Physical Association of Eukaryotic Initiation Factor (eIF) 5 Carboxyl-terminal Domain with the Lysine-rich eIF2β Segment Strongly Enhances Its Binding to eIF3

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The carboxyl-terminal domain (CTD) of eukaryotic initiation factor (eIF) 5 interacts with eIF1, eIF2β, and eIF3c, thereby mediating formation of the multifactor complex (MFC), an important intermediate for the 43 S preinitiation complex assembly. Here we demonstrate in vitro formation of a nearly stoichiometric quaternary complex containing eIF1 and the minimal segments of eIF2β, eIF3c, and eIF5. In vivo, overexpression of eIF2 and tRNA\textsuperscript{Met} suppresses the temperature-sensitive phenotype of tifs-7A altering eIF5-CTD by increasing interaction of the mutant eIF5 with eIF2 by mass action and restoring its defective interaction with eIF3. By contrast, overexpression of eIF1 exacerbated the tifs-7A phenotype because eIF1 forms unusual inhibitory complexes with a hyperstoichiometric amount of eIF1. Formation of such complexes leads to increased GCN4 translation, independent of eIF2 phosphorylation (general control derepressed or Gcd phenotype). We also provide biochemical evidence indicating that the association of eIF5-CTD with eIF2β strongly enhances its binding to eIF3c. Our results suggest strongly that MFC formation is an ordered event involving specific enhancement of eIF5-CTD binding to eIF3 on its binding to eIF2β. We propose that the primary function of eIF5-CTD is to serve as an assembly guide by rapidly promoting stoichiometric MFC assembly with the aid of eIF2 while excluding formation of nonfunctional complexes.

In eukaryotic translation initiation, the 40 S ribosomal subunit binds the eukaryotic initiation factor (eIF)\textsuperscript{1} 2-GTP-Met-tRNA\textsuperscript{Met} ternary complex (TC) to form the 43 S preinitiation complex. Subsequent joining of mRNA carried by eIF4F produces the 48 S preinitiation complex. The eIF3 stimulates recruitment of Met-tRNA\textsuperscript{Met} and mRNA to the 40 S ribosome (for a review, see Ref. 1). The 48 S complex searches for the first AUG codon in the mRNA with the help of small factors eIF1 and eIF1A. Correct base pairing between the Met-tRNA\textsuperscript{Met} anticodon and the AUG codon triggers the hydrolysis of GTP bound to eIF2 by the action of the GTPase-activating protein eIF5, which then leads to dissociation of preassembled eIFs and formation of the 40 S initiation complex. The second GTP-bonding protein eIF5B then stimulates joining of the 40 S initiation complex with the 60 S subunit to form the 80 S initiation complex. The elongation of the polypeptide chain starts from the methionine linked to the 80 S initiation complex. The GDP-bound eIF2 that is released after GTP hydrolysis and 80 S complex formation needs to be recycled to eIF2-GTP because only the GTP form of eIF2 can bind Met-tRNA\textsuperscript{Met}.

This reaction is catalyzed by eIF2B, a five-subunit guanine nucleotide exchange factor.

Substantial evidence indicates that the carboxyl-terminal domain (CTD) of eIF5 serves as a core of ribosomal preinitiation complex formation (2, 3), independent of its GTPase-activating function ascribed to the amino-terminal domain (NTD) (4, 5). The eIF5-CTD binds eIF1, the c-subunit of eIF3, and eIF4G as well as the β-subunit of eIF2, the GTPase-activating protein substrate. Of these interactions, the eIF5-CTD binding to eIF1, eIF2β, and eIF3c has been shown to occur simultaneously (2, 6). These observations led to the discovery of in vivo formation of the multifactor complex (MFC) containing eIFs 1, 2, 3, and 5 and Met-tRNA\textsuperscript{Met} (2). Accumulating evidence indicates that the MFC binds to the 40 S subunit as a preformed unit to form the 43 S complex (7–9). Because yeast mutations relaxing the stringency of start codon selection have been mapped in eIF1, eIF5, and all three subunits of eIF2 (10), it was proposed that the integrity of the MFC on the ribosome is a prerequisite for the function of these factors at the AUG selection step (2, 3). In addition, eIF5-CTD binding to eIF4G was proposed to at least in part mediate mRNA cap-eIF4F complex binding to the 43 S complex (3, 11).

The minimal binding domains for eIF5-CTD are identified in eIF2β (amino-terminal half with three lysine-rich segments or K-boxes) (12–14), eIF3c (amino-terminal serine-rich acidic segment) (2), and eIF4G (an expanded HEAT domain; HEAT named for Huntingtin, Elongation factor 3, A subunit of PP2A, and Target of rapamycin) (11). eIF5-CTD contains a bipartite motif, designated aromatic/acidic amino acid boxes (AA-boxes) 1 and 2 (13, 15) or the W2 domain (16), at its very carboxyl terminus. AA-boxes are also found in carboxyl termini of all eukaryotic eIF2Be (the catalytic subunit of eIF2B) and of mammalian eIF4G (the largest subunit of eIF4F). The three-dimensional structure of the AA-box-containing domain of eIF2Be, equivalent in length to eIF5-CTD, indicates that it is composed of eight α-helices stacked together to form an atypical HEAT domain with the
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### TABLE I
Plasmids used in this study

