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 carboxyl-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 epidermal 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 carboxyl 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 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 vandate 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 (H4IEC) (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 \([\gamma^3\text{P}]\text{ATP}\), and is the only polypeptide reactive with azido-\([\gamma^3\text{P}]\text{ATP}\); 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 KP, 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 disopropyl 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 800 ml of settled DEAE-Phacel (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

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\(^1\) The abbreviations used are: EGFR, epidermal growth factor; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; PAGK, polyacrylamide gel electrophoresis; kinase A, cAMP-dependent protein kinase; kinase C, protein kinase C; DTT, dithiothreitol; EGTA, ethylenebis(oxyethylenenitrilo)tetaacetic acid; HPLC, high pressure liquid chromatography; NEM, N-ethylmaleimide.
a column (5 x 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 (0.05-8 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 (400 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 M NaCl, 0.5% Brij 35, 2 mM β-mercaptoethanol, and 10% glycerol) to a conductivity of <3 mmho, and mixed with 200-250 ml of SP-Sephadex C-50 (Pharmacia) equilibrated with buffer B. After 30 min with occasional agitation, the slurry was transferred into a column (5 x 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 bimodal 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 bimodal 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-glycerophosphate, and 10% glycerol) to a conductivity of <2 mmho, and applied at 1.0 ml/min to a 25-ml (1.5 x 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 x 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-Lys-Arg-Gln-Glu-Lys-n-Ile-Ala-Lys-Arg-Arg-Leu-Ser-Arg-Leu-Ala-Arg-Leu-Thr-Ser-Lys-Ser-Gly-Gly-Ser-Gln-Lys, corresponds to the 32 carboxyterminal amino acids of rat liver ribosomal protein S6 (18). 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 eluted as a single peak between 0.17 and 0.31 M NaCl. 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 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/desorption elution from the Mono Q column. The first Mono Q eluate was diluted by the addition of 3 volumes of buffer C, 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 x 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 (29) and carbonic anhydrase, 29 kDa) are as indicated. S6 kinase and 70 kDa are shown in the closed and open triangles, respectively. This isolation has been carried out >10 times. Recovery of S6 kinase activity varies over a 2-fold range. The final isolates always contain the 68/70-kDa polypeptide as the sole or dominant species.

**Assay of S6 Kinase Activity**—The enzyme was assayed by phosphorylation of 40 S ribosomal subunit (16 A₂₆₀ units/ml) in the presence of [γ-³²P]ATP (0.1 mM) as described previously (9,14), with the addition of 5 mM heat-stable inhibitor of kinase A peptide (17, 18). Assays intended for accurate determination of recovery of enzyme activity during purification were analyzed by standard SDS-PAGE. Assays for routine monitoring of S6 kinase in column effluents during the first three steps (DEAE-Sephacryl, SP-Sephadex, heparin-Sephacryl) also employed 40 S subunits in the phosphorilation reactions, and incorporation was analyzed after separation of the reaction mixture on Pharmacia Phastsystem 10-15% gradient gels. Assays of the elution profiles of the later column steps and the assays employed for certain of the functional characterizations employed a synthetic peptide corresponding to the carboxyl-terminal 32 residues of rat liver S6 (16) which was substituted for 40 S ribosomes at a final concentration of 25 μM in the kinase reaction; these reactions were stopped by pipetting an aliquot onto P-81 filter paper squares that were processed according to the method of Kuenzel and Krebs (19). The S-300 fraction (sequence above) was synthesized on an Applied Biosystems AB430 automatic solid phase peptide synthesizer and purified by desalting followed by reverse-phase HPLC on a Vydac C18 preparative column with a Waters liquid chromatograph. Peptide composition and mass were determined by amino acid analysis, and the peptide was sequenced on an Applied Biosystems gas phase sequenator.

**Determination of Protein**—Routine protein assays during enzyme purification were carried out either by measurement of A₂₆₀ or by the method of Bradford (20) with γ-globulin as standard. Estimates of enzyme purification employed amino acid analysis for determination of protein (Table I); fractions taken at each step were precipitated in acid-washed glass centrifuge tubes by the addition of 85% acetone (v/v) at –20 °C and permitted to stand for 2 h at –20 °C. Precipitates were collected by centrifugation at –20 °C for 30 min at 20,000 × g, rinsed, dried, and hydrolyzed in 6 N HCl for 24 h at 110 °C in vacuo. Amino acid analysis was performed on a Beckman model 121MB analyzer.

