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Conserved bipartite motifs in yeast eIF5 and eIF2Bε, GTPase-activating and GDP–GTP exchange factors in translation initiation, mediate binding to their common substrate eIF2

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In the initiation phase of eukaryotic translation, eIF5 stimulates the hydrolysis of GTP bound to eIF2 in the 40S ribosomal pre-initiation complex, and the resultant GDP on eIF2 is replaced with GTP by the complex nucleotide exchange factor, eIF2B. Bipartite motifs rich in aromatic and acidic residues are conserved at the C-termini of eIF5 and the catalytic (ε) subunit of eIF2B. Here we show that these bipartite motifs are important for the binding of these factors, both *in vitro* and *in vivo*, to the β subunit of their common substrate eIF2. We also find that three lysine-rich boxes in the N-terminal segment of eIF2β mediate the binding of eIF2 to both eIF5 and eIF2B. Thus, eIF5 and eIF2Bε employ the same sequence motif to facilitate interaction with the same segment of their common substrate. In agreement with this, archaea appear to lack eIF5, eIF2B and the lysine-rich binding domain for these factors in their eIF2β homolog. The eIF5 bipartite motif is also important for its interaction with the eIF3 complex through the *NIP1*-encoded subunit of eIF3. Thus, the bipartite motif in eIF5 appears to be multifunctional, stimulating its recruitment to the 40S pre-initiation complex through interaction with eIF3 in addition to binding of its substrate eIF2.

Keywords: eIF2/evolution of eIFs/GAP/GEF/translation initiation complex

Introduction

Formation of the translation initiation complex containing mRNA, methionyl initiator tRNA (Met-tRNA$_i^{Met}$) and the ribosome is stimulated by proteins called initiation factors. The heterotrimeric factor eIF2 delivers the Met-tRNA$_i^{Met}$ to the 43S pre-initiation complex (for review, see Merrick and Hershey, 1996; Sonenberg, 1996; Trachsel, 1996). eIF2 binds GTP through its γ-subunit, and only the GTP-bound form of eIF2 binds Met-tRNA$_i^{Met}$. The eIF2–GTP–Met-tRNA$_i^{Met}$ ternary complex is then recruited to the 40S ribosomal subunit with the help of the complex factor eIF3, which is bound to the 40S subunit. m^{7}G-capped mRNA is recruited to the 40S ribosome by eIF4A, eIF4B and the cap-binding complex eIF4F, again with assistance from eIF3 (Lamphear *et al*., 1995; Imataka and Sonenberg, 1997). Subsequently, selection of the correct AUG codon by Met-tRNA$_i^{Met}$ stimulates hydrolysis of the GTP bound to eIF2 in a reaction requiring eIF5. GTP hydrolysis triggers the ejection of initiation factors, and produces a 40S initiation complex that is competent to bind a 60S subunit and form the 80S initiation complex. For the next round of initiation, the GDP on eIF2 must be exchanged for GTP by the action of the five subunit guanine nucleotide exchange factor, eIF2B.

This reaction scheme was established primarily through biochemical studies using purified mammalian eIFs, and is being tested extensively in the yeast *Saccharomyces cerevisiae* using genetic approaches. Two important phenotypes, Sui$^-$ and Gcd$^-$, have been used to select mutations in yeast translation initiation factors. Sui$^-$ mutations allow translation initiation from a UUG codon in a mutant *HIS4* mRNA, altering the eIFs involved in stringent selection of AUG as the start codon. Such mutations were isolated in all three genes encoding the subunits of eIF2 and also in eIF5, and were shown to affect the function of these proteins *in vitro* (Huang *et al*., 1997). The Sui$^-$ mutant selection also implicated the eIF1 homolog of yeast (encoded by *SUI1*) in the mechanism of stringent AUG selection (Yoon and Donahue, 1992; Kasperaitis *et al*., 1995), although the function of eIF1 is poorly understood. A recent biochemical analysis indicated that eIF1, acting in conjunction with eIF1A, is required to form a stable 48S pre-initiation complex (containing ternary complex, eIFs 3, 4A, 4B and 4F, and the 40S ribosome bound to mRNA) with the ribosome located at the AUG start codon (Pestova *et al*., 1998). Here, we refer to SUI1 as eIF1, based on the nomenclature established for the mammalian eIFs.

The Gcd$^-$ phenotype is indicative of reduced formation of the eIF2–GTP–Met-tRNA$_i^{Met}$ ternary complex, e.g. arising from reduced GDP–GTP exchange on eIF2 catalyzed by eIF2B. In *S.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, 1997). Phosphorylated eIF2, when bound to GDP, forms an inactive complex with eIF2B, thereby reducing the level of eIF2-GTP and, hence, ternary complex formation (Hinnebusch, 1997). Gcd$^-$ mutations mimic this situation and induce translation of *GCN4* mRNA in the absence of GCN2. Accordingly, Gcd$^-$ mutations were isolated in the genes encoding all three subunits of eIF2 and all five subunits of eIF2B, and are predicted to reduce the GDP–GTP exchange on eIF2 (Hinnebusch, 1997). Analyses of complex formation by the yeast eIF2B subunits revealed that eIF2B consists of distinct regulatory (α, β and δ subunits) and catalytic (γ and ε subunits) subcomplexes which bind eIF2 independently (Yang and Hinnebusch, 1996; Pavitt *et al*., 1998). Mutations in the *GCN3* and *GCD7*-encoded regulatory subunit (eIF2Bα

and β) that prevent induction of *GCN4* translation *in vivo* (Pavitt *et al*., 1997) were found to overcome the inhibition of eIF2B by phosphorylated eIF2 *in vitro*. Moreover, extracts from cells overexpressing the ε subunit of eIF2B alone, encoded by *GCD6*, had increased levels of GDP–GTP exchange activity for eIF2 (Pavitt *et al*., 1998), strongly suggesting that eIF2Bε is the catalytic subunit of eIF2B.

Recent biochemical and genetic studies of yeast eIF3 have shed new light on the function of this complex and poorly characterized factor (Naranda *et al*., 1994; Danaie *et al*., 1995; Verlhac *et al*., 1997; Asano *et al*., 1998; Phan *et al*., 1998). Our results indicated that yeast eIF3 consists of only five subunits, homologous to five of the 10 subunits of the mammalian factor (Asano *et al*., 1997, 1998; Phan *et al*., 1998). In addition, eIF1 and eIF5 were shown to co-purify with eIF3 (Naranda *et al*., 1996; Phan *et al*., 1998) and to physically interact with the isolated 93 kDa subunit of eIF3, encoded by *NIP1* (Asano *et al*., 1998; Phan *et al*., 1998). Given its demonstrated function in promoting the binding of the eIF2 ternary complex to the 40S ribosome (Feinberg *et al*., 1982; Danaie *et al*., 1995; Merrick and Hershey, 1996; Trachsel, 1996; Phan *et al*., 1998), eIF3 may play an important role in assembling the factors required for stringent AUG selection, including eIF1, eIF2 and eIF5.

In this study, we focus on the physical interactions that mediate binding of eIF2 to the catalytic subunit of its guanine nucleotide exchange factor, eIF2Bε, and to eIF5, a factor which stimulates GTP hydrolysis in the eIF2 ternary complex. It was noted that the C-termini of eIF2Bε and eIF5 contain a bipartite sequence motif rich in acidic and aromatic residues (Koonin, 1995), and we hypothesized that this shared motif could mediate binding of their common substrate eIF2. As archaea contain eIF2 but appear to lack eIF2B and eIF5, the binding domain in eukaryotic eIF2 for these factors should be absent in the archaeal homologs. Interestingly, the β subunit of eukaryotic eIF2 is considerably larger at the N-terminus than its archaeal counterpart, and contains three lysine-rich segments (K-boxes). Recently, Das *et al*. (1997) presented *in vitro* evidence that the binding domain for mammalian eIF5 resides in the N-terminal half of eIF2β and includes the second of the three K-boxes.

Here we present *in vivo* and *in vitro* evidence that the bipartite motifs conserved at the C-termini of eIF5 and eIF2Bε mediate their binding to the N-terminal half of eIF2β in *S.cerevisiae*, and that the K-boxes in eIF2β are required for both interactions *in vivo*. Thus, the bipartite motifs in eIF5 and eIF2Bε facilitate binding to their common substrate eIF2. Furthermore, we show that the bipartite motif in eIF5 is also required for its interaction with eIF3 via the *NIP1*-encoded subunit of eIF3, and most likely mediates recruitment of eIF5 to the 40S pre-initiation complex. Our findings have important implications for evolution of the guanine nucleotide exchange and GTPase-stimulating factors which regulate eIF2 activity in eukaryotic organisms.

Results

The C-terminal domain of eIF5 specifically binds to eIF2β and eIF3-p93 (NIP1) in vitro

While investigating protein–protein interactions among translation initiation factors of *S.cerevisiae*, we found that eIF5 (encoded by *TIF5*) interacted with both the 93 kDa subunit of eIF3 (encoded by *NIP1*) and the β subunit of eIF2 (encoded by *SUI3*) in the two-hybrid assay (Phan *et al*., 1998; K.Asano and A.G.Hinnebusch, unpublished observations). These interactions were confirmed by *in vitro* binding assays using recombinant proteins: a GST–eIF5 fusion protein specifically interacted with p93/NIP1 and eIF2β, synthesized in rabbit reticulocyte lysates (results described below), but not with any other eIF3 subunits (p90/PRT1, p39/TIF34 and p32/TIF35) (Phan *et al*., 1998) or eIF2 subunits (eIF2α and eIF2γ) (K.Asano and A.G.Hinnebusch, unpublished observations). Accordingly, we used the two-hybrid assay to localize the domain in eIF5 responsible for its binding to eIF3-p93 and eIF2β. A series of GAL4 DNA-binding domain fusions containing N- or C-terminally truncated eIF5 fragments were tested for interaction with GAL4 activation domain fusions containing full-length eIF3-p93 or eIF2β (Figure 1A). The results suggested that the C-terminal one-third of eIF5 is sufficient for binding to both eIF3-p93 and eIF2β. To confirm this conclusion by *in vitro* protein-binding assays, recombinant GST fusions to selected eIF5 fragments (Figure 1A, constructs A6–A9 and B5–B9) were expressed in *Escherichia coli*, bound to glutathione–Sepharose beads and incubated with ^{35}S-labeled eIF3-p93 or eIF2β synthesized in rabbit reticulocyte lysates (see Materials and methods). As shown in the upper panel of Figure 1B, the fusion proteins had the sizes expected, although preparations of the full-length and A6–A9 GST–eIF5 fusions contained another smaller protein that is most likely a degradation product lacking the C-terminal half of eIF5 (lanes 2–12). Only the full-length GST–eIF5 protein, and the B5 and B6 truncated fusions, bound high levels (40% or more) of [^{35}S]eIF3-p93 and [^{35}S]eIF2β (bottom two panels in Figure 1B), consistent with the results of two-hybrid analysis (see summary in Figure 1A). We conclude that the C-terminal 165 amino acids of eIF5 are sufficient for its binding to both eIF2β and eIF3-p93 *in vitro*.

It was conceivable that the *in vitro* translated eIF2β and eIF3-p93 were incorporated into rabbit eIF2 and eIF3, respectively, and that their binding to GST–eIF5 occurred only in the context of these chimeric multisubunit complexes. We believe this is unlikely, however, because we did not observe binding of any other eIF2 or eIF3 subunits to GST–eIF5 in our *in vitro* binding assays. Moreover, only these two subunits of eIF2 or eIF3 interacted with eIF5 in the two-hybrid assay.

