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Protein synthesis in rabbit reticulocytes: A study of the mechanism of action of the protein factor RF that reverses protein synthesis inhibition in heme-deficient reticulocyte lysates

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

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ABSTRACT **A eukaryotic initiation factor 2 (eIF-2)-ancillary protein factor Co-eIF-2 promotes displacement of GDP from eIF-2·GDP and facilitates ternary complex (Met-tRNA$_f$·eIF-2·GTP) formation in the presence of Mg^{2+}. Heme-regulated protein synthesis inhibitor, HRI, phosphorylates the α-subunit of eIF-2 and thus inhibits ternary complex formation as Co-eIF-2 does not displace GDP from eIF-2α(*P*)·GDP. RF, a high molecular weight cell supernatant factor, reverses protein synthesis inhibition in heme-deficient reticulocyte lysates and also reverses HRI inhibition of ternary complex formation. RF contains Co-eIF-2 activity. In addition, an active RF preparation contains excess α-subunit of eIF-2 in the free and unphosphorylated form and this α-subunit of eIF-2 is not phosphorylated by HRI and ATP. In this paper we report (*i*) an active RF preparation contains excess α-subunit of eIF-2 and this α-subunit can be phosphorylated by HRI and ATP in the presence of GDP; (*ii*) RF promotes ternary complex formation by eIF-2·[^{3}H]GDP with accompanying GDP displacement; (*iii*) in the presence of HRI and ATP, RF promotes ternary complex formation by eIF-2·[^{3}H]GDP without accompanying GDP displacement; (*iv*) in the presence of HRI and ATP, the ternary complex formed using RF is active in Met-tRNA$_f$·40S initiation complex formation; (*v*) both the ternary complex and the Met-tRNA$_f$·40S complex formation in the presence of HRI and ATP are completely inhibited by prior incubation of RF with GDP; (*vi*) upon further fractionation of an active RF fraction, a preparation can be obtained that contains HRI-sensitive Co-eIF-2 activity. However, this preparation does not efficiently reverse protein synthesis inhibition in heme-deficient reticulocyte lysates and does not contain excess α-subunit of eIF-2. Based on these observations, we have suggested (*a*) RF provides the unphosphorylated α-subunit to eIF-2α(*P*)·GDP and restores eIF-2 activity. This RF activity is inhibited as the α-subunit in the RF preparation becomes phosphorylated by HRI and ATP in the presence of GDP; (*b*) RF contains Co-eIF-2 activity, which has dual functions: (*i*) stimulation of ternary complex formation by eIF-2 and (*ii*) GDP displacement from eIF-2·GDP during ternary complex formation. In the presence of HRI and ATP, Co-eIF-2 still stimulates ternary complex formation by unphosphorylated eIF-2 but does not displace GDP from eIF-2α(*P*)·GDP.**

Heme-regulated protein synthesis inhibitor, HRI, phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF-2) and inhibits ternary complex (Met-tRNA$_f$·eIF-2·GTP) formation. The following mechanism has been proposed for the inhibition of ternary complex formation: A high molecular weight protein factor that we purify from reticulocyte ribosomal salt wash and term Co-eIF-2 (1) [also termed Co-eIF-2C (2), SF (3), eIF-2·RF (4)] is required for ternary complex formation by eIF-2 in the presence of Mg^{2+}. The bulk of eIF-2 is present as eIF-2·GDP (5). In the presence of Mg^{2+}, GDP remains tightly bound to eIF-2 and prevents ternary complex formation. Co-eIF-2 promotes displacement of GDP from eIF-2·GDP and thus facilitates ternary complex formation (5–10). Co-eIF-2 activity does not displace GDP from eIF-2α(*P*)·GDP (eIF-2 phosphorylated by HRI and ATP) and ternary complex formation is inhibited (1–10).

