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Tandem Affinity Purification Revealed the Hypusine-dependent Binding of Eukaryotic Initiation Factor 5A to the Translating 80S Ribosomal Complex

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Abstract Eukaryotic initiation factor 5A (eIF5A) is the only protein in nature that contains hypusine, an unusual amino acid formed post-translationally in two steps by deoxyhypusine synthase and deoxyhypusine hydroxylase. Genes encoding eIF5A or deoxyhypusine synthase are essential for cell survival and proliferation. To determine the physiological function of eIF5A, we have employed the tandem affinity purification (TAP) method and mass spectrometry to search for and identify the potential eIF5A-interacting proteins. The TAP-tag was fused in-frame to chromosomal *TIF51A* gene and eIF5A-TAP fusion protein expressed at its natural level was used as the bait to fish out its interacting partners. At salt concentrations of 150 mM, deoxyhypusine synthase was the only protein bound to eIF5A. As salt concentrations were lowered to 125 mM or less, eIF5A interacted with a set of proteins, which were identified as the components of the 80S ribosome complex. The eIF5A–ribosome interaction was sensitive to RNase and EDTA treatments, indicating the requirement of RNA and the joining of 40S and 60S ribosomal subunits for the interaction. Importantly, a single mutation of hypusine to arginine completely abolished the eIF5A–ribosome interaction. Sucrose gradient sedimentation analysis of log versus stationary phase cells and eIF3 mutant strain showed that the endogenous eIF5A co-sedimented with the actively translating 80S ribosomes and polyribosomes in an RNase- and EDTA-sensitive manner. Our study demonstrates for the first time that eIF5A interacts in a hypusine-dependent manner with a molecular complex rather than a single protein, suggesting that the essential function of eIF5A is mostly likely mediated through its interaction with the actively translating ribosomes. J. Cell. Biochem. 97: 583–598, 2006.

Key words: eIF5A; hypusine; tandem affinity purification (TAP); 80S ribosome; binding partners

Eukaryotic initiation factor 5A (eIF5A[1]), the only protein in nature that contains hypusine, is evolutionarily conserved from yeast to human. Its homolog aIF5A exists in archaea, but not in eubacteria. Hypusine on eIF5A is formed in two consecutive steps through the action of deoxyhypusine synthase and deoxyhypusine hydroxylase [reviewed by Park et al., 1993, 1997; Chen and Liu, 1997; Chen and Jao, 1999]. Disruption of either eIF5A or deoxyhypusine synthase gene leads to lethality in yeast [Schnier et al., 1991; Sasaki et al., 1996]. Inhibition of deoxyhypusine synthase activity in mammalian cells causes growth arrest [Jakus et al., 1993; Park et al., 1994], cell death [Tome et al., 1997], or tumor differentiation [Chen et al., 1996]. In addition, hypusine formation activity is much higher in virally transformed cells than in their normal counterparts [Chen and Chen, 1997a], and shows a striking attenuation in senescent cells [Chen and Chen, 1997b].

Although eIF5A was considered as an initiation factor based on its in vitro activity in stimulating methionyl-puromycin synthesis [Benne et al., 1978], this notion has been questioned due to the lack of correlation between

Abbreviations used: eIF5A, eukaryotic initiation factor 5A; TAP, Tandem Affinity Purification; CBP, calmodulin binding peptide; TEV, tobacco etch virus; RRE, Rev response element; TCA, trichloroacetic acid; DTT, dithiothreitol; PCR, polymerase chain reaction.

