Eukaryotic translation initiation factor 5 functions as a GTPase-activating protein

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Supratik Das, Rajarshi Ghosh, and Umadash Maitra†

From the Department of Developmental and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

Eukaryotic translation initiation factor 5 (eIF5) forms a complex with eIF2 by interacting with the β subunit of eIF2. This interaction is essential for eIF5-promoted hydrolysis of GTP bound to the 40 S initiation complex. In this work, we show that, in addition to the eIF2β-binding region at the C terminus of eIF5, the N-terminal region of eIF5 is also required for eIF5-dependent GTP hydrolysis. Like other GTPase-activating proteins, eIF5 contains an invariant arginine residue (Arg-15) at its N terminus that is essential for its function. Mutation of this arginine residue to alanine or even to conservative lysine caused a severe defect in the ability of eIF5 to promote GTP hydrolysis from the 40 S initiation complex, although the ability of these mutant proteins to bind to eIF2β remained unchanged. These mutants were also defective in overall protein synthesis as well as in their ability to support cell growth of a ΔTIF5 yeast strain. Additionally, alanine substitution mutagenesis of eIF5 defined Lys-33 and Lys-55 as also critical for eIF5 function in vitro and in vivo. The implications of these results in relation to other well-characterized GAPs are discussed and provide additional evidence that eIF5 functions as a GTPase-activating protein.

Eukaryotic translation initiation factor 5 (eIF5), a monomeric protein of about 49 kDa in mammals (1–3) and 46 kDa in the yeast Saccharomyces cerevisiae (4, 5), in conjunction with GTP and other initiation factors, plays an essential role in initiation of protein synthesis in eukaryotic cells. In vitro studies using purified initiation factors have shown that the overall initiation reaction proceeds with the initial binding of the initiator Met-tRNAf as the Met-tRNAf-eIF2-GTP ternary complex to a 40 S ribosomal subunit followed by the positioning of the 40 S preinitiation complex (40 S-eIF3-Met-tRNAf-eIF2-GTP) at the initiation AUG codon of the mRNA to form the 40 S preinitiation complex (40 S-eIF3-mRNA-Met-tRNAf-eIF2-GTP). The initiator factor eIF5 then interacts with the 40 S initiation complex to promote the hydrolysis of ribosome-bound GTP. Hydrolysis of GTP causes the release of eIF2-GDP (and P) as well as eIF3 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 to form a functional 80 S initiation complex (80 S-mRNA-Met-tRNAf) that is active in peptidyl transfer (for a review, see Refs. 6–8). eIF5-promoted GTP hydrolysis has also been shown to play an important role in the selection of the AUG start codon by the 40 S preinitiation complex (9). The mammalian cDNA and the yeast gene, designated TIF5, encoding eIF5 have been cloned and expressed as functional proteins in Escherichia coli (4, 10).

Biochemical characterization of eIF5-promoted GTP hydrolysis reaction has shown that hydrolysis of GTP occurs only when eIF5 interacts with GTP bound to eIF2 in the 40 S initiation complex and that eIF5, by itself, does not hydrolyze either free GTP or GTP bound to eIF2 as a Met-tRNAf-eIF2-GTP ternary complex in the absence of 40 S ribosomal subunits (10, 11). These observations suggested that eIF5 interacts with one or more components of the 40 S initiation complex to cause hydrolysis of GTP. Subsequent studies showed that eIF5 forms a complex with eIF2 (10), a component of the 40 S initiation complex, and that eIF5-eIF2 complex formation occurs by interaction between the conserved lysine residues at the N-terminal region of eIF2β and the conserved glutamic acid residues at the C-terminal region of eIF5 (12–14). More importantly, mutational analysis of the conserved glutamic acid residues in the C-terminal eIF2β-binding region of eIF5 showed that eIF5-eIF2β interaction plays an essential role in eIF5 function in vitro (14) as well as in vivo (13, 14). However, an important question remained as to whether the interaction of eIF5 with eIF2β is sufficient for eIF5-promoted GTP hydrolysis or other regions in eIF5 are also required for its function.

