Journal of Cellular Biochemistry 75:310–326 (1999)

Characterization of an Activated Ribosomal S6 Kinase Variant From Maturing Sea Star Oocytes: Association With Phosphatase 2A and Substrate Specificity

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Abstract Two ribosomal protein S6 kinases (i.e., pp52^{S6K} and pp70^{S6K}) of the p70 S6 kinase family were markedly activated during meiotic maturation of *Pisaster ochraceus* sea star oocytes. A rapid protocol was developed for the purification from the oocyte cytosol of pp52^{S6K} by ~50,000-fold with a specific enzyme activity of 1.6 μmol per min per mg. The purified enzyme apparently featured the N- and C-terminal regions of pp70^{S6K} as it immunoreacted with antibodies directed to peptides patterned after these amino acid sequences in mammalian pp70^{S6K}. pp52^{S6K} was inhibited by fluoride (IC_{50} ~60 mM), but was relatively insensitive to β-glycerolphosphate, EGTA, dithiothreitol, spermine, heparin, NaCl, and metal ions such as Mn^{2+}, Zn^{2+}, and Ca^{2+}. The consensus sequence for substrate phosphorylation was determined to be RXXSXR, which was partially distinct from mammalian p70^{S6K} in its requirement for an amino-terminal arginine. Phosphorylation of ribosomal protein S6 by p52^{S6K} occurred exclusively on serine on at least five tryptic peptides. Inhibition of sea star p52^{S6K} phosphotransferase activity after treatment with protein serine/threonine phosphatases confirmed that p52^{S6K} was still regulated by phosphorylation. The sea star S6 kinase was purified to near homogeneity with the regulatory and catalytic subunits of protein-serine phosphatase 2A and the heat shock protein 60. The association of an S6 kinase with phosphatase 2A was confirmed by coimmunoprecipitation of S6 kinase activity with phosphatase 2A-specific antibodies. The purified S6 kinase and the sea star oocyte system will be useful for analysis of upstream and downstream signaling events that lead to phosphorylation of the S6 protein and other targets. J. Cell. Biochem. 75:310–326, 1999.

Key words: p70 S6 kinase; p52 S6 kinase; sea star; oocyte maturation

Hyperphosphorylation of the 40S ribosomal protein S6 is one of the most dramatic and consistent responses of many cell types to a variety of mitogenic stimuli. The enzyme responsible for phosphorylation of the S6 protein, the mitogen-activated 70-kDa S6 kinase (pp70^{S6K}) plays an important role in the progression of cells from G1 to S phase of the cell cycle [Chung et al., 1992]. Two isoforms of pp70^{S6K}, the 65-kDa cytosolic form (α2) and the 80-kDa nuclear form (α1) are derived from alternatively spliced products of a single gene [Reinhard et al., 1992]. The mechanism of mitogenic activation of pp70^{S6K} is incompletely understood, but appears to be via a complex series of serine/threonine phosphorylation events by more than one upstream pp70^{S6K} kinase and by interaction with the GTP-binding Rho family proteins Rac1 and Cdc42 [Price et al., 1990;

Abbreviations used: p70/52^{S6K}, 70 kDa S6 protein kinase; PP2A, protein phosphatase 2A; PAP, potato acid phosphatase; MAP kinase, mitogen-activated protein kinase; PKC, protein kinase C; PKB, protein kinase B; TOR, target of rapamycin; PH domain, pleckstrin homology domain; PI 3-kinase, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol-3,4-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PDK1, PIP_3-dependent protein kinase 1; Wm, wortmannin; MOPS, 4-morpholine propanesulfonic acid; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBS, Tris-buffered saline; TTBS, TBS containing 0.05% Tween 20; HTP, hydroxylapatite; FPLC, fast protein liquid chromatography.

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Received 12 February 1999; Accepted 26 April 1999

Chou and Blenis, 1996]. These phosphorylation events result in an apparent increase in the molecular masses of these S6 kinase isforms by approximately 5 kDa on SDS-PAGE gels.

pp70^{S6K} is basally phosphorylated in all types of quiescent cells, and mitogenic stimulation induces increased phosphorylation of the kinase on multiple sites leading to activation of its phosphotransferase activity. Four of the mitogen-induced sites (Ser-411, Ser-418, Thr-421, and Ser-424) lie in the carboxyl terminus within a pseudosubstrate/auto-inhibitory domain [Ferrari et al., 1992]. Deletion of this region, or mutation of these residues to acidic amino acids does not lead to constitutive activation of the kinase, supporting the notion that other phosphorylation events and interactions are required for full activation. Although these mitogen-induced sites contain recognition determinants for proline-directed kinases, MAP kinase fails to activate pp70^{S6K} after phosphorylation of these sites in vitro [Mukhopadhyay et al., 1992], and it is now well established that pp70^{S6K} activation does not lie on the Ras⌒Raf⌒Mek⌒MAP kinase pathway [Ballou et al., 1991]. A growing body of evidence has implicated protein kinase C-ζ(PKC-ζ), phosphatidylinositol 3-kinase (PI 3-kinase), 3-phosphoinositide-dependent kinase, (PDK-1), and protein kinase B (PKB) as upstream signaling molecules in pp70^{S6K} activation in insulin-, PDGF-, EGF-, and IL-2-treated cells [Chou and Blenis, 1995].

The immunosuppressant drug rapamycin, a bacterial macrolide, has become one of the most important and useful tools for dissecting the pathway leading to pp70^{S6K} activation and its regulation. Rapamycin blocks activation of pp70^{S6K} by all known mitogens by preventing the phosphorylation of a specific subset of sites including Thr-229, Thr-389, Ser-404, and Ser-411 [Ferrari et al., 1993]. Except for Ser-411, these sites are flanked by large, aromatic residues, and the principal inhibitory site has been identified as Thr-389 in the linker region coupling the catalytic and auto-inhibitory domains [Pearson et al., 1995]. Mutation of Thr-389 to an acidic amino acid confers high basal activity and rapamycin resistance on the kinase [Pearson et al., 1995].

Rapamycin has been shown to significantly inhibit translation of a select group of genes, including those encoding ribosomal proteins (S3, S6, S14, and S24), translation elongation factors (eEF1A and eEF2), and a secreted peptide growth factor, insulin-like growth factor II (IGF-II) [Terada et al., 1994; Neilsen et al., 1995; Jeffries and Thomas, 1994]. The inhibition appears to be due to structural elements in the 5′ untranslated region (UTR) of certain genes, and may reflect the specific nature of p70^{S6K} inhibition [Jeffries et al., 1994]. Rapamycin does not directly interact with pp70^{S6K}, but binds to FK506-binding protein 12 (FKBP12) and the complex interacts with the target of rapamycin (TOR, also known as FRAP) that acts upstream of pp70^{S6K} [Sabatini et al., 1994]. FRAP is structurally related to the catalytic subunit of PI 3-kinase, but it is thought to function as a protein kinase [Hunter, 1995]. It has recently been shown that PKB (also known as Akt or Rac kinase) can be activated by interaction of its pleckstrin homology (PH) domain with phosphatidylinositol-3,4-bisphosphate (PIP_2) [Franke et al., 1997] generated by the action of PI 3-kinase. Further, PIP_2 activates PDK1, which phosphorylates PKB at Thr-308, increasing its activity 30-fold [Alessi et al., 1997] and Thr-229 of pp70^{S6}. Agents such as wortmannin and LY294002 that inhibit PI 3-kinase activation also block activation of PKB and pp70^{S6K} [King et al., 1997; Cheatham et al., 1994], providing a framework to identify other components of this signaling pathway.

Several studies have recently reported a dissociation of pp70^{S6K} from the known components of the PI 3-kinase pathway. Scheid et al. [1996] found that PI 3-kinase but not pp70^{S6K} is required for cytokine-regulated hemopoetic cell survival, and Dudek et al. [1997] showed that PKB, but not pp70^{S6K} mediates growth-factor induced neuronal cell survival. Additionally, pp70^{S6K} can be activated in a PI 3-kinase-independent manner by phorbol ester treatment, presumably via PKC [Chung et al., 1994]. Recently, phorbol-insensitive PKC-ζ has also been reported to phosphorylate and activate pp70^{S6K} [Romanelli et al., 1999].

The activation of S6 kinases has been previously described during meiotic maturation of frog and sea star oocytes [Meijer et al., 1987; Pelech et al., 1987; Cicirelli et al., 1998]. In the present study, we have purified an ~52 kDa echinoderm homologue of pp70^{S6K} to near homogeneity, and have characterized its enzyme activity with respect to ion dependence, phos-

phorylating activity against the 40S ribosomal proteins and a panel of S6 peptide analogs, and the regulation of the kinase by phosphorylation. We have provided evidence that pp52^{S6K} may interact in vivo with other proteins in a multi-subunit complex in the sea star oocyte.