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eIF5A and the general protein synthesis [Kang and Hershey, 1994; Zuk and Jacobson, 1998]. eIF5A has also been proposed to be a nuclear transport adaptor for Rev or Rex [Ruhl et al., 1993; Katahira et al., 1995]. However, direct interaction between Rev and eIF5A cannot be demonstrated [Henderson and Percipalle, 1997; Mattaj and Englmeier, 1998; Lipowsky et al., 2000]. Moreover, the absence of any dynamic nucleocytoplasmic shuttling activity of eIF5A in an interspecies heterokaryon assay [Jao and Chen, 2002] has cast doubt on any role of eIF5A in nuclear transport. Based on the sequence comparison with other RNA binding proteins such as Rev and NS1, we have proposed that eIF5A may function as a bimodular protein capable of binding to both RNA and proteins [Liu et al., 1997]. The solved crystal structures of aIF5A show that it is composed of two compact β-sheet domains linked by a flexible hinge [Kim et al., 1998; Peat et al., 1998; Yao et al., 2003]. The N-terminal domain contains the hypusine site at the tip of a protruding loop and the C-terminal domain resembles the oligonucleotides-binding fold (OB fold) previously described for RNA binding proteins [Murzin, 1993] such as cold shock protein CspA [Schindelin et al., 1994] and prokaryotic translation initiation factor IF1 [Sette et al., 1997]. These structural features suggest that eIF5A is capable of binding to nucleic acids via hypusine residue or the OB fold in the C-terminal domain. Indeed, we have shown that eIF5A binds to the HIV-1 Rev response element (RRE), U6 RNA [Liu et al., 1997], and post-SELEX RNA [Xu and Chen, 2001]. We have also found that the binding of eIF5A and RNA in vitro depends on the presence of deoxyhypusine or hypusine [Liu et al., 1997; Xu and Chen, 2001].

In addition to RNA, it has been reported that eIF5A interacts with a number of cellular proteins including CRM1 [Rosorius et al., 1999], exportin 4 [Lipowsky et al., 2000], nucleoporins [Hofmann et al., 2001], deoxyhypusine synthase [Thompson et al., 2003], ribosomal protein L5 [Schatz et al., 1998], tissue transglutaminase II [Singh et al., 1998], Lia1 [Thompson et al., 2003], and syntenin [Li et al., 2004]. With the exception of exportin 4, hypusine does not appear to be required for eIF5A to interact with any of these proteins, making them less likely to shed light on the essential functions of eIF5A. Exportin 4, together with CRM1 and nucleoporins, are proteins involved in the nucleocytoplasmic trafficking. Since archaea aIF5A is essential for survival and archaea do not have nuclei, the role of eIF5A in the nucleocytoplasmic trafficking, if any, is unlikely to be its conserved essential function.

Tandem affinity purification (TAP) employs a cassette, consisting of a calmodulin-binding peptide, a tobacco etch virus (TEV) protease cleavage domain, and two IgG-binding domains of *Staphylococcus aureus* protein A, as a dual affinity tag for isolating interacting proteins or multi-component protein complex [Rigaut et al., 1999]. The combination of TAP with mass spectrometry permits the recovery of complexes present at levels as low as 15 copies per cell [Puig et al., 2001]. Here we report the use of TAP procedure to identify the 80S ribosome complex as the eIF5A-binding partner. We show that the eIF5A–ribosome interaction requires the hypusine modification and is mediated through RNA. Importantly, eIF5A prefers to bind to the actively translating ribosome, suggesting that eIF5A has a role in translation but not at the initiation stage.

MATERIALS AND METHODS

Yeast Strains and Plasmids

The wild-type haploid strain used in all experiments is KCY307 (*MATα ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1*). Yeast strains were transformed with the lithium acetate-mediated method [Soni et al., 1993]. To tag the *TIF51A* gene with the TAP tag at its 3′ end on the chromosome in KCY307, a PCR-based genomic tagging technique in yeast [Puig et al., 1998; Rigaut et al., 1999] was used. Integration was confirmed by both PCR and Western blot analysis. The resulting strain expressing the eIF5A-TAP fusion protein is KCY195.