**Autophosphorylation of S6 Kinase**—Aliquots of the fractions from the Mono Q and Sephacryl S-300 columns were autophosphorylated by incubation for 60 min at 30 °C with [γ-³²P]ATP (200 cpm/pmol). Reactions were stopped by the addition of 20% volume of 30% sucrose, 0.5 M EDTA, 2% SDS, 60 mg/ml pyronin Y. The samples were concentrated in an evacuated centrifuge when necessary, heated in a boiling water bath for 2 min, and analyzed by SDS-PAGE on 10-15% gradient gels according to the method of Laemmli (21). The dye front was cut off, and the gels were extensively washed in 40% methanol, 10% acetic acid to remove residual [γ-³²P]ATP. Silver staining was performed.

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![Fig. 1. Mono Q chromatography.](image-url)

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 and washed 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.
Purification of Rat Liver S6 Kinase

Activity of S6 kinase was determined by standard assay with 40 S subunits (see under "Experimental Procedures"). Protein was determined by amino acid analysis of acetone precipitates. The control extract was prepared from a single rat liver, whereas the cycloheximide extract was prepared from 50 rat livers.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity</th>
<th>Volume</th>
<th>Purification</th>
<th>Recovery</th>
<th>Specific activity</th>
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<tbody>
<tr>
<td>Extract, control</td>
<td>266</td>
<td>341</td>
<td>100</td>
<td>1</td>
<td>1.28</td>
</tr>
<tr>
<td>Extract, cycloheximide Rx</td>
<td>10,065</td>
<td>92,590</td>
<td>1,880</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sephasel</td>
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<td>126,140</td>
<td>600</td>
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<td>136</td>
</tr>
<tr>
<td>SP-Sephasdax</td>
<td>56.2</td>
<td>102,240</td>
<td>56.2</td>
<td>198</td>
<td>110</td>
</tr>
<tr>
<td>Heparin-Sephasdax</td>
<td>19.35</td>
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<td>50</td>
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<tr>
<td>Peptide affinity</td>
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<td>63,912</td>
<td>103</td>
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</tr>
<tr>
<td>Mono Q</td>
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<td>32</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>0.014</td>
<td>6,156</td>
<td>6.5</td>
<td>47,795</td>
<td>6.6</td>
</tr>
</tbody>
</table>

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.

by the method of Merrill et al. (22), and the dried gel was exposed to Kodak XAR film for autoradiography.

Determination of ATP Citrate Lyase Phosphorylation Site—ATP citrate lyase was purified from the livers of rats fed a high carbohydrate diet (23). Lyase (final concentration 20 mg/ml) was phosphorylated by the catalytic subunit of kinase A (7.3 µg/ml) or S6 kinase (6 µg/ml) in the presence of 60 mM MOPS, pH 7.4, 12 mM MgCl₂, 1 mM DTT, and 100 mM [γ-32P]ATP (5000 cpm/pmol) for 90 min at 30 °C in a final volume of 0.5 ml. The phosphorylation reaction was stopped by the addition of 0.25 ml of 150 mM ATP, 450 mM NaF, 150 mM KPi, pH 7.5, 4 mM sodium citrate, 10 mM DTT, and 0.3 mM coenzyme A, and incubation was continued for several min in order to discharge the 32P-histidine phosphoenzyme intermediate. The mixture was then desalted over a 1 X 25-cm Sephadex G-25 (coarse) column equilibrated with 10 mM NaF, pH 7.5, 1 mM EGTA, 1 mM DTT. The void volume was pooled, and a portion of the 32P-lyase (~1 mg) was subjected to trypsin digestion and C18 reverse-phase HPLC according to Pierce et al. (15). The single major 32P-phosphoprotein was 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, ~35 pmol of 8-azidoadenosine 5′-triphosphate [γ-32P] (6.7 Ci/mmol) (ICN) was added to ~20 µg 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 dried gel was exposed for autoradiography.

