Direct Binding of Translation Initiation Factor eIF2γ-G Domain to Its GTPase-activating and GDP-GTP Exchange Factors eIF5 and eIF2B*

Received for publication, October 28, 2005, and in revised form, February 13, 2006. Published, JBC Papers in Press, March 7, 2006, DOI 10.1074/jbc.M511700200

Pankaj V. Alone and Thomas E. Dever

From the Laboratory of Gene Regulation and Development, NICHD, National Institutes of Health, Bethesda, Maryland 20892

The GTP-binding eukaryotic translation initiation factor eIF2 delivers initiator methionyl-tRNA to the 40 S ribosomal subunit. The factor eIF5 stimulates hydrolysis of GTP by eIF2 upon AUG codon recognition, whereas the factor eIF2B promotes guanine nucleotide exchange on eIF2 to recycle the factor for additional rounds of translation initiation. The GTP-binding (G) domain resides in the γ subunit of the heterotrimeric eIF2; however, only eIF2γ, and not eIF2γ, has been reported to directly bind to eIF5 or eIF2B. Using proteins expressed in yeast or recombinant systems we show that full-length yeast eIF2γ, as well as its isolated G domain, binds directly to eIF5 and the e subunit of eIF2B, and we map the interaction sites to the catalytically important regions of these factors. Consistently, an internal deletion of residues 50–100 of yeast eIF5 impairs the interaction with recombinant eIF2γ-G domain and abolishes the ability of eIF5 to stimulate eIF2 GTPase activity in translation initiation complexes in vitro. Thus, rather than allosterically regulating eIF2γ-G domain function via eIF2B, our data support a model in which the GTPase-activating factor eIF5 and the guanine-nucleotide exchange factor eIF2B modulate eIF2 function through direct interactions with the eIF2γ-G domain.

The initiation of protein synthesis in eukaryotic cells requires the coordinated activity of at least 10 eukaryotic initiation factors (eIFs), with several of the factors composed of multiple polypeptide chains. Interactions among the initiation factors and the ribosome promote initiator methionyl-tRNA (Met-tRNA\textsuperscript{Met}) and mRNA binding to the 40 S ribosomal subunit and subsequent joining of the large 60 S ribosomal subunit to form the translationally competent 80 S ribosome. The factor eIF2 is responsible for delivering Met-tRNA\textsuperscript{Met} to the 40 S subunit in the first step of translation initiation (reviewed in Refs. 1 and 2). The eIF2 is composed of three polypeptide chains: the core eIF2γ subunit (GCD11 in yeast), to which the eIF2α (SUI2) and eIF2β (SUI3) subunits bind. The eIF2γ subunit contains a consensus GTP-binding (G) domain at its N terminus, and structural studies on the eIF2γ homolog from archaea revealed a three-domain structure that closely resembles the structure of the translation elongation factor EF-Tu (EF1A) (3, 4). The structural similarity between eIF2γ and EF-Tu is consistent with the common function of the two proteins to bind aminoacyl-tRNA in a GTP-dependent manner and to deliver the aminoacyl-tRNA to the ribosome. Mutational analyses of eIF2γ support a similar mode of aminoacyl-tRNA binding by eIF2γ and EF-Tu, wherein the 3′ (aminoacyl) end of the tRNA binds to domain II (3, 5, 6). Interestingly, the C-terminal end of eIF2α binds to an adjacent conserved surface on domain II (3, 7). The N terminus of the eIF2β subunit contains three lysine-rich segments (K-boxes) that are absent from the corresponding archaeal protein and do not appear to be important for binding to eIF2γ (7, 8). Moreover, the binding site for eIF2β on eIF2γ has not been resolved.

Following binding of the eIF2 ternary complex (eIF2-GTP-Met-tRNA\textsuperscript{Met}) to the 40 S subunit and association of other initiation factors, including eIF1, eIF1A, eIF3, and eIF5 (TIF5 in yeast), the so-called 43 S preinitiation complex binds to an mRNA near the 5′ cap structure in a reaction facilitated by the eIF4 family of factors (reviewed in Ref. 1). The 43 S complex then scans the mRNA in a 5′ to 3′ direction in search of an AUG start codon. Base pairing between the 5′-CAU-3′ anticodon of the Met-tRNA\textsuperscript{Met} in the 43 S complex with an AUG codon is thought to halt the scanning ribosome and trigger GTP hydrolysis by eIF2. This GTP hydrolysis reaction is stimulated by the putative GTPase-activating protein (GAP) eIF5 (9, 10). Following GTP hydrolysis, eIF2 and other initiation factors are released from the 40 S ribosome (11, 12), which then joins with the 60 S subunit in a reaction catalyzed by a second GTPase eIF5B (13). The eIF2 is released from the ribosome bound to GDP, and, like a number of G proteins, eIF2 has a higher affinity for GDP than for GTP (~100-fold difference for yeast eIF2). The nonenzymatic exchange of GTP for GDP on eIF2 occurs slowly, and the guanine nucleotide exchange factor (GEF) eIF2B enhances the rate of this reaction and recycles eIF2 for use in subsequent rounds of translation initiation (2). Phosphorylation of the eIF2α subunit on Ser\textsuperscript{51} by one of the stress-responsive eIF2α kinases (GCN2, PERK, PKR, or HR) converts eIF2 from a substrate to an inhibitor of eIF2B and thereby blocks cellular protein synthesis (reviewed in Ref. 14).

The eIF2B is composed of five subunits that can be both physically and functionally separated into two subcomplexes (reviewed in Ref. 2). The regulatory subcomplex, composed of the α (GCD3 in yeast), β (GCD7), and δ (GCD2) subunits of eIF2B, binds to eIF2α in a manner stimulated by Ser\textsuperscript{51} phosphorylation (15). The catalytic subcomplex, composed of the γ (GCD1) and ε (GCD6) subunits of eIF2B, binds to eIF2 and catalyzes guanine nucleotide exchange in vitro (16). Further in vitro studies mapped the eIF2B GEF activity to the C-terminal ~200 residues of eIF2Bε (17), which was found to fold into an eight-stranded α-helical bundle resembling a HEAT repeat (18). Interestingly, the eIF2Bε HEAT domain contains two regions of conserved aromatic and acidic amino acids referred to as AA-boxes (or the W2 domain) that are also found in eIF5 (see Fig. 1) (19, 20). These AA-boxes in both eIF2Bε and eIF5 have been shown...
to directly interact with the K-boxes in the N-terminal half of eIF2β (Fig. 1) (20–22). Multiple alanine substitutions in the AA-boxes of eIF2B and eIF5 disrupt binding to eIF2 (20). Consistently, these AA-box mutations in eIF5 and the corresponding K-box mutations in eIF2β impair initiation complex assembly (23–25).

