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eIF4E/4E-BP dissociation and 4E-BP degradation in the first mitotic division of the sea urchin embryo

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Abstract

The mRNA's cap-binding protein eukaryotic translation initiation factor (eIF)4E is a major target for the regulation of translation initiation. eIF4E activity is controlled by a family of translation inhibitors, the eIF4E-binding proteins (4E-BPs). We have previously shown that a rapid dissociation of 4E-BP from eIF4E is related with the dramatic rise in protein synthesis that occurs following sea urchin fertilization. Here, we demonstrate that 4E-BP is destroyed shortly following fertilization and that 4E-BP degradation is sensitive to rapamycin, suggesting that proteolysis could be a novel means of regulating 4E-BP function. We also show that eIF4E/4E-BP dissociation following fertilization is sensitive to rapamycin. Furthermore, while rapamycin modestly affects global translation rates, the drug strongly inhibits cyclin B de novo synthesis and, consequently, precludes the completion of the first mitotic cleavage. These results demonstrate that, following sea urchin fertilization, cyclin B translation, and thus the onset of mitosis, are regulated by a rapamycin-sensitive pathway. These processes are effected at least in part through eIF4E/4E-BP complex dissociation and 4E-BP degradation.

Keywords: 4E-BP; eIF4E; 4E-BP degradation; Cyclin B; Mitosis; Fertilization

Introduction

The regulation of mRNA translation is a critical feature of entry and progression through the cell cycle (O'Farrell, 2001; Pyronnet and Sonenberg, 2001). The early steps in the development of sea urchin eggs represent an ideal system for studying the relationships between translation regulation and M-phase completion for the following reasons. (i) Sea urchin embryos are naturally synchronized during early development stages. (ii) Fertilization triggers a rapid rise in protein synthesis independent of mRNA transcription and ribosome biogenesis (Brandhorst and Fromson, 1976; Epel, 1967). (iii) De novo protein synthesis is necessary for M-phase completion but is dispensable for progression through S-phase (Dube, 1988; Wagenaar, 1983).

We reported recently the presence of the translation repressor 4E-BP (eIF4E binding protein) in unfertilized sea urchin eggs and have shown that this protein plays an important role in the first mitotic division of sea urchin embryos (Cormier et al., 2001). 4E-BP is a well-characterized inhibitor of cap-dependent translation (Haghighat et al., 1995) that competitively prevents the interaction between the eukaryotic initiation factors (eIF) 4E and 4G (Mader et al., 1995). eIF4E functions in conjunction with eIF4G, a large scaffolding protein that interacts, among others, with eIF4E, eIF4A, and eIF3 (Raught, 2000). eIF4E is the cap-binding protein. eIF4A is an RNA helicase which, in conjunction with eIF4B, is thought to facilitate ribosome binding by unwinding the mRNA secondary structure. eIF3 is a

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ribosome-associated translation initiation factor. Through its interaction with eIF4E and eIF3, eIF4G provides a physical link between the mRNA 5′ end and the ribosome.

In mammals, three 4E-BP homologs exist (4E-BP1, 4E-BP2, and 4E-BP3) (Pause et al., 1994; Poulin et al., 1998), although only one ortholog has been described thus far in insects and in echinoderms (Bernal and Kimbrell, 2000; Cormier et al., 2001; Miron et al., 2001). In mammals, 4E-BPs binding to eIF4E is reversible, and 4E-BPs affinity for eIF4E is determined by the phosphorylation status of 4E-BPs. The underphosphorylated forms of 4E-BPs interact strongly with eIF4E, whereas the hyperphosphorylated forms do not (Pause et al., 1994). Multiple and hierarchical phosphorylation events are required to release 4E-BP1 from eIF4E (Gingras et al., 2001a). A large body of evidence indicates that phosphoinositide 3′-kinase (PI3K) and FKBP12 and rapamycin-associated protein/mammalian target of rapamycin (FRAP/mTOR) signaling kinases effect the release of 4E-BP1 from eIF4E (Gingras et al., 2001b).

Cap-dependent translation plays an essential role in the G1/S transition. For instance, in budding yeast, a conditional mutation in *cdc33* encoding eIF4E, causes arrest at the G1/S boundary, possibly through inefficient synthesis of the cyclin CLN3 (Danaie et al., 1999; Polymenis and Schmidt, 1997). Treatment of cells with rapamycin (Vezina et al., 1975), an immunosupressive macrolide that blocks TOR activity and 4E-BP phosphorylation (Gingras et al., 2001b), causes eIF4E sequestration in a complex with 4E-BP1. Rapamycin also blocks mammalian cells at G1 possibly through inhibition of 4E-BP phosphorylation and consequent repression of cap-dependent translation inhibition (Beretta et al., 1996).

Sea urchin unfertilized eggs are haploid cells that are arrested after completion of their meiotic divisions at the G1 stage. In all eukaryotes, the onset of mitosis is under the control of a heterodimeric complex composed of the CDK1 kinase and a B-type cyclin (Nigg, 2001; O'Farrell, 2001), the maturation-promoting factor (MPF). CDK1 activity is regulated by phosphorylation on certain key tyrosine and threonine residues (Meijer et al., 1991) and depends absolutely on its association with a cyclin (Meijer et al., 1989; Minshull et al., 1989). The mitotic cyclins A and B were first discovered in sea urchin as key proteins which are rapidly synthesized and degraded during M-phase at each cell cycle (Evans et al., 1983; Minshull et al., 1989).

Here, we investigate the mechanism by which 4E-BP functions in the first mitotic cleavage following fertilization of sea urchin eggs. We show that 4E-BP is rapidly degraded following fertilization, and that both 4E-BP degradation and eIF4E/4E-BP dissociation are sensitive to rapamycin. Furthermore, while rapamycin only partially inhibits the increase in protein synthesis that occurs following fertilization, it strongly blocks cyclin B expression and prevents the first mitotic cleavage. Taken together, these data indicate that 4E-BP degradation and eIF4E release from the translational repressor 4E-BP are critical events in the first mitotic division of sea urchin embryos.

