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Phosphorylation of maize eukaryotic translation initiation factor on Ser2 by catalytic subunit CK2

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Abstract Alignment of eukaryotic translation initiation factor 5A (eIF5A) sequences has shown, for plants, in contrast to most other eukaryotes, the presence of N-terminal serine residue (Ser2) which could be phosphorylated by CK2. Using point directed mutagenesis, we demonstrate here that in recombinant maize ZmeIF5Awt Ser2 is exclusively phosphorylated by catalytic subunit of CK2 (CK2α), whereas its mutated variant Ser2Ala is not phosphorylated. To shed light on the physiological significance of this Ser2 phosphorylation, transient expression of fluorescence-labeled proteins was performed in maize protoplast. Wild-type ZmeIF5A was distributed evenly between nucleus and cytoplasm, but the replacement of Ser2 by aspartic acid, which mimics the phosphorylated serine, influences its intracellular localization. We postulate that phosphorylation of Ser2 in maize eIF5A, and most probably in other plant cells, plays a role in specific regulation of nuclear export of eIF5A-bound mRNAs.

Keywords CK2α · eIF5A · Intracellular transport · Phosphorylation of Ser2

Introduction

Eukaryotic translation initiation factor 5A (eIF5A), a highly conserved 18-kDa protein present in all the eukaryotic cells, is involved in a broad spectrum of cellular functions [1]. The exact way whereby eIF5A functions in the cell is not known, but there are some data showing that this factor may selectively recruit specific subsets of mRNA from the nucleus to facilitate their translocation to the cytoplasm for subsequent translation. The export signal in eIF5A appears to be complex and to involve in the hypusine modification that is unique to all the eIF5A [2]. Hypusine is biosynthesized by a two-step post-translational modification involving the reaction of spermidine with a conserved lysine residue of eIF5A. Hypusination of eIF5A is essential for cell proliferation and apoptosis [3].

In plants, like in other eukaryotic organisms, eIF5A is involved in many functions. The three isoforms of eIF5A identified in *Arabidopsis thaliana* (AteIF5A1-3), are active in xylem formation (AteIF5A1), cell division, cell growth, and cell death caused by pathogen infection (AteIF5A2), and the responses to osmotic and nutrient stresses (AteIF5A3) [4–6]. In rice (*Oryza sativa*), two isoforms of eIF5A (OseIF5A1 and OseIF5A2) are regulated during leaf developments, sugar starvation, and environmental stresses [7]. In tomato (*Lycopersicon esculentum,*) there is a correlation between expression of *eIF5A* and programmed cell death [8]. Unfertilized egg cells of maize (*Zea mays*) in G_0 phase contain a high level of *eIF5A* transcript. Following fertilization, the transcript is strongly induced during G_1 phase, but continuously decreases during the next phases [9]. This suggests that unfertilized egg cells of maize are prepared for selective mRNA translation that is quickly triggered after fertilization. The triggering could be regulated by reversible phosphorylation, the key modification of proteins. Indeed, plant and *Saccharomyces cerevisiae*, but not mammalian eIF5As, have been shown to contain a serine residue at the N-terminal position 2, which could be potentially phosphorylated by catalytic subunit of CK2 (CK2α) [10]. The lack of such phosphorylable Ser residue at the N-terminus of animal eIF5A suggests that in plants

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and some yeasts (*S. cerevisiae*), Ser2 phosphorylation plays a unique or an additional function. A role of reversible phosphorylation of Ser2 in ZmeIF5A in the regulation of nuclear export of mRNA is postulated.

Materials and methods

Plant material

Maize seeds (*Zea mays*, cv. Mona), after overnight soaking in water at room temperature, were grown in the dark for 4 days at room temperature on wet filter paper, then in soil in a greenhouse with a daily cycle of 16-h light (250 W/m^2) at 24°C and 8-h dark at 20°C. For preparation of mesophyll protoplast, leaves from 10-day-old plants were collected.