Given the direct interactions of eIF2B and eIF5 with eIF2β and the lack of evidence for a direct interaction of these proteins with the GTP-binding eIF2γ subunit, it could be proposed that these effectors of G protein function modulate eIF2γ nucleotide exchange and hydrolysis indirectly (allosterically) through eIF2β. Alternatively, the interactions with eIF2β may serve a scaffolding role to facilitate the binding of other regions in the GAP (eIF5) and GEF (eIF2B) to eIF2γ. Here we present in vitro evidence that both eIF5 and eIF2B directly interact with eIF2γ. Moreover, we map this interaction to the eIF2γ-G domain, consistent with the notion that these proteins directly modulate guanine nucleotide binding and hydrolysis by eIF2.
**Experimental Procedures**

Yeast Strains—Yeast strains were as follows: H2888 (KAY25), MATa leu2–3,112 ura3–52 ino1 gen2A su13A p[SLI3–FL, LEU2] (20), H2892 (KAY29), MATa leu2–3,112 ura3–52 ino1 gen2A su13A p[SLI3–FL::K12, LEU2] (20), H2894 (KAY35), MATa leu2–3,112 ura3–52 trp1–63A gen2A tfs5::HisG p[TIF5–FL, LEU2] (20); H2895 (KAY36), MATa leu2–3,112 ura3–52 trp1–63A gen2A tfs5::HisG p[TIF5–FL, LEU2] (20); F708, MATa ura3–52 his2–1 tfs5–G62S.

Plasmids—DNA fragments encoding various eIF2γ (GCD11) fragments were amplified by PCR and cloned into the vector pEG2 (25) between the BamHI and Sall sites. The primers used for PCR were as follows: pC2693 (encoding GST–G) (BamHI–GCD11 (5′–CGCGGATC-CCATTCAGACGAGAACTCTAG-3′) plus Sall/TIF5 (5′–CGGATCTTATTCATTTGTTACGATAAGACGATCC-3′)) cloned between the BamHI and SalI sites of the vector pGEX-6P-1 using GGATCCCTAGACGTCTTGAATGATCC-3′ (encoding GST-D2) (D2/BamHI (5′–CACAAGG-3′) plus D2/SalI (5′–CTCTGTTGACAAAGATCGTTATAGCTG-3′)) (28) rabbit polyclonal antibodies against eIF3a (anti-GCD6), eIF5 (anti-TIF5), eIF2B (anti-LEU2), eIF2 (anti-RPS2) or with rabbit polyclonal anti-FLAG M2 peptide (Sigma) antibodies. Anti-yeast eIF2γ antisera was obtained by inoculating rabbits with recombinant His6-tagged eIF2γ purified from Escherichia coli.

**Yeast GST Pull-down Assays**—Yeast cells expressing various GST–eIF2γ fusion proteins were grown in 50 ml of synthetic complete (SC) medium at 30 °C to mid-log phase, harvested, and washed with SGR medium (SC plus 10% galactose and 2% raffinose). The cells were then seeded in 50 ml of SGR medium to A600 ~0.5, grown to A600 ~0.8 to induce expression of the GST fusion proteins, harvested, and frozen at −80 °C until further use. Cells were suspended in lysis buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 25 mM MgCl2, 5 mM NaF, 1 mM EDTA, 2 mM β-ME, containing one tablet of protease inhibitor mixture and 2 μM each aprotinin, leupeptin, pefabloc, 1-chloro-3-tosylamide–7–amino–2–heptanone, and pepstatin (Roche Applied Science)), and whole cell extracts (WCEs) were prepared by homogenizing the cells by vortexing with glass beads. Glutathione-Sepharose 4B beads were washed several times and suspended in 500 μl of binding buffer (lysis buffer plus 0.2% Nonidet P-40 and 1% skimmed milk). 350 μg of yeast WCEs were mixed with ~70 μl of glutathione-Sepharose beads and incubated with rotation at 4 °C for 3 h. Proteins attached to the beads were washed three times with washing buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 25 mM MgCl2, 5 mM NaF, 1 mM EDTA, 2 mM β-ME, 0.2% Nonidet P-40), resuspended in 2× SDS loading buffer, boiled for 5 min, separated by 4–20% SDS-PAGE, and then analyzed by immunoblotting with previously described (28) rabbit polyclonal antibodies against eIF3a (anti-GCD6), eIF2β (anti-GCD6), eIF5 (anti-TIF5), eIF2α (anti-SU12), or RPS2 (anti-RPS2) or with rabbit polyclonal anti-GST (Sigma) antibodies or mouse monoclonal anti-FLAG M2 peptide (Sigma) antibodies. Anti-yeast eIF2γ antisera was obtained by inoculating rabbits with recombinant His6-tagged eIF2γ purified from Escherichia coli.

**Interactions between Recombinant Proteins in Vitro—GST (pGEX–6P-1) or GST–eIF5 fusions (pC2698 (eIF5–FL), pC2699 (eIF5–CTD280–405), pC2700 (eIF5–CTD), pC2701 (GCD11), pC2702 (eIF5–Δ–50), pC2704 (eIF5–Δ–75–100)) were expressed in E. coli BL21 (DE3) pLys-S cells. Cells were grown to A600 ~0.8 at 37 °C, induced with 1 mM isopropyl–1-thio–α–D–galactopyranoside, and incubated at 30 °C for 3 h. The induced cells were harvested, washed with ice-cold 1× Tris-buffered saline, and suspended in 5 ml of sonication buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 25 mM MgCl2, 5 mM NaF, 1 mM EDTA, 2 mM β-ME, 0.5% Triton X-100, and complete protease inhibitor mixture). Cells were sonicated five times with a 2× burst and 2-s cooling, cell debris was removed, and WCEs were clarified by centrifugation at 13,000 × g for 20 min at 4 °C. WCEs were mixed with 0.5 ml of Glutathione-Sepharose beads that had been washed three times and were incubated at 4 °C for 3 h on a nutator. The beads were washed with 20 bed volumes of wash buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 25 mM MgCl2, 5 mM NaF, 1 mM EDTA, 2 mM β-ME, and 1% Triton X-100) and stored at −80 °C. The purified protein bound to the beads was assessed by SDS-PAGE.

