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Identification of a new isoform of eEF2 whose phosphorylation is required for completion of cell division in sea urchin embryos

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ABSTRACT

Elongation factor 2 (eEF2) is the main regulator of peptide chain elongation in eukaryotic cells. Using sea urchin eggs and early embryos, two isoforms of eEF2 of respectively 80 and 83 kDa apparent molecular weight have been discovered. Both isoforms were identified by immunological analysis as well as mass spectrometry, and appeared to originate from a unique post-translationally modified protein. Accompanying the net increase in protein synthesis that occurs in early development, both eEF2 isoforms underwent dephosphorylation in the 15 min period following fertilization, in accordance with the active role of dephosphorylated eEF2 in regulation of protein synthesis. After initial dephosphorylation, the major 83 kDa isoform remained dephosphorylated while the 80 kDa isoform was progressively re-phosphorylated in a cell-cycle dependent fashion. *In vivo* inhibition of phosphorylation of the 80 kDa isoform impaired the completion of the first cell cycle of early development implicating the involvement of eEF2 phosphorylation in the exit from mitosis.

Introduction

Translational control has been shown to be critical for gene expression in response to a number of physiological as well as pathological situations, such as development and differentiation, nervous system function, stress and nutrient deprivation, aging, viral infection and disease (Mathews et al., 2007). Two mechanisms receive overwhelming focus among the well-known examples of translational control, both at the level of initiation (Sonenberg and Hinnebusch, 2009), one involves 4E-BP (eIF4E-Binding protein) the inhibitor of eIF4E (eukaryotic Initiation Factor 4E) (Gingras et al., 1999), the other implicates phosphorylation of eIF2α (α-subunit of eukaryotic Initiation Factor 2) (Dever et al., 2007). Although relatively less attention has been devoted to studying the control of elongation, important discoveries have cast new light on how elongation, the principal phase of protein synthesis, is regulated (Browne and Proud, 2002). Besides its role in the regulation of overall protein synthesis rate, it has been proposed that transitory inhibition of elongation could be a means of specifically regulating the level of short-lived proteins such as the pro-apoptotic proteins (White-Gilbertson et al., 2008) and favoring the translation of mRNAs with weak initiation constants (Ryazanov et al., 1991; Walden and Thach, 1986).

Of the two factors responsible for the translation elongation phase, eEF2 (eukaryotic Elongation Factor 2) is considered to be the major regulatory target for controlling elongation rate (Proud, 2007). The protein eEF2 is a monomeric guanine nucleotide binding protein of about 100 kDa which catalyses the translocation step of peptidyl-tRNA on the ribosome (Merrick and Nyborg, 2000). Phosphorylation of eEF2 (at Thr56 as numbered in the human sequence) inhibits its activity by reducing its affinity for ribosomes, resulting in inhibition of protein synthesis (Ryazanov and Spirin, 1990). Phosphorylation is catalyzed by an unusual enzyme, eEF2 kinase, formerly referred to as CaM kinase III (Nairn and Palfrey, 1987), and dephosphorylation relies on the activity of protein phosphatase 2A (Everett et al., 2001). The specificity of eEF2 kinase for its substrate is very high: no other known kinases are able to phosphorylate eEF2 and eEF2 kinase is unable to phosphorylate a number of typical substrates for other protein kinases (Ryazanov and Spirin, 1990). Consequently, the phosphorylation state of eEF2 and therefore its influence on elongation rely on the activity of eEF2 kinase. It has been proposed that eEF2 kinase acts as a hub for integrating the effect of multiple signaling networks for control of protein synthesis via regulation of the activity of its unique substrate eEF2 (Proud, 2007). An early study reported that eEF2 is substantially phosphorylated in mitotic cells after release from an aphidicolin-induced S-phase block (Celis et al., 1990), which was later confirmed by demonstration that eEF2 is phosphorylated during mitosis in correlation with reduction of translational activity of polysomes (Sivan et al., 2007). Conversely, Smith and Proud (2008) reported a low phosphorylation status of eEF2 via inactivation of eEF2 kinase by cyclin-dependent kinase (CDK1) control in mitotic cells. This apparent

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contradiction has not been resolved. Finally, it has been reported that eEF2 kinase is elevated in cancer cell lines and in human cancer tissue (Arora et al., 2003), which led to the development of a specific eEF2 kinase inhibitor, NH125, as a novel potential anticancer drug (Arora et al., 2003).

Sea urchin embryos have long been a paradigmatic example of translational control (Mathews et al., 2007). Protein synthesis in sea urchin eggs is rapidly and abruptly activated by fertilization in the absence of ongoing transcription. In parallel, fertilization induces cell cycle entry of the eggs formerly blocked in the G1-phase (Epel, 1990). Thus, the regulation of translation achieved by fertilization involves coordinated control of protein synthesis necessary to launch the embryonic development program together with regulation of synthesis of selective proteins implicated in the control of cell cycle, including cyclin B. Cyclin B synthesis is essential for activation of CDK1, the kinase controlling the onset of the G2/M transition (Morgan, 2007). Its disappearance is necessary for mitosis exit (Wolf et al., 2007). The molecular signaling mechanism of fertilization relies on two early ionic events: calcium and pH changes (Epel, 1990). Artificial intracellular elevation of calcium or pH was reported to induce an increase in protein synthesis in unfertilized eggs. However, these treatments designed as parthenogenetic activation do not lead to cell division (Epel, 1990). The fertilization-induced changes in protein synthesis depend on simultaneous increase of translational initiation and elongation rates of masked maternal mRNAs (Humphreys, 1971; Brandis and Raff, 1979; Hille and Albers, 1979; Monnier et al., 2001). At the level of initiation, 4E-BP is rapidly phosphorylated and degraded following fertilization of sea urchin eggs leading to release of eIF4E from its translational repressor 4E-BP (Cormier et al., 2001; Oulhen et al., 2010, 2007; Salaun et al., 2005, 2004, 2003). Recent findings suggest that the de-phosphorylation of eIF2α could participate in the global increase in translation induced by fertilization (Le Bouffant et al., 2008). On the other hand, although specific localization changes of eEF1B (eukaryotic elongation factor B) during the first cell cycle have been reported (Le Sourd et al., 2006), no clear implication of elongation factors has yet been demonstrated.