To generate strains expressing the plasmid-borne eIF5A-TAP fusions proteins, the genomic DNA isolated from KCY195 was served as the template for PCR using high-fidelity *pfu*Turbo® DNA polymerase (Stratagene, La Jolla, CA). The resulting PCR product, covering the region from 1 kilobase upstream of the *TIF51A* coding sequence to the end of the TAP tag, was cloned into the centromeric plasmid pRS414 to yield pRS51ATAP. To generate a construct expressing the hypusine mutant form of eIF5A-TAP, the lysine residue at codon 51 of *TIF51A* gene in pRS51ATAP was mutated to arginine using

Transformer™ Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA) according to manufacturer's instructions, resulting in pRS51A-TAP(K51R). Sequences of both pRS51ATAP and pRS51ATAP(K51R) were confirmed by DNA sequencing from both directions (service performed by UMDNJ Core Facility, Piscataway, NJ). To express both wild-type and K51R mutant forms of eIF5A-TAP proteins under the same genetic background, the wild-type KCY307 cells were transformed with either pRS51ATAP or pRS51ATAP(K51R), giving strains KCY210 and KCY211, expressing the wild-type and K51R mutant forms of eIF5A-TAP, respectively. A strain carrying the pRS414 vector alone (i.e., KCY209) was also established by transforming KCY307 with pRS414.

The yeast strain harboring the *prt1-1* mutant allele used in the study is TP11B-4-1 (*MAT*a *ade1 leu2-3 leu2-112 ura3-52 prt1-1*), originally derived from strain CY2522 [Zhong and Arndt, 1993].

TAP Purification

The purification procedure was based on the previous report with modifications [Rigaut et al., 1999]: Yeast cells were grown at 30°C in 2–4 L of YPD (1% yeast extract, 2% peptone, and 2% glucose) medium (in the case with KCY195) or SD-Trp medium (in the case with KCY210 and KCY211) to mid- to late-log phase and harvested by centrifugation at 4,000 rpm (Beckman JA-14 rotor) for 5 min at 4°C. Cell pellets were resuspended in lysis buffer (20 mM HEPES-KOH at pH 7.9, 10 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol (DTT), and Complete™ protease inhibitors from Roche, Basel, Switzerland). An equal volume of glass beads were added and cells were lysed by 10 cycles of 15-s burst and 2-min rest in a bead-beater. The KCl concentration was adjusted to 150, 125, 100, 75, or 50 mM depending on different experiments. The salt-adjusted homogenate was clarified by centrifugation at 6,000*g* (Beckman JA-17 rotor) for 20 min at 4°C and then added with Tris-HCl to 10 mM at pH 8.0 and NP-40 to 0.1%. 100 μl (bed volume) of IgG beads (Sigma, St. Louis, MO) were then added into the lysate. The solution was agitated for 2 h at 4°C. After incubation, the whole solution was loaded into a 10-ml Econocolumn (Bio-Rad, Hercules, CA) and the beads were washed with 30 ml of IPP buffer (10 mM Tris-HCl at pH 8.0, 0.1% NP-40, and 150, 125, 100, 75, or 50 mM NaCl) followed by 10 ml of TEV cleavage buffer (IPP buffer containing 0.5 mM EDTA and 1 mM DTT). Beads were resuspended in 0.5 ml of TEV cleavage buffer containing 5 μl of TEV protease (about 5 U, Invitrogen, Carlsbad, CA) and agitated for 2 h at 16°C. The eluate was recovered and mixed with 2 ml of CBB buffer (IPP buffer containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM imidazole, and 10 mM β-mercaptoethanol) and 2 μl of 1 M $CaCl_2$ to titrate the EDTA present in the eluate. This solution was agitated with 100 μl (bed volume) of calmodulin beads (Amersham Biosciences, Piscataway, NJ) for 2 h at 4°C. After incubation, the calmodulin beads were washed with 30 ml of CBB buffer. The bound proteins were eluted with 1.5 ml of CEB buffer (IPP buffer containing 3 mM EGTA and 10 mM β-mercaptoethanol). Proteins in the final eluate were trichloroacetic acid (TCA)-precipitated and separated by 4%–16% SDS–PAGE, followed by silver or Coomassie Blue staining.

