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Recycling and phosphorylation of eukaryotic initiation factor 2 on 60S subunits of 80S initiation complexes and polysomes

(regulation of protein synthesis/inhibition of protein chain initiation and elongation/guanine nucleotide exchange factor/heme-regulated inhibitor)

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ABSTRACT **Phosphorylation of the α-subunit (38 kDa) of eukaryotic initiation factor 2 (eIF-2α) regulates initiation of protein synthesis in eukaryotic cells. This phosphorylation is enhanced in cycloheximide-treated heme-deficient reticulocyte lysates in which polysomes are maintained. In early heme deficiency prior to polysome disaggregation, eIF-2(αP) accumulates primarily on the 60S subunits of polysomes. Further, isolated polysomes contain eIF-2α that is efficiently phosphorylated *in vitro* by heme-regulated inhibitor (HRI). Immunoblot analysis of eIF-2 distribution in sucrose gradients of actively protein-synthesizing lysates indicates that eIF-2 is distributed at low levels throughout the polysome profiles. These findings suggest that polysome-bound eIF-2α is a target of HRI under physiological conditions. The presence of eIF-2 on the 60S subunits of polysomes is incompatible with the conventional model in which eIF-2 is recycled during the joining of the 48S preinitiation complex and the 60S subunit to form the 80S initiation complex. A modified model is presented with emphasis on the translocation of eIF-2 from the 40S ribosomal subunit of the 48S preinitiation complex (eIF-2·GTP·Met-tRNA$_f$·40S·mRNA) to the 60S subunit of the 80S initiation complex.**

In eukaryotic protein synthesis, the joining of the 48S preinitiation complex and the 60S subunit to form the 80S initiation complex is accompanied by the hydrolysis of GTP and the formation of a binary complex of eukaryotic initiation factor 2 and GDP [eIF-2·GDP] (1–4). The recycling of eIF-2 requires the dissociation of GDP from eIF-2 and its replacement by GTP to yield [eIF-2·GTP], which forms a functional ternary complex with Met-tRNA$_f$. The GTP/GDP exchange is catalyzed by a factor, now called guanine nucleotide exchange factor (eIF-2B), previously known as reversing factor or GEF (1–6).

We and others have shown that the binary complex [eIF-2·GDP] is associated with the 60S subunits of the 80S initiation complex and with the 60S subunits of polysomes (7–11). These studies suggested that during the joining step, the binary complex is transferred from the 48S preinitiation complex to the 60S subunit of the 80S initiation complex, which is then utilized in polysome formation (7, 9). In heme-deficient reticulocyte lysates, protein chain initiation is inhibited by the activation of the heme-regulated eIF-2α kinase (HRI), the phosphorylation of the eIF-2 α-subunit (eIF-2α), and the sequestration of eIF-2B in a 15S phosphorylated complex eIF-2B·eIF-2(αP) in which eIF-2B is nonfunctional. In early heme deficiency, protein synthesis proceeds at control linear rates for about 5 min and then declines abruptly as polysomes disaggregate (7–9, 12–16). During this initial linear period, eIF-2(αP) is found in polysomes prior to disaggregation; after disaggregation, eIF-2(αP) is found in the 15S complex, on free 60S subunits, and on the 60S subunits of the large 80S peak of 40S/60S ribosome couples, the main storage form of disaggregated polysomes (7–11).

The presence of eIF-2 on the 60S subunits of the 80S initiation complex and of polysomes is contrary to the conventional view of the cycle of eIF-2. As presented in a recent review (17), the eIF-2·GDP complex is depicted as being released in the joining of the 48S preinitiation complex and the 60S subunit and recycled after the exchange of GDP by GTP.

In this paper we present further evidence that eIF-2 is found on polysomes where it can interact with eIF-2B in the process of recycling and where it can also be a target for HRI. We present a model for the cycle of eIF-2 that incorporates this evidence.