Materials and methods

Chemicals and reagents

Sodium orthovanadate, EDTA, EGTA, Histone 1 (type III-SS), β-glycerophosphate, 4-morpholinepropanesulfonic acid (MOPS), dithiothreitol, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), sodium fluoride, *p*-nitrophenyl phosphate, disodium phenylphosphate, leupeptin, aprotinin, soybean trypsin inhibitor, benzamidine, ATP, Tween 20, and Triton X-100 were obtained from Sigma. 4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF) and glycine were purchased from Interchim. [γ^{32}P]ATP (3000 Ci/mmol) and [^{35}S]L-methionine (1000 Ci/mmol) were obtained from Amersham (France). Optiphase Supermix scintillation liquid was purchased from Perkin–Elmer. The purified protein kinase CDK1/cyclin B from starfish and roscovitine were kindly provided by Dr. L. Meijer (Roscoff, France). ECL detection reagents, CNBR-activated Sepharose, 7-methyl-GTP Sepharose 4B beads were obtained from Amersham Pharmacia Biotech. Mouse monoclonal antibody directed against rabbit eIF4E was purchased from Transduction Laboratories (USA). Goat anti-mouse, rabbit anti-guinea pig, and swine anti-rabbit IgG (horseradish peroxidase coupled) were obtained from Dako SA (France). Polyclonal antibody directed against sea urchin cyclin B (Lozano et al., 1998) was generously donated by Pr. Gérard Peaucellier and Dr. André Picard (Banyuls, France).

Handling of gametes and embryos

Sphaerechinus granularis sea urchins were collected in the Brest area, kept in running sea water, and used within 5 days. Spawning of gametes, in vitro fertilization, and culturing of eggs and embryos were performed as described (Marc et al., 2002). Briefly, eggs were collected in 0.22-μm Millipore-filtered sea water (FSW), rinsed twice, and suspended (10% suspension) in FSW containing 0.1% glycine. For 4E-BP and eIF4E analyses, eggs were dejellied as described (Cormier et al., 2001). Sperm was kept dry at 4°C until use. Shortly before use, 10 μl of dry sperm was diluted in 1 ml FSW, and 10 μl was added per milliliter of eggs suspension. More than 90% of the fertilized eggs raised fertilization membranes in all the experiments described in this article. A stock solution of 20 mM rapamycin was made in ethanol and stored at −20°C. A final concentration of 20 μM rapamycin was added 10 min before fertilization to the eggs suspensions or at different times after sperm addition. In some experiments, 100 μM final concentration of emetine was added 10 min following fertilization.

Determination of cleavage rates and nuclear envelope breakdown

Cleavage was scored by observation under a light microscope. The dispersion of the nuclear envelope was followed by Nomarski differential interference contrast (DIC) microscopy. Observation was made on an Olympus BX61 microscope using a ×20 or ×40 UPlanApo objectives and DIC optics. Pictures were taken using a RT monochrome (Diagnostic instrument, Inc.) CCD camera.

Extract preparation for 4E-BP analyses

At the indicated times following fertilization, cells were collected by centrifugation in a Sorvall TC6 centrifuge for 2 min at 2000 rpm and the cell pellet was frozen in liquid nitrogen and stored at −80°C. Cells extracts were prepared as described (Cormier et al., 2001) with the following modifications. Briefly, cells were lysed 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, 100 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF), and 20 μg/ml of aprotinin and leupeptin). Lysates were centrifuged in a Sorvall RMC14 for 15 min at 14,000 rpm at 2°C. For 4E-BP total amount analysis, typically 50 to 100 μg of protein from the supernatant or from the pellet was directly resolved by one-dimensional (15% acrylamide) SDS–PAGE. The sea urchin translation elongation factor EF-1δ that is present in supernatant and pellet fractions was used as a loading control.

Isolation of eIF4E

Isolation from unfertilized eggs and early development stages was performed as described (Cormier et al., 2001) with the following modifications: supernatant of the extract (made as described above) with an OD (260 nm) = 50 were mixed with 100 μl (25 μl bead volume) of m^7GTP-sepharose beads (m^7GTP column). After 60-min incubation at 4°C, unbound material was removed by four washes with 1 ml of binding buffer containing 100 mM NaCl. Laemmli sample buffer was directly added to the beads. Proteins bound to the beads were resolved by one-dimensional (15% acrylamide) SDS–PAGE using the Mini-PROTEAN two-cell system (Bio-Rad Laboratories). Depending on the experiment, proteins bound to the beads were eluted with 40 μl of binding buffer containing 100 mM NaCl and 0.4 mM m^7GTP for 10 min at 4°C. Following centrifugation at 2000 rpm for 2 min at 4°C, the supernatant was collected and stored in liquid nitrogen or mixed with Laemmli sample buffer. Typically, for SDS–PAGE and blot analyses, one-fifth of the purification was loaded.

Immunoprecipitation analyses

Immunoprecipitation analyses were performed as described previously (Cormier et al., 2001). Briefly, 100 μl of unfertilized eggs extracts (15 μg proteins/μl) or 40 μl of proteins eluted as described above from m^7GTP column were incubated with anti-4E-BP2 antiserum (1:250) at least 4 h at 4°C. The antigen–antibody complex was incubated with protein A-Sepharose beads for 1 h at 4°C and the resin was washed three times. Typically, for SDS–PAGE and blots analyses, half of the immunoprecipitation was loaded.

Extract preparation for analysis of de novo synthesis of cyclin B

At the indicated times following fertilization, embryos were collected by centrifugation in a Sorvall TC6 centrifuge for 2 min at 2000 rpm. Laemmli sample buffer was directly added to 200 μl pelleted cells and the samples were boiled for 3 min. After clearing by centrifugation, 10 μl of the embryos extract was resolved by one-dimensional (12% acrylamide) SDS–PAGE.

Western blot analyses

Western blot analyses were performed following electrotransfer of proteins from SDS–PAGE onto 0.22-μm nitrocellulose membranes (Towbin et al., 1992). 4E-BP was analyzed using rabbit polyclonal antibodies directed against human 4E-BP2 (Rousseau et al., 1996) or human 4E-BP1 (Gingras et al., 1998). Polyclonal antibodies directed against the sea urchin elongation factor δ were raised in a guinea pig against a glutathione-*S*-transferase EF-1δ fusion protein (Delalande et al., 1999). eIF4E was analyzed using mouse monoclonal antibody directed against rabbit eIF4E (Transduction Laboratories, USA). Membranes were incubated with anti-4E-BPs (1:2000), anti-eIF4E (1:2000), anti-cyclin B (1:1000), or anti EF-1δ (1:5000) antibodies in 5% skim milk, 0.1% Tween 20, 20 mM Tris–HCl, pH 7.6 at room temperature. The antigen–antibody complex was measured by the chemiluminescence system with peroxidase-coupled secondary antibodies according to the manufacturer's instructions. Signals were quantified using the public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health).

Measurement of CDK1/cyclin B complex activity in vitro

CDK1/cyclin B activity was assayed under standard conditions using histone H1 (Sigma type III-S) as a substrate (Borgne and Meijer, 1996) in the presence of 20 μM rapamycin or 20 μM roscovitine or in the absence of drug. Phosphorylation of histone H1 (50 μg/ml final) was performed for 10 min at 30°C in 50 μl reaction mixture containing 1 μCi/ml [γ-^{32}P]ATP (20 μCI/ml final), 10 μM unlabeled ATP in buffer containing 60 mM β-glycerophos-

phate; 30 mM *p*-nitrophenyl phosphate; 25 mM MOPS (pH 7.2), 5 mM EGTA, 15 mM $MgCl_2$; 1 mM dithiothreitol, 0.1 mM sodium orthovanadate. Reaction was stopped by chilling the tubes in ice. Duplicate aliquots of 10 μl were spotted on Whatman P81 phosphocellulose papers which were washed five times in 1% phosphoric acid and counted in water in a 1450 MicroBetacounter (Wallac, EG&G Instruments).

