Dephosphorylation of eIF2α is essential for protein synthesis increase and cell cycle progression after sea urchin fertilization

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Introduction

Eukaryotic translation initiation factor 2 (eIF2) plays a key role in the regulation of mRNA translation. eIF2 composed of three subunits (α, β, and γ) binds both GTP and initiator methionyl-tRNA to form a ternary complex. eIF2 mediates the binding of initiator methionyl-tRNA to the ribosomes during the initiation step of translation (Pestova et al., 2007; Proud, 2005). Phosphorylation of the alpha subunit of eIF2 at a conserved serine (Ser-51 in mammals) is a widely used mechanism of translational control in many organisms. Phosphorylated eIF2α has an increased affinity to its guanine nucleotide exchange factor eIF2B, leading to the sequestering of eIF2B as an inactive complex with eIF2 and GDP. General protein synthesis is then inhibited because of the decreased overall rate of guanine nucleotide exchange on the remaining unphosphorylated eIF2; paradoxically the translation of a subset of mRNAs containing up-stream open reading frames is stimulated (Jackson et al., 2010). Phosphorylation of eIF2α is performed by four known serine/threonine protein kinases that share a related kinase domain but respond to different stimuli through specific regulatory domains: the general control non-derepressible 2 (GCN2) activated by uncharged tRNA, the PKR-like endoplasmic reticulum kinase (PERK) that is activated by misfolded proteins in endoplasmic reticulum, the double-stranded RNA protein kinase (PKR) that responds to viral infection, and the heme-regulated inhibitor kinase (HRI) that is activated by heme deficiency (Dever et al., 2007). GCN2 is present in all eukaryotes, PERK has been identified in metazoans, HRI is found in vertebrates and in some species of yeasts, insects and invertebrates, PKR is restricted to vertebrates. It is currently accepted that translational control by eIF2α phosphorylation is a conserved adaptation to cell stress that existed since the onset of eukaryotes (Hernandez et al., 2010).

Fertilization of the sea urchin egg offers the opportunity to address translational regulation in a physiological situation and outside the condition of stress. In unfertilized eggs, protein synthesis occurs at a relatively low rate. Within minutes after fertilization, the rate of protein synthesis is stimulated, independently of mRNA transcription and ribosome biogenesis. Furthermore, protein synthesis is necessary for the onset of first cell division (Wagenaar, 1983). Translational up-regulation is exerted through multifactorial mechanisms, including mRNA recruitment into polysomes and increased rates of translation initiation and elongation (Brandis and Raff, 1979; Hile and Albers, 1979). The stimulation in the rate of protein synthesis in sea urchin egg extracts was observed after the addition of exogenous eIF2, eIF2B or eIF4 (Akkaraju et al., 1991; Colin et al., 1987; Huang et al., 1987), suggesting that their activities could play a regulatory role after fertilization. The cap-dependent translation inhibitor 4E-BP...
(Oulhen et al., 2009) is rapidly phosphorylated and degraded following fertilization of sea urchin eggs leading to release of eIF4E (Cormier et al., 2001; Salaun et al., 2003) and its association with the scaffolding protein eIF4G (Oulhen et al., 2007). Therefore eIF4E is now recognized as a crucial actor for the onset of the first mitotic division following fertilization, suggesting that cap-dependent translation is highly regulated during this process (Gilbert, 2006). However translational regulation during fertilization may not be exerted by this one actor only, since the addition of other factors such as eIF2 and eIF2B in sea urchin egg extracts stimulated protein synthesis rate (Akkaraju et al., 1991; Winkler et al., 1985). This observation suggests that the recycling of eIF2 is also an important regulatory step of protein synthesis upon fertilization. Previously, we showed that the phosphorylation of eIF2α subunit is correlated with the inhibition of protein synthesis in response to the treatment of embryos with the DNA-damaging agent MMS (Le Bouffant et al., 2008). In this paper, we analyze the role of the phosphorylation status of eIF2α in the translational activation and in the first mitosis following fertilization.

