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Protein synthesis in rabbit reticulocytes: Characteristics of the protein factor RF that reverses inhibition of protein synthesis in heme-deficient reticulocyte lysates

(translational regulation/polypeptide chain initiation/protein phosphorylation/eukaryotic initiation factor 2–protein complexes)

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ABSTRACT During heme deficiency in reticulocyte lysates, the heme-regulated translational inhibitor of protein synthesis (HRI) is activated and shuts off protein synthesis. In partial reactions, HRI phosphorylates the M_r 38,000 subunit (α subunit) of eukaryotic initiation factor 2 (eIF-2), which forms a ternary complex, Met-tRNA$_f$·eIF-2·GTP. The eIF-2α(*P*) thus formed is not recognized by two eIF-2 ancillary factors, Co-eIF-2B (which promotes the dissociation of the ternary complex at high Mg^{2+}) and Co-eIF-2C (which reverses the inhibition of ternary complex formation), and thus, is presumably inactive in peptide chain initiation. A protein factor, designated RF, which reverses inhibition of protein synthesis in heme-deficient reticulocyte lysates, has been purified from reticulocyte cell supernatant. RF is a high molecular weight ($M_r \approx 450{,}000$) protein complex composed of multiple polypeptides. An active RF preparation contains Co-eIF-2B and Co-eIF-2C activities, and these two activities in RF preparation are not inhibited by HRI and ATP—i.e., eIF-2α(*P*) is recognized. During purification, RF remains associated with eIF-2 activity (eIF-2·RF) and can be freed of this eIF-2 activity by CM-Sephadex chromatography. Both eIF-2·RF and RF contain a M_r 38,000 polypeptide component that is indistinguishable from the M_r 38,000 subunit of eIF-2 by two-dimensional gel electrophoresis. It has been observed that a significant part of this M_r 38,000 polypeptide component in eIF-2·RF and almost the entire M_r 38,000 polypeptide component in RF remain unphosphorylated after prolonged incubation with HRI and ATP. A possible role of this free M_r 38,000 polypeptide in RF action is discussed.

The heme-regulated translational inhibitor of protein synthesis (HRI) becomes activated during heme deficiency in reticulocyte lysates. HRI is also a protein kinase and specifically phosphorylates the α-subunit (M_r 38,000) of eukaryotic initiation factor 2 (eIF-2), which forms a ternary complex, Met-tRNA$_f$·eIF-2·GTP. However, the precise role of such phosphorylated eIF-2 [eIF-2α(*P*)] in overall inhibition of protein synthesis is not fully understood. (For recent reviews, see refs. 1–5.) In partial reactions, HRI inhibits the activities of two eIF-2 ancillary protein factors, Co-eIF-2B (6–9) and Co-eIF-2C (8–19), as these two factors do not recognize eIF-2α(*P*). Co-eIF-2C activity is required for ternary complex formation by eIF-2 in the presence of Mg^{2+} (8, 14, 16). Co-eIF-2B promotes the dissociation of the preformed ternary complex at high Mg^{2+} concentrations (5 mM) and low temperature (0°C) (20). The physiological role of Co-eIF-2B activity in overall initiation of protein synthesis is not apparent.

Several laboratories have reported the isolation of a protein factor, designated RF, from reticulocyte cell supernatant that reverses inhibition of protein synthesis in heme-deficient reticulocyte lysates (16, 19, 21–24). We previously have reported that RF also reverses HRI inhibition of ternary complex formation in partial reactions (24). Purified RF preparation contains a Co-eIF-2C-like activity, and this activity in RF preparation can stimulate ternary complex formation by eIF-2α(*P*) (24). Partial reversal of HRI inhibition of ternary complex formation by a purified RF preparation also has been reported by Siekierka *et al.* (16).