The conserved bipartite motif in eIF5 is required for its interaction with eIF2β and eIF3-p93 (NIP1) in vitro

The sequence of the C-terminal one-third of eIF5 is conserved among yeast, humans, *Caenorhabditis elegans* and *Zea mays*, and contains a bipartite sequence motif also present in the C-terminus of the catalytic (ε) subunit of eIF2B (Koonin, 1995). This motif is composed of two segments, both rich in acidic and aromatic residues, separated by 19–23 less conserved residues (Figure 1A). The two conserved segments in this motif are henceforth called AA (acidic/aromatic)-boxes 1 and 2. The eIF3-p93-and eIF2β-binding domain in eIF5 identified above encompasses the entire C-terminal conserved segment of

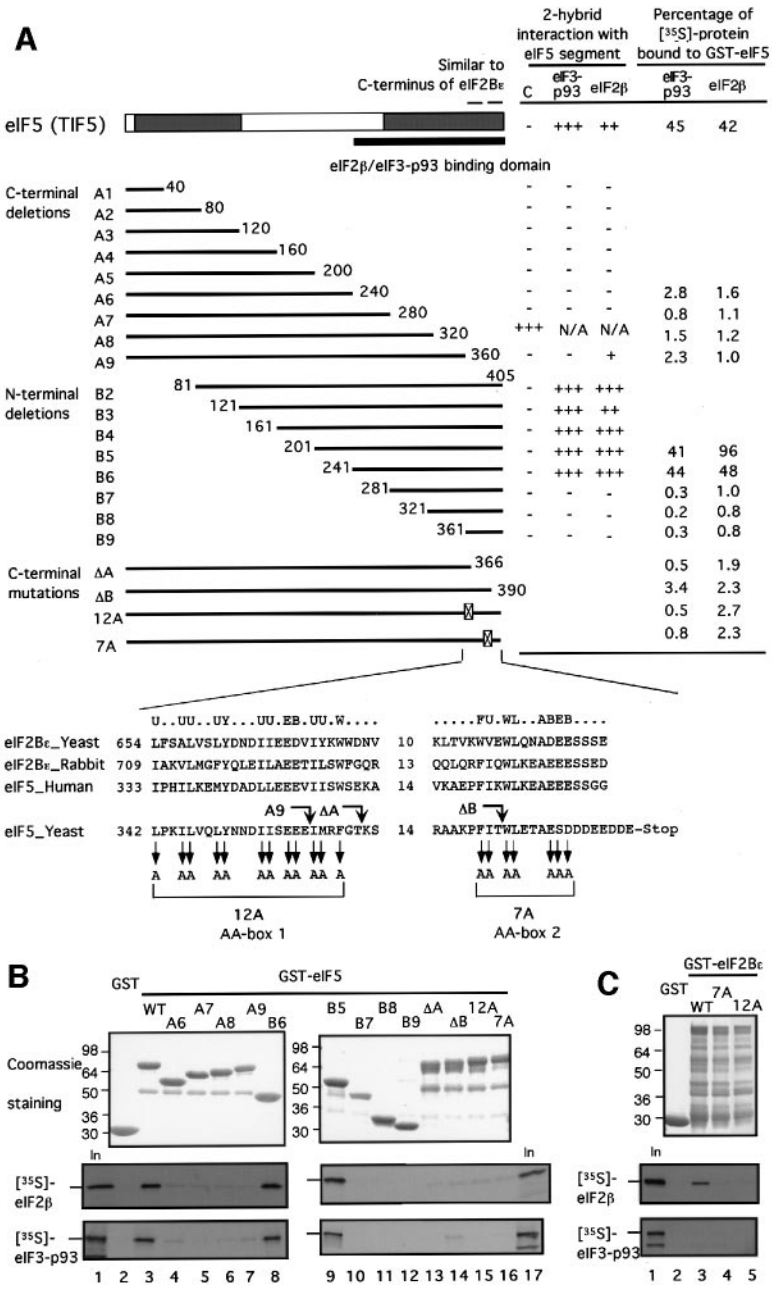

Fig. 1. Conserved bipartite motifs in eIF5 and eIF2Bε are required for their strong interactions with recombinant eIF2β *in vitro*. (**A**) The region of yeast eIF5 responsible for its binding to eIF2β and eIF3-p93 (NIP1). The schematic at the top depicts the primary structure of eIF5 from *S.cerevisiae*. Shaded portions are conserved with its higher eukaryotic homologs (see text). Lines below the schematic depict the segments of eIF5 present in the deletion constructs, with the amino acid positions at the termini indicated above the lines and the clone names indicated on the left. The last two constructs, 12A and 7A, carry multiple alanine substitutions (each shown as a crossed rectangle) in a region conserved with the C-terminal part of eIF2Bε. The amino acid sequences of eIF5 and eIF2Bε from mammals and yeast in the conserved regions are aligned at the bottom. The symbols for conserved amino acids shown at the top of the alignment are according to Koonin (1995). Arrows below the yeast eIF5 sequence indicate the multiple substitutions present in the 12A and 7A constructs; arrows above the sequence indicate the endpoints of the indicated deletions. The results of binding assays are summarized to the right of the schematicized constructs. For *in vivo* two-hybrid assays (first three columns), each mutant protein was expressed as a fusion to the GAL4 DNA-binding domain encoded by pGBT9, and tested for activation of a *GAL–HIS3* reporter in the presence of the GAL4 activation domain alone [column heading C (control)] or with fusions between the GAL4 activation domain and eIF3-p93 or eIF2β. –, no growth at 5 mM 3-AT; ++, growth at 20 mM but not 30 mM 3-AT; +++, growth at 30 mM 3-AT; N/A, results not applicable since the A8 fusion activated *GAL–HIS3* alone. The results of *in vitro* GST pull-down assays shown in (B) are summarized in the last two columns. The percentages of the input amounts of labeled proteins bound to the GST fusions are shown. These values were calculated by quantification of the radioactivity by PhosphorImaging analysis using the STORM model 860 (Molecular Dynamics). (**B**) *In vitro* binding of GST–eIF5 and its derivatives to recombinant eIF2β and eIF3-p93. The wild-type GST–eIF5 protein (lane 3), its mutant derivatives (lanes 4–16) and the GST protein alone (lane 2) were expressed in *E.coli* and immobilized on glutathione–Sepharose beads. The top panel shows the Coomassie Blue staining patterns of the GST fusion proteins in the amounts employed for the binding reactions. The position and size (in kDa) of molecular mass standards are indicated on the left. The GST fusion proteins adsorbed to the resin were incubated with [^{35}S]eIF2β or [^{35}S]eIF3-p93 synthesized by *in vitro* translation. After extensive washing, the bound labeled proteins were visualized by SDS–PAGE followed by autoradiography (middle and bottom panels for eIF2β and eIF3-p93, respectively). Lanes 1 and 17 contain 50% of the input (In) amounts of labeled proteins used in the reactions. (**C**) *In vitro* binding of GST–eIF2Bε and its derivatives to recombinant eIF2β and eIF3-p93. Binding experiments were conducted as described in (B) except that GST–eIF2Bε (lane 3) and its 7A (lane 4) and 12A (lane 5) mutant derivatives were employed. Lane 1, 50% of the input amounts of labeled proteins used in the reactions; lane 2, binding with GST alone.

the protein (Figure 1A). As the eIF5-A9 segment did not bind strongly to eIF3-p93 or eIF2β and lacked part of the bipartite motif (Figure 1A), we examined additional GST–eIF5 fusions containing smaller C-terminal deletions (ΔA and ΔB) and two multiple alanine substitutions (12A and 7A) which replaced all of the conserved residues in AA-boxes 1 or 2, respectively (see lower part of Figure 1A). All four GST–eIF5 fusions carrying these mutations were greatly impaired for binding to eIF3-p93 and eIF2β (Figure 1B, lanes 13–16). These data suggest that the bipartite motif in eIF5 is important for its binding to both of these eIF3 or eIF2 subunits *in vitro*.

To determine whether the bipartite motif was important for interactions between eIF5 and the native eIF2 and eIF3 complexes, we purified these factors from yeast and tested them for interaction with the GST–eIF5 and GST–eIF5-7A fusions purified from bacteria. We found that ~30–50% of the three eIF2 subunits and ~50–100% of the eIF3-p93, -p90 and -p39 subunits in these reactions bound to GST–eIF5, whereas undetectable amounts of these proteins bound to GST–eIF5-7A (Figure 2A). These results confirm that eIF5 interacts directly with eIF3 (Phan *et al*., 1998) and eIF2, dependent on AA-box 2 in eIF5. Similar results were obtained in pull-down assays with GST–eIF5 and GST–eIF5-7A using a whole-cell yeast extract as the source of eIF2 and eIF3 (see below Figure 6A, lanes 1–4).

The AA-boxes of eIF5 are important for its function in vivo

Because eIF5 is essential for protein synthesis *in vivo* (Maiti and Maitra, 1997), the mutations in the bipartite motif which reduced its interaction with eIF2β and eIF3-p93 *in vitro* were expected to impair cell growth. To test this prediction, we introduced the bipartite motif mutations, 7A and 12A, into a *TIF5* allele tagged with the coding sequences for the FLAG epitope on a single-copy *LEU2* plasmid. When we replaced the plasmid-borne *TIF5* allele in strain KAY24 [*ura3 leu2 tif5*Δ p(*TIF5 URA3*)] with the wild-type tagged allele (*TIF5-FL*) by plasmid shuffling, the resulting strain grew indistinguishably from the parental strain containing wild-type *TIF5* at 30 or 36°C (Figure 2B). In contrast, the *tif5-FL-12A* plasmid did not support growth of the *tif5*Δ strain, and the strain bearing *tif5-FL-7A* grew more slowly than the wild-type at 30°C, and not at all at 36°C (Figure 2B). Thus, the *tif5-FL-12A* allele is lethal, whereas *tif5-FL-7A* confers temperature-sensitive (Ts^-) growth in yeast cells (Table I). Western blot analysis of whole-cell extracts (WCEs) showed that the *TIF5-FL* and *tif5-FL-7A* products were expressed at comparable levels (Figure 2B); thus, *tif5-FL-7A* confers a Ts^- phenotype because of impaired eIF5 function.

When the *tif5-FL-12A* allele was present on a single-copy plasmid in a strain containing untagged *TIF5*, the level of eIF5-FL-12A was 10-fold lower than that of eIF5-FL (Table I). To overcome this expression defect, we introduced the *tif5-FL-12A* allele into strain KAY24 on a high-copy *LEU2* vector. The eIF5-FL-12A protein was now 7–8 times higher than wild-type eIF5-FL expressed from a single-copy plasmid (Table I); nevertheless, when the resident *URA3 TIF5* plasmid was evicted, the resulting high-copy *tif5-FL-12A* strain grew very slowly at all temperatures tested (data not shown, see Table I). These results, together with those obtained for *tif5-FL-7A* (Figure 2B), suggest that AA-boxes 1 and 2 of the bipartite motif in eIF5 are crucial for cell growth.

The bipartite motif in eIF5 is required for its binding to native eIF2 and eIF3 complexes in vivo

We next examined the effect of the *TIF5-7A* mutation on co-immunoprecipitation of the native eIF2 and eIF3 complexes with physiological amounts of epitope-tagged eIF5 expressed *in vivo*. WCEs were prepared from KAY24 (*TIF5*), KAY35 (*TIF5-FL*) and KAY36 (*tif5-FL-7A*) and incubated with anti-FLAG affinity resin. After extensive washing, almost all of the eIF5-FL and eIF5-FL-7A remained attached to the resin, whereas untagged eIF5 was absent (Figure 2C, top panel; lanes 2, 5 and 8). Importantly, ~10–20% of all three eIF2 subunits and ~50% of the eIF3-p93, -p90 and -p39 in the WCEs were co-immunoprecipitated with eIF5-FL, whereas much lower amounts of all these proteins were recovered with eIF5-FL-7A (Figure 2C, lanes 5 and 8). These results suggest that AA-box 2 in eIF5 is required for tight binding to the native eIF2 and eIF3 complexes *in vivo*.

Considering the results in Figure 2A–C, we reasoned that the growth defect caused by *tif5-FL-7A* might result, at least partly, from reduced interactions of eIF5 with eIF2 or eIF3. In support of this possibility, the Ts^- phenotype of the *tif5-FL-7A* mutation was partially suppressed by overexpression of all three subunits of eIF2 (Figure 2D, left two panels, rows b–c). Moreover, co-overexpression of $tRNA_i^{Met}$ and eIF2 reduced the growth defect of the *tif5-FL-7A* strain even further (Figure 2D, left two panels, rows c–d). We showed previously that eIF2 and $tRNA_i^{Met}$ are overexpressed from high-copy plasmids by ~10-fold (Dever *et al*., 1995) and ~5-fold (Anderson *et al*., 1998), respectively. These results support the idea that eIF5 functionally interacts with the eIF2 ternary complex *in vivo* in a manner dependent on AA-box 2 in eIF5. When we immunoprecipitated eIF5-FL-7A from WCE from strain KAY36 (*tif5-FL-7A*) overexpressing eIF2 and $tRNA_i^{Met}$, we recovered eIF2 subunits in amounts much greater than were associated with eIF5-FL-7A in the strain bearing vector alone, and comparable with the amounts seen in the wild-type strain (Figure 2D, right panel; lanes 5–8 versus 1–4). As expected, overexpressing eIF2 and $tRNA_i^{Met}$ did not rescue binding of eIF3-p90 to eIF5-FL-7A (Figure 2D, right panel). Thus, overexpression of the ternary complex appeared to compensate by mass action for the reduced interaction between eIF5-FL-7A and eIF2. Nevertheless, the *tif5-FL-7A* strain overproducing eIF2 and $tRNA_i^{Met}$ did not grow as rapidly as did the wild-type (Figure 2D, left panels, rows a and d), suggesting that the weakened interaction between eIF3 and eIF5-FL-7A might also contribute to the Ts^- phenotype of *tif5-FL-7A* cells.

