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Identification of the S6 kinase activity stimulated in quiescent brine shrimp embryos upon entry to preemergence development as p70 ribosomal protein S6 kinase: Isolation of *Artemia franciscana* p70^{S6k} cDNA

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Abstract

We previously demonstrated that a protein kinase responsible for phosphorylating 40S ribosomal subunits is activated in quiescent *Artemia franciscana* embryos within 15 min of restoration of normal tonicity and incubation at 30°C. Here, we identify the activated S6 kinase as *A. franciscana* p70 ribosomal S6 kinase (p70^{S6k}) subsequent to the isolation of an *Artemia* p70^{S6k} cDNA. The protein conceptually translated from cDNA has 70% similarity and 64% identity to both *Drosophila melanogaster* and human p70^{S6k}. Southern blot analysis is consistent with presence of a single p70^{S6k} gene. Two transcripts of 5.4 and 2.7 kb were found. Abundance of both mRNAs increased dramatically around 4 h of preemergence development, and exhibited different steady-state level variation thereafter. Stimulated S6 kinase activity, partially purified by Superose 6 chromatography, correlated best with the slowest migrating, ~65 kDa, form detected by Western analysis using a specific polyclonal antibody made to a peptide from the predicted p70^{S6k} NH₂-terminus. Furthermore, the *A. franciscana* p70^{S6k} was immunoprecipitated with the same antibody, showing in parallel an S6 kinase activity similar to peak profiles. We conclude that the stimulated S6 kinase activity is that of an ortholog of human p70^{S6k} that may be involved in the regulation of protein synthesis during preemergence development in *A. franciscana* species.

Keywords

p70 ribosomal S6 kinase cDNA; brine shrimp; development

Introduction

Artemia franciscana is a crustacean, class Phyllopoda, whose study by developmental biologists has been prompted by its fascinating life cycle (Slegers 1991; Tate and Marshall 1991). In favorable environmental conditions, zygotes can give rise to nauplii (larvae) that are released from the ovisac to the environment. If environmental conditions are unfavorable, embryos may stop development at the gastrulae stage, exit the cell cycle, and undergo specialized changes that result in the formation of cysts. Each encysting embryo is covered by a chitinous shell before release. The released encysted embryos are in diapause, an obligate

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form of developmental arrest, and can remain in this state for long periods. Diapause cysts will not resume development even under otherwise favorable conditions. Release from diapause (activation) requires exposure to specific environmental stimuli or cues (Drinkwater and Clegg 1991). If, after activation, environmental conditions become unfavorable (such as insufficient water or oxygen or low temperature) activated cysts respond with another hypometabolic state, named quiescence. When favorable conditions are restored, normal metabolism resumes and the embryos enter preemergence development (PED). There is a window of about the first 4 h in PED during which a state of quiescence can be regained in response to dessication, but thereafter many of the embryos do not survive such treatment. Among the earliest events of PED are mobilization of energy stores, chiefly the disaccharide trehalose, and restoration of protein synthesis (Slegers 1991; Tate and Marshall 1991).

Regulated translation is a paramount process in PED, as development proceeds without DNA synthesis or cell division until the nauplius is released from the cyst (Clegg 1966). A major focus of research in the developmental biology of *A. franciscana* has been to determine which signals control the rapid acquisition of translational activity, and subsequently which signals control translation of specific stored mRNAs (Brandsma et al. 1997; Moreno et al. 1991; Tate and Marshall 1991). Cysts contain 80S ribosomes as monosomes and tRNA. Also present are seemingly all of the required protein synthetic factors for chain initiation, elongation, and termination. The ribosomes and factors that have been examined appear fully active when purified and tested, and inactive cysts do not contain an inhibitor of translation that explains restriction (Brandsma et al. 1997).

Extracts from inactive cysts fail to translate their mRNAs, whereas extracts from stimulated cysts are active in *in vitro* systems (Brandsma et al. 1997; Moreno et al. 1991). Addition of nuclease-treated reticulocyte-lysate to extracts from undeveloped embryos restores some translational activity, suggestive of a limiting quantity or activity of some key factor(s) (Moreno et al. 1991). The capacity for translation is normally rapidly acquired, suggesting that it is due to signal(s) generated subsequent to rehydration. These signals are unknown. One signal must be for charging of tRNA; levels of aminoacyl-tRNA are very low and rise dramatically when cysts are activated (Brandsma et al. 1997). Moreover, the restriction of *in vitro* translation observed with extracts of quiescent embryo can be partially released by the addition of charged tRNA (Brandsma et al. 1997).

We have been interested in the regulation and function of protein kinases that regulate translation, particularly p70 ribosomal S6 kinase (p70^{S6k}) (reviewed by Dufner and Thomas 1999; Weng et al. 1998). Phosphorylation of ribosomal protein S6, the known physiologic substrate of p70^{S6k}, increases in nauplii in comparison with undeveloped embryos (Kenmochi et al. 1989). In mammalian systems, phosphorylation of ribosomal protein S6 appears to regulate protein synthesis in part by enhancing translation of a specific set of mRNAs, those that possess a 5' tract of pyrimidines (5' TOP mRNAs) (Dufner and Thomas 1999). Many of the 5' TOP mRNAs studied encode proteins involved in translation, and thus one predicted effect of p70^{S6k} activation and S6 phosphorylation is an increase in the translational capacity. However, the amounts of several translation factors are reported not to change dramatically in *A. franciscana* between quiescent and developing embryos (Brandsma et al. 1997; Moreno et al. 1991; Tate and Marshall 1991). Polyribosomes can be detected within 15 min of cyst rehydration; this is consistent with mRNA being rapidly selected and activated for translation (Golub and Clegg 1968).

Previously, we sought to determine whether S6 kinase is activated in the transition from quiescence to active development. We found that phosphotransferase activity towards 40S ribosomal subunits was activated within 15 min in stimulated cysts, remained maximally stimulated for 4 h, and declined slowly thereafter (Malarkey et al. 1998). Protein S6 was also

rapidly phosphorylated in situ within 15 min, and remained maximally phosphorylated for the period examined (1–8 h), as assessed by a gel shift assay and Western blotting. The stimulated S6 kinase activity chromatographed as a single peak with properties similar to p70^{S6k}. It was not possible to use inhibition by rapamycin (Dufner and Thomas 1999) as a criterion, because of the high degree of impermeability of the cyst cuticle. Although strongly suggestive, the isolation of a cDNA for *A. franciscana* remained necessary to definitively prove the relatedness of the stimulated kinase to p70^{S6k}.