Material and methods

Chemicals

Rabbit polyclonal antibody directed against full length eIF2α (FL-315) was obtained from Santa Cruz. Rabbit polyclonal antibody directed against phosphorylated Ser51 eIF2α (9721) was obtained from Cell Signaling. Peroxidase-conjugated secondary antibodies were obtained from DAKO. β2-Methionyl, β-leucinyl and calycin A were purchased from Sigma.

Handling and treatment of eggs and embryos

Sea urchins (Sphaerechinus granularis) were collected in the Brest area (France) and maintained in running seawater. Spawning of gametes was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs, collected in 0.22-μm Millipore-filtered seawater, were dejellied by swirling for 30 s in filtered seawater (pH 5), rinsed twice in fresh seawater and suspended in filtered seawater at 5% (v/v) dilution. Diluted sperm was added to the eggs and withdrawn after 5 min. Experiments were only performed on batches exhibiting more than 80% fertilization of injected eggs. At the indicated time post-fertilization, inhibitors were added to the incubation medium at the indicated concentration. To obtain total protein extracts at different times following fertilization, 0.2 ml of 5% embryo culture was taken, and the proteins were expressed in overnight cultures at 20 °C with vigorous shaking. Protein over-expression was induced during 3 h by adding 1 mM IPTG at 1 OD A600.

Handling and treatment of eggs and embryos

Electrophoretic resolution of proteins was performed under SDS denaturing conditions on a polyacrylamide gel. Proteins were electrotransferred from the gel onto nitrocellulose membranes (Amersham). The membranes were saturated for 1 h in Tris-Buffered Saline (TBS) containing 0.1% Tween 20 and 1–5% Bovine Serum Albumin. Membranes were incubated overnight with antibodies directed against full-length (FL) eIF2α (1:1000), phospho-eIF2α (1:1000). Secondary peroxidase-conjugated antibodies were used at 1:5000 for full-length protein and 1:2000 for phosphorylated proteins. Revelation was done by ECL or ECL+ methods according to the manufacturer’s instructions (Amersham). Quantification was done using the public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health).

In vivo protein synthesis

Protein synthesis activity was measured in vivo: embryos (5% suspension in seawater) were cultured in continuous presence of [35S]-l-methionine at the final concentration of 5 μCi/ml or alternatively, batches of embryos were taken 2 h after fertilization and incubated in the presence of [35S]-l-methionine for 30 min at a final concentration of 10 μCi/ml. [35S]-l-methionine incorporation into proteins was measured on duplicate aliquots after 10% TCA precipitation on Whatman 3 M filters and counting in a scintillation counter in the presence of Optiphase Supermix scintillation liquid as described in Le Bouffant et al. (2008).

Cloning and site directed mutagenesis

Forward (5′-GGGATCCGTCTCAGTGACGGATGATAAGAGCTATCAGATTATTTCCTCTCTCCATGATACGCCTTCTATCCAGCTCACTGAGCAG-3′) and reverse (5′-CCCCCGAATTCTTAGCTTCTTGTCTATGATACGCCTTCTCTCTTCCGTGAGCAGCTCTATTCTCCTACGAG-3′) primers designed from the Strongylocentrotus purpuratus genome were used to amplify a 938-bp fragment by PCR using a S. purpuratus cDNA library. The 938-bp fragment (EMBL sequence XP779939) was digested by EcoRI and BamHI and inserted into plasmid pGEX4T-1 (GE HealthCare) cut with the same enzymes to obtain the wild-type GST-eIF2α clone. Mutants were obtained by site directed mutagenesis of the plasmids pGEX-eIF2α using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer, with the following primers:

SS1D = 5′-ATGATCCTGCTCAGTGACGGATGATAAGAGCTATCAGATTATTTCCTCTCTCTCTCTCTCTCTCTCTCTCAGCTCTATTCTCCTACGAG-3′ and 5′-GATGGATCTGATACGCCTTCTATCCAGCTCACTGATACGATCTATCATC-3′; SS1A = 5′-CTGTCATGGACTGCGAGAGCGGATATCA-3′ and 5′-TGATACGCTCCTCCGCGCCACTGAGCAG-3′.

Mutants and wild type inserts were verified by sequencing on an Applied Biosystems AB3130 automatic sequencer at the Biogenouest® Genomics core facility in Roscoff (France).