Interestingly, we found that overexpression of eIF5 confers a Gcd^- phenotype, dependent on its bipartite motif (Table I, last column, lines 2, 4 and 6). The expression of *GCN4* and amino acid biosynthetic enzymes under its control is increased when cells are starved for histidine by addition of the amino acid analog 3-aminotriazole (3-AT). In wild-type cells, *GCN4* translation is induced when eIF2α is phosphorylated by GCN2, leading to inhibition of eIF2B and a reduction in the ternary complex level. Mutants lacking GCN2 cannot induce *GCN4* transla-

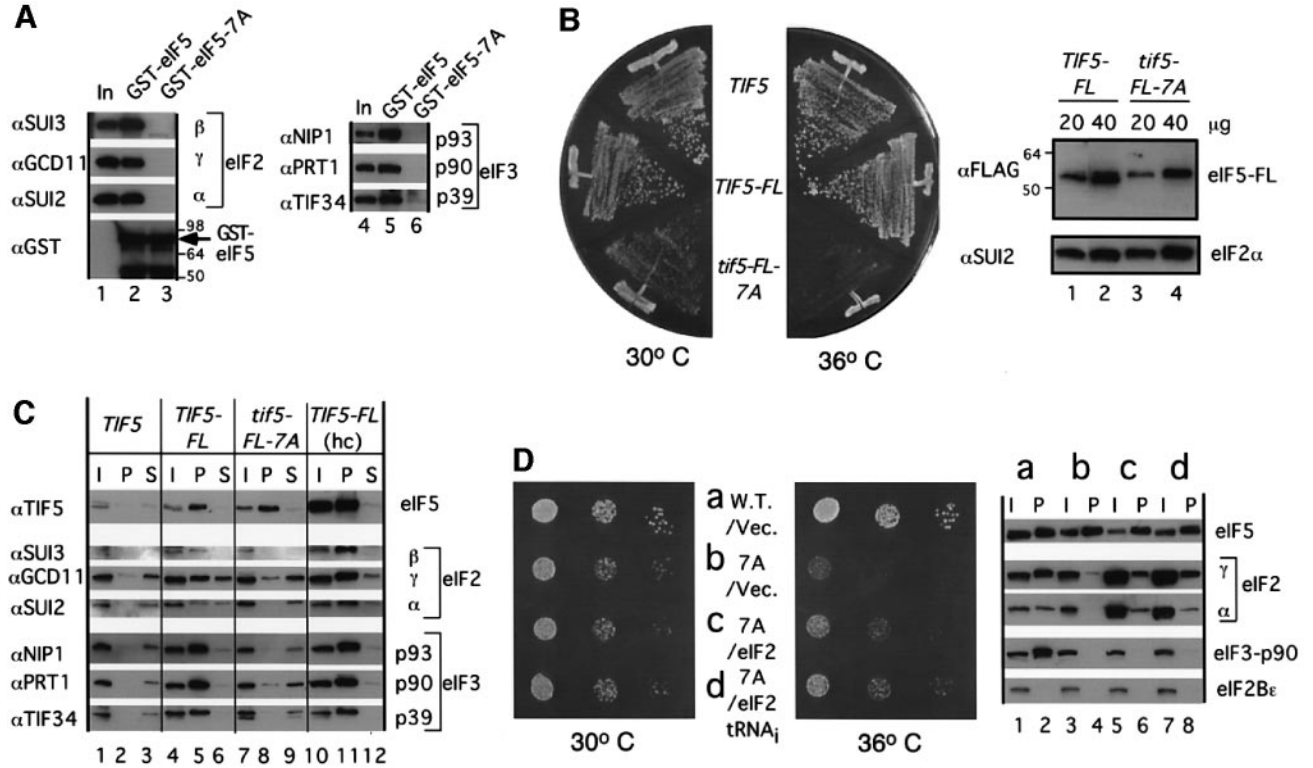

Fig. 2. The 7A mutation in the bipartite motif of eIF5 impairs its essential function and physical interaction with native eIF2 and eIF3 complexes *in vivo*. (**A**) The 7A mutation in GST–eIF5 impairs its binding to purified native eIF2 and eIF3 *in vitro*. Purified eIF2 and eIF3 (1 μg each) was incubated with GST–eIF5 (lanes 2 and 5) or GST–eIF5-7A (lanes 3 and 6) attached to glutathione–Sepharose beads, as described in Figure 1. The bound proteins were analyzed with the antibodies indicated on the left for detection of the proteins indicated on the right. Lanes 1 and 4, 0.2 μg of purified factors used in the binding reactions. (**B**) The *tif5-FL-7A* allele confers temperature sensitivity in yeast cells. Left panel: yeast strain KAY36 carrying the 7A mutation in eIF5 (*tif5-FL-7A*), and the isogenic wild-type strains KAY35 (*TIF5-FL*) and KAY24 (*TIF5*), were streaked on SD media containing tryptophan and uracil, and incubated at the indicated temperature for 2 days. Right panel: 20 and 40 μg of WCE prepared from KAY35 (lanes 1 and 2) and KAY36 (lanes 3 and 4), grown in YPD medium at 30°C, were subjected to Western blot analyses with anti-FLAG and anti-SUI2 antibodies for detection of eIF5-FL and eIF2α, respectively. Detection of immune complexes was performed by chemiluminescence (ECL™, Amersham). (**C**) Evidence that the 7A mutation in eIF5 reduces its interaction with native eIF2 and eIF3 *in vivo*. WCE was prepared from strains KAY24 (*TIF5*) (lanes 1–3), KAY35 (*TIF5-FL*) (lanes 4–6), KAY36 (*tif5-FL-7A*) (lanes 7–9) and KAY39 (*TIF5-FL* in high-copy) (lanes 10–12) grown in YPD medium at 30°C. Aliquots of WCEs were incubated with anti-FLAG affinity resin (Kodak) and, after extensive washing, the bound proteins were analyzed by SDS–PAGE and immunoblotting using the antibodies indicated on the left. Lanes 1, 4, 7 and 10, 20% of input (I) amounts of WCE; lanes 2, 5, 8 and 11, the entire immunoprecipitated (P, pellet) fractions; lanes 3, 6, 9 and 12, 10% of the supernatant (S) fractions. (**D**) Left-panel: co-overexpression of eIF2 and $tRNA_i^{Met}$ partially suppresses the growth defect conferred by *tif5-FL-7A*. KAY36 (*tif5-FL-7A*) was transformed with high-copy plasmid p1780 encoding all three eIF2 subunits (*7A*/eIF2, c), or with high-copy plasmid p1780-IMT, encoding $tRNA_i^{Met}$ and the three eIF2 subunits (*7A*/eIF2 $tRNA_i$, d). As controls, KAY36 and its isogenic wild-type, KAY35, were transformed with an empty vector (WT/Vec. or *7A*/Vec., a and b, respectively). The transformants were grown in SC medium lacking uracil at 30°C to OD_{600} = ~20. Equal OD_{600} units, and 1/10 or 1/100 of these amounts, were spotted from left to right on SD medium containing tryptophan and incubated for 2 days at the indicated temperatures. Right panel: co-overexpression of eIF2 and $tRNA_i^{Met}$ restores binding of eIF2 to eIF5-7A *in vivo*. Aliquots of WCE prepared from transformants a–d analyzed in the left panels were immunoprecipitated with FLAG affinity resin and proteins recovered with eIF5-FL were analyzed by immunoblotting as described in (C). Lanes 1, 3, 5 and 7, 20% of input amounts of WCE (I); lanes 2, 4, 6 and 8, the entire immunoprecipitated (pellet, P) fractions. W.T., wild-type.

tion when starved for histidine and fail to grow on medium containing 3-AT. Gcd⁻ mutations restore growth on 3-AT medium in *gcn2*Δ cells by decreasing ternary complex formation independently of eIF2 phosphorylation. The fact that introducing a high-copy plasmid containing *TIF5-FL* led to 3-AT resistance in a *gcn2*Δ strain (Gcd⁻ phenotype) suggests that the ternary complex level is reduced when eIF5 is overexpressed. As eIF5 binds eIF2 *in vivo*, it seemed possible that the overproduced eIF5 sequestered eIF2 in a non-ribosomal complex, and thereby reduced the concentration of ternary complexes that can participate in translation. In accordance with this idea, when WCE prepared from strain KAY39 overexpressing eIF5-FL was incubated with the anti-FLAG affinity resin, the amount of eIF2 recovered with eIF5-FL increased ~5-fold compared with that observed in an extract with normal amounts of eIF5-FL (Figure 2C, lanes 5 and 11, eIF2 panels). Importantly, the presence of *tif5-FL-7A* or *tif5-FL-12A* on a high-copy plasmid did not confer a Gcd⁻ phenotype (Table I, lines 4 and 6). Presumably, the AA-box 2 mutations in *tif5-FL-7A* eliminated the excessive formation of eIF2–eIF5 complexes that we postulate is responsible for the Gcd⁻ phenotype resulting from overexpression of wild-type eIF5. These results provide additional *in vivo* evidence that eIF5 interacts with eIF2 in a manner requiring AA-box 2 in eIF5.

The bipartite motif in eIF2Bε is important for interaction with the substrate eIF2 in vivo

The catalytic subunit of eIF2B, eIF2Bε (Pavitt *et al.*, 1998), also contains the bipartite motif (Figure 1A). We found previously that *in vitro* translated eIF2β interacted

Table I. Phenotypes of mutations analyzed in this study

Allele	Mutation[a]	Vector[b]	Expression[c]	Growth[d]	Gcd⁻ phenotype[e]
eIF5 mutations					
TIF5-FL	Wild-type	Single-copy	(1)	Wild-type	+
		high-copy	~20	wild-type	–
tif5-FL-12A	AA-box 1	single-copy	~0.1	lethal	
		high-copy	7–8	Slg⁻	+
tif5-FL-7A	AA-box 2	single-copy	1	Ts⁻	+
		high-copy	~20	wild-type	+
eIF2Bε mutations					
GCD6	wild-type	low-copy	(1)	wild-type	+
		high-copy	~20	wild-type	–
gcd6-12A	AA-box 1	low-copy	NT	lethal	
		high-copy	~1[f]	lethal	
gcd6-7A	AA-box 2	low-copy	1	wild-type	–
eIF2β mutations					
SUI3-FL	wild-type	single-copy	(1)	wild-type	+
		low-copy	2–3	wild-type	+
		high-copy	~20	wild-type	–
sui3-FL-K1	K-box 1	single-copy	2–3	wild-type	–
sui3-FL-K2	K-box 2	single-copy	2–3	wild-type	–
sui3-FL-K3	K-box 3	single-copy	~0.8	wild-type	– (weak)
sui3-FL-K12	K-boxes 1,2	single-copy	4–5	wild-type	–
sui3-FL-K13	K-boxes 1,3	single-copy	ND	lethal	
sui3-FL-K23	K-boxes 2,3	single-copy	4–5	wild-type	–
sui3-FL-K123	K-boxes 1–3	single-copy	~0.1	lethal	
		high-copy	4–5	lethal	

LEU2 plasmids encoding *TIF5-FL*, *GCD6* or *SUI3-FL*, or their mutant derivatives, were introduced into strains KAY24 [*gcn2*Δ *tif5*Δ pKA235 (*TIF5 URA3*)], KAY16 [*gcn2*Δ *gcd6*Δ pJB5 (*GCD6 URA3*)] or KAY18 [*gcn2*Δ *sui3*Δ p921 (*SUI3 URA3*)], respectively. Growth and Gcd⁻ phenotypes were tested after evicting the *URA3* plasmid bearing the corresponding wild-type allele on 5-fluoro-orotic acid media.

[a]Alanine substitutions in AA-boxes 1 or 2 of eIF5 or eIF2Bε are depicted in Figure 1A. Ala substitutions in the K-boxes of eIF2β are shown in Figure 5A and B.

[b]Single-copy, YCplac111; low-copy, pRS315; high-copy, YEplac195 for *TIF5*, pRS425 for *SUI3* and *GCD6*.

[c]Expression relative to the wild-type protein (indicated as a value of 1 in parentheses) in WCEs, detected by Western blotting with anti-FLAG antibodies for eIF5-FL and eIF2β-FL, or with anti-GCD6 antibodies for eIF2Bε. Expression from alleles with a lethal or Slg⁻ phenotype was examined in the presence of the *URA3* plasmid carrying the cognate wild-type gene. ND, not detected; NT, not tested.

[d]Ts⁻, temperature-sensitive at 37°C; Slg⁻, slow growth at all temperatures.

[e]Gcd⁻ phenotypes were recognized by suppression of the 3-AT-sensitive phenotype of the *gcn2*Δ allele present in each strain. +, no growth on SC-Leu-Trp-His medium containing 10 mM 3-AT (Asano *et al*., 1998); – (weak), growth on 10 mM 3-AT but not on 30 mM 3-AT; –, growth on 30 mM 3-AT.

[f]When this plasmid was introduced into a wild-type strain, the eIF2Bε protein was detected in amounts twice as high as the endogenous eIF2Bε protein detected in the same strain carrying the vector alone. Thus, the level of the mutant eIF2Bε from this plasmid was judged to be comparable with that of endogenous eIF2Bε.

with a GST–eIF2Bε fusion protein *in vitro*, whereas eIF2α and eIF2γ did not (K.Asano and A.G.Hinnebusch, unpublished results). Thus, we examined whether binding of eIF2β to GST–eIF2Bε is dependent on the bipartite motif in eIF2Bε. As shown in Figure 1C, full-length GST–eIF2Bε and its 12A and 7A derivatives could be purified on glutathione–Sepharose beads, although numerous degradation products (presumably C-terminal truncations) were also evident. Nevertheless, the GST–eIF2Bε preparation specifically bound [^{35}S]eIF2β, and this binding was eliminated by the AA-box mutations (middle panel in Figure 1C). Unlike GST–eIF5, however, GST–eIF2Bε did not bind to eIF3-p93 (lower panel), suggesting that the bipartite motif domain in eIF2Bε interacts specifically with eIF2β.