Here, we report the isolation and characterization of a cDNA encoding *A. franciscana* p70^{S6k} and identification of the stimulated *A. franciscana* S6 kinase as p70^{S6k}, using a specific antibody made to a peptide from the conceptually translated protein. Thus, activation of p70^{S6k} (Malarkey et al. 1998) and charging of tRNA (Brandsma et al. 1997) are two of the identified, early regulated events in translation in PED. The possible role of p70^{S6k} in *A. franciscana* development is briefly discussed, as well as our current understanding of p70^{S6k} regulation from the viewpoint of the new *A. franciscana* sequence.

Materials and methods

Materials

A custom *A. franciscana* cDNA library (in λ ZAP Express) was made by Stratagene (La Jolla, Calif.) from a pellet of frozen *A. franciscana* nauplii. Artemia Revolution™ shell-less cysts were from New Technologies Laboratories Ltd., and were purchased from Carolina Biological Supply Company (Burlington, N.C.). These cysts are from the Great Salt Lake, Utah and are dechorionated and sterilized by treatment with NaOCl. They are sold as a suspension in a high tonicity salt buffer that maintains quiescence. The Artemia Revolution™ cysts were used in all experiments described, except for preparing 40S ribosomal subunits. Other reagents were of the highest grade available.

Design of degenerate primers

Degenerate primers were designed to favorable sequences chosen from an alignment of *Drosophila melanogaster* and rat p70^{S6k}. The forward primer was chosen for sequence (EGIF (L/M)EDT) from subdomain V in the catalytic domain; its sequence was GA(G/A)GG(T/C/A)AT(T/C)T-T(T/C)(A/C)TIGA(G/A)GA(T/C)AC. The reverse primer was chosen for sequence (GFTYVAP) within the COOH-terminus outside the catalytic domain that surrounds a highly conserved phosphorylation site (T389, underlined) in all p70^{S6k} sequences (Dufner and Thomas 1999; Weng et al. 1998); its sequence was GG(A/G/T)GC(A/G/T)AC(A/G/T)T-A(A/G/T)GT(A/G/T)AA(A/G/T)CC.

Amplification of an authentic DNA probe for Artemia p70^{S6k}

PCR was performed with Taq DNA polymerase, degenerate forward and reverse primers (above), and λ phage DNA prepared from an *A. franciscana* λ ZAP Express cDNA library as a template using the following conditions: 94°C, 1 min; 37°C, 1 min; 72°C, 1 min, for 3 cycles; 94°C, 1 min; 42°C, 1 min; 72°C, 1 min, for 30 cycles. A prominent DNA band with the expected ~700 kb size was generated. This specific PCR product was directly cloned into pCR 2.1 vector DNA following a 'TA ligation cloning strategy' (Invitrogen, Carlsbad, Calif.; Cat. No. K2000-0) and the clones were screened by blue-white selection. Positive clones that carried the insert with the expected size were directly sequenced, and the open-reading frame was conceptually translated and compared with protein data banks. One of the positive clones revealed a high homology with the p70^{S6k}, showing the highest similarity with *Drosophila* p70^{S6k}. Its 700-kb DNA insert was used as a probe for cDNA library screening.

Isolation of *A. franciscana* p70^{S6k} cDNA

The above DNA probe was radioactively labeled and used to screen replicate filters of the plated *A. franciscana* λ ZAP Express cDNA library under stringent conditions for *A. franciscana* (42°C, hybridization; 65°C, washing); so far the *A. franciscana* genome appears to be especially AT rich (~68%). Several positive clones were subjected to an in vivo excision protocol, following the manufacturer's recommendations, to rescue the inserts into pBK-CMV. The largest cDNA insert (~1.7 kb) was sequenced, and the conceptually translated protein of the open-reading frame was compared with protein banks. The highest homology was with *D. melanogaster* p70^{S6k}. However, a short 5' sequence was missing in this clone.

The missing 5' end was amplified from the cDNA library by a strategy employing nested PCRs. The two forward primers, Fwd-1 (5'-ATTAACCTC-ACTAAAG; also known as T3 primer) and Fwd-2 (5'-GGGAACAAAA-GCTGGAGC), were chosen to anneal within the λ ZAP Express vector, upstream of the *Eco*RI cloning site. The two reverse primers, Rev-1 (5'-TTCAAATCTTGAGGACC) and Rev-2 (5'-GGAATGGCCTCAACGTCTGG) were chosen within the 5' *A. franciscana* cDNA sequence that had already been cloned, immediately downstream of a unique *Sph*I restriction site. The first PCR reaction (primers Fwd-1 and Rev-1) used a lower annealing temperature (94°C, 1 min, 44°C, 1 min, 72°C, 1 min., for 30 cycles). The second PCR reaction, with primers Fwd-2 and Rev-2 and the product of the first PCR reaction as a template, was set up with a higher annealing temperature (94°C, 1 min, 56°C, 1 min, 72°C, 1 min, for 30 cycles). The strategy led to amplification of three product bands. The two longest products were extracted from the gel and cloned into the pCR 2.1 vector, and DNA from the inserts were sequenced on both strands. The translated aa sequence of one of the two products aligned well with the N-terminus of *D. melanogaster* p70^{S6k}, and also contained identical overlapping nucleotide sequence to that of the incomplete 1.7 kb p70^{S6k} clone already isolated from the library. The 5' end was incorporated into the latter clone contained in pBK-CMV using the *Eco*RI and the *Sph*I sites to obtain *A. franciscana* p70^{S6k} cDNA-pBK-CMV. The sequence of the *A. franciscana* cDNA was deposited in GenBank (Accession No. AF282407).