In the work presented in this paper, using deletion analysis we show that, in addition to the eIF2β-binding region at the C terminus of eIF5, the N-terminal region of eIF5 is also required for eIF5-promoted GTP hydrolysis. Furthermore, consistent with the hypothesis that eIF5 functions as a GTPase-activating protein (GAP), we observed that, like typical well-characterized GAPs, e.g. RasGAPs and RhoGAPs (15, 16), eIF5 also contains an invariant arginine residue (Arg-15) at the N terminus that is essential for eIF5 function both in vitro and in vivo. Mutational analysis of this arginine residue as well as of Lys-33 and Lys-55 of rat eIF5 showed that these residues are critical for eIF5-promoted GTP hydrolysis as well as in the ability of rat eIF5 to functionally substitute for the homologous yeast protein in a ΔTIF5 yeast strain. The implications of these results in understanding the mechanism of eIF5-dependent GTP hydrolysis reaction are discussed.

EXPERIMENTAL PROCEDURES

tRNA, Ribosomes, Purified Proteins, and Antibodies—The preparation of 35S-labeled rabbit liver initiator Met-tRNAf (30,000–50,000 cpm/
pmol), 40 S and 60 S ribosomal subunits from Artemia salina eggs, purified eIF2 from rabbit reticulocyte lysates, and rabbit anti-rat eIF5 antibodies was described previously (2, 10, 11, 17). The mixture of protease inhibitors added to buffer solutions used during purification of recombinant proteins from bacterial cell extracts consisted of leupeptin (0.5 μg/ml), pepstatin A (0.7 μg/ml), aprotinin (2 μg/ml), and freshly prepared phenylmethylsulfonyl fluoride (1 mM).

Construction of Plasmids and Yeast Strains Expressing Wild-type or Mutant Rat eIF5 Proteins—Deletion mutants of eIF5 were generated by one-stage PCR amplification of eIF5 ORF sequences using pGEX-KG-eIF5 as the template and appropriate oligonucleotide primers containing BamHI/EcoRI overhangs. A BamHI/EcoRI restriction fragment of each PCR-amplified deletion mutant was inserted at the same restriction sites of the vector pGEX-KG. The construct pGEX-KG-Δ(18–58)eIF5 was generated by three-fragment ligation of a N-terminal fragment obtained by annealing two single-stranded oligonucleotides containing NdeI/ClaI overhangs that correspond to amino acids 1 to 17, a C-terminal PCR fragment digested with ClaIEcoRI that corresponds to amino acids 59 to 430, and the vector pGEX-KG-digested with NdeI/EcoRI. The resulting constructs expressed deleted eIF5 mutants as GST fusion proteins. The construction of yeast centromeric plasmids pRS316-TIF5, pTM100-EIF5, and pUB-TIF5R and the haploid yeast strains TMY101 (MATα leu2-3, 112 his3-11, 15 ade2-1 trp1-1 ura3-1 can1-100 tif5::TRP1[pRS316-TIF5]) and TMY201R (MATα leu2-3, 112 his3-11, 15 ade2-1 trp1-1 ura3-1 can1-100 tif5::TRP1[pUB-TIF5R]) over the preparation of media for yeast cell growth have been described previously (18). Point mutations within the coding sequence of eIF5 present in the yeast centromeric plasmid pTM100-EIF5 or the bacterial expression plasmid pGEX-KG-eIF5 were constructed as described previously (14). Expression of wild-type or mutant rat eIF5 proteins in yeast cells was detected by immunoblot analysis as described previously (18).

Expression and Purification of Recombinant Wild-type or Mutant Rat eIF5 Proteins—E. coli XL1 Blue cells transformed with recombinant pGEX-KG plasmids containing either the wild-type or mutant eIF5 coding sequences (expressing wild-type or mutant eIF5 as GST fusion proteins) were grown as described previously (14). Untagged wild-type and mutant eIF5 proteins were purified from these cells following the procedures of Das et al. (14). The purified proteins were stored in a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM diethiothreitol, and 60% glycerol at −20 °C. GST-eIF2β Fusion Protein Binding Assay—The binding assays were carried out as described previously (14). A typical binding reaction mixture contained 200 μl of 20 mM potassium phosphate (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 6 μl of a 50% suspension of GST beads containing bound GST-eIF2β (2 μg of total protein), and about 1 μg of either purified wild-type or mutant eIF5 protein.

Other Methods—Yeast cell-free extracts for eIF5-dependent translation of mRNAs were prepared from TMY201R yeast cells as described previously (18). The 40 S initiation complex containing bound [γ-32P]GTP was prepared and isolated free of unreacted reaction components by sucrose-density centrifugation as described (10, 11). Yeast transformations were performed as described by Rose et al. (19). Methods for plasmid and genomic DNA preparations, restriction enzyme digestion, DNA ligation, cloning, and bacterial transformations were according to standard protocols (20).