In this communication, we report that a purified RF preparation that actively reverses the inhibition of protein synthesis in heme-deficient reticulocyte lysates contains Co-eIF-2C activity, and this activity in RF preparation is almost completely resistant to HRI and ATP. Upon further fractionation, this RF preparation loses considerable activity in inhibition reversal of protein synthesis, and the Co-eIF-2C activity in fractionated RF preparation becomes sensitive to HRI. We also report that an active RF preparation that is almost completely devoid of eIF-2 activity contains the M_r 38,000 subunit (α subunit) of eIF-2, and this excess M_r 38,000 subunit in RF preparation is not phosphorylated by HRI and ATP. The significance of this observation is not apparent, at present. A possible role of this excess α subunit in RF action is discussed.

MATERIALS AND METHODS

The sources of the materials used in these studies; preparations of initiation factors eIF-2, Co-eIF-2B, and Co-eIF-2C; and the assay methods have been described (8, 24). Protein concentration was determined by the Bradford procedure with bovine serum albumin as the standard (25).

Purification of RF Activity. We have adopted a procedure for the purification of RF based on the method of Amesz *et al.* (23). In a typical preparation, anemic blood was obtained from 15 rabbits by cardiac puncture. All subsequent operations were performed at 0–4°C. The cells were pelleted by centrifugation at 5,000 rpm for 5 min in a Sorvall SS-34 rotor, and the buffy coat was removed by aspiration. The cells were then pooled and washed twice with 6 volumes of saline solution (130 mM NaCl/5.2 mM KCl/7.5 mM $MgCl_2$/2 mM Hepes·KOH, pH 7.2) per volume of packed cells by centrifugation at 8,000 rpm for 8 min in a Sorvall GSA rotor. The packed cells were lysed by addition of 1.5–2.5 volumes of ice-cold 2 mM Hepes·KOH (pH 7.2). The lysed cells were swirled on ice for 10 min. The cell debris was

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Abbreviations: eIF-2, eukaryotic initiation factor 2, which forms a ternary complex, Met-tRNA$_f$·eIF-2·GTP; Co-eIF-2A, ancillary factor stimulating Met-tRNA$_f$ binding to eIF-2; Co-eIF-2B, ancillary factor promoting dissociation of the ternary complex at high Mg^{2+}; Co-eIF-2C, ancillary factor reversing Mg^{2+} inhibition of ternary complex formation; HRI, heme-regulated translational inhibitor; eIF-2α(*P*), phosphorylated eIF-2 (HRI catalyzed).

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then pelleted by centrifugation at 12,000 rpm for 20 min in a Sorvall GSA rotor. Polysomes were removed from the lysate by centrifugation at 33,000 rpm for 4 hr in a Beckman type 35 rotor. The resulting supernatant S-100 (350 ml) was adjusted to 0.1 M KCl/20 mM Tris·HCl, pH 7.8/1 mM dithiothreitol/0.1 mM sodium EDTA/10% (vol/vol) glycerol (buffer A/0.1 M KCl) by addition of a 5-times concentrated stock solution of buffer A/0.1 M KCl and stored at −4°C overnight. A second preparation was made the following day to yield a total of 675 ml of S-100 (fraction I). The suspension was then loaded onto a heparin-Sepharose column (2.6 × 40 cm) prepared as described (23) and was preequilibrated with buffer A/0.1 M KCl. After the column was loaded, which generally was done overnight, the column was washed thoroughly with buffer A/0.1 M KCl. The adsorbed proteins were eluted with buffer A/0.5 M KCl. The A_{280} peak fractions were pooled and dialyzed against buffer A/0.1 M KCl (fraction II).