Fusion protein purification

Plasmids containing the GST-eIF2αSS1D or GST-eIF2αSS1A or GST alone were inserted into Rosetta competent bacterial strain (Novagen). The proteins were expressed in overnight cultures at 20 °C with vigorous shaking. Protein over-expression was induced during 3 h by adding 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 1 OD A600. Sedimented cells were resuspended in 1× Phosphate-Buffered Saline (PBS) with anti-proteases (1 mM AEBSF, 2 μg/ml leupeptin and 10 μg/ml aprotinin) and disrupted using a French Press. The lysate was treated with 1% Triton X-100 30 min at room temperature. Fusion proteins were purified on glutathione sepharose 4B beads (GE Healthcare; 0.5 μl beads for 1 ml of lysate at 4 °C for 1 h with a gentle agitation). After two washings in 1× PBS and anti-proteases, fusion proteins were recovered in elution buffer (50 mM Tris–HCl pH 6.8, and 10 mM reduced glutathione, pH 8).

Microinjection

Fusion proteins were dialyzed overnight at 4 °C against microinjection buffer (10 mM HEPES pH 7.0, 200 mM KCl, and 550 mM mannitol) and dialuted at 600 ng/μl in the same buffer containing 1 mM Alexa Fluor 488-dextran (Molecular Probes) to allow visualization of injected eggs. The Femtojet microinjection system (Eppendorf) resulted in the injection of approximately 5% of the volume of each egg with an estimated final concentration of the exogenous protein of 0.4 μM. Cleavage was scored by observation under a fluorescence microscope (Olympus IX-51).

Protein synthesis was measured in microinjected eggs using a luciferase reporter mRNA. pT7Luc plasmid (a generous gift of Vincent
Legagneux and Luc Paillard, University of Rennes) contains the firefly luciferase open reading frame cloned into the bgIII site of the pT7 vector (Paillard et al., 1998). Capped mRNA encoding firefly luciferase was produced from BamHI-linearized pT7Luc plasmid using mRNAmessage mMachine T7 kit (Ambion) and was co-injected with fusion proteins in eggs as described above. Two hours after fertilization, injected embryos were collected, lysed in One-Glo luciferase assay buffer (Promega) and luminescence was measured in a TriStar luminometer (Berthold). The statistical analysis for measuring significance levels when comparing the different data sets was done by Mann-Whitney (Wilcoxon) test using Statgraphics Plus 5.1 software.

Results

Phosphorylation of highly conserved site of eIF2α is regulated following fertilization in sea urchin

The sea urchin genome has been made available in 2006 (Sodergren et al., 2006) and one gene encoding for eIF2α is present (SPU_003646; Morales et al., 2006). Primers were designed to amplify the eIF2α open reading frame from a S. purpuratus cDNA library, and yielded a 313 aminoacids protein (EMBL #XP779939, Fig. 1). The sea urchin protein is 70% identical with the human eIF2α. Two cystein residues at positions 69 and 97 (indicated by a star in Fig. 1) are involved in a disulfide bridge thought to stabilize the interaction of two domains in the human eIF2α protein, the OB domain (oligonucleotide-binding domain consisting of a single-stranded anti parallel β-barrel) and the α-helical domain (Nonato et al., 2002). It has been suggested that Cys residue in position 69 is characteristic of vertebrate eIF2α, as it is not conserved in drosophila or yeast (Ganner et al., 2003). The sequence of the sea urchin eIF2α showed that the residue at position 69 is also a cystein. A further search into genome databases from yeast to human showed that this cystein residue is present in deuterostomes from sea urchin to human, in three out four species of nematodes, in cnidarians, but indeed absent in insects and in yeasts. These data therefore exclude the hypothesis that the Cys residue at position 69 is specific of vertebrates and suggest that this residue is conserved throughout metazoans and has been lost in some phyla.