RESULTS

Effect of N-terminal Deletion of eIF5 on Its Function In Vitro—We have shown previously (12, 14) that mammalian eIF5 interacts with mammalian eIF2β through a conserved acidic amino acid-rich C-terminal region and that this interaction is necessary for eIF5-promoted hydrolysis of GTP bound to eIF2 in the 40 S initiation complex (40 S AUG-Met-tRNAeiF2GTP). However, the possibility exists that the interaction between the C-terminal acidic amino acid-rich domain of eIF5 with eIF2β alone is not sufficient for eIF5-promoted GTP hydrolysis.

To determine whether other regions of eIF5 are also essential for eIF5 function, we retained the C terminus of eIF5 containing the eIF2β-binding region and carried out truncations at the N terminus (Fig. 1A). The deletion mutants and wild-type eIF5 were expressed as GST fusion proteins in E. coli XL1-Blue cells and purified as untagged proteins to apparent electrophoretic homogeneity (Fig. 1B), as described under “Experimental Procedures.” These purified mutants were then assayed for their ability to promote hydrolysis of GTP bound to the 40 S initiation complex. Wild-type eIF5 was able to promote rapid and quantitative hydrolysis of GTP as expected (Fig. 1D). However, when the first 98 amino acids from the N terminus of eIF5 were deleted, the resultant mutant Δ(1–98)eIF5 failed to promote hydrolysis of GTP (Fig. 1D), suggesting that the N-terminal end of eIF5 was also essential for eIF5 activity. The mutant Δ(1–98)eIF5 was, however, able to bind to eIF2β as expected (Fig. 1C). To further map the region at the N terminus of eIF5 that is essential for eIF5 function, we constructed two additional deletion mutants. In the first mutant Δ(1–17)eIF5, the first 17 amino acids at the N terminus of eIF5 were deleted whereas in the other mutant Δ(18–58)eIF5, the region between amino acids 18 to 58 was deleted, keeping the rest of the eIF5 ORF intact (Fig. 1A). Both mutants failed to promote hydrolysis of GTP bound to eIF2 in the 40 S initiation complex (Fig. 1D) but were able to bind eIF2β (Fig. 1C). These results indicate that, although the C terminus of eIF5 is involved in binding to eIF2 (via the β subunit of eIF2), this interaction alone is not sufficient for eIF5-promoted GTP hydrolysis. The N terminus of eIF5 is also required for eIF5 activity.

The requirement of the N terminus and the C terminus of eIF5 for its activity is analogous to that of other well-characterized GAPs. Typical GAPs, e.g. RhoGAPs and RasGAPs, contain sequence motifs that are necessary for their GTPase-stimulating activity in addition to motifs that are necessary for binding to their respective GTPase proteins (16). In the case of eukaryotic translation initiation, it is the eIF2 molecule that binds GTP and presumably acts as a GTPase, whereas eIF5 acts as the GAP. The C-terminal domain of eIF5 contains the sequence motifs required for binding of eIF5 to eIF2, the GTPase protein (12–14). It is likely that the activation domain of eIF5 is present at the N terminus and contains sequence motifs required for the GTPase-stimulating activity of eIF5.

Strategy for Mutational Analysis of the N-terminal Region of eIF5—Comparison of the N-terminal region of eIF5 from different species shows that this region of eIF5 is highly conserved (Fig. 2).

The activation domains of GAPs belonging to the well-characterized families of RasGAPs and RhoGAPs have been shown to contain “arginine-finger” motifs consisting of an invariant arginine residue at the N terminus of their catalytic domain that are required for their ability to stimulate GTP hydrolysis (Ref. 16, see also Table I). In rat eIF5, there are two invariant arginine residues at positions 15 and 48. The importance of these arginine residues in eIF5 function was investigated by alanine substitution mutagenesis of these residues. These mutant eIF5 proteins were initially examined for their ability to substitute for yeast eIF5 in a ΔTIF5 haploid yeast strain. The rationale behind this strategy was that, if these invariant arginine residues were required for eIF5 function, eIF5 proteins containing mutations at these residues would be lethal in yeast cells. All mutations were carried out in rat eIF5, because we use mammalian factors in our in vitro assay systems to measure eIF5 function. It should be noted that mammalian eIF5 functionally substitutes for yeast eIF5 in sustaining yeast cell growth and viability (18).