The dialyzed preparation (fraction II) was made 40% saturated in $(NH_4)_2SO_4$ by addition of solid $(NH_4)_2SO_4$, and the precipitate was pelleted by centrifugation at 10,000 rpm for 10 min in a Sorval SS-34 rotor. The supernatant was then made 80% saturated in $(NH_4)_2SO_4$, and the precipitate was pelleted as above. The precipitate was resuspended in buffer A/0.05 M KCl at ≈5 A_{280}/ml and dialyzed overnight against the same buffer with one change (fraction III). The dialyzed preparation was loaded onto a DEAE-cellulose (DE-52, Whatman) 1.6 × 12 cm column preequilibrated with buffer A/0.05 M KCl. The column was then eluted with buffer A containing a 0.05–0.5 M KCl gradient (40 ml). One-ml fractions were collected and assayed for RF and Met-tRNA$_f$ binding activities. The peak RF fractions were pooled and dialyzed against 0.1 M KCl/20 mM potassium phosphate, pH 7.5/1 mM dithiothreitol/0.1 mM sodium EDTA/10% glycerol (buffer B/0.1 M KCl) (fraction IV).

The dialyzed preparation (fraction IV) was loaded onto a phosphocellulose column (P11, Whatman, 1.0 × 7 cm) and preequilibrated with buffer B/0.1 M KCl. The column was washed thoroughly with the same buffer and then was eluted with buffer B containing a 0.1–0.7 M KCl gradient (32 ml); 0.8-ml fractions were collected. The column fractions were assayed for RF and eIF-2 activities. The peak fractions containing RF activity were pooled and concentrated to ≈1 mg/ml by dialysis against buffer A/0.1 M KCl/16% polyethylene glycol (Carbowax 6000, Baker). The RF preparation was then dialyzed extensively against buffer A/0.1 M KCl. The dialyzed preparation was further concentrated by passage through a small DEAE-cellulose column (column volume, 0.5 ml; equilibrated with buffer A/0.1 M KCl) and a one-step elution with buffer A/0.3 M KCl.

Further Fractionation of RF. For further fractionation of RF into component factor activities, such as eIF-2 and Co-eIF-2C, the phosphocellulose-purified RF preparation, as described above, was dialyzed against buffer A/0.18 M KCl and was applied slowly onto a CM-Sephadex (3.0 cm × 1.0 cm) column previously equilibrated with buffer A/0.18 M KCl; 0.5-ml fractions were collected. The RF activity was eluted in the column flow-through and was freed from the bulk of the eIF-2 activity, which remained bound to the column. The eIF-2 activity could be eluted by washing the column with buffer A/0.4 M KCl. The peak fractions were pooled and dialyzed against buffer A/0.1 M KCl (fraction VI).

For density gradient centrifugation, 0.1 ml of fraction VI was applied on top of a linear glycerol gradient (14–30%, vol/vol) in buffer A/0.1 M KCl. The gradients were centrifuged at 45,000 rpm for 11 hr in a Beckman SW-50.1 rotor. The A_{280} profiles of the gradient were monitored, and 0.3-ml fractions were collected. RF activity sedimented as a single symmetrical peak around 20 S. The peak fractions were concentrated as before by using a small DEAE-cellulose column equilibrated with buffer A/0.1 M KCl and a one-step elution of the RF activity from the column with buffer A/0.3 M KCl (fraction VII).

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was performed as described by O'Farrell (26). Ampholines (pH 3.5–10 and pH 5.7) were purchased from LKB. The final pH gradient in the isoelectric focusing gels was linear between pHs 4.5 and 6.6. NaDodSO$_4$/polyacrylamide gel electrophoresis in the second dimension was on a 15% acrylamide/0.09% *N,N'*-methylenebisacrylamide gel. Gels were stained with Coomassie brilliant blue. Autoradiograms were made with Kodak XR-50 medical x-ray film.