Partial Peptide Mapping—A aliquots of S6 kinase from the Mono Q column were autophosphorylated with [γ-32P]ATP as above and subjected to SDS-PAGE. Segments encompassing the 70- and 95-kDa 32P-polypeptide detected by autoradiography of the frozen gel were cut from frozen gels reequilibrated with Laemmli stacking gel buffer and subjected to partial digestion with Staphylococcus aureus V8 protease by coelectrophoresis into the stacking gel of a second SDS gel according to Cleveland et al. (24).

Materials—Histone subfractions were purified according to Bohm et al. (25). Microtubule-associated protein-2 was isolated as in Sloboda et al. (26). Protein phosphatase inhibitor-2 was purified from rabbit skeletal muscle according to Tonks et al. (27). Glycogen synthase I (rabbit skeletal muscle) was a gift from Tom Soderling, and Lee Witters provided acetyl-CoA carboxylase purified from rat liver. The catalytic subunit of the CAMP-dependent protein kinase was purchased from Sigma. Protein kinase C, prepared from rat brain, was purchased from Lipidex, Inc.

RESULTS

S6 kinase is purified from the livers of cycloheximide-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...
At the SP-Sephadex (step 2), heparin-Sepharose (step 3), and eluting, major DEAE peak was taken for further purification. The molecule 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 and slightly later Mono Q elution relative to the 70-kDa polypeptide, 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 promazine 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

![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.](image)

![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.](image)
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, 100 µM [γ-32P]ATP.
For effect of phospholipids, phosphatidyserine was 400 pg/ml, diolein was 40 µg/ml, and Ca²⁺ was 0.25 mM, final concentration.

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>100</td>
</tr>
<tr>
<td>cAMP, 10 µM</td>
<td>93.6</td>
</tr>
<tr>
<td>cGMP, 10 µM</td>
<td>104.3</td>
</tr>
<tr>
<td>Polylysine, 50 µg/ml</td>
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</tr>
<tr>
<td>Heparin, 50 µg/ml</td>
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</tr>
<tr>
<td>Protamine, 25 µg/ml</td>
<td>4.8</td>
</tr>
<tr>
<td>Polyglutamic acid, 50 µg/ml</td>
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</tr>
<tr>
<td>Diolein/phosphatidyserine</td>
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</tr>
<tr>
<td>Diolein/phosphatidyserine/Ca²⁺</td>
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<tr>
<td>CaCl₂, 0.25 mM</td>
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<tr>
<td>Ca²⁺/calcimodulin, 10 µg/ml</td>
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<tr>
<td>MnCl₂, 10 mM</td>
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<tr>
<td>CuSO₄, 10 mM</td>
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</tr>
<tr>
<td>CoCl₂, 10 mM</td>
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<td>Ammonium molybdate, 0.5 mM</td>
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<td>ZnSO₄, 10 mM</td>
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<tr>
<td>DTT, 10 mM</td>
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<tr>
<td>UTP, 1.67 mM</td>
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<td>AMP, 1.67 mM</td>
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</table>

FIG. 6. Mg²⁺/Mn²⁺ concentration dependence of S6 kinase.
S6 kinase was assayed using the S6 peptide (see under "Experimental Procedures") with increasing concentrations of MgCl₂ (O) or MnCl₂ (Δ) at 0.1 mM [γ-32P]ATP. The reaction contained 0.06 mM EGTA.

FIG. 7. Inhibition of S6 kinase by NaF and NaCl.
S6 kinase (20 units/ml) was assayed by standard S6 peptide assay in the presence of increasing levels of NaF ( ●) or NaCl ( □).
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 32P-labeled tryptic peptide isolated from lyase phosphorylated by either kinase A or the S6 kinase is subjected to automated Edman degradation, a burst of 32P 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 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 (29) does not determine 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 these latter two fractions to serve as a substrate for the added kinase was examined before and after a brief heat treatment to inactivate endogenous kinases. The striking observation is that S6 kinase phosphorylates a large number of polypeptides in the nuclear extract (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 32P-polypeptides generated by the endogenous nuclear protein kinase activity.

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.