Analysis of CDK1/cyclin B activation in vivo

The activation state of CDK1/cyclin B was monitored at different times following fertilization. Every 15 min following fertilization, 1 ml of untreated or 20 μM rapamycin or 100 μM emetine-treated embryos were packed by centrifugation for 5 s at full speed in an Eppendorf centrifuge, rapidly frozen in liquid nitrogen, and stored at −80°C until further use. For most experiments, 50 μl pelleted cells were resuspended in 400 μl ice-cold buffer (60 mM β-glycerophosphate; 15 mM *p*-nitrophenyl phosphate, 25 mM MOPS (pH 7.2), 15 mM $MgCl_2$; 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM disodium phenylphosphate, 1 mM AEBSF, 10 μg/ml soybean trypsin inhibitor, leupeptin 10 μg/ml; aprotinin 10 μg/ml, 100 mM benzamidine, 1% Triton) and were homogenized through a 25-G needle. The homogenates were clarified by 14,000 rpm centrifugation for 10 min at 4°C. Using the ability of the 13-kDa product of the suc-1 gene from yeast to interact with CDK1 (Dunphy et al., 1988), CDK1/cyclin B complex was extracted from 300 μl of homogenate by affinity on $p13^{suc-1}$-Sepharose beads, prepared using bacterially produced $p13^{suc-1}$ protein coupled to Sepharose (Arion and Meijer, 1989). Incubation was performed for 45 min under constant rotation at 4°C in the presence of 50 μl of $p13^{suc-1}$-Sepharose beads, and after three washes, the activity of the bound kinase was determined under the standard conditions reported above.

Determination of protein synthesis rates in vivo

A 5% (v/v) suspension of eggs was incubated for 1 h in 10 μCi/ml [^{35}S]-L-methionine at 16°C. Eggs were then rinsed in FSW and fertilized in the presence or absence of 20 μM rapamycin or 100 μM emetine. At the indicated times, 200 μl of cells was pelleted and frozen in liquid nitrogen. Cell extracts were prepared as described above and radioactivity incorporation into proteins was either measured by TCA precipitation or revealed by autoradiography on gel following protein separation by SDS–PAGE (12% acrylamide). [^{35}S]methionine incorporation was measured on duplicate aliquots after 10% TCA precipitation on Whatman 3M filters and counting in the presence of Optiphase Supermix scintillation liquid. Radioactivity on gels was revealed by autoradiography on Hyperfilm-βmax (Amersham).

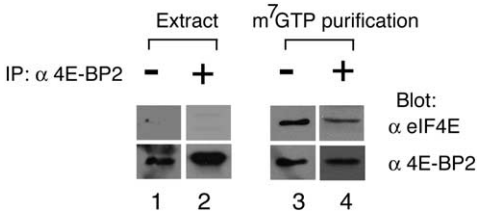

Fig. 1. 4E-BP level is higher than eIF4E level. Extracts were obtained from low-speed centrifugation of unfertilized egg homogenates (lanes 1 and 2). Protein extract before immunoprecipitation (lane 1) or proteins immunoprecipitated using anti-4E-BP2 antibody (lane 2) were resolved on 15% SDS–PAGE and were analyzed by Western blot using anti-eIF4E and anti-4E-BP2 antibodies. Extract was applied to a m7GTP-column and bound proteins were eluted from the column using the cap analogue m7GTP (lanes 3 and 4). Recovered proteins before immunoprecipitation (lane 3) or following immunoprecipitation using anti-4E-BP2 antibody (lane 4) were resolved on 15% SDS–PAGE and were analyzed by Western blot using the same antibodies as above.

Results

Sea urchin 4E-BP is degraded following fertilization

We previously suggested that 4E-BP could act as a repressor of protein synthesis in unfertilized sea urchin eggs and that translational silencing could be in part explained by 4E-BP interaction with eIF4E (Cormier et al., 2001). Higher or equal 4E-BP level relative to eIF4E is certainly required for translational silencing of maternal mRNAs before fertilization. To test this hypothesis, we first compared levels of eIF4E and 4E-BP in unfertilized eggs extracts (Fig. 1, lanes 1 and 2). While eIF4E was undetectable (lane 1, top), 4E-BP was directly detectable in cell extract from unfertilized eggs (lane 1, bottom). Furthermore, eIF4E still remained undetectable following 4E-BP immunoprecipitation from the extracts (lane 2). Codetection of eIF4E and 4E-BP required purification of eIF4E from unfertilized eggs extracts using m^7GTP columns (Fig. 1, lanes 3 and 4). This indicates that, compared to eIF4E, an excess of 4E-BP is present prior to fertilization in sea urchin eggs.

We previously showed that the total amount of 4E-BP decreases rapidly following sea urchin egg fertilization (Cormier et al., 2001). The decrease of 4E-BP could be due either to protein degradation or to redistribution to an insoluble fraction. To distinguish between these two possibilities, the supernatant and pellet obtained from eggs before or at different times after fertilization were analyzed by Western blotting (Fig. 2A) using a polyclonal antibody directed against human 4E-BP2, which was shown to recognize sea urchin 4E-BP (Cormier et al., 2001). While 4E-BP was detected exclusively in the supernatant of unfertilized eggs, the signal disappeared following fertilization (Fig. 2A, top). In contrast, the amount of elongation factor (EF) 1-δ, which served as a control, remained unchanged (Fig. 2A. bottom). Similar results were obtained using another polyclonal antibody, directed against human 4E-BP1, ruling out the possibility that the 4E-BP immunogenic properties could have