RESULTS

The most active and also the most stable RF preparation was obtained after the phosphocellulose chromatographic step (fraction V). This preparation also contained significant amounts of eIF-2, Co-eIF-2A, Co-eIF-2B, and Co-eIF-2C activities. Upon further fractionation with a CM-Sephadex chromatographic procedure, the RF preparation (fraction VI) was almost completely freed from eIF-2 activity. However, such RF preparations contained Co-eIF-2A, Co-eIF-2B, and Co-eIF-2C activities. As shown in Fig. 1, both fraction V (phosphocellulose purified) and fraction VI (CM-Sephadex purified) RF preparations efficiently stimulated protein synthesis in heme-deficient reticulocyte lysates. At higher concentrations, both of the factor preparations increased the initial rate of protein synthesis over that observed in the presence of hemin, and, in both cases, protein synthesis continued over a long period. However, we have observed that the fraction VI RF preparation, which is devoid of eIF-2 activity, is significantly more unstable than the fraction V RF preparation and rapidly loses RF activity upon storage. This loss in RF activity is more pronounced at high dilution. Conceivably, the presence of eIF-2 confers stability upon the RF protein complex. Upon further fractionation of fraction VI RF preparation by using density gradient centrifugation, RF activity was eluted as a single symmetrical peak at 20 S coincident with Co-eIF-2C activity. However, such RF preparation was very inefficient in stimulating protein synthesis in heme-deficient reticulocyte lysates, and the protein synthesis rate declined rapidly after a short period (Fig. 1). The characteristics of such protein-synthesis stimulation observed with this glycerol density-purified RF preparation were comparable to those observed with a purified Co-eIF-2C preparation from ribosomal high-salt wash (Fig. 1).

As reported (2), the phosphocellulose-purified RF preparation (fraction V) contained eIF-2, Co-eIF-2B, and Co-eIF-2C activities, and the Co-eIF-2B and the Co-eIF-2C activities in this RF preparation were almost fully resistant to HRI and ATP. This preparation efficiently formed the ternary complex in the presence of Mg^{2+}, and such complex formation was completely insensitive to HRI and ATP at different concentrations of the fraction tested (Fig. 2). Upon further fractionation, using a CM-Sephadex chromatographic procedure, the RF preparation (fraction VI) could be freed of eIF-2 activity. However, this RF preparation retained the Co-eIF-2C activity, which was partially inhibited by HRI and ATP at low RF concentration and progressively regained insensitivity to HRI with increasing RF concentration (Fig. 2*B*). After glycerol gradient centrifugation, the RF preparation (fraction VI) regained almost complete sensitivity to HRI and ATP, and HRI inhibition of ternary complex formation could not be reversed by increasing concentrations of RF (Fig. 2*C*). These observations suggest that (*i*) some additional factor(s) may be present in active RF preparations and (*ii*) this factor renders Co-eIF-2C activity insensitive to HRI and

