eIF2 independently binds two distinct eIF2B subcomplexes that catalyze and regulate guanine-nucleotide exchange

Graham D. Pavitt,1,4 Kolluru V.A. Ramaiah,1,3 Scot R. Kimball,2 and Alan G. Hinnebusch1

1Laboratory of Eukaryotic Gene Regulation, National Institute of Child Health and Human Development, Bethesda, Maryland 20892 USA; 2Department of Cellular and Molecular Physiology, Pennsylvania State University, College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033 USA

eIF2B is a heteropentameric guanine-nucleotide exchange factor essential for protein synthesis initiation in eukaryotes. Its activity is inhibited in response to starvation or stress by phosphorylation of the α subunit of its substrate, translation initiation factor eIF2, resulting in reduced rates of translation and cell growth. We have used an in vitro nucleotide-exchange assay to show that wild-type yeast eIF2B is inhibited by phosphorylated eIF2 [eIF2(αP)] and to characterize eIF2B regulatory mutations that render translation initiation insensitive to eIF2 phosphorylation in vivo. Unlike wild-type eIF2B, eIF2B complexes with mutated GCN3 or GCD7 subunits efficiently catalyzed GDP exchange using eIF2(αP) as a substrate. Using an affinity-binding assay, we show that an eIF2B subcomplex of the GCN3, GCD7, and GCD2 subunits binds to eIF2 and has a higher affinity for eIF2(αP), but it lacks nucleotide-exchange activity. In contrast, the GCD1 and GCD6 subunits form an eIF2B subcomplex that binds equally to eIF2 and eIF2(αP). Remarkably, this second subcomplex has higher nucleotide-exchange activity than wild-type eIF2B that is not inhibited by eIF2(αP). The identification of regulatory and catalytic eIF2B subcomplexes leads us to propose that binding of eIF2(αP) to the regulatory subcomplex prevents a productive interaction with the catalytic subcomplex, thereby inhibiting nucleotide exchange.

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Guanine-nucleotide exchange factors regulate the activity of G proteins by controlling their rate of conversion from the inactive GDP-bound form to the active GTP-bound state (Bourne et al. 1991; Boguski and McCormick 1993). One regulatory mechanism controlling protein synthesis initiation in eukaryotes involves inhibition of the guanine-nucleotide exchange factor eIF2 in response to starvation or stress conditions. eIF2B converts its substrate, eukaryotic translation initiation factor 2 (eIF2), from an inactive eIF2·GDP binary complex to eIF2·GTP. This active complex binds charged initiator tRNA^Met (Met-tRNA^Met), forming a ternary complex, which interacts with the 40S ribosomal subunit. Following addition of mRNA and the 60S ribosomal subunit, the G-protein cycle is completed by hydrolysis of eIF2-bound GTP and the release of eIF2·GDP (for review, see Merrick 1992; Trachsel 1996). Thus, inhibition of eIF2 activity prevents eIF2 recycling, thereby reducing rates of translation initiation and cell growth.

Although functionally similar to the small GTPases and exchange factors of the Ras superfamily, eIF2 and eIF2B are complex proteins of three (α–γ) and five (α–e) nonidentical subunits, respectively. We considered it likely that the subunit complexity of eIF2B is attributable, at least in part, to its novel mechanism of regulation. eIF2B is inhibited indirectly by phosphorylation of its substrate, eIF2, on the α subunit (eIF2α) at residue serine 51. Three protein kinases, called PKR, HCR, and GCN2, specifically phosphorylate Ser-51 of eIF2α under different stress conditions (Clemens 1996). PKR (previously known as p68 kinase or DAI) is part of the antiviral response and is activated by double-stranded RNA, whereas HCR (also called HRI) is activated in mammalian reticulocytes in response to heme deprivation. Both kinases completely inhibit translation of eIF2α under different stress conditions (Clemens 1996). PKR is also called HRI) is activated in mammalian reticulocytes in response to heme deprivation. Both kinases completely inhibit eIF2B to shut off total protein synthesis. In the yeast Saccharomyces cerevisiae, the protein kinase GCN2 phosphorylates eIF2α in response to amino-acid or purine starvation to induce translation of GCN4 mRNA, encoding a transcriptional activator of amino acid biosynthetic genes (Hinnebusch 1996). General translation and cell growth are inhibited in yeast.
cells expressing mutant hyperactive forms of GCN2 (termed GCN2\(^\gamma\)) (Wek et al. 1990), human PKR, or rabbit HCR (Dever et al. 1993) just as occurs in mammalian cells when eIF2 is highly phosphorylated.

In the one published report demonstrating the catalytic activity of yeast eIF2B in vitro, its regulation by eIF2(\(\alpha\)P) was not examined (Cigan et al. 1993). The results of extensive genetic analysis, however, support the conclusion that yeast eIF2B is inhibited by eIF2(\(\alpha\)P) just as it is in mammalian cells. In addition, these studies have provided evidence for specific functions of eIF2B subunits. All five subunits (encoded by GCN3, GCD7, GCD1, GCD2, and GCD6) show extensive sequence identity with the corresponding mammalian polypeptides (called eIF2B\(\alpha--\epsilon\) respectively) (Bushman et al. 1996a; Pavitt et al. 1997). The four subunits encoded by GCD genes are essential, whereas the GCN3 subunit appears to be dispensable for eIF2B catalytic activity. GCN3 is required for inhibition of translation initiation by eIF2(\(\alpha\)P) in vivo (Hinnebusch and Fink 1983; Hannig and Hinnebusch 1988), however, implying that it mediates the inhibitory effects of eIF2(\(\alpha\)P) on eIF2B catalytic activity. A recent study using recombinant eIF2B expressed in insect cells showed that rat eIF2B\(\alpha\) has a function similar to GCN3, as it was not required for eIF2B catalytic activity, but was needed for inhibition by eIF2(\(\alpha\)P) (Fabian et al. 1997).