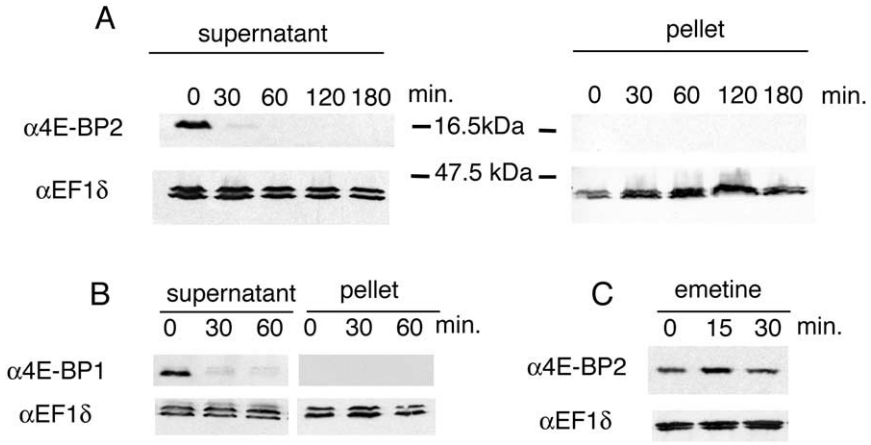

Fig. 2. 4E-BP is degraded following fertilization. (A and B) Supernatant (left) and pellet (right), obtained from low-speed centrifugation of egg homogenates prepared prior or at indicated times following fertilization, were resolved by 15% SDS–PAGE and subjected to Western blot using anti-human 4E-BP2 (A, top), anti-human 4E-BP1 (B, top), or anti-eEF-1δ (A and B, bottom) antibodies. (C) 4E-BP disappearance is independent of inhibition of its synthesis. Unfertilized eggs were incubated with 100 μM emetine during the indicated time, and a Western blot using anti-human 4E-BP2 antibodies was performed as previously described. For all experiment, 75 μg of protein was typically loaded and an anti-sea urchin eEF-1δ antibody was used as loading control (bottom).

been altered by 4E-BP posttranslational modifications following fertilization (Fig. 2B). Because 4E-BP was not recovered in the pellet, its disappearance following fertilization cannot be a consequence of protein redistribution to an insoluble fraction. The amount of a protein in the cell reflects both protein synthesis and degradation. It was thus possible that 4E-BP disappearance reflected an inhibition of its synthesis rather than degradation. However, treatment of unfertilized eggs with emetine, a potent inhibitor of elongation, which at 100 μM rapidly abolishes protein synthesis in sea urchin eggs (Hogan and Gross, 1952), had no effect on 4E-BP amount (Fig. 2C). This rules out the possible involvement of an inhibition of 4E-BP synthesis to explain the 4E-BP disappearance following fertilization.

Rapamycin prevents 4E-BP degradation triggered by fertilization

Following sea urchin egg fertilization, eIF4E is rapidly released from the eIF4E/4E-BP complex (Cormier et al., 2001). In mammalian cells, eIF4E/4E-BP interaction is sensitive to rapamycin, a compound that blocks FRAP/mTOR activity and 4E-BP hyperphosphorylation. To test whether rapamycin also prevents eIF4E/4E-BP dissociation in sea urchin eggs, 4E-BP bound to eIF4E was analyzed after affinity purification of eIF4E on m^7GTP columns from eggs fertilized in the presence or absence of rapamycin. Interestingly, the eIF4E/4E-BP dissociation observed following fertilization is strongly inhibited by rapamycin (Fig. 3A, compare the left and right panels). We next asked whether 4E-BP degradation following fertilization could also be affected by rapamycin. 4E-BP amount was monitored in eggs fertilized in the presence or absence of rapamycin. While the level of 4E-BP decreased dramatically following

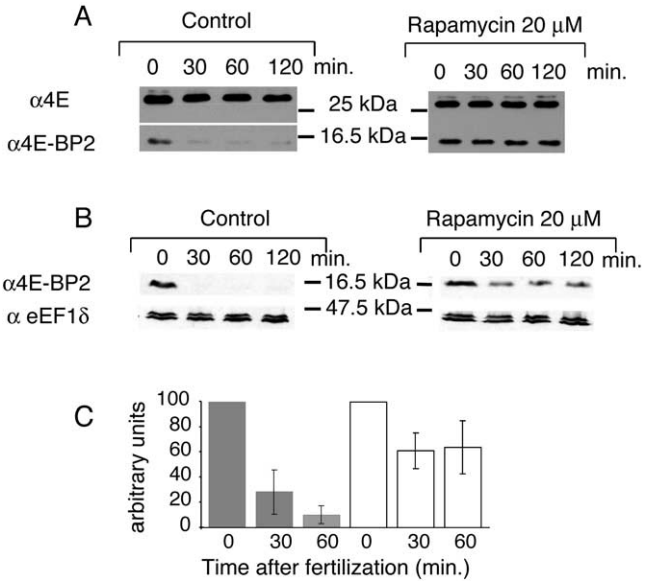

Fig. 3. eIF4E/4E-BP dissociation and 4E-BP degradation following fertilization are both inhibited by rapamycin. (A) Rapamycin inhibits eIF4E dissociation from 4E-BP. Proteins affinity-purified using a m7GTP column prior or at the indicated times following fertilization of untreated (left) or rapamycin-treated (right) eggs were subjected to Western blotting using anti-human 4E-BP2 (bottom) or anti-eIF4E (top) antibodies. (B) Rapamycin inhibits 4E-BP degradation. Total amount of 4E-BP (top) from untreated (left) or rapamycin-treated eggs (right), obtained prior and at the indicated times following fertilization, was analyzed by Western blotting using anti-human 4E-BP2 antibodies. Anti-sea urchin eEF-1δ antibody was used as a loading control (bottom). (C) Quantitation of the results obtained from untreated (gray boxes) or rapamycin treated (white boxes) eggs. 4E-BP amount was normalized against eEF-1δ levels and expressed as a percentage of the value obtained with unfertilized eggs. Vertical bars represent the standard deviation of four independent experiments.