MATERIALS AND METHODS

Experimental Procedures

Pisaster ochraceus were collected from beaches in the Vancouver area. β-glycerol phosphate, EGTA, EDTA, MOPS, β-methylaspartic acid, sodium orthovanadate, ATP, methylsulfonyl fluoride, aprotinin, leupeptin, benzamidine, dithiothreitol (DTT), Coomassie brilliant blue, $_L$-tyrosine agarose, poly-$_L$-lysine agarose, heparin agarose, Q Sepharose, $_L$-arginine agarose and the peptide inhibitor of protein kinase A (PKI) were bought from Sigma. P-81 phosphocellulose filter paper was from Whatman. [γ-^{32}P]ATP, Tris-HCl and glycine were from ICN. Brij 35, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-indolyl phosphate (BCIP), hydroxylapatite (HTP), β-mercaptoethanol, and affinity-purified goat anti-rabbit IgG alkaline phosphatase conjugates were purchased from Bio-Rad. Bromophenol blue was from Schwarz-Mann Biotech. Temed, ammonium sulfate, silver nitrate, and sodium chloride were from Fisher Scientific. The S6 peptides were synthesized at the Biomedical Research Centre, University of British Columbia. The 40S ribosomal subunits were prepared from rat liver by a procedure modified from that of Kreig et al. [1988]. Molecular weight standards for gel electrophoresis and gel filtration were prepared in our laboratory by a procedure modified from that of Griffith (1972). The S6K (C-18) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against the following peptide sequences produced in our laboratory were affinity-purified on peptide-agarose columns: S6K-III, AMIVRNAKDTAHTKAERNILEEVKHPGGC; S6K-CT, CYKKQAFPMISKRPEHLRM; S6K-PNT, MAGVFDIDLDQPEDAC. These antibodies are commercially available from Upstate Biotechnology Inc. (Lake Placid, NY). HSP60 antibody was bought from StressGen Biotechnology (Victoria, BC). Protein phosphate 2A catalytic subunit antibody was the kind gift of Dr. David Brautigan (University of Virginia). Potassium dichromate, glycerol, and all other routine buffer chemicals were from BDH.

Preparation of Mature Sea Star Oocyte Cytosol

Oocytes that were arrested in the G2/M border prior to mitosis were isolated from sea stars as described previously [Pelech et al., 1987]. The immature oocytes were induced to undergo maturation by the addition of the hormone 1-methyladenine for ~2 h at 14°C. At the end of this period over 90% of the oocytes had undergone maturation as judged by the onset of germinal vesicle breakdown (GVBD). Subsequently, a 35% oocyte homogenate was prepared in chilled homogenization Buffer A (50 mM β-glycerol phosphate, 20 mM MOPS, 2 mM EDTA, 5 mM EGTA, 1 mM sodium vanadate, 0.25 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.5 mM β-methyl aspartic acid, pH 7.2) by 2 × 30 sec bursts at the 18,000 setting using a Brinkmann 3000 Polytron Homogenizer. The homogenate was centrifuged at 12,000*g* for 10 min and the supernatant subsequently centrifuged at 100,000*g* for 30 min. The resulting cytosolic (supernatant) fraction was stable for over 1 year when stored at −70°C and the frozen extract was used for all subsequent experiments.

Purification of the Ribosomal S6 Protein Kinase

Cytosol (270 ml, ~15 mg/ml protein) was thawed and diluted 1:1 with chilled 1.6 M $(NH_4)_2SO_4$ in Buffer B (50 mM Tris, 0.5 mM EDTA, 0.1 mM EGTA, 0.25 mM DTT, 1 mM β-methylaspartic acid, pH 7.2). The slurry was gently stirred over ice for 20 min, then centrifuged at 10,000*g* for 10 min in a pre-cooled Beckman rotor. The supernatant was applied at 0.5 ml/min to two 25-ml $_L$-tyrosine agarose columns equilibrated in 0.8 M $(NH_4)_2SO_4$. The columns were washed with 50 ml of 0.8 M $(NH_4)_2SO_4$ and the proteins were eluted with a 400 ml linear 0.8–0.0 M $(NH_4)_2SO_4$ gradient at a flow rate of 1 ml/min. Five ml fractions were collected and assayed for phosphotransferase activity towards a synthetic peptide substrate (AKRRRLSSLRAGGRR) modeled after the C-terminus of S6. The peak activity fractions were pooled, diluted 1:10 with Buffer B and applied at 0.5 ml/min to one freshly poured 25-ml hydroxylapatite (HTP) column equilibrated in Buffer B. The loaded HTP column was washed with 50 ml of Buffer B, and eluted with a 400 ml linear 0.0–0.35 M K_2HPO_4/KH_2PO_4 in Buffer B gradient at a flow rate of 1 ml/min. Five ml fractions were collected, and assayed for phos-

photransferase activity. The peak activity fractions were then pooled and frozen at −70°C for later use. To complete one purification, eight of the pooled HTP fractions (i.e., 16 $_L$-tyrosine agarose elutions) were combined for the following steps which were completed over two days. The fractions were thawed slowly over ice, diluted 1:4 with Buffer B and applied to a 25-ml heparin agarose (used as a reverse column as S6 kinase does not bind to this resin) and a 25-ml poly-$_L$-lysine agarose column linked in series. The S6 kinase was eluted from the poly-$_L$-lysine column with a 400 ml linear 0.0–1.0 M NaCl in Buffer B gradient at a flow rate of 1 ml/min. Five ml fractions were collected and fractions containing S6 kinase phosphotransferase activity were then pooled, diluted 1:3 with Buffer B and applied to a 25-ml Q Sepharose anion exchange column equilibrated in Buffer B. The column was washed with 50 ml Buffer B, and the S6 kinase activity was eluted with a 300 ml linear gradient of 0–0.8 M NaCl in Buffer B at 1 ml/min. Four ml fractions were collected, peak phosphotransferase activity fractions were pooled, diluted 1:3 with Buffer B and loaded on to a 10-ml $_L$-arginine agarose column equilibrated in Buffer B. The column was eluted with a 120 ml linear gradient of 0–0.8 M NaCl in Buffer B, and two ml fractions were collected. The fractions were assayed for kinase activity, peak fractions were pooled, diluted 1:3 with Buffer B, and applied to a 1-ml MonoQ column. The column was eluted with a 20 ml linear gradient of 0–0.8 M NaCl in Buffer B, and 250 µl fractions were collected. The fractions were assayed for S6 kinase activity, pooled, and frozen at −70°C for future use.

Kinase Activity, Autophosphorylation, and Protein Assays

Ribosomal protein S6 kinase phosphotransferase activity was assayed by a procedure described previously [Sanghera et al., 1992]. Briefly, 5 µl of extract was assayed in a final volume of 25 µl containing 0.40 mg/ml of the S6 peptide in Assay Dilution Buffer (ADB: 5 mM $MgCl_2$, 12 mM Mops, pH 7.2) and 50 µM [γ-^{32}P]ATP. The reaction was incubated for 5 min at 30°C, then 20 µl were removed and spotted onto a 1.5 cm^2 piece of Whatman P-81 phosphocellulose paper. The papers were washed extensively in 1% (w/v) H_3PO_4, transferred into 6-ml plastic vials containing 0.5 ml of Ecolume (ICN) scintillation fluid, and the incorporated radioactivity was measured in a Wallac (LKB) scintillation counter. Autophosphorylation of the S6 kinase was performed in a final volume of 50 µl containing 5 mM $MgCl_2$, 12 mM Mops, pH 7.2, and 50 µM [γ-^{32}P]ATP. The reaction was incubated for 30 min at 30°C, and then terminated by the addition of SDS sample buffer (125 mM-Tris-HCl, pH 6.8, 4% SDS, 0.01% Bromophenol Blue, 10% β-mercaptoethanol, and 20% glycerol). Protein was determined by the method of Bradford [1976] using bovine serum albumin as a standard.

Electrophoresis, Silver Staining, and Immunoblotting

SDS/PAGE was performed on 1.5 mm thick gels, with acrylamide at 11% (w/v) in the separating gel and 4% (w/v) in the stacking gel, in the buffer system described by Laemmli [1970]. Samples were boiled for 5 min in the presence of SDS sample buffer and electrophoresed for ~17 h at 10 mA. The separating gels were silver-stained by the method of Merril et al. [1981] or for autoradiography, gels were exposed to Dupont reflection film at room temperature. For immunoblotting, SDS/PAGE gels were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 5 min, sandwiched with a nitrocellulose membrane and transferred at 300 mA for 3 h. The nitrocellulose membrane was stained for proteins with Ponceau S, and subsequently blocked for 2 h at room temperature in Tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 5% skim milk. The membrane was washed 3 × 5 min with TBS containing 0.05% Tween 20 (TTBS) before incubation with p70^{S6K} specific antibodies (in TTBS with 0.05% sodium azide 1:500 dilution) overnight at room temperature. The next day, the membrane was washed extensively (5 × 5 min) with TTBS before incubation with the secondary antibody (goat anti-rabbit IgG coupled to alkaline phosphatase in TTBS with 0.05% sodium azide) for 2 h at room temperature. The membrane was washed 2 × 5 min with TTBS then briefly with alkaline phosphatase buffer (AP: 0.1 M $NaHCO_3$ 10 mM $MgCl_2$, pH 9.8) before incubation with BCIP/NBT color development solution (3% NBT in 70% DMF and 1.5% BCIP in 100% DMF in 100 ml AP buffer). The color reaction was terminated by successive washes in distilled water.