We previously obtained molecular and genetic evidence that the GCN3, GCD7, and GCD2 subunits all play roles in the inhibition of eIF2B by eIF2(\(\alpha\)P). When co-overexpressed, these subunits formed a stable trimeric subcomplex in vivo that could partially suppress the inhibitory effects of eIF2(\(\alpha\)P) (Table 1; Yang and Hinnebusch 1996). This effect implied that GCD2 and GCD7 participate directly with GCN3 in the inhibition of eIF2B by eIF2(\(\alpha\)P), GCN3, GCD7, and the carboxy-terminal half of GCD2 all share sequence similarity (Paddon et al. 1989; Bushman et al. 1993a), suggesting that their homologous regions might be devoted to this regulatory mechanism. This hypothesis was confirmed by the isolation of regulatory mutations in each of the three subunits that were clustered within the shared homologous regions (Vazquez de Aldana and Hinnebusch 1994; Pavitt et al. 1997). Surprisingly, given the apparent redundancy of function, a single missense mutation in any one gene was sufficient to completely eliminate the regulation of eIF2B by eIF2(\(\alpha\)P).

Here we describe a biochemical analysis of the mechanism of inhibition of yeast eIF2B by eIF2(\(\alpha\)P). We have used simple assays to demonstrate that eIF2B complexes with regulatory mutations in the GCN3 or GCD7 subunits overcome inhibition by eIF2(\(\alpha\)P) by accepting it as a substrate for guanine-nucleotide exchange. In addition, we show that the five eIF2B subunits can be divided into two distinct subcomplexes, each of which binds to eIF2 in vitro. One subcomplex, consisting of GCN3, GCD7, and GCD2, binds more tightly to the phosphorylated form of eIF2 and has no exchange activity. The second subcomplex, composed of GCD1 and GCD6, binds equally to eIF2 and eIF2(\(\alpha\)P) and has high-level exchange activity that is insensitive to eIF2 phosphorylation. These results suggest to us that eIF2B has distinct regulatory and catalytic binding surfaces for eIF2 that functionally interact to control eIF2B activity.

### Table 1. Suppression of eIF2\(\alpha\) hyperphosphorylation toxicity in vivo by mutation or overexpression of eIF2B subunits

| Relevant genotype | Growth rate of cells expressing the eIF2\(\alpha\) kinase GCN2\(^{-}\)513 |
|-------------------|----------------------------------------------------------|
| eIF2B mutations\(^a\) |                                          |
| none (wild type)  | 1+                                                        |
| gcn3\(\Delta\)     | 5+                                                        |
| GCD7-S119P (M1)   | 5+                                                        |
| GCD7-I118T, D178Y | 5+                                                        |
| Overexpressed subunits\(^b\) |                                        |
| none (vectors only) | 1+                                                        |
| GCN3, GCD7, GCD1, GCD2, GCD6 | 4+                                                        |
| GCD7, GCD1, GCD2, GCD6 | 5+                                                        |
| GCN3, GCD7, GCD2 | 3+                                                        |
| GCD1, GCD6      | 1+                                                        |
| GCD6            | 1+                                                        |

\(^a\)Data from Pavitt et al. (1997); growth scored on a scale of 0 for no growth to 5+ for wild-type growth, based on colony sizes.

\(^b\)Data taken from Yang and Hinnebusch (1996); growth scored on a scale of 0 for no growth to 6+ for wild-type growth, based on colony sizes.

#### Results

An in vitro assay for yeast eIF2B activity and its inhibition by eIF2(\(\alpha\)P)

To develop an in vitro assay for yeast eIF2B-catalyzed guanine-nucleotide exchange we purified eIF2 from a yeast strain overexpressing all three eIF2 subunits in which eIF2(\(\gamma\)) was modified by addition of six amino-terminal histidine residues (see Materials and Methods). This polyhistidine tag did not affect the function of eIF2(\(\gamma\)) in vivo as determined from growth rates in wild-type and GCN2\(^{-}\) mutant cells; the latter being a sensitive test for the inhibitory effects of eIF2(\(\alpha\)) phosphorylation on eIF2 activity (data not shown). The purified yeast eIF2 formed eIF2·\([\text{H}]\text{GDP}\) binary complexes that were very stable at 10\(^\circ\)C when challenged with a 100-fold excess of nonradio-labeled GDP (Fig. 1A). As a source of eIF2B to stimulate nucleotide exchange on eIF2·\([\text{H}]\text{GDP}\), we used extracts from yeast cells in which all five subunits were overexpressed by -10-fold. We showed previously that the overexpressed subunits form intact eIF2B complexes, as the majority of each subunit could be co-immunoprecipitated with an antibody against the GCD6 subunit (Dever et al. 1995). Addition of 150 µg of cell extract to the eIF2·\([\text{H}]\text{GDP}\) complexes led to rapid dissociation of the bound radionucleotide, whereas an equal amount of an extract from a strain expressing wild-type levels of eIF2B gave a slow rate of nucleotide exchange, only...
slightly above the spontaneous rate with no extract (Fig. 1A). These findings showed that the rapid dissociation of eIF2 $\cdot [3H]GDP$ binary complexes stimulated by the first extract could be attributed to the exchange activity of overexpressed eIF2B.