Southern and Northern blot analysis

Genomic DNA was prepared by conventional methods from *A. franciscana* nauplii and digested (20 μ g) with restriction enzymes, electrophoresed on 0.8% agarose gel in TAE 1 $\times$ (40 mM Tris-acetate, 1 mM EDTA, pH 8.5), blotted to Hybond-N membranes (Pharmacia-Amersham, Piscataway, N.J.) and crosslinked to the membrane with UV light. Restriction digestions appeared complete after DNA staining before transfer. The probe for hybridization was a single-stranded, antisense probe generated by PCR and radio-labeled with ³²P, and included almost the entire cDNA sequence. Hybridization was carried out in Church and Gilbert hybridization buffer (7% SDS, 1 mM EDTA, 0.5 M sodium phosphate buffer, pH 7.2) at 42°C, and then membrane was washed at room temperature with 2 $\times$ SSC/0.1% SDS twice, with 1 $\times$ SSC/0.1% SDS twice and with 0.1 $\times$ SSC/0.1% SDS four times.

Total RNA was prepared from various *A. franciscana* cyst samples incubated at different times in hatching medium (422 mM NaCl, 9.4 mM KCl, 25.4 mM MgSO₄, 22.7 mM MgCl₂, 1.4 mM CaCl₂, 0.5 mM NaHCO₃, 1000 μ g/mL penicillin and 100 μ g/mL streptomycin), by the standard acid guanidinium thiocyanate method. Total RNA (165 μ g per lane) was electrophoresed on 0.8% agarose-2.2 M formaldehyde gels, transferred to Hybond-N membranes, crosslinked, and hybridized to the same probe as above. Hybridization and washes were carried out as described above. The amount of RNA used per lane was normalized first by absorption, and quantified within the experiments by staining with methylene blue. For comparison of mRNA levels in PED, the amount of probe hybridized to each RNA lane was quantified by volume integration using the ImageQuant program (Amersham Pharmacia Bio-

technology, Inc., Piscataway, N.J.), and divided by the amount of rRNA present in that lane, detected by methylene blue staining and quantified by volume integration as well.

Antibody production and immunoblot analysis

The peptide, GVFDIELHEPDDTAHLTC, containing amino acids 3–19 of the *A. franciscana* p70^{S6k}, was synthesized by the University of Virginia Biomolecular Core Facility, conjugated to keyhole limpet hemocyanin by the added C-terminal cysteine and used to produce a rabbit polyclonal antiserum. Specific anti-peptide antibody was affinity-purified on columns of the antigen peptide-coupled to Sulfo-Link beads, according to the manufacturer's recommendations (Pierce; Rockford, Ill.). Portions of chromatography fractions (35 μ L) or extract supernatants (5 μ L) were subjected to 10% SDS-PAGE and transfer to PVDF membranes. Western blot analysis was carried out using the affinity-purified antibody at a 1 : 1000 dilution using the Enhanced Chemiluminescence system (Pharmacia-Amersham, Piscataway, N.J.).

Preparation of extract supernatant

Eleven millilitres of the manufacturer's suspension of Artemia RevolutionTM cysts were combined with 99 mL of hatching medium and incubated at 30°C with rotational mixing (150 rpm) for 2.5 h (activated cysts) or only rehydrated in hatching medium but not incubated (control cysts). At the indicated times, cysts were recovered as a loose pellet by centrifugation (4°C) at $1100 \times g$ for 2 min in an RC-3B Beckman rotor. The supernatant was aspirated and the pellet suspended in 4 mL of ice-cold Superose 6 buffer (50 mM MOPS, pH 7.4, 5 mM β -glycerolphosphate, 0.01% (v/v) Triton X-100, 1 mM Na₄P₂O₇, 1 mM benzamidinium chloride, 1 mM EDTA, 5 mM EGTA, 0.1 mM Na₃VO₄, 1 mM DTT) supplemented with (final concentrations): 1 mM Pefabloc-SC, 0.1% (v/v) Triton X-100, 10 mM Na₄P₂O₇, 0.7 μ g/mL pepstatin, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, and 1 μ M microcystin-LR.

Cyst suspension was homogenized at 4°C in a motor-driven Teflon-glass homogenizer with 30 gentle passes of the pestle. The homogenate was then centrifuged at $100\,000 \times g$ for 20 min. The supernatant including a viscous portion at the bottom of the tube was collected, mixed, and passed through a 0.22 μ m filter prior to any analysis. Total protein was estimated by the Bradford method.

Superose 6 chromatography

Extract supernatant (200 μ L, ~20 mg/mL protein) was applied to a Superose 6 HR 10/30 gel filtration column equilibrated in Superose 6 buffer (specified above), and chromatographed (4°C, 0.3 mL/min); the eluant was collected in 0.6-millilitre fractions. Fractions were kept at 4°C without freezing, and subsequent analysis were carried out immediately.

S6 kinase assay

Portions (11.3 μ L) of each Superose 6 fraction or of extract supernatant (5 μ L) were mixed with 10 μ g of ribosomal 40S subunits diluted into 6.3 μ L of Superose 6 buffer, and incubated for 20 min on ice. Ribosomal 40S subunits were prepared from *A. franciscana* cysts (San Francisco Bay Brand) as described by Zasloff and Ochoa (1974). Assays were performed at 30°C for 20 min in kinase buffer (final concentrations: 25 mM HEPES, pH 7.4, 5 mM β -glycerolphosphate, 3.75 mM EGTA, 30 mM MgCl₂, 0.15 mM Na₃VO₄, 1.5 mM DTT, 6 μ M PKI-tide, 0.5 μ M Microcystin-LR, 300 μ M [γ -³²P]ATP (2000 cpm/pmol)) in a final volume of 25 μ L. Reactions were initiated with [γ -³²P]ATP and stopped with 5 μ L of 6 $\times$ Laemmli SDS sample buffer. Phosphorylated products were resolved by SDS-PAGE (12% gel), Coomassie-stained S6 bands were excised, mixed with counting cocktail, and incorporated ³²P determined by liquid scintillation spectroscopy.