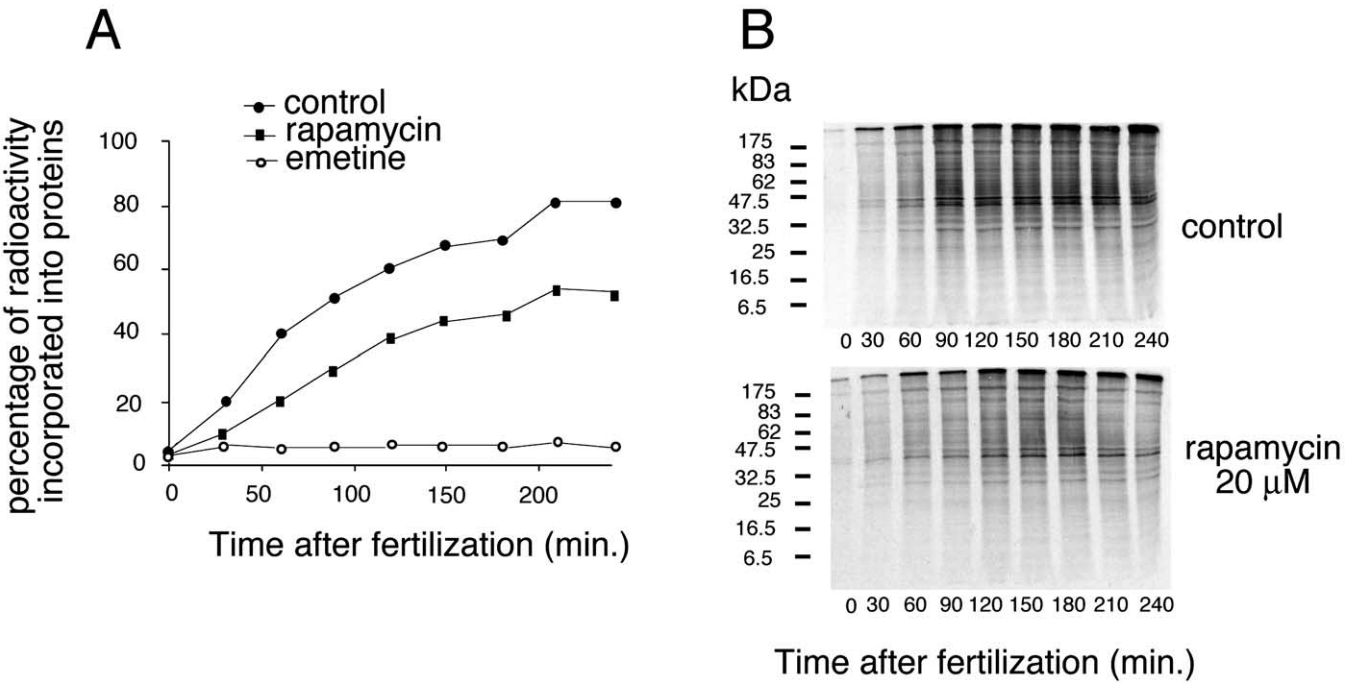

Fig. 4. Rapamycin inhibits the increase in protein synthesis triggered by fertilization. (A) The rate of in vivo protein synthesis was monitored in eggs fertilized in the absence (filled circles) or the presence of 20 μM rapamycin (filled squares) or 100 μM emetine (open circles). Embryos were metabolically labeled in the presence of [^{35}S]methionine and radioactivity incorporation into TCA-precipitated proteins was determined at indicated times after fertilization, as explained under Materials and methods. (B) Pattern of proteins translated after fertilization of untreated (top) or rapamycin-treated (bottom) eggs. Cytosoluble fractions (30 μg of proteins) obtained from embryos at indicated times after fertilization, and labeled in the presence of [^{35}S]methionine, were separated by SDS–PAGE (12%), and neosynthesized proteins were visualized by autoradiography.

fertilization in untreated eggs, 4E-BP levels remained the same in rapamycin-treated fertilized eggs (Fig. 3B, compare the right and left panels). Similar results were obtained with eggs isolated from four different females (Fig. 3C). These data suggest that both rapid eIF4E/4E-BP dissociation and 4E-BP degradation are mediated by a rapamycin-sensitive pathway following sea urchin egg fertilization.

The protein synthesis increase triggered by fertilization is inhibited by rapamycin

As an increase in translation rates generally correlates with 4E-BP/eIF4E dissociation, it is conceivable that rapamycin inhibits the increase in protein synthesis triggered by fertilization in sea urchin. To test this hypothesis, the rate of amino acid incorporation was measured at different times after fertilization in the presence or absence of rapamycin. Translation rates were also monitored in the presence of emetine. As expected, emetine completely blocked [^{35}S]methionine incorporation into proteins (Fig. 4A), while rapamycin inhibited translation only partially (~30%; Fig. 4A). The pattern of proteins synthesized de novo in rapamycin-treated embryos was analyzed following resolution of labeled proteins by SDS–PAGE and autoradiography. Although global translation was reduced by rapamycin treatment, the pattern of synthesized proteins was similar to that observed in untreated eggs (Fig. 4B, compare top to bottom panel). These results show that rapamycin, which blocks both eIF4E/4E-BP dissociation and 4E-BP degradation, also depresses the global increase in protein synthesis that occurs following sea urchin egg fertilization.

Effect of rapamycin on the first mitotic divisions

In sea urchin embryos, a high rate of protein synthesis is necessary to progress through the first mitotic division (Arion and Meijer, 1989; Wagenaar, 1983). Since the results presented above show that translation is sensitive to rapamycin, we asked whether treatment of sea urchin eggs with rapamycin could affect the completion of the first mitotic division. Unfertilized eggs were preincubated in the presence of rapamycin for 15 min and then fertilized and maintained in the presence of rapamycin (Fig. 5). Rapamycin significantly delayed the first mitotic cleavage (Fig. 5). However, fertilization per se was not affected by rapamycin, since the fertilization membranes raised even in the presence of 20 μM rapamycin (Fig. 5). The inhibitory effect of rapamycin on the first mitotic division was maintained up to 2 h after control eggs started to divide (Fig. 5A). Furthermore, rapamycin affected cell-cycle synchrony as judged by changes in the slope of the curve (Fig. 6A). In the presence of rapamycin, embryos which divided once also divided a second time (data not shown). This result suggests that only early events necessary for the first mitotic division were

