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Roles of a 67-kDa polypeptide in reversal of protein synthesis inhibition in heme-deficient reticulocyte lysate

(translational regulation/polypeptide-chain initiation/protein phosphorylation/eukaryotic translation initiation factor 2)

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ABSTRACT **During heme deficiency in reticulocyte lysates, the heme-regulated protein synthesis inhibitor, HRI, phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2) and thus inhibits protein synthesis. Two factors, eIF-2 and a reticulocyte-lysate supernatant factor that we term RF, reverse this inhibition. We now report the following. (*i*) An active eIF-2 preparation contained, in addition to the three subunits (α, β, and γ), a 67-kDa polypeptide. Pretreatment of eIF-2 with polyclonal antibodies against either isolated α subunit or 67-kDa polypeptide almost completely inhibited the reversal activity. Upon further fractionation, three-subunit eIF-2 and the 67-kDa polypeptide were resolved. Neither the three-subunit eIF-2 nor the 67-kDa polypeptide alone was active in protein synthesis inhibition reversal. The activity was, however, restored by combining both the three-subunit eIF-2 and the 67-kDa polypeptide. (*ii*) Active RF preparations contained eIF-2 α (unphosphorylated) and β subunits and the 67-kDa polypeptide. As with eIF-2, prior treatment of the RF preparation with antibodies to either the α subunit or the 67-kDa polypeptide almost completely inhibited the reversal activity. The RF preparation devoid of eIF-2 γ subunit did not form ternary complex (Met-tRNA$_f^{Met}$·eIF-2·GTP). The eIF-2 γ subunit in the free form was isolated, and addition of this isolated γ subunit to RF promoted significant ternary-complex formation. (*iii*) Purified HRI efficiently phosphorylated the α subunit in the three subunit eIF-2. However, the extent of such phosphorylation was significantly reduced when eIF-2 containing the 67-kDa polypeptide was used. The 67-kDa polypeptide apparently protected eIF-2 α subunit from HRI-catalyzed phosphorylation but did not inhibit HRI activity. Based on these results, we suggest that the protein synthesis inhibition reversal activity in both eIF-2 and RF is due to the same components—namely, eIF-2 α subunit and the 67-kDa polypeptide. The 67-kDa polypeptide protects eIF-2 α subunit from HRI-catalyzed phosphorylation and may also be a necessary component of the functioning eIF-2 molecule.**

In heme-deficient reticulocyte lysate, the heme-regulated protein synthesis inhibitor, HRI, is activated and shuts off protein synthesis. HRI phosphorylates the α subunit of eukaryotic peptide-chain-initiation factor 2 (eIF-2). The bulk of reticulocyte eIF-2 is isolated as eIF-2·GDP. Upon phosphorylation, eIF-2[α(*P*)]·GDP forms an abortive complex with another peptide-chain-initiation factor, GEF (guanine nucleotide exchange factor). GEF is presumably present in limiting amounts inside the cell, and protein synthesis is inhibited as 30% of the eIF-2 molecules become phosphorylated and bind to available GEF (for review, see refs. 1–5).

Several laboratories have reported that addition of exogenous eIF-2 (6–9) or the cell supernatant factor preparation enriched in GEF activity (10–15) reverses protein synthesis inhibition in heme-deficient reticulocyte lysate. Ten years ago, our laboratory reported (17) that although a partially purified eIF-2 preparation contained significant protein synthesis inhibition reversal activity, this activity was completely absent from a homogeneous eIF-2 preparation containing the three subunits, α, β, and γ. Our group also reported (18) that RF, the cell supernatant factor that reverses protein synthesis inhibition in heme-deficient reticulocyte lysate, also promotes ternary-complex (Met-tRNA$_f^{Met}$·eIF-2·GTP) formation by HRI-phosphorylated eIF-2. As noted above, this RF preparation is enriched in GEF activity. More recent work provided evidence (19, 20) that the protein synthesis inhibition reversal activity of the RF preparation is not directly related to its GEF activity and is possibly due to the presence of unphosphorylated eIF-2 α subunit. It was postulated (19, 20) that RF reverses protein synthesis inhibition as it donates the unphosphorylated eIF-2 α subunit to eIF-2[α(*P*)]·GDP and reconstitutes active eIF-2 molecules.

