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FUSIDIC ACID-DEPENDENT WHEAT GERM RIBOSOMAL COMPLEXES REQUIRE UNPHOSPHORYLATED ELONGATION FACTOR 2

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Key Word Index—*Triticum aestivum*; Gramineae; phosphorylation; translation; ribosomal complexes; fusidic acid.

Abstract—The effects of the antibiotic fusidic acid on translation and the phosphorylation pattern in a wheat (*Triticum aestivum*) germ cell-free system were investigated. The antibiotic reduces phosphorylation of elongation factor 2 (EF-2) at concentrations at which it efficiently inhibits translation. The interaction of EF-2 with the ribosome occurs in its non-phosphorylated state, as proved by the formation of the fusidic acid-dependent ribosomal complexes.

INTRODUCTION

The changes in the state of phosphorylation of key metabolic enzymes are of great importance in cellular regulation [1]. Regarding protein synthesis, for several years it has been known that certain protein factors involved in polypeptide chain initiation are subjected to phospho-dephosphorylation [2, 3].

Recently, it has also been found that the elongation factor 2 (EF-2) is subjected to phosphorylation [4–7]. Phosphorylation of EF-2 inhibits polypeptide synthesis coded by polyuridylic acid [6, 5] or endogenous globin m-RNA messenger [4]. Very recently, it has been found that the rat liver ribosome-bound EF-2 does not readily undergo phosphorylation [8]. Additionally, phosphorylation seems to impair the interaction of EF-2 with the ribosome thus avoiding the formation of the pretranslocation EF-2·ribosomal complex [8, 9]. As the effect of phosphorylation on the mechanism of interaction of EF-2 with the ribosome is unknown, we addressed the question of whether the phosphorylated factor binds to the ribosome in the post-translocation state which would lead to competition with non-phosphorylated factor, or whether instead it is unable to interact with the ribosome. In both cases, phosphorylation would result in the reduction of functional EF-2 available for translation. To answer this key question we used the antibiotic fusidic acid which is known to stabilize EF-2 (EF-G) on the eukaryotic (prokaryotic) ribosome in a post-translocation state [10–12]. Here we present evidence indicating that fusidic acid reduces the phosphorylation of EF-2 in cell-free extracts from wheat germ. An additional finding is that the fusidic acid-dependent complex formed in the cell-free extract from wheat germ seems to require the non-phosphorylated form of the factor.

RESULTS AND DISCUSSION

To ascertain whether EF-2 interacts with the ribosome either in the non-phosphorylated form or in the phosphorylated one, we formed the fusidic acid-dependent ribosomal complexes with [^{3}H]GDP and thereafter isolated the complexes by chromatography through Sepharose 6B [12]. There was an elution of [^{3}H]GDP in the void volume of the columns despite the presence of fusidic acid (Table 1). This could account for the binding of [^{3}H]GDP to GDP-binding proteins retained in particulate material that eluted into the void volume, presumably some G-proteins present in the plasma membrane and other particulate material. However, in the presence of fusidic acid the label eluted into the void volume was much higher than in its absence, the difference being accounted for by the ribosomal complex [12]; in fact, the extent of the complex formed was *ca* 26% of the ribosomal population. Analogous reaction mixtures in which unlabelled GDP replaced [^{3}H]GDP, but supplemented with [γ-^{32}P]ATP and, either in the absence or the presence of fusidic acid, were chromatographed under exactly the same conditions as above. It was found that the amount of [^{32}P] radioactivity associated with the fusidic acid-dependent ribosomal complex was the same as that found associated with the ribosomes in the absence of the complex. The [^{32}P] radioactivity associated to the ribosomal peak eluted in the void volume was even slightly higher in the absence of fusidic acid than in its presence. This particulate material was analysed by SDS-PAGE and fluorography and no radioactivity was found either at or near the band defined by the EF-2. Taking all these facts into consideration, it seems clear that the complex components are not phosphorylated under our experimental conditions. As the fusidic acid-dependent complex is formed and dissociated continuously, the latter finding also suggests that neither EF-2

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Table 1. Isolation of EF-2·GDP·ribosome·fusidic complexes by column chromatography through Sepharose 6B

Radioactive component present in the reaction mixtures		Radioactivity associated into the ribosomal complexes (pmol/A_{260})		
		+fusidic acid	−fusidic acid	net
[^{3}H]GDP	exp. *a*	8.9	3.0	5.9
	exp. *b*	8.6	2.4	6.2
[γ-^{32}P]ATP	exp. *a*	7 3	9 5	−2.2
	exp. *b*	10.5	15.3	−4.8

Reaction mixts of 60–120 µl containing the components required for complex formation were incubated for 15 min at 30°. Thereafter, the complexes were isolated by column chromatography through Sepharose 6B. The void vols were collected and assayed for radioactivity and A_{260}. Experiment *a* was conducted with reaction mixts twice those used in experiment *b*.

binding, nor its permanence or its release from the ribosome can occur with the phosphorylated form. This also indicates that phosphorylation must take place only as an extra ribosomal event, unless phosphorylation of EF-2 in the complex promotes the very fast decay of the complex and the extremely rapid release of phosphorylated factor from the ribosomal surface, that prevents it being detected by current procedures. These assertions are in close agreement with very recent data which indicate that EF-2·GppCH$_2$p binding to the ribosome in the pretranslocation state and its permanence in such state are events incompatible with EF-2 phosphorylation [8, 9].

