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Correlation between the distribution of the reversing factor and eukaryotic initiation factor 2 in heme-deficient or double-stranded RNA-inhibited reticulocyte lysates

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The recycling of eukaryotic initiation factor eIF-2 requires the exchange of GDP for GTP, in a reaction catalyzed by the reversing factor (RF). Recent studies have suggested that a 60 S ribosomal subunit-bound eIF-2·GDP complex is an intermediate in protein chain initiation. We have monitored the distribution of RF in heme-deficient and dsRNA-inhibited lysates by immunoblot analysis of sucrose gradient fractions and have compared the distribution with that of eIF-2(α-^{32}P). RF and eIF-2(αP) were both found to be tightly associated with 60 S and 80 S ribosomes, as their distribution did not change in gradients containing up to 0.1 M K^+. The association of eIF-2(α-^{32}P) and RF with 60 S and 80 S ribosomes was enhanced in the presence of F^-, indicating the presence of an endogenous ribosome-associated phosphatase activity which is capable of dephosphorylating eIF-2(αP) in the absence of F^-. These observations are consistent with the hypothesis that under physiologic conditions, RF interacts with the 60 S-bound eIF-2·GDP complex to promote the dissociation of GDP from eIF-2 and the release of eIF-2 from the 60 S subunit as a complex with RF.

Protein synthesis; Reticulocyte lysate; Reversing factor; Eukaryotic initiation factor 2; 60 S ribosome; Ribosomal subunit binding

1. INTRODUCTION

The rabbit reticulocyte lysate and purified reticulocyte initiation components utilized in reconstituted in vitro systems have been used as model systems for determining the mechanism by which eukaryotic protein chain initiation occurs [1,2]. These studies indicate that eukaryotic initiation factor 2 (eIF-2) forms a ternary complex with initiator Met-tRNA$_i$ and GTP (eIF-2·Met-tRNA$_i$·GTP), which then binds to the 40 S ribosome. The binding of mRNA to this 43 S complex occurs in the presence of a number of other initiation factors, and is accompanied by the hydrolysis of ATP. The GTP originally present in the ternary complex is subsequently hydrolyzed during joining with the 60 S ribosomal subunit. Therefore, at the final step of initiation, eIF-2 is present as a binary complex with GDP (eIF-2·GDP). The reutilization of eIF-2 in subsequent rounds of initiation requires the exchange of GDP for GTP, in a reaction catalyzed by the reversing factor (RF) [3–8]. RF is composed of 5 subunits with molecular masses of approx. 82, 65, 57, 40 and 34 kDa, and has also been designated GEF, guanine nucleotide exchange factor [6,7] and

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Abbreviations: eIF-2, eukaryotic initiation factor 2; eIF-2α, α(38 kDa)-subunit of eIF-2; eIF-2(α-P), eIF-2 phosphorylated on α-subunit; eIF-2·GDP, binary complex of eIF-2 with GDP; eIF-2(α-P)·GDP, binary complex of eIF-2 with GDP, phosphorylated on the α-subunit of eIF-2; RF, reversing factor (also referred to in the literature as GEF and eIF-2B); RF·eIF-2(α-P), complex of RF with eIF-2 phosphorylated on its α-subunit; HRI, heme-regulated eIF-2α kinase; dsRNA, double-stranded RNA; dsI, dsRNA-activated eIF-2α kinase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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eIF-2B [8] in the current literature. Inhibition of protein chain initiation occurs in the reticulocyte lysate during heme deficiency or in the presence of low levels of double-stranded RNA (dsRNA) due to the activation respectively of a heme-regulated (HRI) or a dsRNA-activated (dsI) protein kinase, both of which phosphorylate the α-subunit of eIF-2 [9]. The inhibition is due to the binding of RF to phosphorylated binary complex [eIF-2(αP)·GDP] to give an RF·eIF-2(αP) complex that is not readily dissociable [10,11]. RF is then unavailable to catalyze GTP/GDP [12] exchange and hence, the recycling of eIF-2 and the initiation of protein synthesis are inhibited.

