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Research Article

Sea urchin elongation factor 1δ (EF1δ) and evidence for cell cycle-directed localization changes of a sub-fraction of the protein at M phase

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Abstract. Eukaryotic elongation factor 1 (eEF1) is a translational multimolecular complex reported in higher eukaryotes to be a target of CDK1/cyclin B, the universal regulator of M phase, but whose role in the cell cycle remains to be determined. A specific polyclonal antibody was produced and used to characterize the delta subunit of sea urchin elongation factor 1 (SgEF1δ) in early embryos, a powerful model for investigating cell cycle regulation. The SgEF1δ protein was present in unfertilized eggs as two isoforms of 35 and 37 kDa, issued from two different mRNAs. The two canonical eEF1δ partners, eEF1γ and eEF1β, were shown to co-immunoprecipitate with the SgEF1δ isoforms. Both isoforms were associated in a macromolecular complex, which resolved upon gel filtration chromatography at a molecular weight > 400 kDa, suggesting association with other yet unidentified partners. After fertilization, the amount as well as the ratio of both SgEF1δ isoforms remained constant during the first cell division as judged by Western blotting. Immunofluorescence analysis showed that a pool of the protein concentrated as a ring at the embryo nuclear location around the period of nuclear envelope breakdown and was visualized later as two large spheres around the mitotic spindle poles. Thus, the eEF1δ protein shows cell cycle-specific localization changes in sea urchin embryos.

Key words. Elongation factor 1B; elongation factor 1; cell cycle; sea urchin early development; protein translation; eEF1δ immunolocalization.

Regulation of mRNA translation is recognized to play an essential role in the control of gene expression. Translational control allows a direct, rapid, reversible and localized fine-tuning of protein levels [1]. The initiation step of translation is a field of much research in translational control and initiation factors such as eIF4F and eIF2 have been shown to be the targets for protein synthesis regulation in a number of signal transduction pathways [2, 3]. Although less investigated, a regulatory role for the elongation step has also been suggested [4], mainly since the discovery of the highly organized macromolecular structure of elongation factor complex, eEF1B [5, 6]. The eEF1B multimolecular complex possesses the guanine nucleotide exchange activity for the G protein eEF1A which catalyzes the first step in elongation, i.e. the attachment of the aminoacyl-tRNA on the ribosome [7]. In most animal species, eEF1B is composed of four subunits: two different guanine nucleotide exchange proteins, eEF1β and eEF1δ, a putative anchoring protein eEF1γ and the valyl-tRNA synthetase. The two proteins eEF1β and eEF1δ are very similar in their C-terminal domain, which contains the guanine nucleotide exchange domain. However, the two domains may be functionally specialized since, surprisingly, a cDNA encoding the C

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terminal of eEF1δ was not able to complement yeast mutants conditional for EF1β [8]. Furthermore, the eEF1δ protein contains a specific N-terminal domain, characterized by the presence of a leucine zipper motif, a feature related to a protein-binding function [9]. Thus, the specific function of eEF1δ requires determination.

Many reports have implicated interactions of eEF1B or its subunits with different cellular constituents. In cultured cells, the major fraction of eEF1B has been shown to localize at the level of the endoplasmic reticulum, correlating with the role of the factor in peptide chain elongation [10, 11]. The protein eEF1A has been shown to be an actin-binding protein as well as a microtubule-associated protein involved in the bundling and severing of F-actin and microtubules [reviewed in ref. 5]. The eEF1γ subunit has long been shown to co-purify with tubulin [12] and *Dictyostelium* EF1β was recently reported to be an actin-binding protein [13]. The entire eEF1B complex was shown to interact with chrome- or transplatine-damaged DNA [14]. Ionizing radiation, which arrests the cell cycle at the G2/M transition by activating the DNA checkpoint, induced overexpression of eEF1δ [15]. These data suggest a role for eEF1B in DNA repair. eEF1δ contains a leucine zipper domain whereas eEF1γ possesses a lysine-rich domain, both structures reminiscent of transcription factor structure and therefore possibly implicated in DNA or RNA binding. Indeed, eEF1γ has been reported to bind the 3′ untranslated region (3′UTR) of vimentin mRNA, a region implicated in the perinuclear localization of this messenger [16]. Altogether, these interactions, which could reflect an efficient targeting of protein synthesis [17], may also be representative of new cellular functions of eEF1 constituents unrelated to their elongation activity.

Several lines of evidence have implicated the elongation factors in the regulation of cell proliferation. Tumor cells were reported to exhibit overexpressed mRNA and/or protein levels of the main eEF1 constituents: eEF1A, eEF1β, eEF1γ and eEF1δ [reviewed in refs 18, 19]. Furthermore, PTP1, corresponding to a mutated and truncated form of eEF1A, is an oncogene [20]. Likewise, eEF1δ is now considered as a true proto-oncogene in the oncogenic response of the cell to cadmium [21, 22]. All the components of eEF1B have been shown to undergo phosphorylation through the action of various kinases involved in the mitogenic signal and the identification of eEF1γ and eEF1δ as major substrates of CDK1/cyclin B, the kinase controling the G2/M transition of the cell cycle, strongly argues for a regulatory function of those factors during the cell cycle [6]. Of note, the site phosphorylated in eEF1δ by CDK1/cyclin B has also been reported to be the target for a conserved protein kinase expressed by all sub-families of herpes viruses [reviewed in ref. 23]. In cells infected by herpes viruses, the cellular eEF1δ protein interacts with a viral regulatory protein (ICP0, Tat) and affects the translational efficiency of host cell mRNA [24]. Could the regulation of eEF1δ by the viral kinase mimic a cellular pathway regulated by CDK1/cyclin B during the cell cycle? Such regulation could affect either the synthesis of a specific protein or proteins [25] or a yet unknown function or functions of eEF1δ.

To gain insight into the functions of eEF1δ in relation to the regulation of the cell cycle, we analyzed eEF1δ during the first mitotic division of the sea urchin embryo, which has proven to be a useful model in cell cycle [26] and translational control [1] studies. This report describes the characterization of EF1δ of sea urchin embryo present as two isoforms encoded from two mRNAs, and the translocation to the mitotic spindle during the first mitotic cell cycle of a pool of the SgEF1δ protein.