**Templates for coupled in vitro transcription and translation of full-length eIF2γ or the isolated G domain were generated by PCR using pC2704 as a template and a common primer, PGK5′ (5′–CGAGACTGATATGCTCCT-3′). The 3′ primer varied for three products: full-length eIF2γ, D3/EcoRI (5′–ACGGGAATTCTTAAAGGCG- GGTTCGTTCAAGTATGAC-3′) and FLASH-tagged G domain, D1/EcoRI (5′–ACGGGAATTCTTAAAGGCG- GGTTCGTTCAAGTATGAC-3′); and FLASH-tagged G domain, D1/EcoRI (5′–ACGGGAATTCTTAAAGGCG- GGTTCGTTCAAGTATGAC-3′). The [35S]methionine (>1000 Ci/mmol at 10 μCi/ml)-labeled proteins were synthesized by programming the TNT rabbit reticulocyte lysate transcription/translation kit (Promega) with the PCR-amplified templates, and the products were frozen at −80 °C. GST or various GST–eIF5 fusions bound to glutathione-Sepharose beads were suspended in 50 μl of binding buffer (20 mM HEPES, pH
7.4, 150 mM KCl, 25 mM MgCl₂, 5 mM NaF, 1 mM EDTA, 2 mM β-ME, 1% Triton X-100, 1% skimmed milk, and complete protease inhibitor mixture), incubated at 4 °C for 1 h on a nutator to block nonspecific binding sites, and then mixed with the full TNT lysates containing 35S-labeled full-length eIF2γ or eIF2γ-G domain and incubated for 3 h at 4 °C. Alternatively, the binding reactions contained 35S-labeled FLAG-tagged eIF2γ-G domain that was purified from rabbit reticulocyte lysates using anti-FLAG M2 agarose (Sigma) followed by elution with FLAG-peptide (100 µg/ml) according to the manufacturer’s instructions. Following binding, the beads were washed four times with 1 ml of binding buffer without skimmed milk, and bound proteins were eluted in 2× SDS loading buffer, boiled for 5 min, and separated by 4–20% SDS-PAGE. Gels were stained with GelCode (Pierce) followed by autoradiography.

**RESULTS**

Direct Interaction of eIF5 and eIF2Bε with eIF2γ—To study the interaction of eIF2γ with other translation initiation factors, we generated a fusion construct to overexpress in yeast cells GST fused to full-length (FL) eIF2γ. Expression of the GST fusion protein was under the control of a galactose-inducible promoter. Following induction of GST or GST-eIF2γ-FL expression, WCEs were prepared, and the GST or GST-eIF2γ fusion protein was immobilized on glutathione beads. Products of the pull-down reactions were separated by SDS-PAGE and analyzed by immunoblot analyses using antibodies specific for various translation factors. As shown in Fig. 2 (top), both GST and GST-eIF2γ were expressed and pelleted with the glutathione beads. The FL GST-eIF2γ fusion protein readily interacted with the α and β subunits of eIF2 (Fig. 2, lane 11), consistent with the idea that eIF2γ is the keystone of the eIF2 complex (3). In addition, GST-eIF2γ (FL) interacted with eIF5 and eIF2Bε (Fig. 2, lane 11) but not with the factor eIF3 nor with the small ribosomal subunit (RPS2p) as a constituent of the yeast 40 S ribosomal subunit.

Previous studies revealed that three lysine-rich segments (K-boxes) located in the N-terminal half of eIF2β mediate a direct interaction with a conserved sequence element consisting of two segments rich in acidic and aromatic amino acids (AA-boxes) and found near the C terminus of eIF5 and eIF2Bε (20–22). Thus, the ability of eIF2γ-FL to pull down eIF5 and eIF2Bε could reflect an interaction bridged by eIF2β rather than a direct interaction of eIF2γ with these two effectors of eIF2 function. It was previously shown that substitution of alanine for all seven lysine residues in the first and second K-boxes of eIF2B (the K12 mutation) impaired the binding of eIF2β to both eIF5 and eIF2Bε (20). In order to test if eIF5 and eIF2Bε bind directly to eIF2γ or whether this interaction is bridged by eIF2β, we examined the binding of eIF5 and eIF2Bε to eIF2γ in strains expressing eIF2β-K12 mutant. However, prior to this experiment, we first tested whether the eIF2β-K12 mutation impaired the binding of eIF5 and eIF2Bε to eIF2γ under the conditions of our pull-down assays. As shown in Fig. 3A (lane 2), eIF2γ, eIF5, and eIF2Bε were readily co-precipitated with FLAG-tagged wild-type eIF2B in FLAG pull-down assays. In contrast, only eIF2γ was co-precipitated with the FLAG-tagged eIF2β-K12 mutant (Fig. 3A, lane 4). Thus, the eIF2β-K12 mutation did not affect eIF2 (α + β + γ) complex formation, but it did impair the interaction between eIF2β and both eIF5 and eIF2Bε under the conditions of our experiments.

Next, we examined, in the same whole cell extracts, the impact of the eIF2β-K12 mutation on the ability of the GST-eIF2γ-FL fusion protein to pull down other factors. As expected, the GST-eIF2γ-FL fusion protein readily interacted with eIF2α and eIF2β-K12 (Fig. 3B, lane 12). Interestingly, GST-eIF2γ-FL also bound to eIF5 and eIF2Bε in the eIF2β-K12 mutant strain (Fig. 3B, lane 12). Thus, disruption of the K-boxes in eIF2β did not impair the interaction between FL eIF2γ and eIF5 or eIF2Bε, consistent with the idea that this interaction is direct and not bridged by eIF2β. Providing further support for this idea, and as will be discussed below, the interaction between eIF2γ and eIF5 was likewise not impaired by mutations in the AA-boxes located near the C terminus of eIF5 that disrupt the interaction between eIF5 and eIF2B.