Mutational Analysis of Putative Arginine-finger Motif in Rat eIF5: Effect on eIF5 Function in Yeast Cells—To identify the invariant arginine residue in eIF5, the eIF5-Ala mutants R15A and R48A were generated using a PCR-based site-directed mutagenesis protocol using the LEU2-based yeast expression plasmid pTM100-EIF5 as the template as described under “Experimental Procedures.” The wild-type rat eIF5 ORF (gene designation EIF5) in the CEN plasmid pTM100-EIF5 is under
the transcriptional control of the galactose-inducible GAL1 promoter (18). To test the function of the rat eIF5-Ala mutants in yeast cells, both the wild-type and the mutant recombinant expression plasmids and the parental vector plasmid pTM100 were transformed separately into the haploid yeast strain TMY101. (The strain TMY101 carries an inactive TIF5 allele disrupted with the TRP1 marker gene and is kept viable by maintenance of an URA3-based CEN plasmid pRS316-TIF5 in which yeast eIF5 is expressed from its natural promoter.) Trp’ Ura’ Leu’ transformants were selected on SGal-Trp-Leu-Ura plates and then replica-plated onto similar SGal plates, which also contained uracil and 5-fluoroorotic acid (5-FOA) to select against retention of the URA3-based plasmid pRS316-TIF5, expressing wild-type yeast eIF5.

Fig. 3A shows that the yeast strain TMY101 transformed with plasmid pTM100-EIF5, expressing wild-type rat eIF5, grew on 5-FOA plates, in agreement with the results reported previously (18), while cells transformed with the parental vector plasmid pRS315 failed to grow, as expected. Under the same conditions TMY101 cells transformed with pTM100-EIF5(R15A), carrying a single point mutation at Arg-15 (Arg to Ala) in rat eIF5, failed to grow on 5-FOA plates (Fig. 3A, right panel) while cells expressing alanine substitution mutation at Arg-48 of rat eIF5 from pTM100-EIF5(R48A) grew as well as the cells expressing wild-type eIF5 from the plasmid pTM100-EIF5. It is known that even a conservative mutation of the invariant arginine residue in RasGAP to lysine dramatically affects its GAP activity (16). We also observed that when TMY101 cells were transformed with the recombinant plasmid pTM100-EIF5(R15K), which expressed the mutant eIF5 protein in which Arg-15 was mutated to Lys, and the resulting transformants were tested for their ability to grow on 5-FOA plates, they failed to grow (Fig. 3A, right panel). Taken together these results suggest that Arg-15 in rat eIF5 plays a critical role in eIF5 function in maintaining yeast cell growth and viability.

In RasGAPs and RhoGAPs, a “secondary” positively charged residue (Arg in RasGAP and Lys in RhoGAP) is also necessary for their GAP hydrolysis-stimulating activity. To identify such a residue in eIF5, we carried out mutagenesis of other invariant positively charged residues at the N-terminal region of rat eIF5 (Fig. 2). The residues Lys-24, Lys-33, Asn-38, and Lys-55 in rat eIF5 were each mutated to alanine. Once again, plasmid shuffling technique was used to test the effect of these mutations on yeast cell growth and viability. Yeast cells expressing either mutant eIF5 K33A (Lys-33 was mutated to Ala) or mutant eIF5 K55A (Lys-55 was mutated to Ala) failed to grow on 5-FOA plates (Fig. 3A, right panel). In contrast, alanine substitution mutation at Lys-24 and Asn-38 of rat eIF5 did not affect cell growth on 5-FOA plates (data not shown). These results indicate that Lys-33 and Lys-55 in rat eIF5 may also play an important role in eIF5 function.

The functional defect of mutant rat eIF5 proteins R15A, R15K, K33A, and K55A was not due to lack of expression of mutant eIF5 in yeast cells. When cell extracts were prepared from Trp’ Ura’ Leu’ transformants harboring both pRS316-TIF5 and pTM100-EIF5(wild-type or mutant) plasmids and analyzed by Western blotting using rabbit anti-rat eIF5 anti-
indicate residues in rat eIF5 that were targeted for mutagenesis in this study to generate the eIF5 mutants.