To examine the regulation of eIF2B activity, we phosphorylated eIF2 in vitro using purified rabbit HCR. Isoelectric-focusing (IEF) PAGE analysis showed that eIF2 was rapidly and completely phosphorylated following the addition of HCR (Fig. 2A). eIF2(aP) $\cdot [3H]GDP$ binary complexes formed with the same efficiency and stability as binary complexes with unphosphorylated eIF2. Addition of the same extract overexpressing all five wild-type eIF2B subunits, however, failed to stimulate release of the bound nucleotide (Fig. 1A). This result demonstrates for the first time that eIF2(aP) is not a substrate for yeast eIF2B in vitro.

eIF2B regulatory mutants can catalyze guanine-nucleotide exchange on eIF2(aP)

Having established an in vitro assay for eIF2B activity that mimics the regulation observed in vivo, we wished to examine guanine-nucleotide exchange using mutant forms of eIF2B to determine their mechanism of action. As indicated above, we showed previously that the reduced cell growth rate resulting from high levels of eIF2(aP) caused by expression of hyperactive GCN2-kinas in yeast can be alleviated by deletion of GCN3 (Dever et al. 1993) or completely suppressed by certain missense mutations in GCN3, GCD7, or GCD2 (Vazquez de Aldana and Hinnebusch 1994; Pavitt et al. 1997). The growth phenotypes of the gcn3A and gcd7 regulatory mutants relevant to this study are summarized in Table 1. We also showed previously by immuno blot analysis that the regulatory mutations did not affect the expression levels of any eIF2B subunits (except, of course, GCN3 in the gcn3A mutant). In addition, a combination of genetic tests and co-immunoprecipitation experiments demonstrated that the GCD7 mutations did not reduce the association of GCN3 with eIF2B, eliminating this possible mode of action (Pavitt et al. 1997). Therefore, it seemed likely to us that these regulatory mutations act by one of two mechanisms: (1) by allowing the inhibitor to act as a substrate for guanine-nucleotide exchange; or (2) by altering the relative binding affinities for phosphorylated and unphosphorylated eIF2 so that eIF2(aP) is rendered an ineffective inhibitor of mutant eIF2B. These models predict different fates for eIF2(aP) $\cdot GDP$ binary complexes. The first model predicts eIF2(aP) $\cdot GDP$ is a substrate for nucleotide exchange, while in the second model it is not. We used our exchange assay to distinguish between these two mechanisms.

To examine the regulatory defect caused by the mutations, we analyzed extracts from cells overexpressing the
four essential subunits of eIF2B [i.e., lacking GCN3 to mimic the effect of a deletion of GCN3 (termed eIF2B*4s)], or overexpressing all five subunits of eIF2B with one of two missense mutations in GCD7 ([GCD7-S119P (termed eIF2B*M1) or GCD7-I118T,D178Y (termed eIF2B*M2)]. We found that all three mutant protein complexes promoted dissociation of [3H]GDP from eIF2 binary complexes independently of eIF2 phosphorylation (Fig. 1B–D). Interestingly, eIF2B*4s catalyzed guanine-nucleotide exchange at a faster rate than did wild-type eIF2B, apparently increasing the rate of exchange by twofold at early time points (Fig. 1A,B).

It was important to demonstrate that eIF2α(P) was not being dephosphorylated during the exchange reactions. Accordingly, we used IEF PAGE analysis to examine the level of eIF2α phosphorylation at three time points during the reaction. The data in Figure 2B revealed that 97%–98% of the eIF2α was phosphorylated at time=0 and that no dephosphorylation occurred over the course of the GDP exchange reactions. These results demonstrate that the regulatory alterations in the mutant eIF2B complexes allow both eIF2α(P) and eIF2 to be used as substrates for GDP exchange with similar efficiencies. These findings can account for the fact that strains bearing these eIF2B mutations maintain protein synthesis and continue growing when a majority of the eIF2 in the cell is phosphorylated (Vazquez de Aldana and Hinnenbusch 1994; Dever et al. 1995; Pavitt et al. 1997), as eIF2α(P) no longer inhibits the guanine-nucleotide exchange activity of eIF2B.

GCD6 has nucleotide-exchange activity in vitro that is enhanced by co-overexpression of GCD1

To overexpress eIF2B in the experiments just described, we used a two-plasmid system, one to overexpress GCD1 and GCD6 and one of a series of second plasmids overexpressing wild-type or mutant versions of GCD2, GCD7, and GCN3. This system allowed us to investigate whether any eIF2B partial complexes have guanine-nucleotide exchange activity. We performed GDP-exchange assays using cell extracts co-overexpressing wild-type GCN3, GCD2, and GCD7 (h.c. GCN3/GCD2/GCD7), the mutants (h.c. GCN3/GCD2/GCD7*M1 or h.c. GCN3/GCD2/GCD7*M2) or the combination of GCD2 and GCD7 (h.c. GCD2/GCD7) (Fig. 3A–D). In each case, no significant nucleotide exchange was observed over background levels, showing that these overexpressed subunits do not possess eIF2B catalytic activity.

In sharp contrast to these results, we found that the overexpressed GCD1 and GCD6 subunits (h.c. GCD1/GCD6) showed full eIF2B catalytic activity (Fig. 3E), identical to the highest activity seen with the extract overexpressing eIF2B*4s (Fig. 1B). This result was unexpected because, unlike overexpressed wild-type eIF2B or eIF2B*4s, co-overexpression of GCD1 and GCD6 alone did not overcome the inhibitory effects of eIF2 hyperphosphorylation in yeast cells (Table 1). To characterize this exchange activity further, we asked whether it was inhibited by prephosphorylation of eIF2α. Similar to our findings on the eIF2B*4s and mutant five-subunit complexes, nucleotide-exchange activity in the h.c. GCD1/GCD6 extracts was unaffected by phosphorylation of eIF2α (Fig. 3E). Importantly, immunoblotting experiments determined that the levels of GCD1 and GCD6 were the same in extracts overexpressing GCD1 and GCD6 as in extracts overexpressing wild-type five subunit eIF2B (data not shown).

We next tested whether GCD1 or GCD6 alone possesses guanine-nucleotide exchange activity. A recent study using recombinant rat eIF2B expressed in insect cells found that eIF2Be alone (the homolog of GCD6) possessed very low-level exchange activity, -30-fold below that of wild-type eIF2B (Fabian et al. 1997). Activity this low would be undetectable in our assay. We found, however, that overexpressed GCD6 alone possessed eIF2B activity only slightly lower than that observed with wild-type five-subunit eIF2B (Fig. 3F). GCD1 and GCD6 show 47% sequence similarity over the entire length of GCD1 (Bushman et al. 1993a). Despite this sequence similarity, we found that GCD1 alone had no exchange activity. Surprisingly, immunoblotting experiments showed that the level of GCD6 was approximately fourfold higher when overexpressed singly compared with co-overexpression with GCD1 (data not shown).
shown). When the relative protein levels are accounted for, these results show that GCD6 is the catalytic subunit of eIF2B and that GCD1 enhances its nucleotide-exchange activity \( \sim 8 \) - to 10-fold. In addition, they imply that GCD1 and GCD6 form a stable subcomplex that binds to eIF2 in the absence of the other three subunits. We provide additional evidence to support the latter assertion below.

