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Mechanism of elongation factor 2 (EF-2) inactivation upon phosphorylation

Phosphorylated EF-2 is unable to catalyze translocation

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Previously we have found that elongation factor 2 (EF-2) from mammalian cells can be phosphorylated by a special Ca^{2+}/calmodulin-dependent protein kinase (EF-2 kinase). Phosphorylation results in complete inactivation of EF-2 in the poly(U)-directed cell-free translation system. However, the partial function of EF-2 affected by phosphorylation remained unknown. Here we show that phosphorylated EF-2, unlike non-phosphorylated EF-2, is unable to switch ribosomes carrying poly(U) and Phe-tRNA in the A site to a puromycin-reactive state. Thus, phosphorylation of EF-2 seems to block its ability to promote a shift of the aminoacyl(peptidyl)-tRNA from the A site to the P site, i.e. translocation itself.

Elongation factor 2; Protein phosphorylation; EF-2 kinase; Calmodulin; Ribosomal translocation; Translational control

1. INTRODUCTION

It has been shown that elongation factor 2 (EF-2) from mammalian cells can be phosphorylated [1] and that up to 50% of the EF-2 purified from cell extracts is phosphorylated [2–4]. Phosphorylation of EF-2 is catalyzed by a Ca^{2+}/calmodulin-dependent protein kinase which is specific for EF-2 and which was called Ca^{2+}/calmodulin-dependent protein kinase III [5,6] or EF-2 kinase [3,4]. Phosphorylation of EF-2 results in its complete inactivation in the cell-free translation system [2,4,6,7] and presumably represents the key mechanism of translational control at the level of polypeptide chain elongation.

It is of interest why phosphorylated EF-2 is inactive in protein synthesis. This is not a simple question, as the EF-2 working cycle consists of several partial reactions (see e.g. [8]) and the blocking of any of them would lead to inactivation of EF-2 in translation. These partial reactions are: (i) binding of EF-G·GTP to the ribosome; (ii) promoting of the peptidyl-tRNA movement from the ribosomal A site to the P site and the release of deacylated tRNA; (iii) ribosome-dependent hydrolysis of GTP; (iv) dissociation of EF-G·GDP from the ribosome; (v) exchange of GDP bound to EF-2 for GTP.

In this work we have obtained evidence that the partial reaction which is arrested by phosphorylation is the EF-2 catalysis of the peptidyl-tRNA shift from the A site to the P site. In other words, the phosphorylation of EF-2 blocks translocation itself.

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Abbreviations: EF-1, elongation factor 1; EF-2, elongation factor 2; GMPPCP, guanosine 5′-(β,γ-methylene)triphosphate; GMPPNP, guanosine 5′-(β,γ-imino)triphosphate

2. MATERIALS AND METHODS

EF-2 was purified from the ribosome-free extract of rabbit reticulocytes by the following procedure. 400 ml (about 50 g of total protein) obtained as in [9] was applied to DEAE-cellulose column (5 × 20 cm, DE-52, Whatman) equilibrated with buffer

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A: 20 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 7 mM β-mercaptoethanol, 10% glycerol. EF-2 was eluted by buffer A with 200 mM KCl and applied to a hydroxyapatite column (2.6 × 30 cm, HA-Ultragel, LKB) equilibrated with 5 mM potassium phosphate buffer, pH 7.0, with 7 mM β-mercaptoethanol and 10% glycerol. EF-2 was eluted by a linear potassium phosphate gradient (5–250 mM). Fractions containing EF-2 activity were collected, diluted with an equal volume of buffer A and applied to a Mono Q HR 10/16 column (Pharmacia) equilibrated with buffer A and connected to the FPLC system (Pharmacia). Non-adsorbed material containing EF-2 was dialyzed overnight against buffer A and chromatographed once again on a Mono Q HR 10/16 column in the same conditions. EF-2 was eluted with linear KCl gradient (10–200 mM).