MATERIALS AND METHODS

HRI and eIF-2 were purified as described (18, 19). L-[^{14}C]Leucine (340 mCi/mmol; 1 Ci = 37 GBq), 8-[^{3}H]GDP (9 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and [^{32}P]orthophosphate (100 mCi/ml) were obtained from DuPont/New England Nuclear. ATP, GTP, GDP, creatine phosphate kinase, fructose-1,6-bisphosphate, NAD$^+$, and 2-deoxyglucose 6-phosphate were purchased from Sigma. Sucrose was purchased from Bio-Rad. Rabbit reticulocyte lysates were prepared as described (20, 21). Protein synthesis reactions were carried out with or without hemin (20 μM) at 30°C, and ribosomes and ribosomal subunits of protein-synthesizing lysates were separated on 10–50% sucrose gradients as described (7, 22). Typically, 100 μl of protein-synthesizing lysates was diluted with an equal volume of dilution buffer containing 20 mM Tris·HCl (pH 7.7), 1 mM Mg(OAc)$_2$, and 100 mM KOAc; samples were layered on gradients and spun at 45,000 rpm for 60 min in an SW50.1 rotor. The gradients were prepared in the dilution buffer containing 1 mM dithiothreitol. The gradient fractions were collected using an ISCO gradient fractionator. Phosphorylation of lysate proteins was carried out under standard protein-synthesizing conditions. Proteins were labeled at various time intervals with [^{32}P]orthophosphoric acid (≈1 mCi/ml) in the presence of an

Abbreviations: eIF-2, eukaryotic initiation factor 2; eIF-2(αP), eIF-2 phosphorylated in the α-subunit; [eIF-2·GDP], binary complex of eIF-2 and GDP; eIF-2B, guanine nucleotide exchange factor (also designated GEF or reversing factor); eIF-2B·eIF-2(αP), 15S complex of eIF-2B and eIF-2(αP); HRI, heme-regulated eIF-2α kinase.

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ATP-regenerating system (1 mM fructose-1,6-bisphosphate and 0.1 mM NAD^+) that provides [γ-^{32}P]ATP of constant specific activity and permits a quantitative analysis of phosphoprotein profiles for any selected time interval (21, 23). Reaction mixtures were concentrated by pH 5 precipitation, and the phosphoproteins were separated by sodium dodecyl sulfate and polyacrylamide gel electrophoresis (SDS/PAGE) and were analyzed by autoradiography (21, 23). Sucrose gradient fractions were also concentrated by pH 5 precipitation and were analyzed by Western blotting to nitrocellulose membranes as described (24). The eIF-2 in Western blot was located using goat polyclonal anti-eIF-2 IgG and rabbit anti-goat IgG horseradish peroxidase and developed with 4-chloro-1-naphthol and H_2O_2. The *in vitro* phosphorylation of polysomal eIF-2α was carried out with purified HRI and 125 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) at 30°C for 15 min as described (19).

RESULTS

Distribution of eIF-2(α^{32}P) in Heme-Deficient Lysates in the Presence and Absence of Cycloheximide. The inhibition of protein synthesis initiation in heme-deficient lysates is characterized in part by the rapid disaggregation of polysomes, which occurs after an initial linear phase of synthesis that persists for about 5 min (Fig. 1). Under normal conditions, the disaggregated polysomes accumulate as 40S/60S couples that sediment in sucrose density gradients as a large 80S ribosome peak as shown in Fig. 1*A*. In the presence of cycloheximide (10 μg/ml), an inhibitor of elongation, the polysomes in heme-deficient lysates are preserved and not degraded (Fig. 1*B*).

We have used this effect of cycloheximide to examine the site(s) of eIF-2α phosphorylation in heme-deficient lysates. When lysates are labeled with ^{32}P for 10 min, the distribution of eIF-2(α^{32}P) is readily determined by sedimentation in sucrose density gradients and electrophoretic separation of each fraction in polyacrylamide gels. As seen in Fig. 2, in normal protein-synthesizing lysates supplemented with hemin (20 μM), little phosphorylation of eIF-2α is found in the 60S, 80S, or polysomal fractions. In contrast, in the first 10 min of incubation in heme-deficient lysates, eIF-2(αP) accumulates in the 15S, 60S, and 80S fractions and, as expected, is barely detectable in the largely disaggregated polysome fraction (Fig. 2). In cycloheximide-treated heme-deficient lysates, however, the polysome profile remains intact, and eIF-2(α^{32}P) is readily detected in the polysome fraction (Fig. 2). A comparison of the 15S, 60S, 80S, and polysome fractions of different gradients representing 1, 2, 5, and 10 min of incubation of heme-deficient lysates is shown in Fig. 3. At 10 min, the levels of polysome-bound eIF-2(α^{32}P) in cycloheximide-treated lysates are comparable to those in the 80S peak of cycloheximide-free lysates (Fig. 3). Since polysomes of heme-deficient lysates are immobilized by cycloheximide treatment, this observation suggests that the α-subunit of eIF-2 bound to polysomes can be directly phosphorylated by HRI.