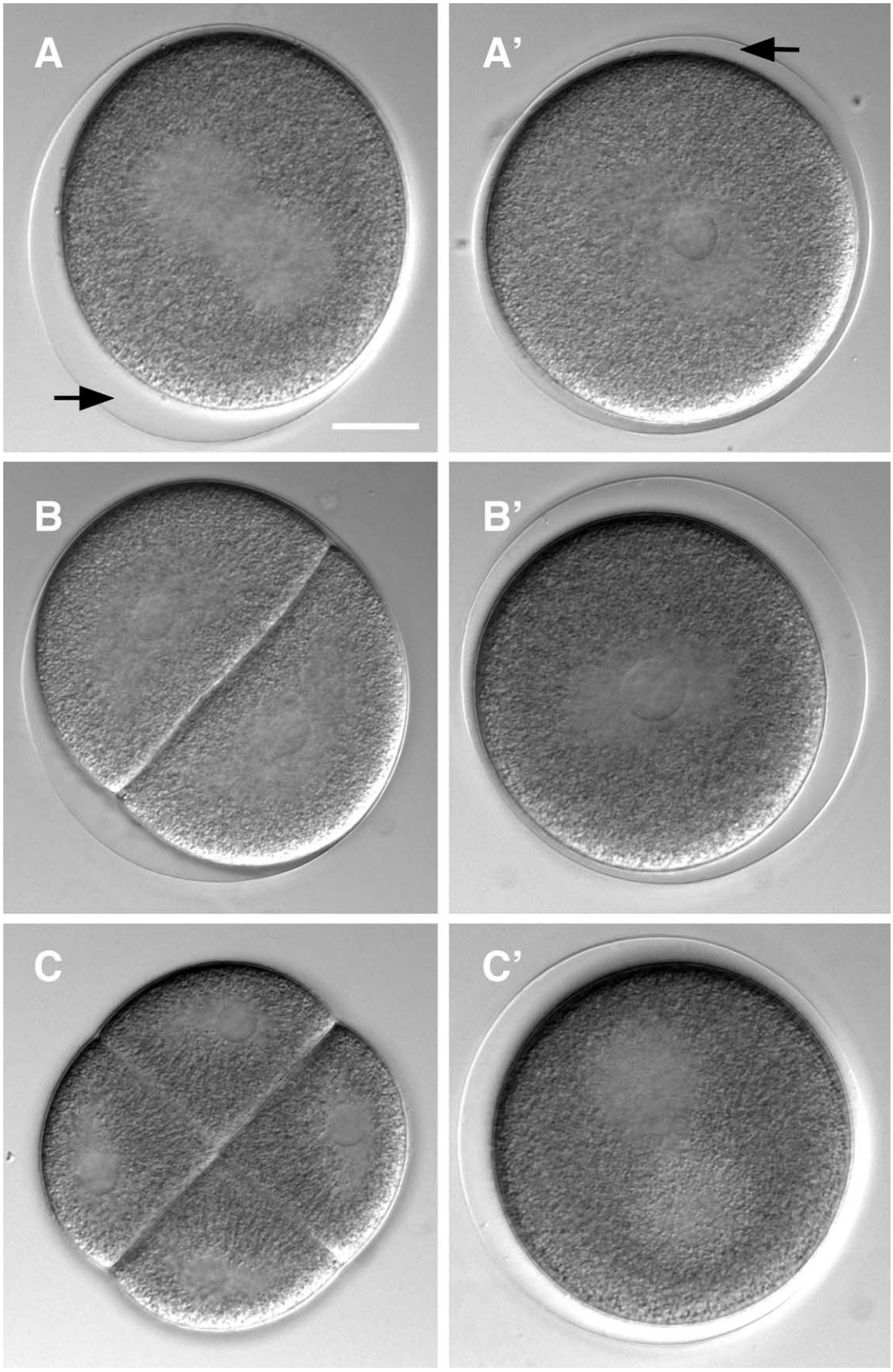

Fig. 5. Rapamycin delays the first mitotic division. Control (A–C) or rapamycin-treated (20 μM) embryos (A′–C′) taken at indicated times following fertilization were observed directly under DIC microscopy. (A–A′) At 100 min after fertilization, control and rapamycin treated eggs raised fertilization membranes (shown by an arrow). While nuclear envelope breakdown was evident in the control (A), nucleus is in center position in rapamycin-treated eggs (A′). (B–B′) At 140 min after fertilization, while control embryos accomplished the first mitotic division (B), rapamycin-treated embryos presented a typical large nucleus in center position of the egg (B′). (C–C′) At 220 min following fertilization, while control eggs divided a second time (C), nuclear envelope breakdown occurred in rapamycin-treated eggs (C′).

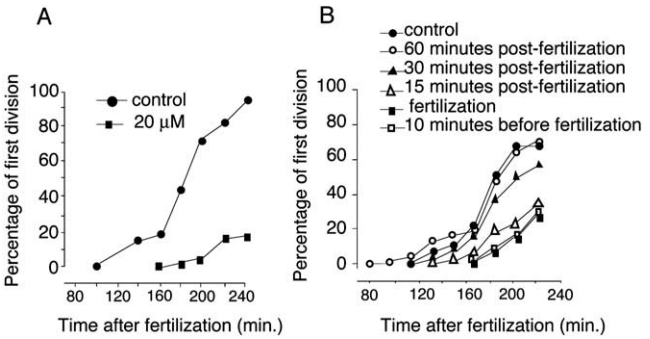

Fig. 6. Effect of rapamycin on kinetic of the first division of early development. (A) Kinetic of the first division of early development. Eggs were fertilized in the absence of rapamycin (filled circles) or following preincubation in the presence of 20 μM rapamycin for 15 min and cleavage rates were scored as described under Materials and methods. These data are representative of at least three independent experiments. (B) Determination of the rapamycin-sensitive period of the embryos. Eggs were incubated with 20 μM rapamycin prior or after fertilization as indicated, and cleavage rates were scored as described under Materials and methods.

affected by rapamycin. Indeed, when added later than 30 min following fertilization, rapamycin had no effect on the cell cycle, but blocked the first mitotic division when added up to 15 min after fertilization (Fig. 6B). Taken together, our results suggest that rapamycin does not affect fertilization per se, but rather prevents an early step required for completion of the first mitosis of the sea urchin embryo.

Rapamycin inhibits cyclin B synthesis which is necessary for CDK1/cyclin B complex activation

Since rapamycin treatment of embryos affects mitotic division, we decided to analyze the effect of the drug on activation of the mitotic kinase complex MPF triggered by fertilization. In mammalian cells, initiation of the cell cycle requires that a critical mass be reached and is dependent on the rate of protein synthesis. Rapamycin is thought to mediate G1 arrest at least in part through its action on 4E-BP via its target FRAP/mTOR and consequent inhibition of translation (Beretta et al., 1996). Unfertilized sea urchin eggs are blocked in G1 and fertilization directly promotes DNA replication in a manner independent of de novo translation (Wagenaar, 1983). Consistent with this, by monitoring [^{3}H]thymidine incorporation, we found that DNA synthesis proceeded in rapamycin-treated eggs (data not shown), suggesting that rapamycin does not act directly on the G1-to-S transition, but rather on the first M-phase of sea urchin embryos.

In sea urchins, CDK1/cyclin B complex activation, and in turn MPF stimulation, is largely dependent on protein synthesis (Arion and Meijer, 1989). Thus, alteration of the CDK1/cyclin B complex may be responsible for the dramatic delay in the first mitotic cleavage caused by rapamycin. We first examined whether rapamycin could directly affect CDK1/cyclin B activity. CDK1/cyclin B complexes were purified by affinity chromatography on p13-suc1-Sepharose beads and used for in vitro histone H1 kinase assays, in the presence of different drugs (Fig. 7A). Rapamycin had no significant effect, whereas roscovitine, a specific inhibitor of CDK1/cyclin B, strongly inhibited H1 kinase activity. This result indicates that rapamycin does not inhibit CDK1/cyclin B kinase activity. This is consistent with the finding that rapamycin had no effect on cell-cycle progression when added later than 30 min following fertilization (Fig. 6B).

We then tested whether rapamycin, or emetine, could affect CDK1/cyclin B activation triggered by fertilization. CDK1/cyclin B complexes were purified from untreated or drug-treated eggs at different times following fertilization by p13-suc1-Sepharose affinity chromatography, and in vitro kinase activity was assayed as described under Materials and methods. As expected, a peak of H1 kinase activity reflecting synchronization of the first mitosis was measured in untreated embryos (Fig. 7B). This peak was markedly reduced when CDK1/cyclin B was purified from eggs fertilized in the presence of rapamycin or emetine. Therefore, these results demonstrate that rapamycin acts upstream of a pathway that leads to CDK1/cyclin B activation.