DISCUSSION

The very large increase in hepatic S6 kinase activity observed 1 h after intraperitoneal injection of cycloheximide

![Fig. 8. C18 reverse-phase chromatography of tryptic digests of 32P-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 32P/subunit, respectively. After desalting on Sephadex G-25, aliquots of 1 mg of 32P lyase were digested with 2 × 10 μg of 1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin; >80% of 32P was released as 32P-peptides soluble in 3% perchloric acid. A portion of the digest was brought to 0.1% trifluoroacetic acid and applied at 0.6 ml/min to a μBondapak C18 column (300 x 3.9 mm) at t = 0 (arrow); at 1 min, a gradient of acetonitrile (in 0.1% trifluoroacetic acid) was started, progressing to 25% over 80 min, followed by 25–80% acetonitrile over the next 30 min. Fractions of 1 ml were collected, and 32P counts were quantitated by Cerenkov counting. Panel A, 32P-peptides from 32P-lyase phosphorylated by the S6 kinase; panel B, 32P-peptides from 32P-lyase phosphorylated by the cAMP-dependent protein kinase; panel C, equal 32P cpm from the major peak shown in A and B were mixed and coinkjeted.](image-url)
Enzyme has greatly impeded purification, and the liver of chromatographic properties to the dominant mitogen-stimulated S6 kinase of rat liver. The very low abundance of this enzyme we have purified corresponds in its functional and phosphorylation occurs in many tissues (except brain) after systemic cycloheximide injection is unknown; increased S6 phosphorylation when added directly to cultured cells (31, 32) is also observed in insulin-treated hepatoma cells (9) and within.

The properties of the rat liver S6 kinase may be compared with those of other purified S6 kinases reported previously. 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.

Each of the first four steps in the purification is indispensable for the success of the procedure; by contrast, the Mono Q and gel filtration steps serve primarily to concentrate and characterize the enzyme and can probably be replaced by a number of other steps, e.g. hydroxylapatite, blue Sepharose, and phosphocellulose, each of which gives comparatively modest purification and recovery in our hands. The use of ion exchange in the early steps has been discussed by several previous reports (14, 33, 34). The ability of the enzyme to bind sequentially to anion and cation exchange resins provides very substantial purification early in the isolation. Although the poorest overall purification is consistently observed at the heparin-Sepharose step, omission of this step has invariably resulted in instability of the enzyme and large losses at the latter steps. We infer that heparin-Sepharose allows separation of the kinase from an inactivating factor (protease or phosphatase) which is not effectively removed by the other steps. The most marked purification is obtained at the peptide affinity step. This outcome was surprising in view of the very basic character of the peptide and its ability to be phosphorylated quite well by protein kinases other than the S6 kinase. Nevertheless, the S6 kinase is retained quantitatively under conditions wherein >96% of the applied protein flows through, and NaCl gradient elution provides a consistent 30–100-fold purification with recovery of ≥70% of the activity applied. After this step, the enzyme activity is stable to storage at −20°C in 50% glycerol for several months, if first concentrated, e.g. by adsorption/elution to Mono Q.

The purified enzyme contains a dominant 70-kDa polypeptide 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.

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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-

**FIG. 10. Inactivation of S6 kinase by heat treatment or N-ethylmaleimide.** (A) S6 kinase (30 units/ml) was inactivated by incubation at increasing temperatures for 5 min in the presence of 0.1 M MOPS, pH 7.4, 20 mM MgCl2, and 2 mM DTT. The heat treatment was terminated by returning the tubes to an ice bath. (B) S6 kinase (30 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 glycogen synthase (GS, Δ, final concentration 8 μg/ml), ATP citrate lyase (ATPCL, O, final concentration 90 μg/ml), 40 S ribosomal subunit (40 S/S6, ●, final concentration 22 μg/ml), and histone H2b (H2b, ◦, final concentration 220 μg/ml). Enzyme reactions were initiated by addition of 100 μM [γ-32P]ATP and terminated after 15 min. SDS-quenched reactions were analyzed by SDS-PAGE in 7% (glycogen synthase, lyase) or 18% (ribosomes, histone H2b) acrylamide gels. 32P content was quantitated by liquid scintillation counting of bands cut from dried gels.
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 residue. 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 phosphorylation 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 apparatus 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 enzymes coincide broadly with those of Maller and Thomas, in that we find extensive deactivation 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**

Purification of Rat Liver S6 Kinase