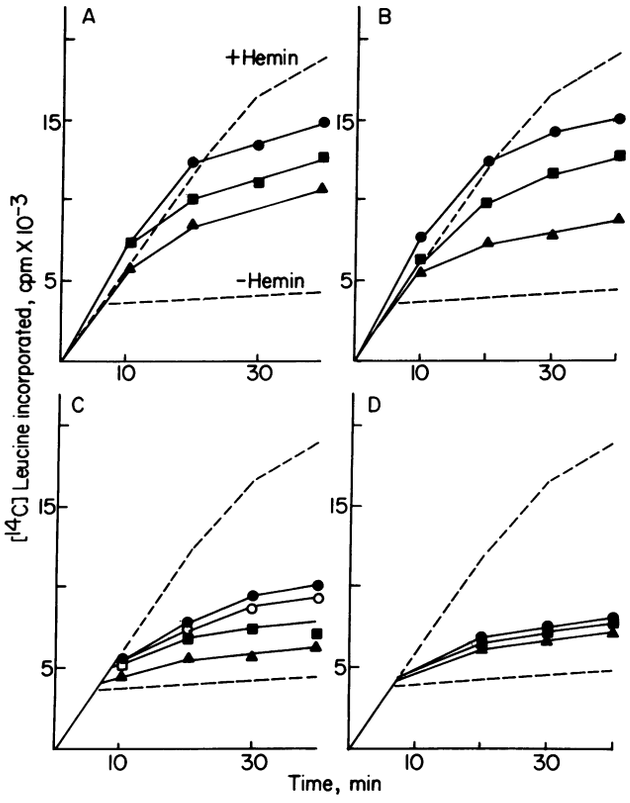

FIG. 1. Inhibition-reversal activities of RF fractions in protein synthesis. (*A*) Fraction V, phosphocellulose-purified RF. (*B*) Fraction VI, CM-Sephadex-purified RF. (*C*) Fraction VII, glycerol gradient RF. (*D*) Co-eIF-2C. Protein synthesis in heme-deficient reticulocyte lysates was assayed by using the standard procedure as described (24) with increasing amounts of the different RF fractions and Co-eIF-2C. (*A*) Phosphocellulose-purified RF at 3 μg (▲), 6 μg (■), and 9 μg (●). (*B*) CM-Sephadex-purified RF at 3 μg (▲), 6 μg (■), and 9 μg (●). (*C*) Glycerol gradient RF at 1 μg (▲), 2 μg (■), 3 μg (○), and 4 μg (●). (*D*) Co-eIF-2C at 8 μg (▲), 16 μg (■), and 24 μg (●). ---, Protein synthesis in the presence or absence of optimal concentrations of hemin (20 μM). [^{14}C]Leucine (specific activity, 112 cpm/pmol) incorporation was determined in 5-μl aliquots of the incubation mixtures.

ATP and is progressively removed by further fractionation with CM-Sephadex chromatography and glycerol density gradient centrifugation as the Co-eIF-2C activity in these fractionated preparations regains sensitivity to HRI and ATP.

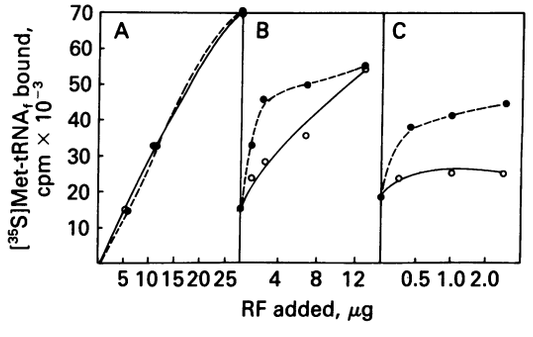

FIG. 2. HRI sensitivity of Co-eIF-2C activities in RF fractions. (*A*) Fraction V. (*B*) Fraction VI. (*C*) Fraction VII. Standard Millipore filtration assay conditions in the presence of 1 mM Mg^{2+} were used to measure the binding of [^{35}S]Met-tRNA$_f$ to Millipore filters; different concentrations of the RF fractions were used as indicated. Fractions VI and VII RF preparations were assayed in the presence of 1 μg of exogenous eIF-2. ---, Without HRI/ATP; ——, with HRI/ATP.

We examined the polypeptide components in fractions V and VI RF preparations. For this experiment, we incubated the RF preparations (fractions V and VI) and a partially purified eIF-2 preparation in the presence of HRI and [γ-^{32}P]ATP and analyzed the polypeptide components in each fraction by NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography (Fig. 3). Both RF preparations (fractions V and VI) showed multiple polypeptide bands, including several prominent polypeptide bands (M_rs $\approx$ 100,000, 67,000, 53,000, 38,000, and 25,000). As reported (17), several of these polypeptide bands are also present in a highly purified Co-eIF-2C preparation from ribosomal salt wash. Both fractions V and VI RF preparations contained significant amounts of M_r 38,000 polypeptide component(s), which moved similarly to the M_r 38,000 polypeptide component of eIF-2 (Fig. 3). However, whereas the M_r 38,000 polypeptide component in eIF-2 and in fraction V RF preparation was extensively phosphorylated by HRI and [γ-^{32}P]ATP, the M_r 38,000 polypeptide component in fraction VI RF preparation remained almost completely unphosphorylated. As noted previously, the fraction V RF preparation contained significant amounts of eIF-2 activity [specific activity (pmol of Met-tRNA$_f$ bound per mg of protein), 550], and the fraction VI RF preparation was almost completely devoid of eIF-2 activity (specific activity, 15). Both of the RF preparations (fractions V and VI) at equivalent microgram concentrations had comparable activities in inhibition reversal of protein synthesis in heme-deficient reticulocyte lysate (see Fig. 1). These results indicate that the fraction VI RF preparation is enriched with a M_r 38,000 polypeptide component that is not a part of intact eIF-2 and does not participate in ternary complex formation. Also, this M_r