Protein Micro-Sequencing, Alignment, and Identification of Sequenced Fragments

Approximately 2.1 ml of purified MonoQ pp52^{S6K} were concentrated by centrifugation in a Centricon 10 tube to a final volume of ~500 µl (~2 h at 4,000 rpm). This was further centrifuged in a microfuge Centricon 10 tube to a final volume of ~150 µl (~15 min at 12,000 rpm). Fifty µl of SDS sample buffer were added, the sample was boiled 5 min and electrophoresed on 11% PAGE. A CD Immobilon membrane was pre-equilibrated in methanol for 15 min followed by equilibration in transfer buffer for 15 min. The proteins were transferred in transfer buffer containing 10% methanol for 3 h at 300 mA, and then for a further hour at 200 mA. The CD membrane was stained with 10 ml of quick stain solution A for 1 min, and then with 25 ml of quick stain solution B for 3 min. The membrane was washed for 1 min in distilled water, and the protein bands were excised, cut into small pieces and placed in 500 µl Eppendorf tubes containing 20 µl of digestion buffer (2.0 M acetic acid containing 0.25 µg pepsin/20 µl). The membranes were incubated for 3 h at 37°C, an additional 0.25 µg pepsin were added, and the samples were incubated for a further 3 h. The proteins were then cleaved in tryptic cleavage buffer (0.1 M Tris, 1 M NaCl, 2 mM $CaCl_2$, 5% acetonitrile (ACN) pH 8.2 containing 0.25 µg bovine trypsin tosylphenylalanylchloromethane (TPCK) treated XII (Sigma)/20 µl) for 5 h at 37°C. After 5 h, 0.25 µg trypsin in 20 µl cleavage buffer containing 2 M urea were added, and the samples were incubated a further 15 h at 37°C. The supernatant was removed, the samples were washed with 20 µl 1% TFA, which was pooled with the supernatant and injected directly onto HPLC. Peaks were collected directly from the HPLC elution, and were analyzed by mass and sequenced in the laboratory of Dr. Ruedi Aebersold (University of Washington, Seattle).

Two-Dimensional Phosphopeptide Mapping

Two-dimensional tryptic-phosphopeptide map analysis of the autophosphorylated S6 kinase was performed by excision of the radio-labelled pp52^{S6K} band from SDS/PAGE gels, digestion of the band with TPCK-treated trypsin in 1 ml of 50 mM NH_4HCO_3, pH 8.0 at 37°C for 24 h. The samples were then dried under vacuum (Speed Vac), washed with 4 × 200 µl of water, re-dried, and then resuspended in 5 µl of electrophoresis buffer (1% NH_4HCO_3, pH8.9). The sample was spotted in the center of a cellulose t.l.c. plate 1.5 cm from the bottom. Electrophoresis was performed in the long dimension at 600 V for 40 min. After drying, the plate was chromatographed in the second dimension in butanol/pyridine/acetic acid/water (13:10:2:8 by volume). The plate was dried overnight and exposed to X-ray film.

Phosphoamino Acid Analysis

The purified pp52^{S6K} was autophosphorylated (or incubated with purified 40S ribosomes), electrophoresed on SDS/PAGE (as described above), and transferred to PVDF membrane for 3 h at 300 mA. The radiolabelled pp52^{S6K} band (or ribosomal protein S6 band) was excised from the membrane and digested in 300 µl of constant-boiling HCl at 105°C for 1 h. The acid-hydrolyzed sample was dried under vacuum (Speed Vac), washed with water, re-dried, and then resuspended in 5 µl of electrophoresis buffer containing pyridine/acetic acid/water (1:10:189 by volume). The sample was spotted on to a cellulose t.l.c. plate next to phosphoserine, phosphothreonine and phosphotyrosine standards (1 mg/ml each), and the plate was electrophoresed for 45–60 min at 1,000 V with cooling. The plate was dried, sprayed with ninhydrin solution (1%) and baked in a 90°C oven for 10 min. The plate was then exposed to X-ray film.

Substrate Specificity of pp52 S6 Kinase

Synthetic peptides were patterned after the C-terminal sequence in the S6 protein known to be phosphorylated by p70^{S6K} [Bandi et al., 1993]. The peptides were synthesized on an A.B.I. 430A peptide synthesizer and cleaved from the resin by hydrofluoric acid. Purity of the peptides was demonstrated by reverse-phase HPLC, and the identity confirmed by ion spray mass spectrophotometry analysis (model API-III). Due to the small size of the peptides and the high purity as confirmed by reverse-phase HPLC, they did not require further purification after lyophilization. The peptides were carefully dried before weighing on an analytical balance. In the experiments shown here, the peptides were solubilized as stock solutions of 2.0 mM in ADB, and serial dilutions from 1 mM to 2 µM final peptide concentrations in a final

volume of 25 µl were assayed in the presence of purified kinase and 50 µM [γ-^{32}P]ATP. For each peptide, K_m was determined using the Michaelis-Menton rate equation, and V_{max} was estimated using Lineweaver-Burke double-reciprocal plots of dose-response data.

Inactivation of pp52 S6 Kinase by Phosphatase Treatment

Purified protein phosphatase 2A (PP2A) was the generous gift of Drs. David Brautigan and Jian Chen (University of Virginia, Charlottesville). The enzyme contained about 1.4 units of activity per ml (1 unit will release 1 nmol of P_i per min from phosphate-labelled phosphorylase). Potato acid phosphatase (PAP) was prepared in our laboratory as active enzyme linked to protein A-Sepharose beads. The purified pp52^{S6K} was pre-incubated in the presence of active PP2A or PAP. The PP2A reaction was terminated by the addition of okadaic acid, while the PAP reaction was terminated by centrifugation of the beads and removal of the supernatant for assay. Phosphotransferase activity of the dephosphorylated purified pp52^{S6K} was assayed with the S6 peptide substrate as described above.

Immunoprecipitation of S6 Phosphotransferase Activity by Anti-PP2A Antibody

One ml of partially purified S6 kinase (tyrosine agarose/HTP/polylysine/Q Sepharose extract was pre-cleared with 100 µl of 1:1 slurry of protein A Sepharose beads for 15 min at 4°C. The beads were pelleted for 2 min at 12,000 in a microfuge and the supernatant was incubated with 10 µl of anti-PP2A A subunit antibody overnight at 4°C. Forty µl of 1:1 slurry protein A Sepharose beads were added for 1.5 h at 4°C to precipitate the antibody, and the samples were washed once with NEFT buffer (1.0 M NaCl, 5 mM EDTA, 50 mM Tris HCl, 50 mM NaF pH 7.4) containing 6% NP40, once with NEFT buffer and once with Buffer B. To the precipitated proteins were added 30 µl ADB, 4 µl purified 40S ribosomes and 50 µM [γ-^{32}P]ATP, and the samples were incubated at 30°C for 35 min. The reaction was terminated by addition of 40 µl SDS sample buffer, the samples were boiled for 5 min and electrophoresed on a 12.5 % PAGE gel. The gel was transferred to a nitrocellulose membrane and exposed to film overnight.

RESULTS

Fractionation and Immunodetection of Maturation-Activated 40S Ribosomal Protein S6 Kinases

We have previous reported increased 40S ribosomal protein S6 phosphotransferase activity during the meiotic maturation of sea star oocytes near the time of germinal vesicle breakdown (GVBD) [Meijer et al., 1987; Pelech et al., 1987]. When a synthetic peptide patterned after the C-terminal phosphorylation sites in S6 was used as a substrate for S6 kinases that were resolved by MonoQ chromatography, three distinct peaks of maturation-stimulated phosphotransferase activity were detected (Fig. 1A). Immunoblotting studies on the MonoQ fractions with an antibody that recognizes the N-terminus of mammalian pp70 S6 kinase demonstrated the presence of two cross-reactive doublets of 50–52 kDa (present in Peaks I and II) and 67–70 kDa (evident in Peaks I and III; Fig. 1B,C). The level of S6 peptide phosphotransferase activity in Peak II was comparable to that detected in Peak III, although Peak II only contained the 50–52 kDa doublet of S6K-PNT immunoreactivity.