In this paper, we report that both active eIF-2 and RF preparations contain unphosphorylated eIF-2 α subunit and a 67-kDa polypeptide and that both these components are necessary for reversal of protein synthesis inhibition. We present the results of our studies of the characteristics of the 67-kDa polypeptide and its roles in reversal of protein synthesis inhibition in heme-deficient reticulocyte lysates.

MATERIALS AND METHODS

The sources of materials and the methods used in these studies were as described (19–21). eIF-2 activity was purified by DEAE-cellulose (fraction II), phosphocellulose (fraction III), and CM-Sephadex (fraction IV) chromatography. The purification procedures were as described by Das *et al.* (21) except that the hydroxylapatite chromatographic step was omitted. NaDodSO$_4$/PAGE of the fraction IV eIF-2 preparation consistently gave a 67-kDa polypeptide band in addition to the three eIF-2 bands (Fig. 1). This fraction IV eIF-2 preparation was used for resolution of the 67-kDa polypeptide and of eIF-2 containing three subunits, as well as for isolation of eIF-2 γ subunit.

Glycerol Density Gradient Separation of eIF-2 and the 67-kDa Polypeptide. Fraction IV eIF-2 preparation (2 mg) was layered on top of a glycerol gradient (10–30%, vol/vol) containing 0.1 M KCl in buffer B (20 mM Tris·HCl, pH 7.8/5 mM 2-mercaptoethanol/50 μM EDTA/10% glycerol). The gradients were centrifuged at 45,000 rpm for 26 hr in a

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Abbreviations: eIF-2, eukaryotic peptide-chain-initiation factor 2, which forms Met-tRNA$_f^{Met}$·eIF-2·GTP (ternary complex); eIF-2[α(*P*)], eIF-2 with phosphorylated α subunit; GEF, guanine nucleotide exchange factor; RF, reticulocyte-lysate supernatant factor that reverses protein synthesis inhibition in heme-deficient reticulocyte lysate; HRI, heme-regulated protein synthesis inhibitor (eIF-2 kinase); dsI, double-stranded-RNA-activated protein synthesis inhibitor (eIF-2 kinase).

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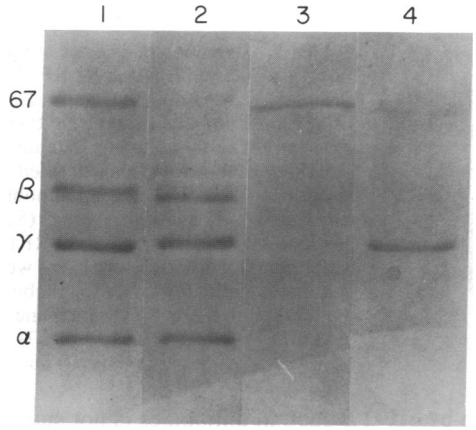

FIG. 1. $NaDodSO_4$/PAGE of fraction IV eIF-2 (8 μg; lane 1), fraction V eIF-2 (6.5 μg; lane 2), isolated 67-kDa polypeptide (2 μg; lane 3), and isolated eIF-2 γ subunit (2.5 μg; lane 4). Samples were analyzed in a 15% polyacrylamide/0.09% *N,N'*-methylenebisacrylamide gel in 0.025 M Tris/0.192 M glycine/0.1% $NaDodSO_4$ (pH 8.3).

Beckman SW 50.1 rotor. The gradients were then fractionated with an ISCO density gradient fractionator and 0.3-ml fractions were collected from the top. Fractions were analyzed by $NaDodSO_4$/15% PAGE. Early fractions containing the 67-kDa polypeptide were pooled, immediately concentrated by dry Sephadex G-200 (Pharmacia), and then dialyzed against buffer B containing 0.1 M KCl. The dialyzed solution was stored frozen in liquid N_2. The late fractions containing eIF-2 activity were pooled, concentrated by dry Sephadex, dialyzed against buffer B containing 0.1 M KCl (fraction V), and stored frozen in liquid N_2.

Purification of γ Subunit. Fraction IV eIF-2 preparation was dialyzed against phosphate buffer (20 mM potassium phosphate, pH 7.6/10 mM 2-mercaptoethanol/50 μM EDTA/10% glycerol) and then loaded onto a hydroxylapatite column that had been equilibrated with the same buffer. The column was washed with the same buffer and proteins were eluted as follows. Free γ subunit was eluted with 0.2 M potassium phosphate (pH 7.6), and eIF-2 containing three subunits (α, β, and γ) and devoid of the 67-kDa polypeptide was eluted with a potassium phosphate gradient (0.2–0.5 M, pH 7.6). The fractions were concentrated by Sephadex, dialyzed against buffer B containing 0.1 M KCl, and stored frozen in liquid N_2.