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 (11–15). We have reported previously that RF also reverses HRI inhibition of ternary complex formation in partial reactions (14, 15). A purified RF preparation contains Co-eIF-2 activity and also can stimulate ternary complex formation by eIF-2α(*P*). We have also reported that an active RF preparation that is almost completely devoid of eIF-2 activity contains the M_r 38,000 (α-subunit) of eIF-2 and this excess M_r 38,000 subunit in the RF preparation is not phosphorylated by HRI and ATP (15). The presence of Co-eIF-2 activity in a purified RF preparation that promotes displacement of GDP from eIF-2·GDP has been reported recently by several laboratories and has been termed GEF (5), RF (9), and eIF-2B (10).

In this communication, we provide evidence that suggests that RF reverses HRI inhibition of ternary complex formation as it provides the unphosphorylated M_r 38,000 subunit to eIF-2α(*P*)·GDP. This ternary complex inhibition-reversal activity of RF is almost completely inhibited by prior incubation of RF with HRI and ATP in the presence of GDP as, under this condition, the excess M_r 38,000 subunit of eIF-2 in RF becomes phosphorylated. Our present results also suggest that RF contains Co-eIF-2 activity that stimulates ternary complex formation by eIF-2 and also promotes GDP displacement from eIF-2·GDP during ternary complex formation. These two activities are separate. In the presence of HRI and ATP, Co-eIF-2 still promotes ternary complex formation by unphosphorylated eIF-2 but does not displace GDP from eIF-2α(*P*)·GDP.

MATERIALS AND METHODS

The sources of the materials used in these studies were the same as described (1, 14–16). Unlabeled HPLC-purified

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Abbreviations: eIF-2, eukaryotic initiation factor 2, which forms ternary complex, Met-tRNA$_f$·eIF-2·GTP; Co-eIF-2, ancillary protein factor that stimulates ternary complex formation by eIF-2; RF, high molecular weight cell supernatant factor that reverses protein synthesis inhibition in heme-deficient reticulocyte lysates; HRI, heme-regulated translational inhibitor; eIF-2α(*P*), phosphorylated eIF-2 (HRI catalyzed).

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GTP and radiolabeled [^{3}H]GDP (specific activity, 10 Ci/mmol; 1 Ci = 37 GBq) were obtained from ICN and New England Nuclear, respectively. Partially purified eIF-2 and Co-eIF-2 preparations were obtained after the CM-Sephadex chromatographic step (1, 16). eIF-2·[^{3}H]GDP was prepared by incubating purified eIF-2 with [^{3}H]GDP as described (1), and the complex formed was further purified by using CM-Sephadex chromatography.

RF activity was purified from the reticulocyte cell supernatant and the procedures up to the phosphocellulose step (fraction V A) were the same as described (15). For further studies, fraction V A was divided into two parts. One part was fractionated directly by using a glycerol density gradient procedure (fraction V B). Another part was further fractionated by using CM-Sephadex chromatography (fraction VI A) followed by glycerol density gradient centrifugation (fraction VI B). The CM-Sephadex chromatography and glycerol density gradient procedures were the same as described (15). Fig. 1 describes a scheme for preparation of different RF fractions.

Standard Millipore filtration methods were used for assays of eIF-2 and RF activities (1, 14, 15). Met-tRNA$_f$·40S initiation complex formation was assayed by using the density gradient centrifugation procedure (2).

NaDodSO$_4$/polyacrylamide gel electrophoresis was performed as described by O'Farrell (17) using a 10% acrylamide/0.26% *N,N'*-methylenebisacrylamide gel. Gels were stained with Coomassie brilliant blue. Autoradiograms were made with Kodak XR-50 medical x-ray film.

RESULTS

RF Preparations. As reported previously (15), both phosphocellulose-purified RF (fraction V A, see Fig. 1) and CM-Sephadex-purified RF (fraction VI A) reversed the inhibition of protein synthesis in heme-deficient reticulocyte lysates. The ability to reverse inhibition was mostly lost upon further purification of fraction VI A by glycerol density gradient centrifugation (to give fraction VI B, formerly called fraction VII). In our present study we used fraction V A RF preparation and fractionated it further by using glycerol density centrifugation (fraction V B). Fig. 2 shows the protein synthesis inhibition-reversal activities of fraction V B and fraction VI B RF preparations. As shown here, fraction V B RF caused complete reversal of protein synthesis inhibition in heme-deficient reticulocyte lysates and the protein synthesis activity in the presence of fraction V B RF was comparable to that observed in the presence of hemin. Under similar conditions, fraction VI B RF preparation at comparable concentration was almost completely inactive in stimulating protein synthesis.