Material and methods

Handling of animals and gametes

Sea urchins of the species *Sphaerechinus granularis* were collected in the Brest area. Eggs and sperm were collected after injection of 0.1 M acetylcholine into the general cavity of the animals. After three washes, eggs were suspended in filtered natural seawater at a 10% (v/v) dilution. For fertilization, diluted sperm were added to an egg suspension in the presence of 0.5 mM 3-amino-1,2,4-triazole (ATA) and 0.1% glycine and were withdrawn after the occurrence of fertilization as judged by membrane elevation around the eggs. Embryos were allowed to develop at 16 °C under gentle agitation. The fertilization membrane was removed after 10 min by treatment with 0.05–0.1 mg/ml pronase (Boehringer Mannheim) in seawater for no more than 5 min. Embryo treatment by microtubule-interfering drugs was performed by adding 10 µM taxol (Sigma) or 10 µM nocodazole (Sigma) to the embryo suspension at the indicated time.

Recombinant proteins and anti-sea urchin EF1δ antibodies

Recombinant sea urchin glutathione S-transferase-EF1δ fusion protein (GST-SgEF1δ) was produced in bacteria and the protein was affinity purified on a glutathione Sepharose column as described elsewhere [27]. The coding region of SgEF1δ [28] was inserted in frame into the *Nco*I and *Eco*RI sites of the isopropyl-1 thio-β-galactopyranoside-inducible fusion vector pET-32 (Novagen) to generate a fusion protein containing a His-tag on both sides of the SgEF1δ protein. The recombinant HIS-SgEF1δ protein was produced in bacteria and purified on a nickel column according to the pET System Manual instructions (Novagen). Polyclonal antibodies against the recombinant proteins were obtained in guinea pigs by a standard immunization protocol (Eurogentec). The sera were kept in aliquots at –20 °C.

Antibodies purified by batch incubation of the serum with protein A sepharose CL-4B (Pharmacia Biotech) were covalently coupled to immobilized protein A as described in Harlow and Lane [29]. The anti-SgEF1δ antibody beads were diluted (20%) in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) and kept at 4°C.

Embryo extracts and protein analysis

Whole-embryo extracts at different times following fertilization were obtained by homogenization of pelleted embryos in 1 vol of buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris HCl, pH 6.8.

Embryo extracts were prepared from large batches of pelleted unfertilized eggs homogenized in 2 vol of buffer containing 50 mM Tris HCl pH 7.4, 75 mM KCl, 50 mM sodium fluoride, 10 mM Na_2HPO_4, 2 mM EDTA, 10 mM ATP, 5 mM paranitrophenylphosphate, 100 µM orthovanadate, 0.3 mM Na-benzoyl-L-arginine methyl ester (BAME), 1 mM benzamidine, 5 µM soybean trypsin inhibitor, 0.3 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM 4-(2-aminoethyl)-benzene sulfonylfluoride hydrochloride (AEBSF), in the presence of 100 mM NaCl. Homogenates were clarified by centrifugation (4000 g for 20 min). The supernatant for gel filtration chromatography and for affinity purification was obtained by further centrifugation at 100,000 g for 30 min. All operations were carried out at 4°C.

Proteins from the 100,000 g supernatant were size-fractionated by chromatography at 0.4 ml/min on a Superose 6 HR 10/30 gel filtration column (Pharmacia Biotech), mounted on an HPLC system (BioCad Sprint; Applied Biosystems) equilibrated with 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 2 mM EDTA. Eighty fractions of 100 µl were collected and analyzed by electrophoresis and Western blot. Calibration of the Superose 6 was carried out with the HMW gel filtration calibration kit from Pharmacia Biotech.

Immunoprecipitation experiments were performed by incubation of the 100,000 g supernatant in the presence of anti-SgEF1δ antibodies at a dilution of 1/200 in 1 ml immunoprecipitation (IP) buffer [50 mM Tris HCl pH 7.4, 500 mM NaCl, 1% Nonidet P40 (NP40), 1% bovine serum albumin (BSA) containing 50 mM sodium fluoride, 10 mM pyrophosphate, 100 µM orthovanadate, 10 mM β-glycerophosphate, 1 mM AEBSF, for one night at 4°C. The immune complex was recovered using Protein A-Sepharose CL-4B and analyzed by gel electrophoresis.

For the affinity purification procedure, the 100,000 g supernatant was incubated in batches with 1% BSA-presaturated anti-SgEF1δ beads (50 µl packed beads), in 1 ml IP buffer for 2 h at 4°C. After washing, the bound proteins were eluted by electrophoresis buffer and resolved on a gel [30].

The sea urchin EF1δ protein was synthesized in vitro in a rabbit reticulocyte lysate (Retic lysate IVT, Ambion) using mRNA transcripts from the SgEF1δ clone as described previously [28]. The reaction proceeded for 1 h at 37°C in the presence of 20 µCi [^{35}S] methionine (1000 Ci/mmol; Amersham). The reaction products were resolved by electrophoresis on a gel, either directly or after immunoprecipitation with the anti-SgEF1δ antibodies. Labeled proteins were detected by autoradiography on Hyperfilm-βmax.

Electrophoretic resolution of proteins was performed under SDS denaturing conditions on a 12% polyacrylamide gel according to Laemmli [30]. Proteins were revealed by Coomassie blue staining. For immunodetection, proteins were transferred from the gel onto nitrocellulose membranes (Schleicher and Schuell). After Ponceau Red staining, membranes were saturated for 1 h in PBS buffer containing 1% BSA and 0.1% Tween and probed with anti-SgEF1δ antibodies diluted 1/2000 in PBS/BSA/Tween. After 2 h to overnight incubation and washing, the bound antibodies were revealed with rabbit anti-guinea pig IgG conjugated to peroxidase (diluted 1/5000; Dako) and a chemiluminescence system (ECL; Pharmacia Biotech) and detected on Kodak X-OMAT film. Loading controls were performed by incubating the membranes with monoclonal anti-PSTAIR antibody (diluted 1/3000; Sigma).

For microsequencing, the proteins were resolved by electrophoresis. Isolated protein bands were excised from the gel after Coomassie staining. In-gel digestion by trypsin was performed and the resulting peptides were separated by reverse-phase microbore HPLC. They were submitted to gas-phase sequencing as already reported [31].

The Bradford assay method [32] was used to quantify protein.

RNA analysis

Total RNA from unfertilized eggs was prepared as described in Chomczynski [33]. Total RNA (500 ng) was hybridized with oligo dT (Sigma) for 10 min at 70°C, and reverse-transcribed for 50 min at 37°C with Superscript Reverse Transcriptase (Invitrogen). Forward primer GAGGCTAAGTACCAGGAACAC and reverse primer GCAAAGGGGTCAAAGTCATCG were used for amplification under the following conditions in a GeneAmp 2700 (Applied Biosystem): 94°C for 3 min, 28 cycles of 94°C 30 s, 48°C 45 s, 72°C 1.5 min, and 74°C for 10 min. PCR fragments were analyzed on 1% agarose/TBE gels, excised from the gel, purified by Genelute (Sigma), cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced on both strands at the Genomer sequencing platform (Roscoff).

