Specific Interaction of Eukaryotic Translation Initiation Factor 5 (eIF5) with the β-Subunit of eIF2

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Eukaryotic translation initiation factor 5 (eIF5) interacts with the 40 S initiation complex (40 S-mRNA-eIF3-Met-tRNAf-eIF2-GTP) and mediates hydrolysis of the bound GTP. To characterize the molecular interactions involved in eIF5 function, we have used 32P-labeled recombinant rat eIF5 as a probe in filter overlay assay to identify eIF5-interacting proteins in crude initiation factor preparations. We observed that eIF5 specifically interacted with the subunit of initiation factor eIF2. No other initiation factors including γ subunit of eIF2 tested positive in this assay. Furthermore, both yeast and mammalian eIF5 bind to the β subunit of either mammalian or yeast eIF2. Binding analysis with human eIF2β deletion mutants expressed in Escherichia coli identified a 22-amino acid domain, between amino acids 68 and 89, as the primary eIF5-binding region of eIF2β. These results along with our earlier observations that (a) eIF5 neither binds nor hydrolyzes free GTP or GTP bound to eIF2 in the initiation complex containing Met-tRNAf-eIF2-GTP ternary complex, and (b) eIF5 forms a specific complex with eIF2 suggests that the specific interaction between eIF5 and the β subunit of eIF2 may be critical for the hydrolysis of GTP during translation initiation.

Initiation of translation in eukaryotic cells occurs by a sequence of partial reactions requiring the participation of a large number of specific proteins called eIFs. An obligatory intermediate step in this overall initiation reaction is the binding of the initiator methionyl-tRNA (Met-tRNAi) as Met-tRNAf-eIF2-GTP ternary complex to a 40 S ribosomal subunit, followed by positioning of the 40 S preinitiation complex (40 S-Met-tRNAf-eIF2-GTP) at the initiation AUG codon of the mRNA to form the 40 S initiation complex (40 S-mRNA-Met-tRNAf-eIF2-GTP). A 60 S subunit then joins the 40 S initiation complex to form the 80 S initiation complex (80 S-mRNA-Met-tRNAf) that is active in peptidyl transfer (for a review, see Refs. 1–3). The subunit joining reaction specifically requires the participation of eIF5, an initiation factor that we have purified and characterized from mammalian cells (4–6) and the yeast Saccharomyces cerevisiae (7). Detailed characterization of the eIF5-catalyzed reaction has shown that eIF5 first interacts with the 40 S initiation complex in the absence of 60 S ribosomal subunits to promote the hydrolysis of ribosome-bound GTP (8). Hydrolysis of GTP causes the release of eIF2 and guanine nucleotide (as an eIF2-GDP complex) from the 40 S subunit, an event that is essential for the subsequent joining of the 60 S ribosomal subunit to the 40 S complex (40 S-mRNA-Met-tRNAf) to form the 80 S initiation complex that is active in subsequent peptidyl transfer reaction (8–11).

The mammalian cDNA (rat and human) and the S. cerevisiae gene encoding eIF5 of calculated Mγ = 48,926 and 45,346, respectively, have been cloned and expressed in Escherichia coli (12–15). An interesting feature of the derived amino acid sequences of mammalian (rat and human) and yeast eIF5 proteins is the presence of sequence motifs that have weak homology to characteristic domains present in proteins of the GTPase superfamily (16) which includes the α subunit of G-proteins, H-Ras and Rab 3A, yeast proteins, CDC42 and SEC4, as well as translation initiation and elongation factors, IF2 and EF1A (formerly called EFTu), EF2 (formerly called EFG), and EF1α. However, unlike these proteins, eIF5 neither binds nor hydrolyzes free GTP or GTP bound to eIF2 as a Met-tRNAf-eIF2-GTP ternary complex (8). eIF5 mediates hydrolysis of GTP only when the nucleotide is bound to eIF2 on the 40 S initiation complex containing bound Met-tRNAf-eIF2-GTP ternary complex (8). The key question therefore is whether interaction of eIF5 with one or more components of the 40 S initiation complex is required for GTP hydrolysis during translation initiation.