...accession numbers Q09689, P55876, and P41375, respectively. The highly conserved amino acid residues between eIF5 of all species are highlighted in dark shading and the moderately conserved residues are highlighted in light shading. Broken lines represent gaps. Arrowheads indicate residues in rat eIF5 that were targeted for mutagenesis in this study to generate the eIF5 mutants.

For this purpose, the mutant eIF5 proteins were expressed in bacteria and purified to apparent homogeneity (Fig. 4A) as described under “Experimental Procedures.” The ability of each purified mutant protein and wild-type eIF5 to bind eIF2β fused to GST and immobilized on GSH-Sepharose beads was examined. Fig. 4B shows that mutant eIF5 protein R15A (lane d), R15K (lane e), K33A (lane f), and K55A (lane g) were all able to bind GST-eIF2β, although the binding efficiency of K55A is somewhat reduced (see legend to Fig. 4B). As expected, wild-type eIF5 did not bind to GST immobilized on GSH-Sepharose beads (lane c).

The purified mutant eIF5 proteins were also tested for their ability to promote in vitro hydrolysis of GTP bound to the 40 S initiation complex. As expected, wild-type rat eIF5 promoted rapid hydrolysis of [γ-32P]GTP bound to the 40 S initiation complex (Fig. 4C). In contrast, under similar experimental conditions the mutant eIF5 protein R15A was unable to hydrolyze [γ-32P]GTP bound to the 40 S initiation complex (Fig. 4C). When the mutant eIF5 proteins R15K, K33A, and K55A were tested in the GTP hydrolysis reaction, we observed 2- and 6-fold reduction in GTP hydrolysis activity (Fig. 4C). Taken together, these results suggest that the eIF5 mutants R15A, R15K, K33A, and K55A were defective in their ability to hydrolyze GTP bound to the 40 S initiation complex, although they retained the ability to bind eIF2β.

The small reduction in binding efficiency of mutant K55A as compared with wild-type eIF5 (see legend to Fig. 4B) is presumably due to some conformational change induced in this eIF5 mutant. However, it should be noted that these binding analyses are at best semi-quantitative and stoichiometric, whereas eIF5-promoted GTP hydrolysis is highly catalytic. Additionally, it is known that the N terminus of eIF5 is not required for binding to eIF2β (14). Thus, the small reduction in the binding efficiency of K55A to eIF2β is unlikely to account for the reduced rate of GTP hydrolysis promoted by this eIF5 mutant.

**Table I**

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bodies, the mutant eIF5 proteins R15A (lane d), R15K (lane e), K33A (lane f), and K55A (lane g) were expressed at levels comparable to wild-type eIF5 (lane b). Extracts prepared from TMY101 cells harboring the plasmid pRS316-TIF5 and the vector plasmid pRS315 and expressing only wild-type yeast eIF5 did not show any immunoreactive polypeptide band with anti-rat eIF5 antibodies, as expected (lane c). It should be noted that rabbit anti-rat eIF5 antibodies do not recognize yeast eIF5 (18).

eIF5 Mutants R15A, R15K, K33A, and K55A Are Able to Bind eIF2β but Are Unable to Promote GTP Hydrolysis—The eIF5 mutants R15A, R15K, K33A, and K55A, which were unable to complement in vivo a genetic disruption in the chromosomal copy of TIF5, were initially tested to confirm that these mutations did not cause a defect in the ability of eIF5 to bind eIF2β. For this purpose, the mutant eIF5 proteins were expressed in...
**FIG. 4.** Analysis of eIF5 point mutants for their ability to bind eIF2β and promote hydrolysis of GTP bound to the 40 S initiation complex. A, recombinant wild-type and mutant eIF5 proteins R15A, R15K, K33A, and K55A were purified from isopropyl-1-thio-β-D-galactopyranoside-induced XL1-Blue cell lysates as described previously (14). Purified recombinant eIF5 proteins (3 μg each) were subjected to SDS-polyacrylamide gel electrophoresis (15% gel) and visualized by Coomassie Blue staining. The arrow indicates the position of purified eIF5. B, purified recombinant wild-type and mutant eIF5 proteins (1 μg each) were separately incubated with 2 μg of GST-eIF2β fusion protein immobilized on GSH beads as indicated. Following incubation at 4 °C with gentle shaking, reaction mixtures were centrifuged and the beads were washed, suspended in 1× Laemmli buffer, and subjected to Western blot analysis using polyclonal anti-eIF5 antibodies. In lane a, purified rat eIF5 was electrophoresed as a marker.