The occurrence of a 52-kDa form of the pp70 S6 kinase has not been previously described, and it was possible that this species was generated by partial proteolysis of pp70^{S6K} kinase. Therefore, antibodies directed against the N- and C-termini of pp70^{S6K} were used to probe cytosolic extracts of freshly lysed sea star oocytes that were either immature or treated for 90 min with 1-methyladenine to induce GVBD (Fig. 2, lanes 2–6). In the immature sea star oocyte-derived samples, the p50^{S6K} and p67^{S6K} forms of these S6 kinases predominated, consistent with inactivation associated with dephosphorylation and increased mobility on the SDS-PAGE gel. As p50^{S6K} and pp52^{S6K} were strongly detected in oocytes extracts that were rapidly prepared and not previously frozen, it is unlikely that they were generated from proteolysis of pp70^{S6K}. Furthermore, following partial purification of the maturation-activated S6 peptide phosphotransferase activity, both the pp52^{S6K} and pp70^{S6K} bands were clearly detected with three different S6 kinase antibodies (Fig. 2, lanes 7–9). Cross-reactivity with antibodies for both termini of pp70^{S6K} would not be

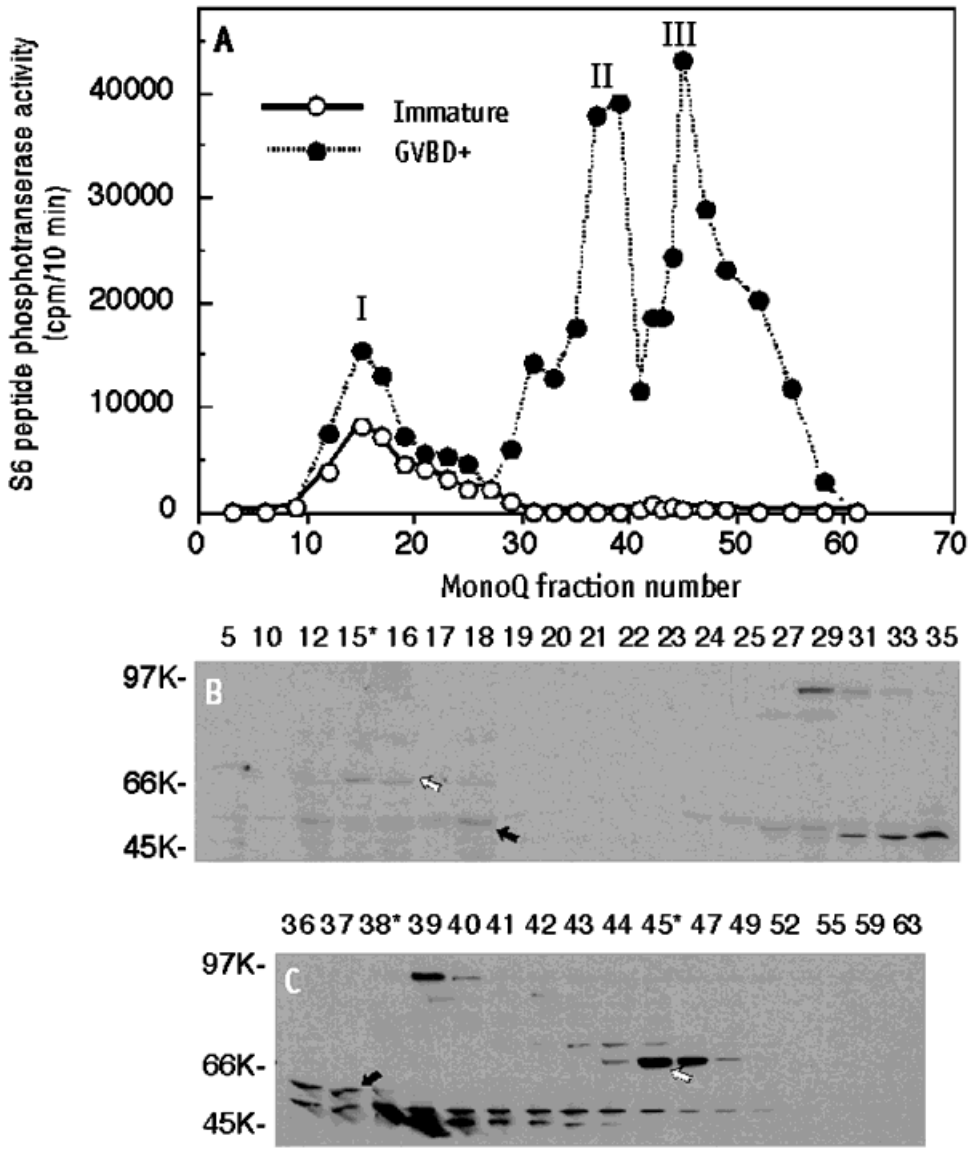

Fig. 1. Identification of S6 phosphotransferase activity in mature sea star extract. **A:** Crude extracts from immature (○) and maturing, GVBD-positive seastars (●; 5 mg protein) were fractionated on a MonoQ ion exchange column with a linear NaCl gradient of 0 to 0.8 M. The fractions were assayed for phosphotransferase activity against a synthetic peptide based on the sequence in the 40S ribosomal protein S6 known to be phosphorylated by murine pp70 S6K. Peak II and Peak III S6 kinase activities eluted from the column at approximately 0.35 M NaCl and 0.55 M NaCl, respectively. **B,C:** The fractions from maturing sea star oocytes were immunoblotted with an anti-peptide antibody generated against the post-amino terminal of $p70^{S6K}$ (S6K-PNT). A white arrow indicates the position of pp70 S6K on the Western blots, whereas a black arrow denotes the location of pp52 S6K. Fractions with peak S6 peptide phosphotransferase activity are asterisked.

expect for $p52^{S6K}$ if it was a degraded fragment of $pp70^{S6K}$.

Purification of Maturation-Activated $pp52^{S6K}$

To further identify and characterize the major S6 peptide kinase from maturing sea star oocytes, we undertook its purification to near homogeneity. The optimized purification procedure (detailed in Experimental Procedures) was completed over 2 days. As the S6 kinase activity was not labile, it was not significantly decreased by the length of purification treatment which ranged up to 4 days. The initial ammonium sulfate precipitation produced a substantial purification as approximately 20% of the total protein was precipitated at 0.8 M $(NH_4)_2SO_4$, while the S6 kinase activity remained in the soluble fraction. This treatment

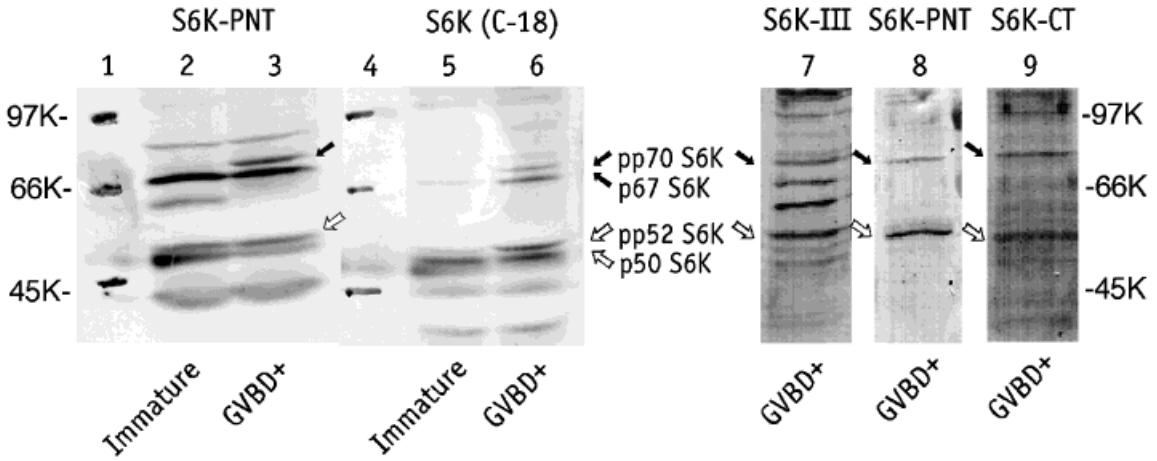

Fig. 2. Immunological identification of partially purified sea star S6 kinases. Cytosolic extracts from immature (**lanes 2** and **5**) and maturing (**lanes 3** and **6**) oocytes were probed with S6K-PNT (lanes 2 and 3) and S6K-C-18 (lanes 5 and 6) antibodies. The positions of pp70 S6K and pp52 S6K are indicated on the Western blots with black and white arrows, respectively. In **lanes 7** to **9**, a partially purified preparation of the S6 phosphotransferase activity from maturing oocytes (following tyrosine agarose and hydroxylapatite chromatography) was immunoblotting with S6K-III (lane 7), S6K-PNT (**lane 8**), and S6K-CT (lane 9) antibodies (UBI). **Lanes 1** and **4** show the positions of molecular weight marker proteins, which have been highlighted with an ink pen.

also raised the ionic strength of the cytosolic fraction sufficiently to allow the kinase to bind to the hydrophobic $_L$-tyrosine agarose resin (Fig. 3A). The hydroxylapatite (HTP) fractionation also provided significant purification as the kinase activity eluted from this resin at ~0.10–0.21 M K_2HPO_4/KH_2PO_4. Heparin-agarose was used to bind contaminating proteins, and the wash-through material that contained the S6 kinase was directly applied to poly-$_L$-lysine agarose. The S6 phosphotransferase activity was eluted from the poly-$_L$-lysine agarose column at approximately 0.46–0.54 M NaCl (Fig. 3C). The S6 kinase activity from this step was subsequently loaded on to an anionic Q Sepharose column, and was eluted with between 0.33 and 0.40 M NaCl (Fig. 3D). From this point on in the purification, it was not possible to measure protein levels in individual fractions, so protein concentrations were estimated from pooled fractions. As the consensus sequence for phosphorylation of the S6 protein by known p70^{S6K} isoforms contains several arginine residues, we reasoned that an $_L$-arginine agarose column might effectively act as an affinity matrix. The S6 kinase activity eluted from this column with between 0.26 and 0.32 M NaCl (Fig. 3E). Finally, the pooled fractions from the $_L$-arginine agarose column were concentrated on a MonoQ column from which the S6 kinase activity was eluted with between 0.30 and 0.35 M NaCl (Fig. 3F). Five 250 µl MonoQ fractions containing the peak of S6 kinase activity were pooled, aliquoted into 100 µl samples and immediately frozen at −70°C. The averaged results of three separate purifications are shown in Table I. Beginning with ~11 g of cytosolic protein, ~5µg of the enzyme was purified over 50,000-fold with a final recovery of ~2% of the starting activity.