Wild-type and mutant eIF2B bind with higher affinity to eIF2(\( \alpha \P \)) than to eIF2

By use of enzyme kinetic methods, it was demonstrated that mammalian eIF2(\( \alpha \P \)) was not a substrate for eIF2B and that eIF2B has a higher affinity for the inhibitor, eIF2(\( \alpha \P \)), than for the substrate, eIF2 (Goss et al. 1984; Rowlands et al. 1988). Rowlands et al. (1988) proposed that eIF2(\( \alpha \P \)) is a competitive inhibitor of nucleotide exchange that acts through repeated noncatalytic binding and release of the eIF2B. Using our exchange assay, we showed above that yeast eIF2(\( \alpha \P \)) was not a substrate for yeast eIF2B. Next, we used the fact that the purified polyhistidine-tagged eIF2 binds to Ni-NTA-agarose affinity resin to devise a pull-down assay to measure stable binding between eIF2B overexpressed in yeast extracts and purified eIF2. The eIF2 - eIF2B complexes formed in solution were captured on the affinity resin, washed, and eluted with imidazole. The fraction of eIF2B subunits bound to eIF2 was assessed by SDS-PAGE and Western blotting. Using this assay, we could show that binding of all five eIF2B subunits to eIF2(\( \alpha \P \)) was reproducibly about 2-fold higher than to unphosphorylated eIF2 and 10-fold higher than the background level of binding seen with no eIF2 added to the reactions (Fig. 4A,B, lanes 2–4). Except GCD2, none of the individual eIF2B subunits present in extracts overexpressing single eIF2B subunits bound to eIF2 or eIF2(\( \alpha \P \)) significantly above background levels (Fig. 5A,B, lanes 5–16 and Fig. 7A,B, lanes 5–12, below). [GCD2 bound to eIF2 but not eIF2(\( \alpha \P \)) (Fig. 5A,B, lanes 10,11); we are currently investigating further this result.] These findings indicate that, like the mammalian enzyme, yeast eIF2B has a higher binding affinity for eIF2(\( \alpha \P \)) than for eIF2, consistent with the idea that eIF2(\( \alpha \P \)) is a competitive inhibitor of eIF2B. Similar re-
results were obtained when we examined the binding of the four-subunit form of eIF2B (eIF2B*4s; Fig. 4A,B, lanes 6–8).

We showed previously that overexpressed GCN3, GCD2, and GCD7 form a stable trimeric complex that can suppress the toxic effects of eIF2 phosphorylation in vivo (Yang and Hinnebusch 1996). To explain this finding we suggested that this trimeric subcomplex binds to eIF2(aP) independently of GCD1 and GCD6. In agreement with this prediction, co-overexpressed GCN3, GCD2, and GCD7 bound to eIF2(aP) at levels approximately 10-fold above background and 3-fold higher than to eIF2 (Fig. 5A,B, lanes 2–4). The implications of these results for the mechanism of action of eIF2B and the trimeric GCN3/GCD2/GCD7 subcomplex are discussed later.

GCD1 forms a subcomplex with GCD6 to stabilize the interaction of GCD6 with eIF2

As the catalytic activity of overexpressed GCD6 was greatly stimulated by the co-overexpression of GCD1, we surmised that GCD1 and GCD6 can form a stable subcomplex in the absence of the other three subunits. To provide direct evidence for this conclusion, we modified GCD1 by adding a carboxy-terminal polyhistidine tag and verified by growth assays that the His-tagged GCD1 gene on a single-copy-number plasmid was fully capable of substituting for wild-type GCD1 in vivo (data not shown, see Materials and Methods). When co-overexpressed with GCD6, His-tagged GCD1 specifically bound a large fraction of the excess GCD6 to Ni–NTA silica resin, while only a small fraction of overexpressed GCD6 was bound to the resin in a control reaction where GCD1 was untagged (Fig. 6A,B, lanes 4,5). These data demonstrated that GCD1 and GCD6 could form a stable subcomplex in vivo. Next we asked if GCD1 could stabilize the binding between GCD6 and eIF2. In a pull-down experiment with extracts overexpressing untagged GCD1 and GCD6 and purified His-tagged eIF2, we found that, when co-overexpressed, GCD1 and GCD6 bound to eIF2 at four- to fivefold higher levels than when each protein was overexpressed singly (Fig. 7, lanes 2–4,6–8) from A relative to the density of the signal in lane 4, which was assigned an arbitrary value of 1. Mean densitometry for all subunits is shown.

Discussion

Regulatory mutations isolated in GCN3, GCD2, and GCD7 overcome the inhibitory effects of eIF2 phosphorylation

Mechanism of action of eIF2B regulatory mutations

Regulatory mutations isolated in GCN3, GCD2, and GCD7 overcome the inhibitory effects of eIF2 phosphorylation.
phorylation on cell growth and activation of GCN4 expression (Vazquez de Aldana and Hinnebusch 1994; Pavitt et al. 1997). These mutations alter residues within homologous regions of each subunit. A single missense substitution in any one subunit is sufficient to disrupt this regulatory function, suggesting that all three subunits act together to mediate the negative regulation of eIF2B by eIF2(αP). Fabian et al. (1997) recently demonstrated that rat eIF2B lacking the α subunit (the homolog of GCN3) was not sensitive to regulation by eIF2(αP), thereby mimicking in vitro the effects of deleting GCN3 in yeast cells. By use of a competition assay in which nucleotide exchange on eIF2-[^3H]GDP is inhibited by the addition of eIF2(αP), however, they could not elucidate the mechanism by which inhibition was overcome.