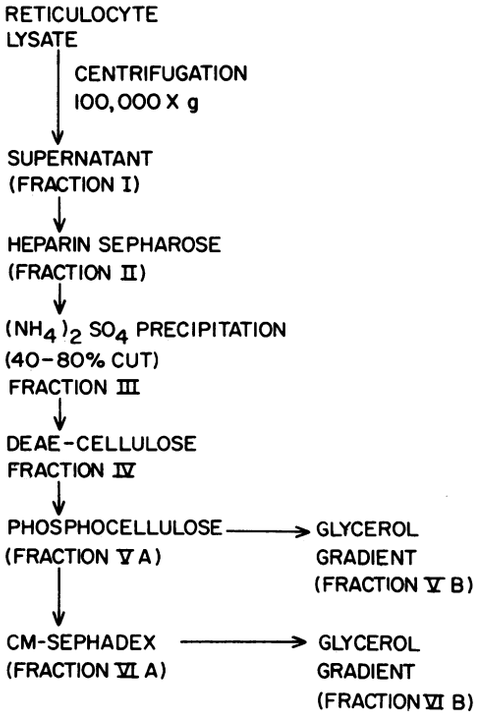

FIG. 1. Purification scheme for RF.

Presence of Excess M_r 38,000 Subunit in the RF Preparation and Its Phosphorylation by HRI and ATP in the Presence of GDP. We have reported previously that fraction V A RF preparation (phosphocellulose purified), which contained eIF-2 activity, was partially phosphorylated by HRI and ATP, and fraction VI A RF preparation (CM-Sephadex purified), which did not contain eIF-2 activity but contained excess M_r 38,000 subunit in free and unphosphorylated form, was not phosphorylated by HRI and ATP (15). Recently, Matts *et al.* have reported that the M_r 38,000 subunit in eIF-2 as it exists in the RF·eIF-2 complex requires GDP for phosphorylation by HRI and ATP (9). We have examined the characteristics of the M_r 38,000 subunit in different factor preparations and the effects of exogeneously added GDP on HRI-catalyzed phosphorylation of this subunit (Fig. 3). As shown here, fraction V A and fraction V B RF preparations contained significant amounts of M_r 38,000 polypeptide, whereas fraction VI B RF and Co-eIF-2 preparations were almost devoid of this polypeptide. All of these preparations, however, contained a slightly lower molecular weight (M_r 36,000) polypeptide component in varying amounts.

