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EFFECT OF CYCLIC AMP AND CYCLIC GMP ON THE AUTOPHOSPHORYLATION OF ELONGATION FACTOR 1 FROM WHEAT EMBRYOS

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Extensively purified EF-1_H (EF-1αββ'γ) from wheat embryos had a protein kinase activity and phosphorylated EF-1β which is one of the two EF-Ts-like factors (EF-1β and EF-1β'). In this reaction ATP and GTP were equally effective as phosphate donors, and threonine residue was phosphorylated. At 10^{-7}M,3',5' cyclic GMP stimulated both the phosphorylation and phe-tRNA binding reactions, whereas 3',5' cyclic AMP inhibited both reactions. These findings indicate that phosphorylation of EF-1_H may play an important role in the translational control of protein biosynthesis at the elongation step.

Regulation of protein biosynthesis by phosphorylation of one of the translational sites, such as messenger RNA protein complex formation (1), aminoacyl-tRNA synthetase activity (2), initiation factor eIF-2 (3,4), and ribosomal protein S-6 (5,6) have been extensively studied. However, little is know regarding the translational control by phosphorylation of elongation factors.

The high molecular weight form of elongation factor 1 (EF-1_H) from animals consists of three subunits (EF-1abc or EF-1αβγ) (7,8), whereas EF-1_H from wheat embryos consists of four subunits (EF-1abb'c or EF-1αββ'γ)(9). EF-1α catalyzes the binding of aminoacyl-tRNA to ribosomes, whereas EF-1βγ (animal) and EF-1ββ'γ (plant) stimulate the binding reaction. Interestingly, both EF-1β and EF-1β' from wheat embryos stimulate equally the binding reaction (9). These results suggest that plant EF-1_H contains two EF-Ts-like factors. However, differences in their functions or the functions of EF-1γ have not been clarified.

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In this communication, we reported that EF-1_H has protein kinase activity and autophosphorylates its EF-1β subunit. Interestingly, both phosphorylation and phe-tRNA binding reactions were stimulated by cGMP and inhibited by cAMP.

MATERIALS AND METHODS

GDP-Sepharose was prepared by the method of Wilchek and Lamed (10) except that Sepharose was activated by the method of March et al. (11).

Heparin-Sepharose was prepared by the method of Funahashi et al. (12).

Preparation of EF-1_H and its subunits. EF-1_H was partially purified by polyethyleneimine and ammonium sulfate fractionations as in Ref (9), and purified by GDP-Sepharose and Heparin-Sepharose column chromatographies as follows. The ammonium sulfate fraction (1,600 mg /20 ml) was diluted with 200 ml of buffer A (50 mM Tris-HCl, pH 7.6, 50 mM KCl, 2 mM 2-mercaptoethanol and 20 % (v/v) glycerol) and applied to a GDP-Sepharose column (2.6 x 16 cm) equilibrated with buffer A. The column was washed with 150 ml of the same buffer and developed with a linear gradient of 0 to 0.3 mM GDP in buffer A in a total volume of 200 ml. EF-1_H and EF-1α were eluted at about 0.15 mM GDP. EF-1 fractions, assayed by phe-tRNA binding reaction, were pooled and applied to a Heparin-Sepharose column (2.2 x 12 cm) equilibrated with buffer A. The column was washed with 150 ml of the same buffer and eluted stepwise with 100 ml each of 90 and 150 mM KCl in buffer A. EF-1_H was eluted at 90 mM KCl, whereas EF-1α was eluted at 150 mM KCl. EF-1_H and EF-1α fractions were separately combined, dialyzed against buffer A, and used in the following experiments. Twenty five mg of EF-1_H and 17 mg EF-1α were obtained from 200 grams of wheat embryos. EF-1ββ'γ was prepared according to the procedure for the preparation of silk gland EF-1βγ (13). EF-1β, EF-1β', and EF-1γ were prepared from EF-1_H as in Ref (8).

High performance liquid chromatography (HPLC). EF-1_H (200 μg/200 μl buffer A) was applied to a TSK-G3000SW column (Toyo Soda, Tokyo) equilibrated with buffer A, and developed with the same buffer at a flow rate of 0.68 ml per min.

Phe-tRNA binding reaction was carried out as in Ref (9).

Phosphorylation reaction. The standard reaction mixture in a final volume of 0.2 ml contained 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 10 % (v/v) glycerol, 4 mM dithiothreitol, 400 pmol [γ-^{32}P] ATP or [γ-^{32}P]GTP (500-1,000 cpm/pmol), and 46 pmol EF-1_H or its subunits, unless otherwise indicated. The reaction mixture was incubated for 30 min at 30°C and the reaction was stopped by adding 10 % trichloroacetic acid. The precipitate was collected on a glass filter (GB 60, Toyo Roshi, Tokyo), washed three times with the same acid and twice with ethanol, dried, and counted with a liquid scintillation spectrometer.

