Embryonic-stage-dependent changes in the level of eIF4E-binding proteins during early development of sea urchin embryos

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Summary

The eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs) inhibit translation initiation by binding eIF4E and preventing recruitment of the translation machinery to mRNA. We have previously shown that fertilization of sea urchin eggs triggers eIF4E–4E-BP complex dissociation and 4E-BP degradation. Here, we show that microinjection of eIF4E-binding motif peptide into unfertilized eggs delays the onset of the first mitosis triggered by fertilization, demonstrating that dissociation of the eIF4E–4E-BP complex is functionally important for the first mitotic division in sea urchin embryos. We also show by gel filtration analyses that eIF4E is present in unfertilized eggs as an 80 kDa molecular mass complex containing 4E-BP and a new 4E-BP of 40 kDa. Fertilization triggers the dissociation of eIF4E from these two 4E-BPs and triggers the rapid recruitment of eIF4E into a high-molecular-mass complex. Release of eIF4E from the two 4E-BPs is correlated with a decrease in the total level of both 4E-BPs following fertilization. Abundance of the two 4E-BPs has been monitored during embryonic development. The level of the two proteins remains very low during the rapid cleavage stage of early development and increases 8 hours after fertilization. These results demonstrate that these two 4E-BPs are down- and upregulated during the embryonic development of sea urchins. Consequently, these data suggest that eIF4E availability to other partners represents an important determinant of the early development of sea urchin embryos.

Key words: Eukaryotic initiation factor 4E (eIF4E), eIF4E-binding protein (4E-BP), Cap-dependent translation, Cell cycle, Sea urchin, Embryonic development

Introduction

Regulation of mRNA translation is an important regulatory step in gene expression in different physiological and physiopathological processes including development (Wickens et al., 2000), apoptosis (Clemens et al., 2000), cell proliferation (Mathews et al., 2000) and cancer pathogenesis (Holland et al., 2004). The eukaryotic initiation factor 4E (eIF4E) is a major target for regulation of translation initiation (Sonenberg and Gingras, 1998; Raught et al., 2000) and plays an important role in the regulation of the cell cycle (Mamane et al., 2004). eIF4E is also thought to have a role during reproduction and embryogenesis (Klein and Melton, 1994; Amiri et al., 2001; Cormier et al., 2001; Robalino et al., 2004).

eIF4E is a mRNA-cap-binding protein (Sonenberg et al., 1978) and is a major target for the regulation of cap-dependent translation (Sonenberg et al., 1979). eIF4E functions in conjunction with eukaryotic initiation factor 4G (eIF4G), a large scaffolding protein that acts as a docking site for several proteins including eukaryotic initiation factors 4A (eIF4A) and 3 (eIF3). eIF4E-binding protein [4E-BP; also called phosphorylated heat-and-stable protein, insulin-stimulated (PHAS-I)], is a small protein that inhibits cap-dependent translation (Haghighat et al., 1995) by competing with eIF4G for a common site on eIF4E (Mader et al., 1995). Although three 4E-BP homologs (4E-BP1, 4E-BP2 and 4E-BP3) exist in mammals (Pause et al., 1994; Poulin et al., 1998), only one ortholog has been described in invertebrates (Bernal and Kimbrell, 2000; Cormier et al., 2001; Miron et al., 2001). In mammals, binding of 4E-BPs to eIF4E is regulated by their phosphorylation state. Underphosphorylated forms of 4E-BPs interact with eIF4E, whereas the hyperphosphorylated forms do not (Pause et al., 1994). 4E-BP is a downstream effector of phosphoinositide (PI) 3-kinase and the Akt/protein kinase B (PKB) (Gingras et al., 1998). In mammalian cells, 4E-BP phosphorylation is also dependent on FRAP/mTOR (for 'FKBP12 and rapamycin-associated protein/mammalian target of rapamycin') (Gingras et al., 2001). In sea urchin, 4E-BP is rapidly phosphorylated (Cormier et al., 2001) and degraded following fertilization, and this involves a rapamycin-sensitive TOR signalling pathway (Salaün et al., 2003).

Previous results suggested that the release of eIF4E from its translational repressor 4E-BP is a crucial event for the first

mitotic division following fertilization (Salaün et al., 2003). Unfertilized sea urchin eggs are haploid cells that are arrested after completion of their mitotic division at the G1 stage. Fertilization triggers entry into S-phase and completion of the first mitotic division. Then a rapid and synchronous cleavage period occurs consisting primarily of successive M- and S-phases corresponding to cell division without an increase in overall mass (Hinegardner et al., 1964). The division synchrony in all blastomeres lasts only until the fourth cell division and a regional synchrony appears that is associated with a progressive increase of the cycle lengths of cell division (Masuda and Sato, 1984). At the blastula stage prior to hatching, the regional synchrony vanishes and the divisions then proceed asynchronously (Masuda and Sato, 1984).

We took advantage of this system to analyse the association of eIF4E with 4E-BP during these different cell divisions following fertilization of eggs and during the early development of sea urchin embryos. Here we show that microinjection of a peptide corresponding to the eIF4E-binding site of 4E-BP1 affects the first mitotic division triggered by fertilization. We report the existence of a heavy 4E-BP-like protein that associates with eIF4E and we show that, during subsequent embryonic development, the amount of 4E-BPs is highly regulated. Taken together, our results suggest that eIF4E release from these two 4E-BPs, and consequently eIF4E availability to other partners, are crucial events for cell divisions occurring during the embryogenesis of sea urchins.

Materials and Methods

Reagents

Acetylcholine, 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF) and glycine were purchased from Interchim. Sodium orthovanadate, EDTA, EGTA, β-glycerophosphate, dithiothreitol (DTT), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), sodium fluoride, *p*-nitrophenyl phosphate, disodium phenylphosphate, leupeptin, aprotinin, Tween 20, protamine sulfate and rapamycin were obtained from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham. Alexafluor488 dextran was purchased from Molecular Probes. ECL detection reagents, 7-methyl-GTP Sepharose 4B beads and a Sephacryl S-200 high-resolution column were obtained from Amersham Pharmacia Biotech. Rabbit polyclonal antibodies directed against human 4E-BP2 have been described previously (Rousseau et al., 1996). Mouse monoclonal antibody directed against rabbit eIF4E was purchased from Transduction Laboratories. Anti-mouse actin and anti-mouse tubulin antibodies were purchased from Sigma. Goat anti-mouse and swine anti-rabbit IgG (horseradish peroxidase-coupled) were obtained from Dako. The eIF4E-binding (wild type) and variant eIF4E-binding peptides were a gift from C. Proud (University of Dundee, UK). The amino acid sequence of the peptide corresponding to 4E-BP1 (wild type) is RIIYDRKFLMEC. The peptide corresponding to a variant peptide in which the tyrosine and the leucine (underlined) are changed to alanine is RIIADRKFAMEC.