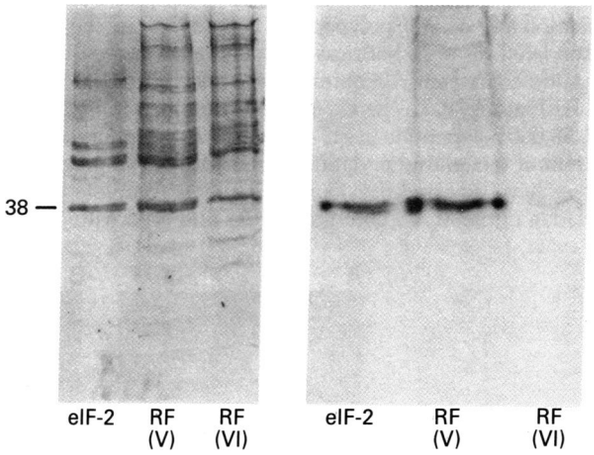

FIG. 3. Phosphorylation of eIF-2 and RF (fractions V and VI). Partially purified eIF-2 and RF preparations (fractions V and VI) were phosphorylated in the presence of HRI and [γ-^{32}P]ATP and then analyzed with NaDodSO$_4$/polyacrylamide gel electrophoresis (*Left*) and autoradiography (*Right*). The reaction mixtures contained (total volume, 0.05 ml) 30 mM Tris·HCl (pH 7.8), 130 mM KCl, 7 μg of bovine serum albumin, 1.8 mM magnesium acetate, 3 mM dithiothreitol, 1 μg of HRI, 0.04 mM [γ-^{32}P]ATP (specific activity, 2,500 cpm per pmol), and, where indicated, 6 μg of eIF-2, 6 μg of phosphocellulose-purified RF (fraction V), and 6 μg of CM-Sephadex-purified RF (fraction VI). The reaction mixtures were incubated at 37° for 10 min. The reactions were terminated by the addition of an equal volume of a denaturing buffer containing 50 mM Tris·HCl (pH 7.0), 50% glycerol (vol/vol), 5% 2-mercaptoethanol, and 2% NaDodSO$_4$. Samples were run on a gel of 15% acrylamide/0.09% *N,N'*-methylenebisacrylamide/NaDodSO$_4$. (*A*) The stained gels are from a duplicate experiment in which bovine serum albumin and HRI were omitted. The band patterns were identical with those observed in the duplicate experiments used for autoradiography but did not contain the contaminating bands of bovine serum albumin and HRI. M_r 38,000 is shown × 10^{-3}.

38,000 polypeptide component is not phosphorylated by HRI and ATP.

To further characterize the M_r 38,000 polypeptide component(s) in fractions V and VI RF preparations, the polypeptide components in eIF-2 and fractions V and VI RF preparations were analyzed by two-dimensional gel electrophoresis and autoradiography (Fig. 4 *A–I*). The M_r 38,000 polypeptide component in eIF-2 moved as a single component with a mobility corresponding to a pI value of ≈5 (Fig. 4*A*). Upon incubation in the presence of HRI and [γ-^{32}P]ATP, a significant part of the original M_r 38,000 polypeptide in eIF-2 moved towards more acidic regions (Fig. 4*D*), and, as expected, this new spot was radioactively labeled (Fig. 4*G*) and was presumably phosphorylated. Comparison of the relative intensities of the phosphorylated and nonphosphorylated spots revealed that ≈80% of the original M_r 38,000 polypeptide was phosphorylated. Under the experimental conditions, fractions V and VI RF preparations contained two M_r 38,000 polypeptide components (Fig. 4 *B* and *C*) of which one had mobility similar to that of the unphosphorylated M_r 38,000 polypeptide of eIF-2. Upon treatment with HRI and [γ-^{32}P]ATP, a significant part (60%) of the original M_r 38,000 polypeptide in fraction V RF moved towards more acidic regions (Fig. 4*E*) and was phosphorylated (Fig. 4*H*). Upon similar treatment, the M_r 38,000 polypeptide in fraction VI remained unchanged (Fig. 4*F*) and was almost completely unphosphorylated (Fig. 4*I*).