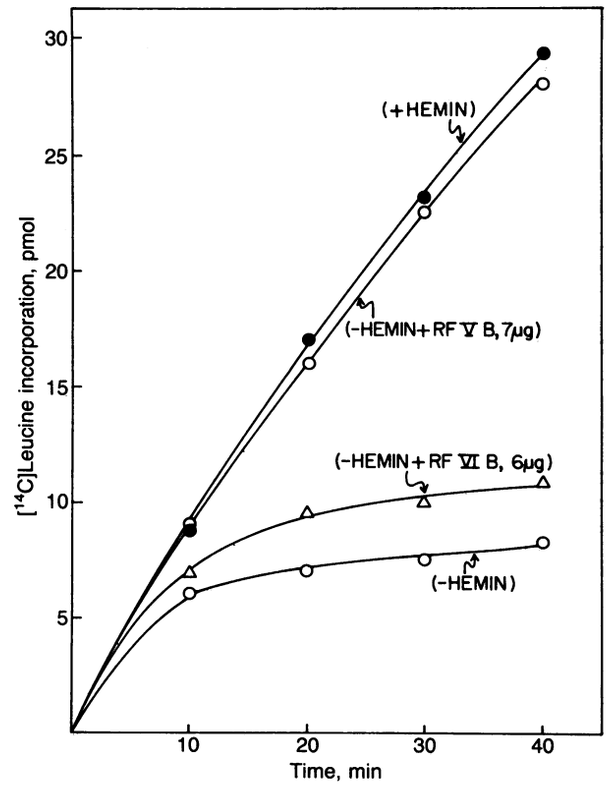

FIG. 2. Inhibition-reversal activities of RF V B and VI B fractions in protein synthesis. Protein synthesis in heme-deficient reticulocyte lysates was assayed by using the standard procedure as described (14, 15). [^{14}C]Leucine (specific activity, 100 cpm/pmol) incorporation was determined in 5-μl aliquots of the reaction. Protein synthesis experiments contained (total volume 25 μl) no hemin (○), 20 μM hemin (●), RF V B, 7 μg (○), and RF VI B, 6 μg (△).

As shown here, the M_r 38,000 subunit in fraction V A RF was phosphorylated by HRI and ATP in the absence of GDP (Fig. 3, lane 1), and the extent of this phosphorylation was significantly enhanced when GDP was added (Fig. 3, lane 2). In contrast, the M_r 38,000 subunit in fraction V B RF was not at all phosphorylated by HRI and ATP in the absence of GDP (Fig. 3, lane 3) but was phosphorylated when GDP was added (Fig. 3, lane 4). As expected, the autoradiograms of fraction VI B and Co-eIF-2 preparations did not show any detectable radioactive band in the M_r 38,000 region (Fig. 3, lanes 5–8).

As noted earlier, fraction V A RF contained significant amounts of eIF-2 activity. In another study (unpublished observation), we have observed that fraction V A RF preparations contain significant amounts of GDP presumably bound to eIF-2 and fraction V B RF preparation is almost free of GDP. These results, therefore, suggest that eIF-2·GDP in fraction V A RF becomes phosphorylated by HRI and ATP, and the excess M_r 38,000 subunit present in both fraction V A and fraction V B preparations requires GDP for subsequent phosphorylation.

Characteristics of Stimulation of Ternary Complex Formation and GDP Displacement Activities of RF V B and VI B Fractions. We used different density gradient fractions obtained during fraction V B and fraction VI B RF preparations and compared their activities in GDP displacement and in stimulation of ternary complex formation by eIF-2·[^{3}H]GDP in the presence and absence of HRI and ATP and the effects of exogeneously added GDP on these activities (Fig. 4). As shown in Fig. 4 *A* and *D*, in both cases, the bulk of eIF-2 activity sedimented as lower molecular weight components and the higher molecular weight components containing Co-eIF-2-like activity contained significantly less eIF-2 activity. The Co-eIF-2-like activity in both fraction V B and fraction VI B RF were equally effective in stimulating ternary complex formation with exogeneously added eIF-2·[^{3}H]GDP (Fig. 4 *B* and *E*), and, in each case, such stimulation was accompanied by concomitant loss of [^{3}H]GDP from eIF-2·[^{3}H]GDP (Fig. 4 *C* and *F*). In the presence of HRI and ATP, RF V B fractions still stimulated ternary complex formation with eIF-2·[^{3}H]GDP but with no accompanying loss of [^{3}H]GDP (Fig. 4 *B* and *C*). Under similar conditions, RF VI B fractions failed to stimulate ternary complex formation with eIF-2·[^{3}H]GDP and, as expected, there was no loss of [^{3}H]GDP from eIF-2·[^{3}H]GDP (Fig. 4 *E* and *F*). These results suggest that fraction VI B RF preparations contain Co-eIF-2 activity. This Co-eIF-2 activity, as reported by other workers, promotes displacement of GDP from eIF-2·GDP during ternary complex formation but does not displace GDP from eIF-2·[^{3}H]GDP in the presence of HRI and ATP as Co-eIF-2 activity does not recognize eIF-2α(*P*)·GDP. On the other hand, the Co-eIF-2-like activity in fraction V B RF preparation can recognize eIF-2α(*P*)·GDP and stimulate ternary complex formation by eIF-2α(*P*)·GDP without accompanying loss of GDP. These results indicate that fraction V B RF preparation provides a component to eIF-2α(*P*)·GDP complex that restores the ternary complex formation ability of eIF-2α(*P*)·GDP and this component binds exogeneously added GTP during ternary complex formation. We suggest that RF provides the excess unphosphorylated M_r 38,000 subunit to eIF-2α(*P*)·GDP and thus reforms eIF-2. Consistent with this suggestion was the observation that the ternary complex inhibition-reversal activity of RF was completely lost when RF was preincubated with HRI and ATP in the presence of 1 μM GDP (Fig. 4*B*). As noted earlier, under the preincubation conditions, the excess M_r 38,000 subunit in the RF preparation becomes phosphorylated by HRI and ATP in the presence of GDP.