Preparation of gametes and determination of cleavage rates

Sphaerechinus granularis sea urchins collected in the Brest area of France were kept in running seawater and used within 5 days. Spawning of gametes, in vitro fertilization, and culturing of eggs and embryos in Millipore-filtered seawater were performed as described previously (Marc et al., 2002). A stock solution of 20 mM rapamycin was prepared in ethanol and stored at –20°C. A final concentration of 20 μM rapamycin was added to eggs 10 minutes before fertilization. In microinjection experiments, dejellied unfertilized eggs were placed in a line on a 1% protamine sulphate-coated dish. 4E-BP1 peptides were diluted at 10 mM final concentration in a microinjection buffer (10 mM HEPES pH 7.0, 200 mM KCl, 550 mM mannitol) containing 1 mM Alexafluor488 dextran to allow visualization of injected eggs. The microinjection system resulted in the injection of approximately 1% of the volume of the egg (De Nadai et al., 1998). Cleavage was scored by observation under a light microscope.

Preparation of cell lysates

At the indicated times following fertilization, cells were collected by centrifugation in a Heraeus Labofuge 4000 centrifuge for 2 minutes at 2000 ***g***; the cell pellet was frozen in liquid nitrogen and stored at –80°C. Cells were lysed by passage through a 25 G syringe in one cell volume of 2× binding buffer [40 mM Hepes pH 7.4, 100 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 100 mM sodium fluoride, 10 mM ATP, 20 mM tetrasodium pyrophosphate (PPi), 100 mM NaCl, 0.4 mM EDTA, 2 mM dithiothreitol, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF), and 20 μg/ml of aprotinin and leupeptin]. For gel filtration experiments and immunopurification of 4E-BPs, cell lysates were centrifuged for 30 minutes at 2200 ***g*** (HS-4 rotor) at 4°C in a Jouan KR 22 centrifuge. For all other experiments, cell lysates were centrifuged for 15 minutes at 16,000 ***g*** at 4°C in an Eppendorf centrifuge 5415R and the supernatants were stored at –20°C before use. Protein quantification was performed in duplicate by the Bradford assay.

Gel filtration experiments

After centrifugation of cell lysates, supernatants were recovered and centrifuged for 1 hour at 100,000 ***g*** at 4°C in a Beckman XL-70 ultracentrifuge. Cytosols were then filtered through a 22 μm filter and loaded onto a Sephacryl-200 high-resolution XK16/70 column equilibrated with 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM DTT, 2 mM EDTA. Gel filtrations were carried out using a High Precision Pump P-500 and a LV-3 valve (Amersham). Fractions were collected in 5 ml plastic tubes using a graduated cylinder and stored at –20°C. Typically, 20 μl of each fraction were loaded for SDS-PAGE and blot analyses.

Isolation of eIF4E and associated proteins

Isolation of eIF4E from the gel filtration elution fractions or from egg extracts was performed using m^{7}GTP beads as described previously (Salaün et al., 2004). Briefly, extracts were mixed with 25 μl of m^{7}GTP-Sepharose beads (m^{7}GTP column). After 60 minutes incubation at 4°C, columns were washed three times with 1 ml of 1× binding buffer containing 100 mM NaCl. Laemmli sample buffer was directly added to the beads. Typically, half of the purified sample was loaded for SDS-PAGE and blot analyses.

Immunopurification of sea urchin 4E-BPs

After centrifugation of cell lysates, supernatants were recovered and centrifuged for 1 hour at 100,000 ***g*** at 4°C in a Beckman XL-70 ultracentrifuge. 500 μl of recovered supernatants were incubated for 2 hours at 4°C in batches with 1% BSA-pre-saturated immobilized protein A beads (50 μl packed beads) covalently coupled to 4E-BP2 antibodies, in 1 ml of IP buffer (50 mM Tris-HCl pH 7.6, 500 mM NaCl, 1% Nonidet P40). Beads were then washed three times in the IP buffer and twice in 500 mM NaCl and 50 mM Tris-HCl (pH 7.6). Laemmli sample buffer was directly added to the beads. Typically, a third of the sample was loaded for SDS-PAGE and blot analyses.

Western blot analyses

Cell extracts, gel filtration-eluted fractions or proteins bound to the m^7GTP beads were resolved by one-dimensional (15% acrylamide) SDS-PAGE using the Mini-Protean 2 cell system (Bio-Rad Laboratories) and analysed by western blotting. Western blot analyses were performed using 0.22 μm nitrocellulose membranes (Towbin et al., 1979). Membranes were incubated with rabbit polyclonal antibodies directed against human 4E-BP2 (1:2000) (Rousseau et al., 1996), mouse monoclonal antibody directed against rabbit eIF4E (1:2000), or mouse monoclonal antibody directed against human tubulin (1:1000) or directed against human actin (1:2000) in 20 mM Tris-HCl (pH 7.6), 5% skimmed milk and 0.1% Tween 20 at room temperature. Antigen-antibody complexes were measured by chemiluminescence using horseradish peroxidase-coupled secondary antibodies and ECL reagent. Signals were quantified using the public domain NIH Image program (written by W. Rasband at the US National Institutes of Health).

Far-western analysis

Far-western blots were performed using ^{32}P-labelled, heart muscle kinase (HMK)-tagged recombinant murine eIF4E, as described previously (Pause et al., 1994). Briefly, proteins were separated on SDS-PAGE and transferred onto a nitrocellulose membrane by western blot. Membranes were pre-incubated in HBB buffer (25 mM HEPES-KOH pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Nonidet P40 and 5% skimmed milk) for 2 hours at 4°C and then incubated in Hyb75 buffer (20 mM HEPES-KOH pH 7.7, 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P40 and 1% skimmed milk) containing ^{32}P-labelled HMK-eIF4E (250,000 cpm/ml) overnight at 4°C. After extensive washing in Hyb75 buffer, the membrane was dried, autoradiographed and quantified as described above.