In our in vitro assays for guanine-nucleotide exchange and eIF2 binding by eIF2B, all of the eIF2 in the preformed eIF2-[^3H]GDP binary complexes was either fully phosphorylated (at least 98% phosphorylated) or unphosphorylated. We began by showing that eIF2(αP) was completely inactive as a substrate for guanine-nucleotide exchange by wild-type eIF2B (Fig. 1). The eIF2 binding experiments demonstrated that eIF2B bound to eIF2(αP) with a higher affinity than to unphosphorylated eIF2 (Fig. 4). Together, these experiments established that

Four-subunit form of eIF2B lacking the α subunit (the homolog of GCN3) was not sensitive to regulation by eIF2(αP), thereby mimicking in vitro the effects of deleting GCN3 in yeast cells. By use of a competition assay in which nucleotide exchange on eIF2-[^3H]GDP is inhibited by the addition of eIF2(αP), however, they could not elucidate the mechanism by which inhibition was overcome.

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Catalytic and regulatory subcomplexes in eIF2B

Our second major finding is the formation of a subcomplex between the GCD1 and GCD6 subunits (Fig. 6), which binds with equal affinity to eIF2α(P) or eIF2α (Fig. 7) and has the full nucleotide exchange activity of eIF2B in vitro (Fig. 3). It was shown recently that rat eIF2Bα alone (the homolog of GCD6) has very low catalytic activity in a recombinant baculovirus expression system (Fabian et al. 1997). Similarly, yeast GCD6 alone has catalytic activity (Fig. 3). In addition, we find that GCD1 alone has no catalytic activity, but stabilizes the binding of eIF2 to GCD6 (Fig. 7), stimulating catalytic activity approximately ninefold. These findings demonstrate that GCD6 is the catalytic subunit of eIF2B and imply that GCD1 serves to stabilize the eIF2 - eIF2B interaction. Consistent with the latter conclusion, we found that several conditional-lethal (temperature-sensitive) mutations in GCD1 were suppressed in vivo by overexpression of all three subunits of eIF2, implying that an increase in eIF2 concentration restores the eIF2 - eIF2B interaction weakened by these gdc1 mutations (Dever et al. 1995; G.D. Pavitt and A.G. Hinnebusch, unpubl.).

There is a striking parallel to this proposed role for GCD1 in the binding of the nucleotide-exchange factor GrpE to the ATPase domain of its substrate DnaK. In the crystal structure of this complex, a GrpE homodimer binds to DnaK, but only one GrpE molecule (GrpE2) makes contacts with DnaK. These interactions promote the structural rearrangements in DnaK required to mediate nucleotide exchange. The second GrpE molecule binds only to GrpE1, thereby stabilizing the productive interaction of GrpE1 with DnaK (Harrison et al. 1997). As GCD1 is similar along its entire length to GCD6 (Bushman et al. 1993a) and as shown here, stabilizes the binding of eIF2 to GCD6 and enhances GCD6 exchange activity, the functional relationship between the GrpE subunits is easy to imagine for GCD1 and GCD6.

We showed previously that GCD2, GCD7, and GCN3 can form a stable subcomplex in vivo that, when overexpressed, mimicked the effect of eIF2B regulatory mutations by suppressing the growth inhibition caused by eIF2 hyperphosphorylation (Yang and Hinnebusch 1996; see Table 1). Using genetic data, we argued that this subcomplex does not possess guanine-nucleotide exchange activity on its own, but can sequester eIF2α(P) and allow
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native eIF2B to catalyze guanine-nucleotide exchange on
the pool of unphosphorylated eIF2 in the cell. In support
of this model, we found that the GCN3/GCD7/GCD2
subcomplex does not possess guanine-nucleotide ex-
change activity (Fig. 3) and that it can interact stably
with eIF2 with a binding preference for eIF2(αP) similar
to that of native eIF2B (Figs. 4, 5). Therefore, when
overexpressed, the GCN3, GCD7, and GCD2 form a regu-
lar subcomplex that could act in vivo to titrate eIF2(αP)
away from native eIF2B.

A second function for eIF2B?

The finding that the GCD1/GCD6 subcomplex has
high-level nucleotide-exchange activity in cell extracts
but is not sufficient to provide the essential function of
eIF2B in vivo is interesting. GCD2 and GCD7 are essen-
tial genes. In addition, as shown in Table 1, overexpress-
ion of the four essential subunits of eIF2B (eIF2B*4s) is
sufficient to overcome the growth-inhibitory effects of
eIF2α hyperphosphorylation (a sensitive, but indirect
measurement of eIF2B activity), whereas co-overex-
pression of just GCD1 and GCD6 has no such effect. In cell
extracts, however, both complexes show identical high-
level eIF2B activity that is insensitive to eIF2 phos-
phorylation. How can we explain this difference between
cell extracts and intact cells?

According to the currently accepted view of protein
synthesis initiation, eIF2 is spontaneously released from
the 48S ribosome following hydrolysis of eIF2-bound
GTP. This allows 60S subunit joining, to form the 80S
initiation complex, and the commencement of transla-
tion elongation. Subsequently, eIF2B catalyzed guanine-
nucleotide exchange on eIF2 occurs free from the ribo-
some (Merrick 1992). Several reports, however, suggest
that, with phosphorylation of mammalian eIF2,
eIF2(αP)-GDP remains associated with ribosomes, and
exogenously added eIF2B can stimulate the release of
eIF2(αP)-GDP (De Benedetti and Baglioni 1983; Thomas
et al. 1985; Gross et al. 1987; Ramalh et al. 1992). This
finding suggests that eIF2B functions to release eIF2
GDP from ribosomal subunits during the initiation
process and that this second activity is also inhibited
by eIF2 phosphorylation. Consistent with this idea,
48S initiation complexes bearing eIF2 were observed in
gcd1 and gcd2 mutants (Cigan et al. 1991; Foiani et al.
1991). Therefore, we speculated that yeast eIF2B simi-
larly performs a second function to release eIF2·GDP
from ribosomes. Although the GCD1/GCD6 subcom-
plex is competent for nucleotide exchange on free
eIF2·GDP in vitro, it is possible that GCD2 and GCD7
are additionally required in vivo to localize eIF2B to the
ribosome or to release eIF2·GDP from ribosomal sub-
units.