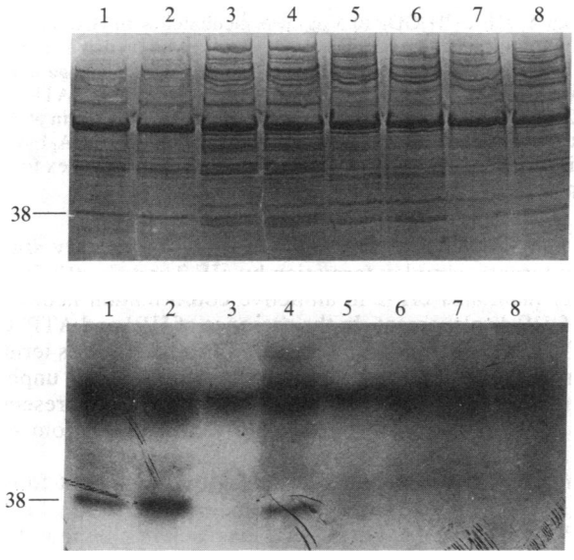

FIG. 3. Analysis for the presence of M_r 38,000 subunit in different factor preparations and the effects of addition of GDP on HRI-catalyzed phosphorylation of the M_r 38,000 subunit. RF preparation fractions V A, V B, and VI B and partially purified Co-eIF-2 were incubated with HRI (3 μg) and [γ-^{32}P]ATP (26 μM; specific activity, 2000 cpm/pmol) under standard incubation conditions as described (15). Where indicated, 1 μM GDP was included in the reaction mixtures. The reaction mixtures were then analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (*Upper*) and autoradiography (*Lower*). Lanes 1, RF V A (4 μg) − GDP; lanes 2, RF V A (4 μg) + GDP; lanes 3, RF V B (14 μg) − GDP; lanes 4, RF V B (14 μg) + GDP; lanes 5, RF VI B (10 μg) − GDP; lanes 6, RF VI B (10 μg) + GDP; lanes 7, Co-eIF-2 (11 μg) − GDP; lanes 8, Co-eIF-2 (11 μg) + GDP. The M_r 38,000 subunit is shown as $M_r \times 10^{-3}$.

Effects of Addition of HRI and GDP on RF (V B and VI B)-Promoted Met-tRNA·40S Initiation Complex Formation. Fig. 5 shows that both RF V B and RF VI B fractions stimulated Met-tRNA$_f$ binding to 40S ribosomes in the presence of eIF-2 and AUG codon. However, whereas fraction V B-promoted Met-tRNA$_f$ binding to 40S ribosomes was almost completely insensitive to HRI and ATP (Fig. 5*A*), fraction VI B promoted-Met-tRNA$_f$ binding to 40S ribosomes was almost completely inhibited by added HRI and ATP (Fig. 5*B*). Again, as shown in Fig. 5*A*, fraction V B RF-stimulated Met-tRNA$_f$ binding to 40S ribosomes in the presence of HRI and ATP was completely inhibited by prior incubation of RF with HRI and ATP in the presence of GDP.

The above results are consistent with our proposed mechanism that suggests that RF V B fractions provide the unphosphorylated M_r 38,000 subunit to eIF-2α(*P*)·GDP and thus reforms eIF-2. This RF-promoted stimulation of ternary complex and Met-tRNA$_f$·40S complex formation is inhibited as the α-subunit becomes phosphorylated by HRI and ATP in the presence of GDP.