We report the existence of two eEF2 isoforms in sea urchin eggs, originating from a unique core protein. We show that phosphorylation of these isoforms is specifically and differentially regulated after fertilization. Both are dephosphorylated shortly after fertilization, the eEF2 83 kDa isoform subsequently remaining in an active dephosphorylated state, while the eEF2 80 kDa isoform is rephosphorylated in correlation with completion of the cell cycle.

Materials and methods

Antibodies

Rabbit polyclonal antibodies directed against epitope mapping at the C-terminus (amino acids 741–858) of human EF-2 (sc-25634) were obtained from Santa Cruz. Rabbit polyclonal antibodies directed against a synthetic phosphopeptide corresponding to residues surrounding Thr56 of human eEF2 (#2331) and rabbit polyclonal antibodies directed against the amino-terminus of human eEF2 (#2332) were from Cell Signaling. Mouse monoclonal antibody directed against the PSTAIR sequence of cyclin dependent kinase-1 (7962) and rabbit polyclonal antibodies directed against human actin (20–33) were purchased from Sigma. Rabbit polyclonal antibodies directed against a synthetic peptide from human elongation factor-1B-gamma subunit (abcam ab72368) were from AbCam. Rabbit polyclonal antibodies directed against the *Sphaerechinus granularis* 4E-BP were previously described (Oulhen et al., 2010). Swine anti-rabbit and goat anti-mouse IgG (horseradish peroxydase-coupled) were obtained from Dako SA.

Preparation of gametes and embryos

S. granularis sea urchins collected in the Brest area (France) were maintained in running seawater. Spawning of gametes, fertilization, calcium ionophore (A23187 Sigma) and NH4Cl activation and cell culture were as described (Oulhen et al., 2010). Where indicated, different concentrations of the inhibitor NH125 (Sigma) from a 10 mM stock solution in DMSO were added to the eggs 20 min before fertilization. Cleavage was scored under a light microscope. Each experiment used gametes from a single female exhibiting greater than 90% fertilization.

Embryo extracts

Crude total extracts were obtained by direct dissolution of a 20 µl cell pellet (corresponding to 12,000 eggs or embryos) with 150 µl of SDS-Fix buffer containing 2% sodium dodecyl-sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris HCl pH 6.8. Dissolved proteins were then analyzed by western blotting.

Egg or embryo lysates were obtained as described (Oulhen et al., 2007). Briefly, one volume of pelleted eggs or embryos was homogenized in one volume of buffer containing 40 mM HEPES pH 7.4, 0.2 mM sodium orthovanadate, 100 mM NaCl, 0.4 mM EDTA, 2 mM DTT, 20 mM pyrophosphate, 100 mM sodium fluoride, 100 mM β-glycerophosphate, 1 mM 4-(2-aminoethyl)-benzene sulfonylfluoride hydrochloride (AEBSF) and 20 µg/ml of aprotinin and leupeptin. Homogenates were clarified by centrifugation for 15 min at 16,000 *g* at 4 °C and the supernatants were used for immunoprecipitation experiments. Protein quantification was performed in duplicate by the Bradford assay (Bradford, 1976).

Immunoprecipitation analysis, electrophoresis and western blotting

Immunoprecipitations from egg or embryo lysates (1 mg protein) were carried out overnight at 4 °C as described (Le Sourd et al., 2006) using antibodies dilution of 1/100 in buffer containing 50 mM Tris HCl pH 7.4, 500 mM NaCl, 1% IGEPAL (Sigma), 1% BSA, 50 mM sodium fluoride, 10 mM pyrophosphate, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM 4-(2-aminoethyl)-benzene sulfonylfluoride hydrochloride (AEBSF). Protein A (CL-4B sepharose beads Sigma) bound proteins were eluted with sample buffer for electrophoretic analyses (Laemmli, 1970).

Electrophoresis and western blotting were performed as reported (Oulhen et al., 2010). Incubation with the primary antibodies was performed overnight at 4 °C in the presence of antibodies directed against the phosphorylated epitope of human eEF2 (1/1000), the C-terminus end of human eEF2 (1/1000), or the N-terminus end of human eEF2 (1/1000). Loading controls were conducted with antibodies against actin (1:1000) or PSTAIR (1:1000). Bands revealed by ECL (Amersham Pharmacia Biotech) detection were quantified after digitization of the films, using the ImageJ 1.43j program (Wayne Rasband, National Institutes of Health, USA). Results are expressed as mean of n experiments with standard error of the mean (SEM).

Peptide mass fingerprinting analysis

Peptide mass fingerprinting by MALDI-TOF MS (Pappin et al., 1993; Yates et al., 1993) was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems). Spectra were acquired in positive ion reflector mode under 20 kV accelerating voltage and a mass range of 450–5000 Da. Internal calibration was performed using trypsin autolysis fragments at *m/z* 1433.70, 2163.05, and 2289.1. Peptide mass maps were searched against the NCBI database sequences using the Mascott program and against the *S. granularis* sequence using the MS-Bridge program of the Protein Prospector suite (Regents of the University of California, CA).

The N-terminus sequence of the protein was obtained by Edman micro-sequencing on the mass spectrometry platform at the Institut de Biochimie et Biophysique Moléculaire et Cellulaire (IBBMC, UMR 8619, Orsay, France).