Recent studies have suggested that a ribosome-bound eIF-2·GDP complex is an intermediate in the protein chain initiation cycle [13–16], and that the eIF-2·GDP complex generated at the final stage of initiation can become associated with the 60 S ribosomal subunit [14,15]. Further evidence suggested that the 60 S subunit may serve as a site of action for RF in the recycling of eIF-2 [14]. To study further the apparent association of RF with the 60 S ribosomal subunit, we have used immunoblotting techniques to compare the distribution of RF in heme-deficient and dsRNA-inhibited lysates fractionated in sucrose density gradients. The distribution of RF was found to be similar to that reported previously for the distribution of eIF-2(αP) [14,15]. The association of RF and eIF-2 with the 60 S ribosomal subunit was found to be tight, being maintained in sucrose gradients containing up to 0.1 M K^+. These observations are consistent with the hypothesis that the 60 S ribosomal subunit is the site at which RF and eIF-2 initially interact in the recycling of eIF-2 in protein chain initiation.

2. EXPERIMENTAL

2.1. *Sucrose density gradient fractionation of rabbit reticulocyte lysates*

Rabbit reticulocyte lysates were incubated for 20 min at 30°C under standard conditions for protein synthesis as described by Ernst et al. [17] but without the addition of [^{14}C]leucine. For analysis of phosphoprotein profiles, samples were pulsed with 30 μCi [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear) from 15 to 20 min. Protein synthesis mixtures (75 μl) were placed on ice and immediately diluted with 125 μl ice-cold buffer containing 25 mM Tris-HCl (pH 7.6), 2 mM $Mg(OAc)_2$, 0.1 mM DTT, 50–100 mM K^+ and 25–75 mM Cl^-, with the remaining anion (Cl^- or F^-) present at 25 mM. Samples were layered over a 5 ml linear 15–40% (w/v) sucrose gradient containing the above buffer, and centrifuged at 45000 rpm in a Beckman SW50.1 rotor for 2.25 h or at 39000 rpm in a Beckman SW65 for 4.25 h at 2°C. Fractions were collected by upward displacement with continuous monitoring at 280 nm and concentrated by precipitation at pH 5 with acetic acid [13].

2.2. *Analysis of the distribution of eIF-2 and RF*

Samples were separated by electrophoresis in SDS-polyacrylamide gels (37.5:1 acrylamide:bis [18]); 8 and 10% gels were used for the analysis of the distribution of eIF-2(α-^{32}P) and RF, respectively. [^{32}P]Phosphoprotein profiles were analyzed directly by autoradiography of dried gels [14] and band intensity was quantitated with a Biorad 620 video densitometer. For analysis of RF, proteins in the gels were electrotransferred to nitrocellulose paper [19] (Schleicher and Schuell, 0.45 μm) in buffer (pH 8.3) containing 20 mM Tris, 150 mM glycine and 20% methanol for 20 h at 125 mA. The nitrocellulose papers (blots) were rinsed with buffer containing 20 mM Tris-HCl (pH 7.6) and 150 mM NaCl (TBS) and soaked for 1 h at 20°C in TBS containing 5% (w/v) non-fat dry milk (TBS/5% NFM). Blots were rinsed twice with TBS, followed by incubation for 16 h at 20°C with 50 ml TBS/5% NFM containing chicken anti-RF antiserum diluted 1:200. Blots were washed consecutively for 10 min each with TBS, TBS/0.5% Triton X-100, TBS and TBS/5% NFM, followed by incubation for 1 h at 20°C with 50 ml TBS/5% NFM containing rabbit anti-chicken IgG antiserum (Miles Scientific) diluted 1:500. After washing as described above, blots were incubated in 75 ml TBS/5% NFM containing 0.1 μCi/ml of ^{125}I-protein A (8–10 μCi/μg, New England Nuclear) for 1 h at 20°C. Blots were again washed, rinsed with deionized water, dried and exposed to Kodak X-Omat AR film for autoradiography. The polyclonal chicken anti-RF antibody readily detects the 82, 65 and 40 kDa subunits of RF. Under the conditions required to transfer quantitatively the 82 kDa subunit, the 34 kDa subunit of RF is usually transferred entirely through the nitrocellulose. Antibody to the 57 kDa subunit is present only at a low level, such that the 57 kDa subunit is not reproducibly detected. Other bands, which are occasionally detected migrating between the 65 and 40 kDa subunits of RF in fractions at the top of the sucrose gradients, are believed to be due to proteolysis upon storage of the fractions prior to their processing for SDS-PAGE.