Immunofluorescence experiments

Eggs and embryos were fixed for at least one night at 4°C in cold 90% methanol, 50 mM EGTA, either directly or

after extraction for 1 h at 4 °C with buffer (25 mM MES pH 6.8, 10 mM EGTA, 0.55 mM $MgCl_2$; 25% glycerol, 1% NP40, 1 mM AEBSF) [34]. After rehydratation in 0.05% Tween in PBS, followed by 1 h saturation in the presence of 1% BSA, eggs were incubated for 2 h in primary antibodies, and then for 1 h in immunofluorescent-coupled secondary antibodies. After washing, cells were mounted in Citifluor AF3 and observed under a fluorescence microscope (Olympus BX60) using a ×10 and ×40 objective (UplanFl, numerical aperture 0.30 and 0.75; Olympus). Pictures were taken using an RT monochrome CCD camera (Diagnostic Instrument, Inc.).

Confocal imaging was performed using a laser scanning confocal microscope (model IX70/Fluoview; Olympus) equipped with two lasers, an argon-krypton laser (643-OLYM-A03 Omnichrome; Melles Griot) to excite fluorescein at 488 nm, and a pulsed laser (Mira900; Coherent) that produces a 760-nm wavelength, which gave an equivalent two-photon excitation around 380 nm for excitation of the DNA dye bis-benzimide. The fluorescence produced by the different fluorochromes was collected at wavelengths below 480 nm and between 510 and 550 nm. Dichroic mirrors used were FVX-DM488/568 for the argon-krypton laser and 650 DCSP XR for the pulsed laser. Confocal images were acquired with a ×40 objective (UplanApo, numerical aperture 0.85; Olympus). The distance between each slice was 0.5 µm.

Immunolocalization of sea urchin EF1δ was performed using anti-SgEF1δ antibodies (diluted 1/100) as primary antibodies and fluorescein-linked goat anti-guinea pig antibody (diluted 1/100) as the secondary antibody. For tubulin detection, monoclonal anti-β-tubulin antibody (Sigma), at a dilution of 1/100, was used as a primary antibody and a fluorescein-linked goat anti-mouse antibody (diluted 1/100) as a secondary antibody. Affinity-purified rabbit polyclonal anti-sea urchin cdk2 antibodies (a gift from A.M. Genevière, Banyuls, France [35]) were used at a dilution of 1/50, with fluorescein-linked goat anti rabbit antibodies at 1/200 as secondary antibodies. All fluorescein-coupled antibodies were from Sigma.

When required, DNA staining was performed using the DNA dye bis-benzimide (1 µg/ml) applied during the last rinse after secondary antibody incubation. Alternatively, eggs or embryos were directly fixed in 75% methanol, 25% glycerol in the presence of 0.1 µg/ml bis-benzimide.

Results

Isolation and characterization of anti-sea urchin EF1δ antibodies

Polyclonal antibodies against sea urchin EF1δ were raised in guinea pigs by immunization with the purified recombinant GST-tagged SgEF1δ [27]. The sera obtained were tested for sensitivity and selectivity. Immunoblotting experiments were performed on different amounts of the purified bacterially produced recombinant protein (fig. 1A). The anti-sea urchin EF1δ antibodies revealed the GST-SgEF1δ protein, migrating at 52 kDa. The lowest amount of antigen that could be detected was 10 ng. Since the polyclonal antibodies could have recognized only the GST-tag, Western blot experiments were done using as the antigen another SgEF1δ fusion protein devoid of the GST-tag and containing a histidine-tag. The antibodies readily revealed the HIS-SgEF1δ protein (fig. 1A), demonstrating that they were also directed against the SgEF1δ moiety of the fusion protein.

The genuine sea urchin EF1δ (SgEF1δ) was synthesized in reticulocyte lysates from the mRNA transcript produced in vitro from the SgEF1δ cDNA [28]. A 35-kDa labeled protein was obtained corresponding to neo-synthesized SgEF1. The labeled 35-kDa protein was readily immunoprecipitated by the anti-SgEF1δ antibodies (data not shown), thus confirming SgEF1δ recognition by the antibodies. Immunoblotting of proteins from embryo total extracts using the anti-SgEF1δ antibodies revealed a doublet at 35/37 kDa (fig. 1B; right panel). The antibodies did not react significantly with other proteins even at very high (30 µg) extract amounts (fig. 1B). As a control, immunoblotting using the pre-immune serum showed no aspecific-reacting proteins (fig. 1B, lane S).

Batches of anti-SgEF1δ antibodies were pre-incubated with increasing amounts (1–300 µg) of purified recombinant GST-SgEF1δ protein and then used to immunoblot equal amounts (3 µg) of embryo extracts. Figure 1C (lane 2) shows that as little as 10 µg GST-SgEF1δ totally abolished the antibody reactivity towards endogenous SgEF1δ. Conversely, pre-incubations using up to 300 µg of BSA did not hinder the antibody-antigen reaction (fig. 1C, lane B).

Therefore, the anti-SgEF1δ antibodies specifically and efficiently recognize native SgEF1δ as a 35/37-kDa doublet.

Characterization of two SgEF1δ proteins in sea urchin embryos:

The anti-SgEF1δ antibodies revealed that SgEF1δ is present in embryo extracts as a 35/37-kDa doublet (see fig. 1B). Among different experiments, the ratio of the lower band versus the upper band was determined to be 2/1 as judged from densitometric analysis of the immunoblots. The 35/37-kDa SgEF1δ doublet was further analyzed after immunoprecipitation from embryo extracts. Beside the two major stained bands corresponding to the heavy (47 kDa) and light (28 kDa) chains of the immunoglobulins, the 35/37-kDa SgEF1δ doublet was recovered (fig. 2A). In addition, the immunoprecipitate contained a thin 45-kDa band just below the heavy immunoglobulin chains, a 30-kDa band scarcely resolved from the light