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### EXPERIMENTAL PROCEDURES

**Plasmids and Yeast Strains**—Plasmids and yeast strains used in this study are listed in Tables I and II, respectively. pKA758 encoding tif5-7A was prepared by replacing the NdeI-SalI fragment of pGEX-TIF5 (13) with the NdeI-SalI segment of pGEX-TIF5-7A. YEpU-eIF3a0l encoding all five core subunits of eIF3 (TIF1a, TIF1b, TIF1p, TIF2p, and TIF3p) on a high copy (hc) URA3 vector was constructed by subcloning the 11.5-kb DrdI-KasI fragment of pLPY-PT1His-TIF34HA-TIF35Flag (7) into the SacI-KasI sites of pLPY-NIP1-TIF32 (7) after the DrdI and SacI sites were blunted by using bean nuclease. pGB-TIF5-B6 or pGB-TIF5-B6a was constructed by cloning the TIF5 segments into pET30-GBFusion1 (kind gift of G. Wagner); the inserted TIF5 segments were created by PCR with primers 5’-GGG GGATCC GTG AAC TCT GAG CTC ACT and 3’-CCC GGA TCC TTC GTC GTC TTC TTC ATC ATC by M. G. C. and E. H. using a T7 promoter. The amounts of bound35S-labeled proteins were quantitated by scanning the gel with STORM/TYPHOON (Amersham Biosciences). Apparent dissociation constants (Kd) for protein interactions assayed in this manner were calculated from the equation: Kd = G(1/f) - 1 where f is the fraction of 35S-protein bound to GST fusion protein and G is the molar concentration of GST fusion protein (11). This holds only when G >> [35S-protein]. Obtained Kd values were averaged from at least three sets of experiments using different amounts of GST fusion protein. GST pull-down assays with GB-eIF5-B6 or -B6a were done similarly with the same buffers (19). Apparent Kd values for these reactions were calculated precisely from initial concentrations of binding components and of the precipitated complex and averaged from independent experiments. In principle, Kd measurement by GST pull-down can underestimate the real values due to the washing step of the reaction. We consistently observed a near hyperbolic inter-
RESULTS

Formation of the Mini-MFC—The eIF5-B6 (CTD with amino acids 241–405) can bridge interactions between eIF1 and eIF2β-N (NTD with amino acids 1–140) and between eIF2β-N and eIF3c-N (NTD with amino acids 1–156) (2) (see Fig. 1A for locations of these segments). Although the formation of the MFC free of the ribosome was demonstrated in vivo (2), it is not clear whether the eIF5-CTD alone is sufficient to mediate a quaternary complex formation with its isolated partners in the MFC. To address this question, we attempted to form a quaternary complex with GST-eIF5-B6, His-eIF3c-N, His-eIF2β-N, and eIF1-FL (carboxy-terminal FLAG-eIF1) that were expressed from bacteria. We affinity-purified this complex first via a glutathione resin and second via anti-FLAG affinity resin. As shown in Fig. 1C, lane 1, this purified complex contained approximately stoichiometric amounts of each of the constituents, which reacted with appropriate antibodies (lane 2). In addition, the purified complex migrated at 130 kDa on a sizing column as expected from its size (Fig. 1D). Immunoblotting with anti-eIF1 (Fig. 1D, bottom panel), anti-eIF5, and anti-His6 (data not shown) antibodies confirmed that the peak fraction contains the four proteins. We designated this complex as Mini-MFC. The stable and nearly stoichiometric formation of this complex strongly suggests that the four parts of the MFC alone can mediate the multi-eIF assembly.

Asymmetric Effect of MFC Partner Overexpression on Yeast tsf5-7A Mutant—Having shown that eIF5-CTD and its isolated partners in the MFC are sufficient to form a tight complex, we considered the mechanism of entire MFC assembly in vivo. The tsf5-7A mutation altering the seven amino acids of eIF5 AA-box (compare Fig. 1A) (also see Fig. 2A) for locations of these segments) impairs its incorporation into MFC (data not shown) antibodies confirmed that the peak fraction contains the four proteins. We designated this complex as Mini-MFC. The stable and nearly stoichiometric formation of this complex strongly suggests that the four parts of the MFC alone can mediate the multi-eIF assembly.

Defective Function of eIF5-CTD in MFC Assembly—Thus, eIF5 alone exacerbated the Ts phenotype due to tsf5-7A mutation. These results suggest strongly that the essential function of eIF5-CTD is primarily caused by the eIF5-CTD mutation. These results suggest strongly that the essential function of eIF5-CTD is primarily caused by the eIF5-CTD mutation. These results suggest strongly that the essential function of eIF5-CTD is primarily caused by the eIF5-CTD mutation. Therefore only the hc TC can suppress the tsf5-7A phenotype. If not and if one interaction is more critical for MFC formation, the overexpression of MFC partner would suppress the tsf5-7A phenotype.