3. RESULTS AND DISCUSSION

Fractionation of dsRNA-inhibited reticulocyte lysates was carried out at various K^+ concentrations in order to examine how tightly eIF-2(α-^{32}P) was associated with 60 S ribosomal subunits and to mimic physiological ionic strength. In sucrose gradients containing up to 100 mM K^+, the association of eIF-2(α-^{32}P) with 60 S ribosomal subunits and 80 S ribosomes is maintained (fig.1). With increasing K^+ concentrations a slight, but significant, shift of eIF-2(α-^{32}P) from the 80 S to the 60 S peak was observed. Relative to the sucrose

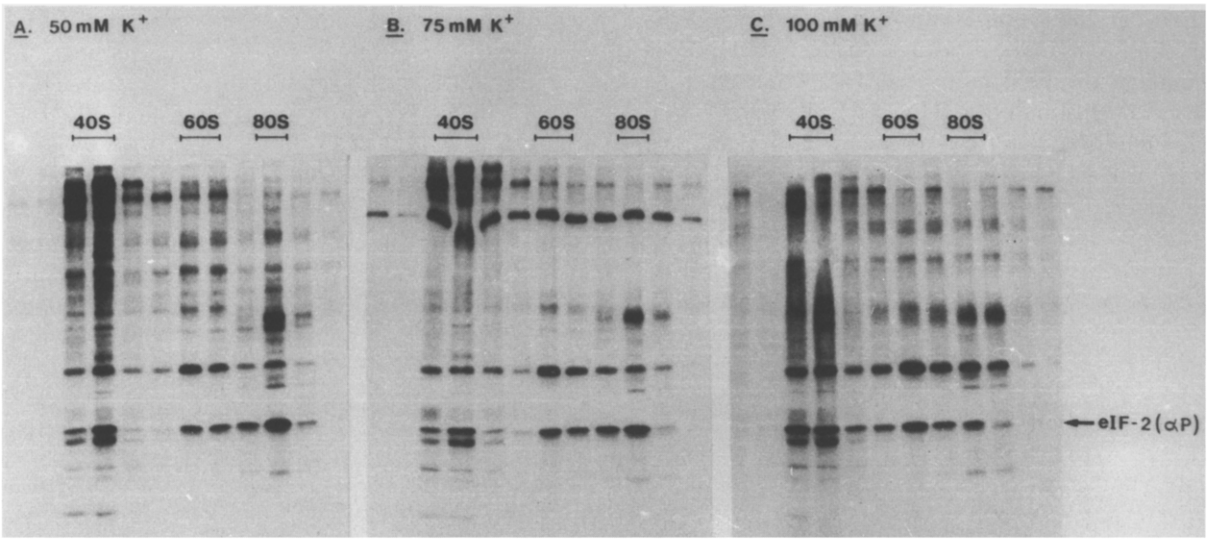

Fig.1. Effect of increasing ionic strength on eIF-2(αP) distribution. dsRNA inhibited lysates were pulsed with [γ-^{32}P]ATP and fractionated in sucrose density gradients containing 25 mM F^- and (A) 50 mM K^+, (B) 75 mM K^+ or (C) 100 mM K^+ as described by Thomas et al. [14]. Fractions corresponding to the sedimentation of the 40 S and 60 S ribosomal subunits and 80 S ribosomes are indicated at the top. The ^{32}P-labelled band corresponding to the migration of the α-subunit of the eIF-2 is indicated to the right.