Separate regulatory and catalytic binding surfaces
for eIF2 mediate inhibition of nucleotide exchange

Our results demonstrate that the catalytic and regula-
tory subcomplexes in eIF2B can both bind eIF2 indepen-
dently in vitro. The stability of the interaction between
eIF2 and the regulatory subcomplex is significantly in-
creased by phosphorylation of eIF2, whereas the catalytic
subcomplex binds equally to phosphorylated and un-
phosphorylated eIF2.

As eIF2γ possesses the GDP/GTP-binding domain
(Hannig et al. 1993), and eIF2α bears the phosphoryla-
tion site, it is tempting to speculate that the eIF2B catalyt-
ic subcomplex interacts with the γ subunit of eIF2,
whereas the regulatory subcomplex binds eIF2α. We pro-
pose that the presence of the regulatory subcomplex in
eIF2B provides a high-affinity interaction with the α sub-
unit of eIF2 that is incompatible with the proper binding
of eIF2 to the catalytic subcomplex required for nucleo-
tide exchange. Thus eIF2 would initially bind to eIF2B in
a nonproductive mode dominated by interactions be-
tween eIF2α and the regulatory subcomplex. When eIF2
is unphosphorylated, as depicted in Figure 8A, an iso-
merization in eIF2B would then shift binding into a pro-
ductive mode involving interactions between eIF2γ and
the catalytic subcomplex. When phosphorylated eIF2
binds, however, the isomerization would be inhibited,
preventing nucleotide exchange (Fig. 8B). The catalytic
subcomplex alone is capable of high-affinity binding to
eIF2, but cannot distinguish between eIF2 and eIF2(αP)
because it does not interact with the α subunit of eIF2
(Fig. 8C). The eIF2B complexes with regulatory muta-
tions in GCN3 and GCD7 analyzed here fail to distin-
guish between eIF2 and eIF2(αP) in the nucleotide ex-
change assays, but at least the eIF2B*4s complex showed
the higher affinity binding of eIF2(αP) versus eIF2 char-
acteristic of wild-type eIF2B. These data suggest that
the mutant eIF2B complexes catalyze nucleotide exchange
with eIF2(αP) because the isomerization from nonpro-
ductive to productive binding is no longer blocked (Fig.
8D).

Equilibrium binding assays will be needed to deter-
mine the dissociation constants for interactions between
eIF2 and the different eIF2B complexes and subcom-
plexes to establish the validity of these interpretations.
In addition, a clearer picture of how eIF2(αP) inhibits
eIF2B will require a deeper understanding of the steps in
the guanine-nucleotide exchange reaction. With the as-
says developed here and identification of the yeast
GCD1/GCD6 subcomplex as a highly efficient exchange
factor, it should be possible to analyze the mechanism
of the exchange reaction in greater detail. It may also be
feasible to obtain structural information on the eIF2B
catalytic subunit, GCD6, or the stable complex formed
between eIF2, GCD1, and GCD6.

Materials and methods

Yeast strains

Standard methods were used for transformation (Ito et al. 1983)
and genetic manipulation of yeast strains (Sherman et al. 1974).
For making extracts containing overexpressed eIF2B subunits,
plasmid transformants of strain BJ1995 (MATa leu2 trp1 ura3-
52 gal2 pep4-3 prb1-1122) (Jones 1991) were employed. Strain
GP3511 (MATa leu2-3110 ura3-52 ino1 gcn2::pep4::LEU2
leu2-1 pep4::LEU2
leu2-3110 ura3-52 ino1 gcn2::pep4::LEU2
leu2-1 pep4::LEU2
leu2-3110 ura3-52 ino1 gcn2::pep4::LEU2
leu2-1 pep4::LEU2
sui2Δ HIS4-lacZ bearing plasmid pAV1089(SU12 SU13 Histagged GCD11 URA3) was used for overexpression and purification of polyhistidine-tagged eIF2. GP3511 was constructed as follows: (1) GCN2 was deleted with p1144 (Dover et al. 1992) in strain H1648 (MATa leu2-3 leu2-112 ura3-52 ino1 sui2Δ HIS4-lacZ bearing plasmid p918(SU12 LEU2) (a gift from Tom Dever), to generate strain H2573; (2) plasmid shuffling (Boeke et al. 1995), generating plasmid Ep517 (Dorris et al. 1995), introducing the polyhistidine tag SGHHHHHHTG (single-letter code) between the first and second codons of GCD11. (3) Subcloning the I–I fragment used in this plasmid failed to complement fully a deletion of GCD11 in some strains when present on a low-copy plasmid (T. Dever pers. comm.). To overcome this defect, an additional 0.8 kb of GCD11 5′ noncoding DNA was subcloned upstream of the GCD11–6×His allele to generate pAV1043. (4) Overexpression of wild-type and regulatory mutant proteins with missense substitutions overexpressing wild-type GCN3 and GCD2 together with GCD7 regulatory mutant proteins with missense substitutions overexpressing all three subunits of eIF2 to the same levels as eIF2B (eIF2B*), making contact with the altered regulatory subcomplex (altered shaped stippled box labeled 2 3 7). The regulatory defect allows the conformational change needed for productive interaction between eIF2γ and the catalytic subcomplex even when eIF2 is phosphorylated.