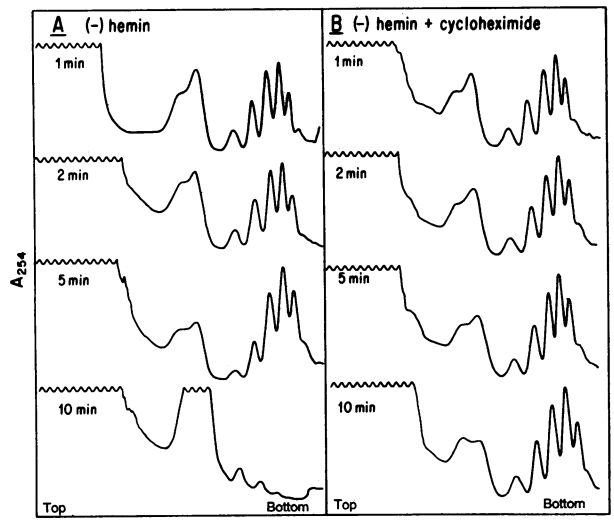


FIG. 1. UV (A_{254}) absorbance profiles of protein-synthesizing reticulocyte lysates. Protein synthesis reaction mixtures (80 μl) were supplemented where indicated with hemin (20 μM) and cycloheximide (10 μg/ml) and were incubated for 1, 2, 5, and 10 min at 30°C. Ribosomes were separated by sucrose density gradients. The top and bottom of the gradients are labeled as such.

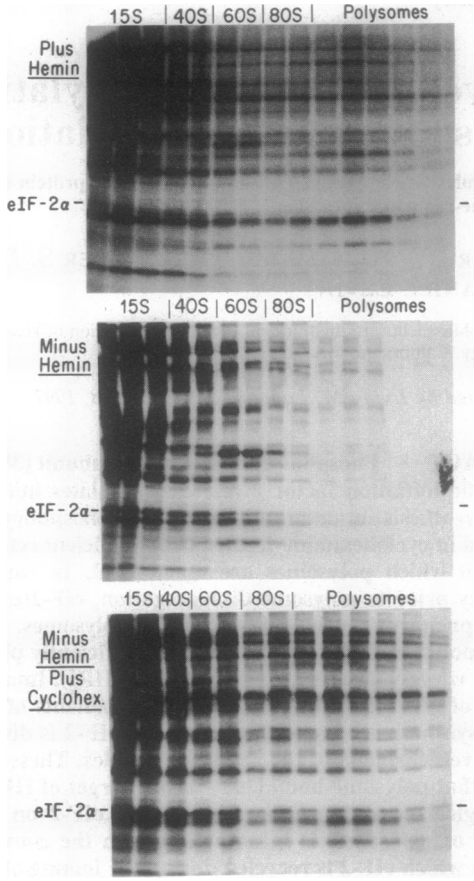


FIG. 2. ^{32}P-labeled phosphoprotein profiles of sucrose density gradients of protein-synthesizing lysates in the presence and absence of cycloheximide (Cyclohex.). Reticulocyte lysates were incubated for 20 min at 30°C under protein-synthesizing conditions containing [^{32}P]orthophosphate. Reaction mixtures were centrifuged in sucrose density gradients as described (7). Fractions were collected as in Fig. 1 and were analyzed as described in the text. The figure is an autoradiogram.