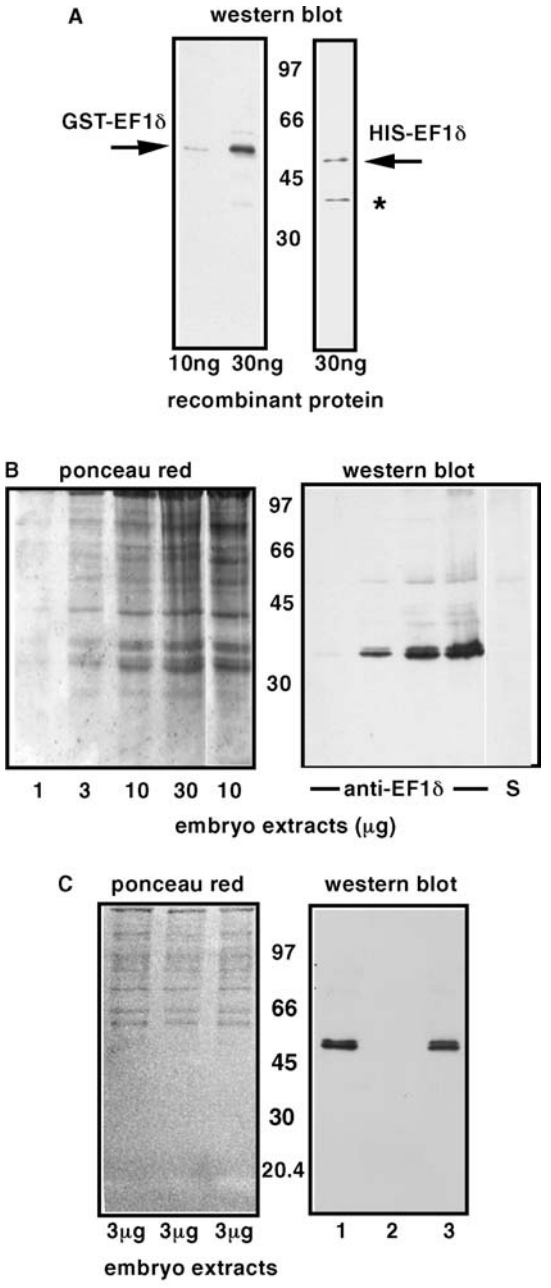

Figure 1. Characterization of SgEF1δ antibodies. (*A*) Western blot of recombinant SgEF1δ proteins. Purified recombinant proteins tagged with glutathione S-transferase (GST) or histidine (HIS) were resolved on 12% polyacrylamide gels and revealed by immunoblotting with anti-SgEF1δ antibodies as described in Materials and methods. Arrows indicate the position of the recombinant proteins. An asterisk points to a 40-kDa truncated form of HIS-SgEF1δ. (*B*) Western blot of egg extracts. Proteins of indicated amounts of egg extract were resolved on 12% polyacrylamide gel and transferred onto nitrocellulose. Left, total proteins revealed by Ponceau Red; right, immunorevelation with anti-SgEF1δ antibodies or with the corresponding pre-immune serum. (*C*) Western blot of egg extract with saturated antibodies. Identical amounts of egg extract proteins (3 μg) were resolved on the gel and immuno-revealed using anti-SgEF1δ antibodies (lane 1), anti-SgEF1δ antibodies pre-incubated with 10 μg recombinant GST-SgEF1δ (lane 2) and anti-SgEF1δ antibodies pre-incubated with 300 μg BSA (lane 3). The positions of molecular-weight markers run in parallel are indicated in kDa.

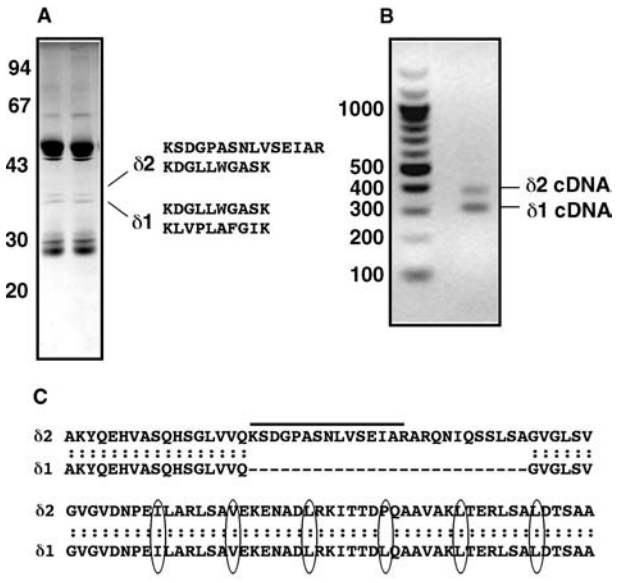

Figure 2. Presence of two SgEF1δ isoforms in sea urchin embryos. (*A*) An anti-SgEF1δ immunoprecipitate from egg extract (1 mg) was resolved on 12% polyacrylamide gel. Proteins were revealed by Coomassie blue staining. The two bands (δ1 and δ2) were excised from the gel and processed for microsequencing (see Materials and methods). The peptide sequences obtained from each band are shown. (*B*) RT-PCR amplification was performed using specific SgEF1δ primers on total RNAs (500 ng) isolated from embryos, as described in Materials and methods. The PCR products were analyzed by electrophoresis on a 1% agarose gel. Fragment sizes were estimated from RNA markers run on the same gel. The lower fragment (δ1 cDNA) and the upper fragment (δ2 cDNA) were cloned and sequenced. (*C*) Comparison of cDNA deduced protein sequences from δ1 and δ2 fragments. The sequence matching the peptide obtained by microsequencing is upperlined. The heptad repeat of the leucine zipper is boxed.

immunoglobulin chains and a faint band migrating at 67 kDa. The 35- and 37-kDa proteins were individually excised from the gel and analyzed by peptide microsequencing after trypsin digestion. Two peptides were obtained from the lower band (δ1), which matched the protein sequence of sea urchin EF1δ and all eEF1δ sequences present in the databases. A peptide identical to that recovered from the 35-kDa band was also recovered from the upper 37-kDa band (δ2). The upper band contained, in addition, a peptide which showed no similarity with any known sequence. Thus, the upper 37-kDa band corresponds either to a post-translational modified SgEF1δ protein co-migrating with another unidentified protein, or to a second SgEF1δ protein different in its amino acid sequence as suggested by the singular peptide.

