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Study of phosphorylation of translation elongation factor 2 (EF-2) from wheat germ

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Phosphorylation of elongation factor 2 (EF-2) by specific Ca^{2+}/calmodulin-dependent kinase is considered as a possible mechanism of regulation of protein biosynthesis in animal cells at the level of polypeptide chain elongation. In this report we show that wheat germ EF-2 can be intensively phosphorylated by the rabbit reticulocyte EF-2 kinase. Phosphorylation results in inhibition of the activity of plant EF-2 in poly(U)-dependent cell-free translation system. Thus, the activity of EF-2 in plant cells can be potentially regulated by phosphorylation. However, we could not detect endogenous EF-2 kinase activity in wheat germ either in vitro or in vivo. Furthermore, EF-2 kinase activity is not displayed in different organs of wheat and other higher plants.

Elongation factor 2; Protein phosphorylation; Regulation of translation; Wheat germ; *Triticum aestivum*

1. INTRODUCTION

The reversible phosphorylation of protein translation factors is considered as one of the main mechanisms of regulation of protein synthesis in eukaryotic cell [1]. It is well known that phosphorylation of only 25–30% of α-subunit of the rabbit reticulocyte translation initiation factor 2 (eIF-2) results in a strong inhibition of protein synthesis [1–4]. Recently it was shown that the elongation factor 2 (EF-2) from animal cells can be phosphorylated in vitro and in vivo by specific Ca^{2+}/calmodulin-dependent EF-2 kinase and phosphorylation makes it inactive in protein synthesis [5–8]. It was assumed that the phosphorylation of EF-2 may play a role in the regulation of protein biosynthesis in animal cells [1,9]. However, the existence of such a possible mechanism of translation regulation in plant cells was not yet investigated.

The present work was undertaken to study the ability of plant (wheat germ) EF-2 to serve as substrate for EF-2 kinase from rabbit reticulocytes. Also, an attempt was made to detect endogenous EF-2 kinase activity in plant cells.

2. MATERIALS AND METHODS

The viable embryos were isolated from wheat grains (*Triticum aestivum*, Kazakhstanskaya 4 varieties) by the method of Johnston and Stern [10]. The cytoplasmic extract of wheat germ (S23) and the ribosome-free cytoplasmic fraction (S100) were obtained as described earlier [11]. To isolate EF-2, S100 was fractionated by ammonium sulfate as described in [12]. The 40–70% $(NH_4)_2SO_4$ protein fraction was dissolved in a minimal volume of buffer A (20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM β-mercaptoethanol (MET), 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma), dialysed and applied onto DEAE-cellulose (Whatman) equilibrated with the same buffer. After washing of non-bound material proteins were eluted with 100 mM KCl and applied onto a column of hydroxyapatite equilibrated with 15 mM potassium phosphate buffer (pH 7.0). The EF-2 was eluted with a 15–300 mM potassium phosphate linear gradient. EF-2 was assayed by the reaction of ADP-ribosylation in the presence of [^{14}C]NAD^+ (8 TBq/mol, Amersham) and diphtheria toxin [13]. Diphtheria toxin was a gift from Dr. E. Davydova (Institute of Protein Research, Pushchino, Russian Federation). Fractions containing EF-2 were loaded onto the Mono-Q column (Pharmacia), and eluted with 50–300 mM KCl linear gradient. Fractions with EF-2 activity were combined and stored at −70°C as a purified preparation of EF-2. The preparation of EF-2 kinase from rabbit reticulocyte obtained by the procedure described earlier [7], was kindly provided by Dr. A.G. Ryazanov (Institute of Protein Research, Pushchino, Russian Federation).

Phosphorylation in vitro was performed in the reaction mixture (40 μl) contained buffer B (30 mM HEPES-KOH, pH 7.6, 10 mM MgOAc, 2 mM DTT, 150 μM $CaCl_2$, 10 mg/ml calmodulin, 0.1 mM ATP, 2 pmol [γ-^{32}P]ATP (37 PBq/mol; Isotope), as well as preparation of substrate proteins and EF-2 kinase as indicated in the legends to figures. After 20 min incubation at 30°C samples were analysed by electrophoresis in SDS-PAAG according to Laemmli [14] or 2D electrophoresis according to O'Farrell [15]. Then gels were stained with Coomassie brilliant blue G-250, dried and exposed to RM-V X-ray film at −70°C.