In the work presented in this paper, we have used 32P-labeled mammalian eIF5 as a probe in filter overlay (“far Western”) assays to demonstrate that eIF5 specifically and directly interacts with the β subunit of eIF2. Similar interaction between yeast eIF5 and yeast eIF2 β was also observed. By systematic deletion analysis, the region of the β subunit of eIF2 involved in binding eIF5 was characterized. The implications of this specific interaction between eIF5 and the β subunit of eIF2 in the eIF5-mediated hydrolysis of GTP during translation initiation are discussed.

EXPERIMENTAL PROCEDURES

Purified Proteins and Immunological Methods—Purified eIF2 and eIF5 from rabbit reticulocyte lysates as well as recombinant rat eIF5 were isolated as described (6, 13, 17). Yeast eIF5 and yeast eIF2 were purified from S. cerevisiae strain, BJ286 as described by Chakravarti et al. (7). IgG antibodies specific for each of the subunits of rabbit eIF2 were isolated from specific rabbit antisera raised against purified rabbit eIF2 (17) by affinity purification using each of the subunits of eIF2 blotted onto aminopropyl thiourea paper (Schleicher and Schuell) as an antigen as described (18). Antibodies specific for the β subunit of yeast eIF2 were a kind gift of Dr. Thomas Donahue of Indiana University. Immunoblot analysis was carried out as described (18). Anti-GST antibodies made in mouse were a kind gift from Charles Weaver of our institution.

Construction of Plasmids—The human cDNA corresponding to the γ
subunit of eIF2 (eIF2-γ-cDNA) was cloned by immunoscreen a HeLa cell cDNA expression library in phage ZAPII (Stratagene) using affinity purified polyclonal anti-eIF2 antibodies as probe. The 1.511-kilobase pair cDNA insert present in the recombinant phage was isolated by in vitro excision as a subclone in the plasmid pBlueScript-SK(+) (19). This eIF2-γ-cDNA was used as a template for the synthesis of the antisense primer used was 5' \text{TCGGAACGAAGATGACCTC-3'}} (19). This antisense primer used was synthesized corresponding to the N-terminal and C-terminal ends of the eIF2-γ-cDNA (21). The N-terminal primer had BamHI-NdeI sites at the 5' end, and the C-terminal primer had an EcoRI overhang. The 1002-base pair long PCR product was sequenced to ensure error-free DNA synthesis and cloned individually into the (a) NdeI-EcoRI sites at the 5'-end of pSET-2B (Pharmacia Biotech Inc.) and (b) BamHI-EcoRI sites of pGEX-2T (Pharmacia Biotech Inc.) to generate the expected plasmids, pET-5a-eIF2β (designated pSET-2β) and pGEX-2T-eIF2β (designated pGEX-2β), respectively. Deletion mutants of eIF2β were generated by one-stage PCR amplification of eIF2β DNA sequences using pET-2β as the template. The appropriate sense strand oligonucleotide primers used for this reaction had BamHI-NdeI overhangs, thereby introducing an in-frame methionine codon at the N-terminal end, and the antisense oligonucleotide primer corresponding to the C-terminal end of eIF2β introduced a translation stop codon followed by an EcoRI restriction site. A BamHI/EcoRI restriction fragment of each PCR-amplified eIF2β deletion mutant was inserted at the same restriction sites of the vector pGEX-2T. The resulting constructs expressed deleted eIF2β mutants as GST fusion proteins. The mutant β construct, β (K23 → A32) in which the polylysine stretch 79KKKKKKTKK87, designated K2, present in wild-type β was substituted with a polyalanine stretch 79AAAAAAATAA87, was generated by a three-step ligation as fol-

