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Mode of action of the heme-controlled translational inhibitor: Relationship of eukaryotic initiation factor 2-stimulating protein to translation restoring factor

(polypeptide chain initiation/protein phosphorylation/eukaryotic initiation factor 2 kinase/eukaryotic initiation factor 2–protein complexes/ternary complex formation)

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ABSTRACT **We have purified the translation restoring factor (RF) and the eukaryotic initiation factor 2 (eIF-2) stimulating protein (ESP) to near homogeneity from the postribosomal supernatant and the ribosomal salt wash, respectively, of rabbit reticulocyte lysate. They were isolated in the form of eIF-2 complexes, apparently in a 1:1 ratio. Their virtually identical NaDodSO$_4$/polyacrylamide gel electrophoretic patterns show, in addition to the eIF-2 α (38,000), β (52,000), and γ (54,000) bands, peptide bands at approximately 80, 65, 57, 40, and 32 kilodaltons. The apparent M_r of either complex is about 450,000, whereas that of free translation restoring factor (RF) is approximately 250,000. At 0.5 mM Mg^{2+}, both ESP and RF stimulate ternary complex (eIF-2·GTP·Met-tRNA$_i$) formation catalytically with unphosphorylated eIF-2. Phosphorylation of the eIF-2 α subunit by preincubation with eIF-2 α kinase and ATP, which virtually blocks eIF-2-ESP interaction, results in only partial blocking of the interaction with RF. This may explain the translation restoring activity of RF.**

In reticulocytes, globin synthesis is governed by the level of heme, the prosthetic group of hemoglobin. Protein synthesis in reticulocyte lysates is maintained only briefly unless heme is added. Heme deficiency activates an inhibitor of polypeptide chain initiation (heme-controlled inhibitor, HCI). Low concentrations of double-stranded RNA (dsRNA) activate a similar inhibitor (dsRNA-activated inhibitor, DAI). HCI and DAI are cyclic AMP-independent protein kinases that phosphorylate the small α subunit (M_r, 38,000) of eukaryotic initiation factor 2 (eIF-2), interfering with its function (for review, see ref. 1). Although HCI and DAI phosphorylate the same sites of the eIF-2 α subunit (2), they are different proteins (3, 4).

The first step in polypeptide chain initiation is the formation of a ternary complex containing equimolar amounts of eIF-2, GTP, and Met-tRNA$_i$. This complex binds to a 40S ribosomal subunit to form a 40S initiation complex (1). At low concentrations of Met-tRNA$_i$ and eIF-2, little or no ternary complex is formed in the absence of a further factor (eIF-2 stimulating protein, ESP) discovered in our laboratory (5, 6). ESP markedly stimulates the formation of ternary and 40S initiation complexes. Phosphorylation of the eIF-2 α subunit does not modify the basic properties of eIF-2 but largely abolishes the enhancement of complex formation by ESP. This supports the view that HCI (or DAI) acts by blocking the eIF-2–ESP interaction. The β subunit of eIF-2 can be phosphorylated by a cyclic AMP-independent protein kinase (casein kinase) in reticulocyte lysates without affecting the reactivity of the factor with ESP. The fact that phosphorylation of the eIF-2 β subunit inhibits neither translation in lysates nor eIF-2–ESP interaction, whereas phosphorylation of the α subunit inhibits both, is consistent with the above view (7, 8). Translational inhibition by HCI (DAI) can be prevented or reversed by addition of (*a*) eIF-2, (*b*) GTP, or (*c*) a supernatant protein (9) called anti-inhibitor (10) or inhibition reversal factor (11). We shall refer to this protein as translation restoring factor (RF). In this paper we present evidence that RF and ESP are closely related proteins. A preliminary report of this work has been published (12).