Protein synthesis in vivo

Every 30 min, embryos (5% suspension in seawater) were radiolabelled by a 10 min pulse in the presence of [^{35}S]-L-methionine (Perkin-Elmer), at a final concentration of 10 µCi/ml. Cells (500 µl) were pelleted and frozen in liquid nitrogen and processed as described (Oulhen et al., 2010).

Results

eEF2 resolves as two phosphorylated isoforms in the sea urchin egg

Immunodetection of eEF2 in sea urchin eggs

Analysis of the full genome sequence of *Strongylocentrotus purpuratus* revealed that sea urchin elongation factor 2 (eEF2) is encoded by a unique gene sharing 75% identity with its human homolog at the primary protein sequence level (Morales et al., 2006). The deduced protein sequence of 841 amino acids includes the conserved threonine residue (Thr-56 as numbered in the human sequence) known to be the site of phosphorylation by eEF2 kinase (Herbert and Proud, 2007).

The presence and phosphorylation status of eEF2 in unfertilized *S. granularis* eggs were analyzed by western blot using commercial antibodies specifically directed against the human elongation factor (Fig. 1). Experiments were conducted using crude extracts obtained from immediate dissolution of eggs or embryos in electrophoretic buffer. An antibody directed against the phosphorylated epitope of human EF2 revealed two proteins at respectively 80 and 83 kDa upon 10% polyacrylamide gel electrophoresis, corresponding to the expected migration for eEF2 (Fig. 1A, lane α P-eEF2). The same extracts were analyzed using antibodies directed against human eEF2 N-terminus end (α N-eEF2) and human eEF2 C-terminus end (α C-eEF2), both known to recognize the eEF2 protein whether phosphorylated or not. The human eEF2 N-terminus antibody decorated a unique specific band at 83 kDa corresponding to the upper band revealed with the phospho-eEF2 antibody (Fig. 1A; compare lane α N-eEF2 and lane α P-eEF2). The human eEF2 C-terminus antibody mainly revealed the 83 kDa protein and faintly but reproducibly an 80 kDa protein corresponding to the lower band present with phospho-eEF2 (Fig. 1A; compare lane α C-eEF2 and lane α P-eEF2). Therefore, the 83 and 80 kDa bands are immunologically related to eEF2 and are present as two phosphorylated isoforms in the unfertilized egg. Using the human eEF2 C-terminus antibody, the signal ratio in ten different experiments using different females was around 10:1 (83/80 kDa). When analyzed with the antibody directed against the phosphorylated epitope of human EF2, the ratio was found to be highly variable between the ten different preparations, but overall in the order of magnitude of 1:1. Therefore, the experiments suggest that the 83 kDa band is quantitatively predominant, while the stochiometry of phosphorylation is in favor of the 80 kDa band.

Unexpectedly, when the extracts were prepared by egg homogenization instead of direct dissolution, different mobility patterns of the 80–83 kDa bands were observed on the immunoblots using the antibody directed against the phosphorylated epitope of eEF2 (Fig. 1B). When homogenized extracts were performed on freezed-pelleted embryos, a unique band at 83 kDa was revealed by the antibody (Fig. 1B, lane H-UE) as compared to the two bands detected at 83 and 80 kDa after direct egg dissolution (see Figs. 1A, lane α P eEF2 and B, lane D-UE). This feature was particularly striking when we analyzed post-fertilized embryos. At 90 min after fertilization, the sole protein detected by the antibody directed against the phosphorylated epitope of eEF2 migrated at 80 kDa in directly dissolved embryo extracts (Fig. 1B lane D–F and see Results section part II.A). By