Protein Identification by Mass Spectrometry

All the mass spectrometry analyses were performed at the Center for Advanced Proteomics Research (CAPR) at UMDNJ-New Jersey Medical School, Newark, New Jersey. The desired protein bands were excised, destained, dehydrated, and then in-gel digested with trypsin at 37°C overnight. After digestion, the peptides were extracted from the gel slices by vortexing with 60% acetonitrile, 5% formic acid, and desalted using C_{18}ZipTips (Millipore, Billerica, MA) according to the manufacturer's instruction. An 1-μl aliquot of samples was taken for matrix-assisted laser desorption ionization (MALDI) using α-cyano-4-hydroxycinnamic acid as the matrix and the time-of-flight (TOF) spectrum was produced. All MALDI-TOF mass analyses were performed on the Applied Biosystems 4700 MALDI-TOF mass spectrometer in the linear delayed-extraction mode with external calibration. The analysis of mass data and identification of proteins were performed through the ProFound website located at Rockefeller University (http://prowl.rockefeller.edu).

In Vivo [^{14}C]Spermidine Incorporation and Autoradiography

Yeast cells were grown at 30°C in 4 ml of SD-Trp medium in the presence of 0.2 μCi/ml of [^{14}C]spermidine (specific activity 4.14 GBq/mmol,112 mCi/mmol, Amersham Biosciences)

to late log phase. The cells were harvested and lysed as described in the TAP purification. About 20 μg of total proteins were separated by 15% SDS–PAGE. The gel was dried and exposed to X-ray film for 6 days at −70°C.

Sucrose Gradient Sedimentation

For each sucrose gradient tube, typically 100 ml of yeast cultures was grown at 30°C to log or stationary phase, depending on experimental designs. For experiments with polyribosomes to be preserved, 15 min before harvesting yeast cultures, 100 μg/ml of cycloheximide was added into the medium to stop translation and to preserve polyribosomes, and the same concentration of cycloheximide was present in all the steps thereafter. Cells were harvested by rapidly pouring into crashed ice and centrifuged at 4,000 rpm (Beckman JA-14 rotor) for 5 min at 4°C. The cell pellet was resuspended in 1 ml of CSB buffer (20 mM HEPES at pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 300 mM sorbitol, 1 mM DTT, and a panel of protease inhibitors). An equal volume of glass beads was added and cells were lysed by vigorously vortexing at room temperature for 20 s and resting on ice for 40 s. The vortexing-resting cycle was repeated 10 times. The homogenate was then centrifuged at 6,000*g* for 20 min at 4°C. Aliquots of 20–25 OD_{260} units or same amounts of proteins of the clarified supernatants were gently loaded on 11 ml of 7%–47% sucrose gradients prepared in 50 mM Tris-HCl at pH 8.0, 10 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM DTT and subjected to centrifugation at 38,000 rpm (Beckman SW40Ti rotor) for 5 h at 4°C. Gradients were fractionated with an ISCO UA-5 absorbance monitor set at 254 nm, and 0.65-ml fractions were collected. To examine the distribution of eIF5A and ribosomal protein L3 in the gradients, total proteins from each sucrose gradient fraction were TCA-precipitated, separated by SDS–PAGE, and subjected to Western blot analysis. To examine the distribution of ribosomal RNA in the gradients, total RNA was isolated from each sucrose gradient fraction. The RNA was electrophoresed in an 1.2% formaldehyde-agarose gel (1.2% agarose and 2% formaldehyde in 20 mM MOPS at pH 7.0, 5 mM sodium acetate, and 1 mM EDTA).

Western Blot Analysis

The procedure was carried out as described previously with appropriate antibodies [Jao and Chen, 2002]. To examine the distribution of eIF5A and ribosomal protein L3 in sucrose gradients, anti-eIF5A polyclonal antibody (1:1,600 dilution, kindly provided by John W. B. Hershey, University of California, Davis) and anti-Tcm1 monoclonal antibody [Vilardell and Warner, 1997] (1:5,000 dilution, kindly provided by Jonathan R. Warner, Albert Einstein College of Medicine) were used, respectively.