Direct Binding of eIF5 and eIF2Bε to the eIF2γ-G Domain—Based upon the archaeal Methanocaldococcus jannaschii and Pyrococcus abyssi eIF2γ crystal structures (3, 4), yeast eIF2γ is predicted to fold into three domains: domain I or G domain (G, residues 1–309), domain II (D2, residues 310–412), and domain III (D3, residues 413–529). To map the eIF2α, eIF2β, eIF5, and eIF2Bε binding sites on eIF2γ, we generated constructs to express GST fusion to the G, D2, D3, and G + D2 + D3 (FL) domains of eIF2γ. The various GST fusion pro-
eIF5 and eIF2β Bind eIF2γ-G Domain

A

![Diagram](image)

**FIGURE 3.** The interaction of eIF2γ with eIF5 and eIF2β is independent of the K-boxes in eIF2β. As described in Fig. 2, GST or various GST-eIF2γ fusion proteins were overexpressed in yeast strains expressing FLAG-tagged eIF2β (H2888) or FLAG-tagged eIF2β-K12, in which the seven lysine residues in the first two K-boxes of eIF2β are substituted by alanines (H2892). A, K-box mutations disrupt the binding of eIF2β to eIF5 and eIF2βα. WCEs from strains H2888 and H2892 overexpressing GST were incubated with anti-FLAG M2-agarose, and proteins bound to the resin were analyzed by immunoblot analysis using anti-FLAG antibodies to detect eIF2β and specific antisera, as indicated and described under “Experimental Procedures,” to detect other translational components. Lanes 2 and 4, the entire pellet (P) from the precipitation reactions; lanes 1 and 3, 10% of the input (In) amounts of the WCEs. Results of two independent co-immunoprecipitation reactions are summarized in the table on the right. B, K-box mutations in eIF2β do not impair the binding of eIF2γ to eIF5 and eIF2βα. WCEs from strain H2892 expressing the indicated GST or GST-eIF2γ fusion proteins were mixed with glutathione-Sepharose beads, and the interacting proteins were analyzed by immunoblot analysis as described in the legend to Fig. 2. Even-numbered lanes, the entire pellet (P) from the pull-down reactions; odd-numbered lanes, 10% of the input (In) amounts of the WCEs. Results of three independent pull-down reactions are summarized in the table on the right.

...

teins were expressed to high levels in yeast and were readily pelleted with glutathione beads (Fig. 2, top). Previously, we and others (3, 7) identified determinants on D2 of eIF2γ that are required for binding of eIF2α. However, in contrast to FL eIF2γ, GST fusion proteins containing a single eIF2γ domain (G, D2, or D3) or G + D2 failed to interact with eIF2α and eIF2β (Fig. 2, lanes 3, 5, 7, and 9). The failure of these individual eIF2γ domains to interact with eIF2α or eIF2β could indicate that stable incorporation of eIF2α and eIF2β in the eIF2 complex requires contacts with multiple domains of eIF2γ or that the epitopes required for eIF2α or eIF2β binding are not folding properly in the context of the isolated eIF2γ domains.

Despite the inability of isolated eIF2γ domains to bind eIF2α or eIF2β, the isolated eIF2γ-G domain (GST-G) as well as the GST-G + D2 fusion protein were able to pull down both eIF5 and eIF2β (Fig. 2, lanes 3 and 9). Consistent with the inability of the GST-G and GST-G + D2 fusion proteins to interact with eIF2β, the binding of eIF5 and eIF2Be to the GST-G and GST-G + D2 fusions was unaffected by the eIF2β-K12 mutation (Fig. 3B, lanes 4 and 10). These results support the idea that the eIF2γ-G domain directly binds to both eIF5 and eIF2β, consistent with their functions to promote GTP hydrolysis and GTP-GDP exchange on eIF2. It is noteworthy that the various GST-eIF2γ fusion proteins did not pull down the 40 S ribosomal subunit (RPS2p) (Fig. 2A, odd-numbered lanes), indicating that the interactions detected in the pull-down assays are direct and not mediated by mutual binding of the translation factors to the same 40 S subunit. Quantification of the results revealed that GST-eIF2γ-FL and GST-G pulled down 1–9% of the eIF5 in the WCEs. This low percentage binding is consistent with the incorporation of eIF5 into the multifactor complex with eIF2 and eIF3 and with the lack of phenotype associated with overexpression of the various GST-eIF2γ fusions in yeast cells (data not shown).

Finally, as shown in Fig. 2 (top panel), the GST-G fusion was expressed at higher levels than the GST-eIF2γ-FL fusion protein (compare lanes 3 and 4 with lanes 11 and 12). Since the GST-eIF2γ-FL fusion is predicted to interact with eIF5 both directly through the G domain and indirectly via eIF2β, the equivalent eIF5 pull-down by GST-eIF2γ-FL and GST-G may be due to the higher expression of the GST-G fusion protein.

**The N-terminal Domain of eIF5 Specifically Binds to the eIF2γ-G Domain**—In addition to promoting GTP hydrolysis by eIF2 in ribosomal complexes bound to an AUG codon, eIF5 plays an important role earlier in the translation initiation pathway by enhancing the binding of the eIF2 ternary complex to the 40 S ribosomal subunit (23–25). This latter function of eIF5 is mediated by the AA-boxes at the C terminus of eIF5, which enable eIF5 to bridge an interaction between eIF2 (via the K-boxes in eIF2β) and eIF3 (23). We confirmed the importance of the eIF5 AA-boxes by examining the ability of wild-type and mutant forms of eIF5 to bind to eIF2 and eIF3 under the conditions used for our GST pull-down experiments. Immunoprecipitation of FLAG-tagged eIF5 from crude yeast extracts co-precipitated eIF3 and eIF2. In contrast, substitution of alanine for seven conserved residues in the second AA-box of eIF5 (eIF5-7A mutant) abolished the interaction with eIF3 and eIF2 (data not shown).