DISCUSSION

Data presented in this paper provide the following information regarding peptide chain initiation:

(*i*) An active RF fraction (fraction V B) stimulates ternary complex formation by eIF-2α(*P*)·GDP without accompanying GDP displacement from the complex. This RF stimulation of ternary complex formation is inhibited as the excess M_r 38,000 (α-subunit) in the RF preparation becomes phosphorylated by HRI and ATP in the presence of GDP. These results are consistent with a proposed mechanism that suggests that RF provides the excess unphosphorylated α-subunit to eIF-2α(*P*)·GDP and thus reforms eIF-2. This RF activity is inhibited as the α-subunit in RF becomes phosphorylated by HRI and ATP in the presence of GDP. The

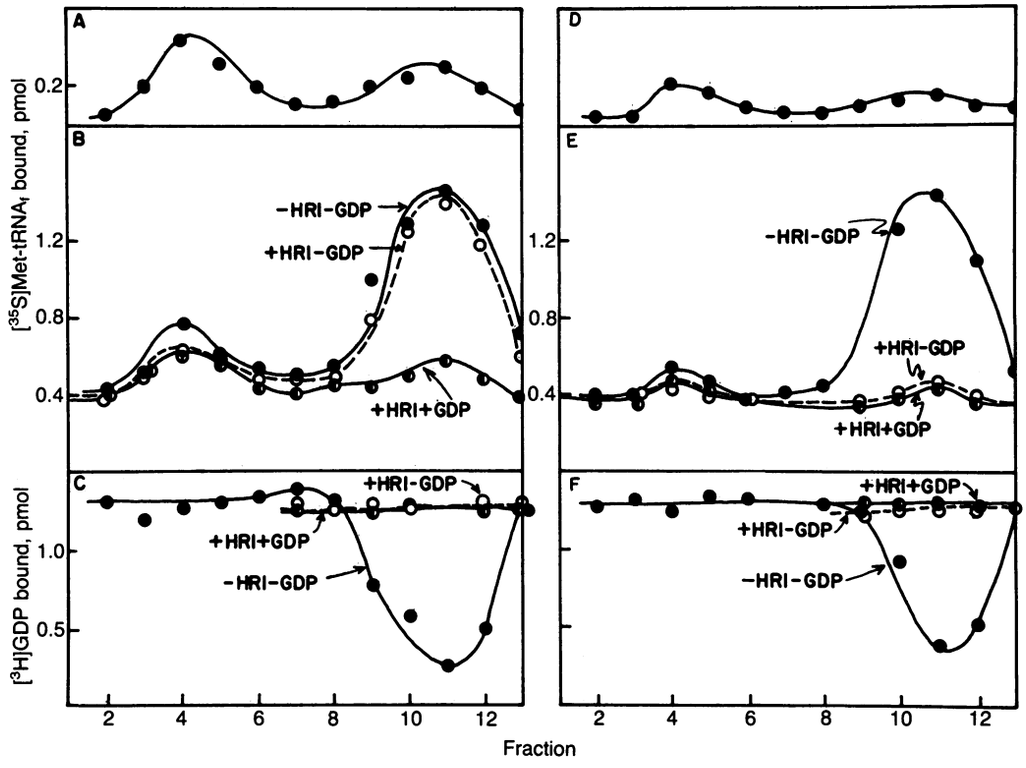

FIG. 4. Analysis of RF V B (*Left*) and RF VI B (*Right*) glycerol density gradient fractions for different activities. RF V A and RF VI A fractions were further fractionated by using glycerol density gradient centrifugation. The individual gradient fractions were assayed for different activities. Fraction I is the top of the gradient. (*A* and *D*) Met-tRNA$_f$ binding activity: Standard Millipore filtration assay conditions in the absence of Mg^{2+} were used. Ten-microliter aliquots of different gradient fractions were used in each experiment. (*B* and *E*) Stimulation of Met-tRNA$_f$ binding to exogeneously added eIF-2·[^{3}H]GDP. Standard Millipore filtration assay conditions were used in the presence of 1 mM Mg^{2+}. Where indicated, HRI (3 μg), ATP (1 mM), and GDP (1 μM) were also included in the reaction mixtures. For studies of HRI effect (●, −HRI −ATP; ○, +HRI +ATP), the experiments were carried out in two stages. In stage 1, eIF-2·[^{3}H]GDP (0.5 μg) was incubated with HRI and ATP for 5 min at 37°C. Stimulation of [^{35}S]Met-tRNA$_f$ binding to eIF-2·[^{3}H]GDP by different gradient fractions was then assayed by using standard Met-tRNA$_f$ binding assay conditions (stage 2). For studies of GDP effect (◐), the experiments were carried out in three stages. In stage 1, eIF-2·[^{3}H]GDP was phosphorylated by incubation with HRI and ATP as described above. The reaction mixtures containing HRI, ATP, and phosphorylated eIF-2·[^{3}H]GDP were then mixed with RF fractions and 1 μM GDP and the incubation was continued for another 5 min at 37°C (stage 2). Stimulation of [^{35}S]Met-tRNA$_f$ binding to eIF-2·[^{3}H]GDP by different gradient fractions was then studied by using Met-tRNA$_f$ binding assay conditions (stage 3). (*C* and *F*) GDP displacement activities. [^{3}H]GDP displacement from eIF-2·[^{3}H]GDP during ternary complex formation in the experiments described in *B* and *F* was used from dual labeling experiments.