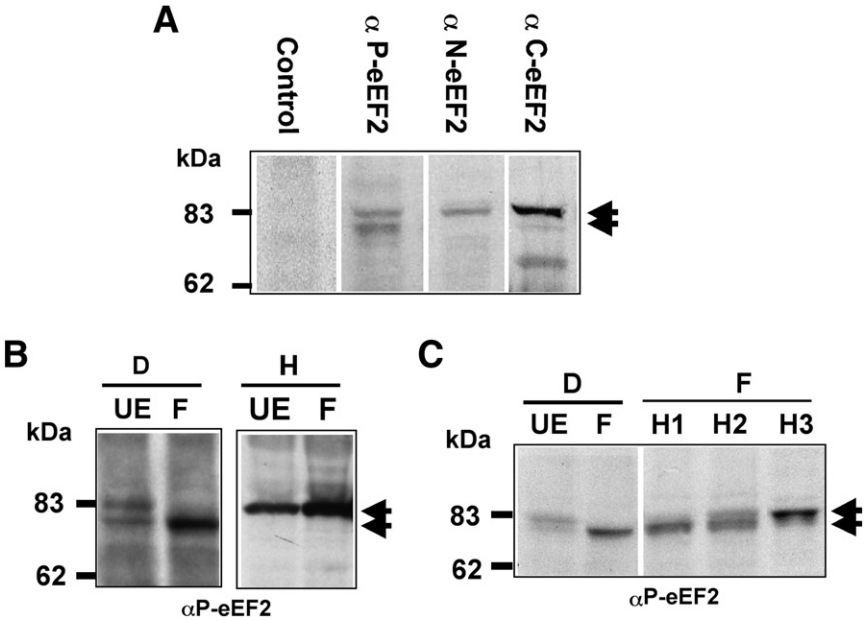

Fig. 1. (A) Sea urchin eggs contain two phosphorylated isoforms immunorelated to eEF2. Total extracts were prepared by direct dissolution of unfertilized eggs. After electrophoresis proteins were analyzed using antibodies directed against the phosphorylated epitope of human eEF2 (α P-eEF2), the N-terminus end of human eEF2 (α N-eEF2), or the C-terminus end of human eEF2 (α C-eEF2). Controls involved incubation with secondary antibodies only. Arrows indicate the position of the two eEF2 related isoforms. Molecular-weight ladders (in kDa) are shown on the left. (B) Eggs homogenization changes 80 kDa isoform migration. Protein extracts were prepared after direct dissolution (D) or homogenization (H) of unfertilized eggs (UE) or of eggs taken 90 min after fertilization (F). After electrophoresis proteins were analyzed using antibodies directed against the phosphorylated epitope of human eEF2 (α P-eEF2). Molecular-weight ladders (in kDa) are shown on the left. Arrows indicate the position of the two eEF2 related isoforms. (C) *In vitro* transformation of the 80 kDa isoform into the 83 kDa isoform. Protein extracts were prepared by homogenization of eggs taken 90 min after fertilization (F). At the different times after homogenization (H1: 0 min; H2: 60 min; H3: 10 h), aliquots were fixed in SDS-buffer. Proteins were analyzed after electrophoresis and immunoblotting using antibodies directed against the phosphorylated epitope of human eEF2 (α P-eEF2) and compared to proteins from immediately dissolved (D) foster embryos (UF: unfertilized, F: fertilized). Molecular-weight ladders (in kDa) are shown on the left. Arrows indicate the position of the two eEF2 related isoforms.

contrast, in embryo homogenates, a unique 83 kDa was revealed by the same antibody (Fig. 1B, lane H–F). These results suggested that the 80 kDa protein had been modified during homogenization, changing its mobility to co-migrate with the 83 kDa band.

Since direct dissolution of the eggs rather reflects the *in vivo* state, we analyzed if an *in vitro* reaction could occur during the homogenization protocol. When homogenization was performed on fresh pelleted embryos and the homogenates immediately fixed by SDS buffer, the 80 kDa band could be readily detected in embryo extracts (Fig. 1C, lane H1). A progressive transformation of the 80 kDa signal in a 83 kDa signal was observed when the extracts were maintained at 4 °C (Fig. 1C, lanes H1 to H3) before the addition of the SDS buffer. The rate of the *in vitro* reaction taking place after homogenization was analyzed in three independent experiments. Surprisingly, 30–50% transformation of the protein from 80 kDa to 83 kDa was observed in one hour at 4 °C. Freezing and thawing the intact eggs (see Fig. 1B) or the eggs extracts immediately after homogenization lead to 100% *in vitro* transformation into the 83 kDa isoform. An overnight incubation of the egg extracts, corresponding to the immunoprecipitation protocol also lead to 100% transformation (Fig. 1C, lane H3).

Identification of the eEF2 immunorelated bands

In order to further characterize eEF2 in sea urchin eggs, cDNA encoding EF2 in *S. granularis* eggs was isolated and sequenced (BankIt1347765 HM151974). The cDNA sequence shared 83% and 82% identity with respectively the *S. purpuratus* (SPU_10829) and *Paracentrotus lividus* (Contig9110) eEF2 nucleotide sequences. The deduced protein sequence presented 83% identity with the sea urchin eEF2 sequences and 75% identity with the human eEF2 sequence. The sequence corresponding to the putative eEF2 kinase phosphorylation motif was recognized and was identical to the human homolog sequence RF^{56}TDTRKDE.

Immunoprecipitation experiments were conducted to purify SgEF2 from unfertilized egg extracts. Neither the human eEF2 N-terminus antibody (α N-eEF2) nor the human eEF2 C-terminus antibody (α C-eEF2) were found to efficiently immunoprecipitate proteins (see for example Fig. 2A, lane 1). Immunoprecipitation with the antibody directed against the phosphorylated epitope of eEF2 led to recovery of a protein detectable at 83 kDa by Coomassie blue staining (Fig. 2A). The immunoprecipitated protein was recognized by the three specific anti-eEF2 antibodies (Fig. 2A). Mass spectrometry analysis was performed on the purified digested protein, using the protein sequence deduced from the *S. granularis* clone for identification. Thirty peptides distributed over the complete protein sequence were found to match the cDNA-deduced sequence. This confirmed that the immunopurified protein corresponded to sea urchin eEF2.

Imunoprecipitation was also performed on the 90 min post fertilization embryo extracts where the unique phosphorylated isoform detected corresponded to the 80 kDa protein (see Fig. 1B, lane D-F). As expected, the mass spectrometry analysis revealed that the immunoprecipitated protein corresponded to eEF2 as judged from the 53 peptides matching the SgEF2 deduced sequence. Furthermore, under Edman sequencing the immunopurified protein produced the N-terminus sequence VXFTGE/D highly identical to the N-terminus from the sea urchin (*S. purpuratus:* MVNFTTD; *P.lividus:* MVNFTID) and human (MVNFTVD) sequences ascertaining identification of the protein.

These experiments demonstrate the presence of eEF2 in sea urchin eggs. The protein appears under two post-translationally modified isoforms: a quantitatively dominant 83 kDa isoform and an 80 kDa isoform. The two isoforms correspond to the same core protein since the lower molecular form could fully transform *in vitro* into the higher isoform. The efficiency of the reaction taking place *in vitro* after homogenization (50% transformation in 1 h at 4 °C) does not for now allow identification of the nature of the post-translational difference between the two isoforms. Immediate dissolution of the eggs in SDS buffer allows the analysis of the *in vivo* phophorylation state of two isoforms and their physiological changes.