We have examined the ability of HRI to phosphorylate 80S- and polysome-bound eIF-2 *in vitro*. As shown in Fig. 4, 80S-bound eIF-2 and polysome-bound eIF-2 in fractions isolated from hemin-supplemented lysates were readily phosphorylated *in vitro* by HRI. A similar result was obtained with comparable fractions from cycloheximide-treated hemin-supplemented lysates (Fig. 4). These data indicate that eIF-2 is present on polysomes of hemin-supplemented lysates and can be phosphorylated by HRI.

Phosphoprotein Profiles of Reticulocyte Lysates Inhibited by Cycloheximide, Pactamycin, and Puromycin. We observed that the levels of total eIF-2(αP) in heme-deficient lysates were increased if cycloheximide (10–20 μg/ml) was also present. This finding suggested that maintenance of the

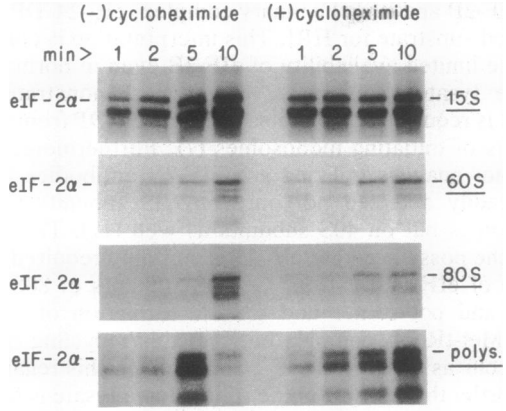


FIG. 3. Distribution of eIF-2(α^{32}P) in heme-deficient lysates in the presence and absence of cycloheximide. Protein synthesis in heme-deficient lysates was carried out with and without cycloheximide (20 μg/ml). Reaction mixtures were separately incubated for 1, 2, 5, or 10 min and then separated in sucrose density gradients. Fractions corresponding to 15 S, 60 S, 80 S, and polysomes (polys.) for each gradient were isolated, concentrated by pH 5 precipitation, and analyzed for eIF-2α phosphorylation. The autoradiograms for the ^{32}P-labeled phosphoproteins of 15 S, 60 S, 80 S, and polysomes display mainly the area containing eIF-2(αP).

polysomes was associated with increased phosphorylation of eIF-2α. To examine this possibility, the levels of total eIF-2(α^{32}P) were compared in lysates incubated with cycloheximide or with other inhibitors of translation that do not preserve polysome integrity, such as pactamycin and puromycin in the presence and absence of hemin (20 μM). Under each set of conditions, protein-synthesizing lysates were labeled with ^{32}P for 4 and 12 min, and the ^{32}P-labeled phosphoprotein profiles of the whole lysate were analyzed in polyacrylamide gels. As shown in Fig. 5, in normal hemin-supplemented lysates, none of the inhibitors gave rise to increased phosphorylation of eIF-2α (tracks 6, 10, and 14) compared to the plus hemin control (track 2), indicating that activation of HRI is not involved in the action of these three inhibitions. However, there is an increase in total eIF-2(α^{32}P) in cycloheximide-treated heme-deficient lysates (tracks 7 and 8) compared to untreated heme-deficient lysates (tracks 3 and 4). In contrast, neither puromycin (tracks 11 and 12) nor pactamycin (tracks 15 and 16) produces a comparable increase in eIF-2α phosphorylation over the minus heme control (tracks 3 and 4). This observation supports the conclusion that the increase in eIF-2α phosphorylation induced by cycloheximide treatment is associated with the maintenance of the polysomes.

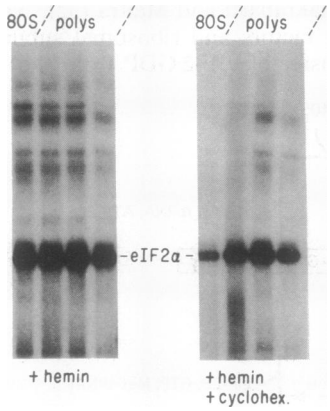


FIG. 4. Phosphorylation of polysome-bound eIF-2α by HRI *in vitro*. Protein-synthesizing lysates (80 μl) were supplemented where indicated with hemin and cycloheximide (cyclohex.) as in Fig. 1, incubated for 20 min at 30°C, and then separated in sucrose density gradients. Aliquots (200 μl) of the 80S and polysome fractions were phosphorylated by partially purified HRI. The figure is an autoradiogram.