reaction sequence is outlined as follows:

$$\text{eIF-2}(\gamma, \beta, \alpha(P))\cdot\text{GDP} + \text{RF}(\alpha) \rightarrow \text{eIF-2}(\gamma, \beta, \alpha)\cdot\alpha(P)\cdot\text{GDP}\cdot\text{RF}$$

$$\text{RF}(\alpha) + \text{GDP} \xrightarrow{\text{HRI + ATP}} \underset{\text{(inactive)}}{\text{RF}\cdot\alpha(P)}$$

(*ii*) Both fraction V B and fraction VI B RF preparations contain Co-eIF-2 activity and stimulate ternary complex formation by eIF-2·[^{3}H]GDP with accompanying GDP displacement. The ternary complex stimulation and also GDP displacement activity observed with fraction VI B RF were completely inhibited by prior incubation of eIF-2·[^{3}H]GDP with HRI and ATP (Fig. 4 *E* and *F*). This observation is consistent with the proposed mechanism that Co-eIF-2 does not displace GDP from eIF-2$\alpha(P)$·GDP and stimulation of ternary complex formation and GDP displacement activities are linked (6–10). However, as shown in Fig. 4 *B* and *C*, fraction V B RF preparation stimulates ternary complex formation by eIF-2·[^{3}H]GDP even in the presence of HRI and ATP, although this preparation does not displace GDP from eIF-2$\alpha(P)$·GDP. These results indicate that the ternary complex stimulatory activity and GDP displacement activity are due to two different activities in RF preparations. This observation supports our previous postulation that Co-eIF-2 protein complex (similar to RF protein complex) contains Co-eIF-2A and Co-eIF-2C activities (1). Co-eIF-2A activity stimulates ternary complex formation by eIF-2 and Co-eIF-2C activity maintains eIF-2 in an active conformation necessary for GDP displacement. In the presence of HRI and ATP, Co-eIF-2A activity in the RF preparation still stimulates ternary complex formation, presumably by reformed and unphosphorylated eIF-2, whereas the Co-eIF-2C activity present in the same preparation does not displace GDP from eIF-2$\alpha(P)$·GDP.

(*iii*) We suggest that RF provides free α-subunit to eIF-2$\alpha(P)$·GDP and this free α-subunit can substitute for the $\alpha(P)$·GDP component of eIF-2. This result implies that the α-subunit in eIF-2 possibly provides the binding site for GDP. Also, the GDP requirement for phosphorylation of the α-subunit in the RF V B preparation may imply that HRI phosphorylates only GDP-bound M_r 38,000 subunit.