EF-2 kinase was purified from rabbit reticulocyte ribosome-free extract as in [4]. The reaction mixture (100 μl) for EF-2 phosphorylation contained 60 μg of EF-2, 2 μg of EF-2 kinase, 1 μg of calmodulin, 50 mM Hepes-KOH, pH 7.6, 10 mM magnesium acetate, 150 μM $CaCl_2$, 5 mM dithiothreitol, 70 μM ATP. The reaction was performed for 30 min at 30°C and stopped by the addition of 10 μl of 1 mM trifluoperazine.

40 S and 60 S ribosomal subunits were obtained as in [10]. 80 S ribosomes carrying poly(U) and [^{3}H]Phe-tRNA in the A site (A-ribosomes) were prepared from 300 pmol of 40 S and 60 S subunits mixed in 1 ml of 20 mM Tris-HCl, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1.2 mM GTP with 0.5 mg poly(U) (Boehringer), 0.52 mg of crude *Escherichia coli* tRNA, charged with [^{3}H]phenylalanine (Amersham) (2000 cpm per 1 μg of crude tRNA) and 30 μg of EF-1. After 5 min incubation at 37°C the reaction mixture was centrifuged through 1 ml of 10% sucrose prepared in the same buffer, without GTP. Centrifugation was performed for 2 h in a type 75 rotor at 45000 rpm (4°C). The ribosomal pellet was dissolved in 400 μl of the same buffer. Ribosomes obtained contained about 1 pmol [^{3}H]Phe-tRNA per 1 mol of 80 S ribosomes.

Translocation reaction was performed with 3.5 pmol of A-ribosomes in 50 μl of the 40 mM Tris-HCl buffer, pH 7.6, containing 100 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and varying amounts of EF-2. In different cases either 0.2 mM GTP, or 0.2 mM GMPPCP, or 0.2 mM GTP and 0.2 mM fusidic acid were added. The reaction mixture was incubated at 37°C.

After the translocation reaction, 50 μl of the 40 mM Tris-HCl buffer, pH 7.8, with 2 mM puromycin (Boehringer), 10 mM $MgCl_2$, 250 mM NH_4Cl, 1 mM dithiothreitol and 1 mM EDTA was added. The reaction mixture was incubated for 1 h at 4°C. [^{3}H]Phe-puromycin was extracted with ethylacetate essentially as in [11]: 100 μl of 2 M sodium acetate, pH 5.5, and 1 ml of ethylacetate was added. Then the mixture was shaken and the phases were separated by centrifugation. Radioactivity was determined in a 0.5 ml of the organic phase in 5 ml of 1:2 Triton X-100/scintillation mixture and multiplied by two. Thus the values in the tables correspond to the whole amount of [^{3}H]Phe-puromycin in the organic phase of each probe.

3. RESULTS

The simplest way to measure the translocation reaction itself is the puromycin test. It is well known that EF-2 is capable of transferring aminoacyl-tRNA from the puromycin-nonreactive state (A site) to the puromycin-reactive state (P site) [12,13]. This reaction can take place in the presence of non-hydrolyzable GTP analogs instead of GTP (GMPPCP or GMPPNP) [12,13]. However, in the presence of non-hydrolyzable GTP analogs EF-2 works stoichiometrically [13].

It is shown in table 1 that the addition of increasing amounts of EF-2 to the A-ribosome preparation in the presence of GMPPCP results in the increase of [^{3}H]Phe-puromycin which can be extracted with ethylacetate. The reaction reaches the plateau level in 15 min which is in accordance with the data reported by Tanaka et al. [13]. Saturation is observed at 0.5 μg of EF-2, i.e. at the ratio of EF-2:ribosomes of 5:3.

Then we studied the ability of the phosphorylated EF-2 to catalyze the shift of [^{3}H]Phe-tRNA into the puromycin-reactive state. As can be seen from table 2, the addition of phosphorylated EF-2 in the presence of GMPPCP does not increase the amount of [^{3}H]Phe-puromycin extracted with ethylacetate, as compared with the control experiment without EF-2 (see table 1). In the presence of GTP or GTP with fusidic acid, the phosphorylated EF-2 promotes an increase of [^{3}H]Phe-puromycin only slightly. This slight increase could be explained if the phosphorylated EF-2 still works, but at a very slow rate. This seems unlikely, however, as prolonged incubations do not lead to an increase of [^{3}H]Phe-puromycin. It is more likely that the slight activity of the phosphorylated EF-2 is due to