RF (fraction VI) was purified according to the procedure of Grace *et al.* (20). As reported (20), RF is a high molecular weight protein complex and contains GEF activity.

Preparation of Polyclonal Antibodies Against Isolated eIF-2 Subunits and the 67-kDa Polypeptide. Fraction IV eIF-2 preparation was subjected to $NaDodSO_4$/15% PAGE. After brief (5 min) staining with Coomassie blue R-250, the gel was thoroughly destained with 25% methanol/10% acetic acid. Stained bands corresponding to the α, β, and γ subunits and the 67-kDa polypeptide were excised with a razor blade and electrophoretically eluted in 100 mM Tris/50 mM Tricine buffer (22). Each electroeluted protein (25 μg) was emulsified with an equal volume of Freund's complete adjuvant and injected into the peritoneal cavity of a mouse. After 15 days, three booster injections of 25 μg of isolated polypeptide in Freund's incomplete adjuvant were given at 1-week intervals. One week after the last injection, blood was collected by tail-bleeding and the antibody titer in the serum was tested by ELISA using a β-galactosidase conjugate. When antibody titer in the serum was 1:2500, blood was drawn frequently by tail-bleeding. The antibodies from immune serum were purified by use of DEAE Affi-Gel Blue, according to the procedure supplied by the manufacturer (Bio-Rad immunoblot assay kit), and were characterized by $NaDodSO_4$/PAGE followed by immunoblotting.

RESULTS

Fraction IV eIF-2 preparation consistently showed a 67-kDa polypeptide in addition to the usual three eIF-2 subunits (Fig. 1, lane 1). Upon further fractionation by glycerol density gradient centrifugation, eIF-2 containing three subunits (fraction V; lane 2) was resolved from the 67-kDa polypeptide (lane 3). The eIF-2 γ subunit (lane 4) was obtained by hydroxylapatite chromatography of fraction IV eIF-2. Small but significant amounts of eIF-2 γ subunit (5–10% of the input eIF-2 γ subunit) were eluted with 0.2 M potassium phosphate buffer (pH 7.6). eIF-2 containing three subunits (α, β, and γ) and devoid of the 67-kDa polypeptide was later eluted from the column with a 0.2–0.5 M potassium phosphate gradient (pH 7.6). All three eIF-2 preparations (fractions IV and V and the hydroxylapatite-purified eIF-2 fraction) formed >70% of the expected stoichiometric amount of ternary complex in the presence of excess Co-eIF-2 (a factor that stimulates ternary-complex formation; ref. 5) and in the absence of Mg^{2+}. The isolation of free eIF-2 γ subunit suggests that this subunit either was present in slight excess in the fraction IV eIF-2 preparation or was loosely bound to the eIF-2 complex.

Both eIF-2 and the 67-kDa Polypeptide Are Required for Reversal of Protein Synthesis Inhibition in Heme-Deficient Reticulocyte Lysate. We tested eIF-2 preparations fractions IV and V and the isolated 67-kDa polypeptide for the ability to reverse protein synthesis inhibition. Whereas fraction IV eIF-2 efficiently reversed protein synthesis inhibition, fraction V eIF-2 was almost completely inactive (Fig. 2*A*). Also, the 67-kDa polypeptide alone was almost completely inactive (Fig. 2*C*). The reversal activity could be reconstituted, however, by combining fraction V eIF-2 and the isolated 67-kDa polypeptide, indicating that both three-subunit eIF-2 and the 67-kDa polypeptide are essential for reversal action (Fig. 2 *B* and *C*).