To investigate the effect of the 7A mutation in eIF2Bε on its interaction with the eIF2 complex, purified eIF2 tagged with the FLAG epitope at the N-terminus of eIF2β was attached to the anti-FLAG affinity resin (Figure 3A, lane 1) and incubated with WCE containing overproduced wild-type or mutant eIF2Bε. We observed that ~15% of wild-type eIF2Bε remained associated with eIF2 after extensive washing and was co-eluted from the resin with FLAG peptide (lanes 2 and 3). In contrast, eIF2Bε-7A was barely detectable in the eluate (lanes 4 and 5). Thus, AA-box 2 of native eIF2Bε is required for its binding to the purified eIF2 complex *in vitro*.

We next examined whether the bipartite motif in eIF2Bε contributes to the function of eIF2B *in vivo*. For this purpose, we introduced the AA-box mutations, 12A and 7A, into a *GCD6* allele carried on a low-copy vector. The resulting *gcd6-12A* and *gcd6-7A* plasmids were introduced into a *gcn2*Δ *gcd6*Δ strain by plasmid shuffling, and Western analysis of the resulting strains revealed that the mutant eIF2Bε-7A was expressed at essentially the same level as the wild-type protein (Figure 3C). Although the *gcd6-7A gcn2*Δ strain grew like the wild-type on minimal medium at all temperatures tested (data not shown, Table I), it had a Gcd⁻ phenotype, conferring the ability to grow on medium containing 3-AT (Figure 3B, left panel, rows 2–3). This phenotype suggests that the AA-box 2 mutation in eIF2Bε impairs eIF2B activity, thereby reducing the level of the eIF2 ternary complex. Co-overexpression of eIF2 and $tRNA_i^{Met}$ suppressed the Gcd⁻ phenotype of *gcd6-7A* (Figure 3B, right panel), supporting the idea that it results from reduced ternary complex levels.

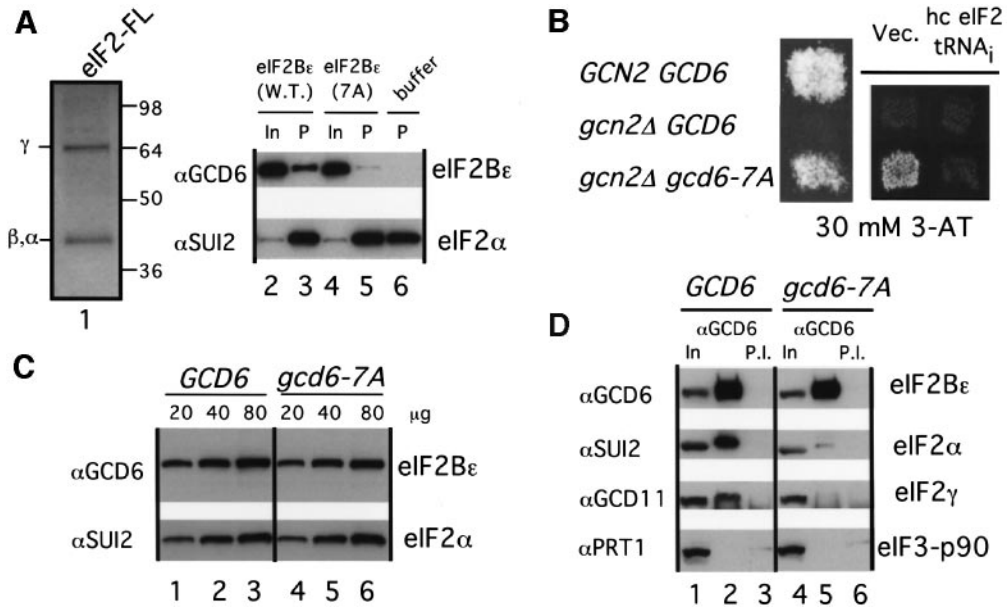

Fig. 3. The bipartite motif in eIF2Bε is required for tight binding of eIF2 to eIF2B *in vivo*. (**A**) Binding of native eIF2Bε to the eIF2 complex is dependent on AA-box 2. FLAG-tagged eIF2 was purified from WCE in one step using anti-FLAG affinity resin as described in Materials and methods. Approximately 500 ng of FLAG-eIF2 (visualized with Coomassie staining in lane 1) attached to the resin was incubated with WCE from the transformants of BJ1995 overexpressing wild-type eIF2Bε (lanes 2 and 3) or eIF2Bε-7A (lanes 4 and 5), or with buffer alone (lane 6). After extensive washing, the eIF2–eIF2Bε complex was eluted with FLAG peptide, and 10% of the eluate (P), along with 5% of the input amount of WCE (In), was analyzed by Western blotting using antibodies against eIF2Bε or eIF2α. (**B**) *gcd6-7A* confers a Gcd$^-$ phenotype. Isogenic strains H1902 (*GCN2 GCD6*), KAY33 (Δ*gcn2 GCD6*) and KAY34 (Δ*gcn2 gcd6-7A*) (left), or transformants of the latter two strains bearing YEp24 (Vec.) or p1780-IMT (hc eIF2 $tRNA_i$) (right), were grown to confluence on SD medium containing minimal supplements, replica-plated to SC-Leu supplemented with 30 mM 3-AT, and incubated for 2 days at 30°C. (**C**) Expression of the *gcd6-7A* product. Samples of WCE (20, 40 or 60 μg) from KAY33 (Δ*gcn2 GCD6*) and KAY34 (Δ*gcn2 gcd6-7A*) were separated by SDS–PAGE, blotted and probed with the antibodies indicated on the left for detection of eIF2Bε or eIF2α, as described in Figure 2B. (**D**) Interaction between eIF2 and eIF2B *in vivo*. WCEs prepared from KAY33 (*GCD6*) and KAY34 (*gcd6-7A*) were immunoprecipitated with anti-eIF2Bε antibodies and the precipitated proteins were analyzed by Western blotting using antibodies against eIF2Bε, eIF2α, eIF2γ and eIF3-p90. In, 30% input amount of WCE (lanes 1 and 4); αGCD6 or P.I., the entire precipitate with anti-eIF2Bε (lanes 2 and 5) or its pre-immune serum (lanes 3 and 6).

The effect of the *gcd6-7A* mutation on the physical interaction between eIF2B and eIF2 *in vivo* was examined directly by co-immunoprecipitation analysis. Cell extracts from yeast strains containing the wild-type or 7A derivative of eIF2Bε were immunoprecipitated with antibodies against GCD6, and the precipitates were probed by Western blotting for other subunits of eIF2B and for the α and γ subunits of eIF2. Nearly all of the α, β, γ and δ subunits of eIF2B were co-immunoprecipitated with wild-type or the 7A mutant eIF2Bε, indicating that formation of the eIF2B complex was not affected by the mutation (data not shown). Furthermore, ~30–40% of the α and γ subunits of eIF2 were co-immunoprecipitated with wild-type eIF2Bε (Figure 3D, lane 2). By contrast, only trace amounts of eIF2α and eIF2γ were co-immunoprecipitated with eIF2Bε-7A (Figure 3D, lane 5). We conclude that the AA-box 2 mutation in *gcd6-7A* reduces the eIF2–eIF2B interaction *in vivo*, impairing the conversion of eIF2-GDP to eIF2-GTP.

The *gcd6-12A* allele on a low-copy plasmid did not support the growth of the *gcd6*Δ strain (Table I). Although we did not epitope-tag the *GCD6* alleles, it was clear that the mutant eIF2Bε protein was poorly expressed, since the total amount of eIF2Bε protein in a wild-type strain increased by only a factor of ~2 when the *gcd6-12A* allele was present in high-copy (Table I), whereas wild-type *GCD6* on a high-copy plasmid increased the eIF2Bε level ~20-fold (Table I). Even in high-copy, however, *gcd6-12A* did not rescue growth of the *gcd6*Δ deletion strain (Table I). Thus, the AA-box 1 mutation in eIF2Bε is lethal, supporting our conclusion that the bipartite motif in eIF2Bε is important for eIF2B function *in vivo*.

The lysine-rich boxes (K-boxes) in the N-terminal segment of eIF2β are required for binding of eIF2β to eIF5 and eIF2Bε in vitro

Having established the importance of the bipartite motifs in eIF5 and eIF2Bε for their interaction with eIF2, we set out to locate the segment of eIF2β responsible for its binding to these proteins. For this purpose, we produced the [^{35}S]eIF2β peptides shown in Figure 4A, and tested them for interaction with the GST–eIF5 and GST–eIF2Bε proteins described above. All four C-terminally truncated eIF2β peptides, but neither N-terminally truncated peptide, bound at high levels to GST–eIF5. As expected, binding of the smallest N-terminal peptide (eIF2βΔS) to GST–eIF5 was abolished by the 7A mutation in eIF5 (Figure 4B; summarized in A). Likewise, GST–eIF2Bε bound the N-terminal peptide eIF2βΔS, but not the largest C-terminal peptide (eIF2βΔX), and the interaction with eIF2βΔS was abolished by the 7A mutation in GST–eIF2Bε (Figure 4C). These results indicate that the eIF2βΔS fragment (amino acids 1–140) is sufficient for binding to both eIF5 and eIF2Bε *in vitro* in a manner dependent on the bipartite motifs in both proteins.

Since the N-terminal segment of eIF2β contains three lysine-rich boxes (K-boxes) (Donahue *et al*., 1988; Pathak *et al*., 1988; see Figures 4A and 5A) and the bipartite

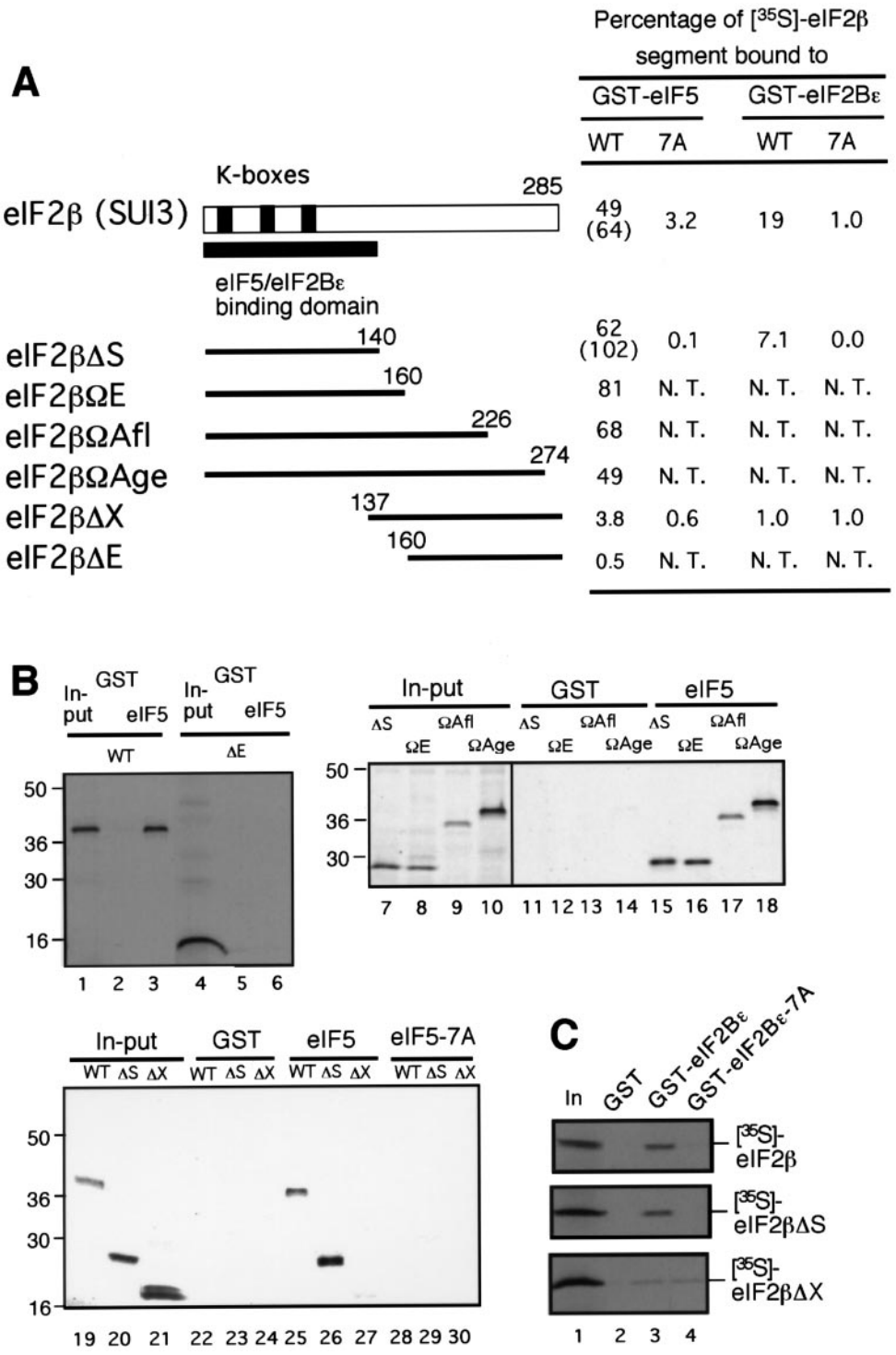

Fig. 4. The N-terminal domain of eIF2β is sufficient for its interaction with both eIF5 and eIF2Bε *in vitro*. (**A**) The segment of yeast eIF2β responsible for its binding to eIF5 and eIF2Bε *in vitro*. The schematic at the top depicts the primary structure of eIF2β from *S.cerevisiae*. Black rectangles denote the lysine boxes (see text and Figure 5A). Deletion derivatives of eIF2β are depicted as for eIF5 in Figure 1A. The results of binding experiments shown in (B) and (C) using these radiolabeled eIF2β peptides and GST–eIF5, GST–eIF2Bε or their 7A derivatives are summarized to the right, as in Figure 1A. Values in parentheses indicate results of independent experiments. (**B**) ^{35}S-Labeled eIF2β peptides, listed in (A), were synthesized *in vitro* and incubated with GST alone, GST–eIF5 or GST–eIF5-7A, as described in Figure 1B. Lanes 1–30 show the results of GST pull-down experiments: input, 50% of the input amounts of the full-length wild-type (WT) or truncated [^{35}S]eIF2β proteins described in (A); GST, the entire samples recovered with GST alone; eIF5 or eIF5-7A, the entire samples recovered with GST–eIF5 or GST–eIF5-7A, respectively. eIF2βΔS and eIF2βΩE co-migrate (lanes 8, 15 and 16), since the former contains an additional 19 amino acids at the C-terminus, encoded by the vector. (**C**) Binding experiments with GST–eIF2Bε or its 7A derivative. The GST proteins employed in binding reactions are indicated across the top. Each panel shows the recovery of ^{35}S-labeled eIF2β or its ΔS or ΔX derivative with the different GST fusions (lanes 2–4). Lane 1, 50% of the input amounts of labeled proteins in the binding reactions.

