as kinase A. Among the other purified proteins tested, acetyl-CoA carboxylase, histones H1 and H4 were phosphorylated by S6 kinase at a rate ≤5% that of kinase A; protein phosphatase inhibitor-2, casein, and phosphorylase \(b\) were not modified by S6 kinase. In addition to these relatively purified proteins, we examined the ability of these three kinases to phosphorylate several crude mixtures of unidentified proteins, including a preparation of adipocyte heat-stable proteins enriched for the insulin-stimulated 22-kDa phosphoprotein (28), a preparation of crude cytosolic protein from liver, and the protein mixture extracted from rat hepatoma cell nuclei by a 0.4 M NaCl wash, a preparation known to be enriched in nonhistone nuclear proteins (29). The ability of heat treatment as well as NEM to inactivate endogenous kinases. The striking observation is that S6 kinase was compared with the effect of these treatments on the kinase activity toward the other protein substrates (Fig. 9), both before and after heat treatment, under conditions wherein kinases A and C added at comparable "S6 peptide kinase units" show virtually no phosphorylation of nuclear proteins. A number of nuclear substrates for S6 kinase comigrate with \(^{32}\)P-polypeptides generated by the endogenous nuclear protein kinase activity.

**DISCUSSION**

The ability of heat treatment as well as NEM to inactivate S6 kinase was compared with the effect of these treatments on the kinase activity toward the other protein substrates (Fig. 10). Inhibition of S6 kinase by NEM or elevated temperature is accompanied by a parallel loss of kinase activity toward each of the major substrates. Thus, we conclude that the phosphorylation of ATP-citrate lyase, glycogen synthase, and histone H2b is carried out by the same enzyme as catalyzes the phosphorylation of S6.

**FIG. 8.** C18 reverse-phase chromatography of tryptic digests of \(^{32}\)P-labeled ATP citrate lyase. ATP citrate lyase was purified from rat liver, phosphorylated by either kinase A or S6 kinase to overall stoichiometry of 0.09 and 0.06 mol of \(^{32}\)P/subunit, respectively. After desalting on Sephadex G-25, aliquots of 1 mg of \(^32\)P lyase were digested with 2 × 10 \(\mu\)g of 1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin; >80% of \(^{32}\)P was released as \(^{32}\)P peptides soluble in 3% perchloric acid. A portion of the digest was brought to 0.1% trifluoroacetic acid and applied at 0.5 ml/min to a Bondapak C18 column (300 × 3.9 mm) at \(t = 0\) (arrow); at 1 min, a gradient of acetonitrile (in 0.1% trifluoroacetic acid) was started, proceeding to 25% over 80 min, followed by 25-80% acetonitrile over the next 30 min. Fractions of 1 ml were collected, and \(^{32}\)P counts were quantitated by Cerenkov counting. Panel A, \(^{32}\)P-peptides from \(^{32}\)P-lyase phosphorylated by the S6 kinase; panel B, \(^{32}\)P-peptides from \(^{32}\)P-lyase phosphorylated by the cAMP-dependent protein kinase; panel C, equal \(^{32}\)P cpm from the major peak shown in A and B were mixed and coinjected.

**FIG. 9.** Phosphorylation of nuclear extracts by S6 kinase and/or P kinase. Nuclear extracts from H35 hepatoma cells were prepared by the procedure of Dignam et al. (29) using 0.42 m NaCl in the nuclear extraction step, with subsequent dialysis into their buffer D. Nuclear extracts (left, right, unheated; center, heated at 80°C) were then phosphorylated before or after a 5-min incubation at 80°C (to inactivate endogenous protein kinases) by endogenous kinases, added S6 kinase, and cAMP-dependent protein kinase catalytic subunit. S6 kinase and cAMP-protein kinase were added at equal S6 peptide phosphorylating activity. After incubation in the presence of 60 mM MOPS, pH 7.4, 10 mM MgCl\(_2\), 1 mM DTT, and 100 \(\mu\)M [\(\gamma\)-\(^{32}\)P]ATP at 30°C for 20 min, reactions were stopped by addition of SDS and subjected to SDS-PAGE. Autoradiography was carried out for 24 h (left and center panels) or 3 h (right panel, which is the same gel as in the left panel).
Purification of Rat Liver S6 Kinase