Phosphorylated amino acid analysis was performed according to the method of Hunter and Soften (14).

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (15) using 12.5 % gel, and phosphorylated EF-1_H subunit was detected by an autoradiography using an X-ray film (Fuji, Kokyo).

RESULTS AND DISCUSSION

Protein kinase activity associated with EF-1_H. EF-1_H was purified by GDP-Sepharose and Heparin-Sepharose column chromatography to apparent homogeniety as judged by polyacrylamide gel electrophoreses. EF-1_H thus obtained was found to have a protein kinase activity without added phosphate acceptors. About 0.1 pmol phosphate was incorporated per pmol EF-1_H in the standard phosphorylation reaction. The reaction was time dependent and about 0.5 pmol phosphate per pmol EF-1_H was incorporated during the incubation of 2 hr in the presence of 200 μM [γ-^{32}P] ATP. EF-1_H did not phosphorylate histone H2A, casein, or 80S ribosomes which are known to be phosphorylated by various protein kinases. These results suggest that EF-1_H has autophosphorylating activity. To confirm this, and to identify the subunit(s) to be phosphorylated, purified EF-1_H was subjected to HPLC. Fig. 1 shows the elution profiles of protein, protein kinase activity (without added phosphate acceptors), and phe-tRNA binding activity. They coincided well. These results indicate that the protein kinase activity is associated with EF-1_H. Electrophoretic analyses of the active fractions revealed the presence of four protein bands, α, β, β', and γ. Out of the four EF-1_H subunits, only EF-1β was phosphorylated (Fig. 1C). Since EF-1β and EF-1β' have nearly the same specific activity in stimulating EF-1α-dependent phe-tRNA binding reaction and they have quite similar tryptic peptide maps (9), it is inreresting that only one of the two EF-Ts-like factors was phosphorylated. The fact that EF-1β formed a firm complex with EF-1γ, while EF-1β' did not (Kawamura and Ejiri, unpublished results) is likely to account for the preferential phosphorylation of EF-1β. In the autophosphorylation reaction, [γ-^{32}P]ATP and [γ-^{32}P]GTP were equally effective as phosphate donors. Acid hydrolysis of the