Despite disrupting the eIF5-eIF2β interaction, the eIF5-7A mutation did not impair the binding of eIF5 to eIF2γ. The GST-eIF2γ fusion proteins were expressed in the isogenic strains expressing FLAG-tagged eIF5 or eIF5-7A, and pull-down assays were used to assess eIF2γ binding to other translation factors. As shown in Fig. 4A, the GST-FL eIF2γ fusion readily bound eIF5, eIF2βε, and eIF2α (lane 12), whereas the GST-G and GST-G + D2 fusions bound both eIF5 and eIF2βε (lanes 4 and 10). Importantly, all of these interactions were not impaired by the eIF5-7A mutation (Fig. 4B, lanes 4, 10, and 12). Interestingly, the GST-G fusion pulled down a greater percentage of eIF5-7A than of eIF5 (lane 4 in Fig. 4, A and B). To account for this enhanced interaction, we propose that the eIF5-7A mutation, which weakens the binding to eIF3 and eIF2β, may liberate eIF5 from the multifactor complex. Accordingly, this free eIF5-7A is then available to readily interact with the GST-G fusion protein, resulting in the increased binding in the pull-down assay. We conclude that the AA-boxes in eIF5 that mediate a direct interaction with eIF2β are not critical for the binding of eIF5 to eIF2γ. Thus, these results provide further support for the idea that the eIF2γ-G domain directly binds to both eIF5 and eIF2βε.

Since the GST pull-down assays employed in these studies rely on the co-expression of the GST fusion and the interacting protein in the same yeast cell, it is possible that other yeast proteins may bridge the eIF2γ-eIF5 interaction. To test whether eIF5 can bind to eIF2γ in vitro, we expressed in bacteria and purified GST-eIF5 fusion proteins consisting of eIF5 residues 1–405 (FL), residues 1–279 (NTD), or residues 280–
directly binds to eIF5 and that the primary determinants for this binding are located in the NTD of eIF5.

Consistent with the results of these in vitro pull-down assays, previous studies implicated the NTD of eIF5 in regulating eIF2γ activity. It was proposed that Arg15 in the eIF5 NTD functions as an "arginine finger" to catalytically stimulate the hydrolysis of GTP by eIF2γ (9, 10). Consistent with this possibility, substitution of alanine, lysine, or methionine for Arg15 in mammalian eIF5 significantly impaired GTP hydrolysis by eIF2γ in 48 S initiation complexes (9, 10). To further map the eIF2γ-G domain binding site on eIF5, we expressed in bacteria and purified GST-eIF5 fusion proteins lacking the N-terminal residues 1–50 (GST-eIF5A–50), lacking residues 50–100 (GST-eIF5Δ50–100), or lacking residues 75–100 (GST-eIF5Δ75–100). Whereas GST-eIF5A–50 and GST-eIF5Δ75–100 readily interacted with the eIF2γ-G domain expressed in reticulocyte lysates, pulling down 6 and 4%, respectively, of the input (Fig. 5B, lanes 5 and 7), the GST-eIF5Δ50–100 fusion protein failed to interact with the eIF2γ-G domain above the background levels observed with GST (Fig. 5B, compare lanes 2 and 6; <1% binding). These results indicate that the primary binding determinants for the eIF2γ-G domain are present in the NTD of eIF5 and map between residues 50 and 100.

To test the ability of the various eIF5 deletion mutants to stimulate GTP hydrolysis by eIF2γ, the eIF5 portion of the GST-eIF5 fusion proteins was liberated by protease cleavage. Following purification, wild-type or mutant forms of eIF5 were mixed with 40 S ribosomal subunits, a model mRNA, the factors eIF1 and eIF1A, and preformed eIF2γ-[γ-32P]GTP-Met-tRNA ternary complexes. Measurements of the rate of GTP hydrolysis in the presence of saturating amounts of wild-type eIF5 revealed an observed rate constant of 0.12 s⁻¹ (Fig. 5D), which is comparable with what has been reported previously (30). Interestingly, the eIF5 NTD (residues 1–279) was sufficient for stimulating GTP hydrolysis (kobs = 0.11 s⁻¹), whereas the CTD (kobs = 0.01 s⁻¹) lacked activity (Fig. 5D). Consistent with its defective binding to the eIF2γ-G domain, eIF5Δ50–100 failed to stimulate eIF2 GTPase activity (kobs = 0.01 s⁻¹; Fig. 5D). In contrast, despite their abilities to bind to the eIF2γ-G domain, both eIF5A–50 (0.03 s⁻¹) and eIF5Δ75–100 (0.02 s⁻¹) failed to stimulate GTP hydrolysis by eIF2 (Fig. 5D). Interestingly, the results obtained with the eIF5A–50 mutant resemble the abolished GAP function and partially impaired eIF2γ binding activity associated with mutating the putative arginine-finger (R15A) in full-length eIF5 (data not shown). Taken together, these results reveal that the eIF5 NTD is both necessary and sufficient for binding to eIF2γ and stimulating eIF2 GTPase activity. Moreover, deletion of eIF5 residues 50–100, which leaves Arg15 intact, impaired both eIF2γ binding and GAP activities, supporting the idea that the direct binding of eIF5 to eIF2γ identified in this paper is important for stimulating eIF2 GTPase activity. Like the "arginine finger" mutations in eIF5, the ssu2-1 mutation in yeast eIF5 impaired the stimulation of eIF2 GTP hydrolysis activity (24). Originally identified as a suppressor of the temperature-sensitive phenotype of the ssu1-17 mutation in yeast eIF1, the ssu2-1 mutation was proposed to restore translational fidelity by lowering the rate of GTP hydrolysis by eIF2 at non-AUG start sites (24). It is noteworthy that the ssu2-1 mutation substitutes Ser for Gly62 (G62S) in the region of eIF5 (residues 50–100) that we showed was important for binding to the eIF2γ-G domain. To test whether the eIF5-G62S mutation affected the binding of eIF5 to eIF2γ, we expressed the various GST-eIF2γ fusion proteins in an ssu2-1 mutant yeast strain, which only expresses the mutant form of eIF5. As shown in Fig. 4C, eIF5-G62S readily bound to GST-G (lane 4), GST-G + D (lane 10), and GST-FL (lane 12) eIF2γ fusion proteins in vivo. These results indicate that the Gly62 residue in eIF5 is not critical for bind-