Immunoprecipitation and immune-complex kinase assays

Portions of the Superose 6 fractions (100 μ L) were first precleared by incubating with 20 μ L of protein A/G beads (Pierce, Rockford, Ill.; Cat. No. 20421). Cleared supernatants were removed, mixed with 1 μ L immunopurified antibody (1 mg/mL) to the predicted N-terminus of *A. franciscana* p70^{S6k}, and incubated for 1 h on ice. Immune-complexes were isolated using protein A/G-beads. Beads were washed three times with Superose 6 Buffer plus 75 mM NaCl, 1% NP-40, and then heated in 6 $\times$ Laemmli SDS-buffer for SDS-PAGE (10% gel) for Western analysis performed on PVDF membranes with the immunopurified polyclonal antibody against the *A. franciscana* p70 S6 kinase N-terminus.

For immune-complex kinase assays, the immunoprecipitates were washed once in Superose 6 buffer plus 75 mM NaCl and 1% NP-40, and twice in S6 kinase buffer (without ATP), and finally resuspended in 18 μ L S6 kinase assay buffer (minus ATP) to be assayed for S6 kinase activity essentially as described above. In these assays, resuspended complexes were incubated on ice for 20 min with 2 μ L ribosomal 40S subunits (8.3 mg/mL), and then 20 μ L S6 kinase buffer with 600 μ M [γ -³²P]ATP (4000 cpm/pmol) was added and the mixture incubated for 20 min at 30°C.

Data analysis

All experiments described were repeated 2 or 3 times, and results from a representative experiment are shown.

Results and discussion

Molecular cloning of *Artemia* p70^{S6k} cDNA

PCR with degenerate primers was used to isolate a specific DNA fragment that could be labeled and used to probe a cDNA library. The forward primer was chosen to a favorable 8 aa sequence (EGIF(L/M)EDT) found between subdomains V and VIA in the catalytic domain that is conserved for human and *D. melanogaster* p70^{S6k} with one substitution (L/M), but is not highly conserved with respect to other kinases. The reverse primer was based on a sequence (GFTYVAP) that contains T389, a highly conserved phosphorylation site in p70^{S6k} (Dufner and Thomas 1999; Weng et al. 1998). We obtained a 700 bp DNA fragment that was compatible with the origin from *A. franciscana* p70^{S6k} cDNA by PCR, using λ DNA from an *A. franciscana* cDNA library as a template. This fragment was used to isolate a nearly full-length cDNA clone (1.7 kb), and the missing 5'-end was amplified from library λ DNA by consecutive, nested PCR reactions. This strategy is illustrated in Fig. 1. The correctness of the amplified 5' end was assured by an exact match of overlapping sequence in the DNA fragment with sequence in the 1.7 kb clone already isolated, and by similarity of the translated aa sequence to the N-terminus of p70^{S6k}. The 5' end was then incorporated at the *Sph*I site to complete the cDNA clone. The initiating ATG is in a Kozak consensus sequence, and it must represent the actual start site because two in-frame stop codons are present 12 nucleotides upstream of ATG in the 5' sequence (tga taa atc ttt tac aaa ATG).

The assembled *A. franciscana* p70^{S6k} cDNA (~1.8 kb) (GenBank Accession No. AF282407) contains an open reading frame of 1452 nucleotides predicted to encode a polypeptide of 484 residues, with a calculated molecular mass of 54.7 kDa (Fig. 2). The sequence shows ~70% overall similarity and ~64% identity with both *D. melanogaster* (Stewart et al. 1996; Watson et al. 1996) and human (Grove et al. 1991) p70^{S6k}. Residues from mammalian p70^{S6k} to be discussed will be referred to with residue numbers for the shorter, non-nuclear form of human p70^{S6k} (Grove et al. 1991) now known as S6K1. The cytoplasmic and nuclear forms of p70^{S6k} derive from one gene, the *S6K1* gene (Gout et al. 1998), and differ by 23 additional amino acids at the N-terminus of p85^{S6k}. A second *S6K2* gene encoding a highly similar

p70^{S6k} protein, termed S6K2/p70^{S6kb}, also with two forms (b1, b2), has been identified in mammalian species (human and mouse) (Gout et al. 1998; Shima et al. 1998). *D. melanogaster* contains a single p70^{S6k} gene (Stewart et al. 1996; Watson et al. 1996). Recent data indicate regulation of S6K2 differs from S6K1; compound U0126 (which inhibits activating kinases for ERKs) antagonizes S6K2 activation (Martin et al. 2001).

Sequence comparison and its discussion

A. franciscana and *D. melanogaster* S6Ks are aligned with human S6K1 in Fig. 2. The NH₂-terminal domain of *A. franciscana* S6K, like that of other sequences reported is highly acidic, but is much less well conserved between species, displaying 46% and 44% similarity with *D. melanogaster* S6K and human S6K1 sequences, respectively. The catalytic domain shows the highest degree of identity (83% with *D. melanogaster* S6K, and 80% with human S6K1). The residues surrounding the site of regulatory phosphorylation in the activation loop of S6K1 (T229) are identical in the multiple alignment (Fig. 2). Phosphorylation of T229 by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for S6K to be active (Dufner and Thomas 1999; Weng et al. 1998). The COOH-terminal tail, from Hanks-Hunter alignments of protein kinases, is the sequence following HPFF (residues 326–329 of S6K1). The COOH-terminal tail contains a region (shaded in gray) that also exhibits high similarity and is often referred to as the linker region (Dufner and Thomas 1999; Weng et al. 1998).

In the linker region, residues surrounding S371 and T389 are identical and T367 is also conserved in the *A. franciscana* protein homolog. This region has 78% similarity with *D. melanogaster* S6K and 75% similarity with human S6K. This region is centrally important to mitogen-stimulated activation of p70^{S6k} (Dufner and Thomas 1999; Weng et al. 1998). T389 is a rapamycin-sensitive site of regulatory phosphorylation whose phosphorylation in response to mitogens appears to be rate-limiting for p70^{S6k} activation (Weng et al. 1998). S371 phosphorylation does not appear to be rapamycin sensitive, but phosphorylation of S371 is stimulated by mitogens and its replacement by Ala or Glu makes p70^{S6k} inactive (Moser et al. 1997). S371 appears necessary for T389 phosphorylation in situ because T389 is not detectably phosphorylated in S371A or S371E forms. The mechanisms and enzymes (protein kinases and protein phosphatases) involved in controlling S371 and T389 phosphorylation in p70^{S6k} in vivo have not been identified, but two reports show that mTOR (mammalian target of rapamycin) can directly phosphorylate T389 (Burnett et al. 1998; Isotani et al. 1999). Studies show that phosphorylation of T229 is strongly stimulated by phosphorylation of T389, that concurrent phosphorylation of both sites is required for maximal p70^{S6k} activity, and that activation and deactivation of p70^{S6k} in vivo correlates well with T389 phosphorylation (Dufner and Thomas 1999; Weng et al. 1998). It is not understood if in a structural sense this region is a linker, but it lies between the catalytic domain and a more divergent sequence containing a putative autoinhibitory domain (Dufner and Thomas 1999; Weng et al. 1998).