gradients containing 50 mM K^+, the eIF-2(α-^{32}P) associated with the 80 S ribosomes decreased by 17 and 31%, and that associated with the 60 S ribosomal subunits increased by 10 and 28% in the gradients containing 75 mM and 100 mM K^+, respectively (fig.1). This observation indicates that eIF-2(α-^{32}P) remains tightly associated with 60 S ribosomal subunits upon the dissociation of 80 S couples, which occurs to a degree at the higher ionic strengths. The association of eIF-2(α-^{32}P) with 60 S and 80 S ribosomes was maintained in gradients containing physiological salt concentrations only in the presence of F^- (fig.2A,B). In addition, sample preparation time was found to be an important variable. In experiments in which centrifugation time was shortened by approx. 50% (fig.2C,D), loss of eIF-2(α-^{32}P) from the 60 S and 80 S ribosomes in the absence of F^- (fig.2A,B) was significantly reduced. These observations emphasize the need to limit the action of reticulocyte phosphatase [20], when lengthy fractionation procedures are utilized to study the distribution of eIF-2.

Previously, we reported that, upon addition of purified RF to heme-deficient lysates, several of the subunits of RF were observed in association with 60 S and 80 S ribosomes [14]. In this report, endogenous RF distribution in dsRNA-inhibited and heme-deficient lysates was monitored by immunoblot analysis [20], utilizing chicken anti-RF antibodies and ^{125}I-protein A, under conditions which readily detect the 82, 65 and 40 kDa subunits of RF. While the bulk of the RF was detected in fractions at the top of the sucrose gradient as reported in [13], RF was also detected bound to both the 60 S and 80 S ribosomes (fig.3). This association was maintained in sucrose gradients at physiological ionic strength (fig.3C,D). A small amount of RF was also observed associated with the 40 S region of the gradient. A decrease in the amount of RF associated with 60 S and 80 S ribosomes was observed in the absence of F^- (fig.3A,B). This correlated with the decrease in eIF-2(α-^{32}P) bound to 60 S and 80 S ribosomes also observed under these conditions (fig.2A,B). Similarly, under conditions where more eIF-2(α-^{32}P) was associated with 60 S and 80 S ribosomes (fig.2A,C,D), higher levels of ribosome-associated RF were also detected (fig.3A,C,D). Therefore, the association of RF with 60 S ribosomal subunits and 80 S ribosomes was maintained at physiological salt concentrations under conditions

where the dephosphorylation of bound eIF-2(αP) was limited. These findings suggest that RF is bound to the 60 S ribosome as a complex with eIF-2(α-^{32}P), and that a ribosome-associated phosphatase activity is present in the reticulocyte lysate [20] which is capable of dephosphorylating eIF-2(αP), possibly at the level of the RF-eIF-2(αP) complex.

The data reported here on the distribution of reticulocyte lysate eIF-2(αP) and RF in sucrose gradients at physiological ionic strength, agree with earlier findings [14,15], and support the hypothesis that the binding of eIF-2 and RF to 60 S and 80 S ribosomes is physiologically relevant. The ability of F^- to maintain high levels of both ribosome-associated eIF-2(αP) and RF suggests that phosphorylation of eIF-2 decreases the rate at which the eIF-2·GDP complex is released from the ribosome by RF. In the absence of F^-, eIF-2(αP) can be rapidly dephosphorylated