To clarify this point, we overproduced eIF1 or all five eIF3 subunits in the tsf5-7A mutant in addition to all TC components. Fig. 2A confirms that the TC overexpression from the hc TC plasmid p1780-IMT suppresses the Ts phenotype of tsf5-7A (compare rows 3 and 5). By contrast, overexpression of all five eIF3 subunits from YEpU-eIF3all did not suppress the phenotype (row 6). More interestingly, overexpression of eIF1 alone exacerbated the Ts growth defect of the tsf5-7A mutant (row 4) even though hc eIF1 does not retard cell growth of wild-type cells (row 2) (also see Fig. 2B for effects on doubling time). To test whether these negative results are due to the expression of only one of the two factors (eIF1 and eIF3) known to bind tightly together, we co-overproduced eIF1 and eIF3 in the mutant. However, we still did not observe the suppression of tsf5-7A but instead observed the exacerbation of the mutant phenotype as observed with eIF1 alone (Fig. 2A, rows 3 and 9). Therefore only the hc TC can suppress the Ts phenotype caused by the eIF5-CTD mutation. These results suggest strongly that the essential function of eIF5-CTD is primarily mediated by the eIF2/eIF5 interaction.
tibodies specific for FLAG-tagged eIF5-7A mutant. As shown in Fig. 3A, panel a, lanes 4–9, immunoprecipitation with anti-FLAG antibodies confirms that hc TC increases eIF2/eIF5 interaction as reported previously (13). Under these conditions, immunoprecipitation of HA-eIF3 with anti-HA antibodies shows concomitant increase in the amount of eIF5 associated with HA-eIF3 (Fig. 3A, panel b, lane 8). Thus, the increase in the eIF2/eIF5 interaction by mass action appears to be sufficient to at least partially restore the defective interaction of the mutant eIF5 with eIF3 (see Fig. 3C for schematic illustration). Alternatively it is possible that hc TC increased its direct interaction with eIF3, as was previously shown (8), thereby recruiting eIF5-7A to eIF3 by mutual cooperativity effects. However, we believe that this is not the case because hc eIF3 increased the amount of eIF2a (a) in complex with HA-eIF3 (see below, Fig. 3B, lane 8) but did not suppress the Ts− phenotype of tif5-7A (Fig. 2A, row 6). The latter results suggest that the increase in HA-eIF3/eIF2 complex due to hc eIF3 did not result in increase in functional MFC including eIF5-7A. Therefore we conclude that the eIF2/eIF5 interaction is required specifically for the eIF2/eIF5/eIF3 linkage in the MFC.

Ligandwise communoprecipitation studies in Fig. 3B show that hc eIF3-HA in tif5-7A increases its interaction with eIF5-7A by mass action as well as that with eIF2a (compare lanes 2 and 8). The amount of eIF2a precipitated in lane 8 is comparable to the amount of the same factor precipitated with wild-type cell extracts (lane 14), whereas the amount of eIF5-7A in lane 8 is one-third of wild-type eIF5 precipitated with TIF5 cell extracts in lane 14. However, the fact that this hc eIF3 did not suppress the Ts− phenotype of tif5-7A (Fig. 2A) suggests that the observed increase in the eIF5-7A/eIF3 interaction did not lead to an increase in functional MFC formation. In addition, nearly half of the overproduced eIF3-HA was immunoprecipitated by anti-HA (Fig. 3B, compare lanes 7–9 versus lanes 1–3 or lanes 13–15, top three panels); thus, the amount of eIF2a and eIF5-7A precipitated in this fraction appears to be stoichiometric to that of HA-eIF3. A feasible interpretation for these observations would be that hc eIF3-HA only increased its partial subcomplexes separately with eIF5-7A and eIF2 rather than functional MFC containing all four eIFs (see Fig. 3C). If so, the partial eIF2/eIF3 subcomplex observed here is likely mediated by direct eIF2/eIF3 linkage independently of eIF5-CTD (8). These results are consistent with the idea that only the eIF5-eIF2 subcomplex, but not the eIF5-eIF3 or eIF2/eIF3 subcomplex, serves as a core of functional MFC formation.

Overexpression of eIF1 Exacerbates the tif5-7A Phenotype by Disturbing Normal Preinitiation Complex Formation—As shown in Fig. 2A, hc eIF1 in the tif5-7A mutant exacerbates the Ts− growth of the tif5-7A mutant (row 4). To test whether this inhibition is associated with a reduced rate of translation initiation, we conducted sucrose gradient analyses of the mutant extracts to obtain polysome profiles. The results in Fig. 2B, left panels, confirmed that the polysome content of the Ts− tif5-7A mutant strain KAY36 was modestly decreased shortly (1 h) after the cells were shifted to a high temperature as shown previously with a different tif5-7A strain KAY51 (2). As shown in Fig. 2B, right panels, the polysome content was further decreased by eIF1 overexpression, and this trait was enhanced at the restrictive temperature, indicating that the exacerbation of the mutant phenotype by hc eIF1 results from a defect in translation initiation.

Communoprecipitation studies indicated that hc eIF1 restores HA-eIF3 binding to eIF2, eIF5-7A, and eIF1 itself (Fig. 3B, compare lanes 2 and 5) in support of the idea that eIF1 can serve as a core of MFC assembly by bridging eIF2 and eIF3, a
were prepared and resolved by velocity sedimentation on 5–45% sucrose gradients. Fractions were collected while scanning continuously at 1h (bottom panels)

20 mM 3-aminotrizole for 4 days as in (rif5-7A/Vec1, YEplac195; rif5-7A/Vec2, YEplac181; rif5-7A/eIF1, YEplac181; rif5-7A/eIF3, YEplac181) were tested as in rows 1–6 except that cells were spotted on synthetic dextrose medium. The rif5-7A mutants KAY36 (Trp/Ura1) and KAY38 (Leu/Ura1) grow at nearly identical rates as shown with vector controls in rows 3 and 7. B, KAY36 transformants carrying YEplac195 (left panels, 7A/Vec) or YEplac181 (right panels, 7A/eIF1) grown exponentially in synthetic complete medium lacking Ura at 30 °C were shifted to 37 °C for 1 h (bottom panels) or kept at 30 °C as a control (top panels). Cycloheximide was added to the cultures for 5 min prior to harvesting the cells. WCEs were prepared and resolved by velocity sedimentation on 5–45% sucrose gradients. Fractions were collected while scanning continuously at 1h (bottom panels)

Amino acid synthesis enzymes (3-AT)