The purified MonoQ fractions were electrophoresed on SDS-PAGE (11%) and the proteins in the purified preparation were visualized by staining with silver (Fig. 4A). Several distinct protein bands coincided with the peak of S6 kinase activity, and these included apparent molecular masses of ~60 kDa, 55 kDa, and 52 kDa as well as more intensely silver-stained proteins of 65 kDa, 90 kDa, and 95 kDa. The major protein in the 49–53 kDa range in the purified preparation was identified as pp52^{S6K} by immunoblotting with the S6K-PNT antibody (Fig. 4B). The kinase appeared to be subject to degradation or dephosphorylation during the purification procedure, as immunoblot analysis of the MonoQ fractions after purification showed immunoreactivity with several protein bands ranging in size from ~49 to 53 kDa, perhaps reflecting differences in phosphorylation state or possibly proteolyzed fragments of the pp70^{S6K}. Many kinases have been shown to undergo autophosphorylation and this often correlates with changes in phosphotransferase activity. Autoradiography of the purified kinase after

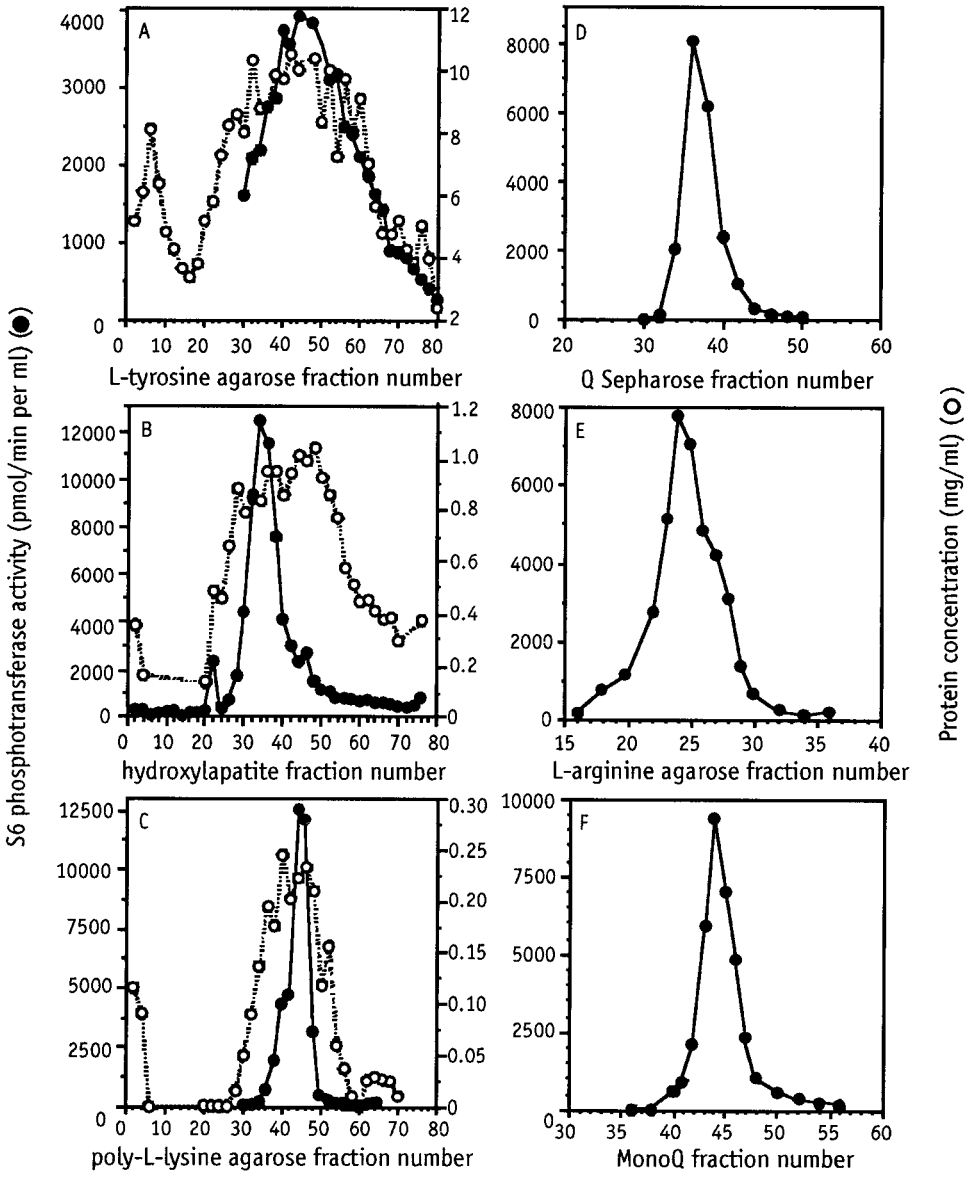

Fig. 3. Purification of sea star pp52 S6 kinase. The S6 kinase activity (●) was purified to near homogeneity over a series of chromatography columns. The tyrosine agarose (**A**), hydroxylapatite (**B**) and polylysine agarose (**C**) fractionations include protein concentrations measured by Bradford assay (○). The protein levels were too low to measure in the final three purification steps: Q Sepharose (**D**), arginine agarose (**E**), and MonoQ (**F**). See Experimental Procedures for the complete purification protocol.

incubation with [γ-^{32}P]ATP showed that p52^{S6K} underwent autophosphorylation (Fig. 4C).

Association of S6 Kinase With Protein Phosphatase 2A

As mentioned above, several contaminating protein bands were noted in the purified enzyme preparation. Two of these had apparent molecular masses of ~60 kDa and 55 kDa. Immunoblot and microsequence analysis identified the ~60 kDa band as HSP-60, an abundant heat shock protein that co-purified with the kinase and may have been recognized by the p70^{S6K} antibodies (Table II and Fig. 5A). Four separate peptides were sequenced (T4, T5, T6, and T7). Of these, T4 was identical in 10 of 12 amino acids to the published sequence of human HSP 60. T5 was identical in three of the three amino acids that were unambiguous, T6 was identical in eight of eight residues, and T7 was identical in six of eight. In addition, the ~55 kDa band was identified as the regulatory

TABLE I. Purification of S6 Kinase From Mature Sea Star Oocytes[a]

Column step	Volume (ml)	Total activity (nmol/min)	Protein (mg)	Specific activity (nmol/min/mg)	Recovery (%)	Purification (-fold)
Homogenate	720	350	10,800	.032	100	1.0
L-tyrosine agarose	640	286	6,000	.048	82	1.5
Hydroxylapatite	320	240	320	.75	69	23
Poly-L-lysine agarose	35	175	7	25	50	772
Q Sepharose	28	130	1.5	87	37	2,690
L-arginine agarose	10	56	.05	1,120	16	34,600
MonoQ	1.25	8	.005	1,600	2	50,000

[a]Seven-hundred-twenty ml of crude extract (~15 mg/ml protein) were used to purify S6 kinase in a final volume of 1.25 ml containing ~5 μg of protein. Sixteen L-tyrosine agarose elutions were pooled onto eight hydroxylapatite columns and the eluate was subsequently loaded on to a single poly-L-lysine column. The final recovery was less than 2% with a specific activity of 1,600 nmol/min/mg. The kinase was purified about 50,000-fold.