FIGURE 4. The binding of eIF2γ to eIF5 is independent of the AA-boxes required for the interaction of eIF5 with eIF2B and eIF3. As described in the legend to Fig. 2, GST or various GST-eIF2γ fusion proteins were overexpressed in yeast strains expressing FLAG-tagged eIF5 (H2894), FLAG-tagged eIF5-7A in which seven conserved acidic and aromatic residues in the second AA-box in eIF5 were substituted by alanines (H2895), or untagged eIF5-G62S (F708). A and B, 7A mutation in eIF5 does not impair binding to eIF2γ. WCEs from strain H2894 (A) and H2895 (B) expressing the indicated GST or GST-eIF2γ fusion proteins were mixed with glutathione-Sepharose beads, and the interacting proteins were analyzed by immunoblot analysis as described in the legend to Fig. 2 (except eIF5 was detected using anti-FLAG antibodies). Even-numbered lanes, the entire pellet (P) from the pull-down reactions; odd-numbered lanes, 10% of the input (I) amounts of the WCEs. C, the ssu2-1 mutation in eIF5 does not impair the interaction with eIF2γ. WCEs from strain F708 expressing eIF5-G62S (ssu2-1 mutant defective in promoting eIF2 GTPase activity) and the indicated GST or GST-eIF2γ fusion proteins were mixed with glutathione-Sepharose beads. The binding of eIF5 (anti-TIF5) and eIF2β (anti-GC6δ) to the GST fusion proteins was analyzed by immunoblot analysis as indicated. Even-numbered lanes, the entire pellet (P) from the pull-down reactions; odd-numbered lanes, 10% of the input (I) amounts of the WCEs. Results are representative of two independent experiments. 405 (CTD). The GST fusion proteins were added to rabbit reticulocyte lysates programmed to express either eIF2γ-FL (Fig. 5A) or the isolated eIF2γ-G domain (Fig. 5B). Purified GST-eIF5-FL and GST–eIF5-NTD, but not GST alone, readily interacted with FL eIF2γ (Fig. 5A, lanes 3 and 4 versus lane 2). Moreover, GST-eIF5-FL and GST–eIF5-NTD bound the isolated eIF2γ-G domain (Fig. 5B, lanes 4 and 8). Quantification of the binding, taking into account the molar amounts of GST fusion proteins recovered in the pellets, revealed that ~3% of the total input eIF2γ-FL or eIF2γ-G was bound by the GST-eIF5-FL and GST–eIF5-NTD fusion proteins. In contrast, the GST-eIF5-CTD fusion displayed a very weak ability to pull down the eIF2γ-G domain (Fig. 5B, lane 3; <1% binding). These results suggest that eIF2γ directly binds to eIF5; however, it is possible that a component in the reticulocyte lysates is bridging the interaction between these two factors. To test this possibility, we expressed FLAG-tagged eIF2γ domain in reticulocyte lysates and then purified the protein using anti-FLAG resin. The purified eIF2γ-G domain bound to purified recombinant GST–eIF5-FL but not to GST (Fig. 5C, lane 3 versus lane 2). Again, GST-eIF5-FL bound ~3% of the input eIF2γ-G domain. Thus, we conclude that eIF2γ
**eIF5 and eIF2β Bind eIF2γ-G Domain**

**FIGURE 5. Binding between recombinant eIF5 and eIF2γ in vitro.** A and B, in vitro binding of GST-eIF5 or its derivatives to recombinant FL-eIF2β (A) or eIF2γ-G domain (B). GST or the indicated GST-eIF5 fusion proteins were expressed in E. coli and immobilized on glutathione-Sepharose beads. The GST fusion proteins bound to the resin were incubated with rabbit reticulocyte lysates programmed to express [35S]eIF2-FL (A) or [35S]eIF2γ-G domain (B). After extensive washing, the bound proteins were separated by SDS-PAGE. The upper panels show the GelCode Blue staining pattern of the bound proteins. The positions of the various GST fusion proteins are indicated as follows. Black arrowhead, GST; dark gray arrowhead, GST-eIF5-NTD; light gray arrowhead, GST-eIF5-FL and deletion mutants; white arrowhead, GST-eIF5-CTD. The lower panels show autoradiograms of the same gels. The input (In, lane 1) represents 16% (A) or 20% (B) of the input amounts of labeled proteins. C, in vitro binding of recombinant GST-eIF5 to purified eIF2γ-G domain. [35S]eIF2γ-G domain was synthesized in reticulocyte lysates, bound to anti-FLAG M2-agarose, and eluted with FLAG-peptide. The purified [35S]eIF2γ-G domain was then mixed with recombinant GST or GST-eIF5-FL proteins bound to glutathione-Sepharose beads. After washing, bound proteins were separated by SDS-PAGE, followed by GelCode Blue staining (top) and autoradiography (bottom). Black arrowhead, GST; light gray arrowhead, GST-eIF5-FL. Input (In, lane 1) represents 16% of the input amount of purified [35S]eIF2γ domain. D, ribosome-dependent GTP hydrolysis assay catalyzed by eIF2 in the presence of the indicated derivatives of eIF5. Preformed eIF2γ-[33P]GTP-Met-tRNA<sub>i</sub> ternary complexes were incubated with 40 S ribosomal subunits, elf1, elf1A, a model mrRNA, and the indicated forms of eIF5. The fraction of GTP hydrolyzed was determined after the indicated times of incubation.