For analysis of EF-2 phosphorylation in vivo, wheat embryos (1 g) were labeled at 25°C for 36 h in 3 ml of 1% glucose solution containing antibiotics (500 units/ml penicillin, 25 μg/ml levomycetine, 55 μg/ml nistatin) and 37 PBq [^{32}P]orthophosphate (185 PBq/mol). After labeling the wheat embryos were washed and homogenized in a buffer containing 20 mM Tris-HCl, pH 7.8, 10 mM MET, 1 mM PMSF, 4 mM Na_2MoO_4 and 20% glycerol. Sodium molibdate was added to the buffer as an inhibitor of endogenous phosphatases. Then rapid isolation of the EF-2 was performed starting from S23 fraction and DEAE-chromatography step was omitted. After fractionation by ammonium sulfate subsequent chromatographies on hydroxyapatite and Mono-Q

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were performed using stepwise elution. The crude EF-2 preparation obtained by this rapid procedure was analysed as described above.

The effect of phosphorylation on the EF-2 activity was examined in the poly(U) directed cell-free system as described in [7]. Purified wheat germ EF-2 (2 μg) was preincubated for 4 min at 30°C in buffer B with different additions (see Table I) and then mixed with a cell-free translation system (100 μl) containing rabbit reticulocyte ribosomes (washed twice with 0.6 M KCl to remove endogenous EF-2), 10 μg poly(U) (Sigma), 2 μg rabbit reticulocyte EF-1, 50 μg of total *E. coli* tRNA aminoacylated with [^{3}H]phenylalanine (150 Bq/μg, Amersham), 1 mM GTP in the buffer of 20 mM Tris-HCl, pH 7.6, 75 mM KCl, 10 mM $MgCl_2$, 5 mM MET. The reactions were performed for 20 min at 30°C and stopped by addition of 5% trichloroacetic acid. After 10 min hydrolysis at 100°C the mixture was passed through GF-C filters (Whatman), the filters were dried and radioactivity was measured in a liquid scintillation counter LS-100C (Beckman).

Non-specific phosphatase activity in wheat germ cytoplasmic extracts (S23 or S100) was determined by dephosphorylation of *p*-nitrophenylphosphate [16]. Samples (100 μl) were added to 2 ml of a buffer containing 1 M Tris-HCl, pH 8.0, and 0.2 mg/ml *p*-nitrophenyl-phosphate and change of the absorbance at 410 nm was measured. An increase in A_{410} by 0.0162 o.u. per 1 min corresponds to one unit of the phosphatase activity.

3. RESULTS AND DISCUSSION

As seen in Fig. 1A, wheat germ EF-2 has M_r of about 100,000 and is represented by at least three charge isoforms with isoelectric points (p*I*) over 6.1–6.4 range: two main components with p*I* 6.1 and 6.2 and a minor one with p*I* 6.4. The p*I* values were determined using carbamylated carboanhydrase (Carbamylyte, Pharmacia). The position of the most left spot corresponds to p*I* 6.7 and for every next spot to the right the p*I* value decreases by 0.1. Several isoforms (up to four) were described also for EF-2 from animal cells [17,18]. The existence of different isoforms of wheat germ EF-2 can also be due to covalent modification such as diphtamide formation [19], ADP-ribosylation [18], and/or phosphorylation [17].

To determine the ability of wheat germ EF-2 to serve as substrate for phosphorylation the purified EF-2 was incubated with EF-2 kinase from rabbit reticulocyte in the presence of [γ-^{32}P]ATP, and then analysed by 2D electrophoresis (Fig. 1B and C). As seen in Fig. 1B after phosphorylation the p*I* of one of the main isoform of

Table I

Inhibition of wheat germ EF-2 activity by phosphorylation

Additions	[^{3}H]Phe polymerized (pmol)
None	23.0
EF-2 kinase	8.5
EF-2 kinase + trifluoperazine	25.3
EF-2 kinase − (Ca^{2+}/calmodulin)	22.8

The EF-2 preparation (2 μg) was preincubated with indicated additions (2 μg of EF-2 kinase, 60 μM trifluoperazine in buffer B) at 30°C for 4 min in a final volume of 20 μl. Then an aliquot (10 μl) was added into poly(U)-directed cell-free translation system (see section 2).