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FIG. 2. Asymmetric effect of MFC partner overexpression on rif5-7A. A, KAY36 (rif5-7A gcn2Δ) or KAY35 (TIF5 gcn2Δ) transformants carrying the indicated plasmids were grown overnight in synthetic dextrose medium supplemented with tryptophan. Equal A_{600} units and its 1:10 and 1:100 dilutions were spotted from left to right on the same medium and incubated at the indicated temperature for 3 days. The following plasmids were introduced: YEplac195 (vector), YEplac181 (eIF1), Yeplac182 (eIF2 TC), or Yeplac181 (eIF3/eIF1). In rows 7–9, KAY38 (rif5-7A gcn2Δ) or KAY37 (TIF5 gcn2Δ) transformants carrying YEplac195 and YEplac181 (eIF2 TC) were tested as in rows 1–6 except that cells were spotted on synthetic dextrose medium. The rif5-7A mutants KAY36 (Trp/Ura1) and KAY38 (Leu/Ura1) grow at nearly identical rates as shown with vector controls in rows 3 and 7. B, KAY36 transformants carrying YEplac195 (left panels, 7A/Vec) or YEplac181 (right panels, 7A/eIF1) grown exponentially in synthetic complete medium lacking Ura at 30 °C were shifted to 37 °C for 1 h (bottom panels) or kept at 30 °C as a control (top panels). Cycloheximide was added to the cultures for 5 min prior to harvesting the cells. WCEs were prepared and resolved by velocity sedimentation on 5–45% sucrose gradients. Fractions were collected while scanning continuously at A_{600}.

Positions of 40 and 60 S subunits, 80 S ribosomes, and polysomes are indicated. P/M, ratio of A_{600} in the combined polyosomes fractions to that in the 80 S peak; d. t., cell doubling time in hours. C, KAY38 (rif5-7A gcn2Δ) or KAY37 (TIF5 gcn2Δ) transformants carrying plasmids listed in the table to the left were assayed as in A except that the cells were spotted onto synthetic dextrose medium supplemented with leucine and 20 mM 3-aminotrizole (3-AT) and incubated for 5 days. Schematics to the right illustrate the signaling pathway leading to amino acid synthesis activation either induced by amino acid starvation or Gcd mutation. Crossing indicates that the Gcn2p kinase is deleted in all the strains used. D, KAY38 (rif5-7A) transformants carrying two plasmids, indicated in the table to the left, were spotted and grown on synthetic dextrose medium containing leucine and 20 mM 3-aminotrizole for 4 days as in C. Vec1, YEplac195; Vec2, YEplac181; eIF1, YEplac181; eIF2 TC, Yeplac182; Gen4, gcn2 mutation. Crossing indicates that the Gen2p kinase is deleted in all the strains used.
subunit (3) (Fig. 4, A, panel b, and B, columns 7A/Vec).2 As shown in Fig. 4A, panel c, eIF1 overexpression in tif5-7A cells allows ∼10 times more eIF1 to be associated with the 40 S subunit than eIF2 and eIF3 that remain associated with the 40 S subunit in the same cells (Fig. 4A, panel c and B, columns 7A/eIF1). Therefore, eIF1 overexpression in the absence of eIF5-CTD function appears to cause direct eIF1 binding to the 40 S subunit by mass action. Together with the results shown in Fig. 3, we propose that the mutant eIF5, HA and FL refer to HA and FLAG epitope tags introduced to eIF3 and eIF5, respectively. Direct contacts indicate interactions. “??” in the schematics indicates that the proposed configuration is merely one of different alternative possibilities.

Overexpression of eIF1 in tif5-7A Shows a Gcd− Phenotype—To verify the physiological relevance of unusual MFC with an excessive amount of eIF1 observed in hc eIF1 tif5-7A cells (Fig. 3B), we tested the Gcd− (general control derepressed) phenotype of the strain. If the observed complexes are inhibitory, they are expected to sequester eIF2 TC from the active pool involved in the initiation process. This would result in slower TC binding to the 40 S subunits migrating down the GCN4 mRNA leader, allowing them to bypass upstream open reading frames to reinitiate GCN4 translation independently of the action of Gcn2p eIF2 kinase (20). Because Gcn4p activates amino acid biosynthesis genes, this event rescues growth inhibition caused by amino acid starvation due to the drug 3-aminotriazole. For this reason, mutations impairing TC formation or its binding to the 40 S subunit confer 3-aminotriazole resistance in the absence of Gcn2p kinase (Gcd− phenotype, see also schematics in Fig. 2C). As shown in Fig. 2C, row 4, hc eIF1 in the tif5-7A mutant indeed confers a Gcd− phenotype, although hc eIF1 in the wild-type yeast does not (row 2). We confirmed that this phenotype is due to limiting TC binding to the 40 S subunit by showing that TC co-overexpression suppressed the

Fig. 3. Coimmunoprecipitation studies. A and B, 200 μg of WCEs prepared from KAY50 (TIF34-HA TIF5-FL), KAY51 (TIF34-HA tif5-7A-FL), and KAY37 (TIF34 TIF5-FL) transformants carrying YEplac195 (Vector), p1780-IMT (eIF2 TC), YEplU-SUI1 (eIF1), or YEplU-eIF3all (eIF3) grown in synthetic complete medium lacking Ura was used for immunoprecipitation with anti-FLAG (A, panel a) and anti-HA (A, panel b, and B) affinity resin. The entire pellet fractions (P) were analyzed together with 10% input (I) and supernatant (S) fractions with immunoblotting with anti-eIF5 (28), anti-eIF2α (29), anti-eIF2α (30), anti-eIF3b (31), anti-eIF3g (7), and anti-eIF1 (32) antibodies indicated to the right. The “anti-HA” label under eIF3i-HA indicates that HA-tagged eIF3i was detected with anti-HA antibodies (BabCO). C, model of MFC assembly in the strains tested. Circles indicate individual eIF. The filled circle indicates the mutant eIF5. HA and FL refer to HA and FLAG epitope tags introduced to eIF3 and eIF5, respectively. Direct contacts indicate interactions. “??” in the schematics indicates that the proposed configuration is merely one of different alternative possibilities.