MATERIALS AND METHODS

Assays. Translation was assayed in 25-μl samples essentially as described by Hunt *et al.* (13) with 10 μl of 1:1 lysate and [^{14}C]lysine as the labeled amino acid, without or with 20 μM hemin and RF or ESP as specified. Incubation was for 30 min at 30°C. One unit of RF was taken as the amount causing a 1.5-fold increase in lysine incorporation in the absence of added hemin (cf. ref. 10). Ternary complex formation was measured in 50-μl samples by assay A (7) with eIF-2 CM-350 (see below) and 5 μg of bovine serum albumin added as a stabilizer. This assay was used routinely for purification of ESP and RF. One unit is the amount of factor promoting the binding of 1 pmol of Met-tRNA$_i$. Specific activity is expressed throughout as units/mg of protein. Protein was determined by the Bradford (14) procedure with bovine serum albumin as the standard.

Preparations. Rabbit reticulocyte lysate for translation assays was prepared as described by Hunt *et al.* (13). Lysate used for preparation of eIF-2, ESP, and RF was made in a similar way with cells purchased from Pel-Freez. High-speed (postribosomal) and ribosomal fractions were prepared as described (6). Free RF (uncomplexed with eIF-2) was prepared by a slight modification of the method of Amesz *et al.* (10). Postribosomal supernatant was step 1, phosphocellulose chromatography was done at pH 6.8 rather than 7.2, and sucrose density gradient centrifugation was substituted for the corresponding glycerol gradient centrifugation step. Three different preparations of eIF-2 were used as specified, eIF-2 CM-350 (7) of about 25% purity (eIF-2 a), a preparation obtained by Sepharose-heparin chromatography of the CM-350 fraction and about 75% pure (eIF-2 b), and >90% pure preparation (eIF-2 c) made by the procedure of Benne *et al.* (15). eIF-2 in eIF-2–RF and eIF-

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Abbreviations: eIF-2, eukaryotic initiation factor 2; Met-tRNA$_i$, eukaryotic initiator methionyl transfer RNA; HCI, heme-controlled translational inhibitor (an eIF-2 α kinase); dsRNA, double-stranded RNA; DAI, dsRNA-activated inhibitor (another eIF-2 α kinase); ESP, eIF-2 stimulating protein; RF, translation restoring factor.

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Table 1. Purification of RF from reticulocyte lysate

Step	Protein mg	Translation assay			Ternary complex assay		
		Amt. used, μg	Units	SA*	Amt. used, μg	Units	SA*
Postribosomal supernatant	93,600						
Sepharose-heparin I	194						
DEAE-cellulose	73						
CM-200	42	4	26,250	625	300	123,500†	2,940
Sepharose-heparin II	4.4	0.6	18,330	4,166	40	77,640	17,645
Phosphocellulose	1.3	0.3	16,250	12,500	20	36,110	27,776
Sucrose gradient	0.48	0.2	12,000	25,000	20	20,000	41,667

* Specific activity.
† High value might be due to the presence of proteins that stimulate ternary complex formation nonspecifically (see ref. 7).

2–ESP was phosphorylated in the α subunit by incubation with eIF-2 α kinase and [γ-^{32}P]ATP. After phosphorylation (see legend to Fig. 2) the samples were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis (see legend to Fig. 1) and autoradiography. Partially purified HCI (eIF-2 α kinase) was prepared as described (6, 7). For full activation it was incubated with 6 mM *N*-ethylmaleimide for 10 min at 30°C and the unreacted ethylmaleimide was neutralized with 9 mM dithiothreitol. GDP-free GTP and Met-tRNA$_i$ were prepared as described (7).

RESULTS

RF was isolated from the reticulocyte cytosol; ESP was derived from the ribosomal salt wash. We found no ribosome-associated RF activity but we do not know at this time whether or not the cytosol contains ESP.