was anticipated on the basis of earlier reports that cycloheximide injection produced a large increase in hepatic S6 phosphorylation; we infer that the new peak of hepatic S6 kinase detected on the DEAE column after cycloheximide injection actually catalyzes the cycloheximide-induced S6 phosphorylation (3, 30). Increased S6 phosphorylation and the concomitant appearance of an S6 kinase with very similar chromatographic and functional properties are also observed in insulin-treated hepatoma cells (9) and within second of two major DEAE peaks of S6 kinase activity (>736,000 units/ml) was dialyzed into buffer C minus DTT; aliquots of the enzyme were incubated in the presence of NEM at the concentrations indicated for 30 min at 30 °C. Treatment was terminated by the addition of DTT to 2 mM. Remaining enzyme activity was assayed with gelatin as its substrate (GS, A, final concentration 5 μg/ml), ATP citrate lyase (ATPCL, O, final concentration 90 μg/ml), 40 S ribosomal subunit (40 S/S6, ■, final concentration 40 μg/ml), and histone H2b (H2b, ○, final concentration 220 mg/ml). Enzyme reactions were initiated by the addition of 100 μM [γ-32P]ATP and terminated after 15 min. SDS-quenched reactions were analyzed by SDS-PAGE in 7% (gelatin synthase, lyase) or 18% (ribosomes, histone H2b) acrylamide gels. 32P content was quantitated by liquid scintillation counting of bands cut from dried gels.

The purified enzyme contains a dominant 70-kDa poly peptide that is certainly an S6 kinase; in some isolates, a minor 95-kDa polypeptide is also present, the latter contributing perhaps 5% of the total mass and overall 32P incorporation in autophosphorylation. The two polypeptides are not physically associated. The 70-kDa polypeptide is always present in fractions that exhibit S6 kinase activity and binds azido-ATP; the 95-kDa protein is not reactive with azido-ATP and has a 32P-peptide map entirely distinct from the 70-kDa polypeptide. The 95-kDa polypeptide is probably a contaminating protein that is structurally and functionally unrelated to the S6 kinase but which can serve as a substrate for the kinase in vitro. Its relevance as a "physiologic" substrate is moot. The 70-kDa polypeptide is usually visualized as a 70/68-kDa doublet; slightly smaller 32P-labeled polypeptides are also seen. These species may include proteolytic products derived from the 70-kDa polypeptide, post-translationally modified (e.g., phosphorylated) forms, and/or minor isozymic variants.

An idiosyncratic feature of the enzyme is an apparent requirement for EGTA in the initial extraction; this requirement is lost during purification. The present results suggest that the requirement for EGTA may arise from the sensitivity of the enzyme to inhibition by transition metals, probably mediated by one or more enzyme — SH groups critical for activity.

The properties of the rat liver S6 kinase may be compared with those of other purified S6 kinases reported previously. Eriksen and Maller (35) purified to near homogeneity the second of two major DEAE peaks of S6 kinase activity ("S6 kinase II") extracted from Xenopus eggs; this enzyme is a 92-kDa polypeptide that phosphorylates Kemptide slightly but was otherwise specific for S6 among the substrates examined. Antibodies to the purified Xenopus S6 kinase II (36) or to the recombinant protein kinase described by Jones et al. (37) are reactive with the S6 kinase activated in insulin-treated oocytes as well as the S6 kinase activated in chick embryo fibroblasts transformed by Rous sarcoma virus. Thus, avian S6 kinase has been extensively but incompletely purified and corresponds to a 65-kDa polypeptide (33). By contrast, exper-
items we have carried out in collaboration with E. Erikson and J. Maller indicate that these antisera immunoprecipitate neither the S6 kinase activity nor the $^{32}$P-autophosphorylated polypeptides associated with the rat liver S6 kinase as described in this report, under conditions wherein the Xenopus S6 kinase II is immunoprecipitated. Moreover, these antibodies do not detect the rat liver S6 kinase in an immunoblot reaction. Thus, the rat liver S6 kinase described in the present report is immunologically distinct from both the Xenopus and avian enzymes.

Tabarini et al. (38) reported purification of an S6 kinase from bovine liver which corresponds to a 67-kDa polypeptide. The similar $M_\text{r}$ and high activity toward 40 S S6 suggest that their enzyme and the rat liver S6 kinase are related. However, the bovine S6 kinase was purified from livers that had not undergone prior activation of S6 kinase activity. Moreover, although both enzymes are retained by Fast S columns, many other chromatographic properties differ; in addition, the bovine enzyme exhibits substantial cascin kinase activity (perhaps due to contaminating casein kinase II), a marked contrast to the rat liver S6 kinase. Further study will be required to establish the relationship between these two hepatic S6 kinases. Thomas and colleagues (39) have purified an S6 kinase from vanadate-treated 3T3 cells. This enzyme is a 70-kDa polypeptide whose S6 kinase activity can be extensively inactivated by treatment with protein phosphatases (2A $>>$ 1). Moreover, they have shown that when purified from $^{32}$P-labeled 3T3 cells stimulated by serum addition prior to extraction, the enzyme is recovered as a 70-kDa $^{32}$P-peptide. We have shown recently that our purified hepatic "cytochrome-a-activated" S6 kinase can be completely deac- tivated by treatment with highly purified protein phosphatase 2A. The similarities in chromatographic properties, $M_\text{r}$, and susceptibility to deactivation by phosphatase 2A all indicate that the murine S6 kinase of Thomas and co-workers (39) is most closely related to the rat liver enzyme described herein.