Protoplast transient expression assay

The pLNU expression vector (pLNU is pUC-based vector carrying a constitutively active ubiquitin promoter) was used to express *ZmeIF5A* and its mutated forms in maize protoplasts. Constructs of wild-type and mutated forms of *ZmeIF5A* tagged with *enhanced yellow fluorescent protein* (*EYFP*) were prepared as described before [10]. Maize mesophyll protoplasts were obtained according to Sheen [11] and transformed separately with 50 μg of plasmid DNA (*ZmeIF5Awt-EYFP, ZmeIF5AS2A-EYFP,* or *ZmeIF5AS2D-EYFP*) and about 50 μg of carrier DNA per ca. 2×10^5 protoplasts. The transformed protoplasts were incubated at 22°C for 16 h and viewed by confocal microscopy (Eclipse TE200-E, Nikon).

Phosphorylation of eIF5A by CK2

The pGEX-4T-1 expression vector (Amersham Pharmacia Biotech) was used to express ZmCK2α, ZmeIF5A and its mutated form in *E. coli*.

The constructs GST-ZmCK2α-1, GST-ZmeIF5Awt and its mutated form GST-ZmeIF5AS2A were generated as described previously [10]. All the constructs were transformed into *E. coli* BL21(DE3), expressed and purified using gluthathione-Sepharose 4B according to the manufacturer's instruction.

In vitro phosphorylation of GST-ZmeIF5Awt and GST-ZmeIF5AS2A by GST-ZmCK2α-1 was performed in a final volume of 50 μl. The incubation mixture contained: 0.1 μg of purified ZmCK2α-1, 3 μg of ZmeIF5Awt or ZmeIF5AS2A, 20 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, and 50 μM γ[^{32}P]ATP (100–200 cpm pmol^{-1}). The mixture was incubated for 20 min at 30°C, 40 μl of the mixture was spotted onto 3MM filter strip, and then the filters were washed in 5% TCA five times for 10 min each. Next, the filters were washed in 96% ethanol, dried, and the radioactivity was quantified using a liquid scintillation counter. Efficiency of phosphorylation was calculated from the specific radioactivity of ^{32}P incorporated into known amounts of proteins. Alternatively, reaction products were subjected to SDS-PAGE and visualized by autoradiography.

Results and discussion

A comparison of eIF5A amino acid sequences from different organisms showed that eIF5A proteins are conserved with a lower amino acid similarity at the terminal positions [10]. The eIF5A N-terminal extension has a high content of acidic residues. In the plants analyzed and a yeast (*Saccharomyces cerevisiae*), but not in another yeast (*Schizosaccharomyces pombe*) or animals (*Homo sapiens* and *Drosophila melanogaster*), eIF5As contain a serine residue at position 2 (Fig. 1). Ser2 is followed by acidic amino acids which suggests that can be phosphorylated by CK2, because the characteristic feature of CK2 phosphorylation sites are multiple acidic residues located mostly downstream of the phosphorylable amino acid [10, 12]. To confirm the prediction that Ser2 in maize eIF5A can be phosphorylated by CK2, recombinant ZmeIF5Awt, ZmeIF5AS2A, and ZmCK2α proteins were produced in *E. coli*. Wild-type ZmeIF5A was phosphorylated efficiently (about 1 M of phosphate per mole of protein) by CK2α, whereas the protein with Ser2 converted to Ala was not phosphorylated (Fig. 2). The lack of phosphorylation of the Ser2Ala variant indicates that only Ser2 can be phosphorylated by CK2α.

Growing evidence indicates that eIF5A appear to facilitate translation of specific sets of mRNA required for cell division and cell death (see "Introduction" section). This raises the possibility that the eIF5A isoforms are elements of a biological switch responding to a variety of physiological and environmental signals [6, 13].

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                                ↓
Homo sapiens                    MAD---DLD-FET-GDAGASAT
Drosophila melanogaster         MADM--DDH-FET-TDSGASST
Schizosaccharomyces pombe       MAE--EEHVDFEG-GEAGASLT
Saccharomyces cerevisiae        MSD---EEHTFET-ADAGSAAT
Arabidopsis thaliana            MSD---DEHHFEA-SESGASKT
Oryza sativa                    MSDS--EEHHFESKADAGASKT
Zea mays                        MSDS--EEHHFESKADAGASKT
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Fig. 1 Comparison of N-terminal sequences of eIF5A from different kingdoms. The sequences are from *H. sapiens* (NP001961), *D. melanogaster* (NP611878), *S. pombe* (NP596130), *S. cerevisiae* (NP010880), *A. thaliana* (AAF07023), *O. sativa* (NP0011051336), and *Z. mays* (Y07920). *Arrow* indicates position 2 in the amino acid sequences of eIF5As. *Dashes* indicate gaps introduced to maximize the alignment