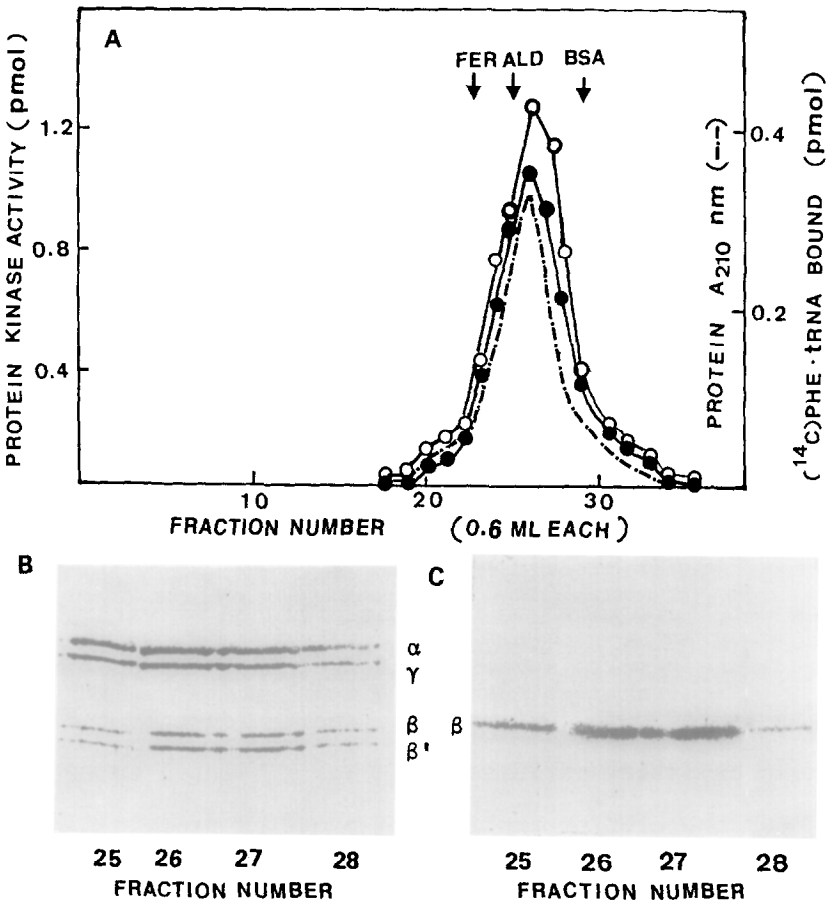

Fig. 1. Identification of protein kinase activity associated with EF-1_H on an HPLC. Purified EF-1_H (200 µg/200 µl) was applied to a TSK-G3000SW column and developed as described under "Materials and Methods." (A): aliquots (100 µl) of the column fractions were assayed for protein kinase activity without added substrate (○), or for [^{14}C]phe-tRNA binding to ribosomes (●) as described under "Materials and Methods." The following protein markers were used : FER (ferritin, 450,000), ALD (aldolase, 158,000), and BSA (bovine serum albumin, 68,000). (B): aliquots (200 µl) of the active fractions in experiment (A) were incubated with [γ-^{32}P]ATP in the standard protein kinase assay system. After reaction for 30 min at 30°C, the reaction were subjected to an SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue. (C): autoradiogram was made by placing the dried gel in experiment (B) in contact with an X-ray film and exposed for 2 days.

radioactive EF-1β resulted in the formation of phosphothreonine and small amount of phosphoserine (Fig. 2).

Fig. 3 shows the autophosphorylating activity of EF-1_H subunits. EF-1ββ'γ had about half the activity as that of EF-1_H,

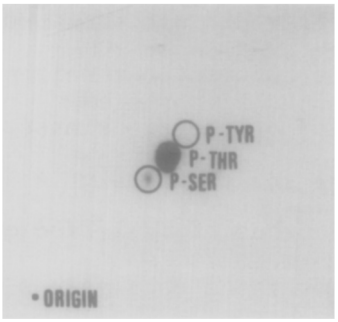

Fig. 2. Identification of phosphoamino acids in the phosphorylated EF-1β. The EF-1_H phosphorylated under the standard reaction condition with [γ-^{32}P]ATP was subjected to SDS-polyacrylamide gel electrophoresis and EF-1β was extracted with 70 % formic acid. The extract was evaporated to dryness, and hydrolyzed with 6 N HCl at 110°C for 1.5 hr. After removal of HCl, the sample was analyzed on cellulose thin layer plates (10 x 10 cm, Chromagram, Eastman) using isobutyric acid/0.5 N NH_4OH (5 : 3) in two dimensions. Autoradiography was performed as in the Fig. 1 legend. Unlabeled phosphoserine (P-SER), phosphothreonine (P-THR), and phosphotyrosine (P-TYR) were used as markers and they were detected by staining with ninhydrin.

whereas EF-1β, EF-1β', and EF-1γ had no activity even when they were used in combinations. EF-1α, when added to EF-1ββ'γ in an equimolar ratio, restored the activity. These results indicate that EF-1ββ'γ which corresponds to EF-Ts has protein kinase activity. EF-1β and EF-1β' prepared under denaturing condition restored full activity in stimulating EF-1α-dependent binding

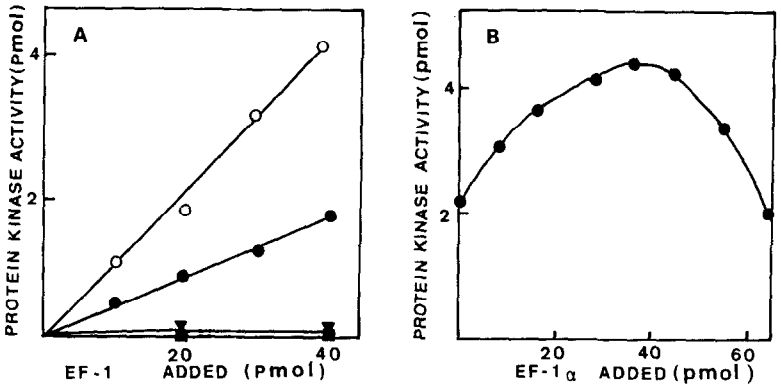

Fig. 3. Autophosphorylating activities of EF-1_H and its subunits. Protein kinase activity was assayed as described under "Materials and Methods" using [γ-^{32}P]ATP as a phosphate donor. (A): 46 pmol each of EF-1_H (○), EF-1ββ'γ (●), mixtures of α, β, β', and γ (▼), or each of the four subunits (■). (B): 46 pmol EF-1ββ'γ and EF-1α as indicated (●).

reaction (9); however, they did not show protein kinase activity (Fig. 3). These results suggest that neither EF-1β nor EF-1β' bear the catalytic center of protein kinase. Finally, EF-1γ remains as the candidate for the catalytic subunit of the protein kinase. Since the functions of EF-1γ are quite unknown, the suggestion that EF-1γ may bear the catalytic site is of interest.