The autoinhibitory domain is so-called because in the initial characterization of the conceptually translated rat S6K1 cDNA, a region in the COOH-terminal tail (residues 400–436 of S6K1) was found to partially align with a sequence from the phosphorylated region of S6 substrate. The 37-mer peptide from this domain competitively ($K_i = 20 \mu\text{M}$) inhibited kinase activity but did not serve as a substrate; this is consistent with the possibility that this region is structurally a pseudosubstrate (Price et al. 1991). The domain contains a series of S/T-P phosphorylation sites thought to be required for S6K1 activation, but proven not essential for mitogen-stimulated activation in a S6K1- Δ CT104 truncation mutant that removes the carboxy terminal 104 amino acids, including the autoinhibitory domain. The role of phosphorylation of S/T-P sites in this region is not satisfactorily understood, but these sites are still considered important in a model for hierarchical phosphorylation and activation of p70^{S6k}. Replacement of S/T residues in this domain with acidic residues (Glu/Asp), such as in the p70^{S6k}D3E-type

mutant, increases basal activity of enzyme recovered from unstimulated cells and replacement with Ala suppresses mitogen-stimulated activity (Dufner and Thomas 1999; Weng et al. 1998). The p70^{S6k}D3E-type mutant also shows enhanced phosphorylation by PDK1 in vitro. Phosphorylations in the autoinhibitory domain are thought on this basis to control its interactions with other portions of the p70^{S6k} molecule that modulate suitability (possibly accessibility) of other required sites, chiefly T229, for phosphorylation.

In the multiple alignment, the autoinhibitory region (residues 400–436 of S6K1) is divergent between *A. franciscana*, *D. melanogaster*, and human p70^{S6k} except for the motif for phosphorylation of S411 (underlined). S411 is phosphorylated by cdc2-cyclinB during mitosis (Papst et al. 1998). The residues (S/T-P-X-K/R) surrounding S411 that are critical for substrate selection by cdc2-cyclin B are also conserved in *A. franciscana* p70^{S6k}. S411 is not thought to be essential for p70^{S6k} activation since several p70^{S6k} mutants with S411 substitutions are activated in quiescent cells stimulated with mitogens. S411 is also highly phosphorylated in quiescent cells and its phosphorylation, unlike S229 and T389, is not robustly stimulated by mitogens (Weng et al. 1998). Conservation of S411 suggests it may have an unidentified function. There is one other S-P site in the autoinhibitory domain (S429, in human S6K1) that is present in *A. franciscana* p70^{S6k}. The rest of the mammalian phosphorylation sites in the autoinhibitory domain (S418, T421, S424) are absent in *A. franciscana* and *D. melanogaster* sequences. The remainder of the COOH-termini displayed no significant homology in the multiple alignment nor with proteins in the protein data bases, indicating that these segments are unique to each protein.

Southern analysis

Southern blot analysis revealed only a few bands with most of the restriction reactions, indicative of p70^{S6k} or closely related genes present in a low copy number (Fig. 3). Depending on the specific restriction enzyme used, the specific *A. franciscana* p70^{S6k} cDNA antisense probe hybridized to only one (with *Bgl*I), two (with *Xho*I), three (with *Eco*RI), or seven (with *Hind*III) fragments of genomic DNA. These data are consistent with the presence of a single *A. franciscana* p70^{S6k} gene. First, the size of the gene calculated by the sum of the fragments is ~26–29 kb, which is the average size of *A. franciscana* genes. There is one band with *Bgl*I digestion. The *Eco*RI/*Bgl*I double restriction doesn't reveal additional fragments. The *Eco*RI/*Xho*I double restriction also does not produce a new hybridizing fragment because the two new bands from this digest are accounted for by disappearance of the 9.1 kb *Eco*RI band. The *Eco*RI/*Hind*III double digest is more complex. All three bands from *Eco*RI single digestion disappear but they must because our *A. franciscana* p70^{S6k} cDNA contains a *Hind*III site. Two weakly hybridizing bands from the *Hind*III single digestion (~6.1 kb and 3.5 kb) disappear in the *Eco*RI/*Hind*III double digest, and are not readily explained since no cleavage site exists for *Eco*RI within the isolated cDNA, but *Eco*RI sites could be present in introns of the gene. The *A. franciscana* gene should have an *Eco*RI site in either introns, or in additional 5' untranslated region not in our cDNA, to explain three *Eco*RI bands if there is a single *A. franciscana* p70^{S6k} gene as we suspect. This will require characterization of the *Artemia* p70^{S6k} gene in a future study.

Northern analysis

Northern blot analysis of total RNA (Fig. 4A) revealed two putative transcripts of 5.4 and 2.7 kb, both of them large enough to code for the complete p70^{S6k} polypeptide chain. Northern blot analysis of poly (A)⁺ RNA also revealed the presence of the same transcripts (data not shown). Approximately 73% of the larger and 46% of the smaller transcript are predicted to represent untranslated sequences. The two mRNAs may represent either alternatively spliced forms or mRNAs that use either alternative promoters or distinct polyadenylation signals. The single copy *D. melanogaster* p70^{S6k} gene produces three mRNAs (5, 3.7, and 2.8 kb).

As a first step in understanding how levels of p70^{S6k} mRNAs are controlled, we performed Northern analysis to estimate steady state levels of the two mRNAs at different times of PED. The amount of rRNA present per lane was used to correct for possible differences in the amount of RNA (see Materials and methods) because the amount of rRNA present per cell does not change during *A. franciscana* PED (Koller et al. 1987). The normalized quantification of both transcripts at different stages of development is shown in Fig. 4B. The amount of 5.4 kb transcript was detectably increased by 2 h, reaching its maximal value between 4–16 h. The increase in 5.4 kb mRNA was more than 5-fold at 12 h of PED and thereafter the amount of this transcript decreased. The 2.7 kb transcript reached its maximal levels (~1.5 fold) at 4 h of PED, and decreased slowly thereafter.