RESULTS

Dys1p Co-Purifies with eIF5A Under 150 mM Salt Concentrations

To purify proteins associated with the yeast eIF5A, one of the yeast eIF5A genes, *TIF51A*, was fused with the TAP tag at its 3′-end by integrating a DNA cassette into the genome of a haploid cell strain [Puig et al., 1998]. The TAP tag consists of two IgG-binding domains of *Staphylococcus aureus* protein A and a calmodulin binding peptide (CBP) separated by a TEV protease cleavage site. The 3′-end tagging strategy ensures that the fusion protein is expressed at its natural level under the control of *TIF51A* promoter. The eIF5A-TAP fusion protein along with its binding partners were purified from the cell extracts by sequential IgG and calmodulin affinity columns [Rigaut et al., 1999]. Figure 1A shows a typical SDS–PAGE profile of proteins eluted after the two columns when the TAP procedure was performed with the salt concentrations maintained at 150 mM at all steps. The three proteins co-purified with eIF5A-TAP were excised from the gel and subjected to MALDI-TOF peptide mapping. The protein migrating with an apparent molecular weight of 48 kDa was identified as Dys1p (i.e., deoxyhypusine synthase), the enzyme that modifies eIF5A in the first step of the biosynthesis of hypusine. The other two proteins with the apparent molecular weights around 66 and 116 kDa turned out to be the dimer and tetramer of eIF5A-CBP, respectively.

Ribosome Complexes Specifically Bind to eIF5A Under Low Salt Conditions

Since deoxyhypusine synthase was the only protein co-purified with eIF5A-TAP at the salt concentrations of 150 mM, we further examined whether other specific interactions might exist at lower salt concentrations. As shown in Figure 1B, as the salt concentrations were lowered below 125 mM, we observed the