Fertilization triggers a complex pattern of SgEF2 isoform phosphorylation

Physiological changes following fertilization

The level and phosphorylation status of the two SgEF2 isoforms (80 and 83 kDa) were analyzed following fertilization in crude embryo extracts. Fig. 3A shows the western blot patterns obtained every 15 min during 180 min after fertilization. The amount and ratio of the two eEF2 isoforms remained unchanged after fertilization as shown by western blotting using the human eEF2 C-terminus antibody (Fig. 3A; α C-eEF2). By contrast, fertilization triggered

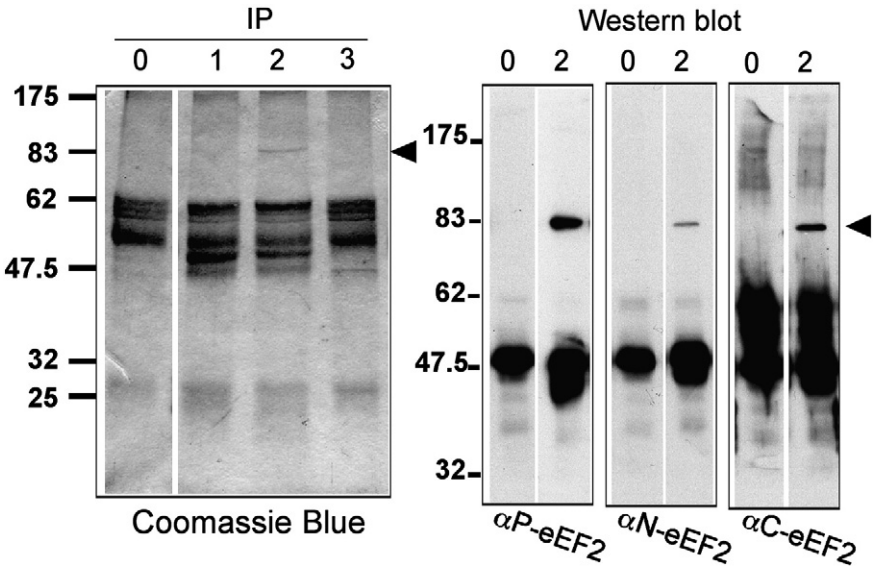

Fig. 2. Purification and identification of eEF2 immunorelated isoforms. Proteins (1 mg) from homogenates prepared from unfertilized eggs were immunoprecipitated with antibodies directed against the C-terminus end of human eEF2 (1), the phosphorylated epitope of human eEF2 (2), or a peptide from human elongation factor-1B-gamma subunit (3). For the control experiment (0), antibodies were not added. The left panel (IP) corresponds to the coomassie blue stained gel, and the right panel to western blot using antibodies directed against the phosphorylated epitope of human eEF2 (α P-eEF2), the C-terminus end of human eEF2 (α C-eEF2) or the N-terminus end of human eEF2 (α N-eEF2). Arrows indicate the position of the immunoprecipitated protein. Molecular-weight ladders (in kDa) are showed on the left.

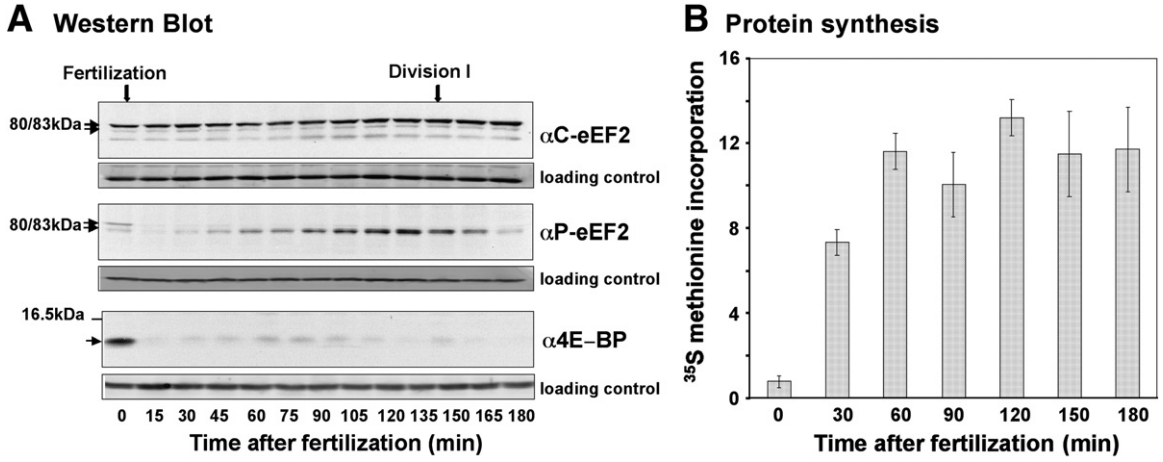

Fig. 3. Changes in the phosphorylation of eEF2 isoforms during early development. Pelleted embryos (20 µl) were sampled at the indicated times following fertilization and dissolved as indicated in the methods section. After electrophoresis proteins were analyzed using antibodies directed against the C-terminus end of human eEF2 (α C-eEF2), the phosphorylated epitope of human eEF2 (α P-eEF2) or the 4E-BP protein. Loading controls were anti-PSTAIR for eEF2 analyses and anti-actin for 4E-BP analysis. In this experiment, first cleavage occurred at 140 min. The figure is representative of experiments performed with embryos from 20 different females. (B) Kinetic of protein synthesis after fertilization. The figure shows the mean (± SEM) of the data obtained from experiments performed with embryos from 4 different females.

important modifications in the phosphorylation pattern of the two eEF2 isoforms as demonstrated by western blotting using the antibody directed against the phosphorylated epitope of eEF2. By 15 min dephosphorylation of both isoforms was reproducibly observed (Fig. 3A; α P-eEF2). The dephosphorylation of eEF2 isoforms correlated with a net increase in protein synthesis activity measured by ^{35}S-methionine incorporation into proteins (Fig. 3B). The reported disappearance of the protein synthesis initiation inhibitor, 4E-BP (Salaun et al., 2003) was also observed (Fig. 3A; α4E-BP).