Presence of eIF-2 Subunits and the 67-kDa Polypeptide in eIF-2 and the RF Preparation. Besides eIF-2, the reticulocyte

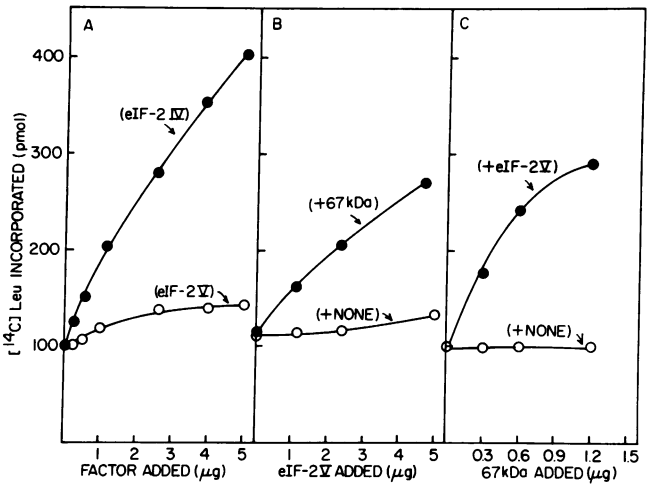

FIG. 2. Reversal activity of fraction IV eIF-2, fraction V eIF-2, and 67-kDa polypeptide. Protein synthesis in heme-deficient reticulocyte lysate was assayed by the standard procedure (19). [^{14}C]Leucine (specific activity, 160 cpm/pmol) incorporation was determined in 20-μl aliquots of the reaction mixture. Mixtures contained (in a total volume of 25 μl) various amounts of fraction IV eIF-2 or fraction V eIF-2 (*A*), various amounts of fraction V eIF-2 with or without a fixed amount (1.2 μg) of 67-kDa polypeptide (*B*), or various amounts of 67-kDa polypeptide with or without a fixed amount (4.8 μg) of fraction V eIF-2 (*C*).

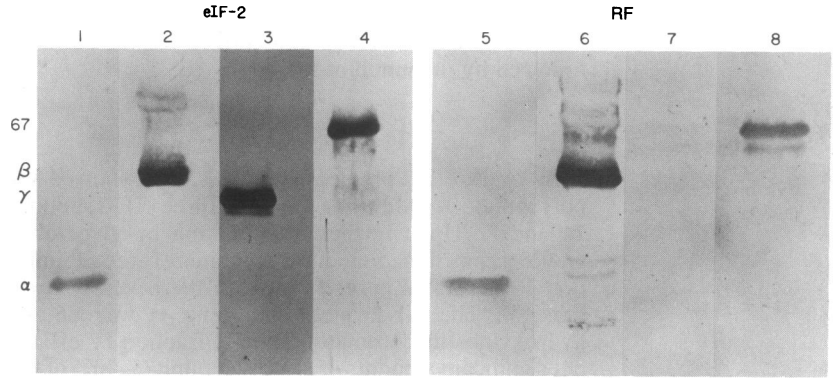

FIG. 3. Immunoblot analysis of fraction IV eIF-2 and fraction VI RF. The proteins were subjected to NaDodSO$_4$/PAGE and electrophoretic transfer to nitrocellulose sheets. The nitrocellulose sheets were then treated as described in the Bio-Rad immunoblot kit. Lanes 1–4, fraction IV eIF-2 (5 μg per lane); lanes 5–8, fraction VI RF (30 μg per lane). Nitrocellulose strips were incubated with antibodies to α subunit (lanes 1 and 5), to β subunit (lanes 2 and 6), to γ subunit (lanes 3 and 7), or to 67-kDa polypeptide (lanes 4 and 8).

cell supernatant factor preparation RF (18–20) actively reverses protein synthesis inhibition in heme-deficient reticulocyte lysates. To compare the characteristics of the components in both eIF-2 and RF preparations that are responsible for reversal activity, we analyzed both active eIF-2 (fraction IV) and RF preparations for the presence of eIF-2 subunits and the 67-kDa polypeptide (Fig. 3). For this experiment, we used polyclonal antibodies against individually isolated eIF-2 α, β, and γ subunits and the 67-kDa polypeptide in an immunoblotting assay. As reported (20), the RF preparation contains several polypeptides besides the eIF-2 α subunit. Fig. 3 shows that the polyclonal antibodies reacted specifically with the corresponding polypeptides, although the antibodies against β subunit showed some crossreactivity. The results show that the active RF preparation contains eIF-2 α and β subunits and the 67-kDa polypeptide but is devoid of eIF-2 γ subunit. Complete absence of the eIF-2 γ subunits in the active RF preparation explains the lack of ternary-complex-forming activity of RF.