Results

Microinjection of an eIF4E-binding motif peptide into unfertilized eggs affects the onset of the first mitotic division of the sea urchin embryos

We investigated the implications of the release of eIF4E from the translational repressor 4E-BP in the control of the first mitotic division of sea urchin eggs. We used a peptide that has been developed based on the conserved eIF4E-binding motif within human 4E-BP1 (Herbert et al., 2000). First, we tested the ability of the peptide to inhibit the association between endogenous 4E-BP and eIF4E in sea urchin extracts (Fig. 1A). Unfertilized egg extracts were incubated with 10 μM of eIF4E-binding peptide (RIIYDRKFLMEC), corresponding to the eIF4E-binding sequence of the human 4E-BP1, or with 10 μM of a variant eIF4E-binding peptide (RIIADRKFAMEC). After 1 hour incubation, 4E-BP bound to eIF4E was analysed after affinity purification of eIF4E on m^7GTP columns. The eIF4E-binding peptide (wild type) affected the association between eIF4E and 4E-BP, whereas the variant eIF4E-binding peptide did not affect the association between the two proteins (Fig. 1A). On the basis of this result, we tested the effects of the peptides microinjected into unfertilized eggs on the first mitotic division following fertilization (Fig. 1B). Peptides were microinjected at a final concentration of 10 μM. Fertilization per se was not affected by microinjection, since the fertilization membranes were raised normally after microinjection (data not shown). Strikingly, the eIF4E-binding peptide (wild type) microinjected at this concentration delayed the first cleavage efficiently, whereas the variant eIF4E-binding peptide did not affect the first mitotic division compared with the control. These data provide a direct demonstration that the release of eIF4E from 4E-BP is necessary for the onset of the first cell cycle of sea urchin embryos and underlines the importance eIF4E/4E-BP complex regulation following the fertilization of sea urchin eggs.

Fertilization induces the recruitment of eIF4E from an 80 kDa complex to a high-molecular-mass complex

eIF4E functions in conjunction with other partners. The hypothesis that eIF4E, released from 4E-BP, could be recruited into a high-molecular-mass complex was therefore tested by gel filtration. Extracts from unfertilized eggs and eggs 30 minutes post-fertilization were separated on a Sephacryl-200 column (Fig. 2A) and aliquots from collected fractions were analysed after electrophoresis by immunoblotting with anti-4E-BP2 and anti-eIF4E antibodies. 4E-BP (monomer size16 kDa) eluted as a single peak of 80 kDa from unfertilized eggs

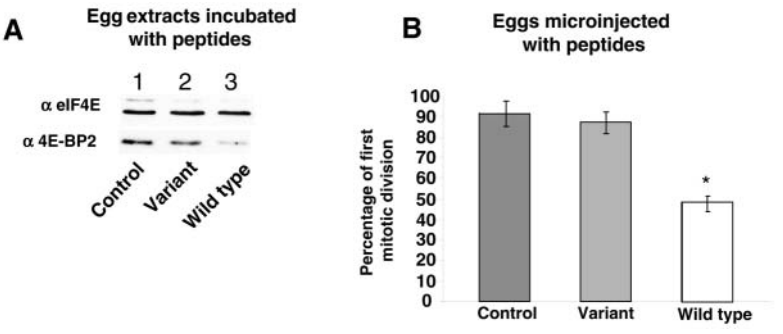

Fig. 1. 4E-BP peptide affects eIF4E–4E-BP association and the first mitotic division of sea urchin embryos. (A) Treatment of extracts obtained from unfertilized eggs with the 4E-BP peptide leads to dissociation of eIF4E from 4E-BP. Control extract (lane 1) or extract treated with 10 μM variant eIF4E-binding peptide (lane 2) or 10 μM eIF4E-binding peptide (wild type) (lane 3) were incubated for 1 hour at 4°C. After incubation, eIF4E was purified from each extract using 50 μl m^7GTP columns as described in the Materials and Methods. Proteins bound to the beads were separated by SDS-PAGE and subjected to western blotting using antibodies to eIF4E (top panels) or 4E-BP (bottom panels). Data are representative of three independent experiments. (B) Microinjection of 4E-BP peptide into unfertilized eggs impedes the first mitotic division triggered by fertilization. The eIF4E-binding peptide (wild type; white bar) or variant eIF4E-binding peptide (light grey bar) were injected at a final intracellular concentration of 10 μM. Control corresponds to unfertilized eggs microinjected with buffer (dark grey bar). Cleavage was scored by observation under a light microscope at 180 minutes after fertilization. An average of 100 unfertilized eggs were injected for each compound in each experiment and vertical bars represent the standard deviation of three independent experiments. Significance was assessed using Fisher's *F*-test and Student's *t*-test. An asterisk indicates a significant difference between eggs microinjected with eIF4E-binding peptide (wild type) and eggs microinjected with variant eIF4E-binding peptide or control (* corresponds to $P<0.01$).