To compare the ability of S6 kinase, kinase A, and kinase C to phosphorylate the same protein, we chose to normalize these kinase activities using the synthetic peptide based on that the murine S6 kinase of Thomas and co-workers (39) is most closely related to the rat liver enzyme described herein. Although isolated by virtue of its S6 protein kinase activity, these kinase activities using the synthetic peptide corresponded to the multiple sites of phosphorylation (4). Thus, to achieve comparable peptide phosphorylation, it was inferred that an "access" of kinase A or kinase C catalytic activity would be required in comparison to S6 kinase. This reasoning may not in fact be correct inasmuch as all three kinases appear to phosphorylate the synthetic S6 peptide almost equally well per mol of kinase catalytic subunit (estimated from the protein mass); moreover, both kinases A and C (especially the latter) phosphorylate the synthetic S6 peptide much more efficiently than they phosphorylate S6 in the 40 S subunit, whereas the reverse is true for the S6 kinase. Nevertheless, the comparison shown in Table III and Fig. 9 demonstrates clearly that the purified S6 kinase, although isolated by virtue of its S6 protein kinase activity, can phosphorylate at a substantial rate, a wider range of protein substrates than anticipated from earlier reports and studies employing less highly purified enzyme (33, 39). The parallel inactivation of kinase activity toward each of these protein substrates by treatment of the enzyme with heat or NEPl indicates that the phosphorylation of each of these protein substrates is catalyzed by the same enzyme, previously considered to be an S6 kinase.

The phosphorylation of glycogen synthase by S6 kinase, e.g. is quite rapid, and further characterization of this reaction is underway. The ability of the S6 kinase to phosphorylate ATP citrate lyase is of particular interest; insulin, EGF, and glucagon each stimulate the phosphorylation of ATP citrate lyase in intact hepatocytes at precisely the same serine resi- due. This residue can be phosphorylated selectively by the CAMP-dependent protein kinase in vitro, and it is likely that the cAMP-dependent protein kinase mediates the stimulation of ATP citrate lyase phosphorylation observed in response to glucagon treatment of the intact cell (23). The identities of the ATP citrate lyase kinase(s) responsive to insulin and EGF have, by contrast, remained elusive. We have argued previously that the insulin (and EGF)-regulated phosphorylation of ATP citrate lyase in the intact cell, although probably irrelevant per se to the cellular program of hormone action, is important as a reflection of the activation of insulin/EGF-responsive serine/threonine-specific protein kinases (15). Inasmuch as the purified rat liver S6 kinase described herein probably corresponds to the enzyme activated by insulin and EGF in the intact hepatocyte, the ability of this kinase to phosphorylate ATP citrate lyase selectively on the same serine residue phosphorylated in response to insulin (and glucagon) in vivo, at a rate 15-fold greater than catalyzed by kinase A in vitro, makes the S6 kinase a prime candidate for the insulin/EGF-activated ATP citrate lyase kinase.

It now remains to identify the physiologic substrates of this kinase in addition to S6. The ability of S6 kinase to phosphorylate a large number of nonhistone nuclear proteins extracted from H4 cells at a vigorous rate in comparison to comparable catalytic concentrations of kinase A and kinase C, two acknowledged multifunctional kinases, raises the possibility that the ubiquitous growth factor-activated S6 protein kinase may play a wider role in the cellular program initiated by insulin and growth factors than anticipated by its phos- phorylation of S6. It will be of interest to examine the ability of S6 kinase to phosphorylate specific transcriptional regulatory factors as well as components of the translational appa- ratus other than the 40 S ribosomal subunit.

Finally, the availability of substantial quantities of the mammalian S6 kinase should facilitate studies directed at the mechanism of its activation by hormones. Our preliminary observations with the rat liver enzyme coincide broadly with those of Maller and Thomas, in that we find extensive deac- tivation by treatment of the kinase with protein phosphatase 2A. The role of enzyme phosphorylation in situ and the identity of the relevant S6 kinase-kinases remain to be established.