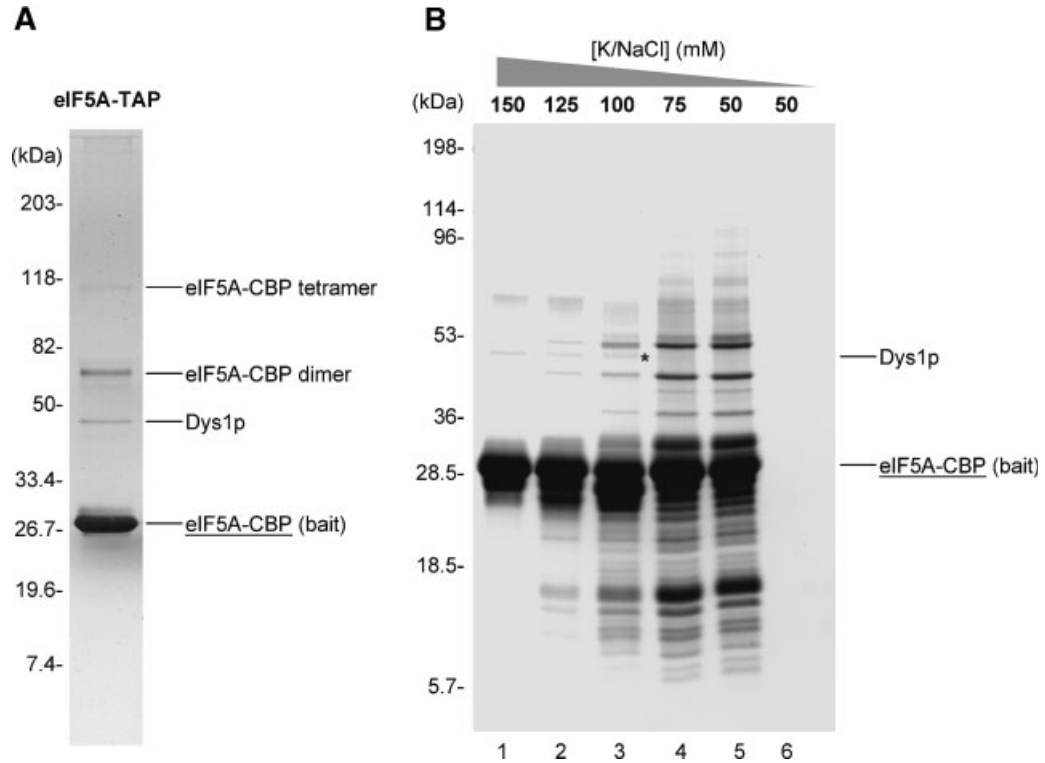

Fig. 1. Tandem affinity purification of eIF5A-associated proteins under different salt concentrations. **A**: The TAP procedure using strain KCY195 was performed under 150 mM salt concentrations. **B**: Five parallel TAP procedures using strain KCY195 were performed under different salt concentrations (i.e., 150, 125, 100, 75, and 50 mM; lanes 1–5). Another TAP procedure using the untagged wild-type strain KCY307 was also performed under 50 mM salt concentrations (lane 6). Fractions eluted from the calmodulin affinity resin were analyzed on 4%–16% SDS–PAGE followed by Coomassie Blue staining. Note that a set of proteins was co-purified with eIF5A-CBP as the salt concentrations were lowered below 125 mM in the TAP procedure. The eIF5A-CBP (bait) was underlined.

appearance of a set of proteins co-purified with eIF5A-CBP. The interaction between these proteins and eIF5A-CBP appeared to be specific because no protein was recovered if the untagged wild-type strain was used in a parallel TAP procedure (Fig. 1B, lane 6). Given the history of possible roles of eIF5A in translation, we suspected that the ribosome might be the complex co-purified with eIF5A-TAP. To assess this possibility, we isolated the ribosome fraction from yeast extracts and compared its protein composition with that of the eluate from the TAP procedure. As shown in Figure 2A, the compositions of proteins from these two sources were quite similar. To further determine the identities of the proteins co-purified with eIF5A-TAP, we excised all the major protein bands from the gel for MALDI-TOF peptide mapping analysis. The results of the MALDI-TOF analysis are detailed in Table I. The protein bands that were identified are indicated in Figure 2B. Altogether, 14 of 19 proteins are ribosomal proteins from either large or small ribosomal subunits, and they are Rpl3, Rpl4a, Rpl4b, Rpp0, Rps4a, Rpl2a, Rpl8a, Rpl8b, Rps1b, Rps14b, Rpl6a, Rpl17b, Rps19b, and Rps22a. The other proteins identified include elongation factor 1A (eEF1A) and two chaperone proteins, zuotin and ssb2p, both present in the nascent peptide chain complex [Gautschi et al., 2002; Hundley et al., 2002]. In addition, two high-molecular-weight proteins (i.e., Clu1/TIF31 and YPL207W) with unknown functions were also identified. Together, these results suggest that the binding partner of eIF5A-TAP is in the form of a large complex, consisting of the 80S ribosome and its associated proteins.

The eIF5A–ribosome Interaction Requires RNA and Intact 80S Ribosomes

Given that the all three major RNA species (rRNA, mRNA, and tRNA) converge at the ribosome and that eIF5A is capable of binding to synthetic RNA in vitro [Xu and Chen, 2001],