Residues in the phosphorylation loop region surrounding the eIF2α regulatory phosphorylation site (Ser-51 in the human protein and Ser-50 in the sea urchin protein) are highly conserved among eIF2α orthologs (Fig. 1; Dever et al., 2007) suggesting a possible control of translation at this level in sea urchin. We detected the sea urchin full length eIF2α protein by Western blot using an antibody directed against the human protein: a single band at the expected size of 32 kDa was detected (Fig. 2A). In order to study the eIF2α phosphorylation status following fertilization, we used an antibody directed against the phospho-serine 51 of the human protein. The antibody recognized a band in sea urchin unfertilized egg extract at the expected size (32 kDa; Fig. 2A). Fertilization triggered a rapid dephosphorylation with only 50% of the phosphorylated form remaining at 15 min and maximal dephosphorylation was reached at 60 min following fertilization (Figs. 2A and B). The dephosphorylation of eIF2α is associated with the increase of protein synthesis activity as shown by the incorporation of labeled methionine into proteins (Fig. 2B).

eIF2α phosphorylation correlates with the inhibition of protein synthesis activity and cell cycle progression

To test whether phosphorylation status at serine residue 51 of eIF2α could play a role in the activation of the protein synthesis induced by fertilization and required for the first mitotic division of the sea urchin embryo, we decided to impose changes on the phosphorylation state of this translational factor. It has been shown previously that the phosphatase inhibitor, calyculin A, inhibits the phosphorylation of eIF2α in mammalian cell (Boyce et al., 2005; Brush et al., 2003). Calyculin A was used to manipulate the phosphorylation of eIF2α in sea urchin eggs, and the phosphorylation of eIF2α was analyzed by Western blot using the antibody directed against the phospho-serine 51 of the human protein. In the unfertilized sea urchin eggs, the phosphorylation status of eIF2α remained unchanged by calyculin A treatment suggesting low turnover for the phosphorylation/dephosphorylation mechanism (data not shown). The effect of calyculin A, added 20 min

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**Fig. 1. Sequence alignment of eIF2α proteins.** Amino acid sequences of eIF2α from Stronglylocentrotus purpuratus (XP_779939.1), Ciona intestinalis (XP_002130619.1), human (P05198) and mouse (Q6ZWX6) were aligned using ClustalW. Identical residues are highlighted in black and conserved ones in grey. The phosphorylation site corresponding to the serine 51 is indicated by an arrow. The stars indicate the conserved cysteins at positions 69 and 97, involved in the disulfide bridge connecting the oligonucleotide-binding OB domain (black line) and the helical domain (grey line).
post-fertilization, was therefore analyzed in fertilized eggs when most eIF2α was dephosphorylated. Treatment of the embryos with 1 μM calyculin A increased the level of eIF2α phosphorylation (Fig. 3A). Protein synthesis activity was inhibited by 68% (s.d. ± 2.8) in the presence of calyculin A, when compared to control embryos taken at 2 h post-fertilization (Fig. 3B). Furthermore, incubation of embryos with calyculin A induced a complete inhibition of the first cell division triggered by fertilization (Fig. 3C).

Previous reports showed that alcoholic derivatives of amino acids were able to induce the phosphorylation of eIF2α (Kimball et al., 1991; Zhang et al., 2002). Sea urchin embryos taken at 20 min post-fertilization (see Fig. 1) were exposed to the alcohol derivatives of methionine and leucine, methioninol and leucinol, and monitored for eIF2α phosphorylation status. Incubation of embryos with methioninol or leucinol increased the phosphorylation of eIF2α as judged by Western blot using the antibody directed against phospho-serine 51 eIF2α (Fig. 4A). Furthermore, protein synthesis activity was dramatically inhibited in presence of 10 mM methioninol or 4 mM leucinol (Fig. 4B). Treatment of the embryos with aminoacid alcohols induced a dose-dependent inhibition of the first cell division triggered by fertilization, with an IC50 on cell division of 6 mM methioninol and 2.5 mM leucinol respectively (Fig. 4C). It is noteworthy that the minimal concentration of amino alcohol acid that induced the maximal eIF2α phosphorylation was the concentration that inhibited drastically the protein synthesis activity and the first cell division.

Taken together, these data suggest that eIF2α must be dephosphorylated to allow the increase of protein synthesis and cell division triggered by fertilization.