The possibility that the two isoforms of sea urchin EF1δ issued from two mRNAs was tested by RT-PCR on total RNAs. The primers were designed from the known sequence of sea urchin EF1δ cDNA, chosen in the open reading frame of the cDNA. The products of amplification resolved on the gel showed two fragments migrating at 300 and 380 bp (fig. 2B). Upon sequencing, the lower

fragment was found to contain 305 bp and to be 100% identical to the already known SgEF1δ sequence (EMBL Y14236 [28]) and therefore issued from the corresponding messenger. The upper fragment contained 383 bp and presented a similarity of 97% at the nucleotide level with the 305-bp fragment. The extra 78 bp were present as an inserted sequence located in phase from the 52nd position. The peptide encoded by the 383-bp fragment was identical to that encoded by the 305-bp fragment, apart from an insertion of 26 amino acids (fig. 2C). The unknown peptide issuing from the microsequencing of the 37-kDa protein was found in this 26 amino acid-inserted sequence (upperlined in fig. 2C). The 383-bp sequence contained the leucine zipper motif characteristic of the delta subunit. Therefore, the 383-bp fragment issues from a new SgEF1δ messenger (EMBL data bank AJ541051; SgEF1δ2 partial sequence)

Thus, the sea urchin embryo contains two isoforms of SgEF1δ which differ by the presence in one of them of a stretch of 26 amino acids resulting in a 2-kDa difference in electrophoretic mobility. The two protein isoforms are encoded by two distinct mRNAs, both present in the early embryo.

Presence of sea urchin EF1δ in a high molecular-weight complex

Quantification of SgEF1δ1/δ2 in the embryos was performed by immunoblotting increasing amounts of embryo extract and comparing them to increasing amounts of purified GST-SgEF1δ fusion protein (fig. 3). Revelation was performed with the antibody directed against the HIS-SgEF1δ recombinant protein to avoid interference of the GST-tag with the signal of the antibody. The same signal intensity as judged by densitometric analysis was obtained with 30 ng pure GST-SgEF1δ and 2 µg embryo extract. This result showed that SgEF1δ1/δ2 in the embryo accounted for approximately 1.5% of the total proteins.

The proteins from an embryo extract were resolved by gel filtration chromatography under non-denaturing conditions. The presence of SgEF1δ was screened by immunoblotting of the different fractions with anti-SgEF1δ antibodies. Both isoforms (35 and 37 kDa) were recovered in the same fractions eluting around 440 kDa (bar in fig. 4A). No signal was ever detected at the level of free SgEF1δ, around 35–37 kDa. Therefore, in sea urchin embryo, the entire SgEF1δ1/δ2 pool is enclosed in a macromolecular complex or complexes with a molecular weight greater than 400 kDa.

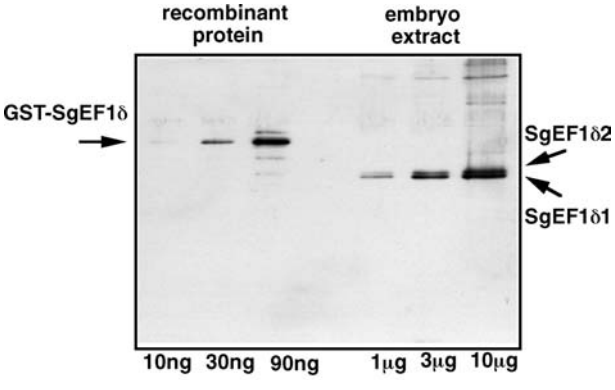

Figure 3. Quantification of SgEF1δ in sea urchin embryo. Increasing amounts of recombinant GST-SgEF1δ (10–90 ng) and of egg extract (1–10 µg) proteins were resolved on a 12% polyacrylamide gel and revealed by immunoblotting using anti-HIS-SgEF1δ antibody. Arrows point to the position of recombinant protein (left) and of the two native sea urchin EF1δ isoforms (δ1 and δ2) (right).

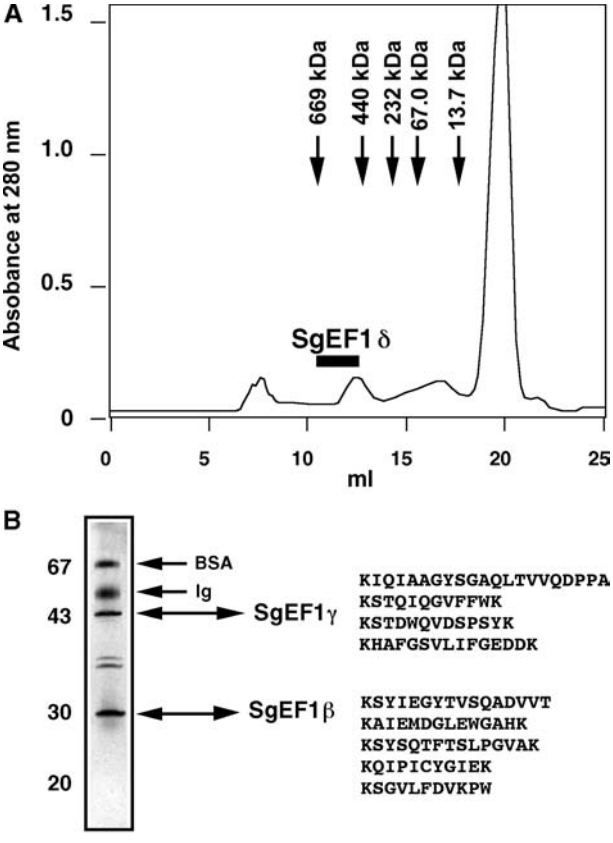

Figure 4. Proteins associated with SgEF1δ. (*A*) Analysis of the proteins from embryo extract by gel filtration. Proteins (1 mg) of the 100,000 g supernatant from unfertilized eggs were fractionated by chromatography on a Superose 6 column and monitored by absorbance at 280 nm. The bar shows the position of SgEF1δ as determined after Western blot analysis of each fraction (100 µl). The positions of the molecular mass standards (in kDa) used to calibrate the column are indicated by arrows. (*B*) Affinity purification of SgEF1δ and its associated proteins. The 100,000 g supernatant was incubated in the presence of anti-SgEF1δ antibody coupled to Sepharose beads. The proteins fixed on the beads were eluted by electrophoresis buffer, resolved on a 12% polyacrylamide gel and revealed by Coomassie blue staining. The bands arrowed SgEF1γ and SgEF1β were excised from the gel and microsequenced for their identification. The peptides obtained from the microsequencing procedure are shown. The positions of molecular-weight markers run in parallel are indicated in kDa.