Expression and Purification of Recombinant Wild-type or Mutant eIF2β Proteins—The pET5a-eIF2β series of plasmids containing either the wild-type or mutant eIF2β coding sequence and pSET-eIF2β plasmid containing the coding sequence of eIF2β were generated at the position of eIF2β were fused at its N terminus to GST were transformed into E. coli DH5α cells (Life Technologies, Inc.). In each case, a single ampicillin-resistant colony was grown to mid-logarithmic phase in 3 ml of 2YT medium (22), induced with 1 mM IPTG, and grown for an additional 2–3 h. The cells were centrifuged and bacterial pellets suspended directly in 1 × Laemmli buffer and boiled for 4 min at 100 °C, and the proteins in the boiled extract were resolved by SDS-polyacrylamide (15%) gel electrophoresis. The GST or GST-eIF2β fusion protein was isolated by inducing E. coli DH5α cells harboring either the pGEX-2T or pGEX-2T-eIF2β plasmids as described above. The harvested cells were suspended (at approximately A260 = 1.0) in buffer A (10 mM potassium phosphate, pH 7.0, 150 mM NaCl, 5 mM 2-mercaptoethanol, and protease inhibitor mixture containing 0.5 mM phenylmethanesulfonyl fluorid, pepstatin A (0.7 μg/ml), leupeptin (0.5 μg/ml) and sonicated 10 times for 20 s each with cooling on ice between bursts. Following addition of Triton X to 1% final concentration, the cell lysate was centrifuged by centrifugation at 15,000 × g for 10 min. The lysate containing either GST or GST fusion proteins was incubated with glutathione-Sepharose 4B beads (Pharmacia) in buffer A containing 0.1% Nonidet P-40 for 1 h at 4 °C and then washed three times with 1 ml of the same buffer. The amount of protein present in the washed beads containing either GST or GST-eIF2β fusion protein was measured by Bio-Rad method. The beads were stored in small aliquots at −70 °C until use.

Preparation and Isolation of 32P-Labeled eIF5—Recombinant rat eIF5 (70–80 μg) was phosphorylated with [γ-32P]ATP (6,000 cpm/pmol) and casein kinase II in a reaction mixture similar to that described previously for phosphorylation of rabbit reticulocyte eIF5 (8). 32P-Labeled eIF5 was isolated by subjecting the reaction mixture to phosphocellulose chromatography as described (8). Similar procedures were used to prepare 32P-labeled yeast eIF5. It should be noted that eIF5 phosphorylated in vitro by casein kinase II retains full in vitro activity (18).

Filter Overlay Method (Far Western Analysis) for Detection of eIF5 Binding Proteins—eIF5 binding proteins were identified by an adapta-

RESULTS

Identification of eIF5-interacting Proteins in Crude Initiation Factor Fractions—To identify eIF5-interacting protein(s), partially purified initiation factor preparations obtained from rabbit reticulocyte lysates as well as a crude cell-free HeLa cell extract were subjected to far Western blot analysis using 32P-
labeled eIF5 as probe (as explained under “Experimental Procedures”). Fig. 1, panel A, shows that in partially purified

quenced using U.S. Biochemical Corp. kit to ensure error-free DNA synthesis.
Identification of eIF5-binding proteins in cell-free extracts. Protein fractions were denatured and electrophoresed in a SDS-15% gel, and the resolved proteins were transferred to PVDF membranes as indicated under “Experimental Procedures.” The membrane blots were probed with 32P-labeled eIF5 (~10^6 cpm of radioactivity in 20 ml of blotto buffer) overnight as outlined under “Experimental Procedures” under “Filter Overlay Method (Far Western Analysis) for Detection of eIF5 Binding Proteins.” The washed membrane blots (panels A and B) were autoradiographed, and the autoradiograms are presented. Panel A, lanes a and b, partially purified initiation factor fractions obtained by step-wise elution of 0.5 M KCl-wash proteins of rabbit reticulocyte ribosomes from a DEAE-cellulose and a phosphocellulose column, respectively (20 μg each); lane c, crude HeLa cell extract (30 μg). Panel B, purified rabbit reticulocyte eIF2 (4 μg), eIF3 (10 μg), and eIF2-eIF2B (10 μg). Panel C, purified eIF2 (2 μg) and purified eIF2-eIF2B (5 μg) were subjected to Western blot analysis using 1:2000 dilution of polyclonal anti-eIF2 antibodies as probes. The positions of the eIF2 subunits, α, β, and γ are indicated.