Following its initial dephosphorylation, the 83 kDa isoform remained almost undetectably phosphorylated for up to 3 h after fertilization (Fig. 3A; α P-eEF2). Strikingly, the 80 kDa isoform underwent differential phosphorylation changes during the first cell divisions. After its dephosphorylation by 15 min, the 80 kDa form was progressively rephosphorylated, reaching a maximum level about 30 min before the cell division, and was again dephosphorylated shortly after the cell division (Fig. 3A; α P-eEF2). Subsequently, phosphorylation increased again during the second cell cycle (Supplementary data 1). Quantification of experiments conducted with eggs from nine different females demonstrated that the maximum increase in phosphorylation of the 80 kDa form reached 3.55 fold (SEM = 0.43) around 90–120 min after fertilization, and in all cases preceeding by 30 min the cell division judged by cytokinesis. The peak in phosphorylation of the 80 kDa isoform coincided with a previously reported transient decrease (Monnier et al., 2001) in the rate of protein synthesis (Fig. 3B).

The two eEF2 isoforms therefore appear to both be dephosphorylated early after fertilization on the eEF2 kinase phosphorylation site and subsequently differentially phosphorylated/dephosphorylated on the same site during early development.

Insights in the origin of the eEF2 phosphorylation changes

Artificial elevation of intracellular calcium by ionophore or alkalinization in the presence of NH4Cl were both reported to parthenogenetically activate unfertilized eggs and to induce an increase in protein synthesis, although cell division was not observed in these conditions (Epel, 1990; Oulhen et al., 2010). The treatment of unfertilized eggs with calcium ionophore or NH4Cl induced the dephosphorylation of both eEF2 isoforms at 15 min after treatment (Fig. 4A), and, as in fertilized embryos, was correlated with the increase in protein synthesis (Fig. 4B). After the initial decrease in eEF2 phosphorylation, the 83 kDa isoform remained in its dephosphorylated form while phosphorylation of the 80 kDa isoform increased over a time-course comparable to that of fertilized embryos, as early as 30 min post treatment (Fig. 4A). However, unlike the control fertilization, phosphorylation of the 80 kDa isoform remained at a high level for at least 3 h in the activated eggs in correlation with the absence of cell division.

Therefore, during the first 15 min after fertilization, dephosphorylation of eEF2 isoforms is mediated through calcium and pH increases, as is rephosphorylation after 30 min of the 80 kDa isoform. At 150 min after fertilization, dephosphorylation of the 80 kDa isoform relies on completion of the cell cycle.

Rephosphorylation of the eEF2 80 kDa isoform during the first cell cycle is required for completion of cell division

The role of eEF2 phosphorylation was investigated using a specific eEF2 kinase inhibitor, NH125 (Arora et al., 2003). Cytological monitoring revealed that the inhibitor appeared to be toxic to eggs when applied at 30 µM or higher. At lower concentrations, the addition of NH125 to unfertilized eggs for as long as 3 h was not toxic to the eggs as demonstrated by cytological monitoring and by their potential to be fertilized. The phosphorylation status of both eEF2 isoforms remained unchanged in the unfertilized eggs suggesting low turnover for the phosphorylation/dephosphorylation mechanism (Supplementary data 2). The effect of the inhibitor was analyzed in fertilized eggs. The inhibitor reproducibly inhibited the re-phosphorylation of the 80 kDa isoform that normally occurs from 30 min after fertilization (Fig. 5). The effect of the inhibitor was dose-dependent in a narrow range of concentrations. From 10 µM the inhibitor significantly affected the increase in phosphorylation of the 80 kDa form with a maximum effect observed at 20 µM (Fig. 5A). Quantification analyses were conducted with eggs from different females. At 90 min after fertilization, phosphorylation of the 80 kDa form was found to be increased by 2.60 (SEM 0.43, $n = 8$) in embryos incubated in the presence of 20 µM NH125 as compared to the 3.55 (SEM 0.43; $n = 9$) increase in control fertilized embryos (Fig. 5B). Therefore, at non-toxic doses, NH125 provoked a significant 27% inhibition of the physiological phosphorylation increase of the 80 kDa isoform. The effect of NH125 on cell division was investigated for the same range of concentrations. As shown in Fig. 6, NH125 inhibited the occurrence of the first cell division in the same dose-dependent range, thus correlating phosphorylation of the 80 kDa eEF2 isoform with completion of cell division.