2 Unlike this previous finding, however, we did not observe an increase in eIF2 and eIF3 levels on the 40 S subunit in tif5-7A cells but that a part of eIF2 and eIF3 dissociate more readily from the 40 S subunit and migrate in loose complex with the mutant eIF5 in the sucrose gradient under our laboratory conditions.
In Vitro Protein Interaction Assays between Mini-MFC Components—An attractive model to explain the specific requirement of eIF2 association for eIF5 binding to eIF3 is that the eIF5-binding partner in eIF2 (the lysine-rich eIF2/H9252 segment) specifically enhances eIF5-CTD for its binding to the partner in eIF3 (the amino-terminal eIF3c segment). To test this idea, we attempted to establish protein-protein interaction assays with recombinant protein segments that mediate formation of the MFC. Fig. 1B summarizes the results of our determination of equilibrium dissociation constants (KD) for binary interactions between GST-fused MFC segments and 35S-labeled partners (see “Experimental Procedures”). The position of the MFC subunit or segment used in the binding assays is described in Fig. 1A and identical to that of the segments used to form Mini-MFC in Fig. 1, C and D. For each of the binary MFC component interactions, we constructed GST fusions to both partners, each of which was allowed to bind its 35S-labeled counterpart synthesized in rabbit reticulocyte lysates. For all except the interactions of eIF1 with eIF5 and eIF2/H9252, we obtained comparable KD values for the two different reactions measuring the same interaction. Thus, GST fusion does not affect these interactions. On the other hand, GST fusion to eIF1 abolished its binding to eIF5 and eIF2/H9252 as observed previously with amino-terminal FLAG tagging of eIF1 (6).

The major obstacle to assaying the interaction between the eIF2/H9252/eIF5 complex and eIF3c was to obtain a high quality recombinant eIF5-CTD segment since the His-eIF5-B6 construct used in previous studies (2) did not efficiently bind its partners, eIF3c-N and eIF2/H9252-N, even though it could bridge interaction between them.3 As shown in Fig. 5A, however, a new construct of eIF5-B6 designated GB-eIF5-B6 (lane 1 for 40% input amount) bound efficiently to eIF2/H9252-N (lane 5) and eIF3c-N (lane 7) that were fused to GST but not to GST alone (lane 3). This construct was an amino-terminal fusion of the eIF5-B6 segment to the highly soluble streptococcal protein G B1 domain (residues 1–65) as a solubility enhancement tag (21). More quantitative binding assays with GB-eIF5-B6 (examples shown in Fig. 5B) indicated the KD for the GST-eIF2/H9252-N/GB-eIF5-B6 interaction to be 1.5 ± 0.5 μM and that for the GST-eIF3c-N/GB-eIF5-B6 interaction to be 0.3 ± 0.1 μM as summarized in Fig. 1B in parentheses. These values are quite

3 C. R. Singh and K. Asano, unpublished data.
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FIG. 5. Specific enhancement of eIF5-CTD binding to eIF3c by the lysine-rich eIF2β segment. A, 5 µg each of GST alone (lanes 3 and 4), GST-eIF2β-N (lanes 5 and 6), and GST-eIF3c-N (lanes 7 and 8) were incubated with 2.5 µg of GB-eIF5-B6 (lanes 3, 5, and 7) or GB-eIF5-B6Δ (lanes 4, 6, and 8). Then the complex was one-step purified via glutathione resin and analyzed by SDS-PAGE. Shown is the Coomassie-stained gel separating the complexes. Lanes 1 and 2, 1 µg of GB-eIF5-B6 and GB-eIF5-B6Δ, respectively. B, the indicated amounts of GST fusion proteins were allowed to bind 0.15 µg of GB-eIF5-B6. The complex was analyzed by SDS-PAGE followed by immunoblotting with anti-polyhistidine antibodies (Santa Cruz Biotechnology) (bottom panel). Top panel, GST fusion proteins were visualized by Ponceau S staining. Lanes 1 and 7, 30 ng of GB-eIF5-B6. C, schematics illustrate two different interpretations for the results of GST pull-down assays using a GST fusion (protein Y, empty circle), a bridging component (protein X, filled circle), and a third, peripheral component (protein Z, gray circle). Arrows indicate direct interactions. Crossed arrows indicate no interaction. In panels a, the apparent affinities of GST-Y-X complex against Z are measured. The apparent affinities of GST-X against Z and of GST-Y against Z were separately measured in panels b and c, respectively. D and E, GST pull-down assays using eIF5-B6 (5), eIF2β-N (2β), and eIF3c-N (3c). Schematics at the top depict the components in the binding reaction as defined in C. Apparent Kd values (App. Kd) for GST-Y-X complex against Z were calculated from the results of the experiments (see “Experimental Procedures”) and are given below the schematics. 5 µg of indicated GST or GST fusion proteins were adsorbed to the glutathione-Sepharose resin and incubated with 35S-labeled partner in the presence of the indicated amounts of GB-eIF5-B6. After washing the resin, GST complexes were analyzed by SDS-PAGE. The amounts of GB-eIF5-B6 bound to GST fusions (shown by a dotted square) were measured by the NIH Image software using the Coomassie-stained pattern (top panel) and indicated below. The bottom panel shows the autoradiography of the Coomassie-stained gel with 20% input amount (In) of 35S-labeled partner (lane 1). Shown below is the percentage of 35S-labeled partner bound to GST fusion protein in each lane. Arrows indicate the positions of the proteins used in each experiment. F, the fraction of [35S]eIF3c-N or [35S]eIF2β-N bound to GB-eIF5-B6/GST-eIF2β-N or GB-eIF5-B6/GST-eIF3c-N complex was plotted against the molar concentration of the complexes. Data from independent experiments (shown in D and E and also not shown) are combined.