Isolation of eIF-2 Complexes with RF and ESP. Reticulocyte lysate was prepared from 1.5 liters of blood obtained from phenylhydrazine-treated rabbits (Pel-Freez). The reticulocytes were washed twice with 120 mM NaCl/50 mM KCl. After they were centrifuged down, the washed cells were lysed with 1.5 vol of water and centrifuged for 20 min at 15,000 rpm in a Sorvall GSA rotor. The lysate was centrifuged for 14 hr at 30,000 rpm in a Beckman 35 Ti rotor. The postribosomal supernatant (800 ml) used for the preparation of RF was decanted and loaded directly onto a 2.5 × 40 cm Sepharose 6B-heparin column equilibrated in 20 mM Tris·HCl, pH 7.5/5 mM mercaptoethanol/0.2 mM EDTA, 10% (vol/vol) glycerol (buffer A) containing 100 mM KCl. The column was loaded at a rate of 25 ml/hr, and washed extensively with buffer until $A_{280\,nm}$ was 0.1 or less. The retained protein was eluted with buffer A containing 500 mM KCl, and the eluate was dialyzed against buffer A containing 100 mM KCl. The dialyzed solution was loaded onto a 1.5 × 20 cm DEAE-cellulose column (Whatman DE-52) and equilibrated in buffer A containing 100 mM KCl. Protein was eluted with Buffer A containing 250 mM KCl.

The eluted protein was diluted with buffer A to give a final KCl concentration of 200 mM and was directly loaded onto a 0.9 × 10 cm CM-Sephadex G-50 column equilibrated in buffer A containing 200 mM KCl. The protein not retained was pooled and loaded onto a second Sepharose 6B-heparin column (1.5 × 22 cm) equilibrated in buffer A containing 200 mM KCl. eIF-2, which remains bound to CM-Sephadex, was eluted with buffer A containing 350 mM KCl and stored in liquid nitrogen. Protein was eluted from the Sepharose 6B-heparin column with a 150-ml linear gradient of 200–600 mM KCl in buffer A. A single peak of activity eluted at 400 mM KCl. Active fractions were pooled and dialyzed against 20 mM sodium PO$_4$, pH 6.8/5 mM mercaptoethanol/0.2 mM EDTA/10% glycerol (buffer B) containing 100 mM KCl. The dialyzed protein was loaded onto a 0.9 × 8.5 cm phosphocellulose column (Whatman P11) equilibrated in buffer B containing 100 mM KCl. Protein was eluted with a 40-ml gradient of 100–700 mM KCl. RF activity eluted at 500 mM KCl.

The active fractions were pooled and dialyzed against buffer A containing 100 mM KCl and 5% glycerol. After dialysis, the protein was concentrated to 1.0 ml by ultrafiltration with an Amicon XM50 membrane. The concentrated protein was loaded onto two 12-ml 10–30% sucrose density gradients in buffer A with 100 mM KCl. The gradients were centrifuged at 38,000 rpm for 22 hr in a Beckman SW 40 rotor. Gradients were analyzed by using a model 640 ISCO density gradient fractionator. The absorbance was monitored at 254 nm, and 0.3-ml fractions were collected and assayed. RF sedimented as a single symmetrical peak at a position corresponding to M_r 450,000.