**DISCUSSION**

Previous studies established the direct interaction between the N-terminal K-boxes in eIF2β and the C-terminal regions of eIF2β and eIF5 containing the AA-boxes (20–22). It was proposed that these interactions facilitate an additional, and perhaps transient, interaction between eIF5 or eIF2β and eIF2γ; however, this latter interaction was never observed. Here we showed that eIF5 and eIF2β directly interact with eIF2γ. When expressed in yeast, a GST-eIF2γ-FL fusion protein was able to pull-down both eIF5 and eIF2β as well as the α and β subunits of eIF2 (Fig. 2). The ability of the GST-eIF2γ-FL fusion to pull down eIF5 and eIF2β was unaffected by eIF2β-K12 and eIF5-7A mutations that impair direct interactions between eIF2β and eIFβ2 or eIF5 (Figs. 3 and 4). Thus, the interaction between eIF2γ-FL and eIF5 and eIF2β is direct and not bridged by eIF2β. Further supporting this idea, GST fusion proteins consisting of the eIF2γ-G or G + D2 domains were able to pull down the native eIF5 and eIF2β in yeast cells but not eIF2α or eIF2β (Figs. 2–4). Supporting the significance of these interactions, the eIF2γ-G domain-binding site was mapped to the C-terminal catalytic HEAT domain of eIF2β (Fig. 6). Finally, using recombinant proteins,
we confirmed the direct interaction between eIF5 and the eIF2γ-G domain and we mapped the eIF2γ-G domain-binding site to the N-terminal portion of eIF5 (Fig. 5) that was previously implicated in GAP activity. Thus, our data support a model in which eIF5 and eIF2β directly bind to the eIF2γ-G domain, consistent with their functions to promote GTP hydrolysis and GDP-GTP exchange on eIF2.

Our results help clarify the role of the eIF2β interaction with eIF5 and eIF2β. Previously, it was proposed that the direct interaction between the N-terminal K-boxes in eIF2β and the C-terminal AA-boxes of eIF5 facilitated recruitment of eIF5 to the 40 S subunit to trigger GTP hydrolysis by eIF2 following start codon recognition (20–22). Alternatively, since the slow growth phenotype of a yeast strain expressing the eIF5-7A mutant was partially suppressed by overexpression of eIF2 (20), the eIF5-eIF2 binding interaction may facilitate (together with an eIF3-eIF2 interaction) eIF2 binding to the ribosome (prebound with eIF5 and eIF3) (20, 25, 32). The finding that both the GAP (eIF5) and GEF (eIF2β) for eIF2 bound to the eIF2β subunit was initially surprising, given the presence of the G domain in the eIF2γ subunit. Thus, two models could be proposed. 1) Binding of eIF5 or eIF2β allosterically activates eIF2β to promote GTP hydrolysis and/or GTP-GDP exchange on eIF2γ, or 2) the N terminus of eIF2β serves as a high affinity binding (docking) site for eIF5 and eIF2β and recruits these latter partners to eIF2γ, where they directly promote GTP hydrolysis or nucleotide exchange.

The allosteric model, in which the eIF2β subunit activates a GAP and GEF activity inherent in eIF2γ, is consistent with the lack of eIF5, eIF2β, and K-boxes in eIF2β in archaea. Whereas homologs of eIF2α, eIF2β, and eIF2γ were readily identified in archaea and shown to form an eIF2 complex (7, 33), functional homologs of eIF5 and eIF2B have not been identified. Accordingly, the K-boxes in eIF2β evolved along with the requirement for an external factor to promote GTP hydrolysis and GDP-GTP exchange. It is notable that all experiments examining eIF2 GTPase activity as well as nucleotide exchange have utilized eIF2 holo-complexes rather than the isolated eIF2γ subunit (6, 9, 10, 16, 17, 31). Whereas this may reflect protein solubility problems with free eIF2γ, it also is consistent with the idea that eIF2β activates GAP and GEF activities in eIF2γ.

Our results, revealing the direct binding of eIF5 and eIF2β to the G domain of eIF2γ, support the second hypothesis that eIF2β simply serves as a high affinity docking site for eIF5 and eIF2β, which in turn directly regulate eIF2γ function. Whereas our data do not rule out a regulatory function for eIF2β, the fact that both eIF5 and eIF2β bind to eIF2γ supports the idea that these factors directly modulate eIF2γ function. Since the binding of eIF5 or eIF2β to eIF2γ was only detected when one of the proteins was overexpressed, these direct interactions are apparently weak. Accordingly, we propose that the interaction of the C-terminal regions of eIF5 and eIF2β with eIF2β serves to recruit these factors to the eIF2 complex (Fig. 1, black arrows). Following their recruitment, the effector regions in the N-terminal half of eIF5 and the C-terminal segment of eIF2β then interact with the eIF2γ-G domain to promote GTP hydrolysis or nucleotide exchange (Fig. 1, gray arrows). The idea that eIF2β directly catalyzes guanine-nucleotide exchange on eIF2γ is consistent with the identification of critical “exchange” residues in eIF2β (17, 18, 31). Similarly, a direct and catalytically important interaction between eIF5 and eIF2β is supported by the identification of a putative “arginine-finger” in eIF5 (9, 10). In analogy to other GAPS (34), this conserved Arg residue in eIF5 is predicted to insert into the GTP-binding site of eIF2γ and help catalyze GTP hydrolysis. However, our results indicate that the putative arginine-finger (Arg 15) is not essential for eIF3 binding to eIF2γ (Fig. 5B, lane 3; GST–Δ1–50-eIF5 binds eIF2γ-G) (data not shown). Moreover, whereas our results in Fig. 5D reveal that the N terminus of eIF5 is both necessary and sufficient to promote eIF2 GTPase activity, additional experiments are required to prove that Arg 15 in eIF5 functions as a direct catalytic residue in the GTP hydrolysis reaction by eIF2γ.