Confirmation that the eIF5-interacting protein was indeed the subunit of eIF2 was derived from the following observations. First, the open reading frame of human eIF2 β-cDNA (21) was subcloned into the E. coli expression plasmid, pET-5a under the control of T7 RNA polymerase promoter. When E. coli BL21 (DE3) cells, harboring the pET5a-eIF2β (pET-2β) expression plasmid induced with IPTG and proteins in induced cell lysates, were subjected to Western blot analysis using anti-eIF2 antibodies as probes, the synthesis of eIF2β was easily observed (Fig. 2, panel A, lane c). The same polypeptide interacted with 32P-labeled eIF5 when these induced cell lysates were subjected to far Western blot analysis (Fig. 2, panel B, lane c) which comigrated with the same mobility as the eIF5-reacting band of eIF2 (Fig. 2, panel B, lane a). In contrast, no eIF5-interacting polypeptide was detected in E. coli cell lysates that did not synthesize the β subunit of eIF2 (Fig. 2, compare lane b of panel A with lane b of panel B). It should be noted here that bacterially expressed eIF2β undergoes proteolytic degradation. The lower molecular weight eIF5-reacting band in panel B (lane c) is very likely an N-terminal fragment of eIF2β which is weakly detected in immunoblot analysis (panel A, lane c) as the anti-eIF2 antibodies recognize the N terminus of eIF2β very poorly. Additionally, when lysates of E. coli cells overexpressing the γ subunit of eIF2 were subjected to far Western blot analysis, 32P-labeled eIF5 probe did not detect any eIF5-interacting polypeptide band (Fig. 2, compare lane d
Western blot analysis using 32P-labeled recombinant rat eIF5 as a probe. A, lane b
(eIF2) interacted with a single polypeptide band in the purified yeast system. To determine whether yeast eIF5 and eIF2 plays an essential role in the function of eIF5 in mammals, if the interaction between eIF5 and the initiation is highly conserved from the unicellular yeast to mammals. If the interaction between eIF5 and the subunit interact with each other, we performed far Western blot analysis of a purified yeast eIF2 preparation (see “Experimental Procedures”). The GST-eIF2 fusion protein interacted very weakly with eIF5 compared with GST-eIF5-(27–170). The GST-eIF2-(88–170) fusion protein did not bind 32P-labeled eIF5. Further deletion of amino acids 1–26 from eIF2β-(1–170) did not affect the binding ability of the fusion protein β-(27–170) to 32P-labeled eIF5. However, in the context of β-(1–170), when amino acids 1–87 were deleted, the resulting GST-β-(88–170) fusion protein interacted very weakly with eIF5 compared with GST-β-(1–170). The GST-β-(27–123) fusion protein was also recognized by eIF5 quite efficiently although slightly less than β-(27–170), whereas the GST-β-(88–123) fusion protein did not interact with eIF5. Taken together, these results suggested that the region of eIF5 encompassing amino acids 27–87 is sufficient for conferring major eIF5-binding properties to eIF2β. This was confirmed by showing that GST-β-(27–89) bound 32P-labeled eIF5 very efficiently. The eIF5-binding region of eIF2β was further delimited by showing that the deletion mutant GST-β-(68–95) bound 32P-labeled eIF5 quite efficiently. Thus, the eIF5-binding region of eIF2β is a 22-amino acid stretch that lies between amino acids 68 and 89, whereas the region between amino acids 124 and 170 also had low eIF5 binding activity.

**Mutational Analysis of the Conserved Polysynthetic Stretches in the eIF5-binding Region of eIF2β**—Comparison of the amino acid sequence of human eIF2β with those from other species showed the presence of three highly conserved stretches of six to eight lysine residues, designated K1 (amino acids 14–21), K2 (amino acids 79–87), and K3 (amino acids 124–129) in the N-terminal region of eIF2β (Fig. 5). The minimal eIF5-binding
region (amino acids 68–89) of eIF2β contains the conserved poly lysine stretch K2, whereas the region comprising amino acids 124–170 which exhibits low eIF5 binding activity contains the conserved polylysine stretch K3 (Fig. 5). To investigate the role of these lysine-rich domains of eIF2β in eIF5 binding, we substituted each polylysine stretch with a polyalanine stretch of the same length as described under “Experimental Procedures.” Fig. 6, left panel, shows that all the mutant proteins were expressed in comparable amounts. Substitution of the polylysine stretch K2 of eIF2β with a polyalanine stretch A2 in the mutant β (K2 → A2) reduced by about 4-fold (from that for wild-type eIF2β) the binding affinity of 32P-labeled eIF5 (Fig. 6, right panel, compare lane a with lane c) as determined by densitometric scanning (not shown). In contrast, the mutants β (K1 → A1) and β (K3 → A3) in which the polylysine stretches K1 and K3 have been replaced by polyalanine stretches A1 and A3, respectively, showed no significant change in their ability to bind 32P-labeled eIF5 (Fig. 6, right panel, lanes b and d). Furthermore, when both the polylysine stretches K2 and K3 were replaced with the polyalanine stretches A2 and A3, the resulting β mutant β (K2 → A2, K3 → A3) had similar reduced binding ability to 32P-labeled eIF5 as β (K2 → A2) (Fig. 6, right panel, lane e). These results suggest that the polylysine stretch K2, which lies in the minimal eIF5-binding region comprising amino acids 68–89 of eIF2β, is required for optimal eIF5 binding.