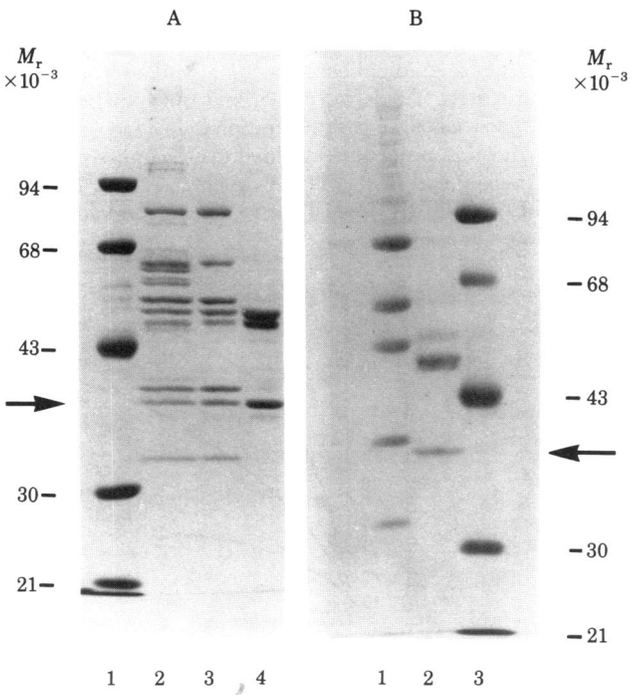

FIG. 1. NaDodSO$_4$/polyacrylamide slab gel electrophoresis of the eIF-2 complexes of ESP and RF (*A*) and of free RF (*B*). Electrophoresis was run in 0.1% NaDodSO$_4$/10% acrylamide/0.26% *N,N'*-methylenebisacrylamide for 6 hr (*A*) or 5 hr (*B*) at 4 mA per gel track (7). Gels were stained with 0.2% Coomassie blue. (*A*) Tracks: 1, markers, each at 2 μg (phosphorylase B, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; and soy bean trypsin inhibitor, 21,000); 2, ESP–eIF-2 complex, 4.2 μg; 3, RF–eIF-2 complex, 4 μg; 4, eIF–2 c, 2 μg. (*B*) Tracks: 1, free RF, 3 μg; 2, eIF-2 c, 3 μg; 3, markers as in *A*. Arrows indicate the position of the eIF-2α subunit (38,000).

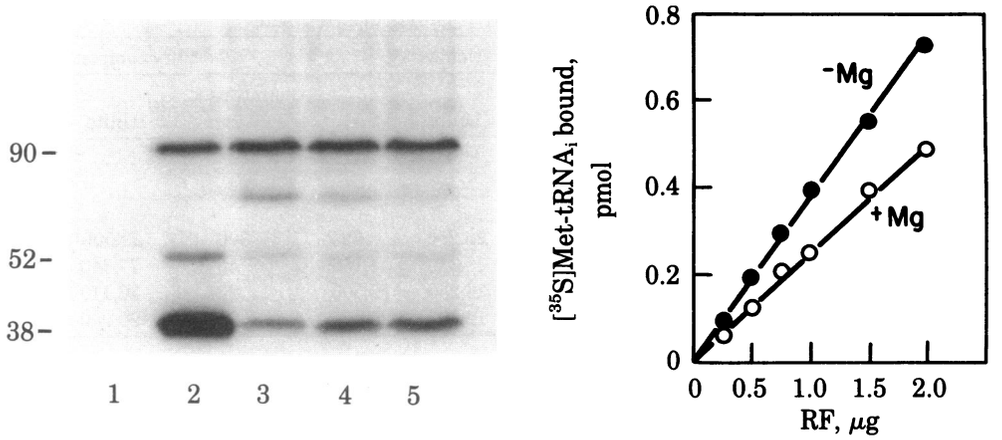

FIG. 2. (*Left*) Phosphorylation of the α subunit of eIF-2 in the RF–eIF-2 complex. Samples (75 μl) containing 20 mM Tris·HCl, pH 7.5/2.0 mM $Mg(OAc)_2$/1.0 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (1140 cpm/pmol), and sucrose-gradient purified (Table 1) RF–eIF-2 complex as indicated below, with or without eIF-2 c (4.5 μg; ≈32.6 pmol) and with or without HCl (eIF-2 α kinase), 7 μg, were incubated for 10 min at 30°C and then subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Tracks: 1, eIF-2; 2, eIF-2 plus HCl plus [γ-^{32}P]ATP; 3–5, RF–eIF-2 complex, 3.8, 7.5, and 11.2 μg, respectively, plus HCl plus [γ-^{32}P]ATP. eIF-2 is contaminated with traces of casein kinase. This explains phosphorylation of the 52-kilodalton eIF-2 β subunit in track 2. The phosphorylated 90 kilodalton band is eIF-2 α kinase itself. (*Right*) Increasing amounts of purified RF–eIF-2 complex were assayed for ternary complex formation in the presence or absence of added 0.5 mM $Mg(OAc)_2$.

Peak fractions were pooled directly from the sucrose gradient and stored in liquid nitrogen at a protein concentration of 0.2 mg/ml. Under these conditions, RF is stable for several months. Table 1 summarizes the purification procedure. RF cannot be assayed prior to separation from free eIF-2 by CM-Sephadex chromatography (step 4); the extent of purification from the lysate is thus unknown.

ESP was prepared from the ribosomal salt wash through the CM-Sephadex step (CM-200) as described (7). This was followed by Sepharose-heparin chromatography, ammonium sulfate precipitation, sucrose gradient centrifugation, and a second Sepharose-heparin step. Yields were poor and the specific activity was lower than that of the RF preparation.