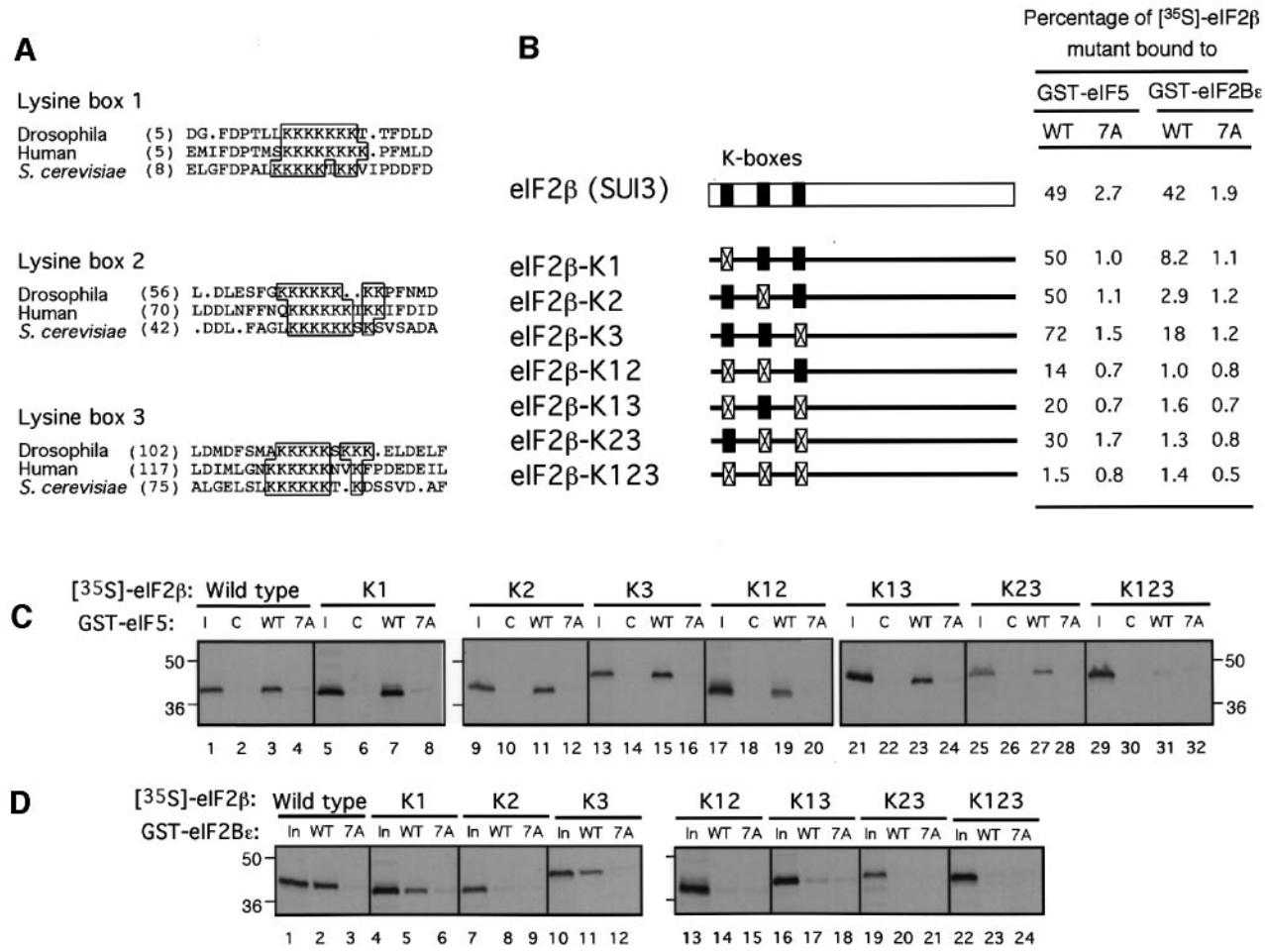

Fig. 5. The K-boxes in eIF2β make additive contributions to its binding to eIF5 and eIF2Bε *in vitro*. (**A**) The sequences of lysine-rich K-boxes conserved in eIF2β from *Drosophila*, human and *S.cerevisiae*. The amino acid number of the first residue in each segment is in parentheses, and the clustered lysine residues are boxed. (**B**) The K-box mutations made in eIF2β. The schematic at the top depicts the primary structure of wild-type eIF2β and below it are depicted the mutant forms of eIF2β, with filled rectangles indicating wild-type K-boxes and crossed rectangles indicating mutant boxes with all seven lysine residues substituted by alanines. The results of *in vitro* binding experiments shown in (C) and (D) for the different [^{35}S]eIF2β polypeptides with GST–eIF5 or GST–eIF2Bε fusion proteins, and their 7A derivatives, are summarized on the right, as described in Figure 1A. (**C**) and (**D**) *In vitro* binding of mutant or wild-type [^{35}S]eIF2β polypeptides, synthesized *in vitro*, to GST–eIF5 or GST–eIF2Bε fusions. The [^{35}S]eIF2β peptides indicated above the horizontal bars were incubated with GST alone, the wild-type GST–eIF5 or GST–eIF2Bε fusion, or the corresponding 7A mutant derivative (C, WT or 7A below the horizontal bars, respectively) attached to glutathione–Sepharose beads. The recovered labeled proteins were analyzed as in Figure 1. In (D), more wild-type eIF2β was recovered with GST–eIF2Bε than in the previous experiment described in Figures 1C and 4C because we used a higher concentration of both wild-type and mutant GST–eIF2Bε fusion proteins here.

motif is rich in acidic residues (Figure 1A), we suspected that the K-boxes were involved in the interaction with the bipartite motif domains. To test this possibility, we changed all seven lysine residues in each box to alanines, designating these multiple mutations as K1, K2 and K3 (Figure 5A and B). We found that none of the single mutations (K1, K2 or K3) affected *in vitro* binding between GST–eIF5 and eIF2β (Figure 5C), whereas all three double mutations (K12, K13 and K23) reduced the binding by 40–70%, and the triple mutation (K123) completely eliminated the binding (Figure 5C). These results indicate that the K-boxes make additive contributions to the interaction between eIF2β and eIF5 *in vitro*.

The interaction between GST–eIF2Bε and eIF2β was also reduced by the K-box mutations in eIF2β (Figure 5D). In contrast to the eIF5–eIF2β interaction where double K-box mutations were required to reduce binding, each single K-box significantly reduced the interaction between GST–eIF2Bε and eIF2β (Figure 5D, lanes 5, 8 and 11). Moreover, whereas K-box 2 seemed to be most critical for the eIF2Bε–eIF2β interaction (Figure 5D, lanes 5, 8 and 11), this was not the case for eIF5–eIF2β binding (Figure 5C, lanes 19, 23 and 27). We conclude that the K-boxes in eIF2β are required for its interactions with eIF2Bε and eIF5, but that the relative contributions of the different K-boxes are not identical for these two interactions.

The K-boxes in eIF2β are important for the function of eIF2 in vivo

We then asked whether the K-box mutations in eIF2β affect cell growth, or cause a Gcd$^-$ phenotype, indicative of a weakened interaction between eIF2 and eIF5 or eIF2B. When we inserted the K-box mutations into the *SUI3-FL* allele (encodes FLAG-tagged eIF2β) on a single-copy plasmid and introduced the resulting plasmids into