**FIG. 3.** Effect of mutations in rat eIF5 on growth of haploid yeast transformants expressing rat eIF5. A, haploid yeast strain TMY101 (18) that carries inactive TIF5, disrupted with TRP1 and harboring the URA3 plasmid pRS316-TIF5 that expresses the yeast wild-type TIF5 gene under the control of its natural promoter, was transformed separately with different mutant eIF5-expressing plasmids as indicated. Transformants selected on SGal-Trp-Leu-Ura plates (left panel) were replica-plated on SGal-Trp-Leu-Ura + 5-FOA plates (right panel). Cells were allowed to grow on 5-FOA plates for 5 days. B, immunoblot analysis of eIF5 in lysates of yeast cells expressing both yeast eIF5 and wild-type or mutant mammalian eIF5 proteins from the recombinant plasmids. Yeast cells harboring both the URA3 plasmid pRS316-TIF5 and the different recombinant LEU2-expression plasmids expressing either the wild-type or mutant mammalian eIF5 were grown to mid-logarithmic phase in synthetic medium containing 2% galactose as the sole source of carbon. Cell lysates were prepared as described previously (18) and analyzed by Western blotting using rabbit polyclonal anti-rat eIF5 antibodies. In lane a, purified recombinant rat eIF5 was electrophoresed as a marker.

**FIG. 4.** Analysis of eIF5 point mutants for their ability to bind eIF2β and promote hydrolysis of GTP bound to the 40 S initiation complex. A, recombinant wild-type and mutant eIF5 proteins R15A, R15K, K33A, and K55A were purified from isopropyl-1-thio-β-D-galactopyranoside-induced XL1-Blue cell lysates as described previously (14). Purified recombinant eIF5 proteins (3 μg each) were subjected to SDS-polyacrylamide gel electrophoresis (15% gel) and visualized by Coomassie Blue staining. The arrow indicates the position of purified eIF5. B, purified recombinant wild-type and mutant eIF5 proteins (1 μg each) were separately incubated with 2 μg of GST-eIF2β fusion protein immobilized on GSH beads as indicated. Following incubation at 4 °C with gentle shaking, reaction mixtures were centrifuged and the beads were washed, suspended in 1× Laemmli buffer, and subjected to Western blot analysis using polyclonal anti-eIF5 antibodies. In lane a, purified rat eIF5 was electrophoresed as a marker and probed with anti-eIF5 antibodies. In lane c, wild-type rat eIF5 was incubated with GST alone. It should be noted here that densitometric scanning using NIH IMAGE 1.62b7 (not shown) indicated that mutants R15A and K33A bound GST-eIF2β with similar efficiency. Mutant R15K bound GST-eIF2β with about 30% more efficiency, whereas mutant K55A bound GST-eIF2β with about 30% less efficiency. C, eIF5-promoted GTP hydrolysis. Each reaction mixture (90 μl) was prepared as described under the legend to Fig. 2 using 20 ng of either purified or mutant eIF5 proteins as indicated. Following incubation at 25 °C, aliquots (10 μl) were removed at each indicated time point and the amount of 32P released by the hydrolysis of [γ-32P]GTP was measured by the ammonium phosphomolybdate method as described (11). A reaction lacking eIF5 was also included, and the amount of 32P released in this control reaction mixture is also shown. The results shown represent the total amount of 32P formed per 90-μl reaction mixture. It should be noted here that the results shown are representative of three independent experiments. The average margin of error is ±5%.
eIF5 Functions as a GAP

6725

FIG. 5. Effect of eIF5 mutations on in vitro translation of total yeast RNA. eIF5-depleted cell-free translation extracts were prepared from TMY201R cells (18), incubated and analyzed for \([^{35}S]\)methionine incorporation into proteins as described in Ref. (18). Each reaction mixture (50 \(\mu\)l) contained 15 \(\mu\)Ci of \([^{35}S]\)methionine (11 Ci/mmol) and 25 \(\mu\)g of total yeast RNA and where indicated 100 ng of either purified recombinant rat eIF5 or purified mutant eIF5 proteins. Following incubation at 25°C for 40 min, aliquots (10 \(\mu\)l) were withdrawn and analyzed for \([^{35}S]\)methionine incorporation into proteins. A control reaction mixture lacking total yeast RNA and exogenously added eIF5 was also incubated and analyzed. The amount of \([^{35}S]\)methionine incorporated into proteins in this control reaction mixture was subtracted from the results shown. It should be noted that data similar to these presented in the figure were obtained in several independent experiments.