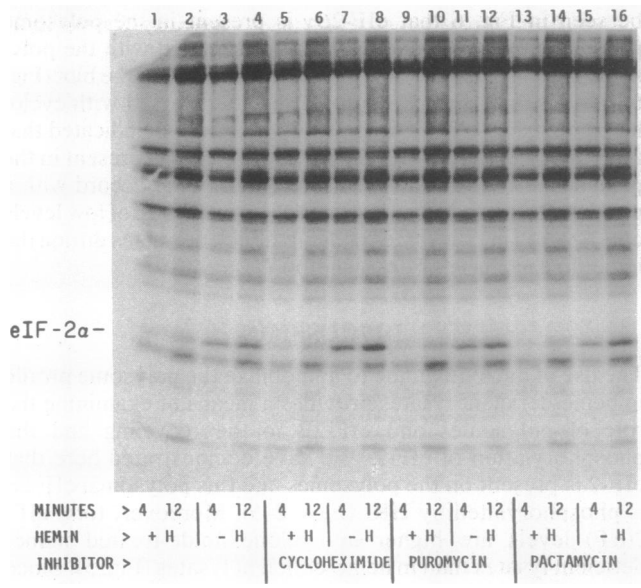


FIG. 5. ^{32}P-labeled phosphoprotein profile of reticulocyte lysates treated with cycloheximide, pactamycin, and puromycin. Protein synthesis reaction mixtures (25 μl) were supplemented where indicated with cycloheximide (20 μg/ml), pactamycin (2 μM), or puromycin (10 μg/ml) and incubated in the presence or absence of hemin (20 μM). All incubations contained 20 μCi of [^{32}P]orthophosphoric acid. Lysates were incubated under each set of conditions for 4 or 12 min at 30°C, concentrated by pH 5 precipitation (23), and separated by electrophoresis in 10% polyacrylamide/0.1% SDS as described (23). The figure is an autoradiogram.

Distribution of eIF-2 in Reticulocyte Lysates. Results presented in Figs. 2 and 3 indicated that eIF-2 is present in the polysomes as determined by the phosphorylation of eIF-2α. To determine the distribution of total eIF-2, Western blot analysis of sucrose gradient fractions was carried out. The anti-eIF-2 antibodies react strongly with eIF-2β and eIF-2γ but weakly with eIF-2α, which is not discernible in Fig. 6. It can

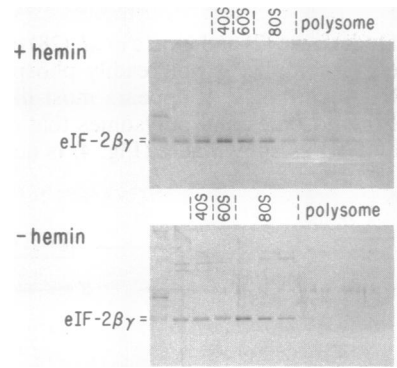


FIG. 6. Western blot analysis of eIF-2 distribution in reticulocyte lysates. Protein-synthesizing reticulocyte lysates (80 μl) were incubated for 20 min at 30°C in the absence and presence of hemin (20 μM). The reaction mixtures were centrifuged in sucrose density gradients (10–50%). Fractions were collected and separated by 10% SDS/PAGE. The protein was transferred to nitrocellulose membrane and then eIF-2 in the immunoblots was located as described in the text. (*Upper*) Gradient fractions of hemin-supplemented lysates. (*Lower*) Heme-deficient lysate fractions. The locations of 40S and 60S ribosomal subunits, 80S ribosomes, and polysomes are indicated. Lane 1 in each panel contains purified eIF-2 and serves as marker.

be seen in Fig. 6 that eIF-2$\beta\gamma$ is present in the polysome profile. The proportion of eIF-2$\beta\gamma$ associated with the polysomes represents only 5–10% of the total eIF-2 in the blot (Fig. 6). This is in agreement with the results obtained with cycloheximide-treated heme-deficient lysates, which indicated that a small percentage (5–10%) of total eIF-2(αP) is present in the polysomes (Fig. 2). This distribution also is in accord with a previous study that demonstrated the appearance of low levels of eIF-2(αP) in polysomes of heme-deficient lysates during the initial linear phase prior to shutoff (7).