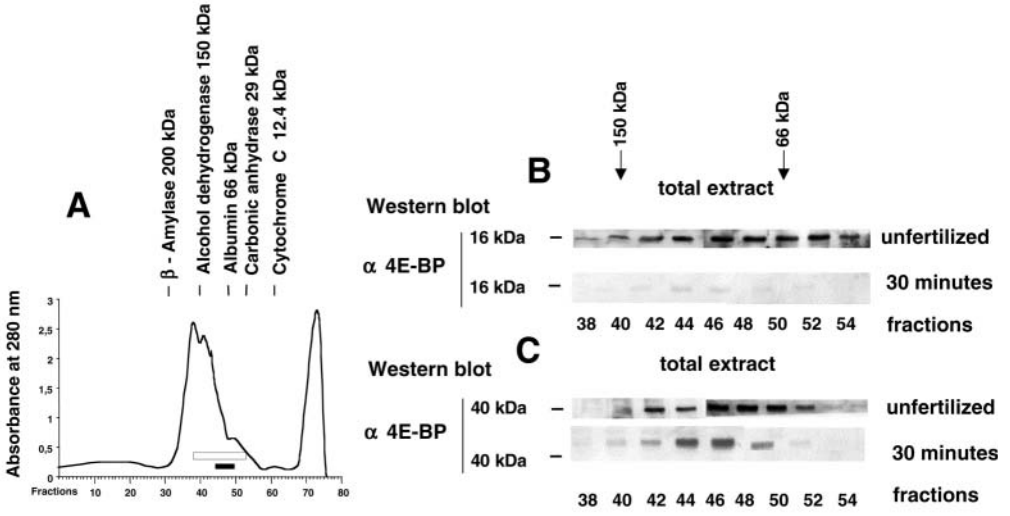

Fig. 2. 4E-BP is present in an 80 kDa complex in unfertilized eggs. (A) Proteins (5 mg) from extracts prepared from unfertilized or from extracts from 30 minutes post-fertilization eggs were separated by chromatography on a Sephacryl-200 column and were monitored by absorbance at 280 nm. The bars show the position of 4E-BP from unfertilized (open bar) and from 30 minutes post-fertilization (filled bar) eggs as determined by western blot analysis of each fraction using an anti-human 4E-BP2 antibody. The column was calibrated with the molecular mass standards indicated at the top. (B,C) Aliquots of each eluted fraction were resolved by electrophoresis and analysed by western blotting using 4E-BP2 antibodies. The position of the molecular mass standards (in kDa) used to calibrate the gel filtration column is indicated by arrows at the top of the figure. Data are representative of two independent experiments.

and, as expected, the amount of the protein decreased dramatically after fertilization (Fig. 2B, compare bottom and top panels). Strikingly, the anti-4E-BP2 also recognized a second protein, with an apparent molecular mass of 40 kDa, that co-eluted with 4E-BP in unfertilized eggs (Fig. 2C, top panel). Furthermore, the amount of the 40 kDa protein also decreased in the extracts from 30 minutes post-fertilization (Fig. 2C, compare top and bottom panels). We have previously shown by western blot analysis that eIF4E is undetectable in egg extracts of sea urchins (Salaün et al., 2003). As sample dilution is inevitable when using gel filtration, eIF4E was consequently never detectable in the eluted fractions. Sephacryl-200 eluted fractions were applied to m^7GTP-Sepharose columns and, therefore, the amount of enriched eIF4E was monitored in each fraction after purification of the protein on m^7GTP columns (Fig. 3). In unfertilized eggs, eIF4E was present in fractions 40-50 as a single peak of 80 kDa (Fig. 3A, top panels). In 30 minutes post-fertilization embryos, although part of the eIF4E was still recovered from fractions 40-50, a signal was detected in fractions corresponding to proteins eluting at around 180 kDa (Fig. 3A, bottom panels), indicating that part of the eIF4E was recruited to a high-molecular-mass complex (Fig. 3B, grey arrow). The 40 kDa protein and 4E-BP co-purified with eIF4E on m^7GTP columns and eluted as a peak of 80 kDa in unfertilized eggs (Fig. 3A, top panels). As expected, in 30 minutes post-fertilization embryos, the total amount of 4E-BP and the 40 kDa protein that co-purified with eIF4E on m^7GTP columns decreased dramatically and the main remaining proteins co-purified with eIF4E in the 80 kDa complex (Fig. 3A, bottom panels).

Taken together, these data suggest that, in unfertilized eggs, eIF4E is present in a complex of about 80 kDa containing 4E-BP and the 40 kDa protein. Fertilization induces dissociation of the 16 kDa 4E-BP and the 40 kDa protein from eIF4E that recruits other partners and consequently shifts to high-molecular-mass complexes.

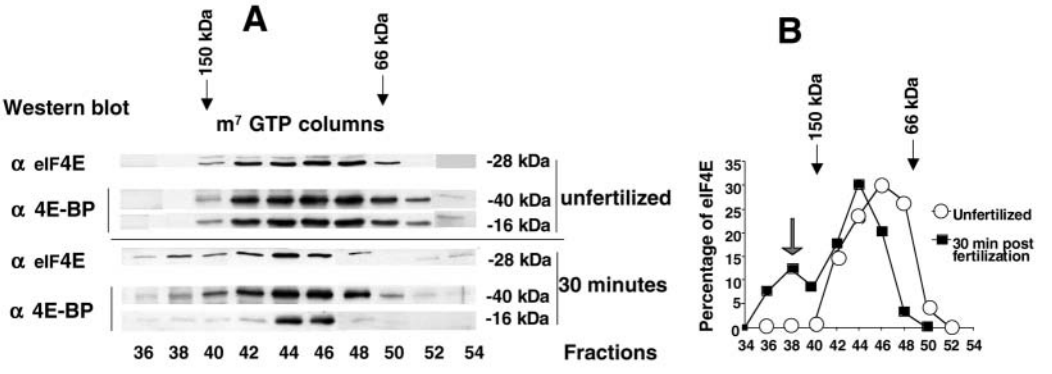

Fig. 3. eIF4E shifts from a 80 kDa complex in unfertilized eggs to a high-molecular mass complex following fertilization. (A) Eluted fractions from the Sephacryl-200 gel filtration purification of unfertilized (top) or 30 minutes post-fertilization (bottom) extracts were applied to a m^7GTP column and bound proteins were subjected to western blotting using anti-human eIF4E and anti-human 4E-BP2 antibodies. Arrows at the top of the figure correspond to the position of molecular mass standards (in kDa) used to calibrate the gel filtration column and the numbers at the bottom correspond to the fraction from the gel filtration purification showed in Fig. 2A. (B) Quantification of the results obtained from unfertilized (open circle) or 30 minutes post-fertilization (filled square) extracts. eIF4E amounts in each fraction were expressed as a percentage of the total amount of eIF4E contained in unfertilized or 30 minutes post-fertilization extracts, respectively. Arrows at the top of the figure correspond to the position of the molecular mass standards (in kDa) used to calibrate the gel filtration column showed in Fig. 2A. These data are representative of more than two independent experiments.