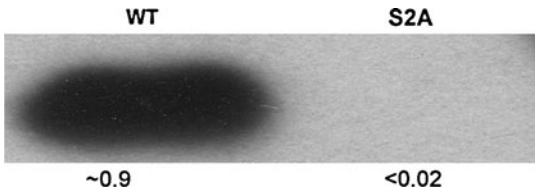

Fig. 2 Phosphorylation of recombinant ZmeIF5Awt and its Ser2Ala mutant by CK2α. Incorporation of phosphate (mole per mole of the protein) is indicated in the *bottom* of the figure

To study the role of Ser2 phosphorylation, ZmeIF5A and its variants mutated at Ser2 (Ser2Ala and Ser2Asp) were expressed in maize protoplasts as fusion proteins with EYFP and their location was visualized by confocal microscopy. The relative intensity of fluorescence in the nucleus and cytoplasm was determined. Wild-type ZmeIF5A and its Ser2Ala variant were distributed evenly between the nucleus and the cytoplasm, whereas the variant with Ser2 replaced by aspartic acid, which mimics a

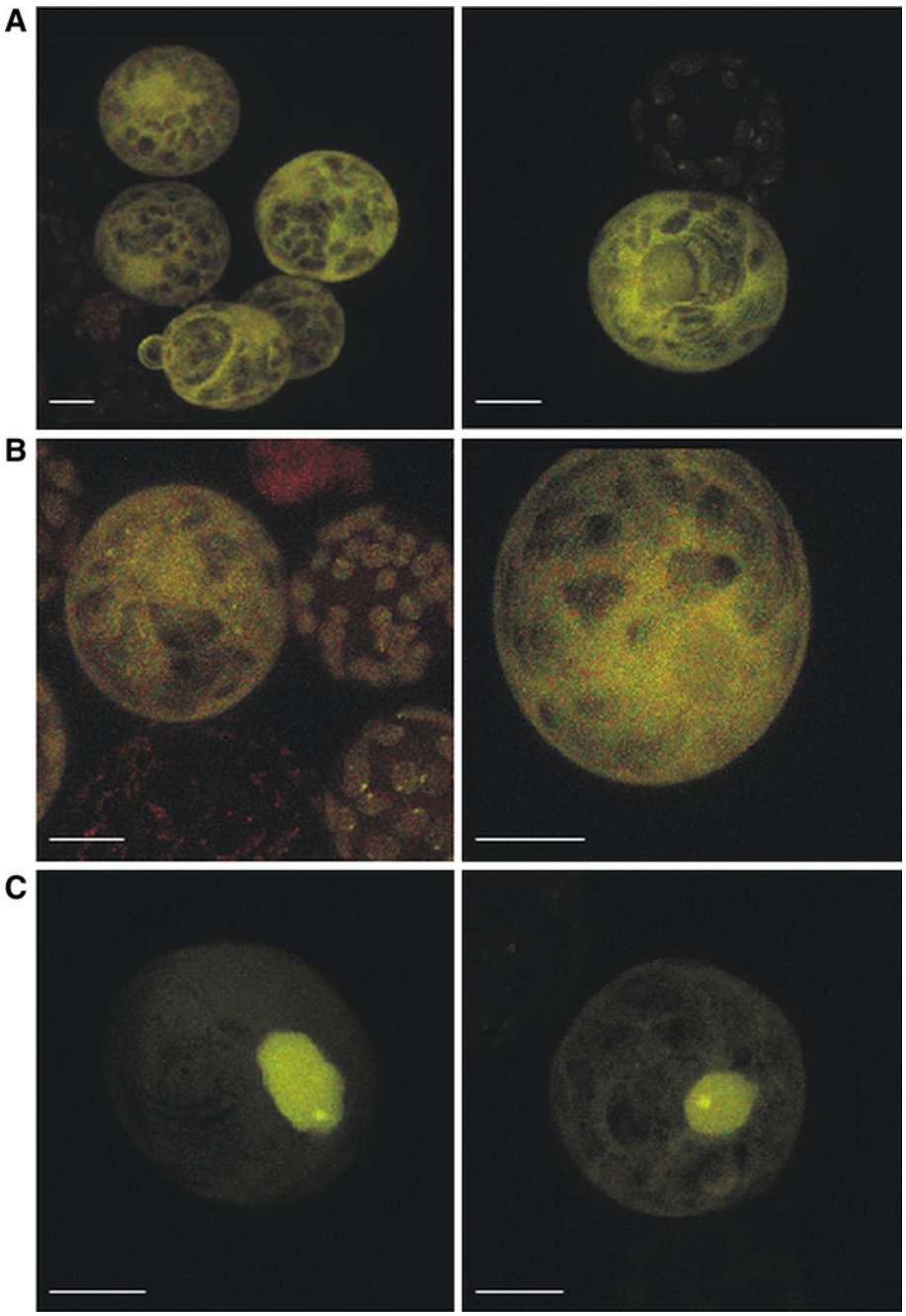

Fig. 3 Localization of eIF5A-EYFP and its mutated variants. Spatial fluorescence was analyzed by confocal microscopy in whole maize protoplasts. **A** eIF5A-wt-EYFP, **B** eIF5A-S2A-EYFP, **C** eIF5A-S2D-EYFP. Constructs are described in "Materials and Methods" section. In the case of A and B, fluorescence was evenly distributed between the nucleus and cytoplasm, in contrast to C, where most of fluorescence was located in nucleus and fluorescence in cytoplasm was only slightly visible. *White bars* represent scale 10 μm

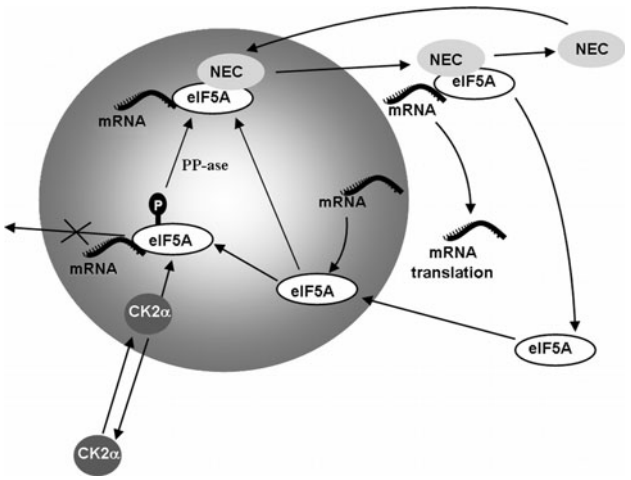

Fig. 4 Postulated role of ZmeIF5A phosphorylation on Ser2 by CK2α. NEC hypothetical "Nuclear Exporting Complex," PP-ase protein phosphatase

phosphorylated serine, was sequestered in the nucleus (Fig. 3). This confirmed the previously obtained results from the measurement of fluorescence of optically sectioned protoplasts [10], which indicated that phosphorylation of Ser2 plays a role in the nuclear export of plant