Plasmids

Standard methods were used for the manipulation of DNA (Sambrook et al. 1989). pRS425 and pRS426 are LEU2- and URA3-marked 2μ plasmids, respectively (Christian et al. 1992). Plasmid p1873 is a derivative of pRS425 for overexpressing GCD1 and GCD6 (Dover et al. 1995). Derivatives of pRS426 for overexpressing different combinations of eIF2B subunits were: p1871 (GCD2, GCD7, GCN3) and p1872 (GCD2, GCD7) (Dover et al. 1995), and p2297 (GCD2), p2305 (GCD7), p2304 (GCN3), p2301 (GCD1), p2300 (GCD6), and p2302 (GCD1, GCD6) (Yang and Hinnebusch 1996).

pAV1089 is a 2μ URA3 plasmid used to overexpress all three subunits of eIF2 [SU12 (α), SU13 (β), and GCD11 (γ)] with polyhistidine-tagged GCD11 that was constructed in three steps. (1) Site-directed mutagenesis (Altered Sites mutagenesis kit, Promega) introduced the polyhistidine tag SGHHHHHHHTG (single-letter code) between the first and second codons of GCD11 in plasmid Ep517 (Dorris et al. 1995), generating plasmid pAV1027 (CEN LEU2 GCD11–6×His). This plasmid complemented the gcd11Δ allele in strain EY551 (MATa leu2-3 leu2-112 ura3-52 gcd11::hisG CEN LEU2 GCD11–6×His bearing plasmid Ep291[YPc50 GCD11]) (Dorris et al. 1995) when Ep293 was eliminated by plasmid shuffling. (2) The GCD11 fragment used in this plasmid failed to complement fully a deletion of GCD11 in some strains when present on a low-copy plasmid (T. Dever pers. comm.). To overcome this defect, an additional 0.8 kb of GCD11 5′ noncoding DNA was subcloned upstream of the GCD11–6×His allele to generate pAV1043. (3) Subcloning the GCD11–6×His allele from pAV1043 on an Nhel–Xbal fragment into p1778, the 2μ plasmid overexpressing eIF2α, eIF2β (as in A with an added filled circle), and eIF2γ (as in A) permissive exchange with GDP for GTP (grey filled circle with dark center). (B) Inhibition of nucleotide exchange by phosphorylated eIF2. eIF2α(P) (as in A with an added filled circle) labeled –P binds to the eIF2β regulatory subcomplex with high affinity (bold arrow), this inhibits the conformational change in eIF2B, preventing nucleotide exchange even with eIF2α(P). (D) Regulatory mutant eIF2B can perform nucleotide exchange with eIF2α(P). eIF2α(P) binds to mutant eIF2B (eIF2B*), making contact with the altered regulatory subcomplex (altered shaped stippled box labeled 2 3 7). The regulatory defect allows the conformational change needed for productive interaction between eIF2γ and the catalytic subcomplex even when eIF2 is phosphorylated.

Figure 8. A sequential binding model for eIF2B catalyzed guanine-nucleotide exchange and its inhibition by eIF2α(P). (A) Proposed two-binding surface scheme for GDP/GTP exchange with unphosphorylated eIF2. The eIF2 (unfilled oval labeled α, β, γ) in a binary complex with GDP (light grey filled circle with a light center) makes initial contact with eIF2B via a surface on the regulatory GCN3/GCD7/GCD2 subcomplex (stippled shape labeled 2 3 7). A hypothetical conformational change in eIF2B (movement indicated by grey arrows) occurs to promote correct contact between eIF2γ and the eIF2B catalytic subcomplex (filled black with subunits labeled 6 1), permitting exchange of GDP for GTP (grey filled circle with dark center). (B) Inhibition of nucleotide exchange by phosphorylated eIF2. eIF2α(P) (as in A with an added filled circle) labeled –P binds to the eIF2β regulatory subcomplex with high affinity (bold arrow), this inhibits the conformational change in eIF2B, preventing nucleotide exchange even with eIF2α(P). (D) Regulatory mutant eIF2B can perform nucleotide exchange with eIF2α(P). eIF2α(P) binds to mutant eIF2B (eIF2B*), making contact with the altered regulatory subcomplex (altered shaped stippled box labeled 2 3 7). The regulatory defect allows the conformational change needed for productive interaction between eIF2γ and the catalytic subcomplex even when eIF2 is phosphorylated.
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1989), the 2.1-kb C1al—Bsp120 l fragment containing either GC7-S119P (M1) from pAV1079 (Pavitt et al. 1997) or GC7-1118T, D178Y (M2) from p1563 (Vazquez de Aldana and Hinnebusch 1994), and the high-copy GCN3 URA3 plasmid p2304, cut with Spel and Noti. These plasmids contained restriction frag-ments equivalent to those present in p1871, from which wild-type GC7, GCD2, and GCN 3 were co-overexpressed (Dever et al. 1999).

General protein methods

Protein concentrations were determined by the Bradford assay (BioRad) using BSA as a standard. eIF2 and eIF2B proteins were resolved by 12% SDS-PAGE (Sambrook et al. 1989) and detected by Western blotting as described (Dever et al. 1995; Yang and Hinnebusch 1996) with the appropriate rabbit polyclonal pri-mary antisera (Cigan et al. 1989, 1991; Bushman et al. 1993b), an HRP-conjugated anti-rabbit secondary antibody, and the enhanced chemiluminescence system from Amersham.

Purification of His-tagged eIF2

His-tagged eIF2 was purified by use of Ni-NTA–agarose (Qiagen) and heparin–Sepharose (Pharmacia) chromatography as de-scribed by Erickson and Hannig (1996) with the following modi-fi-cations. We used the plasmid pAV1089 to overexpress His-tagged eIF2 in the protease deficient ( pep4), gcn2A strain GP3511 described above. We added phosphatase inhibitors NaF (50 mm) and Na3VO4 (100 µm) to breaking buffer and Ni-col-umn-loading buffer, and NaF only (5 mm) to all subsequent buff-ers. Cell lysis was done in a bead-beater (Biospec Products) with ice-cold acid-washed glass beads with 5×45-s beardings and 1-min intervals for cooling. Column fractions containing eIF2 were identified by 12% SDS-PAGE and by assaying for ternary complex formation activity (Hannig et al. 1993) with yeast [3H]Met-tRNAMet in 50-µl aliquots. Subunit integrity of the final preparation was confirmed by 12% SDS-PAGE, Western blotting with anti-sera to the α, β, and γ subunits, IEF PAGE to determine the phosphorylation state of eIF2γ (see below), and assaying for ternary complex formation. The yield was ~5 mg of eIF2 from 100 grams (wet weight) of yeast cells, at 75%–80% purity.