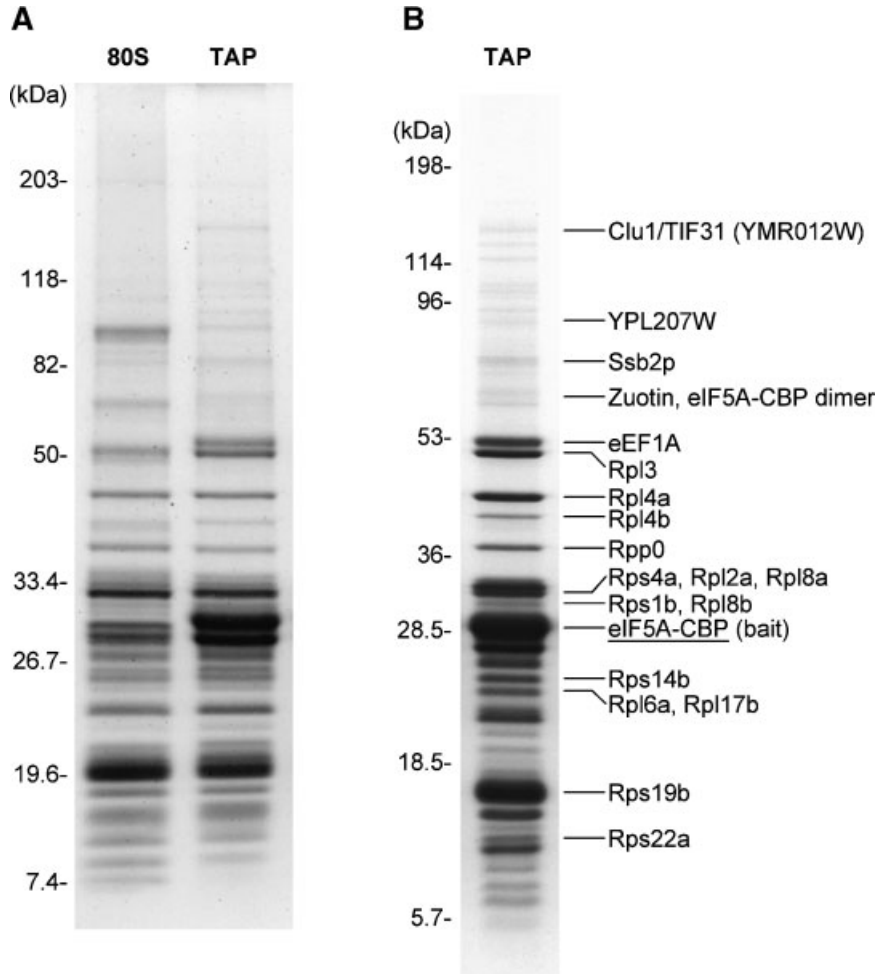

Fig. 2. Identification of the 80S ribosome and its associated proteins as the complex associated with eIF5A. **A**: The purified ribosome fraction (80S) and the fraction eluted from the calmodulin affinity resin of the TAP procedure using strain KCY195 under 50 mM salt concentrations (TAP) were analyzed on 4%–16% SDS–PAGE followed by Coomassie Blue staining. Note that the protein compositions from these two fractions are very similar. **B:** Mass spectrometry analysis of the proteins associated with eIF5A after the TAP procedure under 50 mM salt concentrations. Identified proteins were indicated on the right of the gel. The eIF5A-CBP (bait) was underlined. Note that a vast majority of those eIF5A-associated proteins are ribosomal proteins.

we asked whether RNA plays any role in this interaction. To address this question, we included RNase A in the IgG binding step of the TAP procedure to disrupt RNA and then determined whether the binding still exists. As shown in Figure 3, the amount of proteins co-purified with eIF5A from the RNase-treated sample was reduced by more than 90% as compared to the control (Fig. 3A, lane 1 vs. lane 3). The result suggests that RNA is directly involved in the eIF5A–ribosome interaction. Alternatively, it is possible that the loss of interaction is caused by the disruption of the global structure of the ribosome due to the degradation of rRNA. To determine whether this is the case, we performed the sucrose gradient sedimentation analysis. The sedimentation coefficient of the RNase-treated ribosomes remained to be 80S, same as that of the control (Fig. 3B, panel 2 vs. panel 1), suggesting that the RNase treatment did not significantly alter the size, shape, and composition of the ribosome. Consequently, certain RNA species is likely to mediate, either directly or indirectly, the interaction between eIF5A and the ribosome. Since RNase A targets at all three RNA species, we cannot tell which one is responsible for mediating the interaction. However, since yeast tRNA added in excess during the TAP procedure did not affect the binding of the 80S ribosome to eIF5A-TAP (data not shown), we concluded that tRNA is not directly involved the eIF5A–ribosome interaction.