According to the proposed mechanism outlined above, RF promotes transfer of unphosphorylated α-subunit to eIF-2$\alpha(P)$·GDP and reforms eIF-2. The mechanism of this transfer reaction is not known. This transfer reaction is possibly mediated by some "putative" factor that confers a catalytic role for RF action. *In vitro* data for ternary complex formation can possibly be explained by stoichiometric transfer of α-subunit in RF to eIF-2$\alpha(P)$·GDP to form ternary complex. However, RF action in reticulocyte lysates has been reported to be catalytic (10). We postulate that reticulocyte lysates

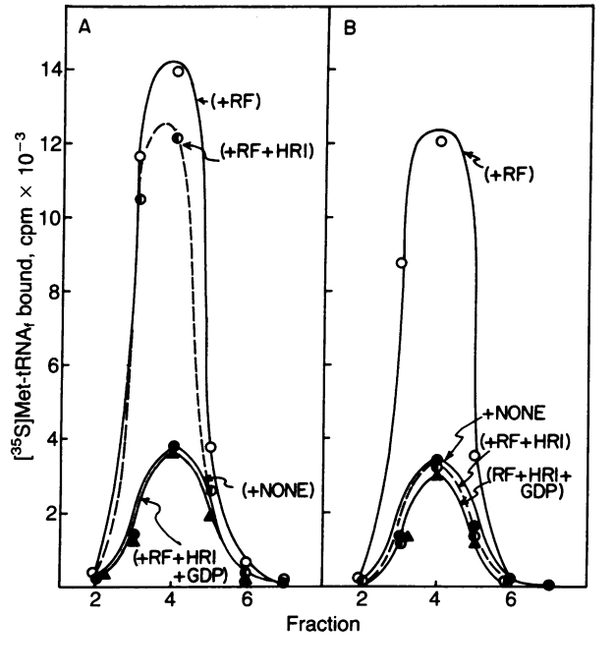

FIG. 5. Stimulation of Met-tRNA$_f$·40S·AUG complex formation by fraction V B and VI B RF preparations and the effects of HRI, ATP, and GDP on this complex formation. Standard density gradient centrifugation methods were used for studies of [^{35}S]Met-tRNA$_f$ binding to 40S ribosomes in the presence of AUG codon (2). Peak V B (*A*) and VI B (*B*) RF fractions were concentrated by dialysis against solid sucrose and were freed from sucrose by further dialysis against buffer A containing 0.1 M KCl (15). The standard reaction mixture contained, in addition to the usual salt components, 1 μg of purified eIF-2 and 8 μg of RF V B or 8 μg of RF VI B fraction. As in the experiments described in Fig. 3, a two-stage procedure was used for studies of HRI effect and a three-stage procedure was used for studies of HRI and GDP effect. As before, in stage 1, eIF-2 was phosphorylated by incubation with HRI and ATP. The reaction mixtures were then mixed with 1 μM GDP and RF fractions and were further incubated at 37°C for 5 min. [^{35}S]Met-tRNA$_f$ binding to 40S ribosomes was then studied by using glycerol density gradient centrifugation followed by Millipore filtration as described (2).

contain a pool of excess M_r 38,000 subunit in the unphosphorylated form and RF promotes transfer of this α-subunit to eIF-2α(*P*)·GDP and reforms unphosphorylated eIF-2. The pool of unphosphorylated α-subunit can possibly be maintained by release of the excess phosphorylated M_r 38,000 subunit from the initiation complex at the end of initiation cycle and subsequent dephosphorylation by eIF-2 phosphatase (18).†

†Our postulation for the presence of free eIF-2 α-subunits in active RF preparations is based on the fact that these RF preparations contain eIF-2 α-subunits but do not form corresponding amounts of ternary complexes. At this time, we do not exclude an alternative possibility that active RF preparations contain eIF-2 (all three subunits) in an inactive form and this eIF-2 in RF preparations provides the unphosphorylated α-subunit to eIF-2·α(*P*)·GDP to reform unphosphorylated eIF-2 active in ternary complex formation.

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