As reported above (see fig. 2A), several proteins co-immunoprecipitated with sea urchin EF1δ, in particular a 45-kDa protein and a 30-kDa protein scarcely distinguishable from the immunoglobulin chains. To characterize these SgEF1δ-associated proteins, SgEF1δ was purified by affinity chromatography on beads of immobilized antibodies. The purified proteins were resolved on gels (fig. 4B). The two SgEF1δ-associated proteins migrating at 45 and 30 kDa could be easily isolated from the immunoglobulin chains and from the BSA (present in the medium as a saturating tool). Upon microsequencing, the 45-kDa protein was identified as sea urchin EF1γ and the 30-kDa protein as sea urchin EF1β by comparison with the known sequences in other species (fig. 4B).

Subcellular localization changes of SgEF1δ during the first mitotic cell cycle

The amount of SgEF1δ proteins during the first cell cycle was quantified by immunoblot on whole-embryo extracts prepared at different times after fertilization (fig. 5). Both SgEF1δ isoforms were detected at comparable levels and ratios throughout the cell cycle.

The subcellular localization of SgEF1δ was studied as the embryo progressed through the first cell cycle. The different stages of the cell cycle were monitored by DNA staining with bis-benzimide and microtubular network staining with anti-tubulin antibodies. Twenty to 30 min after fertilization, the fusion of the male and female pronuclei had occurred as reported [36] and a microtubular interphasic network was present (fig. 6; tubulin). In such embryos, SgEF1δ staining was found distributed throughout the cell (fig. 6; EF1δ). Controls using a non-immune serum (data not shown) or the second antibody alone revealed limited faint background fluorescence (fig. 6, FITC). The staining appeared more or less granular among different experiments (compare fig. 6 and 10). Since diffusible material has been extracted with detergent before fixation of the embryos (see Materials and methods), one can assume that the staining revealed an association of SgEF1δ with sub-cellular structures. Of note, comparison of tubulin and SgEF1δ staining indicated that SgEF1δ was not co-localized with the interphasic stable microtubules (fig. 6; compare tubulin and EF1δ). Confocal microscopy analysis revealed that the nucleus was almost devoid of SgEF1δ staining. Since at 30 min post-fertilization, nuclear envelope breakdown could have begun, we addressed the nuclear presence of SgEF1δ at earlier times. Analysis of SgEF1δ at the time of fertilization could not be performed using detergent-extracted embryos, since the treatment led to lysis of the cell

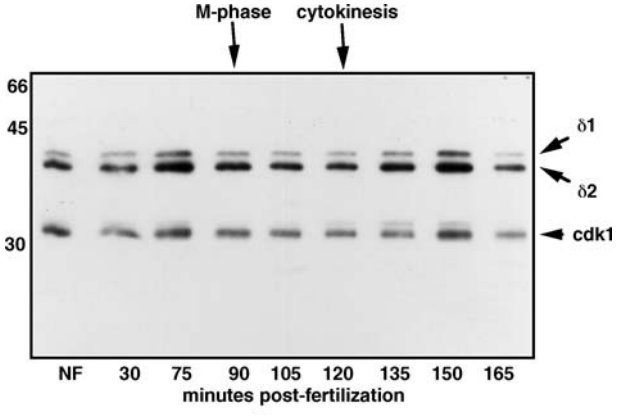

Figure 5. Quantification of SgEF1δ in sea urchin embryo during the first cell cycle. Whole extracts from identical numbers of embryos taken before (NF) and at different times (as indicated in min) after fertilization were resolved on a 12% polyacrylamide gel. The proteins transferred onto nitrocellulose were revealed by immunoblotting with anti-SgEF1δ antibody. The positions of molecular-weight markers run in parallel are indicated in kDa. As a loading control, the membrane was incubated with anti-PSTAIR antibody revealing CDK1. Periods of M phase and cytokinesis of the first cell cycle are indicated.

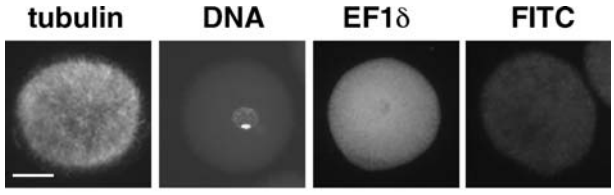

Figure 6. Subcellular localization of SgEF1δ in fertilized sea urchin embryos. Embryos taken 30 min after fertilization were detergent extracted and fixed in methanol/EGTA (see Materials and methods). Whole embryos were observed by fluorescence microscopy after staining with anti-tubulin antibody (tubulin), a DNA dye (DNA) or SgEF1δ antibody (EF1δ). Embryo staining by the secondary fluorescein-linked goat anti-guinea pig antibody alone is also shown (FITC). Bar, 30 µm.

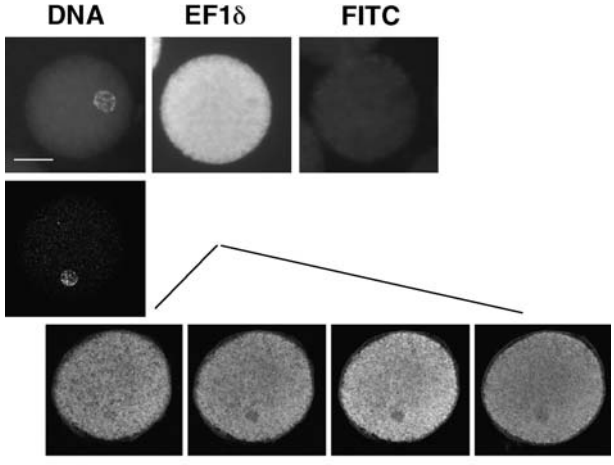

Figure 7. Subcellular localization of SgEF1δ in unfertilized sea urchin embryos. Unfertilized sea urchin embryos were fixed in methanol without prior detergent extraction. Whole embryos were stained with the DNA dye (DNA), SgEF1δ antibody (EF1δ) or the secondary antibody alone (FITC) and observed by fluorescence microscopy (upper images) and confocal microscopy of slices separated by 2 µm (lower images). Bar, 30 µm.

when applied to unfertilized eggs or early fertilized embryos (0–20 min). Therefore, we studied SgEF1δ distribution in embryos fixed by methanol without detergent extraction. In unfertilized eggs, SgEF1δ staining was distributed homogenously throughout the cytoplasm (fig. 7) as reported for the 30-min embryos. The different slides from confocal analysis clearly showed that the germinal vesicle was devoid of staining (fig. 7, lower panel). Therefore, SgEF1δ distribution is restricted to the cytoplasmic compartment in unfertilized eggs and through the first minutes post-fertilization at least until nuclear envelope breakdown.