eIF5 Mutants R15A, R15K, K33A, and K55A Are Also Defective in Translation of mRNAs in Vitro—We have previously demonstrated that, in a yeast cell-free translation system depleted of endogenous eIF5, translation of mRNAs in vitro can be restored by the addition of exogenous yeast or mammalian eIF5 (18). We used such an eIF5-depleted yeast cell-free translation system to test the ability of the eIF5 mutants to restore translation of yeast mRNAs in vitro. Fig. 5 shows that these extracts showed poor translation activity in the absence of exogenously added eIF5. As expected, translation in these extracts was restored by the addition of purified wild-type rat eIF5 (Fig. 5). In the absence of mRNA, the addition of eIF5 had virtually no effect (data not shown). In contrast to wild-type rat eIF5, the mutant eIF5 protein R15A was virtually inactive in restoring translation of mRNAs in these extracts, whereas the mutant eIF5 proteins R15K, K33A, and K55A were about 50, 40, and 20% as active as the wild-type protein, respectively (Fig. 5).

DISCUSSION

Several lines of evidence presented in previous reports (1, 11–14) and summarized below suggested that eIF5 functions as a GTPase-activating protein (GAP) in translation initiation. First, eIF5 promotes GTP hydrolysis only when the protein interacts with the 40 S initiation complex (40 S AUG-Met-tRNA \(_{\text{eIF2-GTP}}\), eIF5, by itself, neither binds nor hydrolyzes either free GTP or GTP bound as a Met-tRNA \(_{\text{eIF2-GTP}}\) ternary complex in the absence of 40 S ribosomal subunits. Second, eIF5 interacts with the heterotrimERIC GTP-binding protein eIF2 (10). This interaction, which occurs between the conserved lysine residues at the N-terminal region of eIF2\(\beta\) and the conserved glutamic acid residues at the C-terminal region of eIF5 (12–14), is essential for eIF5 activity both in vitro (14) and in vivo in yeast cells (13, 14).

In this paper, we present additional evidence that shows that eIF5 indeed functions as a GAP in translation initiation. Typical GAPs like RhoGAPs and RasGAPs, which have been characterized extensively, contain “arginine-finger” motifs consisting of an invariant arginine residue at the N terminus of their catalytic domains that are necessary for their GTPase-stimulating activity in addition to motifs that are necessary for interacting with their respective G proteins (Ref. 16 and Table I). Biochemical and structural studies carried out with RasGAP have shown that in RasGAP the arginine at position 789 is the “primary” element required for stimulating GTP hydrolysis (16). The positive charge of the invariant arginine residue stabilizes the transition state of the GTP hydrolysis reaction by neutralizing the negative charges developing in the transition state (16). In addition, hydrogen bonding between Ras-GDP and GAP334 (the catalytic domain of RasGAP) also stabilizes the transition state (21). A secondary arginine residue, Arg-903 in GAP334 (Lys-122 in the case of RhoGAP) stabilizes the finger loop carrying the primary arginine residue (16).

Comparison of the N-terminal amino acid sequences of eIF5 from different species revealed the presence of invariant arginine residues at positions 15 and 48 that are conserved in all species of eIF5 identified so far (Fig. 2). Like RasGAP and RhoGAP (22), the Arg-15 residue in eIF5 is preceded by two conserved hydrophobic residues, phenylalanine and tyrosine. It has been suggested that the function of these two hydrophobic residues is to anchor the catalytic loop into the hydrophobic core of the GAP (22). Thus, in eIF5, Arg-15 is in a better sequence context than Arg-48. Based on this analysis, we observed that mutation of Arg-15 in rat eIF5 to Ala or even to conservative Lys resulted in mutant proteins that were unable to substitute for yeast eIF5 in maintaining yeast cell growth and viability in a \(\Delta tif5\) yeast strain (Fig. 3). In contrast, \(\Delta tif5\) yeast cells expressing eIF5 mutant R48A were able to maintain growth and viability (Fig. 3). In agreement with these results obtained in vitro, we observed that the purified eIF5 mutants R15A and R15K were severely defective in their ability to promote GTP hydrolysis. Whereas mutant R15A showed virtually no activity, mutant R15K showed a low level of activity (<20%). Thus, although the lysine residue at position 15 of eIF5 mutant R15K was able to compensate for the positive charge of arginine, it was probably unable to correctly position itself with respect to the guanine nucleotide or the active site of the GTPase protein (presumably the conserved histidine residue at position 138 in the \(\gamma\) subunit of human eIF2 (23)) in the transition state. We also observed that mutation of Lys-33 and Lys-55 of rat eIF5 to alanine also caused a severe defect in eIF5 function both in vitro and in vivo in yeast cells. In analogy with RasGAP and RhoGAP, it is likely that either Lys-33 or Lys-55 or both in rat eIF5 constitute the secondary element required for eIF5 GAP function.