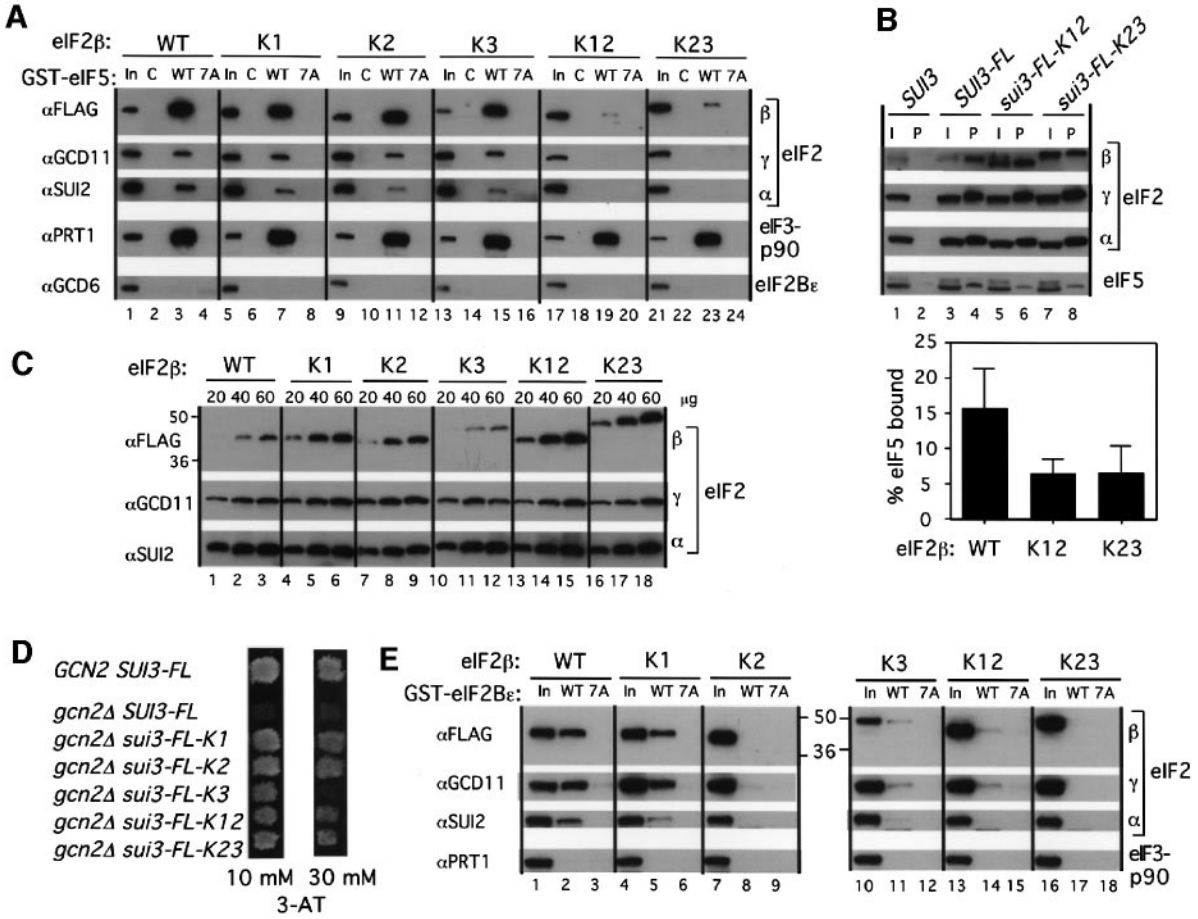

Fig. 6. K-box mutations in eIF2β reduce the interaction of eIF2 with eIF5 and eIF2Bε, and confer Gcd⁻ phenotypes *in vivo*. (**A**) Effect of the K-box mutations in eIF2β on binding of native eIF2 complex in WCE to GST–eIF5. Samples of WCE from KAY25 [*SUI3-FL* (WT; lanes 1–4)], KAY26 [*sui3-FL-K1* (K1; lanes 5–8)], KAY27 [*sui3-FL-K2* (K2; lanes 9–12)], KAY28 [*sui3-FL-K3* (K3; lanes 13–16)], KAY29 [*sui3-FL-K12* (K12; lanes 17–20)] and KAY30 [*sui3-FL-K23* (K23; lanes 21–24)] were incubated with GST alone (C), GST–eIF5 (WT) or GST–eIF5-7A (7A) immobilized on glutathione–Sepharose beads. The entire fraction recovered with the GST proteins, together with 20% of the input amounts of WCE (In), were separated by SDS–PAGE, blotted and probed with the antibodies indicated on the left to detect the proteins indicated on the right. (**B**) Interaction between eIF2 and eIF5 *in vivo*. WCEs prepared from strains KAY33 (*SUI3*), KAY25 (*SUI3-FL*), KAY29 (*sui3-FL-K12*) and KAY30 (*sui3-FL-K23*) were immunoprecipitated with FLAG affinity resin and the precipitated proteins were analyzed by Western blotting using antibodies against eIF2β, eIF2α, eIF2γ and eIF5. In, 20% input amount of WCE (lanes 1, 3, 5 and 7); P, the entire precipitated fraction (lanes 2, 4, 6 and 8). The percentages of eIF5 in the WCEs that were immunoprecipitated in this and two other replicate experiments were plotted for the wild-type (WT) *SUI3-FL*, *SUI3-FL-K12* (K12) and *SUI3-FL-K23* (K23) extracts. (**C**) Expression of eIF2β proteins bearing K-box mutations. Samples of WCE (20, 40 or 60 μg) from the same strains described in (A) were separated by SDS–PAGE, blotted and probed with the antibodies indicated on the left for detection of the eIF2 subunits listed on the right. (**D**) K-box mutations in eIF2β confer Gcd⁻ phenotypes. Isogenic strains KAY25 (*SUI3-FL*), KAY26 (*sui3-FL-K1*), KAY27 (*sui3-FL-K2*), KAY28 (*sui3-FL-K3*), KAY29 (*sui3-FL-K12*) and KAY30 (*sui3-FL-K23*) were grown to confluence on SD medium containing minimal supplements, replica-plated to SC-Leu supplemented with 3-AT (10 mM, left, or 30 mM, right), and incubated for 2 days at 30°C. (**E**) Effect of the K-box mutations in eIF2β on binding of native eIF2 complex in WCE to GST–eIF2Bε. This experiment was conducted exactly as in (A) except that GST–eIF2Bε was used in place of GST–eIF5 and 10% of the input amounts of WCE (In) were loaded.

a *sui3*Δ strain by plasmid shuffling, we found that the K13 and K123 mutations were lethal, whereas the other mutations did not affect cell growth at 25, 30 and 36°C (Table I). Western blot analysis of the cell extracts with anti-FLAG antibodies showed that the eIF2β-FL proteins carrying K1, K2, K3, K12 and K23 were expressed in amounts comparable with, or even higher than, that of the wild-type (Figure 6C). Western analysis of transformants containing both wild-type *SUI3* and the lethal *sui3-FL-K13* or *sui3-FL-K123* alleles showed that the *sui3-FL-K13* product was undetectable, most probably explaining its lethal phenotype, whereas *sui3-FL-K123* was expressed at 1/10 of the wild-type level (data not shown, see Table I). A high-copy plasmid containing *sui3-FL-K123* could not rescue the *sui3*Δ strain even though the mutant protein was expressed at a level 4- to 5-fold higher than wild-type (Table I). Therefore, we conclude that the K123 mutation abolishes an essential function of eIF2β *in vivo*.

To investigate whether the K12 and K23 double mutations in eIF2β reduced binding between eIF2 and eIF5 *in vitro*, we conducted pull-down assays with GST–eIF5 and wild-type or mutant eIF2 present in WCEs. As shown in Figure 6A, the K12 and K23 mutations substantially reduced the binding between eIF2 and GST–eIF5. As expected, the interaction between GST–eIF5 and eIF3-p90/PRT1 was unaffected by the K-box mutations in eIF2β (Figure 6A). [We noted that greater fractions of eIF2β versus eIF2α or eIF2γ were recovered with GST–eIF5. This may have occurred because eIF2β makes direct contact with eIF5, such that greater amounts of eIF2α and eIF2γ compared with eIF2β may have dissociated from the GST–eIF5/eIF2 complex during the washing steps.

The same phenomenon was observed to a lesser extent in the binding of purified eIF2 with GST–eIF5 (Figure 2A).] The results in Figure 6A indicate that the K-boxes in eIF2β make additive contributions to the interaction between the eIF2 complex and eIF5 *in vitro*. To show that the K-boxes are important for binding of eIF2 to eIF5 *in vivo*, we examined the effects of the K12 and K23 double mutations on co-immunoprecipitation of eIF5 with FLAG-tagged eIF2β from WCEs. As shown in Figure 6B, these mutations (encoded by *SUI3-FL-K12* and *SUI3-FL-K23*) reduced the co-immunoprecipitation of eIF5 with FLAG-tagged eIF2 by about a factor of three.

Interestingly, all the K-box mutations conferred Gcd$^-$ phenotypes (Figure 6D and Table I), suggesting that they impaired ternary complex formation *in vivo*. As the overexpression (~10-fold) of wild-type eIF2β causes a Gcd$^-$ phenotype in itself (Dever *et al*., 1995), and considering that all the viable K-box mutations except K3 increased the level of the mutant eIF2β by a factor of 2–5 (Figure 6C and Table I), it was important to determine whether a moderate increase in eIF2β levels is sufficient to confer a Gcd$^-$ phenotype. To answer this question, we introduced *SUI3-FL* on the low-copy plasmid YDpSUI3 into the *sui3*Δ *gcn2*Δ strain by plasmid shuffling and determined that wild-type eIF2β-FL was expressed from this plasmid at a level 2–3 times higher than from the single-copy vector; however, YDpSUI3 did not confer a Gcd$^-$ phenotype (Table I). These results imply that the Gcd$^-$ phenotypes of the K1, K2 and K3 single mutations in *SUI3* arose from a defect in eIF2 function, or in its recycling from the GDP- to GTP-bound form by eIF2B, rather than merely from overexpression of eIF2β. Consistent with this last interpretation, the eIF2 complexes present in WCEs from the *sui3-FL-K1*, *sui3-FL-K2* and *sui3-FL-K3* strains were defective for binding to the GST–eIF2Bε fusion protein (Figure 6E). As observed for binding of recombinant eIF2β proteins to GST–eIF2Bε (Figure 5D), the K2 mutation led to the greatest reduction in binding of native eIF2 to GST–eIF2Bε among the single K-box mutations. These data support the idea that tight binding of eIF2B with its substrate eIF2 is dependent on the K-boxes in eIF2β in addition to the bipartite motif in eIF2Bε.

Discussion

Roles of the bipartite motifs of eIF5 and eIF2Bε in the translation initiation pathway of S.cerevisiae

Bipartite motifs rich in aromatic and acidic residues (designated AA-boxes 1 and 2) are conserved at the C-termini of eIF5 and the catalytic subunit of eIF2B (eIF2Bε) from all eukaryotes examined so far (Koonin, 1995). In this study, we found that the bipartite motif in the C-terminal domain of *S.cerevisiae* eIF5 is required for its binding to eIF2 and eIF3 both *in vivo* and *in vitro* (Figures 1 and 2). We propose that eIF5 is recruited to the 40S ribosome, at least partly, by its interaction with eIF3, which involves the bipartite motif in eIF5 and the p93 (NIP1) subunit of eIF3 (Figure 7A). This idea is based on our previous finding that eIF5 co-purified with the eIF3 complex in nearly stoichiometric amounts (Phan *et al*., 1998) and the results presented here indicating that a large fraction of the total eIF3 could be co-immunoprecipitated with epitope-tagged eIF5 in a manner dependent on the bipartite motif of eIF5 (Figure 2C). Previously, we proposed that recruitment of eIF1 to the 40S ribosome is also assisted by its interaction with eIF3-p93 (Asano *et al*., 1998); although, in this case, eIF1 is either less tightly associated with eIF3 or is present in excess, such that a considerable amount of eIF1 occurs free of eIF3 in cell extracts (Naranda *et al*., 1996; Phan *et al*., 1998).

Following recruitment to the 43S pre-initiation complex of both the ternary complex and the m^{7}G-capped mRNA bound to eIF4F, it is believed that correct AUG selection by Met-tRNA$_i$ stimulates the eIF5-dependent hydrolysis of the GTP bound to eIF2 in the ternary complex (Figure 7A). We propose that the bipartite motif in eIF5 stabilizes its interaction with eIF2 at this step in the initiation pathway based on the reduction in eIF2–eIF5 complex formation *in vivo* conferred by the 7-Ala mutation in eIF5 (Figure 2C). It is also possible that the interaction between eIF5 and eIF2β provides a second pathway for recruitment of eIF5 to the pre-initiation complex, in addition to that involving eIF5–eIF3 interactions.

The segment of eIF5 containing the bipartite motif is required for its interaction with both eIF3 and eIF2 (Figures 1 and 2). It remains to be determined whether both interactions can occur simultaneously through different surfaces on this segment of eIF5. They could be sequential instead, with the eIF5–eIF3-p93 interaction involved in recruiting eIF5 to the pre-initiation complex giving way to the eIF5–eIF2β interaction required for stimulating GTP hydrolysis on eIF2. The possibility of simultaneous interaction is consistent with the fact that the binding domain for eIF5 in eIF3-p93 (NIP1) does not contain K-boxes. Therefore, NIP1 may contact a surface of the C-terminal domain in eIF5 containing the bipartite motif distinct from that which binds to the K-box region of eIF2β (K.Asano and A.G.Hinnebusch, unpublished results). If so, the postulated role of eIF3 in anchoring eIF5 to the initiation complex could persist until the GTP in the ternary complex is hydrolyzed and eIF5 and eIF2-GDP are released.

Our proposal that the bipartite motif in eIF5 is important for its stable interaction with eIF2 *in vivo* is consistent with the finding that the conditional lethal phenotype of *tif5-FL-7A* was fully suppressed by increasing the level of the mutant protein (Table I) and partially suppressed by increasing eIF2 ternary complex levels by overexpressing eIF2 and tRNA$_i^{Met}$ (Figure 2D). As the 7-Ala mutation in eIF5 weakens its interaction with both eIF3 and eIF2, it should impair both of the potential pathways for recruiting eIF5 to the pre-initiation complex discussed above. It is possible that recruitment of eIF5-7A was partially rescued through an increase in the ternary complex levels by formation of an eIF5–eIF2–GTP–Met-tRNA$_i^{Met}$ quaternary complex that would bind to the 43S pre-initiation complex and initiate the scanning process. While there is evidence for specific complex formation by mammalian eIF2 and eIF5 *in vitro* (Chaudhuri *et al*., 1994), it is unknown whether eIF2 can simultaneously form a stable complex with both tRNA$_i^{Met}$ and eIF5 free of the ribosome. The fact that overproducing eIF2 and tRNA$_i^{Met}$ only partially suppressed the *tif5-FL-7A* mutation could reflect the inefficiency of recruiting eIF5 via the ternary complex