The localization of SgEF1δ was further analyzed during cell cycle progression (fig. 8). A striking redistribution of SgEF1δ was found to take place at the onset of M phase. First, around 60 min, a pool of SgEF1δ concentrated around the nucleus location, as shown by the appearance of a ring of higher labeling distinct from the overall cytoplasmic and nucleoplasmic staining (fig. 8; 60 min EF1δ). The ring of higher labeling was observed while chromatin was still uncondensed (fig. 8; 60 min DNA) and the asters were positioned at opposite poles of the nucleus (fig. 8; 60 min tubulin), suggesting that the concentration of SgEF1δ occurs after S phase when the centrosome duplicates and splits into two asters and before the first chromatin modifications of M phase.

Later, at the metaphase stage, SgEF1δ staining appeared as two large spheres surrounding the mitotic apparatus poles with no obvious fibrous pattern (fig. 8; 90 min EF1δ). No SgEF1δ concentration was apparent at the level of the chromatin. As a comparison, the localization of cdk2 was analyzed on foster embryos. The cdk2 protein has been shown to be specifically associated with chromatin during the S and M phase [35]. Figure 9 shows that cdk2 did indeed co-localize with the condensed chromosomes at metaphase, with no staining of the astral or spindle microtubules. The EF1δ staining was concentrated around the astral microtubules and not around the spindle microtubules. The specific co-localization of SgEF1δ with the astral microtubular region was further evident during the two next stages, anaphase and telophase, with the two EF1δ spheres migrating apart from each other, associated with each pole of the spindle (fig. 8, 120 min EF1δ). The specificity of EF1δ recognition by the antibody was again ascertained. When the anti-SgEF1δ antibodies were saturated by pre-incubation in the presence of 200 µg of purified recombinant GST-EF1δ, no staining of the embryos could be detected, while a parallel pre-incubation in the presence of the same amount of BSA, did not abolish the staining (fig. 10).

To analyze further the co-localization of SgEF1δ with aster structures, the embryos were treated with microtubule-interfering drugs. When embryos were incubated with taxol just after fertilization, a giant spermatic monopolar aster developed (fig. 11A, tubulin), as already described [37]. The anti-SgEF1δ antibodies decorated the giant aster, again visible as a diffuse labeling (fig. 11A; EF1δ). On the other hand, incubation of embryos at the time of metaphase, in the presence of the microtubule-depolymerizing drug nocodazole, induced the destruction of all microtubular structures together with the disappearance of the characteristic double-spherical staining for SgEF1δ (fig. 11B). Thus, a close relationship exists between a pool of SgEF1δ and active dynamically polymerizing tubulin.

The major part of SgEF1δ appears to be homogenously distributed in the cytoplasm and excluded from the nucleus in the interphase of the cell cycle. The major pool

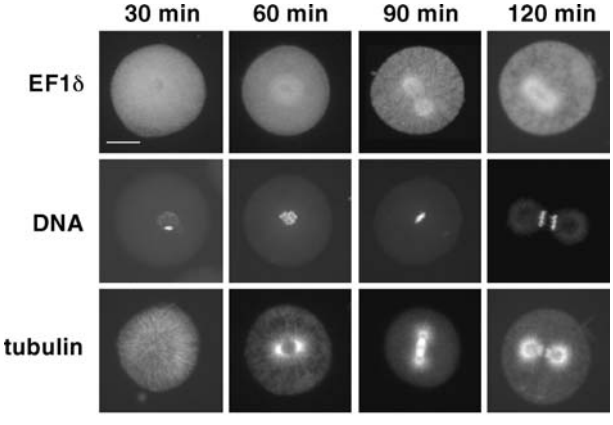

Figure 8. Localization of SgEF1δ during the first mitotic cell cycle. Embryos were taken at the indicated different times after fertilization, extracted by detergent and fixed with methanol. Whole embryos were stained with anti-SgEF1δ antibody (EF1δ), the DNA dye (DNA) or anti-tubulin antibody (tubulin) and observed by fluorescence microscopy. Bar, 30 µm.

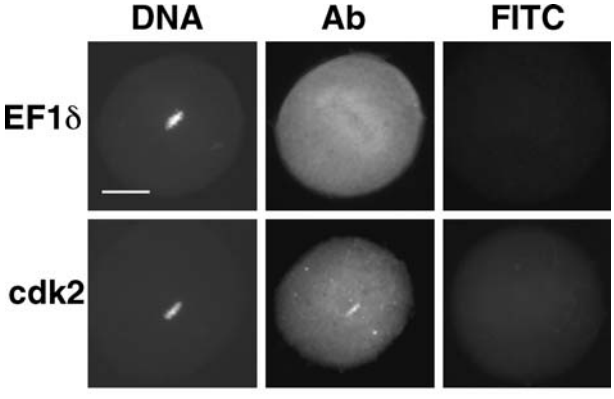

Figure 9. Comparison of SgEF1δ and cdk2 localization in fertilized embryos. Embryos taken at the time of metaphase were extracted by detergent and fixed with methanol. Whole embryos were stained with a DNA dye (DNA), with antibody (Ab) against SgEF1δ (EF1δ) or cdk2 (cdk2) or with the corresponding secondary fluorescein-linked antibody (FITC). Bar, 30 µm.

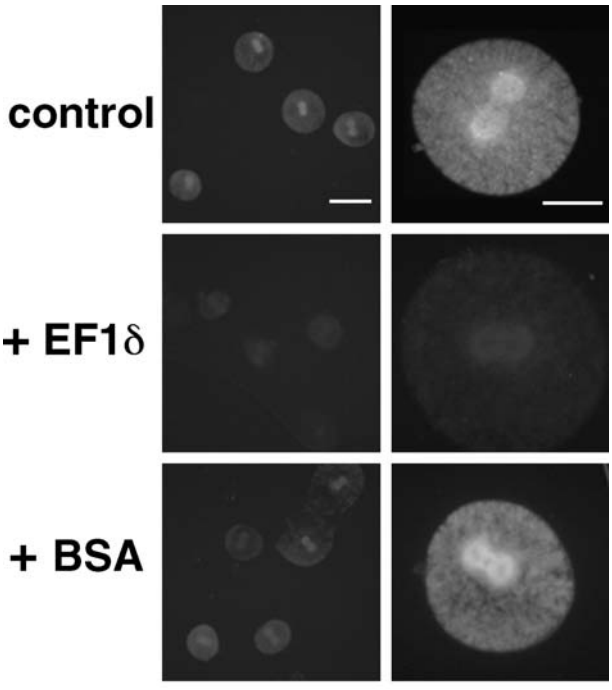

Figure 10. Specificity of SgEF1δ labeling. Embryos taken at the time of metaphase were stained by SgEF1δ antibody (control) or by the antibody which had been previoulsy saturated in the presence of either 200 µg purified recombinant GST-SgEF1δ (+ EF1δ) or 200 µg BSA (+ BSA). Two magnifications are shown: bar – 90 µm on left, 30 µm on right.