DISCUSSION

The use of cycloheximide to immobilize the polysome profile in heme-deficient lysates provides a means of examining the role of polysomes and eIF-2B in the recycling and the phosphorylation of eIF-2. We have demonstrated here that eIF-2 is present on the polysomes and this polysomal eIF-2α is phosphorylated by HRI (Figs. 2–5). Moreover, total eIF-2(αP) levels are higher in cycloheximide-treated heme-deficient lysates than in heme-deficient lysates (Fig. 5). Since elongation is inhibited and polysomes are maintained but immobilized in the presence of cycloheximide, we are led to the conclusion that polysomal eIF-2 is a target for HRI and that maintenance of the polysome profile is associated with increased phosphorylation of eIF-2α. This correlation between polysome integrity and increased eIF-2(αP) levels is emphasized by studies in heme-deficient lysates using puromycin and pactamycin. As in the case of cycloheximide, these antibiotics do not affect HRI activity but, unlike cycloheximide, they do not maintain polysomes and do not cause an increase in eIF-2(αP) beyond control levels (Fig. 5). Our interpretation that eIF-2 in the 60S subunits of 80S initiation complexes and of polysomes is a target for HRI in reticulocyte lysates is consistent with the recent findings that yeast eIF-2α kinase, GCN2, is associated with 60S ribosomes of 80S initiation complexes (25).

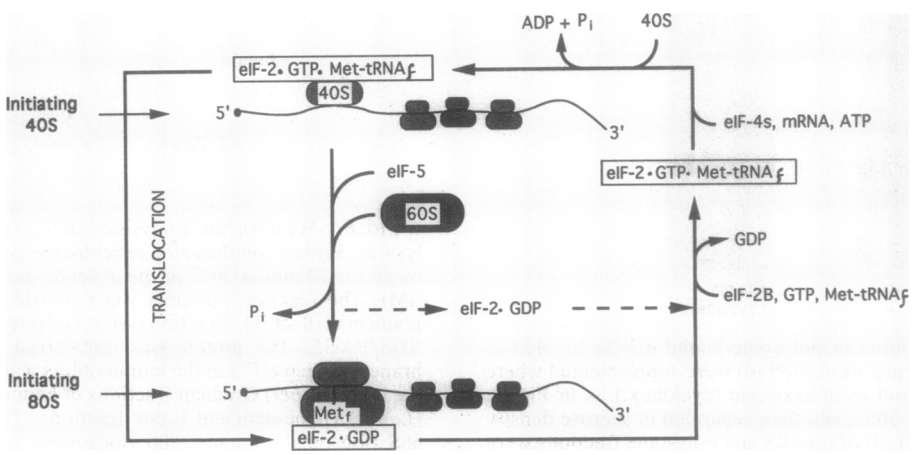
Our findings of increased phosphorylation of eIF-2α when polysomes are maintained by the addition of cycloheximide may also serve to explain the observation of Clemens and coworkers (26, 27) that a diminished rate of chain elongation that results from diminished amino acid tRNA synthetase activity is associated with increased phosphorylation of eIF-2α but with no change in eIF-2α kinase or phosphatase activity.