These results indicate that the fraction VI RF preparation, which has negligible eIF-2 activity, contains the M_r 38,000 polypeptide (α subunit) of eIF-2 in the unphosphorylated form, which is not phosphorylated by HRI and ATP. Both partially purified eIF-2 and a fraction V RF preparation (which also contains significant amounts of eIF-2 activity) contain a unique and identical M_r 38,000 polypeptide component in the unphosphorylated form; in both cases, a significant part of this polypeptide component becomes phosphorylated in the presence of HRI and ATP. In the experiment described in Fig. 4*D*, the M_r 38,000 polypeptide in eIF-2 was ≈80% phosphorylated. The extent of this phosphorylation varied with eIF-2 preparations; as we have reported previously (8), almost complete phosphorylation of the M_r 38,000 subunit was observed with an extensively purified eIF-2 preparation. The variations in the degree of phosphorylation of the M_r 38,000 subunit of eIF-2 with different eIF-2 preparations could possibly be due to the presence of associated RF activity in the eIF-2 preparations (27). It is conceivable that such RF activity in eIF-2 preparations, as in fraction VI RF preparation reported in this paper, contains excess free M_r 38,000 subunit that is not phosphorylated by HRI and ATP. It should be noted that the M_r 38,000 polypeptide in fraction V RF preparation was significantly less (60%) phosphorylated than was eIF-2. As noted earlier, this RF preparation also contains significant amounts of eIF-2 activity. This observation is consistent with the postulation that HRI phosphorylates only the M_r 38,000 subunit present in intact eIF-2 molecules and that RF contains excess free M_r 38,000 subunits that do not participate in ternary complex formation and are not phosphorylated by HRI and ATP.

DISCUSSION

An RF fraction (fraction V) obtained after phosphocellulose chromatography during purification actively reversed inhibition of protein synthesis in heme-deficient reticulocyte lysates and also contained significant amounts of eIF-2, Co-eIF-2B, and Co-eIF-2C activities. The Co-eIF-2B and Co-eIF-2C activities in this preparation are resistant to HRI and ATP. Therefore, this observation provides a rationale for the activity of RF in reversing inhibition of protein synthesis.

However, the precise mechanism of RF action and the altered recognition of Co-eIF-2C in RF preparation is not clear. A relevant observation reported in this paper is that active RF preparations contain excess of the M_r 38,000 subunit (α subunit) of eIF-2 in the free form, which is not phosphorylated by HRI and ATP. The significance of the presence of free α subunit of eIF-2 in RF preparation is not apparent and may be related to the basic mechanism of RF action. A likely explanation for RF action is that RF promotes replacement of the phosphorylated α subunit in eIF-2 with the nonphosphorylated α subunit in RF preparation, and the nonphosphorylated eIF-2 thus reformed is recognized by Co-eIF-2B and Co-eIF-2C and is active in protein synthesis initiation.