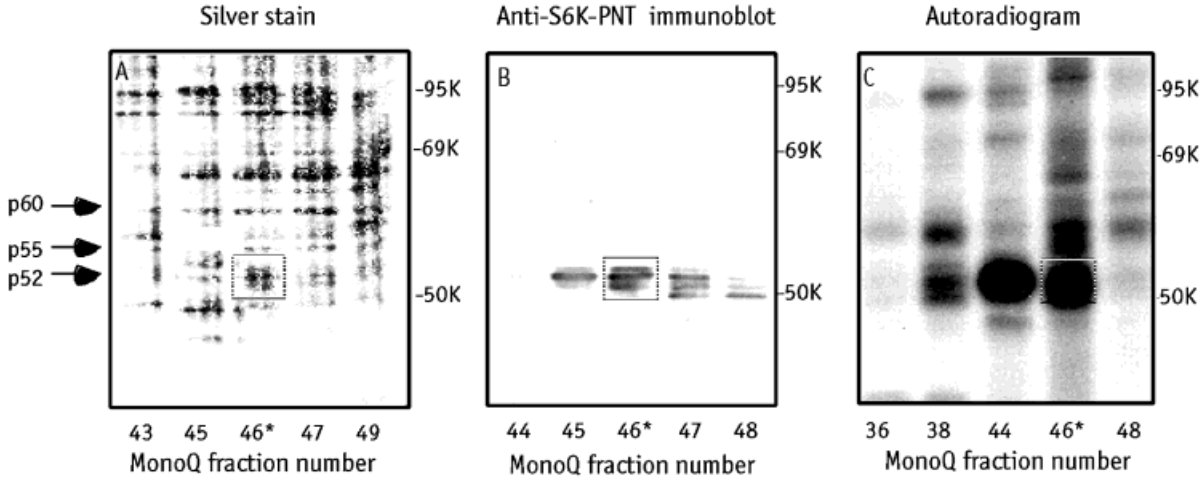

Fig. 4. Silver stain, immunoblot and autophosphorylation of purified sea star pp52 S6 kinase. The MonoQ fractions containing S6 phosphotransferase activity were stained with silver (**A**). The protein bands that were subjected to protein microsequence analysis are marked with arrows. The same fractions were immunoblotted with the S6K-PNT antibody (**B**) and subjected to autoradiography (**C**). The peak S6 kinase activity in fraction 46 was recognized by the antibody and underwent autophosphorylation. Migration positions of molecular weight standards are shown.

subunit of protein phosphatase 2A (PP2A) both by immunoblotting and by direct protein microsequence analysis (Table II and Fig. 5B). The T1 peptide was a perfect match to *Drosophila* PP2A alpha subunit in all 16 amino acids sequenced, while the T2 peptide was identical in eight of 11 residues. The T3 peptide was identical in nine of 10 residues to the *Porcine* PP2A beta subunit.

To further explore the relationship between PP2A and S6 kinase, PP2A was immunoprecipitated from partially purified sea star extract with antibody directed against its regulatory alpha subunit (Fig. 6). The immunoprecipitates were found to exhibit phosphotransferase activity against the 40S ribosomal S6 protein. There was no kinase activity evident in the control lane (antibody and beads in the absence of sea star extract). These findings supported the association of PP2A and an S6 kinase in a high molecular weight complex. Gel filtration analysis demonstrated that most of the pp52^{S6K} and pp70^{S6K} behaved as monomers in cytosolic extracts from maturing oocytes (Fig. 7). However, the pp70^{S6K} isoform was also detected in a high molecular mass complex with an apparent size of approximately 90 kDa. In view of the aforementioned findings, and our recent discovery that pp70^{S6K} and PP2A are physically associated in rat brain [Westphal et a*l*., 1999], it would appear that these enzymes are physiological partners.

TABLE II. Alignment and Identification of Tryptic Peptides[a]

Band ID protein MW	Peptide number	Sequence	Identification
Band 1:52 kDa	T1	GEYNVYSTFQSHEPEF	PP2A-alpha
Band 1:52 kDa	T2	MFEEPEDPSNR	PP2A-alpha
Band 2:55 kDa	T3	LSSGDWFTSR	PP2A-beta
Band 3:60 kDa	T4	IGGSSDVEVNEK	HSP60
Band 3:60 kDa	T5	XXXFXDN	HSP60
Band 3:60 kDa	T6	GANPVEIR	HSP60
Band 3:60 kDa	T7	VQDAMNAT	HSP60

Band 1. Alignment with PP2A-alpha

PP2A	[61]ANPRRGEYNV YSTFQSHEPE FDYLKSLEIE EKINKIRWLQ QKNPVHFLLS[110]
T1	GEYNV YSTFQSHEPE F
PP2A	[181]YSSSKGTIRL CDMRSAALCD RHSKQFEEPE NPTNRSFFSE IISSISDVKL[230]
T2	MFEEPE DPSNR

Band 2. Alignment with PP2A-beta

PP2A	[121]PSDLEAHFVA LVKRLASGDW FTSRTSACGL FSVCYPRVSS AVKAELRQYF[170]
T3	LSSGDW FTSR

Band 3. Alignment with HSP60

HSP60	[121]RSIAKEGFEK ISKGANPVEI RRGVMLAVDA VIAELKKQSK PVTTPEEIAQ[170]
T6	GANPVEI R
HSP60	[301]KAPGFGDNRK NQLKDMAIAT GGAVFGEEGL TLNLEDVQPH DLGKVGEVIV[350]
T5	XXXFXDN
HSP60	[391]NERLAKLSDG VAVLKVGGTS DVEVNEKKDR VTDALNATRA AVEEGIVLGG[440]
T4/T7	IGGSS DVEVNEK VQDAMNAT

[a]Identification of co-purifying proteins by micro-sequencing. The purified S6 kinase preparation contained several distinct protein bands that were subjected to protein micro-sequencing. A concentrated aliquot of the purified preparation was electrophoresed on 11% PAGE, transferred to CD membrane, stained, and destained with quick stain. The proteins were excised, digested with pepsin, cleaved with trypsin, and injected onto HPLC. The peaks were collected, analyzed by mass spectrometry, and micro-sequenced in the laboratory of Dr. Ruedi Aebersold at the University of Washington.

Sea Star S6 Kinase Is Regulated by Phosphorylation

Purified pp52^{S6K} was pre-incubated in the presence of PP2A or potato acid phosphatase (PAP) on protein A Sepharose beads. The phosphatase reactions were subsequently inactivated either by the addition of okadaic acid (for PP2A) or by pelleting the beads in a centrifuge (for PAP), and phosphotransferase activity was measured in the presence of a peptide substrate of S6 kinase (Fig. 8). The kinase became dephosphorylated and inactivated by pretreatment with PP2A and PAP, indicating that pp52^{S6K} was regulated by phosphorylation.

Co-Factors and Inhibitors of the 40S Ribosomal S6 Kinase

All known protein kinases require metal cofactors, usually magnesium, for phosphotransferase activity. The purified pp52^{S6K} utilized either magnesium or manganese for the phosphotransferase reaction. To further characterize the requirement of pp52^{S6K} for various ions, and the effect of inhibitors on kinase activity, the purified kinase was incubated in the presence of a variety of molecules, and the concentrations that caused 50% maximal inhibition (IC_{50}) were determined (Table III). The pp52^{S6K} was inhibited by sodium fluoride, but was relatively insensitive to high concentrations of β-glycerolphosphate, EGTA, dithiothreitol, spermine, heparin, NaCl, and metal ions such as Mn^{2+}, Zn^{2+}, and Ca^{2+}.

Autophosphorylation and Phosphorylation of the 40S S6 Ribosomal Protein and Other Substrates

The purified pp52^{S6K} was incubated in the presence of a variety of conventional protein kinase substrates, electrophoresed on SDS-PAGE, and the gel was exposed to X-ray film (Fig. 9A). The kinase efficiently phosphorylated

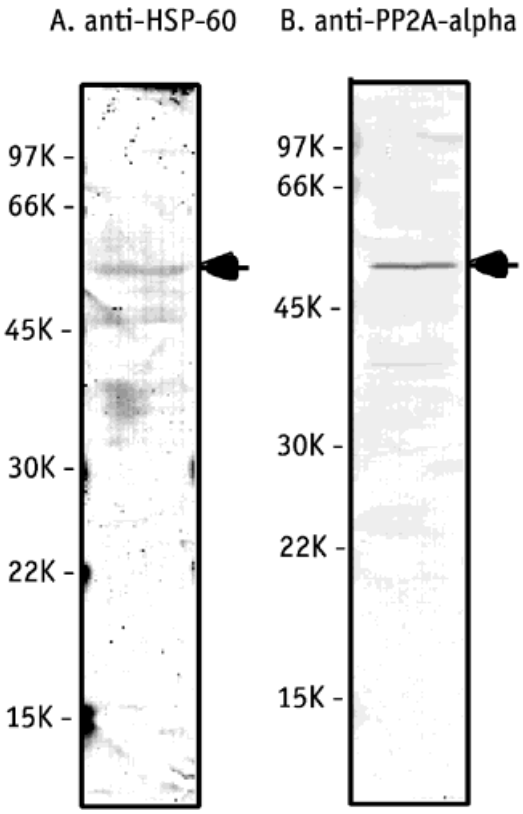

Fig. 5. Immunological identification of PP2A and HSP 60 in purified sea star S6 kinase preparation. The purified S6 kinase preparation was electrophoresed on 11% PAGE, transferred to nitrocellulose and probed with antibodies directed against the catalytic and regulatory subunits of HSP60 (**A**) and PP2A (**B**).