In earlier studies, we (2) and Grace *et al.* (28) observed that the eIF-2B·eIF-2 complex is not readily phosphorylated *in vitro* by HRI. Accordingly, it appears most likely that the eIF-2 on 80S monosomes and polysomes that can be phosphorylated by HRI as shown here (Fig. 4) is not complexed with eIF-2B and is in the binary complex, eIF-2·GDP, which is a good substrate for HRI. This interpretation is consistent with the limited availability of eIF-2B even in normal reticulocyte lysate. Previously, we have demonstrated that eIF-2B is required for the release of eIF-2·GDP from the 60S subunits of initiating monosomes (7). Furthermore, by immunoblot analysis utilizing anti-eIF-2B antibodies, eIF-2B was readily detected not only on 60S subunits and 80S monosomes but on 40S subunits as well (11). This finding raises the possibility that eIF-2B is not only required for the release of eIF-2·GDP from the 60S subunits of 80S monosomes and polysomes and for the formation of the eIF-2·GTP·Met-tRNA$_f$ complex but that this recycling of eIF-2 may occur as an eIF-2B·eIF-2 complex. In this regard, it is noteworthy that eIF-2B in the reticulocyte lysate is found in complex with eIF-2 and the purification of eIF-2B free of eIF-2 is accomplished with difficulty (1–3, 29, 30). Recently, the yeast equivalent of mammalian eIF-2B has been characterized (31). The yeast eIF-2B is also found to be complexed with eIF-2; subunits of yeast eIF-2B (GCD1, GCD2, and GCN3) and eIF-2α are found comigrating with the 48S preinitiation complex at restrictive temperature of three GCD mutants. These findings are consistent with our suggestion described above that the recycling of eIF-2 may occur as an eIF-2B·eIF-2 complex.

Based on the findings presented here and previous studies, we propose the following model for the recycling of eIF-2 (Fig. 7). The recycling of eIF-2 by eIF-2B-catalyzed exchange of GTP for GDP in the eIF-2·GDP complex may occur only partially from the initiating 80S monosomes due to the limiting amount of eIF-2B, thus permitting the remaining eIF-2·GDP to stay bound to the 60S subunits of ribosomes that are involved in the process of elongation. The polysome-bound eIF-2·GDP is a target for eIF-2B and also for HRI. The eIF-2(αP)·GDP can bind and sequester eIF-2B, which is then unavailable for GTP/GDP exchange, but the eIF-2B·eIF-2(αP) complex can dissociate from the ribosomes and then appear as a 15S complex in the nonribosomal cytosol (8). The recycling of eIF-2 or of an eIF-2B·eIF-2 complex may be viewed as a translocation from the 60S to the 40S subunit, a process that is very efficient and, perhaps, may be effected by eIF-2B alone.

The translocation of eIF-2 from the 40S subunit to the 60S subunit in the joining reaction that is catalyzed by eIF-5 involves the hydrolysis of GTP to GDP. The GTPase activity is intrinsic to eIF-5 (32). It remains to be determined whether the translocation is a property of eIF-5 alone.

Recently, Chakrabarti and Maitra (32), working with purified initiation factors and ribosomal subunits, have confirmed the transfer of eIF-2·GDP from the 40S to the 60S



subunits in an eIF-5-catalyzed reaction. They claim, however, that if the 60S subunits are pretreated with eIF-2 or, eIF-2·eIF-2B, this transfer to the 60S subunits does not occur and that, on release from the 40S subunits, the eIF-2·GDP is recycled and is not found in the 80S initiation complex. They claim further that although there is a specific binding site on the 60S subunit for eIF-2, the binding of eIF-2·GDP to the 60S subunits is diminished by 80% at 50 mM K^+, thus by implication raising a question of the significance of the binding that has been observed. Under far more physiologic conditions in whole reticulocyte lysates, however, we have found that eIF-2 and eIF-2B are tightly associated with 60S subunits and 80S ribosomes in gradients containing up to 100 mM K^+ (11). Furthermore, their model of recycling of eIF-2 would require the prior saturation of all 60S subunits with eIF-2 or eIF-2·eIF-2B complexes. However, as is shown in Fig. 6, the ratio of eIF-2 to ribosomes in polysomes is decreased as the size of the polysomes is increased. Therefore, under physiologic conditions, the 60S subunits of ribosomes are not all saturated with eIF-2.

From the present available evidence we conclude that under physiologic conditions, the 60S subunits of 80S initiation complexes and of polysomes are an integral part of the cycle of a significant portion of eIF-2. Our model does not preclude the possibility that some eIF-2 may be recycled during the joining of the 48S preinitiation complex and the 60S subunit.

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