The 40 kDa protein is an eIF4E-binding protein

The results presented above, indicate that a protein of 40 kDa co-purifies with eIF4E and that the 4E-BP2 antibody recognizes this 40 kDa protein. We checked that the same antibody also detected this 40 kDa protein in a total extract from unfertilized eggs (Fig. 4A, lane 1). We then asked whether this 40 kDa protein detected in the extracts might interact directly with eIF4E. To address this question, total extract was tested by far-western blotting using recombinant ^{32}P-labelled HMK-eIF4E as a probe, which corresponds to an exogenous eIF4E (Pause et al., 1994). In addition to the 16 kDa 4E-BP, a 40 kDa protein interacted with radiolabelled eIF4E (Fig. 4A, lane 2). It was then important to check whether the 40 kDa protein detected by the 4E-BP2 antibody was also able to directly associate with exogenous eIF4E. Proteins from unfertilized eggs were immunopurified using 4E-BP2 antibodies coupled to Sepharose beads. After electrophoresis, immunopurified proteins were analysed by western and far-western blotting (Fig. 4B, lanes 1). In addition to 4E-BP, the 40 kDa protein was efficiently immunopurified by the 4E-BP2 antibody (Fig. 4B, left panel, lane 1). Furthermore, exogenously added, radiolabelled eIF4E also bound to the immunopurified 40 kDa protein (Fig. 4B, right panel, lane 1). Proteins purified from unfertilized eggs using m^{7}GTP columns were analysed by western and far-western blotting (Fig. 4B, lanes 3). In addition to 4E-BP, the 40 kDa protein co-purified with endogenous eIF4E after m^{7}GTP column purification (Fig. 4B, left panel, lane 3) and bound to the exogenous radiolabelled eIF4E (Fig. 4B, right panel, lane 3). Taken together, these data demonstrate that the 40 kDa protein is a eIF4E-binding protein.

The results shown in Fig. 3A suggested that this new eIF4E-binding protein was partially dissociated from eIF4E in eggs taken 30 minutes after fertilization. We then asked whether the amount of the 40 kDa protein might be modified following fertilization of the eggs. The 40 kDa protein was monitored in extracts obtained from unfertilized eggs and from embryos 60 minutes after fertilization. Interestingly, the amount of the 40 kDa protein decreased dramatically in embryos harvested at 60 minutes after fertilization (Fig. 5A, compare lanes 1 and 2). We have previously shown that both dissociation of the eIF4E–4E-BP complex and 4E-BP degradation are mediated by a rapamycin-sensitive mTOR pathway induced by fertilization (Salaün et al., 2003). We therefore tested whether rapamycin could affect the disappearance of the 40 kDa protein and/or its dissociation from eIF4E triggered by fertilization. Strikingly, rapamycin prevented both the decrease in the 40 kDa protein in total extracts (Fig. 5A, compare lanes 1 and 3) and its dissociation from eIF4E (Fig. 5B) following fertilization. We demonstrated that a 4E-BP peptide affects the association between eIF4E and 4E-BP (Fig. 1A). It was then interesting to test whether the 4E-BP peptide could also affect the association between the endogenous 40 kDa protein and eIF4E (Fig. 5C). The 40 kDa protein bound to eIF4E was analysed after incubation of the peptide and affinity purification of eIF4E on m^{7}GTP from unfertilized egg extracts. The eIF4E-binding peptide (wild type) affected the association between eIF4E and the 40 kDa protein (Fig. 5C, compare lanes 1 and 3), whereas the variant eIF4E-binding peptide had no significant effect (Fig. 5C, compare lanes 1 and 2). Taken together, these data show that this new 40 kDa eIF4E-binding protein behaves similarly following fertilization to the sea urchin 4E-BP already identified.

4E-BP and the 40 kDa protein are upregulated during the late embryonic development of sea urchin

The results presented above demonstrate that the amount of the translational repressor 4E-BP and the amount of the 40 kDa protein is downregulated during early development of sea urchin embryos. The amount of the two proteins was then monitored during later stages of the sea urchin embryonic development. Extracts prepared from unfertilized eggs and from embryos taken every 2 hours until 14 hours after fertilization were analysed by western blotting (Fig. 6A). As expected, fertilization triggered a decrease in both the 4E-BP and the 40 kDa proteins. The level of the two proteins remained very low for 4 hours. Strikingly, the amount of both proteins

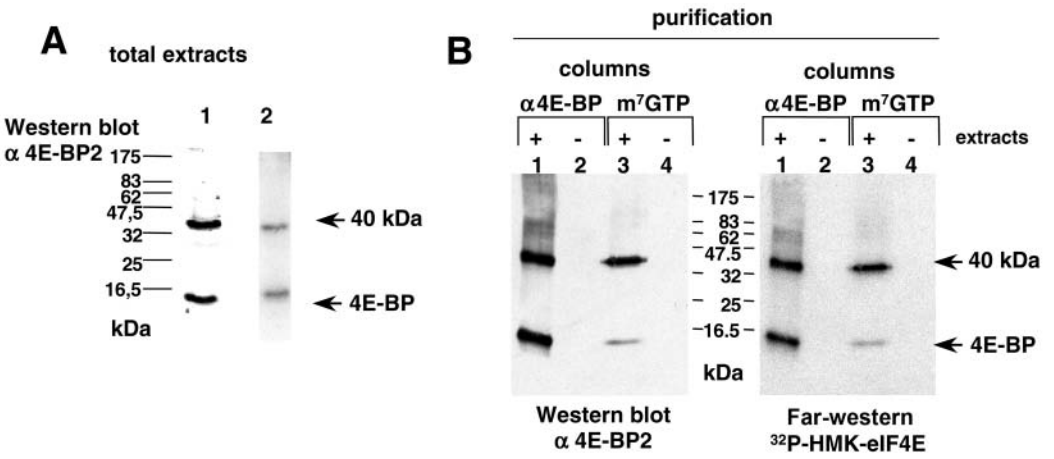

Fig. 4. The 40 kDa protein associates with eIF4E. (A) Total extract obtained from unfertilized eggs was subjected either to western blotting using 4E-BP2 antibody (lane 1) or to far-western analysis (see Materials and Methods) using ^{32}P-labelled mouse recombinant HMK-eIF4E as a probe (lane 2). (B) Western blot and far-western analyses of the purified 40 kDa protein. Proteins from unfertilized egg extracts were immunopurified using 4E-BP2 antibodies coupled to Sepharose beads (1) or using a m^{7}GTP column (3). The proteins fixed on the beads were resolved either by western blotting using 4E-BP2 antibodies (left panel) or by far-western analysis using an eIF4E-HMK probe (right panel). 4E-BP2-coupled Sepharose beads alone (lane 2) or m^{7}GTP-column beads alone (lane 4) were used as controls. Arrows point to the positions of 4E-BP and the 40 kDa protein. Data are representative of at least three independent experiments.