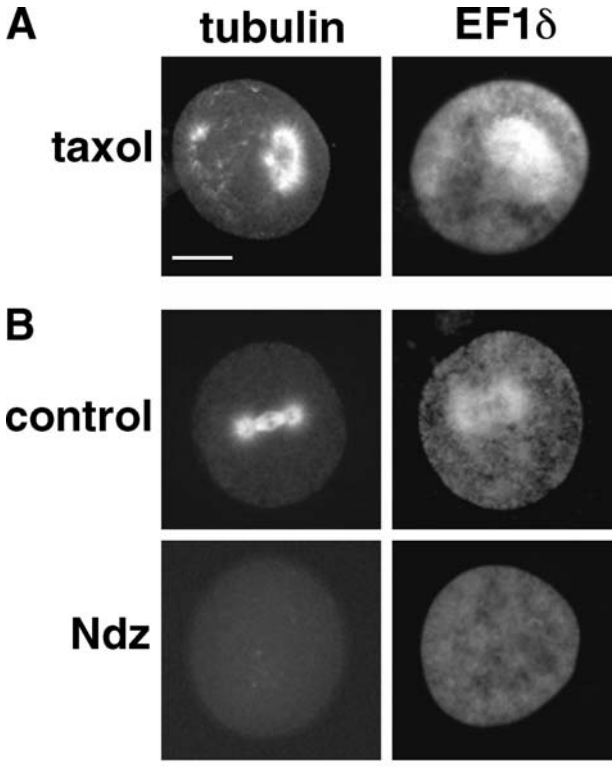

Figure 11. Effect of microtubule-interfering drugs on SgEF1δ localization. (*A*) Fertilized embryos were incubated in the presence of 10 µM taxol (taxol). After 90 min, whole embryos were fixed and treated for tubulin (tubulin) or SgEF1δ (EF1δ) localization by immunofluorescence observation. (*B*) At the time of metaphase, embryos were treated for 15 min in the presence of 10 µM nocodazole (Ndz). Whole embryos were fixed, stained for immunofluorescence as in **A** and compared to untreated foster embryos (control). Bar, 30 µm.

of the protein is associated with cytoplasmic structures, as judged from resistance to detergent extraction. A pool of the protein concentrates as a diffuse ring at the nucleus location at the time of G2/M transition and further separates into two spherical aster-associated structures. At anaphase, the two sub-fractions of the SgEF1δ pool move apart with their corresponding spindle pole and subsequently distribute between the two daughter cells

Discussion

The eEF1δ protein, a subunit of elongation factor 1, was discovered in *Xenopus* [6] and is specific to higher animals [9]. The protein eEF1δ, although sharing an activity redundant with eEF1β, is original both at the structural level by the presence of a leucine zipper motif and at the functional guanine nucleotide exchange domain which cannot substitute for the eEF1β counterpart (see Introduction). Since the protein and its partners could be involved in cell cycle regulation, we investigated EF1δ in sea urchin early development, a convenient model for the study of cell division.

We produced an antibody specific for the SgEF1δ protein, which revealed the existence in sea urchin embryos of two isoforms of the protein. The two isoforms appear to be encoded by two mRNAs, which differ by the existence of a 78-base stretch inserted in the open reading frame, in front of the leucine zipper-encoding sequence. The co-existence in the same cell of two EF1δ proteins was previously found in *Xenopus laevis* oocytes [38] and had been assigned to the known genome duplication in this species. However, the existence of two closely related proteins corresponding to EF1δ in sea urchin embryo argues for a universal feature in higher eukaryotes and strongly suggests a specific, yet unidentified, role for each eEF1δ protein. In the sea urchin egg, the two isoforms represents 1.5% of the total proteins, which fits with the high amount of the protein found in other cell types [6]. The two isoforms are found in a 1:2 ratio and in the same high molecular-weight complex in association with SgEF1β and SgEF1γ. Purification of the complex (or complexes) is currently under investigation and preliminary observations have revealed the co-purification of a set of proteins in addition to SgEF1β and SgEF1γ.

The protein SgEF1δ was shown to associate with detergent-resistant cytoplasmic structures, which most proba-

bly correspond to the endoplasmic reticulum, as described in other species [10, 11]. This localization undoubtedly relates to the role of the protein in translation as a sub-unit of the nucleotide exchange complex, eEF1B. In unfertilized eggs and up to 30 min post-fertilization, the nucleus appears devoid of EF1δ, in concordance with the exclusion from the nucleus of almost all translation factors reported to contribute to confine translation to the cytoplasm in other cell types [39].

Interestingly, a pool of SgEF1δ protein was found to concentrate around the nucleus at the period of the nuclear envelope breakdown, and to further surround each of the asters as two diffuse spherical structures. The behavior of EF1δ was dependent on the presence of dynamic microtubular structures as judged from taxol and nocodazole experiments. Likewise, EF1A has been reported to be a component of the mitotic spindle poles or centrospheres in sea urchin dividing embryos [40, 41]. One can propose that the presence of these two elongation factors is related to a requirement for efficient protein synthesis at this area. On the other hand, as eEF1δ is recognized as a multifunctional factor [5] and the specific function of eEF1δ is yet unknown, the presence of high levels of the two factors at this area could be related to any other function. Whatever its physiological function, the accumulation of EF1δ takes place where two major mitotic events (nuclear envelope breakdown and mitotic spindle organization) occur, and therefore strengthens the existence of a link between EF1δ and the cell cycle.

In conclusion, our results demonstrate on the one hand increasing complexity of so-called elongation factor 1B by the simultaneous presence of two EF1δ isoforms encoded by two mRNAs and differing by a 26 amino-acid stretch located in front of the leucine zipper domain. They establish on the other hand, the existence of a physiological cell cycle-related relocalization of a sub-fraction of the total SgEF1δ protein. An attractive hypothesis could be that only one SgEF1δ isoform corresponds to the mitotic-related pool. The major challenge is now to isolate the pool which shows cell cycle-dependent localization and to analyze both the SgEF1δ isoforms and the associated proteins. Investigation of the molecular cause, as well as the molecular consequences of the behaviour of EF1δ in sea urchin should provide new insights into the structure-function relationship between the cell cycle process and/or regulation and the increasing structural complexity of of EF1 complex.

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