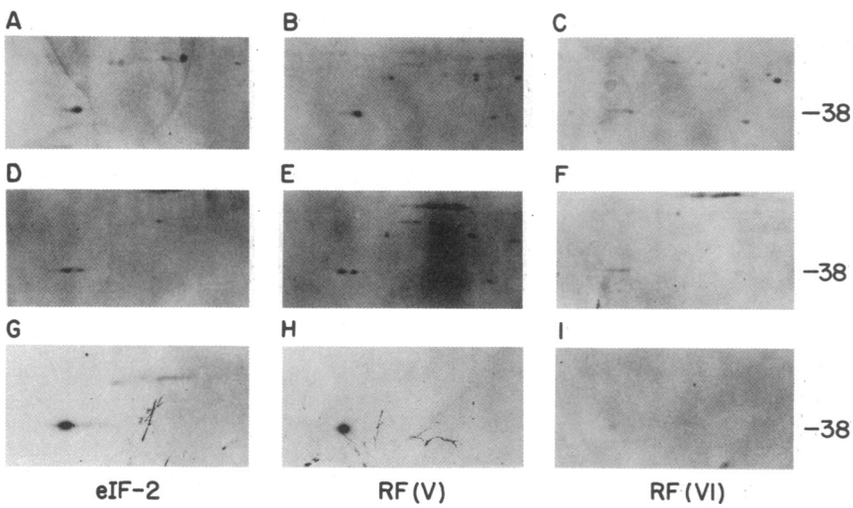

FIG. 4. Two-dimensional gel electrophoresis and autoradiography of partially purified eIF-2 and partially purified RF fractions (fractions V and VI). Phosphorylation of partially purified eIF-2 (10 μg) and RF [fractions V (12 μg) and VI (9 μg)] with [γ-^{32}P]ATP and HRI was as described in Fig. 3. The reactions were terminated by the addition of an equal volume (50 μl) of denaturing buffer and 28 mg of urea. The reaction mixtures (100 μl) were then loaded on the first-dimension tube gels. The autoradiograms were exposed for 12 hr. The segments of the stained gels (*Top* and *Middle*) and the autoradiograms (*Bottom*) around the M_r 38,000 (shown × 10^{-3}) polypeptide regions are indicated. (*Top*) Unphosphorylated eIF-2 (*A*), RF(V) (*B*), and RF(VI) (*C*). (*Middle*) Phosphorylated eIF-2 (*D*), RF(V) (*E*), and RF(VI) (*F*). (*Bottom*) Corresponding autoradiograms of *Middle* gels (*G–I*).

Thus, this paper provides a rationale for RF action and the relationship of RF to two eIF-2 ancillary factor activities—namely, Co-eIF-2B and Co-eIF-2C. Recently, Siekierka *et al.* have reported (19) isolation of an RF factor, which they term eIF-2·SP. This eIF-2·SP preparation presumably contains both eIF-2 and Co-eIF-2C activities and promotes ternary complex formation by exogenously added eIF-2 in the presence of Mg^{2+}. According to these authors (19), eIF-2·SP relieves Mg^{2+} inhibition of ternary complex formation by eIF-2 as it promotes displacement of GDP from firmly bound eIF-2·GDP complex. However, the precise relationship of eIF-2·SP to our Co-eIF-2C and RF factors is not clear. The eIF-2·SP-promoted ternary complex formation by eIF-2 is inhibited by HRI and ATP; therefore, this factor, as such, does not provide a rationale for RF activity as it relates to reversal of protein-synthesis inhibition in heme-deficient reticulocyte lysates. We believe that the extensively purified reticulocyte supernatant factor eIF-2·SP is probably similar to our glycerol density gradient RF preparation (fraction VII; see Figs. 1 and 2) which contains HRI sensitive Co-eIF-2C activity.

Finally, the observation in RF preparations of the presence of a pool of free α subunit of eIF-2 that is not phosphorylated by HRI and ATP may provide a rationale for several recent reports, which indicate that only 25–30% of eIF-2 becomes phosphorylated under conditions of almost total inhibition of protein synthesis in heme-deficient reticulocyte lysates (28, 29). The presence in reticulocyte lysates of a pool of free α subunit of eIF-2 that is not phosphorylated by HRI and ATP could reasonably explain this lack of correlation between the extent of phosphorylation of α subunits (eIF-2 bound and free) and the degree of protein-synthesis inhibition in heme-deficient reticulocyte lysates. Our present results suggest that only eIF-2 bound α-subunit is phosphorylated by HRI and ATP.

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