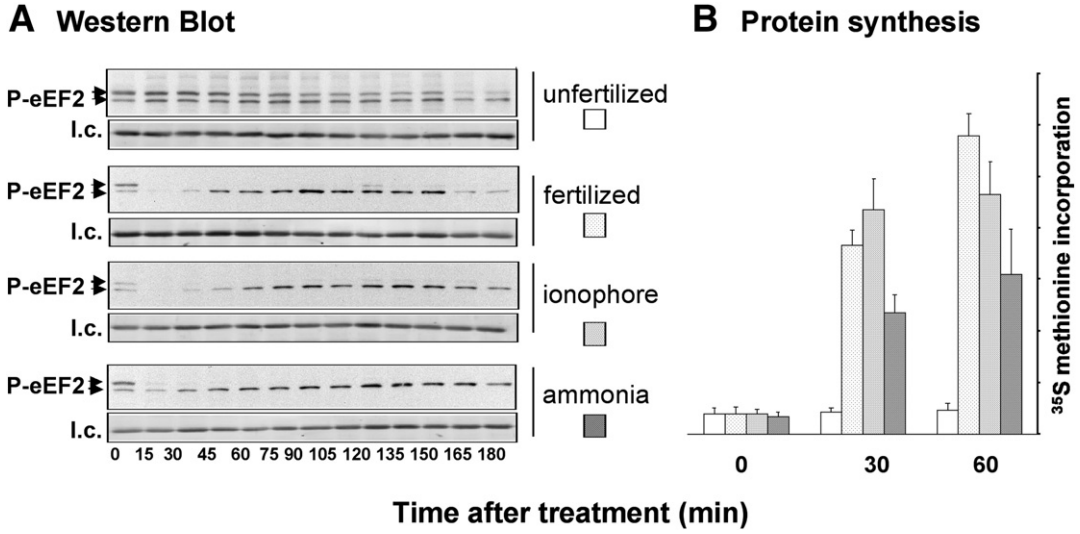

Fig. 4. Phosphorylation of eEF2 isoforms after parthenogenetic activation of eggs. Batches of eggs from the same female were incubated in the absence (unfertilized) or the presence of 10 μM calcium ionophore (ionophore), or of 10 mM NH4Cl pH 8 (ammonia) and incubated in parallel with fertilized embryos (fertilized). (A) Total dissolved extracts were prepared at the indicated times. After electrophoresis, proteins were analyzed with the antibody directed against the phosphorylated epitope of human eEF2. Arrows indicate the position of eEF2 isoforms. The loading control (l.c.) used anti-PSTAIR. The figure is representative of experiments performed on at least 15 different females. (B) Time course of protein synthesis. The figure shows the mean (± SEM) of the data obtained from experiments performed with embryos from 4 different females.

Discussion

The protein eEF2, the unique substrate for eEF2 kinase (Herbert and Proud, 2007), was identified in sea urchin eggs based on immunological detection with specific antibodies directed against human eEF2 and mass spectrometric analysis of the protein purified by immunoprecipitation using an antibody specific for the phosphorylated epitope of eEF2 kinase.

We show that the protein is present as two isoforms migrating at 80 and 83 kDa which are phosphorylated in eggs and the phosphorylation of which is specifically and differentially regulated during early development. The resolution of eEF2 as two different molecular weight bands was unexpected in light of current knowledge (Herbert and Proud, 2007). We demonstrate that a reaction taking place *in vitro* readily transforms the 80 kDa isoform into the 83 kDa isoform. Existence of two distinct eEF2 isoforms might have escaped detection in other cell types depending on the experimental conditions of homogenization. The *in vitro* transformation of the 80 kDa into the 83 kDa isoform indicates that proteins observed *in vivo* originate from the same core protein and are not the result of alternative splicing or proteolytic truncation.

The two eEF2 isoforms underwent differential *in vivo* changes in their phosphorylation status during early development. In the 15 min following fertilization, and concomitant with a global increase in protein synthesis, both isoforms underwent dephosphorylation. Since eEF2 was reported to be active in elongation under its dephosphorylated form (Herbert and Proud, 2007), the dephosphorylation of both eEF2 isoforms may contribute to the net increase in protein synthesis that follows fertilization (Epel, 1990). The contribution of eEF2 dephosphorylation to the net increase in protein synthesis appears limited since most of the eEF2 in unfertilized eggs was already in the dephosphorylated form. The main contribution to the increase in protein synthesis would depend on degradation of the regulator of initiation, the protein 4E-BP (Gingras et al., 1999), which is degraded upon fertilization (Salaun et al., 2003). Fertilization is known to involve early calcium and pH changes (Parrington et al., 2007). We show that the dephosphorylation of both eEF2 isoforms is mediated through calcium and pH signaling pathways as

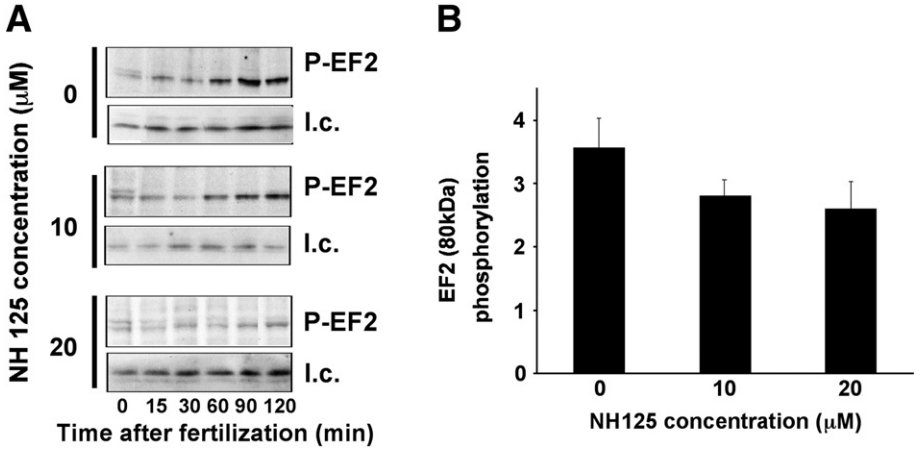

Fig. 5. Effect of NH125 on phosphorylation of the 80 kDa eEF2 isoform. Eggs were fertilized in the absence (0) or presence of NH125 at 10 μM or 20 μM. The inhibitor was added to the incubation medium 20 min before fertilization. (A) Dissolved proteins were analyzed using the antibody directed against the phosphorylated epitope of human eEF2 (α P-eEF2). Loading controls were anti-PSTAIR. The figure shows the result from a representative experiment performed on one female (B) Quantitative analysis of the effect of NH125 was performed by measuring the fold increase between time 0 and time 90 min of 80 kDa phosphorylation determined by quantification of the western blot analysis. The figure shows the results (mean ± SEM) from independent experiments performed on different females ($n = 9$, 7 and 8 for respectively 0, 10; 20 μM NH125).