De novo synthesis of cyclins is necessary for CDK1/cyclin B activation following sea urchin egg fertilization. Since rapamycin partially inhibits translation (Fig. 4), cyclin B synthesis may have been affected by rapamycin treatment. To investigate this, we analyzed cyclin B expression in egg extracts following fertilization (Fig. 7C). In untreated embryos (Fig. 7C, left and top), cyclin B protein appeared as early as 45 min after fertilization, and as time proceeded shifted up into a slower migrating form, and disappeared as the cells exit from mitosis. In rapamycin-treated embryos (Fig. 7C, right and top), cyclin B appeared with a delay of over 60 min following fertilization. Thus, these results demonstrate that rapamycin affects cyclin B synthesis, an event necessary for completion of the first mitotic division in sea urchin early development.

Discussion

We have previously shown that the amount of 4E-BP, and its association with eIF4E, decreases rapidly following sea urchin egg fertilization (Cormier et al., 2001). In this study, we demonstrate that 4E-BP disappearance is due to degradation of the protein. This is the first demonstration that 4E-BP degradation plays an important role in the control of protein synthesis. We also show that 4E-BP degradation and eIF4E/4E-BP dissociation induced by fertilization are both under the control of a rapamycin-sensitive pathway. Furthermore, while rapamycin treatment of embryos only partially inhibits the increase in translation rate triggered by fertilization, de novo synthesis of cyclin B is strongly blocked by rapamycin. Consequently, completion

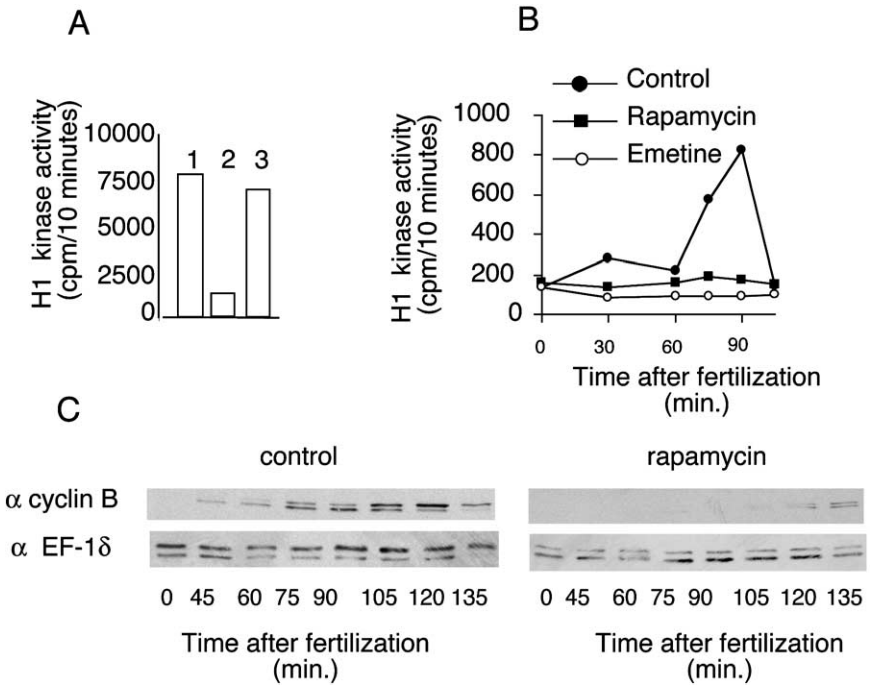

Fig. 7. Rapamycin blocks CDK1/cyclin B complex activation through inhibition of cyclin B synthesis. (A) Rapamycin has no direct effect on CDK1/cyclin B activity. H1 kinase activity of CDK1/cyclin B complex purified on p13-sepharose beads was measured in vitro in the absence (lane 1) or the presence of 20 μM roscovitine (lane 2) or 20 μM rapamycin (lane 3), as described under Materials and methods. (B) Rapamycin inhibits CDK1/cyclin B complex activation in vivo. Eggs were fertilized in the absence (full circles) or the presence of 20 μM rapamycin (full squares) or 100 μM emetine (open circles), and H1 kinase activity of CDK1/cyclin B complex purified on p13 sepharose beads was measured at indicated times postfertilization, as described under Materials and methods. (C) Rapamycin inhibits cyclin B synthesis. Eggs were fertilized in the absence (left) or the presence of rapamycin (right). Soluble fractions obtained from low-speed centrifugation of embryo homogenates prepared at indicated times following fertilization were resolved by 12% SDS–PAGE and subjected to Western blotting using anti-sea urchin Cyclin B (top), or anti-sea urchin eEF-1δ antibodies (bottom).

of the first mitotic division is dramatically delayed when sea urchin eggs are fertilized in the presence of rapamycin.

Proteolysis, a new means to regulate 4E-BP function

During oogenesis and maturation of sea urchin eggs, maternal mRNAs accumulate but remain untranslated until fertilization (Davidson et al., 1982). While precise quantitation of 4E-BP relative to eIF4E was not determined, our results suggest that an excess of 4E-BP is probably required for translational silencing of maternal mRNAs before fertilization. Our results also demonstrate that 4E-BP degradation is a posttranslational mechanism that could relieve cap-dependent translation repression following fertilization. A few reports suggest that such posttranslational regulation of 4E-BP function may also occur in other systems. For instance, in mammals, while 4E-BP2 mRNA is ubiquitously expressed in most tissues (Tsukiyama-Kohara et al., 1996), 4E-BP2 protein is present at high levels in testis and colon, but is not detected in kidney and heart (Tsukiyama-Kohara et al., 2001). Therefore, it will be interesting to determine whether the lack of 4E-BP2 in such tissues is due to inefficient translation or rapid degradation of the protein. Since it has been shown that in mammals, overexpression of 4E-BP1 or 4E-BP2 partially reverts the transformed phenotype of eIF4E-, *ras*-, or *src*-transformed cells (Rousseau et al., 1996), and since several colon carcinoma cell lines exhibit a low 4E-BP/eIF4E ratio (Dilling et al., 2002), an intriguing hypothesis is that 4E-BP proteolysis plays a role in cell transformation. Thus, posttranslational degradation may represent a new mechanism for the regulation of 4E-BP function. The proteolysis pathway responsible for 4E-BP degradation following sea urchin egg fertilization remains to be identified. Proteolysis has been shown to be important for the initiation of the first mitotic division in sea urchins. Indeed, inhibition of the proteasome activity specifically delays the onset of nuclear envelope breakdown in embryonic cell cycle (Kawahara et al., 2000). Nevertheless, the suppression of entry into mitosis in proteasome-inhibited embryos is caused by a defect in the regulatory mechanism of CDK1 activation rather than by any effect on cyclin synthesis (Kawahara et al., 2000). Therefore, 4E-BP degradation is unlikely to be dependent of the proteasome pathway. The presence of trypsin-like proteases has been reported in both membrane and cytoplasmic fractions of sea urchin (Penn et al., 1976). Since specific inhibitors of these proteases delay mitotic division in sea urchin embryos (Penn et al., 1976), it would