The purification procedures yielded eIF-2 complexes of RF (Fig. 1A, track 3) and ESP (Fig. 1A, track 2). The α, β, and γ chains of eIF-2 (Fig. 1A, track 4), at 38, 52, and 54 kilodaltons, respectively, were present in both cases along with five polypeptides at approximately 80, 65, 57, 40, and 32 kilodaltons. The calculated M_r for the RF and ESP complexes, if each contains 1 mol each of the eight subunits listed, would be 418,000. The apparent M_r from sucrose gradient centrifugation was about 450,000. Free RF (Fig. 1*B*, track 1) had the 80-, 65-, 57-, 40-, and 32-kilodalton peptides but lacked the ones corresponding to eIF-2. The calculated M_r, 274,000, agrees with the apparent M_r, about 250,000, from sucrose gradient centrifugation. The ESP preparation showed some additional peptide bands but, with the exception of two or three bands between peptides at 71 and 56 kilodaltons, they were very faint. Whether some or all of the extra peptides seen in the ESP dissociating gel electrophoresis pattern are impurities (in line with the lower specific activity of the ESP preparation) or true structural elements must await further purification of this factor.

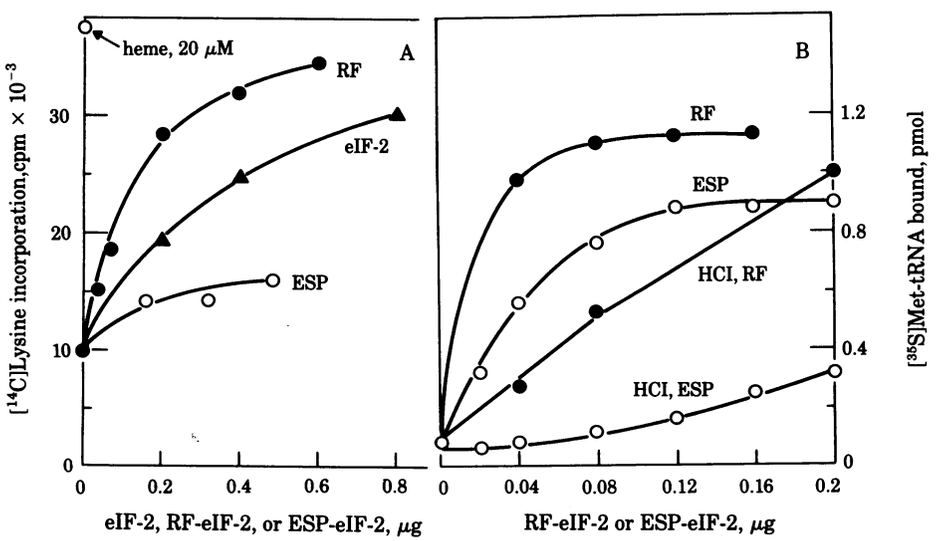

FIG. 3. (*A*) Effect of RF–eIF-2, ESP–eIF-2, and free eIF-2 on translation in the heme-deficient reticulocyte lysate as a function of the concentration of factor. (*B*) Effect of RF–eIF-2 and ESP–eIF-2 on ternary complex formation, without or with phosphorylation of the eIF-2 α subunit by preincubation with eIF-2 α kinase and ATP, as a function of the concentration of factor. Ternary complex formation was measured by de Haro and Ochoa's (7) assay B. The samples (final volume, 50 μl) contained 20 mM Tris·HCl, pH 7.6/100 mM KCl/0.5 mM $Mg(OAc)_2$/22 μM GTP/1.0 mM dithiothreitol, 5 μg of bovine serum albumin, 3 pmol of eIF-2 a, 3 pmol (71,000 cpm/pmol) [^{35}S]Met-tRNA$_i$, and RF–eIF-2 (Table 1, sucrose gradient) or ESP–eIF-2 as indicated. When present, the preincubation samples (30 μl) contained 0.3 μg of eIF-2 α kinase and 80 μM ATP.