eIF5A. Lipowsky et al. [2] have shown that exportin 4 (Exp4) functions as a nuclear receptor for eIF5A specifically assembling into the trimeric eIF5A–Exp4–RanGTP complex, which results in efficient export of eIF5A from the nucleus. The positively charged amino acid hypusine facilitates eIF5A binding to Exp4, thereby enhancing its nuclear export. The negative charge of phosphorylated Ser2 may cause an opposite effect, preventing the eIF5A interaction with the nuclear exporting complex. The consequence is sequestration of eIF5A in the nucleus. CK2α is predominantly localized to the nucleus, but is also present in the cytoplasm. The nuclear localization of CK2α is a signal-mediated process, and the nuclear localization signal present in this subunit is partly responsible for the localization [14]. In turn, eIF5A can enter the nucleus via passive diffusion [15]. The postulated regulation of translocation of eIF5A by reversible phosphorylation is presented in Fig. 4. We propose that dephosphorylation of Ser2 phosphorylated by CK2α would reactivate the nuclear export of ZmeIF5A. The phosphorylated form of ZmeIF5A could be dephosphorylated by maize protein phosphatase 2A (PP2A) [16], which is regulated by direct interaction with CK2α [17]. Assuming that the essential function(s) of eIF5A is conserved in eukaryotes and archae (which have no nuclei), nuclear export of macromolecules seems not to be a primary function of eIF5A. However, eukaryotic eIF5As could as well be multifunctional proteins with the regulation of plant eIF5A export by phosphorylation being a late evolutionary acquisition.

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Conflict of interest None.

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