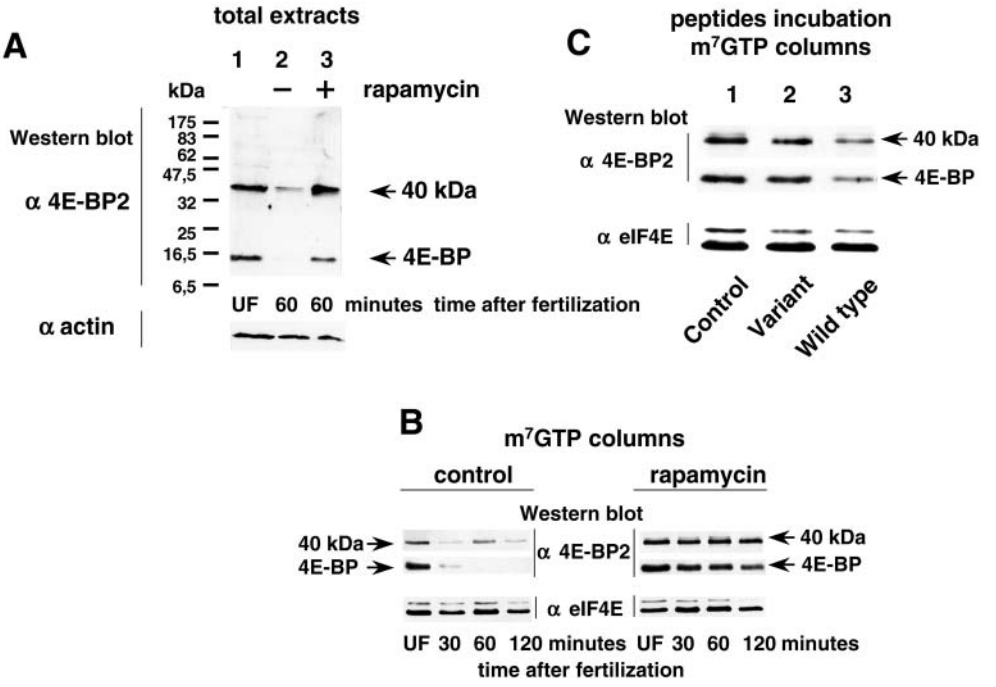

Fig. 5. The new 40 kDa eIF4E-binding protein presents behaviour similar to that of the sea urchin 4E-BP already identified. (A) Rapamycin inhibits the disappearance of the 40 kDa protein following fertilization. The total amount of the 40 kDa protein from unfertilized eggs (lane 1) and from untreated (lane 2) or rapamycin-treated (lane 3) eggs obtained 60 minutes following fertilization was analysed by western blotting using 4E-BP2 antibodies (top panel). A western blot anti-actin was also performed (bottom panel) as loading control. (B) Rapamycin inhibits the dissociation of the 40 kDa protein from eIF4E. Proteins affinity-purified using m^7GTP columns prior to fertilization or at the indicated times following fertilization of untreated (left) or rapamycin-treated (right) eggs were subjected to western blotting using anti-4E-BP2 (top) or anti-eIF4E (bottom) antibodies. The data are representative of at least five independent experiments. (C) Treatment of extracts obtained from unfertilized eggs with the 4E-BP peptide also leads to the dissociation between eIF4E and the 40 kDa protein. Control extract (lane 1) or extract treated with 10 μM variant eIF4E-binding peptide (lane 2) or 10 μM eIF4E-binding peptide (lane 3) were incubated for 1 hour at 4°C. After incubation, eIF4E was purified using a m^7GTP column. Proteins bound to the beads were separated by SDS-PAGE and subjected to western blotting using 4E-BP (top panels) or eIF4E (bottom panels) antibodies.

increased gradually until 10 hours post-fertilization, demonstrating that the two proteins are upregulated during late development of the sea urchin embryo. The same extracts were then tested by far-western blotting (Fig. 6B). Exogenous radiolabelled eIF4E interacted both with 4E-BP and with the 40 kDa protein that re-appeared in late development. To test whether 4E-BP and the 40 kDa protein reassociated with the endogenous eIF4E during late development, 4E-BP and the 40 kDa protein bound to eIF4E were monitored after affinity purification of eIF4E on m^7GTP columns at the indicated times after fertilization (Fig. 6C). Although the amount of eIF4E that bound to the column did not change significantly during the development of the embryos (Fig. 6C, top panel), the amount of 4E-BP (bottom panel) and the 40 kDa protein (middle panel) that is associated with purified eIF4E decreased dramatically following fertilization and then increased strongly in late development, around 8-10 hours post-fertilization. Taken together, these data demonstrate that 4E-BP and the 40 kDa protein re-appear during late development and reassociate with eIF4E.

Discussion

A peptide corresponding to the eIF4E-binding site of the human 4E-BP1 protein was able to affect the association of eIF4E–4E-BP complex in extracts from unfertilized sea urchin eggs. These data are consistent with the binding of mammalian eIF4E to sea urchin 4E-BP in far-western analyses and demonstrate that functional binding sites between eIF4E and 4E-BP are highly conserved throughout deuterostome evolution. The nonpolar amino acid residues of eIF4E that are likely to interact with 4E-BP (Marcotrigiano et al., 1997) are conserved in starfish eIF4E (Lee et al., 2000). However, interaction between the two proteins 4E-BP and eIF4E has not been tested in starfish. This point is important since, in zebrafish, although two eIF4E proteins contain all the known residues required for interaction with the 4E-BPs, only one is able to interact with 4E-BP (Robalino et al., 2004). Whether several eIF4E isoforms exist in echinoderms remains unknown.