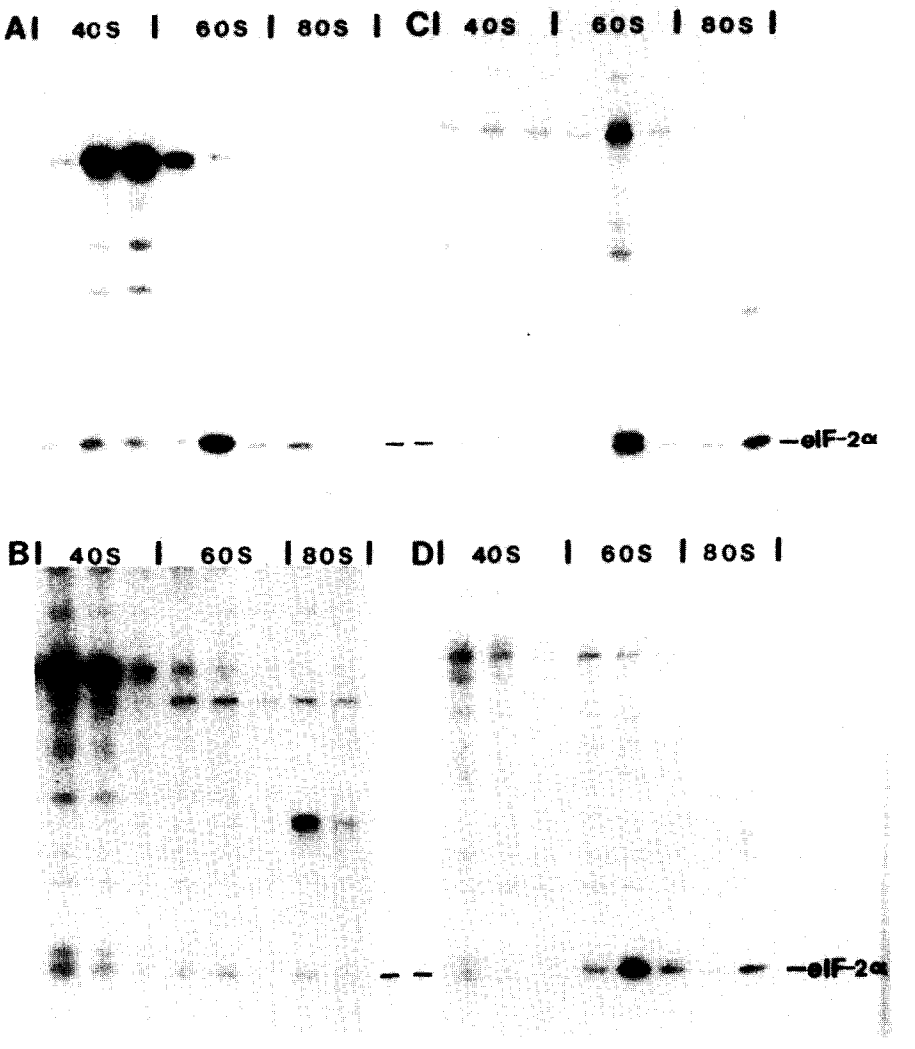

Fig.2. Effect of F^- and sample preparation time on the distribution of eIF-2(αP) in dsRNA-inhibited and heme-deficient lysates. dsRNA-inhibited lysates were pulsed with [γ-^{32}P]ATP and centrifuged for 4.25 h at 39000 rpm in sucrose density gradients containing (A) 100 mM K^+ and 50 mM F^- or (B) 100 mM K^+ and no F^-. Heme-deficient lysates were pulsed with [γ-^{32}P]ATP and centrifuged for 2.25 h at 45000 rpm on sucrose density gradients containing (C) 100 mM K^+ and 25 mM F^-, or (D) 100 mM K^+ and no F^-. Figure is an autoradiogram of gradient fractions monitored as described in section 2.