A major feature of the profile is the clear increase of both transcripts by 4 h, whereas they were detected with difficulty in control cysts (0 h of rehydration). The transcripts were barely detectable in the 0 h total RNA fraction, but were more readily detected in the 0 h poly (A)⁺ RNA fraction (data not shown). The results are consistent with a global activation of RNA synthesis that occurs during PED at around 4–5 h for several mRNAs studied, including mRNAs for actin and Na⁺/K⁺ and Ca²⁺ ATPases (Escalante et al. 1994). Steady-state levels of mRNAs for EF-1 α and eL12 and eL12' (two 60S subunit ribosomal proteins) were also coordinately increased at ~4 h of PED (Maassen et al. 1985). In addition to a global increase in mRNA expression from many genes, other genes are differentially transcribed and expressed in PED. For example, actin mRNAs increase during PED, but the increase is not accompanied by an increase in actin protein (Escalante et al. 1994).

Mammalian p70^{S6k}, via phosphorylation of the ribosomal S6 protein, is thought to recruit mRNAs marked in their 5' untranslated region with oligopyrimidine tracts into polysomes (Dufner and Thomas 1999). Nothing is known about existence or translation of 5' TOP mRNAs during PED in *A. franciscana*. It is known that mRNAs are stored in undeveloped cysts as cytoplasmic and nuclear messenger ribonucleoprotein particles (mRNP), and both poly (A)⁻ and poly (A)⁺ fractions of mRNAs are present in cysts (Brandsma et al. 1997; Moreno et al. 1991; Slegers 1991; Tate and Marshall 1991). mRNA from undeveloped cysts may be largely inactive, as assessed by in vitro translation, but a subset of mRNAs can be translated when transferred to heterogeneous systems or when extracts from undeveloped cysts are supplemented with RNAase-treated reticulocyte lysate (Moreno et al. 1991). The translatability of mRNAs is regulated and increases during PED. The mRNA in mRNP is associated with potential regulatory proteins, including a p38 kDa poly(A)-binding protein, which are substrates for protein kinases and phosphatases. Readers are referred to discussions in Moreno et al. (1991) and Brandsma et al. (1997) for a review. Our previous finding that S6 is rapidly phosphorylated (by ~15 min) in PED together with this report showing that the phosphorylation is likely due to p70^{S6k} provide a motivation for future studies of mRNA selection in early PED.

Identification of stimulated S6 kinase activity as p70^{S6k}

Analysis of fractions from Superose 6 chromatography by kinase assay and Western blotting—To characterize the S6 kinase activity activated in PED, we fractionated proteins in *A. franciscana* extract supernatants by gel permeation chromatography, and assayed portions of the fractions for phosphotransferase activity towards 40S ribosomal subunits. When we used cysts that had been incubated in hatching medium for 2.5 h as starting material (referred to here as activated cysts), we detected two peaks of S6 kinase activity (Fig. 5A). This is in contrast to what had been previously observed (Malarkey et al. 1998), wherein only one S6 kinase peak was detected. The major peak of activity from activated cysts (Fig. 5A) eluted with ~70,000 Mr just prior to a BSA standard. In our previous study, the stimulated S6 kinase activity (with 40S subunits as substrate) behaved as a single ~70,000 Mr peak with Superose 6 chromatography and also generated single peaks with MonoQ and phosphocellulose

chromatographies. For this reason, we concluded that the major peak of S6 kinase activity in Fig. 5A that elutes with an apparent Mr of ~70 kDa is the rehydration-stimulated S6 kinase observed by Malarkey et al. (1998). Consistent with this conclusion, the 70,000 Mr peak was reduced when extract supernatant from unactivated (control) cysts was used (Fig. 6A).

A minor peak of S6 kinase activity was observed in chromatographic profiles from activated cysts that consistently eluted near the void volume with a very large Mr, greater than that of thyroglobulin (669,000). We did not characterize this activity peak further. This peak may contain enzymes other than p70^{S6k} that are capable of phosphorylating ribosomal protein S6. Western blotting (Figs. 5B, 6B) revealed immunoreactive forms of p70^{S6k} in these fractions, but none of the most up-shifted phospho-forms of p70^{S6k}.

S6 kinase activity was also detected in the high Mr and ~70,000 Mr fractions produced by chromatography of supernatants of control cysts (Fig. 6A). This is in contrast with a previous study in which no S6 kinase activity was detected in extract supernatants from non-activated cysts (Malarkey et al. 1998). In our study, more concentrated supernatants (~20 mg/mL) were prepared to facilitate Western blotting (Figs. 5B, 6B) and included all of the mixed, post ~100,000 g supernatant, including a viscous portion at the bottom of the tube. The high Mr peak from control cysts (Fig. 6) contained more immunoreactive p70^{S6k} (panel B) and more S6 kinase activity (panel A) in comparison with the high Mr peak from activated cysts (Fig. 5). The presence of immunoreactive forms of p70^{S6k} in the high Mr peak may represent binding or aggregation of p70^{S6k} with RNA, other proteins, or protein–detergent complexes under these conditions, but none of the most up-shifted phosphoforms were detected in this peak. The S6 kinase activity in the high Mr peak likely represents other enzymes detectable with these conditions because only the up-shifted forms of p70^{S6k} are active in mammalian systems. This conclusion is tentative pending additional study of the conditions that result in observation of the high Mr peak. The presence of protein aggregates in cyst extracts has been previously reported. For example, the translation factors EF1 and EF2 have been found in large complexes that dissociate with development (Slegers 1991).