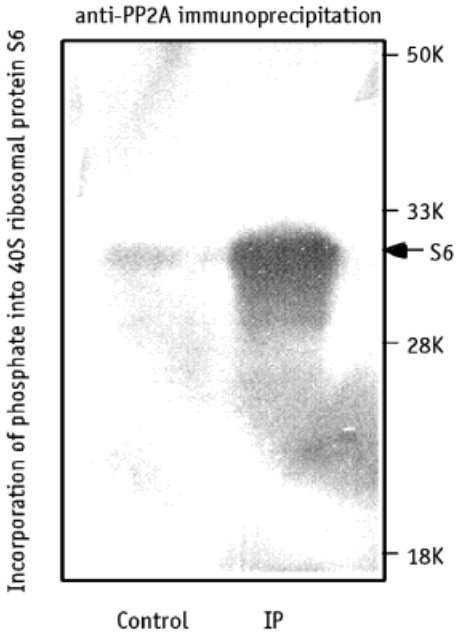

Fig. 6. Immunoprecipitation of S6 kinase phosphotransferase activity by anti-PP2A antibody. Anti-PP2A alpha antibody was incubated with crude extract from maturing, GVBD-positive sea stars. The immunoprecipitate was assayed for kinase activity against 40S ribosomal protein S6 and phosphorylation was visualized by exposure to X-ray film.

myelin basic protein (MBP) as well as 40S ribosomal proteins. Purified 40S ribosomes phosphorylated with 50 μM [γ-^{32}P]ATP and purified pp52^{S6K} were separated by SDS-PAGE, transferred to PVDF membranes, and subjected to phosphoamino acid and 2D phospho-peptide analysis. The S6 protein was phosphorylated exclusively on serine residues (Fig. 9B), and the 2D phosphopeptide map generated by the tryptic digest of the phosphorylated S6 protein had five distinct radio-labeled spots (Fig. 9C) indicating several phosphorylation sites, consistent with other reports for mammalian pp70^{S6K} [Bandi et al., 1993].

Substrate Specificity of the 40S Ribosomal S6 Kinase

The substrate consensus sequence of a previously characterized mammalian S6 kinase has been determined [Flotow and Thomas, 1992] and the minimal sequence required for recognition was deduced to be RRRXXSX, where X could be any amino acid. Purified sea star pp52^{S6K} was also analyzed using synthetic peptides based on the C-terminus of the S6 protein. These peptides were constructed by step-wise alteration of specific amino acid residues surrounding the phosphorylation site. The true K_m values of purified S6 kinase for the peptides were estimated from Lineweaver-Burke plot analysis (Table IV). The optimum sequence for phosphorylation by p52^{S6K} was deduced to be RXXSXR, where the +1 position may not be an acidic amino acid (see Discussion).

DISCUSSION

In this paper, we describe for the first time the purification of a ribosomal S6 kinase from an echinoderm source. At least two isoforms of this kinase were identified by immunological analysis following fractionation of sea star oocyte extracts by MonoQ chromatography. An S6 phosphotransferase activity that became activated during maturation was purified to near homogeneity through a series of chromatography steps. The simple, six column-step procedure produced hyperactive enzymatic phosphotransferase activity, although protein yields were very low. Immunoblot analysis of the S6

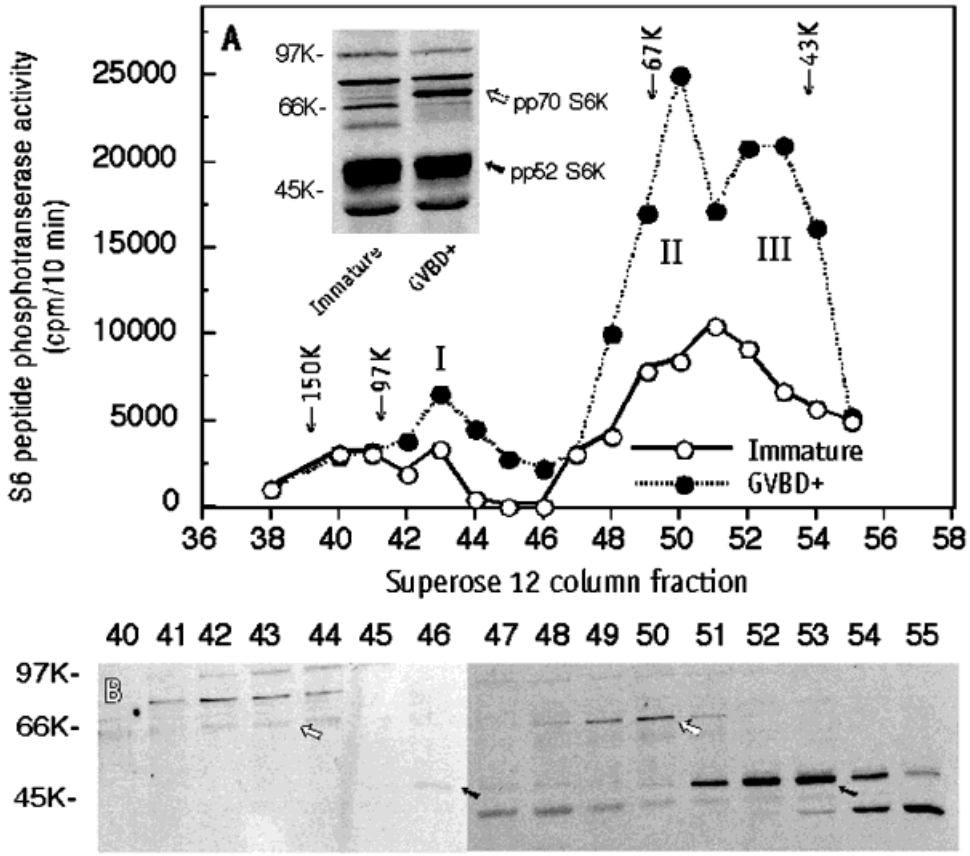

Fig. 7. Gel filtration chromatography of S6 peptide phosphotransferase activities in sea star oocytes. **A:** Crude extracts from immature (○) and maturing, GVBD-positive sea star oocytes (●; 4 mg protein) were fractionated on a Superose 12 column. The fractions were assayed for phosphotransferase activity against a synthetic peptide based on the C-terminus of S6. Elution positions of molecular weight marker proteins are indicated. A inset: A Western blot of the cytosolic extracts from immature and GVBD-positive oocytes probed with the S6K-PNT antibody. **B:** An immunoblot of selected Superose 12 fractions with the S6K-PNT antibody. A white arrow indicates the position of p70 S6K (in Peak II), whereas a black arrow denotes the location of p52 S6K (in Peak III).

kinases from early stages of the purification revealed immunoreactive proteins of around 50–52 kDa and 67–70 kDa with three different antibodies directed against the amino terminal, carboxyl terminal, and internal sequences of rat pp70^{S6K}. A 52 kDa isoform of pp70^{S6K} was ultimately purified from the sea star oocytes. The purified pp52^{S6K} was hyperactive towards the S6 peptide substrate and the 40S ribosomal protein S6 and the activity was stable for months at −70°C, allowing us to analyze it over a period of time. We further characterized pp52^{S6K} with respect to ion and co-factor dependence and showed that the kinase was inhibited by fluoride, but relatively insensitive to many other compounds. The kinase autophosphorylated in the presence of [γ-^{32}P]ATP, although the effect of autophosphorylation on phosphotransferase activity was not determined. The purified pp52^{S6K} efficiently utilized myelin basic protein and the 40S ribosomal protein S6 as substrates, and phosphoamino acid analysis of the S6 protein showed that the kinase phosphorylated S6 exclusively on serine. Two-dimensional phosphopeptide mapping of the 40S ribosomal proteins revealed phosphorylation of five distinct tryptic peptides, consistent with characteristics of previously identified pp70^{S6K} isoforms [Bandi et al., 1993]. Following submission of this manuscript, we learned of two mammalian 54-kDa isoforms (β1 and β2) of pp70^{S6K} that are highly related in structure to pp70^{S6K} and similarly regulated (J. Blenis, personal communication). It is likely that pp52^{S6K} is a seastar homolog of these 54-kDa S6 kinases.

We went on to examine the substrate specificity of echinoderm pp52^{S6K} using a series of synthetic peptides modeled after the sites in the S6 protein known to be phosphorylated by

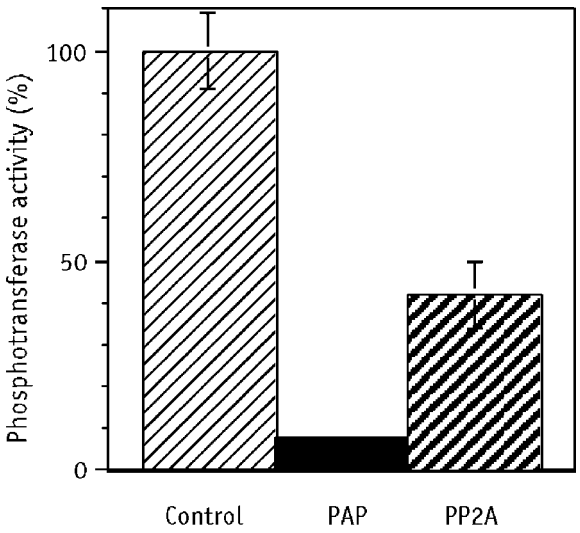

Fig. 8. Purified pp52^{S6K} is dephosphorylated and inactivated by potato acid phosphatase and protein phosphatase 2A. Purified pp52^{S6K} was pre-incubated in the presence of PAP or PP2A, then assayed for phosphotransferase activity against the S6 peptide substrate. The experiment shown here is the average of three separate assays ± S.E.M.