To examine the specificity of the interaction, we removed the last 15 amino acids of the eIF5 portion of GB-eIF5-B6, creating GB-eIF5-B6Δ (Fig. 5A, lane 2). This deletion mutation is identical to W391Δ as described below in Fig. 7. GB-eIF5-B6Δ bound to GST-eIF3c-N but not to GST-eIF2β-N (Fig. 5A, lanes 6 and 8), consistent with the finding that this mutant is defective in binding to eIF2 but not defective in binding to eIF3 (see below, Fig. 7).

The eIF5-CTD Association with eIF2β Segment Specifically Enhances Its Binding to eIF3c—With this high quality eIF5-CTD segment, we proceeded to measure the affinity between the eIF2β-N-eIF5-B6 subcomplex and eIF3c-N. In principle, if all interactions between three proteins (X, Y, and Z) are considered, the binding of the XY subcomplex to Z is stronger than the separate binding of X to Z due to mutual cooperativity effects (Fig. 5C, model I). However, if X cannot bind Z and the affinity of the XY complex to bind Z is higher than that of X alone to bind Z, this affinity increase can be attributable to the specific effect of X on the binding of X to Z (Fig. 5C, model II).

Because eIF3c-N cannot bind eIF2β-N, we can test the specific effect of the eIF2β-N segment on the binding of eIF5-B6 to eIF3c-N. For this purpose, we allowed GST-eIF2β-N to bind [35S]eIF3c-N in the absence or presence of different amounts of
GB-eIF5-B6 as shown in Fig. 5D, lanes 2–8. Subsequently the complex containing GST-eIF2β-N-GB-eIF5-B6-[35S]eIF3c-N ternary complex was one-step purified by glutathione resin and analyzed by SDS-PAGE followed by Coomassie staining and autoradiography (top and bottom panels, respectively). Assuming a 1:1 binary complex, we calculated the molar concentration of [35S]eIF3c-N to GST-eIF2 binding a 1:1 binary complex, we calculated the molar concentration of [35S]eIF3c-N to GST-eIF2 dividing it by the reaction volume. The fraction of bound [35S]eIF3c-N to GST-eIF2β-N-GB-eIF5-B6 complex was plotted against the molar concentration of the latter subcomplex as shown by triangles in Fig. 5F. Together with data from independent experiments, the binding curve reached a plateau at about 40% [35S]eIF3c-N binding (Fig. 5F). Thus, it appears that nearly half of the trimeric complex had dissociated during the washing step. Importantly only 10 nM (on average) GST-eIF2β-N-GB-eIF5-B6 complex was sufficient to bring eIF3c-N binding to half of this level, indicating that the \(K_D\) for eIF2β-N-eIF5-B6 to bind eIF3c-N is about 10 nM. This value was significantly smaller than the \(K_D\) of 1.5–3 \(\mu\)M for eIF2β-N alone to bind eIF3c-N (Figs. 1B and 5B), suggesting a specific effect of the eIF2β-N segment on eIF5-CTD binding to eIF3c-N.

In sharp contrast, Fig. 5E indicates that the GB-eIF5-B6 bound to GST-eIF3c-N was only able to retain \([35S]eIF2β-N\) at a \(K_D\) comparable to that for GB-eIF5-B6 alone to bind \([35S]eIF2β-N\) (\(K_D = 0.5–1 \mu\)M). Therefore, eIF3c-N does not enhance eIF5-CTD binding to eIF2β-N. The contrasting results shown in Fig. 5, D–F, strongly suggest that the association of the eIF5-B6 segment with GST-eIF2β-N, but not with GST-eIF3c-N, strongly enhances its affinity to the third component in the heterotrimeric eIF2β-eIF5-eIF3 complex. The results also argue against the possibility that our assay is strongly biased toward measuring dissociation rate due to the washing step of the assay; because we started the washing step with the same trimeric complex in Fig. 5, D–F, and its dissociation rate should be the same between the two experiments, the great difference in the pull-down of \(35S\)-labeled partner would most likely arise from the difference in association rates. Finally, to rule out the possibility that the observed activation was due to suboptimal folding of the eIF5-B6 deletion construct, we tested the affinity of GST-eIF2β-N-full-length eIF5 complex to bind [35S]eIF3c-N. As shown in Fig. 6, we confirmed that the \(K_D\) for this interaction was \(-10\) nM. In conclusion, we propose that the specific enhancement of eIF5 binding to eIF3 by the lysine-rich eIF2β segment at least partially accounts for the critical function of eIF5-CTD mediated by the eIF2β/eIF5 interaction as observed in vivo earlier in this study.