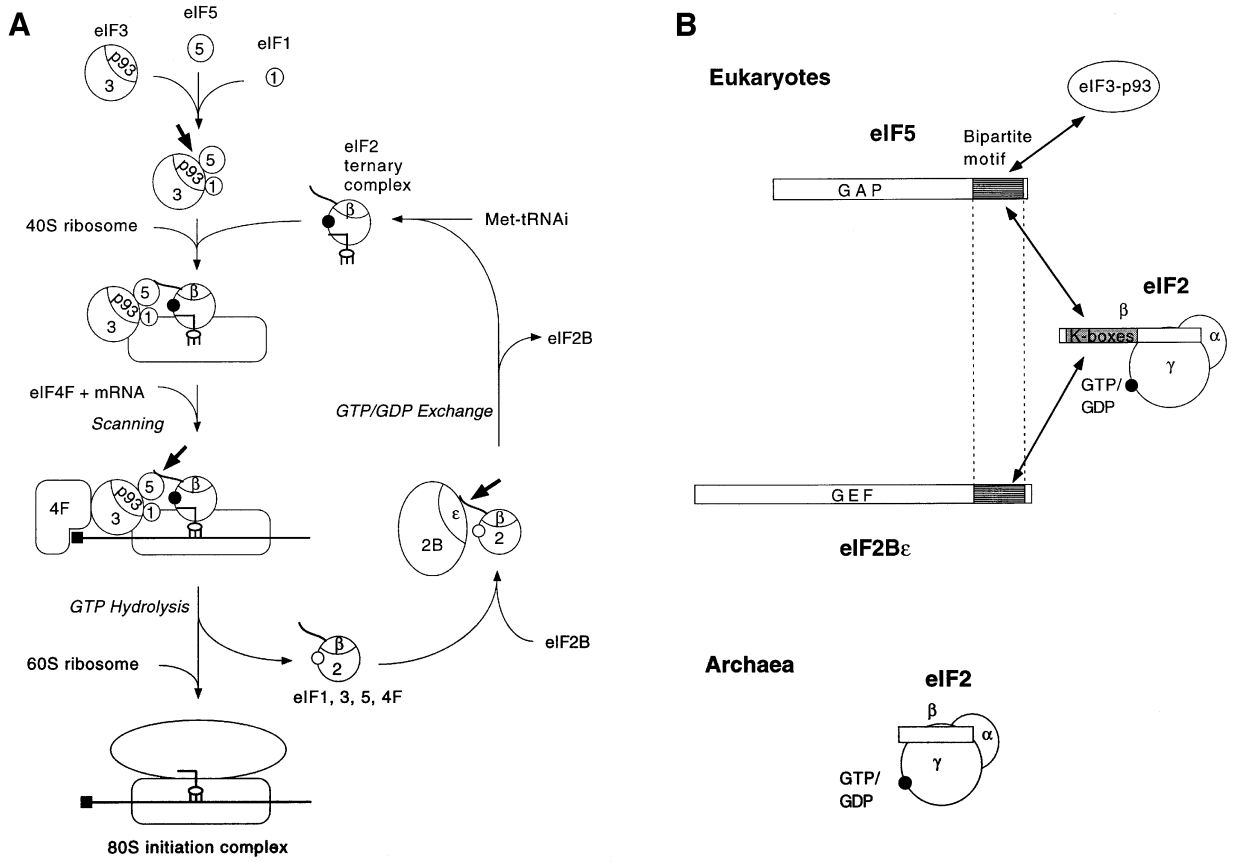

Fig. 7. The role of conserved bipartite motifs in eIF5 and eIF2Bε in promoting interactions between initiation factors involved in recognition of the AUG codon by $tRNA_i^{Met}$. (**A**) Hypothetical model for assembly of the 80S initiation complex in yeast *S.cerevisiae*. Based on results presented here and elsewhere (Asano *et al*., 1998; Phan *et al*., 1998), we propose that eIF1 (1) and eIF5 (5) are recruited to the 40S ribosome, at least partly, through their interactions with the 93 kDa subunit of eIF3, encoded by *NIP1* (3). The interaction between eIF5 and eIF3-p93 is dependent on the bipartite motif in eIF5 (see B). It is generally believed that the eIF2 ternary complex binds to the 40S ribosome subsequent to the binding of eIF3 (Merrick and Hershey, 1996). However, the data presented in Figure 2C and our preliminary results are consistent with the model that the ternary complex binding occurs in concert with the binding of the eIF1–eIF3–eIF5 complex. Subsequently, eIF4F (4F) delivers the mRNA to the 40S ribosome and the GTP on eIF2 is hydrolyzed upon AUG recognition. The bipartite motif in eIF5 is also important at this step for promoting the interaction with its substrate, eIF2-GTP. The GDP on eIF2 is exchanged to GTP by the action of eIF2B (2B). The bipartite motif in eIF2Bε, the catalytic subunit, promotes interaction with its substrate eIF2-GDP. The N-terminal region of eIF2β, the common binding site for eIF5 and eIF2B, is represented as the thick wavy line. The three thick arrows highlight the protein–protein interactions identified in this study. Filled circle, GTP; empty circle, GDP; plug, Met- $tRNA_i^{Met}$; thick line with a filled box (cap) at one end, m7G-capped mRNA. (**B**) Proposed evolution of the GTPase-activating and GDP–GTP exchange factors in eukaryotic translation initiation. Upper panel: boxes denote the primary structures of eIF5, eIF2Bε and eIF2β from eukaryotes (not drawn to scale). The conserved regions in eIF5 and eIF2Bε are hatched and connected by dotted lines. The N-terminal region of eIF2β, bearing the conserved K-boxes, is boxed in gray. Arrows denote the interactions revealed in this study. Circles represent the other two eIF2 subunits, γ and α. The open ellipse denotes eIF3-p93. Lower panel: archaea lack eIF5 and eIF2B, and, consistent with this fact, archaeal eIF2β lacks the K-box domain.

alone, in the absence of the complementary interaction between eIF5 and eIF3-p93 which is additionally impaired by this mutation.

An alternative explanation for the suppression of *tif5-FL-7A* by overexpression of the ternary complex would be that ribosome-bound eIF5 assists in recruitment of the ternary complex through its ability to interact directly with the eIF2β subunit of eIF2. This interaction would be weakened by the 7-Ala mutation in eIF5 and then rescued by mass action through overexpression of the ternary complex. An argument against this alternative explanation is that the *tif5-FL-7A* mutation should have a Gcd^- phenotype if eIF5 is normally required for efficient recruitment of the ternary complex to the pre-initiation complex, and this was not observed (Table I).

Based on the findings that mutations in the bipartite motif of eIF2Bε abolished its binding to both the eIF2β polypeptide and to native eIF2 (Figures 1C, 3A and 6E) and reduced the interaction between eIF2 and eIF2B *in vivo* (Figure 3D), we propose that the bipartite motif in eIF2Bε is essential for binding of eIF2 to eIF2B in a manner required for the GDP–GTP exchange reaction (Figure 7A). Consistent with this conclusion, the 7-Ala mutation in eIF2Bε conferred a Gcd^- phenotype that could be suppressed by overexpression of the ternary complex (Figure 3B and C), suggesting a defect in the recycling of eIF2 by eIF2B.

The K-boxes in eIF2β as the common binding site for eIF5 and eIF2B

Three lysine-rich stretches (K-boxes) in the N-terminal segment of eIF2β are required both *in vitro* (Figures 4, 5, 6A and E) and *in vivo* (Figure 6B–D) for strong interactions of the eIF2 complex with both eIF5 and eIF2B. Our co-immunoprecipitation results in Figures 2C and D, and 3D suggest that these interactions are mutually exclusive.

Thus, eIF2Bε did not co-immunoprecipitate with the eIF5 complex(es) containing eIF2 and eIF3 subunits (Figure 2D, lane 2), and eIF3 (and presumably eIF5) did not co-immunoprecipitate with the eIF2B–eIF2 complex (Figure 3D, lane 2). The exclusivity of these interactions is consistent with the fact that eIF5 and eIF2B promote opposing reactions on the guanine nucleotide bound to eIF2 (Figure 7A). As eIF2γ binds the guanine nucleotide (Merrick and Hershey, 1996), additional (perhaps transient) interactions between eIF5 or eIF2B and the γ subunit could be important for promoting GTP hydrolysis or guanine nucleotide exchange, by inducing conformational changes within the GTP-binding domain of eIF2γ. In addition, there is evidence that the δ (Kimball *et al*., 1998) and γ (Pavitt *et al*., 1998) subunits of eIF2B also contribute to the binding of eIF2.

Evolution of the control of GTP binding and hydrolysis on eIF2 in eukaryotic translation initiation

Only three initiation factors are known in eubacteria (IF1, IF2 and IF3), and base pairing between the 3′ end of 16S rRNA and the Shine–Dalgarno sequence in the mRNA plays a prominent role in selection of the start codon (Voorma, 1996). In contrast to eukaryotic systems, initiation at non-AUG triplet occurs frequently in eubacteria (Voorma, 1996). The translation initiation systems in eukaryotes devote much energy and many additional factors to the binding of mRNA to the small ribosomal subunit and to stringent selection of AUG as the start codon (see Introduction). Presumably, many of the complex reactions involved in these two processes have been added to the more basic reactions involving the 40S ribosome and the initiation factors eIF1, IF1/eIF1A (Kyrpides and Woese, 1998) and IF2 (Choi *et al*., 1998), which appear to be universally conserved in all three kingdoms of life.

Interestingly, archaea contain all three subunits of eIF2, but appear to lack eIF5 (Bult *et al*., 1996; Klenk *et al*., 1997; Smith *et al*., 1997). It was reported that archaea contain one or two homologs of the regulatory subunits (α and δ) of eIF2B. However, our analysis indicates that these archaeal proteins belong to a new protein family, which contains hypothetical eukaryotic and eubacterial proteins distinct from eIF2Bα or eIF2Bδ (data not shown). Accordingly, we believe that archaea lack all five subunits of eIF2B. Consistent with this conclusion, archaeal eIF2β lacks the K-box domain which, as shown here, is crucial for interactions of eIF2 with eIF5 and eIF2B (Figure 7B).

It is tempting to speculate that during the course of eukaryotic evolution the primordial eIF5 and eIF2Bε acquired domains containing the bipartite motifs, whereas their common substrate eIF2 acquired the K-box domains in the β subunit for interaction with the bipartite motif-containing domains (Figure 7B). This would provide a high affinity binding site on eIF2 for the proteins that regulate its GTPase activity and catalyze GDP–GTP exchange, without compromising the basic functions of eIF2 in transferring $tRNA_i^{Met}$ to the small ribosomal subunit and in AUG selection. The dependence on eIF5 for hydrolysis of GTP in the eIF2 ternary complex appears to provide a proofreading capability, as Sui^- mutations in eIF5 increase the probability of initiation at non-AUG triplets (Huang *et al*., 1997). The GDP–GTP exchange factor eIF2B confers the ability to regulate the concentration of the active GTP-bound form of eIF2 (Trachsel, 1996). Inhibition of eIF2B by phosphorylation of eIF2 is a mechanism for down-regulating protein synthesis in response to starvation or stress that is employed from yeast to humans (Clemens, 1996). In yeast cells, it allows for specific translational induction of the transcriptional activator GCN4 during an amino acid or purine limitation (Hinnebusch, 1996). Thus, the appearance of eIF5 and eIF2B in eukaryotic evolution increased the accuracy of start codon selection and provided the means to regulate translation at the $tRNA_i^{Met}$-binding step of initiation.

Materials and methods

Plasmids

Plasmids pGAD-NIP1(Asano *et al*., 1998) and pGAD-SUI3 encode the GAL4 activation domain fusions with eIF3-p93 and eIF2β. pGBT-TIF5, pGBT-A1 to -A9 and pGBT-B2 to -B9 encode the GAL4 DNA-binding domain fusions with truncated versions of eIF5 (Figure 1A). These plasmids, employed for two-hybrid analyses, were constructed by synthesizing DNA containing the corresponding coding regions by PCR using oligonucleotides that introduced restriction enzyme sites at both ends (5′-*Bam*HI–*Pst*I-3′ for pGAD-SUI3, 5′-*Eco*RI–*Bam*HI-3′ for pGBT-TIF5 and pGBT-A1 to -A9, and 5′-*Bam*HI–*Sal*I-3′ for pGBT-B2 to -B9), and by subcloning the resulting DNA fragments into pGAD424 or pGBT9 (Bartel *et al*., 1993).

pGEX-TIF5 (Phan *et al*., 1998) and its mutant derivatives, constructed as below, were employed for bacterial expression of GST–eIF5 or its mutant derivatives. pGEX-A6 to -A9 and pGEX-B5 to -B9 were constructed by transferring the truncated *TIF5* coding regions from the corresponding pGBT-TIF5 derivatives into pGEX-4T-1 (Smith and Johnson, 1988). pGEX-ΔA and pGEX-ΔB were constructed by truncating the *TIF5* open reading frame (ORF) of pGEX-TIF5 at *Asp*718I and *BsaA*I sites, respectively. The AA-box 1 mutant (12A) derivative of pGEX-TIF5 was constructed by replacing the 82 bp *Bst*YI–*Asp*718I fragment of pGEX-TIF5 with the corresponding fragment containing all of the Ala substitutions shown in Figure 1A (12A), with both strands chemically synthesized (Gibco-BRL) and annealed together. The derivative of pGEX-TIF5 containing the AA-box 2 Ala substitutions (7A) was constructed by synthesizing the mutant *TIF5* ORF by PCR using an oligonucleotide complementary to the mutated 3′-terminal region of the *TIF5* ORF and tagged with the *Sal*I site, and by subcloning the resulting fragment into pGEX-4T-1.

pGEX-GCD6 (a gift of Weimin Yang) encoding the GST–eIF2Bε fusion lacking residues 1–15 was constructed by subcloning the 2.3 kb *Nco*I–*Not*I fragment of pJB85 (Bushman *et al*., 1993a) (with the *Nco*I site filled in with Klenow enzyme) between the *Sma*I and *Not*I sites of pGEX-4T-2 (Smith and Johnson, 1988). The derivative of pGEX-GCD6 containing the AA-box 1 Ala substitutions (12A) was constructed by replacing the 625 bp *Afl*II–*Not*I fragment (encoding residues 506–712 of eIF2Bε) of pGEX-GCD6 with the 444 bp *Afl*II–*Sau*3AI fragment (residues 506–654) and the 181 bp *Sau*3AI–*Not*I segment (residues 654–712) containing the entire mutations, which were introduced by PCR with an oligonucleotide containing the entire mutation. For the convenience of subcloning, a silent ATT to ATC base change was introduced at Ile654 to produce the *Sau*3AI site. The derivative of pGEX-GCD6 containing the AA-box 2 Ala substitutions (7A) was constructed by replacing the 625 bp *Afl*II–*Not*I fragment of pGEX-GCD6 with the corresponding 7A mutant segment, synthesized by PCR with an oligonucleotide complementary to the mutated 3′-terminal region of the *GCD6* ORF.