Identification of the Polypeptide Components in Both eIF-2 and RF Preparations Responsible for Reversal of Protein Synthesis Inhibition. To determine which polypeptide components in eIF-2 and RF preparations are responsible for the reversal activity, we pretreated these preparations with polyclonal antibodies against α, β, γ, and the 67-kDa polypeptide for 1 hr at 0°C and then analyzed the pretreated factor preparations for reversal of protein synthesis inhibition (Table 1). Pretreatment of both eIF-2 and RF preparations with polyclonal antibodies against α subunit and the 67-kDa polypeptide almost completely inhibited the reversal activity. Pretreatment with antibodies against eIF-2 γ subunit showed significant inhibition when eIF-2 was used but had negligible effect when RF, which is devoid of γ subunit, was used. Under similar conditions, pretreatment of either fraction with antibodies against the β subunit had very little effect. Lack of inhibition by eIF-2 β-subunit antibodies is not surprising, as previous studies have indicated that the β subunit is not essential for eIF-2 activity (21, 23, 24). However, in this experiment β-subunit antibodies served as a control in place of normal or preimmune mouse IgG.

Table 1. Effects of antibodies on protein synthesis inhibition reversal by eIF-2 and RF preparations

	[^{14}C]Leucine incorporated, pmol			
		− Hemin		
Antibodies added	+ Hemin	Control	+ eIF-2 (IV)	+ RF (VI)
None	396	100	340	248
Anti-α	—	—	115	109
Anti-β	—	—	303	213
Anti-γ	—	—	176	216
Anti-67-kDa	—	—	127	97

Protein synthesis in heme-supplemented and heme-deficient reticulocyte lysate was assayed by the standard procedure (19). [^{14}C]Leucine incorporation was determined in 20-μl aliquots of the reaction mixture. Where indicated, 4.5 μg of fraction IV eIF-2, 7.5 μg of fraction VI RF, and 10 μg of Affi-Gel Blue-purified antibodies against specific polypeptides were added.

These results suggest that both eIF-2 and RF preparations require eIF-2 α subunit as well as the 67-kDa polypeptide for reversal activity.

Reconstitution of eIF-2 from its Subunits. As noted above, the RF preparation, devoid of eIF-2 γ subunit, does not form a ternary complex. We tested the possibility that the addition of free eIF-2 γ subunit to RF would restore the ternary-complex-forming activity (Fig. 4). Neither eIF-2 γ subunit alone nor the RF preparation formed a ternary complex. However, when eIF-2 γ subunit was added to the RF preparation, significant ternary-complex formation was ob-

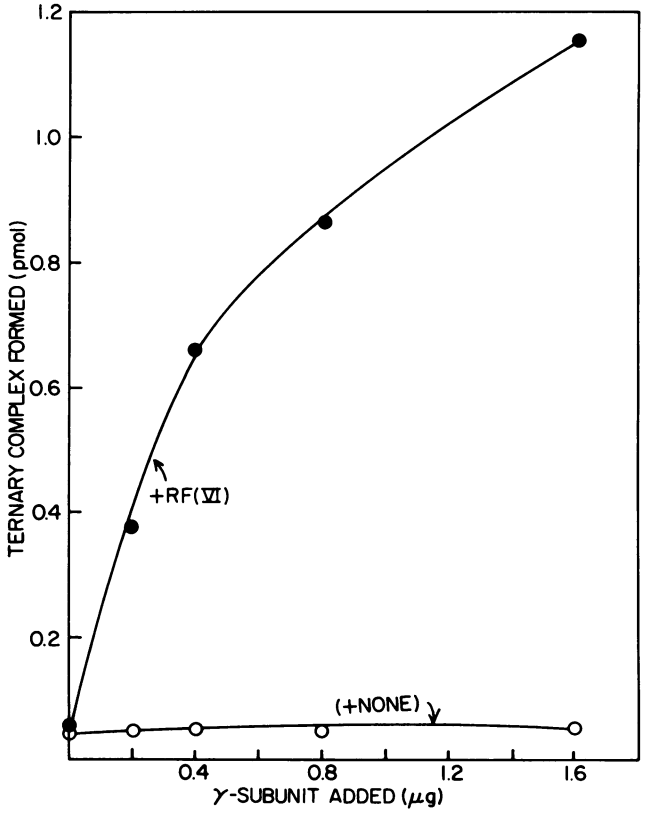

FIG. 4. Reconstitution of ternary complex formation (Met-tRNA$_f^{Met}$ bound in pmol) by fraction VI RF with increasing concentration of added eIF-2 γ subunit. Reaction mixtures contained (in a total volume of 0.075 ml) 20 mM Tris·HCl (pH 7.5), 100 mM KCl, 10 μg of bovine serum albumin, 2 mM dithiothreitol, 7.5 μg of fraction VI RF, 15 pmol of [^{35}S]Met-tRNA$_f^{Met}$ (10,000 cpm/pmol), and various concentrations of eIF-2 γ subunit.