A Novel eIF5-CTD Mutation, Defective Only in eIF2 Binding in Vitro, Reduces Its Binding to eIF3 in Vivo—To further study the role of eIF2/eIF5 interaction, we focused on a new eIF5-CTD mutation, isolated by Ts mutant screening, that was found to abolish its binding to eIF2β, but not to eIF3c, in vitro. This mutation designated W391Δ removes the carboxyl-terminal 15 amino acids of eIF5, including the last five of AA-box 2 amino acids (see Fig. 7A). Consistent with the binding defect with isolated subunits, Fig. 5B indicates that W391Δ reduced the GST-eIF5 binding to eIF2 by ~5-fold (panel a, lanes 3 and 4) but did not reduce the binding to eIF3 (panel b, lanes 3 and 4). In contrast, tif5-7A reduced both the interactions with eIF2 and eIF3 by ~10- and ~3-fold (Fig. 7B, lane 3 versus lane 5), respectively, consistent with previous finding (13). Note that both eIF2 and eIF3 used in these assays were present in WCEs isolated from yeast overexpressing all the corresponding subunits, hence considered to be native (see Fig. 7 legend). Therefore, the W391Δ mutation provided us with an opportunity to examine in vivo interaction between eIF3 and eIF5 in the absence of the eIF2/eIF5 linkage.

As shown in Fig. 7C, coimmunoprecipitation studies using strains carrying HA-eIF3 and tif5-7A or W391Δ eIF5 mutant indicated that the amount of eIF5-W391Δ immunoprecipitated with HA-eIF3 is 3 times smaller than that of wild-type eIF5 associated with HA-eIF3 (compare lanes 5 and 11 in the fourth panel; quantitation is shown in Fig. 7D). Consistent with the defect in eIF2 binding as observed in vitro, eIF5-W391Δ did not bridge HA-eIF3 and eIF2 (Fig. 7C, lanes 5 and 11 in the bottom panel) nor did it bind FLAG-eIF2 in other coimmunoprecipitation studies using strains carrying FLAG-eIF2 and eIF5 mutants (Fig. 7E, lanes 5 and 11). Thus, the ability of eIF5 to bind eIF2 appears to be critical for achieving the wild-type level of eIF5 binding to eIF3 in vivo, although we cannot exclude the possibility that W391Δ in itself has a greater impact on direct eIF3 binding in vivo than in vitro. Under these conditions, little or no eIF5-7A was found to be precipitated with HA-eIF3 and FLAG-eIF2, confirming a more severe impact caused by its

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defect in binding to both eIF2 and eIF3 (Fig. 7, C and E, lanes 8). These results provide further evidence that the eIF2/eIF5 linkage is critical for cooperative MFC formation.

**DISCUSSION**

Substantial evidence from studies on yeast factors supports the idea that the constituents of MFC comprising eIF1, eIF3, eIF5, and the eIF2 TC bind to the 40 S ribosome as a preformed unit (2, 6, 9). While evidence suggests that the integrity of MFC on the 40 S subunit is critical for postassembly processes including scanning (3, 22), the physiological significance of MFC formation in 43 S complex assembly has not been clear. In this study, we showed that eIF1 and the segments of eIF2β (amino acids 1–140), eIF3c (amino acids 1–156), and eIF5 (amino acids 241–405) can form a nearly stoichiometric quaternary complex (Fig. 1, C and D). These results not only extend the previous observations that stable ternary eIF1–eIF2β–eIF3c, eIF1–eIF2β–eIF5, and eIF2β–eIF3c–eIF5 complexes can be formed from the same fragments (2, 6) but also suggest that these parts of MFC alone have a potential to initiate the entire MFC assembly. To delineate the mechanism of MFC assembly, we advanced the characterization of the tsf5-7A mutant altering AA-box 2 at the eIF5 carboxyl terminus (13). We found that overexpression of either TC or eIF3 increased the mutant eIF5 binding to both eIF2 and eIF3 (Fig. 7, C and E, lanes 8). This limited TC binding likely resulted from dsd-PAGE followed by immunoblotting with antibodies against factors indicated to the right. GST fusion proteins were visualized by Ponceau S staining (top panel). Lane 1, 10% input amount; lane 2, GST alone (C); lane 3, GST–eIF5 (wild type (WT)); lane 4, GST–eIF5–W391Δ (Δ); lane 5, GST–eIF5–7A (ΔA). *, carboxyl-terminal deletion of GST–eIF5 due to proteolytic cleavage in bacteria. C, immunoprecipitation of HA–eIF5 using KAY37 (Control), KAY50 (TIF5), KAY51 (tsf5–7A), and KAY123 (W391Δ). The results are presented exactly as in Fig. 3A, panel b, and B, D, the level of eIF5 binding to HA–eIF3 was compared with the input amount and averaged from three independent experiments with error bars representing S.D. C, control; WT, wild type TIF5. E, immunoprecipitation of FLAG–eIF2 using KAY17 (Control), KAY127 (TIF5), KAY138 (tsf5–7A), and KAY284 (W391Δ). The results are presented exactly as in Fig. 3A, panel a.  