**DISCUSSION**

The GTP/GDP cycle involved in eukaryotic translation initiation resembles many aspects of prokaryotic translation initi-
lation (1, 2), prokaryotic and eukaryotic translation elongation (1–3), as well as protein translocation, G-protein-mediated signal transduction, microtubule assembly, Ras and Rho-mediated neoplastic transformation, Rab group of small GTPase-directed vesicular trafficking, and many other regulated processes (16). An important feature of these GTP-binding proteins is that they all possess intrinsic GTPase activity which is often stimulated by their interaction either with an appro-

priate acceptor or a separate protein molecule. For example, in both prokaryotic translation initiation and elongation, the GTP-bound form of IF2 and EF1A mediates the transfer of Met-tRNA and aminoacyl-tRNAs, respectively, to ribosomes with the concomitant hydrolysis of GTP (1, 2). However, it has been observed that both IF2 and EF1A have very low intrinsic GTPase activities that are activated when the GTP-bound form of the protein interacts with 50 S ribosomal subunits that act as effectors of GTPase activity (25, 26). Likewise, in eukaryotic translation initiation, eIF2-GTP is directly involved in transferring Met-tRNA to a 30 S ribosomal subunit to form the 30 S initiation complex. However, hydrolysis of GTP is not activated by the interaction of the 60 S ribosomal subunit with the 30 S initiation complex. Rather, prior to the joining of the 60 S ribosomal subunit, eIF5 interacts with the 30 S initiation complex in the absence of 60 S ribosomal subunits to promote GTP hydrolysis (8, 9). The key question, therefore, concerning the role of eIF5 in translation initiation is the mechanism by which the interaction of this factor with the 30 S initiation complex causes the hydrolysis of bound GTP.

Previous results on the properties of mammalian eIF5 (8) have shown that eIF5, by itself, does not hydrolyze either free GTP or GTP bound to the Met-tRNA, eIF2-GTP ternary complex in the absence of 40 S ribosomal subunit. These results suggest that eIF5 does not directly interact with GTP to catalyze its hydrolysis. Rather, it is likely that the interaction of eIF5 with the 40 S initiation complex causes a conformational change in the 40 S subunit-bound eIF2 which then acts as a GTPase catalyzing the hydrolysis of bound GTP. In this sense, eIF5 acts as a GTPase-activating protein, and both this initiation factor and possibly 40 S ribosomal subunit act as effectors in GTP hydrolysis catalyzed by eIF2 during translation initia-

### Fig. 6. Effect of substitution of conserved polylysine stretches with polyalanine stretches in eIF2β on its eIF5 binding property.

Crude extracts of E. coli BL21 (DE3) cells expressing equivalent levels of wild-type and mutant eIF2β proteins were boiled in 3x Laemmli buffer. Equal amounts of sample were loaded onto two separate gels run simultaneously and then transferred to a PVDF membrane. One blot was analyzed by immunoblotting using polyclonal antibodies as probes (left panel), and the other blot was subjected to far Western analysis using 32P-labeled eIF5 as probe (right panel) as described under “Experimental Procedures.”