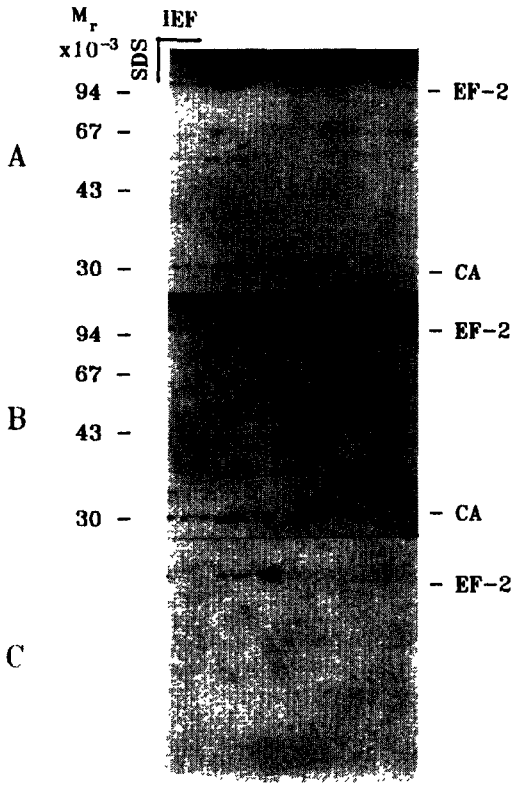

Fig. 1. Two-dimensional analysis of the non-phosphorylated (A) and phosphorylated (B) wheat germ EF-2. (A,B) Stained gels. (C) Radioautogram of gel (B). CA, carbamylated carboanhydrase.

EF-2 (p*I* 6.1) is shifted to the acidic region (p*I* 5.8). The radioautogram of the same gel (Fig. 1C), shows that this new EF-2 isoform is phosphorylated most intensively. A small amount of radioactivity is present also in two other forms (with p*I* 6.2 and 6.4). Nevertheless, they almost do not change their electrophoretic mobilities. It should be noted also that: (i) under standard conditions wheat germ EF-2 is phosphorylated to the same extent as EF-2 from rabbit reticulocytes (data not shown); (ii) the purified wheat germ EF-2 does not possess the ability for autophosphorylation (see Fig. 3); (iii) phosphorylation of wheat germ EF-2 by rabbit reticulocyte EF-2 kinase is highly specific (Fig. 2B and C) and is Ca^{2+} and calmodulin dependent (Table I).

We have studied also the effect of phosphorylation on the activity of wheat germ EF-2 (Table I). The phosphorylated EF-2 from wheat germ like its analogue from animal cells, loses its activity in poly(U)-directed cell-free translation system. Such inhibition is not observed in the presence of calmodulin antagonist, trifluoperazine.

Thus, EF-2 from wheat germ is an authentic substrate for EF-2 kinase from animal cell, and its activity can be regulated by phosphorylation. Recently the amino acid sequence of plant (green alga *Chlorella kessleri*) EF-2 has been determined from the nucleotide sequence of

	52	60
Plant	Gly-Asp-Gln-Arg-Leu-Thr-Asp-Thr-Arg	
	51	59
Animal	Gly-Glu-Thr-Arg-Phe-Thr-Asp-Thr-Arg	

Fig. 2. Comparison of the amino acid sequences of the same region of plant (alga) and animal (rabbit) EF-2 molecule. Phosphorylation of animal EF-2 proceeds at two threonine residues (Thr-56 and Thr-58).

the cloned gene [20]. Comparison of this sequence with that of mammalian EF-2 [21] reveals rather good similarity within the phosphorylation domain (Fig. 2).