**A mutant mimicking phosphorylated eIF2α impairs first cell division occurrence and affects protein synthesis activity in sea urchin embryo**

The above data suggested that the eIF2α phosphorylation status was important for protein synthesis increase after fertilization and for the subsequent occurrence of the first cell division, however we cannot rule out eIF2α-independent effects of the drug treatments on protein synthesis activity and on cell cycle progression. Therefore, we decided to assess directly the effect of eIF2α phosphorylation by using a phoso-mimetic variant of eIF2α. eIF2α from sea urchin was cloned in fusion with the GST protein, and two mutant proteins on the residue corresponding to the phospho-serine were produced using site directed mutation: S51D, mimicking a constitutively phosphorylated protein, and S51A, mimicking a non-phosphorylatable protein. The introduction of the exogenous protein was done by microinjection into unfertilized eggs, and the first division was scored during 3 h after fertilization. To assess for aspecific effects of the microinjection, the GST moiety alone was injected and showed no effect on the kinetics of the first cell division as compared to non injected eggs after fertilization. While the injection of the non-phosphorylatable mutant (S51A) had no significant effect on the cell division, the injection of the mutant mimicking the phosphorylated protein (S51D) affected the kinetics of cell division (Fig. 5A). The S51D mutant induced a significant inhibition (51.9%, s.d. ± 9.4) on the first cell division (Fig. 5B). The efficiency of protein synthesis influences the kinetic or occurrence of cell division, we therefore directly analyzed protein synthesis activity using co-microinjection of a reporter mRNA producing a luminescent protein, with the eIF2α fusion protein. As shown in Fig. 5C, the mutant mimicking the phosphorylated protein (S51D) led to a drastic inhibition of protein synthesis.
A typical experiment out of three is shown here. B—i-leucinol and α-methioninol inhibit protein synthesis. Incorporation of [35S]-l-methionine into proteins is expressed as a percentage of total methionine, measured at 2 h after fertilization (a mean of three independent experiments). C—Dose response of i-leucinol and α-methioninol on the first cell division of sea urchin embryos are shown as a mean of 6 (i-leucinol) or 4 (α-methioninol) independent experiments.

(73%, s.d. ± 12, three independent experiments) in the S51D-injected embryos as compared to S51A-injected embryos.

Discussion

The mechanism by which the rate of protein synthesis increases after fertilization is a long standing and fundamental question in biology. Two decades ago, it was suggested that eIF2 activity could play a role in the protein synthesis regulation occurring at fertilization in sea urchin (Akkaraju et al., 1991; Dholaik et al., 1990). We showed in this report physiological changes of phosphorylation of the eIF2α subunit during fertilization of the sea urchin egg. Induction of the phosphorylation of eIF2α by phosphatase inhibitor or by activating a kinase signaling pathway leads to the inhibition of protein synthesis, and to subsequent cell cycle arrest. By using a phosphomimetic mutant of eIF2α, we showed that dephosphorylation of eIF2α is necessary for protein synthesis increase and for cell cycle division that follow fertilization of sea urchin eggs. Therefore, our data demonstrate that eIF2α in sea urchin contributes to the regulation of protein synthesis necessary for the onset of the first cell division.

One major regulation of protein synthesis activity resides on the assembly of initiation factors onto mRNAs (reviewed in Jackson et al. (2010)). In sea urchin eggs, protein synthesis stimulation upon fertilization has been shown to depend on the availability of the cap binding protein eIF4E through the release from and degradation of its translational repressor 4E-BP (Cormier et al., 2001; Oulhen et al., 2007; Salaun et al., 2003). One other major regulation of protein synthesis activity resides on the assembly of the 43S complex (Lorsch and Dever, 2010). The initiation factor eIF2α plays also a role in the
transcription activation following fertilization, since inducing its phosphorylation is sufficient to inhibit protein synthesis activity in embryos, when the cap binding eIF4E is available for translation. Since both complexes formations are the rate-limiting steps in translation (Hershey and Merrick, 2000), we hypothesize that the unfertilized egg is maintained translationally inactive by two major regulators, namely eIF4E and eIF2α, to ensure that no inappropriate translation occurs without fertilization.