By microinjection of 4E-BP peptide into sea urchin eggs, we have provided a direct demonstration that eIF4E release from the translational inhibitor 4E-BP is required for the onset of the first mitotic division triggered by fertilization of sea urchin eggs. This result is consistent with the fact that eIF4E–4E-BP complex dissociates rapidly following fertilization of the eggs (Cormier et al., 2001) and that a rapamycin-sensitive pathway is involved in the completion of the first mitotic division of sea urchin embryos (Salaün et al., 2003). Taken together, these results suggest that eIF4E is required for the dramatic rise in the rate of translation initiation that occurs rapidly after fertilization of sea urchin eggs (Lopo et al., 1988). Our results also suggest that 4E-BP is required for translational silencing of maternal mRNA before fertilization. The premise for this hypothesis is the fact that 4E-BP levels are the same or higher than eIF4E levels prior to fertilization (Salaün et al., 2003), however precise quantitation of 4E-BP relative to eIF4E remains to be determined. The suggestion of a role for the cap-binding protein eIF4E in the increase of protein synthesis induced by fertilization is underscored by our observation that fertilization leads to the recruitment of the eIF4E protein to high-molecular-mass protein complexes. In mammals, the initiation factor eIF4E is known to form the eIF4F complex by

association with eIF4A and eIF4G (Raught et al., 2000). In a cell-free translation system using heterologous proteins, the eIF4F complex has been shown to be a crucial factor whose activity is stimulated following fertilization in sea urchin (Jagus et al., 1992). Therefore, it is expected that eIF4G and eIF4A are associated with eIF4E following fertilization. The existence of these two proteins in sea urchin eggs is supported by the identification of partial cDNA encoding eIF4A and a complete cDNA encoding eIF4G (EMBL, accession number AJ634049) from a sea urchin library (our unpublished data). However, the fact that sea urchin fertilization induces eIF4F formation needs to be confirmed. Although the mammalian eIF4F is described classically as a complex of approximately 300 kDa, our data indicate that a fraction of eIF4E is recruited into a complex of around 180 kDa. We cannot exclude that several complexes are formed and that other proteins associate with eIF4E following fertilization of eggs. Therefore, identification of the proteins associated with eIF4E in the 180 kDa complex remains to be carried out.

It has been shown previously that introduction of eIF4E-binding peptides into mammalian cells induces rapid cell death, with characteristics of apoptosis (Herbert et al., 2000). The mechanism by which the peptide induces apoptosis remains unclear but cell death might be a result of interference of the peptide with a nuclear function of eIF4E (Herbert et al., 2000). The effect on cell viability can be explained by the fact that eIF4E-binding peptides were linked to the penetratin peptide-carrier sequence, which mediates the rapid transport of peptides across cell membranes. Sea urchin eggs possess functional apoptotic machinery (Voronina and Wessel, 2001). Yet, the microinjection of 4E-BP peptide into unfertilized sea urchin eggs did not affect cell viability. This apparent discrepancy could be explained by the fact that 4E-BP peptide was not linked to the penetratin peptide-carrier in our experiments and that, consequently, microinjected peptide could not interfere with the nuclear function of eIF4E. Therefore, it would be interesting to test the effect of 4E-BP peptide linked to the penetratin peptide-carrier sequence on the induction of apoptosis in sea urchin eggs.

In this study, using convergent approaches, we provide a demonstration that a 40 kDa protein associates with eIF4E in unfertilized sea urchin eggs. Furthermore, we show that this 40 kDa protein is recognized by an antibody directed against human 4E-BP2 and that the 40 kDa protein shares several characteristics with the 4E-BP (Cormier et al., 2001; Salaün et al., 2003). We propose to name this protein H4E-BP for 'heavy molecular mass eIF4E-binding-like protein'.

Finding new regulators of eIF4E is crucial to understanding how eIF4E functions are modulated during different processes such as the cell cycle, cell differentiation or development (von der Haar et al., 2004). In addition to the well-described eIF4E-binding proteins represented by 4E-BPs and eIF4G, new eIF4E partners have recently been discovered. In *Xenopus laevis* oocytes, the protein maskin prevents eIF4F formation by binding eIF4E (Stebbins-Boaz et al., 1999). Similarly, in *Drosophila*, the anterior determinant bicoid is hypothesized to bind simultaneously to eIF4E and the *caudal* 3′ untranslated region, thereby preventing recruitment of eIF4G to *caudal* mRNA (Niessing et al., 2002). More recently, the protein cup has been shown to bind eIF4E and, consequently, to trigger smaug-mediated translational repression (Nelson et al., 2004) and translational repression of the posterior determinant oskar (Wilhelm et al., 2003). In mammals, eIF4E-T is another functional 4E-binding protein, which is involved in the nucleocytoplasmic shuttling of eIF4E (Dostie et al., 2000). In humans, the proline-rich homeodomain protein (PRH) has been identified as binding partner of eIF4E that seems to serve as a negative regulator of the eIF4E-dependent export of a subset of mRNAs (Topisirovic et al., 2003). These eIF4E-binding proteins share a common eIF4E recognition motif, YxxxxLΦ (where x is any amino acid and Φ is an aliphatic residue, usually L, M or F), although the tyrosine of this motif is occupied by a threonine residue in the maskin protein (Stebbins-Boaz et al., 1999) and the PRH contains a related motif in which the hydrophobic Φ is exchanged for a polar glutamine (Topisirovic et al., 2003). The protein H4E-BP is recognized by the anti-4E-BP2 antibody. This rules out the possibility that H4E-BP corresponds to one of these novel eIF4E-binding proteins. It has recently been demonstrated that