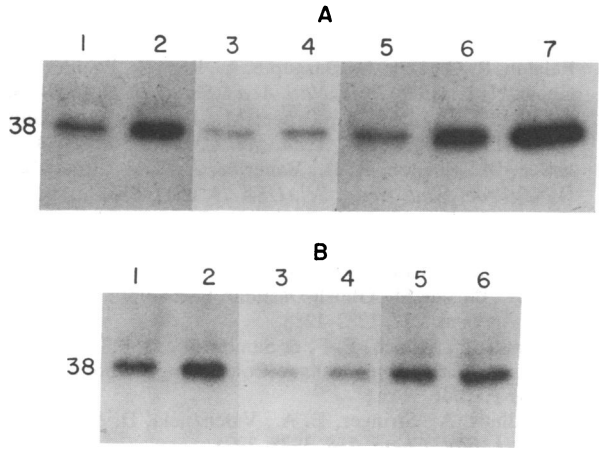

FIG. 5. Phosphorylation of eIF-2 α subunit (38 kDa) with ATP and HRI in the presence or absence of 67-kDa polypeptide. The reaction conditions were as described (19). The reaction mixtures were incubated at 37°C for 10 min, except for the samples in lanes 6 and 7 (in *A*), which were incubated at 37°C for 20 min. Reactions were terminated by adding an equal volume of 2% $NaDodSO_4$/50 mM 2-mercaptoethanol/50 mM Tris·HCl, pH 7.0/50% glycerol/0.2% bromophenol blue. Samples were heated at 100°C for 3 min, subjected to $NaDodSO_4$/15% PAGE, and autoradiographed on Kodak X-Omat AR-5 film for 12 hr with DuPont intensifying screen. (*A*) Lanes 1 and 2, fraction V eIF-2 (3.5 and 7.0 pmol, respectively); lanes 3 and 4, fraction IV eIF-2 (3.5 and 7.0 pmol); lane 5, fraction V eIF-2 (7.0 pmol) plus 67-kDa polypeptide (1 μg) added at zero time of incubation; lane 6, fraction V eIF-2 (7.0 pmol) plus 67-kDa polypeptide (1 μg) added after 10 min of incubation and then incubated at 37°C for another 10 min; lane 7, fraction V eIF-2 (7.0 pmol) incubated at 37°C for 20 min. (*B*) Lanes 1–4, same as in *A*; lanes 5 and 6, fraction V eIF-2 plus fraction IV eIF-2 (3.5 pmol of each and 7.0 pmol of each, respectively).

served and the complex formation increased with the amount of γ subunit added.

The 67-kDa Polypeptide Inhibits HRI-Catalyzed Phosphorylation of eIF-2 α Subunit. HRI efficiently phosphorylated the α subunit present in fraction V three-subunit eIF-2 (Fig. 5*A*, lanes 1 and 2) and the extent of such phosphorylation was significantly lowered when fraction IV eIF-2, containing the 67-kDa polypeptide, was used (lanes 3 and 4). Also, the phosphorylation of the three-subunit eIF-2 was significantly inhibited when fraction V was mixed with the isolated 67-kDa polypeptide (lane 5). This 67-kDa polypeptide does not contain eIF-2[$\alpha(P)$] phosphatase activity; addition of the 67-kDa polypeptide after phosphorylation of fraction V eIF-2 did not dephosphorylate eIF-2[$\alpha(P)$] (lane 6). However, such addition, as expected, prevented further phosphorylation of the remaining unphosphorylated eIF-2 (lane 6), whereas a control experiment without added 67-kDa polypeptide showed continued phosphorylation during the additional incubation period (lane 7). The 67-kDa polypeptide in fraction IV eIF-2 protected the eIF-2 α subunit from HRI-catalyzed phosphorylation but did not inhibit HRI activity; the added HRI in the reaction mixture did not phosphorylate the α subunit in fraction IV eIF-2 (Fig. 5*B*, lanes 3 and 4) but efficiently phosphorylated the α subunit in fraction V eIF-2 when the two eIF-2 preparations were added together (lanes 5 and 6). These results suggest that the 67-kDa polypeptide protects the eIF-2 α subunit from HRI-catalyzed phosphorylation.