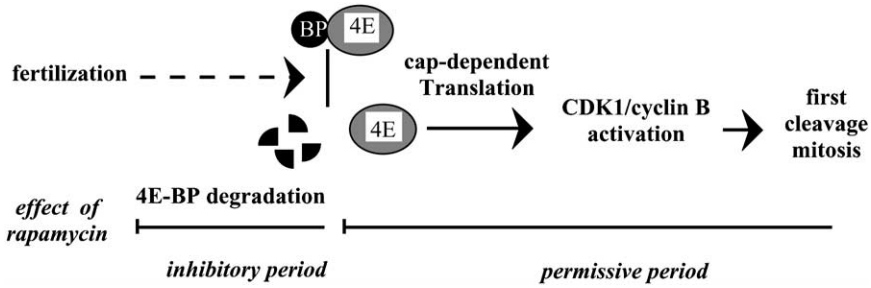

Fig. 8. Schematic diagram illustrating the relationship between 4E-BP function and completion of the first mitotic cleavage. The model posits that fertilization induces eIF4E/4E-BP dissociation and 4E-BP degradation through a rapamycin-sensitive signaling cascade. Consequently, global translation rate increases, thus allowing the synthesis of mitotic cyclins which are required for CDK1/cyclin B complex activation and completion of the first mitotic cleavage.

be interesting to test whether 4E-BP degradation is controlled by such proteases.

Rapamycin effects on 4E-BP degradation and eIF4E release from 4E-BP following fertilization

In mammals, the 12-kDa FKBP12 (FK506-binding protein) is the primary intracellular rapamycin receptor (Harding et al., 1989; Siekierka et al., 1989). The rapamycin/FKBP12 complex binds to, and inhibits, the protein kinase FRAP/mTOR, which is the best candidate for 4E-BP phosphorylation in vivo (Gingras et al., 2001a). In sea urchin, FKBP12 and TOR partial cDNAs have been isolated and are now accessible in an EST-database (Cameron et al., 2000). Therefore, as rapamycin prevents eIF4E/4E-BP dissociation in sea urchins, it is most probable that this event is mediated by the TOR signaling cascade following fertilization. It is conceivable that 4E-BP destruction is sensitive to rapamycin because when it is bound to eIF4E, it is protected against destruction, but becomes rapidly degraded once the eIF4E/4E-BP complex dissociates. However, the alternative possibility that the protease(s) responsible for 4E-BP degradation is directly under the control of a rapamycin-sensitive pathway cannot be excluded.

Effect of rapamycin on M-phase completion

Our results demonstrate the involvement of a rapamycin-sensitive pathway for the completion of the first mitotic division of sea urchin embryos. These results are at an apparent variance with the evidence that rapamycin arrests mammalian or budding yeast cells at the G1 phase of the cell cycle. However, our findings are consistent with the fact that following meiotic maturation, sea urchin eggs are blocked at G1, and de novo protein synthesis is required for M-phase completion, but is dispensable for progression through S-phase following fertilization (Dube, 1988; Wagenaar, 1983). Thus, cap-dependent translation is required for the first mitotic cleavage in sea urchin (this work), while in mammalian cells in culture, cap-dependent translation is required for G1 progression but is in contrast inhibited during mitosis (Pyronnet and Sonenberg, 2001). Once the first mitotic division is achieved, sea urchin embryos continue to divide. However, the size of the embryo remains unchanged until blastula, and consequently cells, are rendered progressively smaller (see Fig. 5). Indeed, during the early stages of sea urchin development, cells divide through a fast consecutive sequence of S- and M-phases, without the G1 growth phase. Thus, there is no need for an alternation between high and low cap-dependent translation rates, as is normally the case later during development (Pyronnet and Sonenberg, 2001). Consistent with this, 4E-BP levels dramatically fall during the first mitotic cleavage and remain very low thereafter. Rapamycin, which likely targets 4E-BP through the TOR signaling pathway, has no effect when added after 4E-BP destruction. It has been published that rapamycin does not affect the meiotic maturation in *Xenopus* (Morley and Pain, 1995) or in starfish oocytes (Lee et al., 2000), further supporting the idea that rapamycin effects are specific to the first mitotic division in early development stages.

We also demonstrated that rapamycin treatment only modestly prevents the increase of protein synthesis following fertilization. This result suggests a multicomponent nature of the translation silencing in sea urchin unfertilized eggs. However, rapamycin markedly affects cyclin B synthesis, which indicates that compared to other proteins, translation of cyclin B is preferentially stimulated following fertilization. Recently, a combined microarray and proteomic approach has uncovered novel genes affected by rapamycin (Grolleau et al., 2002). While a large number of ribosomal proteins and elongation factors was found to be repressed by rapamycin, only a few growth-related proteins were affected by the drug. In addition to its effect in translational control, rapamycin has been shown to affect transcript levels of several growth-related genes, which could explain the inhibition of proliferation observed in rapamycin-treated T cells (Grolleau et al., 2002). During early development of sea urchins, the cleavages of cells are naturally synchronized and first mitotic divisions are independent of mRNA transcription and ribosome biogenesis. Therefore sea urchin eggs could present an elegant system to help clarify the effect of rapamycin on translation of cell-cycle-related proteins. We thus propose a model to

explain the interplay between increased translation rates and entry into the first mitosis following fertilization (Fig. 8). The model posits that, in unfertilized eggs, cap-dependent translation is inefficient because eIF4E is sequested by the translational repressor 4E-BP. Fertilization triggers eIF4E/4E-BP dissociation and 4E-BP degradation through a rapamycin-, and thus, TOR-sensitive pathway (inhibitory period). Cyclin B is then translated in a cap-dependent manner and entry into mitosis can occur. However, once eIF4E/4E-BP is dissociated and 4E-BP is degraded following fertilization, cap-dependent translation of cyclin B is no longer sensitive to rapamycin and, consequently, mitosis occurs even in the presence of rapamycin (permissive period).

A teratogenic activity of rapamycin has been reported in early development of mouse embryo (Hentges et al., 2001). Since early development, including gastrulation, could be readily studied in sea urchin, our model should offer the opportunity to further analyze the teratogenic activity of rapamycin and the relationships between translation initiation and cell-cycle regulations during embryonic development.

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