It was of interest whether there is the functional analogue of EF-2 kinase in plant cell. To answer this question, cytoplasmic extracts from dormant and germinating (6–48 h at 25°C) wheat embryos were incubated with [γ-^{32}P]ATP in the presence of calmodulin and Ca^{2+} ions and then were subjected to electrophoresis (Fig. 3, lanes 4–7). In some variants a purified wheat germ EF-2 was added to the incubation mixture to enhance the sensitivity of endogenous EF-2 kinase detection (Fig. 3, lanes 5 and 7). As shown in Fig. 3, endogenous EF-2 kinase activity was not observed in any cases. A similar result was obtained using extracts from maturating wheat embryos (data not shown).

Presence of non-specific phosphatase activity in wheat germ extracts can be one of the reasons of failure to detect endogenous phosphorylation of EF-2. In fact, according to our data, the level of specific phosphatase activity in extracts from dormant embryos is 0.086 units/mg protein and is increased 3-fold (to 0.231 units/mg protein) during germination. This can also be seen from a decrease in the level of total phosphorylation of proteins in vitro (see lanes 4,5 and 6,7 in Fig. 3B). To investigate this possibility wheat embryos were germinated in the presence of [^{32}P]orthophosphate. Then using a rapid isolation procedure (see section 2) crude preparation of EF-2 was obtained and analysed by electrophoresis (Fig. 4). The radioautogram of the gel (Fig. 4B, lane 1), shows the absence of any incorporation of ^{32}P in EF-2 band. In the control experiment, 20 μg of purified wheat germ EF-2 prephosphorylated by rabbit reticulocyte EF-2 kinase was added to extract (S23), incubated for 10 min at 25°C, and then subjected to a similar isolation procedure. As seen in Fig. 4B (lane 2), the phosphorylated EF-2 retains ^{32}P-radioactivity during all the isolation procedure despite an increased phosphatase level in a cytoplasmic extract.

Thus, we have failed to detect endogenous EF-2 phosphorylating activity in wheat germ either in vitro or in vivo. This suggests that though the activity of EF-2 from wheat germ can be potentially regulated by phosphorylation, this mechanism of regulation is not realized in wheat germ. However, recently it was found that protein with M_r of about 100 kDa is phosphorylated by endogenous kinases in extracts from plants *Triticum aestivum* [22], *Vicia sativa* [23] and *Cucumis sativus* [24]. Since this phosphorylation decreased in the presence of fusidic acid which is known to trap the EF-2 as a complex with the ribosomes [25], this protein was suggested to be EF-2. However, there was no any direct evidence for this suggestion. It should be noted also that in these experiments very high concentrations of fusidic acid was used (up to 3 mM) resulting in non-specific decrease of phosphorylation of many other proteins. So it is difficult to conclude whether the 100 kDa protein mentioned above is actually the EF-2. We also observed several phosphorylated proteins with M_r of about 100