TABLE III. Summary of Various Inhibitors of pp52 S6K[a]

Compound	IC_{50}
Zinc	15 mM
Calcium	40 mM
Manganese	15 mM
Dithiothreitol	>100 mM
EGTA	300 mM
Spermine	>40 mM
NaCl	>5 M
NaF	60 mM
β-Glycerophosphate	>800 mM
Heparin	>200 mg/ml
cAMP	>20 mM

[a]The purified p52^{S6K} was assayed with the S6 peptide substrate in the presence of a variety of common kinase inhibitors and the IC_{50} was determined.

pp70^{S6K} (R^{232}RLS*S*LRAS*TSKS*ESS*QK). Kinetic analyses of the K_m and V_{max} for phosphorylation of various peptides (see Table IV) provided some intriguing differences from previously reported consensus sequences for pp70^{S6K}. Flotow and Thomas [1992] determined the consensus sequence of p70^{S6K} purified from the livers of cycloheximide-treated rats to be R(R)RXXSX. They found that arginine residues in the -3 and -5 positions were essential for activity, while arginine in the -4 position was not as critical. In our analyses, we found that an arginine residue in the -3 position was also critical for kinase activity (K_m and V_{max} of 0.30 mM and 3700 pmol/min per mg), but that arginine in the -4 position was dispensible and an additional arginine at the -5 position was actually inhibitory (K_m and V_{max} of 0.77 mM and 1,800 pmol/min per mg; compare peptide number 4, 5, and 9 in Table IV). In addition, we found that an arginine residue at the +2 position was essential for kinase activity (compare peptide number 5 with 12). Replacing the hydrophobic leucine at the +1 position with an acidic residue (compare peptide number 5 with 8) was also inhibitory to kinase activity. The sites in the S6 protein are phosphorylated sequentially in vivo in the order Ser-236 > Ser-235 > Ser-240 > Ser-244 > Ser-247 [Krieg et al., 1988; Martin-Perez and Thomas, 1983], so we tested peptides with Ser-235 changed to alanine (compare peptide number 3 with 5), and found that Ser-236 was also preferentially phosphorylated by pp52^{S6K}. Another interesting observation was that changing Ser-236 to threonine (compare peptide number 5 with 7) resulted in K_m and V_{max} being affected in opposite directions. The S6 protein becomes phosphorylated strictly on serine residues in vivo, and clearly threonine was not efficiently phosphorylated by pp52^{S6K} in our assay. The consensus sequence for phosphotransferase activity of the purified p52^{S6K} was found to be RXXSXR where the amino acid in the +1 position is ideally not an acidic residue.

Interestingly, two proteins that co-purified with pp52^{S6K} have been identified by immunoblot analysis as well as by direct protein micro-sequencing, as a heat shock protein (HSP-60) and the regulatory subunit of PP2A. Further, we have co-immunoprecipitated S6 kinase phosphotransferase activity with anti-PP2A specific antibodies. These results raise the intriguing possibility that p52^{S6K} may form a multi-protein complex in vivo and the activity of the S6 kinase may be regulated by it's association with PP2A and/or HSP60. In collaboration with Dr. Brian Wadzinsky (Vanderbilt University, Nashville), we have observed that purified rat brain protein phosphatase 2A also complexes with p70^{S6K} [Westphal et al., 1999]. Complexes of this sort could represent yet another level of regulation of signaling during cell division, proliferation, or differentiation.

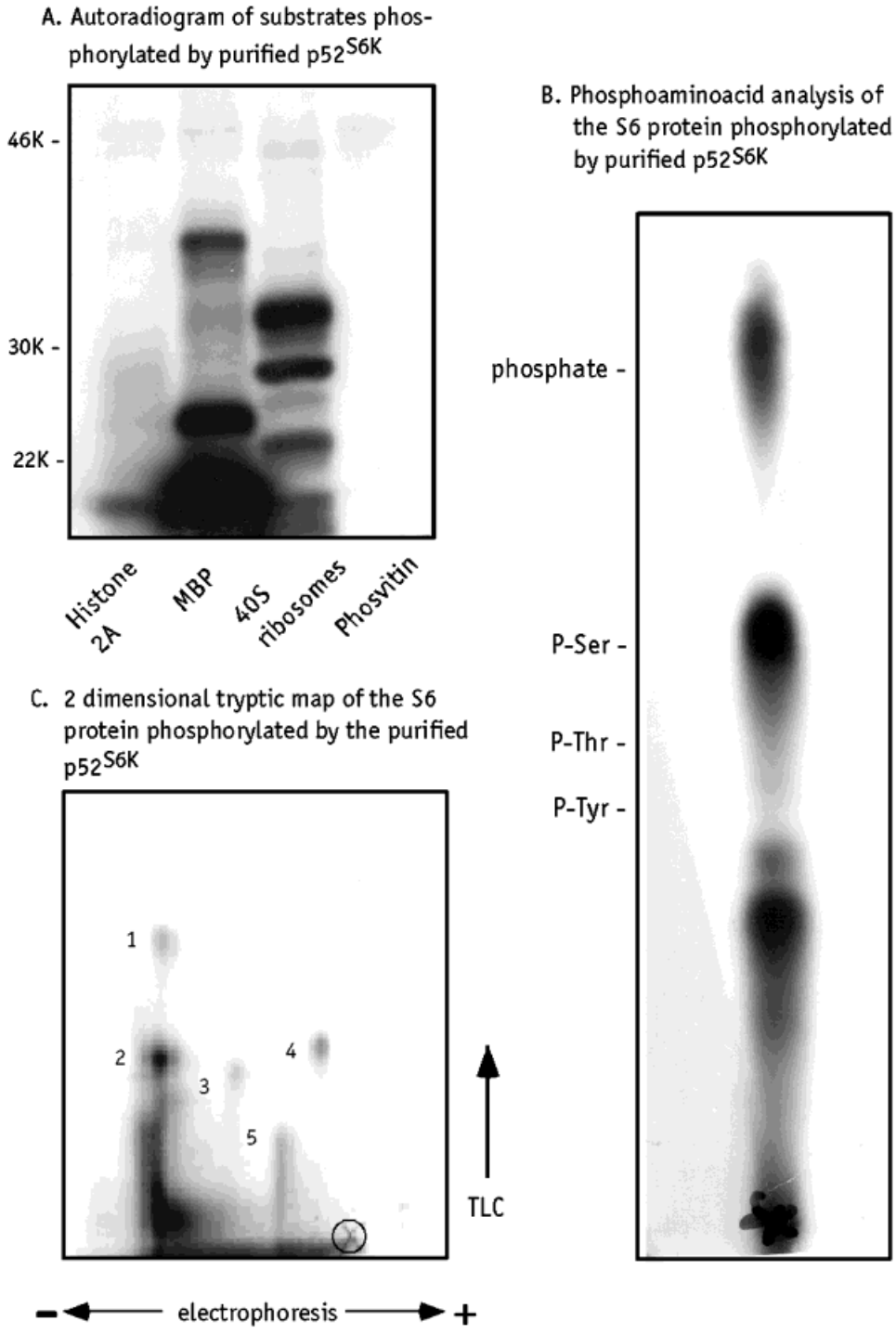

Fig. 9. Phosphorylation of the 40S ribosomal proteins and other substrates. The purified pp52 S6 kinase was used to phosphorylate histone 2A, myelin basic protein, the 40S ribosomal proteins and phosvitin. The proteins were separated by electrophoresis and exposed to film (**A**). The phosphorylated 40S ribosomal proteins were subjected to phosphoamino acid analysis (**B**) and two-dimensional phosphopeptide analysis (**C**).

As the phosphorylation of the S6 protein is an integral and obligatory step in the initiation of translation of specific proteins required for mitosis, the purification of p52^{S6K} described here will provide us the means to further dissect the pathways leading to cell division and growth in a variety of systems. In addition, studies are ongoing to determine the role, if any, of HSP60 and PP2A in the regulation of p52^{S6K}. Specific kinase-phosphatase complexes may represent

TABLE IV. Summary of Substrate Specificity of pp52 S6K

Peptide sequence	K_m (μM)	V_{max} (nmol/min per mg)	V_{max}/K_m
1. AKRRRLSSLRASTSKSESSQK	0.22	7.3	33
2. RRRLSSLRASTSKSESSQK	0.19	5.7	30
3. RRLSSLRAGGRR	0.27	3.6	13
4. RLASLRAGGRR	0.30	3.7	12
5. RRLASLRAGGRR	0.31	3.5	11
6. AAQKRPSQRTKYLA	0.34	3.6	11
7. RRLATLRAGGRR	3.91	23.8	6.1
8. RRLASERAGGRR	0.40	1.4	3.6
9. RRRLASLRAGGRR	0.77	1.8	2.4
10. AKRRRASA	0.79	0.9	1.1
11. AKRRRLS-amide	2.07	1.0	0.5
12. RRLASRAGGRR	8.40	0.7	0.08

a novel means by which cellular signaling may be regulated.

ACKNOWLEDGMENTS

We thank Dr. David Brautigan for the purified protein phosphatase 2A and phosphatase 2A antibodies. This work was supported by an operating grant from the Medical Research Council of Canada. L.A.C. was the recipient of a National Sciences and Engineering Council of Canada Studentship and an Izaak Walton Killam Predoctoral Fellowship. I.C.-L. and S.L.P. were recipients of Medical Research Council of Canada Scientist Awards.

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