The presence of eIF-2 in the RF complex was also demonstrated by phosphorylation of its α subunit with [γ-^{32}P]ATP in the presence of eIF-2 α kinase (Fig. 2 *Left*). The same was true (not shown) of the ESP complex. Comparison with the eIF-2 control showed that the bound eIF-2 was phosphorylated less efficiently, perhaps because the α subunit is less accessible in the complex. The presence of eIF-2 in the RF preparation was further established by direct assay for ternary complex formation in the absence of added eIF-2 (Fig. 2 *Right*). About 40% of the calculated eIF-2 was accounted for in the incubations with no added Mg^{2+}.

Properties of RF–eIF-2 and ESP–eIF-2. The inhibition of translation caused by heme deficiency in reticulocyte lysates was largely prevented by the RF–eIF-2 complex but was not prevented or was prevented to a much lesser extent by the ESP–eIF-2 complex (Fig. 3*A*). The effect of free eIF-2 is shown for comparison. It is evident that eIF-2, which makes up about one-third of the complex, cannot by itself account for the bulk of the RF–eIF-2 effect. As would be expected, RF also reversed the inhibition produced by the addition of dsRNA or oxidized glutathione to heme-containing lysates (data not shown).

The effect of RF–eIF-2 and ESP–eIF-2 on ternary complex formation was examined both without and with preincubation of eIF-2 with eIF-2 α kinase and ATP. We confirmed that ternary complex formation is inhibited by Mg^{2+} and ESP relieves this inhibition (16, 17). Ternary complex formation was nearly maximally inhibited by 0.5 mM Mg^{2+}, and the inhibition was largely prevented by ESP or RF. Mn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+} are also inhibitory but ESP and RF prevent only the inhibition produced by Mg^{2+}, Mn^{2+}, and Ca^{2+} (18). All samples in the experiments of Fig. 3*B* contained 0.5 mM Mg^{2+}. RF–eIF-2 and ESP–eIF-2 caused a pronounced stimulation of ternary complex formation by 3 pmol of eIF-2 (Fig. 3*B*). The specific activities, calculated for the initial parts of the curves were 33,000 and 14,000 for RF–eIF-2 and ESP–eIF-2, respectively, corresponding to a catalytic ratio (mol of excess ternary complex/mol of factor) of 14 and 6, respectively. Preincubation with eIF-2 α kinase and ATP strongly decreased the effect of ESP–eIF-2 but that of RF–eIF-2 still was substantial. Nevertheless, the specific activity of the latter then was 6000 rather than 33,000. This corresponds to a catalytic ratio of about 2, so that under these conditions the reaction is essentially stoichiometric.

DISCUSSION

The results presented in this paper show that ESP, the eIF-2 stimulating protein, and RF, the translation restoring factor, are similar if not identical proteins. Both can be isolated in the form of eIF-2 complexes ($M_r \approx 450{,}000$) and both contain polypeptide chains of approximately 80, 65, 57, 40, and 32 kilodaltons besides the three eIF-2 peptides (Fig. 1). This is a significant finding that may help elucidate the mode of action of these factors in polypeptide chain initiation. Preliminary observations indicate the occurrence of an exchange between free and RF–complexed eIF-2.

Both ESP and RF promote ternary complex formation with intact eIF-2 (Fig. 3*B*). However, preincubation of eIF-2 with eIF-2 α kinase and ATP, which markedly decreases the interaction of eIF-2 with ESP, causes only partial blocking of the RF–eIF-2 interaction (Fig. 3*B*). The reasons for this difference are not understood. RF has been reported not to block α phosphorylation of eIF-2 or enhance the dephosphorylation of eIF-2 (αP) (10, 11). Although this matter requires further investigation, the ability of RF to promote (with low efficiency) ternary complex formation in the presence of eIF-2 α kinase and ATP probably explains its translation restoring activity (Fig. 3*A*). This requires relatively large amounts of RF and it is doubtful whether this effect has any physiological significance. That RF may in reality be ESP and that the protein we had called ESP may be a modified form of the factor have been suggested (12). The data in this paper seem to lend some support to this suggestion.

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