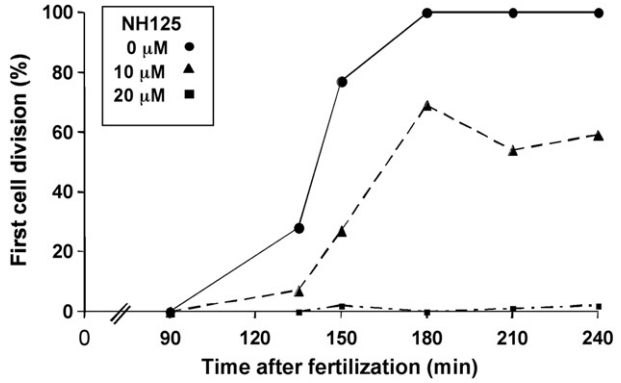

Fig. 6. Effect of NH125 on the first cell division. Sea urchin eggs in solution in filtered seawater (5% w/w) were incubated 20 min prior to fertilization in the presence of NH125 at 0 μM (circles), 10 μM (triangles) or 20 μM (squares). One hundred embryos were scored for the first cleavage by binocular microscope observation as a function of time after fertilization. The kinetic is representative of six experiments performed with gametes from four different females.

demonstrated by parthenogenetic egg activation experiments. The eEF2 kinase was identified as a calcium-calmodulin dependent kinase (Ryazanov and Spirin, 1990), therefore the increase in calcium would be expected to activate the enzyme and hence inhibit eEF2 dephosphorylation. In contrast, the increase in pH following fertilization (Parrington et al., 2007) may have contributed to a decrease in eEF2 kinase activity since this enzyme has been shown to be inhibited by increasing pH (Dorovkov et al., 2002). In unfertilized eggs eEF2, phosphorylation/dephosphorylation turnover is a slow rate process since the specific inhibitor of eEF2 kinase, NH125, had little or no effect on eEF2 phosphorylation over a 180 min period. A change in the activity of eEF2 kinase would rather lead to slow changes in the level of eEF2 dephosphorylation, therefore the rapid dephosphorylation observed after fertilization suggests an active role for a protein phosphatase, possibly protein phosphatase 2A, the enzyme responsible for dephosphorylation of eEF2 (Redpath and Proud, 1989). Such changes in protein phosphatase 2A activity have been recorded in sea urchin eggs shortly after fertilization and proposed to be a mechanism for regulating rapid dephosphorylation (Kawamoto et al., 2000).

Surprisingly, from 15 min after fertilization, the phosphorylation state of the eEF2 isoforms radically differed, the 83 kDa isoform remaining dephosphorylated while the 80 kDa isoform was gradually re-phosphorylated reaching a 3.5 fold higher level around 30 min before cell division, as compared to the initial level in unfertilized eggs. Since both isoforms contain the identical phosphorylation motif, target of the unique eEF2 kinase, the difference in their phosphorylation state must involve another feature such as their cellular localization, possibly related to the unidentifed post-translational modification. The increase in phosphorylation of the 80 kDa eEF2 isoform following its initial dephosphorylation was also recorded in parthenogenetically activated eggs. In this case, however, the phosphorylation remained high and eggs did not divide as previously reported (Epel, 1990). In parthenogenetically activated eggs, the cell cycle regulator CDK1/cyclinB is not activated (Epel, 1990). This shows that dephosphorylation of the 80 kDa isoform following completion of the cell cycle (Fig. 3; after 150 min) is under the direct or indirect control of previous CDK1/cyclinB activation.

The simultaneous presence in one cell of two eEF2 isoforms subject to specific differential regulation through phosphorylation could contribute to explanation of previously reported contradictory data on eEF2 phosphorylation during mitosis (Sivan et al., 2007; Smith and Proud, 2008). eEF2 kinase down-regulation during mitosis as reported by Smith and Proud (Smith and Proud, 2008) would be required to keep the major eEF2 83 kDa isoform under its dephosphorylated active form, whereas the phosphorylated eEF2 protein described by Sivan et al. (Sivan et al., 2007) could correspond to the 80 kDa eEF2 isoform responsible for ribosomal slow-down during mitosis.

The existence of two isoforms differentially regulated by phosphorylation/dephosphorylation suggests a specific role for each not only as house-keeping elongation factor regulators. Maintenance of a major fraction of eEF2 (the 83 kDa isoform) in its dephosphorylated and therefore active form would contribute to allowing, in synergy with the absence of 4E-BP for cap-dependent initiation, the high level of protein synthesis required for embryo development, but what could be the role of re-phosphorylation of the 80 kDa isoform following its initial dephosphorylation? Of note, when the re-phosphorylation of eEF2 80 kDa was inhibited by the specific inhibitor NH125 cell division was also impaired. Since the eEF2 80 kDa isoform represents a small fraction (1:10) of total eEF2, its phosphorylation could inhibit at the most 10% of overall elongation activity. Either the small fraction of protein elongation inhibited may affect specific proteins involved in cell cycle regulation, or eEF2 80 kDa affects the cell cycle by an as yet unknown mechanism. A low level of inhibition of global elongation could more efficiently affect proteins with rapid turnover such as cyclin B, the regulatory subunit of the universal M-phase factor CDK1 (Morgan, 2007) and anti-apoptotic proteins (White-Gilbertson et al., 2008). Concerning cyclin, such a mechanism could participate to the disappearance of cyclin B whose degradation is necessary for mitosis exit (Wolf et al., 2007). Pauses in the increase in protein synthesis have been demonstrated to occur during mitosis, both in sea urchin eggs (Monnier et al., 2001) and other dividing cells (Pyronnet and Sonenberg, 2001). The phosphorylated, and thus inactivated eEF2 80 kDa isoform could be involved in the observed pauses in translation efficiency. The inhibition of elongation would stall messengers on ribosomes protecting them from degradation and allowing rapid resumption of active translation (Sivan and Elroy-Stein, 2008). Alternatively, the eEF2 80 kDa isoform may be localized differentially from the 83 kDa isoform. Its phosphorylation could be related to local translation suppression in a specific cell compartment as suggested in neuronal cells (Sutton et al., 2007). In such a compartment, translation inhibition could be complete for certain specific mRNAs involved in cell cycle regulation. The sub-localisation of each eEF2 isoform would have to be analyzed to assess this theory.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.12.015.

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