DISCUSSION

Data presented in this paper show that eIF-2 and RF, which have been reported to reverse protein synthesis inhibition in heme-deficient reticulocyte lysates (6-20), possibly reverse inhibition by the same mechanism; in both cases the active components include the eIF-2 α subunit and the 67-kDa polypeptide. The active components in eIF-2 preparations include the 67-kDa polypeptide in addition to the usual three subunits contained in eIF-2. The active RF preparation contains the same 67-kDa polypeptide and the eIF-2 α and β subunits. In the presence of the 67-kDa polypeptide, the eIF-2 α and β subunits in RF join with added γ subunit to form active eIF-2 molecules competent in ternary-complex formation.

We suggest the following model for regulation of protein synthesis initiation in reticulocyte lysates containing eIF-2 and the 67-kDa polypeptide. The active components of the eIF-2 molecules in reticulocyte lysates include the 67-kDa polypeptide as well as the usual three subunits, α, β, and γ. The 67-kDa polypeptide is postulated to be present in limiting amounts. The reticulocytes, therefore, contain varying amounts of four-subunit eIF-2 (α, β, γ, and 67 kDa), three-subunit eIF-2 (α, β, and γ), and pools of free subunits. The eIF-2 kinases (HRI, activated in the absence of hemin, and dsI, activated by double-stranded RNA) phosphorylate the three-subunit eIF-2 but not the four-subunit eIF-2 (Fig. 5) or free eIF-2 α subunits (25). Protein synthesis is inhibited as phosphorylated three-subunit eIF-2 effectively competes with the four-subunit eIF-2 for some limiting component of the protein synthesis machinery or as eIF-2[$\alpha(P)$]·GDP binds to the available 67-kDa polypeptide and inactivates it. Addition of excess 67-kDa polypeptide and unphosphorylated eIF-2 α subunit leads to the formation of excess four-subunit eIF-2, which can compete effectively with endogenous eIF-2[$\alpha(P)$]·GDP to form active ternary complex. Protein synthesis can, therefore, be restored without significant change in the level of eIF-2 α-subunit phosphorylation. It should be noted, however, that addition of either the 67-kDa polypeptide or three-subunit eIF-2 alone did not reverse protein synthesis inhibition in heme-deficient reticulocyte lysate (Fig. 2). Apparently, under the experimental conditions, (*i*) the added 67-kDa polypeptide failed to inhibit the limiting eIF-2 α-subunit phosphorylation necessary for protein synthesis inhibition and (*ii*) the concentration of unphosphorylated eIF-2 α subunit became limiting. It may be that in the presence of inhibitory, phosphorylated eIF-2 α subunit, the affinity of the 67-kDa polypeptide for the three-subunit eIF-2 is reduced so that higher concentrations of both 67-kDa polypeptide and three-subunit eIF-2 are necessary for interaction and subsequent formation of four-subunit eIF-2.

Finally, we emphasize that there is now convincing evidence that mammalian cells extensively use eIF-2 α-subunit phosphorylation to regulate protein synthesis and possibly to promote preferential translation of different mRNAs (26–29). However, activation of eIF-2 kinases leading to complete phosphorylation of eIF-2 α subunit should result in a total loss of protein synthesis activity of the cells and eventual cell death. There are, however, mechanisms available whereby the cells can inactivate or inhibit the eIF-2 kinase(s), preferably at a certain developmental stage, and thus promote preferential translation of mRNAs available at that stage. For example, Siekierka *et al.* (27) and Schneider *et al.* (28) reported that synthesis of excess double-stranded RNA inhibits activation of dsI during adenovirus infection of animal cells. Similarly, Rice and Kerr (29) reported the synthesis of an inhibitor of dsI during the late phase of vaccinia virus infection. The characteristics of this inhibitor of eIF-2 kinase are not known. The results presented here provide evidence that the 67-kDa polypeptide protects the eIF-2 α subunit from phosphorylation by eIF-2 kinase. The levels of this 67-kDa polypeptide at different developmental stages may thus be a critical factor in regulating protein synthesis in animal cells and also in promoting preferential translation of mRNAs.

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