**Fig. 7.** Effect of tsf5–W391Δ, a new eIF5 mutation, on MFC partner binding in vitro and in vivo. A, yeast eIF5 amino acids from positions 339–400 are arranged to show secondary structures predicted from the eIF2B–CTD structure (17). Those predicted to participate in α-helices are boxed with the helix number in Roman numeral. Dotted arrows denote van der Waals contacts predicted for the highly conserved tyrosine residue. Thick arrows indicate tsf5–7A mutation sites (gray arrows) or the region deleted by tsf5–W391Δ (solid arrows). Highlighted with gray squares are AA-box amino acids (AA box 1 in helices 6 and 7; AA-box 2 in helix 8). Conserved acidic residues are typed in bold. The dotted line indicates a possible K-box interface. B, effect on in vitro binding to eIF2 and eIF3. 5 μg of GST fusion constructs indicated across the top were attached to glutathione resin and allowed to bind eIF2 or eIF3 present in 100 μg of WCEs prepared from KAY51 (tsf5–FL–7A) transformants carrying p1780–IMT (panel a) or YeplU–eIF3all (panel b) as described previously (13). After extensive washing of the resin, the complexes were analyzed by SDS-PAGE followed by immunoblotting with antibodies against factors indicated to the right. GST fusion proteins were visualized by Ponceau S staining (top panel). Lane 1, 10% input amount; lane 2, GST alone (C); lane 3, GST–eIF5 (wild type (WT)); lane 4, GST–eIF5–W391Δ (Δ); lane 5, GST–eIF5–7A (ΔA). *, carboxyl-terminal deletion of GST–eIF5 due to proteolytic cleavage in bacteria. C, immunoprecipitation of HA–eIF3 using KAY37 (Control), KAY50 (TIF5), KAY51 (tsf5–7A), and KAY123 (W391Δ). The results are presented exactly as in Fig. 3A, panel b, and B, D, the level of eIF5 binding to HA–eIF3 was compared with the input amount and averaged from three independent experiments with error bars representing S.D. C, control; WT, wild type TIF5. E, immunoprecipitation of FLAG–eIF2 using KAY17 (Control), KAY127 (TIF5), KAY138 (tsf5–7A), and KAY284 (W391Δ). The results are presented exactly as in Fig. 3A, panel a.
and the 40S subunit (23) but also forms a ternary complex with eIF2β and eIF3c amino termini (6), we suggest that the defective interactions of eIF5-7A with the partners in the MFC might allow eIF1 to uncoordinatedly bind its partners when eIF1 is present in excess, resulting in formation of nonfunctional translation initiation intermediates. Understanding the mechanism of specific and efficient formation of functional multiprotein complexes from the entire proteome (by avoiding nonspecific complexes) is an important question under extensive investigations (24–26). We believe that our results provide us with a clue to understanding how eIF5-CTD coordinates the assembly of one of the major cellular multiprotein complexes. It could be proposed that the primary function of eIF5-CTD is to serve as an assembly guide by rapidly promoting stoichiometric MFC formation with the aid of eIF2 while excluding formation of nonfunctional complexes.

Our in vitro binding assays, shown in Figs. 5 and 6, suggest that the specific requirement for the eIF2/eIF5 linkage in the MFC assembly is attributable to the enhanced affinity of eIF5-CTD for eIF3c on its binding to the lysine-rich eIF2β segment. This point was substantiated further by our characterization of a new eIF5 mutant designated W391A that can bind to eIF3 but not to eIF2 in vitro (Figs. 5A and 7A). We found that the binding of mutant eIF5 to eIF3 was significantly reduced compared with the wild-type binding level in vivo (Fig. 7C), supporting (although not proving) the model that eIF2 association enhances eIF5 binding to eIF3.

Based on the data presented here, we propose two hypothetical pathways for the MFC assembly in which eIF2 TC first binds eIF5 for its assembly activation as shown in Fig. 8. The activated eIF5 then binds either eIF2/eIF1 complex (A) or eIF3 alone (B). In the latter case, the trimeric eIF3/eIF5/TC complex would provide high affinity binding site for eIF2. Although eIF1 can bind to the 40 S subunit at a high affinity (Kd = 16 nM) (23), we propose that the strong enhancement of eIF5 binding to eIF3 by the eIF2β segment (Figs. 5–7) and the mutual cooperativity of eIF1 interactions with each of the other MFC segments (2, 6) would make the MFC one of the major pathways for eIF1 recruitment to the 40 S subunit. The direct eIF1 binding to the 40 S subunit might only be possible in the absence of eIF5-CTD function as suggested by overloading of eIF1 to the 40 S subunit observed with h eIF1 tis7-7A cells (Fig. 4A, panel c). Furthermore recent studies suggest that eIF5 (9) and eIF1 (6) might substantially enhance MFC binding to the 40 S subunit, while eIF1A can stimulate eIF1 alone to bind the 40 S subunit (23).

Finally an attractive model to explain the rapid release of MFC constituents on AUG recognition would be to propose that the critical eIF5-CTD/eIF2β-NTD linkage is somehow displaced on or prior to AUG recognition, thereby decreasing the affinity of eIF5-CTD against eIF3. We previously showed that the eIF5/eIF2β interaction is exclusive with the eIF5/eIF4G interaction in vitro (3), and this eIF5/eIF4G interaction was recently confirmed in vivo (11). Therefore, the eIF5/eIF2β linkage might indeed be displaced by the time eIF5 binds eIF4G in the 48 S complex, thereby mobilizing the factor linkage prior to AUG recognition. The weakened factor linkage by eIF5-CTD “inactivation” would certainly favor rapid factor dissociation on AUG pairing. According to this model, eIF5-CTD could be proposed to function as a molecular switch regulated by binding to the lysine-rich segment of eIF2β. Thus, we believe that further characterization of interactions involving eIF5-CTD is important for understanding the mechanism of coordinated assembly of the ribosome preinitiation complex and its rapid dissociation on precise recognition of the start codon.
Role of eIF5-CTD in Cooperative MFC Assembly

Physical Association of Eukaryotic Initiation Factor (eIF) 5 Carboxyl-terminal Domain with the Lysine-rich eIF2 β Segment Strongly Enhances Its Binding to eIF3
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