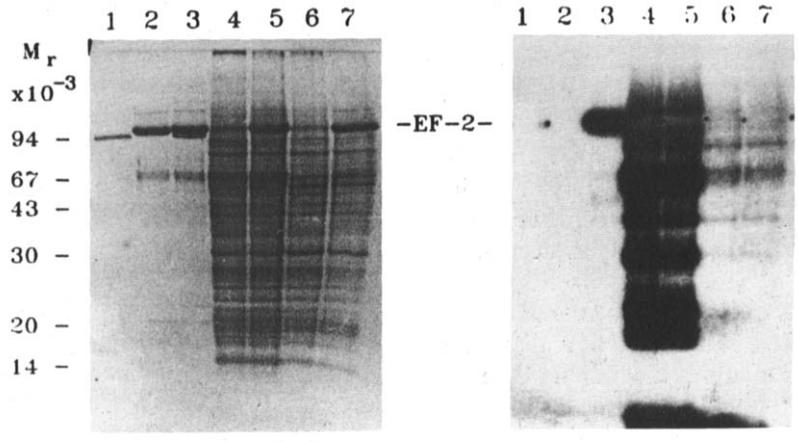

Fig. 3. Phosphorylation of the wheat germ EF-2 by the EF-2 kinase from rabbit reticulocyte. (A) Stained gel. (B) Radioautogram of gel (A). Lane 1, 2 μg of rabbit reticulocyte EF-2 kinase; lane 2, 5 μg of purified wheat germ EF-2; lane 3, 2 μg of rabbit reticulocyte EF-2 kinase + 5 μg of purified wheat germ EF-2; lane 4, extract (S23) from dormant wheat germ; lane 5, S23 (dormant wheat germ) + wheat germ EF-2; lane 6, extract (S23) from germinated wheat germ; lane 7, S23 (germinated wheat germ) + wheat germ EF-2.

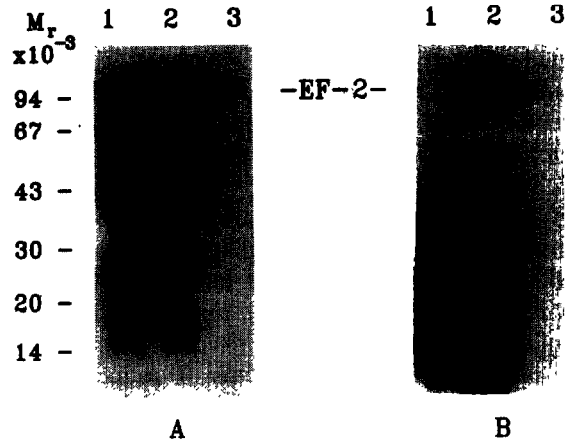

Fig. 4. The in vivo analysis of EF-2 phosphorylation. Wheat embryos were labeled 36 h in the presence of [^{32}P]H_3PO_4. (A) Stained gel. Lane 1, crude preparation of EF-2 isolated from labeled wheat germ; lane 2, 20 μg of [^{32}P]EF-2 added to the extract before isolation; lane 3, purified wheat germ EF-2. (B) Radioautogram of gel (A).

kDa in extracts from wheat germ (see Fig. 3) and many other plants, but detailed analysis revealed that they did not relate to EF-2. Moreover, though in many plant objects different protein kinases [26], including Ca^{2+}/calmodulin-dependent ones [27,28], were described, none of them phosphorylated EF-2. Thus, phosphorylation of wheat germ translation machinery components (including EF-2) by protein kinase isolated from the same source was studied; however, EF-2 was not a substrate for this kinase [29,30]. Also the study of phosphorylation of proteins in a cytoplasmic extract from *Artemia salina* embryos in the presence of [γ-^{32}P]ATP did not reveal any significant incorporation of ^{32}P into EF-2 [31]. Nevertheless, endogenous EF-2 phosphorylating activity was detected by an indirect method in a cytoplasmic extract from yeast *Saccharomyces cerevisiae* [32].

It is known that in animal cells the rate of protein synthesis declines during mitosis and one of the reasons for such a decline can be phosphorylation of EF-2 due to activation of the EF-2 kinase [17]. For wheat germ the existence of such a mechanism is unlikely since though the division of cells proceeds synchronously for at least 100 h, it is not accompanied by decrease of protein synthesis [33].

One cannot exclude a possibility that the activity of EF-2 kinase in wheat germ is inhibited at a given stage of ontogenesis in some manner making its detection difficult. There are two examples of such inhibition (in PC-12 cells [34] and in *Xenopus laevis* oocytes [35]), that are presumably important for regulation of differentiation and cell development [36]. In this respect, we have examined the phosphorylation of EF-2 in cytoplasmic extracts from differentiated organs of wheat: leaves, roots, normal and etiolated seedlings, pollen, as well as extracts from other plants including leaves and pollen of tobacco, embryos, shoots and roots of corn and pea. However, EF-2 phosphorylating activity could not be observed (data not shown).

Taken together these data suggest that the protein synthesis in plants is not regulated by phosphorylation of EF-2. Nevertheless, it cannot be excluded that this mechanism is realized only at certain phases of plant development (for instance, during gametogenesis) and such a possibility requires further study.

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