Guanine-nucleotide exchange assays

Total yeast cell protein extracts (10–15 µg/µl) containing the indicated overexpressed subunits of eIF2B were prepared from cells grown in synthetic complete media. Cells were grown to A600 = 0.5–1.0, harvested by centrifugation, washed with sterile deionized water, resuspended in breaking buffer (20 mm Tris-HCl at pH 7.5, 100 mm KCl, 10 mm 2-aminopurine (Sigma), 3 mm MgCl2, 5 mm NaF, 1 mm DTT, 10% vol/vol glycerol, 1 mm PMSF, 1 µg/ml of leupeptin, 0.7 µg/ml of pepstatin, and 1 µg/ml of aprotonin) and broken with acid-washed glass beads (0.4 to 0.52-mm diameter) in a Braun homogenizer (B. Braun) as described (Moehtie and Hinnebusch 1991).

Binary complexes of eIF2·[3H]GDP (50 µM) were formed in 12 x 75 mm glass tubes containing binary complex buffer (20 mm Tris-HCl at pH 7.5, 100 mm KCl, 2 mm MgCl2, 2 mg/ml of creatine phosphokinase, 3 mm MgCl2, 5 mm NaF, 1 mm DTT, 0.1 mm ATP, 0.1 mm EDTA, and 15% [vol/vol] glycerol) with the addition of 8–10 µg (~50 µl) of purified yeast eIF2 and 20 µl of [3H]GDP (11.6 Ci/mmmole) (Amersham). After 12 min at 23°C, reactions were held on ice. Under these conditions, –7 pmole of eIF2·[3H]GDP formed. In experiments where the effect of eIF2γ phosphorylation was assessed, eIF2 in binary complex buffer was first incubated with or without 1 µl (~0.1 µg) of purified rabbit reticulocyte HCR kinase (Jackson and Hunt 1985) for 10 min at 23°C. The presence of HCR did not affect binary complex formation.

To measure dissociation of binary complexes, reactions (from above) and 150 µg of cell extracts overexpressing eIF2B subunits, as indicated in Figures 1 and 3, were held at 10°C for 4 min, a 100-fold excess of unlabeled GDP was added to the binary com-plex, and, 1 min later, the cell extract (or extract buffer as a control) was added to start the reaction. Ten-microliter samples were removed immediately (assay time=0) and at the indicated times, added to 2 ml of ice-cold wash buffer (20 mm Tris-HCl at pH 7.5, 100 mm KCl, 5 mm MgCl2, 0.1 mm EDTA), filtered through nitrocellulose filters (Whatman) using a vacuum mani-fold (Millipore), and washed twice with 4 ml each of ice-cold wash buffer. Filters were dried and counted by liquid scintilla-tion in Econofluor 2 (Packard). Counting efficiency was ~56%.

Assays were done in duplicate or triplicate and in at least three separate experiments. Mean results of typical experiments are shown. Variation in values was ±0%–6% per time point with a mean variation for all time points of ±2.1% (t = 0 excluded).

IEF PAGE

Samples of purified eIF2 (~1 µg in 5 µl) in binary complex buffer (as above) were diluted with 150 µl of IEF sample buffer (Dever 1997). Fifty microliters of each sample was resolved on a vertical IEF polyacrylamide slab gel (Dever 1997).

Ni pull-down assays with His-tagged eIF2

In these assays purified His-tagged eIF2 is kept at a constant concentration and is in large molar excess over eIF2B and eIF2 present in cell extracts. Thus the fraction of total eIF2 bound is a function only of the dissociation constant (Phizicky and Fields 1995). Purified yeast eIF2 (2.5 µg) was incubated with or without HCR (1 µl) at 30°C for 10 min in 25 µl of pull-down buffer (20 mm Tris-HCl at pH 7.5, 100 mm KCl, 2 mm magne-sium acetate, 0.4 mg/ml of creatine phosphokinase, 5 mm NaF, 5 mm 2-mercaptoethanol, 0.1 mm ATP, 0.1 mm EDTA, and 7% [vol/vol] glycerol). Control reactions without eIF2 contained pull-down buffer only. Reactions were added to 100 µg of whole-cell extracts (prepared as described for nucleotide exchange as-says) from yeast strains overexpressing different eIF2B subunits in 35 µl of PD buffer (20 mm Tris-HCl at pH 7.5, 100 mm KCl, 2 mm magnesium acetate, 5 mm NaF, 5 mm 2-mercaptoethanol, 1 mm PMSF, 1 µg/ml of leupeptin, 0.7 µg/ml of pepstatin, and 1 µg/ml of aprotonin). eIF2 and cell extracts were incubated together at 10°C for 10 min then added to 50 µl of Ni-NTA-agarose beads (Qiagen) prewashed in PD buffer. Reactions were mixed by rotating on a Nutorator (Becton-Dickinson) at 4°C for 30 min. The agarose beads were collected by low-speed centrifuga-tion and washed 3x with 500 µl of buffer PD-I-5 (PD buffer with 5 mm imidazole added) by rotation on a Labquake shaker (Barn-stead-Thermolyne). Finally, bound material was eluted with 60 µl of buffer PD-I-250 by rotation for 30 min at 4°C and collection of the beads by centrifugation. The eluted material was mixed with 4x Laemmli sample buffer and heated to 37°C for 10 min prior to fractionation by 12% SDS-PAGE.

Ni pull-down assays with His-tagged GCD1

Assays were performed as described above with the following...
changes: No purified eIF2 was added, 400 µg of cell extract was used, and 10 µl of Ni-NTA-silica resin (Qiagen) was used in place of Ni-NTA-agarose, as this gave lower nonspecific binding of yeast proteins.

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