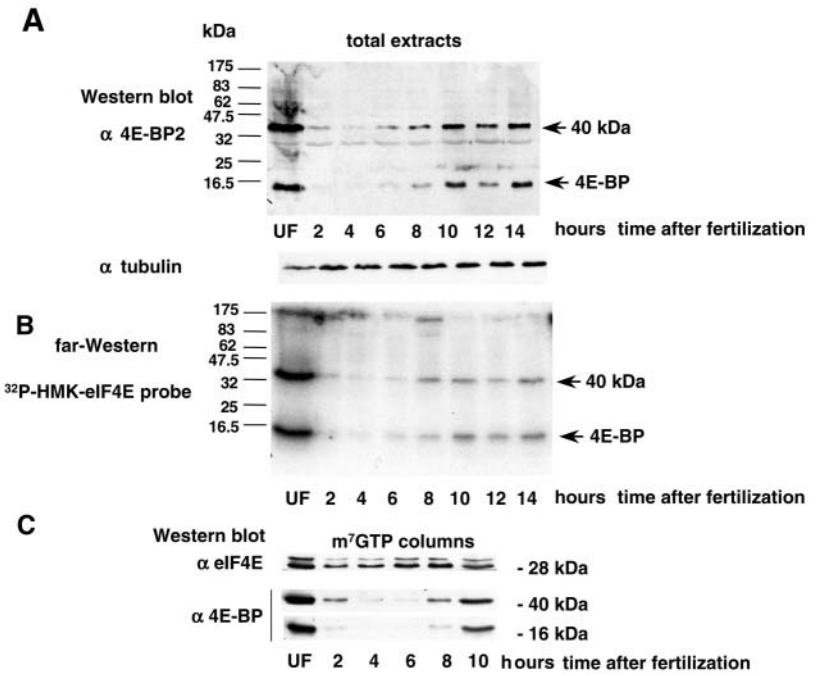

Fig. 6. 4E-BP and the 40 kDa proteins re-appear during late embryogenesis of sea urchin. (A) Total extracts of unfertilized eggs or eggs at the indicated times following fertilization were subjected to western blotting using 4E-BP2 or eIF4E antibodies. Tubulin antibody was used as a loading control. Data are representative of at least five independent experiments. (B) Total extracts prior to fertilization or at the indicated times following fertilization were subjected to far-western blotting using an eIF4E-HMK probe. Data are representative of two independent experiments. (C) Proteins affinity purified using an m^7GTP column prior to fertilization or at the indicated times following fertilization were subjected to western blotting using 4E-BP or anti-eIF4E antibodies. Data are representative of at least two independent experiments.

a transcript arises from the fusion of 4E-BP3 with the mammalian homologue of the *Drosophila MASK* (for 'multiple ankyrin repeats, single KH domain') gene (Poulin et al., 2003). This transcript encodes the protein MASK-BP3^{ARF} using an alternative reading frame. Therefore, two different proteins are encoded by the same exon of 4E-BP3, with the protein MASK-BP3^{ARF} not presenting any similarity to the protein 4E-BP3 (Poulin et al., 2003). By contrast, H4E-BP presents many similarities with the sea urchin 4E-BP, ruling out the possibility that the 4E-BP-like sequence is translated in an alternative reading frame. One hypothesis is that H4E-BP could be the result of the fusion of two genes encoding two different protein domains that are in the same reading frame, one containing an unknown protein domain and a second containing the 4E-BP domain. Such a possibility is suggested in a few reports that describe fused proteins resulting from intergenic splicing between neighbouring genes in eukaryotes (Fears et al., 1996; Magrangeas et al., 1998; Thomson et al., 2000; Pradet-Balade et al., 2002). Cloning of the cDNA that encodes H4E-BP is now important to give insight into the mechanism involved in H4E-BP production and to analyse further the role of the heavy form of 4E-BP in sea urchin. Furthermore, since a cDNA encoding 4E-BP from echinoderms is not yet available and there is no 4E-BP sequence accessible in the available sea urchin EST-database (Cameron et al., 2000), cloning of these two cDNAs remains an important goal in the study of early development in marine invertebrates.

We investigated the temporal expression of 4E-BP and H4E-BP during early development. The two eIF4E-binding proteins are abundant in unfertilized eggs, decline abruptly following fertilization and remain low during the rapid cleavage stage. The re-appearance and accumulation of both 4E-BP and H4E-BP in late embryogenesis is progressive until 8-10 hours post-fertilization, which corresponding to the blastula stage of the embryos. This is the first observation that the translational repressor 4E-BP can be down- and upregulated at the protein level during embryonic development. Whether mRNAs coding for these two proteins are also regulated during development remains to be elucidated. Thus, studies on sea urchin early development provide a promising model to investigate the importance and mechanism of regulation of gene expression of these eIF4E-binding proteins.

In mammalian cells, association between eIF4E and its translational repressor 4E-BP is regulated during the cell cycle (Heesom et al., 2001; Pyronnet and Sonenberg, 2001). Consistent with this, eIF4F, and thus cap-dependent translation initiation, plays an essential role in the G1/S transition (Pyronnet and Sonenberg, 2001). Unfertilized sea urchin eggs are haploid cells that are blocked at the G1 stage. We showed previously that cap-dependent translation is crucial for the first mitotic division of sea urchin following fertilization (Salaün et al., 2003). Here we show that the abundance of 4E-BP and H4E-BP is very low during the following cell cycles that alternate rapidly between S-phase and M-phase. The abrupt decrease in the abundance of these 4E-binding proteins triggers a release of eIF4E and could be an important molecular event that allows rapid cell divisions to occur.

Interestingly, the two eIF4E-binding proteins re-appear and associate progressively with eIF4E later during development. Indeed, there is no true mid-blastula transition in sea urchin development, as was shown in amphibians for example (Signoret and Lefresne, 1971; Newport and Kirschner, 1982), but there is a spatially and progressive process. Echinoderm embryos show asymmetric cleavage in the fourth and fifth divisions that cause the formation of different size cells: mesomeres, macromeres and micromeres (Hörstadius, 1973). The cell-cycle lengths progressively increase during the development of the embryo for these different kinds of cells (Masuda and Sato, 1984; Yasuda and Schubiger, 1992). The first cells in which the cell cycle slows down are the micromeres in which lengthening of the fifth cell cycle is due to the acquisition of a G2-phase (Lozano et al., 1998). Macromere and mesomere lineages always undergo shorter cell cycles and continue cycling longer than micromere lineage (Masuda and Sato, 1984). At the blastula stage prior to hatching, the division proceeds in an asynchronous way (Parisi et al., 1978) and this embryonic stage corresponds to a normal cell-cycle length with the presence of gap phases. Therefore, the progressive re-appearance and accumulation of these two eIF4E-binding proteins could play an important role in the control and organization of cell-cycle length during early embryonic development. Since these eIF4E-binding proteins are well recognized by the antibodies, it would be interesting to monitor the level and localization of these proteins during the embryonic development in time-course immunofluorescence experiments.

Our work demonstrates that the association between eIF4E and 4E-BP is highly regulated during sea urchin development. These data indicate that eIF4E is temporally available to other partners in sea urchin embryos and, thus, suggests that eIF4E can play a role in the control of gene expression during development. Other reports have implicated eIF4E in nematode spermatogenesis (Amiri et al., 2001), and embryogenesis in zebrafish and amphibians (Klein and Melton, 1994; Fahrenkrug et al., 1999; Robalino et al., 2004). Furthermore, eIF4E was shown to associate with a protein involved in pattern formation during early *Drosophila* embryogenesis (Niessing et al., 2002; Wilhelm et al., 2003). Therefore, it will be interesting to determine whether sea urchin eIF4E is able to bind to factors involved in patterning in the sea urchin embryos (Angerer and Angerer, 2003).

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