### Fig. 7. Sequence homology between the C-terminal region of eIF2β and the N-terminal region of eIF5. The amino acid sequences of the C-terminal region of human, rabbit, Drosophila, and yeast eIF2β are aligned with the N-terminal region of eIF5 from a wide variety of species using the programs PRODOM and DNASTAR as shown. The sequences of human and yeast eIF2β are from Refs. 21 and 29, respectively, and those of rabbit and Drosophila are from SWISSPROT (accession numbers P41035 and P41375, respectively). The sequences of human, rat, and yeast eIF5 are from Refs. 12, 14, and 15, respectively, and those of S. pombe and Phavu were obtained from SWISSPROT (accession numbers Q09689 and P41375, respectively). Highly conserved amino acid residues of eIF2β and eIF5 are indicated by an asterisk (*), and the conserved cysteine residues of C-4 type zinc finger motif in both eIF2β and eIF5 are indicated by a filled circle (●). The consensus sequence between the C-terminal portion of eIF2β and the N-terminal portion of eIF5 is also shown. Residues moderately conserved to the consensus are highlighted with light shading. The portion of the G1 domain of eIF5 which falls within this conserved region is underlined.
ion. According to this hypothesis, protein-protein interaction between eIF5 and the 40S subunit-bound eIF2 may be critical for the hydrolysis of GTP bound to the 40S initiation complex. Such an interaction between eIF2 and eIF5 has been directly demonstrated (13).

Experiments presented in this paper clearly demonstrate that interaction of eIF5 with eIF2 occurs through the β subunit of eIF2. This observation was somewhat unexpected in view of the recent demonstration that the γ subunits of both mammalian and yeast eIF2 contain consensus GTP-binding domains (19, 20, 27), and this subunit of eIF2 is presumably involved in guanine nucleotide binding although this has not yet been experimentally demonstrated. In proteins of the GTPase superfamily, the subunit that binds GTP also possesses the latent GTPase activity that is activated by its interaction with an effector molecule (16). We, therefore, expected eIF5, the effector protein to interact directly with the γ subunit of eIF2. It is likely, however, that interaction of eIF5 with the β subunit of eIF2 induces a conformational change in the eIF2 molecule resulting in the activation of the latent GTPase activity of the γ subunit which in conjunction with the 40S ribosomal subunit can then act as a GTPase hydrolyzing the bound GTP. Further work is clearly necessary to understand the mechanism of eIF5-mediated hydrolysis of GTP bound to the 40S initiation complex.

We have characterized a 22-amino acid region in the human eIF2β (amino acids 68–89) which appears necessary and sufficient for interaction with eIF5 although the region encompassing amino acids 124–170 also has some weak eIF5 binding activity and may, in fact, be required for optimal binding of eIF5 to eIF2β. Experiments presented in this paper also demonstrate that the polylysine stretch K2, KKKKKKKKKKKK, present in the primary eIF5-binding region of eIF2β is critical for binding eIF5. The polylysine stretch K3, present in the secondary eIF5-binding region was, however, found to be non-essential for binding. In agreement with these results, the polylysine stretch K2 has been found to be conserved in all species of eIF2β so far examined, and the polylysine stretch K3 is absent in wheat eIF2β (28). Further mutational analysis in the eIF5-binding region of eIF2β is clearly necessary to identify the critical amino acid residues involved in the interaction of eIF5 with eIF2β. It will also be important to carry out systematic deletion and point mutations in eIF5 and use the mutant proteins both for their ability to bind eIF2β as well as in mediating the hydrolysis of GTP bound to the 40S initiation complex. These studies will then allow us to correlate the binding of eIF5 to eIF2 (through the β subunit) with the function of eIF5 in GTP hydrolysis during translation initiation. These studies are currently underway in our laboratory.

Finally, scanning the available sequence data base for eIF2β and eIF5 from a wide variety of eukaryotic species revealed a high degree of sequence conservation between the N-terminal region of eIF5 and the C-terminal region of eIF2β (Fig. 7). This sequence conservation spanned over a stretch of about 95 amino acid residues. The significance of this sequence conser-

vation between the two proteins which have distinct functions in translation initiation is not completely clear. However, it is tempting to speculate that this conserved region in each protein may be involved in binding to a common component of the translation initiation machinery, perhaps the 40S ribosomal subunit. Additionally, we also observed that eIF5 from different species has a conserved zinc finger motif of the type Cys-X5-Cys-X5-Cys-X5-Cys (Fig. 7). The presence of a similar zinc finger motif in yeast eIF5 has been implicated in ribosomal start site selection during the scanning process in which eIF2 plays an important role (29). The significance of the presence of such a sequence motif in both mammalian and yeast eIF5 is currently unknown.

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