pT7-SUI3 and its mutant derivatives, constructed as described below, and pT7-NIP1 (Asano *et al*., 1998) were employed for synthesizing ^{35}S-labeled eIF2β, its mutants and eIF3-p93 in reticulocyte lysates, respectively. pT7-SUI3 was constructed by subcloning the *Nde*I–*Pst*I fragment containing the *SUI3* ORF, synthesized by PCR, into pT7-7 (Tabor and Richardson, 1987). pT7-SUI3 derivatives encoding the truncated eIF2β proteins shown in Figure 4 were constructed by frameshifting the *SUI3* ORF by digestion of pT7-SUI3 with *Eco*RI (eIF2βΩE), *Afl*II (eIF2βΩAfl) or *Age*I (eIF2βΩAge), followed by filling-in and self-ligation, or by subcloning the *Nde*I–*Ssp*I (eIF2βΔS), *Eco*RI–*Pst*I (eIF2βΔE) or *Xba*I(filled-in)–*Pst*I (eIF2βΔX) fragments of pT7-

SUI3 into pT7-7. The pT7-SUI3 derivative encoding eIF2β-K1, -K2 or -K3, in which the boxed lysine residues in Figure 5A were replaced with Ala residues, were constructed by subcloning the following mutant segments, synthesized by PCR, between the *Nde*I and *Sty*I or *Mlu*I sites of pT7-SUI3: the 280 bp *Nde*I–*Mlu*I fragment containing the entire K1 or K3 mutation, and the 223 bp *Nde*I–*Sty*I fragment containing the entire K2 mutation. Double or triple K-box mutant derivatives of pT7-SUI3 were constructed similarly, starting from the above single mutant derivatives as the template for the PCR reactions.

pKA234 (*TIF5 LEU2*) and pKA235 (*TIF5 URA3*) were prepared by subcloning the 2.2 kb *Eco*RI–*Sal*I fragment of *TIF5*, which was synthesized by PCR from the yeast chromosomal DNA and contained the *TIF5* ORF plus the flanking 0.5 kb regions, into YCplac111 and YCplac33 (Gietz and Sugino, 1988), respectively. YCpTIF5 (*TIF5-FL LEU2*), encoding eIF5 tagged by the FLAG epitope at its C-terminus, was constructed by subcloning into YCplac111 the following three DNA fragments with modified ends, generated by PCR: the 0.5 kb *Eco*RI–*Nde*I and *Sal*I–*Xho*I fragments harboring the 5′ and 3′ untranslated regions (UTRs), respectively, and the 1.2 kb *Nde*I–*Sal*I fragment bearing the *TIF5-FL* ORF. Thus, unique *Nde*I and *Sal*I sites were introduced into YCpTIF5, flanking the *TIF5-FL* ORF. The AA-box mutant derivatives of YCpTIF5 were constructed by replacing the *Nde*I–*Sal*I fragment containing the *TIF5-FL* ORF with the corresponding mutant fragments, generated by PCR using the cognate pGEX-TIF5 derivative as template. High-copy *LEU2* plasmid YEpTIF5 bearing *TIF5-FL* and its mutant derivatives were constructed by transferring the 2.2 kb *Eco*RI–*Hin*dII fragments from the cognate YCpTIF5 derivatives into YEplac181 (Gietz and Sugino, 1988).

pJB5 (*GCD6 URA3*) was described previously (Bushman *et al*., 1993a). YDpGCD6 and its mutant derivatives were constructed by replacing the 0.8 kb *Afl*II–*Not*I fragment of pJB102, a low-copy *GCD6 LEU2* plasmid (Bushman *et al*., 1993a), with the following two fragments synthesized by PCR with modified ends: the 0.6 kb *Afl*II–*Not*I fragment from wild-type or mutant pGEX-GCD6 and the 0.2 kb *Not*I–*Eag*I fragment containing the 3′ UTR. High-copy plasmid YEpGCD6 and its mutant derivative encoding wild-type or mutant eIF2Bε were generated by transferring the 2.7 kb *Xho*I–*Sac*I fragment from the cognate YDpGCD6 derivative into pRS425 (*LEU2*) (Christianson *et al*., 1992).

p921 (*SUI3 URA3*) was described previously (Dever *et al*., 1995). pKA257 (*SUI3 LEU2*) was constructed by subcloning into YCplac111 the following three DNA fragments: the 0.5 kb *Sac*I–*Nde*I fragment containing the *SUI3* 5′ UTR (synthesized by PCR), the 0.8 kb *Nde*I–*Age*I fragment of pT7-SUI3 containing the *SUI3* ORF lacking the 3′-terminal region, and the 0.3 kb *Age*I–*Hin*dIII fragment of p921 containing the remainder of the *SUI3* ORF plus the 3′ UTR. YCpSUI3 or its K-box mutant derivative, encoding wild-type or mutant eIF2β tagged with the FLAG epitope at the N-terminus, was constructed by replacing the 0.3 kb *Nde*I–*Mlu*I fragment of pKA257 with the corresponding fragment generated by PCR using the cognate pT7-SUI3 derivative as template and modified at its 5′ end with the FLAG tag-coding sequence. The 2.6 kb *Sac*I–*Alw*NI fragment of YCpSUI3 was subcloned between the *Alw*NI–*Sac*I sites of pRS425 or pRS315 (Sikorski and Hieter, 1989) to generate YEpSUI3 or YDpSUI3, respectively.

The *URA3* plasmid p1780 encoding all three subunits of eIF2 was constructed previously (Dever *et al*., 1995). p1780-IMT was prepared by inserting the 170 bp *Xho*I fragment of *IMT4* encoding $tRNA_i^{Met}$, generated by PCR, into the *Xho*I site of p1780. p1780-FL was constructed by replacing the 1.4 kb *Bsi*WI(filled-in)–*Mlu*I fragment of p1780 with the 1.3 kb *Ehe*I–*Mlu*I fragment of YCpSUI3 containing a part of *SUI3-FL*.

Yeast strains

TIF5 was deleted in strain H1894 (*MATa ura3-52 leu2-3 leu2-112 trp1-*Δ*63 gcn2*Δ) (Kawagishi-Kobayashi *et al*., 1997) exactly as described for the deletion of *TIF34* (Asano *et al*., 1998). Briefly, the DNA fragment containing the *tif5*Δ*::hisG::URA3::hisG* disruption allele was integrated into the *TIF5* locus of H1894 carrying plasmid pKA234 (*TIF5 LEU2*), and the *URA3::hisG* portion was evicted to generate strain KAY23 [*MATa ura3-52 leu2-3 leu2-112 trp1-*Δ*63 gcn2*Δ *tif5*Δ pKA234 (*TIF5 LEU2*)]. KAY24 is a derivative of KAY23 in which pKA234 was replaced by pKA235 (*TIF5 URA3*). YCpTIF5, YEpTIF5 and their AA-box 2 mutant derivatives were introduced into KAY24 in place of pKA235 by plasmid shuffling (Boeke *et al*., 1987) to generate KAY35 (*TIF5-FL*), KAY36 (*tif5-FL-7A*), KAY39 (*TIF5-FL* in high-copy) and KAY40 (*tif5-FL-7A* in high-copy).

The *gcd6*Δ strain H1905 [*MAT*α *ura3-52 leu2-3 leu2-112 ino1 gcd6*Δ *<HIS4-lacZ ura3-52>* pJB102 (*GCD6 LEU2*)] was described previously (Bushman *et al*., 1993a). The *GCN2* allele of this strain was deleted using pHQ414 (*gcn2::hisG::URA3::hisG*) as described (Qiu *et al*., 1998) to generate KAY15 [*MAT*α *ura3-52 leu2-3 leu2-112 ino1 gcd6*Δ *gcn2*Δ *<HIS4-lacZ ura3-52>* pJB102 (*GCD6 LEU2*)]. KAY16 is a derivative of KAY15 in which pJB5 (*GCD6 URA3*) replaces pJB102. YDpGCD6, its AA-box 2 mutant derivative and YEpGCD6 were introduced in place of pJB5 into KAY16 by plasmid shuffling to generate KAY33 (*GCD6*), KAY34 (*gcd6-7A*) and KAY41 (*GCD6* in high-copy).

The *sui3*Δ strain H1650 [*MAT*α *ura3-52 leu2-3 leu2-112 ino1 sui3*Δ *<HIS4-lacZ ura3-52>* p920 (*SUI3 LEU2*)] was a gift of Tom Dever. *GCN2* was deleted in this strain using pHQ414 as above to generate KAY17 [*MAT*α *ura3-52 leu2-3 leu2-112 ino1 sui3*Δ *gcn2*Δ *<HIS4-lacZ ura3-52>* p920 (*SUI3 LEU2*)]. KAY18 is a derivative of KAY17 in which p921 (*SUI3 URA3*) replaces p920. YCpSUI3 (*SUI3-FL*) and its K-box mutant derivatives were introduced in place of p921 into strain KAY18 to generate KAY25 (*SUI3-FL*), KAY26 (*sui3-FL-K1*), KAY27 (*sui3-FL-K2*), KAY28 (*sui3-FL-K3*), KAY29 (*sui3-FL-K12*) and KAY30 (*sui3-FL-K23*).

Strain BJ1995 (*MAT*α *leu2-3,-112 trp1 ura3-52 gal2 pep4-3 prb1-1122*) was described previously (Jones, 1991). Strain KAY42, employed as the source for FLAG-tagged eIF2, was the transformant of KAY34 (*gcd6-7A*) carrying p1780-FL (*SUI2 SUI3-FL GCD11* in high-copy).

Materials

Yeast eIF2 and eIF3 were purified as described (Pavitt *et al*., 1998; Phan *et al*., 1998). Yeast WCEs were prepared as follows: 50 ml of yeast cells growing exponentially in YPD medium were collected by centrifugation and suspended in 2–3 cell volumes of buffer A [20 mM Tris–HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), Complete™ protease inhibitors (Boehringer Mannheim) and 1 μg/μl each of pepstain A, leupeptin and aprotinin], except that a previously described breaking buffer (Pavitt *et al*., 1998) was used for strains KAY33, KAY34, KAY41 and the transformants of BJ1995 overexpressing eIF2Bε (Figure 3). Suspended cells were broken with acid-washed glass beads (425–600 μm, Sigma) by three 15 s pulses in a Braun homogenizer (B.Braun) at 4°C, with 30 s of cooling between pulses. Homogenized cell extracts were clarified by centrifugation, and the recovered supernatants were employed as WCEs for the binding assays described below.

Protein–protein interaction assays

Yeast two-hybrid assays and GST pull-down assays with ^{35}S-labeled proteins, synthesized in rabbit reticulocyte lysates, were conducted as described previously (Asano *et al*., 1998).

For GST pull-down assays with native eIF2 or eIF3, either purified or present in WCEs, the GST–eIF5 or GST–eIF2Bε fusion proteins expressed in *E.coli* strain BL21(DE3) carrying the appropriate pGEX plasmids were immobilized on glutathione–Sepharose beads (Pharmacia) as instructed by the manufacturer, and incubated with 200 μg of WCEs or 1 μg of purified eIF2 or eIF3 in 300 μl of buffer A containing 1% non-fat dry milk and 0.05% NP-40. Alternatively, 1.2 mg of WCE in 100 μl of the same buffer was incubated with the GST–eIF2Bε fusion proteins. After incubation for 2 h at 4°C, the protein complexes attached to the beads were washed with 0.5 ml of phosphate-buffered saline (PBS) four times, and eluted in 10 μl of 2× loading buffer (Laemmli, 1970) for 2 min at 95°C. The eluted proteins were separated by SDS–PAGE, blotted to PVDF membranes (NOVEX) and probed with the following rabbit polyclonal antibodies: anti-SUI2 and anti-SUI3 (Dever *et al*., 1995), anti-GCD11 (Hannig *et al*., 1992), anti-NIP1 (Greenberg *et al*., 1998), anti-PRT1 (Cigan *et al*., 1991), anti-TIF34 (Asano *et al*., 1998), anti-GCD6 (Bushman *et al*., 1993b) and anti-GST (Santa Cruz Biotechnology); or with mouse anti-FLAG antibodies (Kodak). Detection of immune complexes was performed by enhanced chemiluminescence (ECL, Amersham).

Co-immunoprecipitation with anti-FLAG antibodies was conducted by incubating 200 μg of WCE in 300 μl of buffer A with 15 μl wet volume of the FLAG affinity resin (Sigma) for 2 h at 4°C. The protein complexes adsorbed to the resin were washed with 0.3 ml of buffer A four times, and eluted and analyzed by SDS–PAGE and Western blotting, as described above for the GST pull-down assays with native proteins. Co-immunoprecipitation with anti-GCD6 antibodies was conducted as described previously (Dever *et al*., 1995; Pavitt *et al*., 1997), except that we used 400 μg of WCE instead of the ribosomal salt wash fraction.

For analyzing interaction between purified eIF2 and native eIF2Bε, 1 mg (per reaction) of WCE from strain KAY42 (*gcd6-7A* p1780-FL) was incubated with 10 μl wet volume of anti-FLAG affinity resin (Sigma) in 200 μl of buffer A for 2 h at 4°C. FLAG-eIF2 attached to

the resin was washed with 0.3 ml of buffer B (Phan *et al*., 1998) containing 350 mM KCl four times. The yield of purified eIF2-FL was ~500 ng per reaction. The eIF2-FL resin was then incubated with 200 µg of WCE from the transformant of BJ1995 carrying YEpGCD6 or YEpGCD6-7A in 100 µl of breaking buffer (Pavitt *et al*., 1998) for 30 min at 25°C. The proteins on the resin were washed with 0.2 ml of the same breaking buffer four times and eluted with the FLAG peptide solution as recommended by the manufacturer.

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