An important property of eIF5-dependent GTP hydrolysis reaction is that, in addition to eIF2 and eIF5, 40 S subunits also play an essential role in GTP hydrolysis. This is analogous to the essential requirement of 50 S ribosomal subunits in IF2-, EF-Tu-, and EF-G-catalyzed GTP hydrolysis reaction in prokaryotes (7, 8). A similar requirement of ribosomes in GTP hydrolysis reaction catalyzed by the signal recognition peptide receptor subunit SR\(\beta\) has been reported (24). Biochemical studies of GAP mutants and crystal structure analysis of GTPase-GAP complexes (22) have shown that GAPs provide two functions to the transition state of a GTP hydrolysis reaction. First, it physically binds to the G protein and causes a conformational change in the G protein resulting in the stabilization of the switch I and switch II regions in the G protein (22). This results in the correct positioning of the active site glutamine residue of the G protein in the transition state and
its activation. Second, GAP also provides an arginine residue to the pentacoordinate transition state and stabilizes it. In the case of eIF5-dependent GTP hydrolysis reaction, although eIF5 provides the essential arginine residue of the GAP, the question arises: how are the switch regions in eIF2γ (the polypeptide that contains the conserved GTP binding domains (23, 25) and is the presumed GTPase) stabilized in the absence of direct physical interaction between eIF5 and eIF2γ? Clearly, although interaction between eIF5 and eIF2γ anchors eIF5 to eIF2 and eIF2γ is also known to physically interact with eIF2γ (26), the fact remains that these interactions, although necessary, are not sufficient for GTP hydrolysis, because eIF5 cannot promote hydrolysis of GTP bound to eIF2 in the MetRNAeIF2-GTP ternary complex in the absence of 40 S ribosomal subunits. Thus, the possibility exists that the 40 S subunit directly interacts with eIF2γ and causes the conformational change necessary to stabilize the switch regions in eIF2γ. In this respect, the 40 S ribosomal subunit also behaves as a GAP in eIF5-dependent GTP hydrolysis reaction during translation initiation. Such a distribution of dual functions of a GAP in two different proteins has also been suggested for GTP hydrolysis reaction mediated by ADP-ribosylation factor and Gα signal transduction protein (27).

Finally, both mammalian and yeast eIF5 have been reported (3, 4) to contain sequence motifs that are somewhat homologous to G1–G4 domains that are characteristic of members of the GTPase superfamily (28). However, unlike the well-characterized GTPases, these domains in eIF5 are quite “imperfect” (Table II). The conserved G1 domain GXXGXXG(S/T) is present in rat eIF5 as 27GKGNGIKT34 and in yeast eIF5 as 27GRGGNGIKT34. Clearly, insertion of an extra amino acid, isoleucine, into the consensus phosphate-binding loop makes the G1 domain an imperfect consensus sequence. Additionally, the first four amino acids in the G1 domain in eIF5 are GXXG instead of the consensus GXXG. Furthermore, the spacings between the four domains are also not conserved in eIF5 as they are in members of the GTPase superfamily. Finally, unlike GTPases like Ras, where a conserved glutamine residue (histidine in some GTPases) in the G3 domain is the active site of the GTPase (28), such a conserved positively charged residue at a similar position is absent in eIF5. These observations along with the results of our mutational analysis (data not shown)

showing that mutation of conserved residues in the G1 and G4 domains of eIF5 did not affect eIF5 function in vitro and in vivo in yeast cells suggested that these domains are not critical for eIF5 function.

REFERENCES


### Table II

<table>
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<th>Motifs</th>
<th>G1</th>
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<th>G3</th>
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<td>(N/T)V/K(Q)XD</td>
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Eukaryotic Translation Initiation Factor 5 Functions as a GTPase-activating Protein
Supratik Das, Rajarshi Ghosh and Umadas Maitra

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