Finally, our results reveal unique properties for the segments of eIF5 and eIF2β containing the conserved AA-boxes. The x-ray structure of the C-terminal fragment of eIF2β, which we show here binds to the eIF2γ-G domain, revealed a bundle of eight α-helices arranged in a so-called HEAT repeat previously observed in the nuclear cap-binding protein CBP80 and the translation factor eIF4G (18). The first AA-box in the eIF2β structure is located near the core of the HEAT domain and is predicted to be important for the structural integrity of the fold. Likewise, the second AA-box in eIF2β is located in helix VIII and may help pack this helix to the core of the structure. It is likely that the C-terminal segment of eIF5, which, like eIF2β, contains two AA-box motifs, folds into a similar HEAT domain-like structure. Presumably, the HEAT domains in eIF2β and eIF5 interact with the N-terminal region of eIF2β (containing the K-boxes) in a structurally similar manner. However, whereas the HEAT domain of eIF2β binds both eIF2β and eIF2γ, the HEAT domain of eIF5 binds only to eIF2β, whereas the N-terminal portion of eIF5 binds to eIF2γ. Presumably, a common surface on the HEAT domains of eIF5 and eIF2β provides a docking site for the N terminus of eIF2β, whereas a distinct and unique surface on the eIF2β HEAT domain binds to eIF2γ and promotes guanine-nucleotide exchange. Thus, whereas the HEAT domain is commonly thought of as a protein-docking site, the HEAT domain in eIF2β has, in addition, acquired a catalytic function.

Acknowledgments—We thank Jon Lorsch and members of the Lorsch laboratory for reagents and advice, Alan Hinnebusch for comments on the manuscript, and members of the Dever and Hinnebusch laboratories for useful discussions.

REFERENCES

1. Hershey, J. W. B., and Merrick, W. C. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 33–88, Cold
**eIF5 and eIF2β Bind eIF2γ-G Domain**

Spring Harbor Laboratory, Cold Spring Harbor, NY

2. Hinnebusch, A. G. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 185–243, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

3. Roll-Mecak, A., Alone, P., Cao, C., Dever, T. E., and Burley, S. K. (2004) *J. Biol. Chem.* 279, 10634–10642

4. Schmitt, E., Blanquet, S., and Mechulam, Y. (2002) *EMBO J.* 21, 1821–1832

5. Erickson, F. L., and Hannig, E. M. (1996) *EMBO J.* 15, 6311–6320

6. Kapp, L. D., and Lorsch, J. R. (2004) *J. Mol. Biol.* 335, 923–936

7. Yatime, L., Schmitt, E., Blanquet, S., and Mechulam, Y. (2004) *J. Biol. Chem.* 279, 15984–15993

8. Thompson, G. M., Pacheco, E., Melo, E. O., and Castilho, B. A. (2000) *Biochem. J.* 347, 703–709

9. Das, S., Ghosh, R., and Maitra, U. (2001) *J. Biol. Chem.* 276, 6720–6726

10. Paulin, F. E., Campbell, L. E., O’Brien, K., Loughlin, J., and Proud, C. G. (2001) *Curr. Biol.* 11, 55–59

11. Maag, D., Fekete, C. A., Gryczynski, Z., and Lorsch, J. R. (2005) *Mol. Cell* 17, 265–275

12. Unbehaun, A., Borukhov, S. I., Hellen, C. U., and Pestova, T. V. (2004) *Genes Dev.* 18, 3078–3093

13. Pestova, T. V., Lomakin, I. B., Lee, J. H., Choi, S. K., Dever, T. E., and Hellen, C. U. T. (2000) *Nature* 403, 332–335

14. Dever, T. E. (2002) *Cell* 108, 545–556

15. Krishnamoorthy, T., Pavitt, G. D., Zhang, F., Dever, T. E., and Hinnebusch, A. G. (2001) *Mol. Cell. Biol.* 21, 5018–5030

16. Pavitt, G. D., Ramaiah, K. V. A., Kimball, S. R., and Hinnebusch, A. G. (1998) *Genes Dev.* 12, 514–526

17. Gomez, E., Mohammad, S. S., and Pavitt, G. D. (2002) *EMBO J.* 5292–5301

18. Boesen, T., Mohammad, S. S., Pavitt, G. D., and Andersen, G. R. (2004) *J. Biol. Chem.* 279, 10584–10592

19. Aravind, L., and Koonin, E. V. (2000) *Genome Res.* 10, 1172–1184

20. Asano, K., Krishnamoorthy, T., Phan, L., Pavitt, G. D., and Hinnebusch, A. G. (1999) *EMBO J.* 18, 1673–1688

21. Das, S., Maiti, T., Das, K., and Maitra, U. (1997) *J. Biol. Chem.* 272, 31712–31718

22. Das, S., and Maitra, U. (2000) *Mol. Cell. Biol.* 20, 3942–3950

23. Asano, K., Clayton, J., Shalev, A., and Hinnebusch, A. G. (2000) *Genes Dev.* 14, 2534–2546

24. Asano, K., Shalev, A., Phan, L., Nielsen, K., Clayton, J., Valašek, L., Donahue, T. F., and Hinnebusch, A. G. (2001) *EMBO J.* 20, 2326–2337

25. Singh, C. R., Yamamoto, Y., and Asano, K. (2004) *J. Biol. Chem.* 279, 49644–49655

26. Mitchell, D. A., Marshall, T. K., and Deschenes, R. J. (1993) *Yeast* 9, 715–722

27. Ung, T. L., Cao, C., Lu, J., Orzato, K., and Dever, T. E. (2001) *EMBO J.* 20, 3728–3737

28. Valašek, L., Phan, L., Schoenfeld, L. W., Valašková, V., and Hinnebusch, A. G. (2001) *EMBO J.* 20, 891–904

29. Algire, M. A., Maag, D., Savio, P., Acker, M. G., Tarun, S. Z., Jr., Sachs, A. B., Asano, K., Nielsen, K. H., Olsen, D. S., Phan, L., Hinnebusch, A. G., and Lorsch, J. R. (2002) *RNA* 8, 382–397

30. Algire, M. A., Maag, D., and Lorsch, J. R. (2005) *Mol. Cell* 20, 251–262

31. Gomez, E., and Pavitt, G. D. (2000) *Mol. Cell. Biol.* 20, 3965–3976

32. Nielsen, K. H., Szamecz, B., Valašek, L., Jivotovskaya, A., Shin, B.-S., and Hinnebusch, A. G. (2004) *EMBO J.* 23, 1166–1177

33. Pedulla, N., Palermo, R., Hasenoehrl, D., Blasi, U., Cammarano, P., and Londei, P. (2005) *Nucleic Acids Res.* 33, 1804–1812

34. Schefzik, K., Ahnadian, M. R., and Wittinghofer, A. (1998) *Trends Biochem. Sci.* 23, 257–262