We performed Western analyses on portions of the fractions and extract supernatant (Figs. 5B, 6B) to assess whether the S6 kinase activity detected after Superose 6 chromatography is attributable to the *A. franciscana* p70^{S6k} whose cDNA we cloned. The blots were probed with a polyclonal antibody, prepared to the predicted N-terminus (aa3–19) of *A. franciscana* p70^{S6k}, and immunopurified before use. The antibody detected a broad ~58–65 kDa band in supernatants from both control and activated cysts. This band consists of several closely spaced bands that increased in number by activation (consistent with the behavior of mammalian p70^{S6k}), and was best seen with the actual developed film. An unidentified cross-reactive band of 45 kDa was also detected, possibly representing a p70^{S6k} fragment formed by proteolysis. The antibody also cross-reacted with an unidentified protein of ~94 kDa.

The 58–65 kDa complex of bands eluted in parallel with the stimulated S6 kinase activity, and its properties are consistent with those expected for *A. franciscana* p70^{S6k}. The theoretical mass of *A. franciscana* p70^{S6k} is 54.7 kDa, which corresponds quite well to this protein. Five bands with approximate molecular masses of 64.7, 63, 61, 60 and 58 kDa are visible in the 58–65 kDa region across fractions 25–30 in the original film. Mammalian p70^{S6k} is multiply phosphorylated in growth-factor stimulated cells and, like the *A. franciscana* protein, is resolved into a ladder of bands. Within the 58–65 kDa region of the blot, the elution profile of the most up-shifted protein band (~65 kDa) matches almost exactly the envelope of stimulated S6 kinase activity. This behavior also matched that of mammalian p70^{S6k} (Dufner and Thomas 1999; Weng et al. 1998). This result strongly supports our hypothesis that the ~70,000 Mr peak of S6 kinase activity is p70^{S6k}.

Immunoprecipitation and immune complex kinase assay of *Artemia* p70^{S6k}—To ensure that the S6 kinase activity can be attributed to an *A. franciscana* p70^{S6k} ortholog, we carried out additional experiments wherein fractions from activated cysts were immunoprecipitated with the specific antibody, and the immunoprecipitates were assayed for S6 kinase activity and subjected to Western blot analysis. The antibody immunoprecipitated an S6 kinase activity from the fractions, and the profile of immunoprecipitated activity exactly matched the S6 kinase profile from a direct assay of the fractions (Fig. 7A). In the immunoblot, the same five bands previously seen were also immunoprecipitated, and the profile correlated with the activity peak (Fig. 7B). The most up-shifted immunoprecipitated band was again observed to correlate best with S6 kinase activity. The common stained ~55 kDa band across the blot is an immunoglobulin heavy chain, detected because of its abundance in the samples. The antibody was not able to detectably immunoprecipitate the cross reacting ~94 kDa band. The S6 kinase activity and immunoreactive bands from the >670 kDa M_r peak (fractions 13–15) were also not observed. The inability to detect these bands in immunoprecipitates may be due to the relatively small amounts of immunoreactive protein eluted in these fractions. The inability to detect activity correlates with the absence of the most up-shifted p70^{S6k} band in these fractions (Fig. 5B).

The ribosomal protein S6 kinase activated by rehydration of quiescent *A. franciscana* embryos at onset of PED is p70^{S6k}. The *A. franciscana* p70^{S6k} has conserved sequences for phosphorylation sites (T229, S371, T389, S411) found in both mammalian and *D. melanogaster* p70^{S6k} and elucidating the kinases which activate *A. franciscana* p70^{S6k} will contribute to understanding the regulation of eukaryotic p70^{S6k} in general. The role of p70^{S6k} in regulation of cell growth and cell size has been clearly shown by the disruption of the gene in *D. melanogaster*, which produces smaller cells and a smaller but otherwise phenotypically normal fly (Montagne et al. 1999). The mechanism is still under study but appears to be explained by the ability of p70^{S6k} to regulate translational capacity by mRNA selection. Activation of p70^{S6k} may play an important regulatory role in early PED of *A. franciscana* when there is a biologic need to rapidly reestablish growth while favorable conditions last and in translation of stored mRNAs.

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Fig. 1.

Scheme for identification and assembly of *A. franciscana* p70^{S6k} cDNA. The missing 5' end of the p70^{S6k} cDNA was isolated by nested PCR reactions from the library as template, and the complete clone assembled at a unique *SphI* site. Sense primers were annealed within the λ left arm in the λ ZAP Express vector, upstream of the EcoRI cloning site; the antisense primers were annealed within the 5' *A. franciscana* p70^{S6k} cDNA sequence that had been already cloned, immediately downstream of the *SphI* site.