We next studied the pattern of phosphorylation of the S-30 wheat germ. Incubation of the extracts with [γ-^{32}P]ATP led to the phosphorylation of several proteins (Fig. 1). Among these there is a band with an apparent M_r near to 100 000 which coincides exactly with the position of the highly phosphorylated EF-2 band from rabbit reticulocyte lysates [4]. Because fusidic acid complexes EF-2 on the ribosomes and this factor is not phosphorylated in such complexes (Table 1), the effect of the antibiotic on this phosphorylation pattern was studied. The addition of fusidic acid to the reaction mixtures led to a dose-dependent reduction in the labelling of the M_r 100 000 band; this being fully abolished at 3 mM fusidic acid (Fig. 1).

Finally, to ascertain whether fusidic acid, under the conditions of the above-mentioned dephosphorylation does in fact behave as an effective inhibitor in the wheat germ cell-free system, we investigated its effects on translation. The antibiotic fully inhibits translation at 3 mM (Fig. 2). This indicates that all the ribosomes that were available and active in protein synthesis were engaged with EF-2 as a fusidic·EF-2·GDP·ribosome complex [10]. This in turn could considerably reduce the concentration of free EF-2 in the cell-free extract. At 1 mM fusidic acid, translation and phosphorylation of the M_r 100 000 band occurred. Since complete inhibition of translation does not seem to lead to complete dephosphorylation of EF-2, we suggest that EF-2 must be in excess of the requirements for translation in this cell-free system. This agrees with the hypothesis raised by other workers concerning rabbit reticulocyte lysates [4].

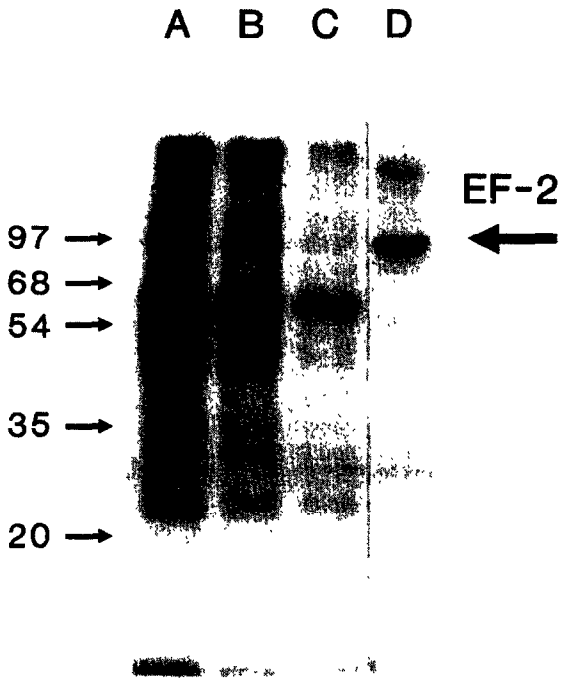

Fig. 1. Influence of fusidic acid on the protein phosphorylation pattern of a cell-free extract from wheat germ. Protein phosphorylation from a cell-free extract (S-30) of wheat germ was studied as described in the text. Incubations contained: no further additions (A), 1 mM fusidic acid (B), 3 mM fusidic acid (C). A protein phosphorylation pattern obtained from rabbit reticulocyte lysate in the same conditions (D) is included for comparative purposes. The amount of protein in lanes A–C was 32 and 250 µg in lane D; the total amount of radioactivity added per lane ranged from 10 000 to 20 000 dpm. Numbers on the left indicate the M_r scale in thousands.

In conclusion, our results show that the fusidic acid-dependent ribosomal complex requires non-phosphorylated EF-2. Concerning the increased fusidic acid-dependent dephosphorylation of EF-2, at present we have no information to ascertain the exact mechanism by which