Effect of cyclic nucleotides on the phosphorylation and phe-tRNA binding reactions. Fig. 4 shows the effect of cAMP and cGMP on the autophosphorylation and phe-tRNA binding reactions. Interestingly, 10^{-7}-10^{-6}M cGMP stimulated both reactions, whereas the same concentration of cAMP inhibited both the reaction. The effect of cyclic nucleotides were lost with higher concentrations of them. As a possible explanation of this fact, we postulate stimulatory (S) and inhibitory (I) sites which have a higher affinity to cGMP and cAMP, respectively. At 10^{-7}-10^{-6}

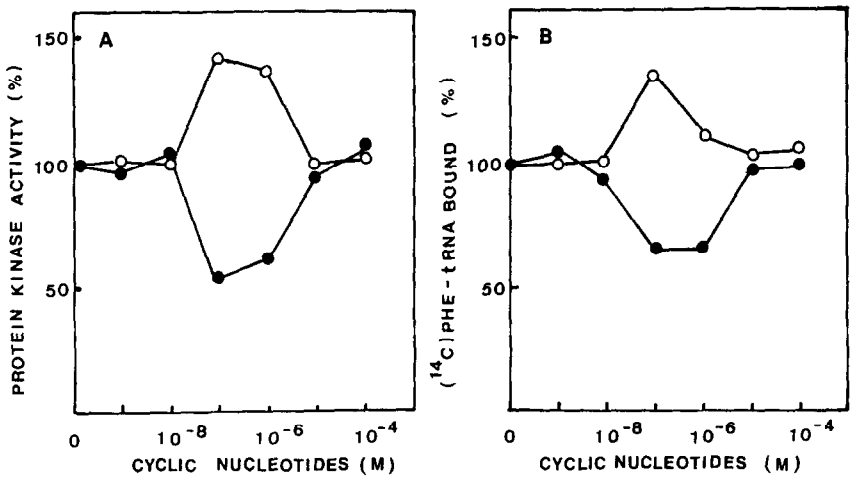

Fig. 4. Effect of 3',5'cyclic AMP and 3',5'cyclic GMP on the EF-1$_H$-dependent autophosphorylation and phe-tRNA binding reactions. (A): phosphorylation was carried out as described under "Materials and Methods" in the presence of 400 pmol [γ-^{32}P] ATP, 46 pmol EF-1$_H$, and cyclic nucleotides as indicated. (B): the phe-tRNA binding reaction in a final volume of 250 µl was carried out for 30 min at 30°C as described in Ref (9) in the presence of 10 pmol [^{14}C]phe-tRNA, 10 pmol EF-1$_H$, 10 µg poly(U), 0.3 mM GTP, 1.0 A_{260} unit of ribosomes and cyclic nucleotides as indicated. [^{14}C]phe-tRNA, bound to ribosomes was assayed by a nitrocellulose membrane filter.

M, cGMP (cAMP) will bind to the S-site (I-site) and will stimulate (inhibit) the phosphorylation. At higher cGMP (cAMP) concentrations, however, it will bind also to the I-site (S-site) and will abolish the stimulatory (inhibitory) effect. Stimulation of polypeptide elongation by cGMP was observed as early as 1973 by Varrone et al. (16). Since then a large numbers of unsuccessful attempts have been carried out to elucidate the action mechanism of cGMP. The present results may facilitate further works on the subject.

Recently, Davydova et al. (17) reported that EF-1α in $EF\text{-}1_H$ from rabbit reticulocytes was phosphorylated when $EF\text{-}1_H$ was incubated with [γ-^{32}P]ATP and ribosomes at low ionic strength. However, $EF\text{-}1_H$ from wheat embryos and silk gland did not show such a ribosome-dependent protein kinase activity. Although potent cyclic nucleotide-independent protein kinase was detected in wheat embryos, it was not absorbed to the GDP-Speharose column. The kinase had no autophosphorylating activity, and it did not phosphorylate $EF\text{-}1_H$ or its subunits. These results indicate that $EF\text{-}1_H$ kinase is a unique protein kinase distinct from known protein kinases. Since silk gland $EF\text{-}1_H$ purified by the same procedures also had autophosphorylating activity (Takizawa and Ejiri, unpublished results), it is likely that eukaryotic $EF\text{-}1_H$ in general has protein kinase activity.

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