Art	MAGVFDIELH	EPDDTAHLTI	QSE..LQSD	DSIEVEDVSM	LDQGSYYETF	48
Dm	MADVSDPSEL	FDLELHDLEL	QDDKARDSDD	DRIELDDVD.	LEPELCINLH	49
Hs	MAGVFDIDLD	QPEDAGSEDE	LEEGGQLNES		MDHGGVGPYE	40
Art	VNQPDVEAIP	ISEQNVNQGK	EKTGPQDFEL	RKVLGKGGYG	KVFQVRKLTG	98
Dm	QDTEGQETIQ	LCEENVNPGK	IKLGPQDFEL	KKVLGKGGYG	KVFQVRKTAG	99
Hs	LGMEHCEKFE	ISSETSVNRGP	EKIRPECFEL	LRVLGKGGYG	KVFQVRKVTG	90
Art	QDSGHTFAMK	VLKKATIVRN	QKDTAHTKAE	RNILEAVKHP	FIVDLIYAFQ	148
Dm	RDANKYFAMK	VLKKASIVTN	QKDTAHTRAE	RNILEAVKHP	FIVELVYAFQ	149
Hs	ANTGKIFAMK	VLKKAMIVRN	AKDTAHTKAE	RNILEEVKHP	FIVDLIYAFQ	140
Art	TGGKLYLILE	YLSGGELFMH	LEREGIFMED	TACFYLSEII	LALHLHHQG	198
Dm	TDGKLYLILE	YLSGGELFMH	LEREGIFLED	TTCFYLSEII	LALGHLHKLK	199
Hs	TGGKLYLILE	YLSGGELFMQ	LEREGIFMED	TACFYLAELS	MALGHLHKGK	190
Art	IIYRDLKPEN	ILLDAQGHVK	LTDFGLCKES	VIEGVSHTTF	CGTIEYMAPE	248
Dm	IIYRDLKPEN	ILLDAQGHVK	LTDFGLCKEH	IQEGIVHTTF	CGTIEYMAPE	249
Hs	IIYRDLKPEN	IMLNHQGHVK	LTDFGLCKES	IHDGTVHTTF	CGTIEYMAPE	240
Art	ILTRSGHGKA	VDWWSLGALM	YDMLTGAPPF	TAENRKKTIE	KILKGKLNLP	298
Dm	ILTRSGHGKA	VDWWSLGALM	FDMLTGVPFF	TAENRKKTIE	TILKAKLNLP	299
Hs	ILMRSGHNRA	VDWWSLGALM	YDMLTGAPPF	TGENRKKTID	KILKCKLNLP	290
Art	PYLSPDARDL	IRKLLKRTVN	QRLGSGPEDG	ESLKDHPFFR	LVNWNVDLMR	348
Dm	AYLTPEARDL	VRLMKRQEP	QRLGSGPEDA	AAVQIHPFFK	HVNWDDVLAR	349
Hs	PYLQTPEARDL	LKKLLKRNAA	SRLGAGPGDA	GEVQAHPFFR	HINWEELLAR	340
Art	RVDPPFPKPAL	TSEDDVSQFD	TRFTKQTPVD	SPDESTLSES	ANLVFQGFITY	398
Dm	RLEPPPIKPLL	RSEDDVSQFD	TRFTRQIPVD	SPDDTTLSES	ANLIFQGFITY	399
Hs	KVEPPFPKPLL	QSEEDVSQFD	SKFTTRQTPVD	SPDDSTLSES	ANQVFLGFITY	390
Art	VAPSVLEDVN	RPHVI..KAR	SPRK.PTFHL	TRMNSQPFSP	RQTTYQHFP.	444
Dm	VAPSILEDMH	RANRM..PAR	SPRR.TPRQL	PDSSFRLLQFP	SANVGANAPL	446
Hs	VAPSVLESVK	EKFSEFEPKIR	SPRRFIGSPR	TPVSPVKFSP	GDFWGRGASA	440
Art	.CFNSATRSS	VDIGDTMDTG	MPSIVNNATT	DFQQQRFMPM	L~~~~~	484
Dm	AMHGHQSQRSG	MFARATPPHH	MQTFAPRPSP	AQDEMMDVQG	LPMV~~~~~	490
Hs	STANPQTPVE	YPMETSGIEQ	MDVTMSGEAS	APLPIRQPN	GPYKKQAFFM	490
Art	~~~~~	~~				
Dm	~~~~~	~~				
Hs	ISKRPHELRM	NL	502			

Fig. 2.

Predicted amino acid sequence of *A. franciscana* S6 kinase (Art) and its multiple alignment with *D. melanogaster* p70 S6 kinase (Dm) and human p70 S6K1. Multiple alignment was carried out by PILEUP (GCG). Numeration of the protein sequences starts with the first Met of *A. franciscana* and *D. melanogaster* S6 kinases and S6K1. The catalytic domain is boxed, the linker region is boxed and shaded, and the pseudosubstrate autoinhibitory domain in human p70 S6K is underlined. Residues known to be phosphorylated in the human protein are boxed, and comparable residues conserved in *D. melanogaster* and (or) *A. franciscana* are boxed as well. The *A. franciscana* sequence is GenBank (Accession No. AF282407).

Fig. 3. Southern blot analysis of *A. franciscana* p70 S6 kinase. The figure shows that a small number of restriction fragments hybridize when a single-stranded *A. franciscana* p70^{S6k} cDNA is used as a probe. Markers (bp) are indicated at the left. Twenty micrograms of *A. franciscana* genomic DNA was digested with the restriction enzymes indicated above the gel lanes.

Fig. 4. Northern blot analysis of *A. franciscana* p70^{S6k}. (A) Expression of two transcripts of p70^{S6k} mRNA during *A. franciscana* development. Total RNA (~170 µg) from quiescent embryos (lane 0) and from cysts incubated in hatching medium at 30°C for the indicated time (h), were analyzed. (B) Quantification of p70^{S6k} mRNA expression during *A. franciscana* PED (see Materials and methods).

Fig. 5.

Superose 6 chromatography of supernatant from activated cysts and S6 kinase activity and Western analyses of the fractions. (A) S6 kinase activity from activated cysts. *A. franciscana* extract supernatant (AES) (200 μL, 18–25 mg/mL total protein) from cysts incubated in hatching medium for 2.5 h (activated cysts), was chromatographed at 0.3 mL/min on a Superose 6 HR 10/30 column equilibrated in Superose 6 Buffer, and 0.6-millilitre fractions were collected. Fractions were assayed for S6 kinase activity (closed circles) and protein elution (solid line). 1–3, molecular-mass standards: thyroglobulin (669 kDa), BSA (67 kDa), and RNase A (13.7 kDa), respectively. (B) Western blot analysis for activated cyst. Portions of the Superose 6 fractions (35 μL) or 5 μL of AES were subjected to Western blot analysis with an immunopurified polyclonal antibody against an N-terminal *A. franciscana* p70^{S6k} synthetic peptide. Left, protein standards: phosphorylase B (103.1 kDa), BSA (67.5 kDa), and ovalbumin (44.45 kDa); Right, position of 5 bands (1–5) visible on the original film.

Fig. 6. Superose 6 chromatography of supernatant from control cyst' and S6 kinase activity and Western analyses of the fractions. (A) S6 kinase activity. (B) Western blot. Experiment and labeling as in Fig. 5, except that AES was prepared from quiescent cysts that had been suspended in rehydration medium and homogenized immediately after recovery of the pellet by centrifugation.

Fig. 7. Western blotting and S6 kinase assays after immunoprecipitation with anti-p70^{S6k} antibody. (A) Immune complexes were isolated from portions (100 μ L) of Superose 6 fractions from activated cysts using purified antibody against an N-terminal *A. franciscana* p70 S6 kinase synthetic peptide and subjected to Western analysis with the same antibody. Labeling, as in Figs. 5B, 6B. The band underneath the five specific bands is the IgG heavy chain present in all of the immune complexes. (B) Immune complexes were assayed for S6 kinase activity. Labeling as in Figs. 5A, 6A.