Our results suggest furthermore that regulators of eIF2α phosphorylation (kinase and phosphatase activities) are modulated at fertilization. Phosphatase inhibitor calyculin A increased the phosphorylation of eIF2α following treatment of fertilized eggs, whereas it did not affect significantly the level of phosphorylated eIF2α in unfertilized eggs, suggesting that a phosphatase activity acting upon eIF2α rises after fertilization. In mammalian cells, eIF2α can be dephosphorylated by PP1 (protein phosphatase 1) associated with two different regulatory proteins: the stress-inducible Ppp1r15a/GADD34 (Brush et al., 2003; Novoa et al., 2001) and the constitutive Ppp1r15b/CREP (Jousse et al., 2003). Salubrinal induces eIF2α phosphorylation of mammalian cells and inhibits eIF2α dephosphorylation mediated by both ER stress-induced and constitutive phosphatase complexes (Boyce et al., 2005). In contrast to calyculin A, no effect on eIF2α phosphorylation is induced by incubation of sea urchin embryos with salubrinal. It should be noted that no homologues of GADD34 or CREP were found in the sea urchin genome database and web site. Nucleic Acids Res. 37, D750–D754.

An interesting question is which kinase(s) is/are involved in the regulation of eIF2α phosphorylation and hence protein synthesis and occurrence of cell cycle at fertilization. Three eIF2α kinases have been described in the sea urchin genome, namely Sp-GCN2, Sp-HRI and Sp-PERK (Morales et al., 2006). Transcripts encoding for the three kinases have been detected in early embryo (Cameron et al., 2009), however there is no data available for the stored maternal kinases in the unfertilized eggs. An attractive candidate for the eIF2α kinase in sea urchin eggs could be GCN2 kinase, based on evidence in mouse oocytes. Recently, Alves et al. (2009) showed that GCN2 is highly expressed and active in mouse unfertilized eggs, correlated with a high level of eIF2α phosphorylation. Moreover eIF2α phosphorylation is rapidly reduced after fertilization and in two-cell embryos (Alves et al., 2009). In the sea urchin unfertilized egg extract, a high molecular weight protein of the expected size that could correspond to GCN2 reacts with an antibody raised against a phospho-peptide recognizing the active mouse GCN2 kinase (Supplementary data), suggesting that GCN2 could be implicated in the phosphorylation changes in eIF2α. Further work is needed to definitely identify the eIF2α kinase involved at fertilization. The full cascade of events acting upon the regulators of eIF2α remains to be elucidated.

Because eIF2α phosphorylation has been regarded mainly as a stress-induced event, our finding that eIF2α regulation is involved in cell cycle progression during fertilization in sea urchin is appealing. Other studies have recently demonstrated physiological role of eIF2α regulation in various developmental processes, as in mouse meiotic maturation (Alves et al., 2009), epidermal morphogenesis in Caenorhabditis elegans (Nukazuka et al., 2008) and aggregation and proliferation in Dictyostelium (Bowman et al., 2011; Fang et al., 2003). Impairment of regulated eIF2α dephosphorylation leads to growth arrest and degeneration of mouse embryos, consistent with the known role of uncontrolled eIF2α phosphorylation in promoting cell death (Harding et al., 2009; Srivastava et al., 1998).

Several reports show that a subset of mRNAs containing upstream short open reading frames are selectively translated in response to eIF2α phosphorylation, as exemplified by GCN4 in yeast during amino acid starvation or ATF4 during stress in mammals (reviewed in Jackson et al., 2010). Similarly, during Dictyostelium development, translation of mRNAs encoding for chalones are upregulated in response to eIF2α phosphorylation (Bowman et al., 2011). Selective mRNA translation is crucial to the proper developmental process, from fertilization to organogenesis; the complex series of events that orchestrate the recruitment of mRNA is still under investigation. Our results point out to new potential regulations of gene expression in early development, which could implicate selective translation via upstream open reading frames.

The regulation that we demonstrated opens the possibility of selective translation in the control of gene expression post-fertilization which can be investigated in the sea urchin model. An evaluation of the mRNAs that are recruited into polysomes before and after fertilization will bring insights on the role of eIF2α phosphorylation on global and selective translation in sea urchin embryos, and more generally on the translational controls at fertilization and early cleavage stages of embryonic development.

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