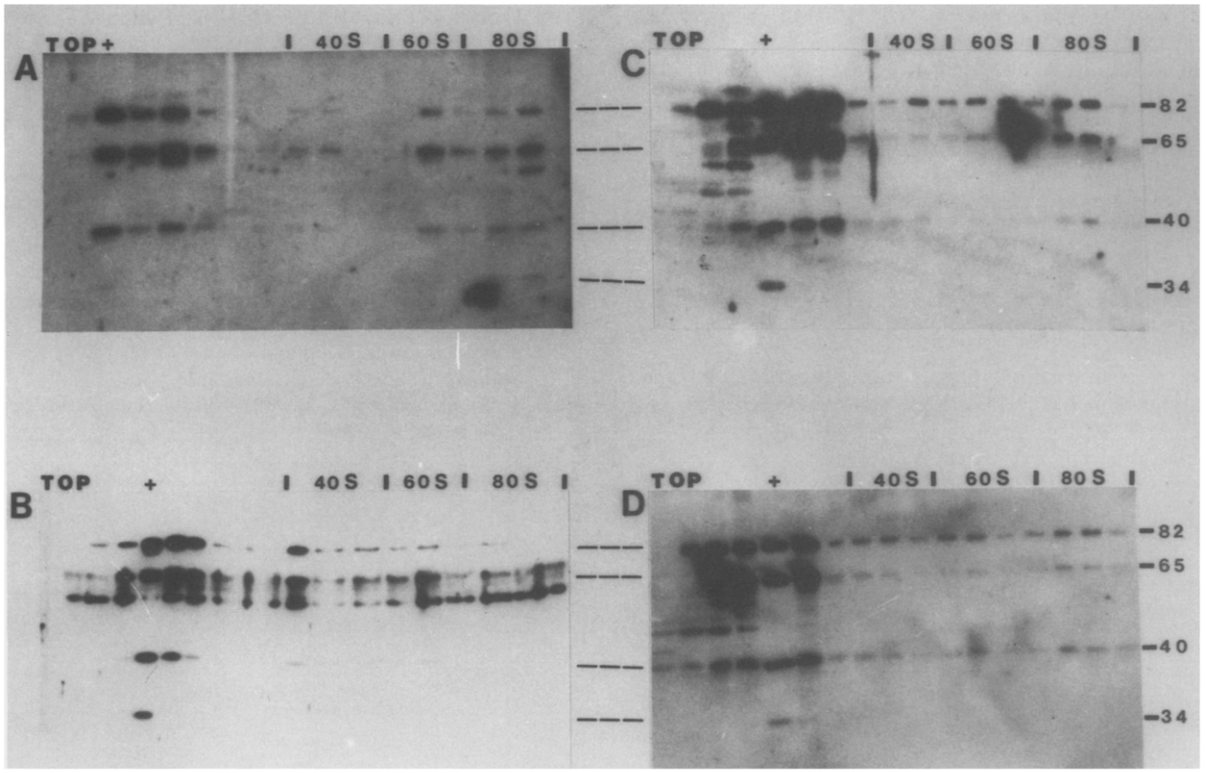

Fig.3. Effect of ionic strength and F^- on RF distribution with varying centrifugation times. dsRNA-inhibited lysates were incubated for 20 min at 30°C and fractionated in sucrose density gradients containing (A) 50 mM K^+ and 25 mM F^-, or (B) 100 mM K^+ and no F^-, as described in fig.2. Heme-deficient lysates were incubated for 20 min at 30°C and fractionated in sucrose density gradients containing (C) 100 mM K^+ and 25 mM F^-, or (D) 100 mM K^+ and no F^-, as described in fig.2. Figure is an autoradiogram of gradient fractions monitored by immunoblot analysis utilizing anti-RF antibodies and ^{125}I-protein A as described in section 2. The positions to which the 82, 65, 40 and 34 kDa subunits of RF migrate upon SDS-PAGE are indicated at the right-hand side; (+) lane containing purified RF standard.

[21,22], leading to the rapid dissociation of the RF·eIF-2 complex from the ribosome. These findings are consistent with the proposal that 60 S-bound eIF-2·GDP is an intermediate in the cycle of protein chain initiation and that the complex is released from the ribosomal subunit upon its interaction with RF [14,15]. The function of ribosome-bound eIF-2·GDP in the initiation of protein synthesis and the role which RF-catalyzed GDP exchange plays in the release of this complex from the 60 S ribosomal subunit are currently under investigation.

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