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REFERENCES
1. Wool, I. G. (1979) Annu. Rev. Biochem. 48, 719–754
2. Gressner, A. M., and Wool, I. G. (1974) J. Biol. Chem. 249, 6917–6922
3. Gressner, A. M., and Wool, I. G. (1974) Biochem. Biophys. Res. Commun. 60, 1482–1490
4. Krieg, J., Hofsteenge, J., and Thomas, G. (1988) J. Biol. Chem. 263, 11473–11477
5. Gordon, J., Nielsen, P. J., Manchester, K. L., Towbin, H., Jimenez de Asua, L., and Thomas, G. (1982) Curr. Top. Cell. Regul. 21, 89–99
6. Smith, C. J., Rubin, C. S., and Rosen, O. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2641–2645
7. Novak-Hofer, I., and Thomas, G. (1984) J. Biol. Chem. 259, 5955–6000
8. Tabarini, D., Heinrich, J., and Rosen, O. M. (1985) Proc. Natl.
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9. Nemenoff, R. A., Gunsalus, J. R., and Avruch, J. (1986) Arch. Biochem. Biophys. 245, 196–203
10. Cobb, M. H. (1986) J. Biol. Chem. 261, 12994–12999
11. Pelech, S. L., and Krebs, E. G. (1987) J. Biol. Chem. 262, 11596–11606
12. Maller, J. L., Pike, L. J., Freidenberg, G. R., Cordera, R., Stith, B. J., Olefsky, J. M., and Krebs, E. G. (1986) Nature 320, 459–461
13. Blenis, J., and Erikson, R. L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1733–1737
14. Nemenoff, R. A., Price, D. J., Mendelsohn, M. J., Carter, E. A., and Avruch, J. (1988) J. Biol. Chem. 263, 19455–19460
15. Pierce, M. P., Palmer, J. L., Keutmann, H. T., Hall, T. A., and Avruch, J. (1982) J. Biol. Chem. 257, 10681–10686
16. Chan, Y.-L., and Wool, I. G. (1988) J. Biol. Chem. 263, 2891–2896
17. Scott, J. D., Fischer, E. H., Takio, K., Demaille, J. G., and Krebs, E. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5732–5736
18. Grove, J. R., Price, D. J., Goodman, H. M., and Avruch, J. (1987) Science 238, 530–533
19. Kuenzel, E. A., and Krebs, E. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 737–741
20. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
21. Laemmli, U. K. (1970) 227, 680–685
22. Merrill, C. R., Sutcliffe, J. B., and VanKemenen, M. L. (1980) Anal. Biochem. 105, 361–365
23. Pierce, M. W., Palmer, J. L., Keutmann, H. T., and Avruch, J. (1981) J. Biol. Chem. 256, 9867–9870
24. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102–1106
25. Bohn, E. L., Strickland, W. H., Strickland, M., Thwais, B. H., Van Der Westhuizen, D. R., and Von Holtz, C. (1973) FEBS Lett. 34, 217–221
26. Sloboda, R. D., Dentler, W. L., and Rosenbaum, J. L. (1976) Biochemistry 15, 4497–4505
27. Tonks, N. K., and Cohen, P. (1984) Eur. J. Biochem. 145, 65–70
28. Blackshear, P. J., Nemenoff, R. A., and Avruch, J. (1982) Biochem. J. 204, 817–824
29. Dignam, J. D., Leibowitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1445–1453
30. Nielsen, P. J., Manchester, K. L., Towbin, H., Gordon, J., and Thomas, G. (1982) J. Biol. Chem. 257, 12316–12321
31. Kaelin, M., and Korak, I. (1978) Eur. J. Biochem. 90, 463–469
32. Lastick, S. M., and Melkonian, E. H. (1980) Biochem. Biophys. Res. Commun. 95, 917–923
33. Blenis, J., Kuo, C. J., and Erikson, R. L. (1987) J. Biol. Chem. 262, 14373–14376
34. Jeno, P., Ballou, L. M., Novak, I., and Thomas, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 406–410
35. Erikson, E., and Maller, J. L. (1986) J. Biol. Chem. 261, 350–355
36. Erikson, E., Stefanovic, D., Blenis, J., Erikson, R. L., and Maller, J. L. (1987) Mol. Cell. Biol. 7, 3147–3155
37. Jones, S. W., Erikson, E., Blenis, J., Maller, J. L., and Erikson, R. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3377–3381
38. Tabarini, D., Garcia de Herreros, A., Heinrich, J., and Rosen, O. M. (1987) Biochem. Biophys. Res. Commun. 144, 891–899
39. Jeno, P., Jaggi, N., Luther, H., Siegmann, M., and Thomas, G. (1989) J. Biol. Chem. 264, 1293–1296
40. Ballou, L. M., Siegmann, M., and Thomas, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7154–7158