Table 1

Dependence of [^{3}H]Phe-puromycin yield on the amount of EF-2 and incubation time

EF-2 (μg)	Yield of [^{3}H]Phe-puromycin (pmol)	Incubation time (min)
–	0.10	15
0.13	0.70	15
0.26	1.20	15
0.52	2.24	15
1.24	2.52	15
0.80	1.98	5
0.80	2.44	15
0.80	2.40	30

Table 2

Comparison of ability of native and phosphorylated EF-2 to catalyze translocation

Factor (0.8 μg)	Addition	Yield of [^{3}H]Phe-puromycin (pmol)	Incubation time (min)
EF-2	GTP	3.02	15
EF-2	GTP, fusidic acid	2.88	15
EF-2	GMPPCP	2.30	15
P-EF-2	GTP	0.42	15
P-EF-2	GTP, fusidic acid	0.22	15
P-EF-2	GMPPCP	0.16	15
P-EF-2	GMPPCP	0.20	60
P-EF-2	GMPPCP	0.20	120

Table 3

Catalysis of translocation by native EF-2 in the presence of phosphorylated EF-2

Factor (μg)	Yield of [^{3}H]Phe-puromycin (pmol)
EF-2 (0.15)	0.82
EF-2 (0.30)	1.76
EF-2 (0.60)	2.22
EF-2 (1.20)	2.46
EF-2 (2.40)	2.44
EF-2 (0.15), P-EF-2 (0.45)	1.34
EF-2 (0.30), P-EF-2 (0.90)	2.08
EF-2 (0.60), P-EF-2 (1.80)	2.20

a small contamination of nonphosphorylated EF-2 in the preparation.

We also studied the effect of the phosphorylated EF-2 on the activity of the nonphosphorylated EF-2 in this system. Previously, we have found that in poly(U)-directed system of polyPhe synthesis the phosphorylated EF-2 inhibits the activity of nonphosphorylated EF-2 [4]. However, in the case of the translocation reaction measured by puromycin reactivity the phosphorylated EF-2 does not inhibit the nonphosphorylated EF-2, and even noticeably stimulates it (see table 3).

4. DISCUSSION

Previously we have shown that the phosphorylated EF-2 is completely inactive in poly(U)-directed polyPhe synthesis [2,4,7]. However, the partial function of EF-2 arrested by phosphorylation remained unknown. Here we have found that the phosphorylated EF-2 is unable to shift Phe-tRNA into the puromycin-reactive state. In other words, the phosphorylated EF-2 is unable to catalyze translocation. This rules out the possibility that the partial reaction of EF-2 arrested by phosphorylation is the step of dissociation of EF-2 from the ribosome (as in the presence of fusidic acid). Hence, the phosphorylated EF-2 is either unable to bind GTP, or the phosphorylated EF-2 complexed with GTP is unable to bind to the ribosome, or the phosphorylated EF-2 is unable to catalyze translocation after binding to the ribosome. The third explanation seems to us the most likely as we have found (unpublished experiments) that the phosphorylated EF-2 can bind to 80 S ribosomes in the presence of GMPPCP. We believe that phosphorylated EF-2 can bind to GTP as well as to the ribosome, but the binding to the ribosome is not correct, or something has happened to the EF-2 structure which makes EF-2 unable to catalyze translocation.

The ability of the phosphorylated EF-2 to bind to the ribosome also follows from the fact that it competes with the nonphosphorylated EF-2 in poly(U)-directed translation [4]. Translocation experiments reported in this paper clearly show that this binding is not enough to promote translocation.

In any case it is clear that phosphorylation affects EF-2 activity at the level of the interaction of EF-2 with the pretranslocation state ribosomal complex. It is noteworthy that the sites of phosphorylation: Thr 53, Thr 56 and Thr 58 (Ovchinnikov et al., in preparation) are located in the so-called E-domain [14] (between amino acids 53 and 77); this domain is homologous in all elongation factors and presumably is crucial for interaction of the factors with the ribosome or with peptidyl-tRNA [14].

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