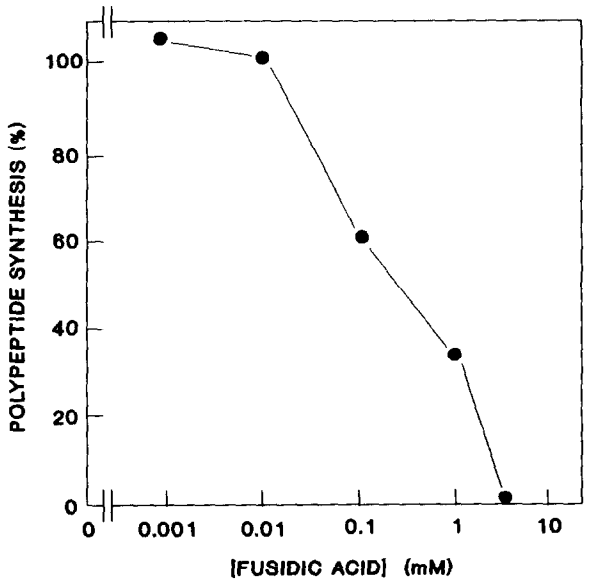

Fig. 2. Effect of fusidic acid on polypeptide synthesis carried out by a cell-free system isolated from wheat germ. The experiment was carried out as indicated in the Experimental section. The antibiotic was prepd fresh as a 50 mM soln of the Na fusidate. 100% represents 241 000 dpm mg^{-1} of protein in the cell-free extract.

fusidic acid exerts its effects. A plausible hypothesis would be that both forms of the phospho- and non-phosphorylated factor are in equilibrium and that fusidic acid is able to induce the shift of equilibrium between both forms. Such a perturbation of this potential equilibrium might be promoted by entrapment of the non-phosphorylated form as ribosomal complex. However, the possibility that fusidic acid might interact with the enzymes related to the phospho-dephosphorylation process, namely Ca^{2+}-dependent protein kinase III and type-2A phosphatase [4, 7] thus distorting the normal level of phosphorylation, cannot be ruled out so far.

EXPERIMENTAL

Materials. L-[^{3}H]GDP (333 GBq $mmol^{-1}$), L-[^{3}H]valine (2.4 TBq $mmol^{-1}$), [γ-^{32}P]ATP (111 TBq $mmol^{-1}$) were purchased from New England Nuclear. Nucleotides, enzymes, phosphoenolpyruvate and Tris were purchased from Boehringer. Salts, current chemicals and biochemicals were purchased from E. Merck. Na fusidate was obtained from Sigma. Sepharose 6B and Sephadex G-25 were purchased from Pharmacia.

Cell-free extracts. The wheat germ cell-free system was obtained as indicated elsewhere [13]. The extracts were filtered through Sephadex G-25 to remove small M_r compounds and stored in small aliquots under liquid N_2. Rabbit reticulocyte lysates were prepd as in ref. [14].

Protein synthesis. Protein synthesis in the wheat germ cell-free system (S-30) was measured as described previously [13]. The reaction mixts (50 µl at 30°) contained 1.11 kBq of L-[^{3}H]valine (sp. act. 2.4 TBq $mmol^{-1}$) and were incubated for 30 min. Thereafter, they were processed for incorporation of radioactivity into hot trichloroacetic acid-insoluble material [15].

Isolation of ribosomal complexes. Fusidic acid-dependent ribosomal complexes were obtained from the wheat germ cell-free extracts (S-30) in 60–126 µl reaction mixts containing 25mM KCl, 9 mM $MgCl_2$, 25 mM NH_4Cl, 20mM Tris–HCl (pH 7.8), 5 mM dithiothreitol, 9.4 µM L-[^{3}H]GDP (sp.act. 333 GBq $mmol^{-1}$), 3 mM Na fusidate, 47 µM ATP, and 40–80 µl S-30 from wheat germ and 5–10 µl of purified wheat germ ribosomes. After incubation of these mixts for 15 min at 30°, the ribosomal complexes were isolated by chromatography through Sepharose 6B (15.5 × 0.6 cm) with a flow rate of 124 µl per min [12]. From the eluate, aliquots of 370 µl were taken and 40 µl of each was added to 1.5 ml of H_2O and used to measure the A_{260}. The rest of the aliquot was added to 2.25 ml of liquid scintillation cocktail (Ready Safe, Beckman) and counted for radioactivity. For the analysis of phosphorylation of the components of the complex, 47 µM [γ-^{32}P]ATP (sp. act. 74 TBq $mmol^{-1}$) replaced the cold ATP in the reaction mixts for the formation of the ribosomal complexes, omitting the L-[^{3}H]GDP, and the mixts were processed exactly in the same way as for the analysis of the complex.

Protein phosphorylation pattern in the S-30 wheat germ extract. At a vol. of 34 µl, the reaction mixts contained the same components as for the complex formation except that GDP was unlabelled and was present at a final concn of 1 mM. [γ-^{32}P]ATP (sp. act. 70 TBq mol^{-1}) was present at a final concn of 50 µM. At the end of incubation, 11 µl of sample buffer containing dithiothreitol and SDS were added to each sample, thereafter boiling the mixture for 90 sec to stop the phosphorylation reaction [4]. Following this, 13 µl of these solns were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis and fluorography. One dimensional SDS-PAGE was carried out in gels of 10% as described in ref. [16]. Gels were treated for fluorography with Amplify (Amersham) and subjected to fluorography with the Kodax X-Omat film, for 2 days at −80°.

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