TABLE I. Yeast eIF5A Co-purified Proteins Identified by Mass Spectrometry

Standard name/ (rp-name)[a]	ORF[b]	Length (amino acids)	Function
Dys1p[c]	YHR068W	387	Deoxyhypusine synthase, hypusine biosynthesis
Clu1	YMR012W	1,277	Unknown
YPL207W	YPL207W	810	Unknown
Ssb2p	YNL209W	613	ATPase activity; unfolded protein binding
Zuotin	YGR285C	433	Unfolded protein binding
eEF1A	YPR080W, YBR118W	458	Translation elongation
Rpl3/(L3)	YOR063W	387	Structural constituent of the 60S ribosomal subunit
Rpl4a/(L4A)	YBR031W	362	Structural constituent of the 60S ribosomal subunit
Rpl4b/(L4B)	YDR012W	362	Structural constituent of the 60S ribosomal subunit
Rpp0/(P0)	YLR340W	312	Structural constituent of the 60S ribosomal subunit
Rps4a/(S4A)	YJR145C	261	Structural constituent of the 40S ribosomal subunit
Rpl8a/(L8A)	YHL033C	256	Structural constituent of the 60S ribosomal subunit
Rpl8b/(L8B)	YLL045C	256	Structural constituent of the 60S ribosomal subunit
Rpl2a/(L2A)	YFR031C-A	254	Structural constituent of the 60S ribosomal subunit
Rps1b/(S1B)	YML063W	255	Structural constituent of the 40S ribosomal subunit
Rps14b/(S14B)	YJL191W	138	Structural constituent of the 40S ribosomal subunit
Rpl6a/(L6A)	YML073C	176	Structural constituent of the 60S ribosomal subunit
Rpl17b/(L17B)	YJL177W	184	Structural constituent of the 60S ribosomal subunit
Rps19b/(S19B)	YNL302C	144	Structural constituent of the 40S ribosomal subunit
Rps22a/(S22A)	YJL190C	130	Structural constituent of the 40S ribosomal subunit

[a]rp-name, ribosomal protein name.
[b]ORF, open reading frame.
[c]Co-purified with eIF5A at 150 mM salt concentrations; all other proteins were co-purified with eIF5A at salt concentrations lower than 150 mM.

To test whether eIF5A can also bind to either of the 40S or 60S ribosomal subunits, we studied the effect of ribosome dissociation on the binding pattern. EDTA was used in the IgG binding step to remove Mg^{2+}, an obligate factor to keep the 80S ribosome from dissociation [Ramirez et al., 1991]. As can be seen in Figure 3A (lane 2 vs. lane 3) there was a significant decrease in the binding intensity of the co-purified proteins under the EDTA-treated condition, suggesting that dissociation of the 80S ribosome into its component free 40S and 60S subunits reduced the eIF5A–ribosome interaction. Sucrose gradient sedimentation analysis confirmed that the EDTA treatment caused more than 50% of the 80S ribosomes to dissociate as indicated by the reduction of the 80S peak of the UV absorbance (Fig. 3B, panel 3 vs. panel 1). We therefore concluded that eIF5A binds only to the intact 80S ribosome, but not the 40S or 60S ribosomal subunit.

Endogenous eIF5A Co-sediments with Ribosomes

To further explore the physical interaction between eIF5A–TAP and the 80S ribosome detected by TAP procedure, we investigated whether endogenous eIF5A could co-sediment with the 80S ribosome and polyribosomes in the sucrose gradient and whether the co-sedimentation would be affected by any changes in the ribosomal integrity. To preserve the polyribosome profile, cycloheximide was added to "freeze" the translation elongation. Figure 4A shows that the distribution of ribosomal protein L3 and the rRNA species, 25S and 18S, were consistent with the UV absorbance profile, indicating a continuous distribution of various ribosomal species along the sucrose gradient. Under this condition, about 10%–12% of total cellular eIF5A proteins appeared in the 80S ribosome and polyribosome fractions (from fractions 11 to 17 of the gradient), consistent with the observation that eIF5A binds to the 80S ribosome under the TAP procedure.

To mimic the RNase treatment used in the TAP procedure, we treated the cell extract with RNase A before loading it onto the sucrose gradients. The results shown in Figure 4B showed that RNase A disrupted the polyribosomes into the 80S ribosomes, resulting in a large increase of the 80S peak in the UV profile. However, eIF5A was completely absent in the fractions corresponding to the 80S peak (i.e., from fractions 11 to 17), suggesting that eIF5A did not bind with the 80S ribosome, consistent with the results obtained from the TAP procedure (Fig. 3A, lane 1 vs. lane 3). Although the rRNAs in the RNase-treated ribosomes appeared to be partially degraded (Fig. 4B), the distribution of L3 and the sedimentation coefficient of the RNase-treated ribosomes, which remained to be at 80S, suggest that the

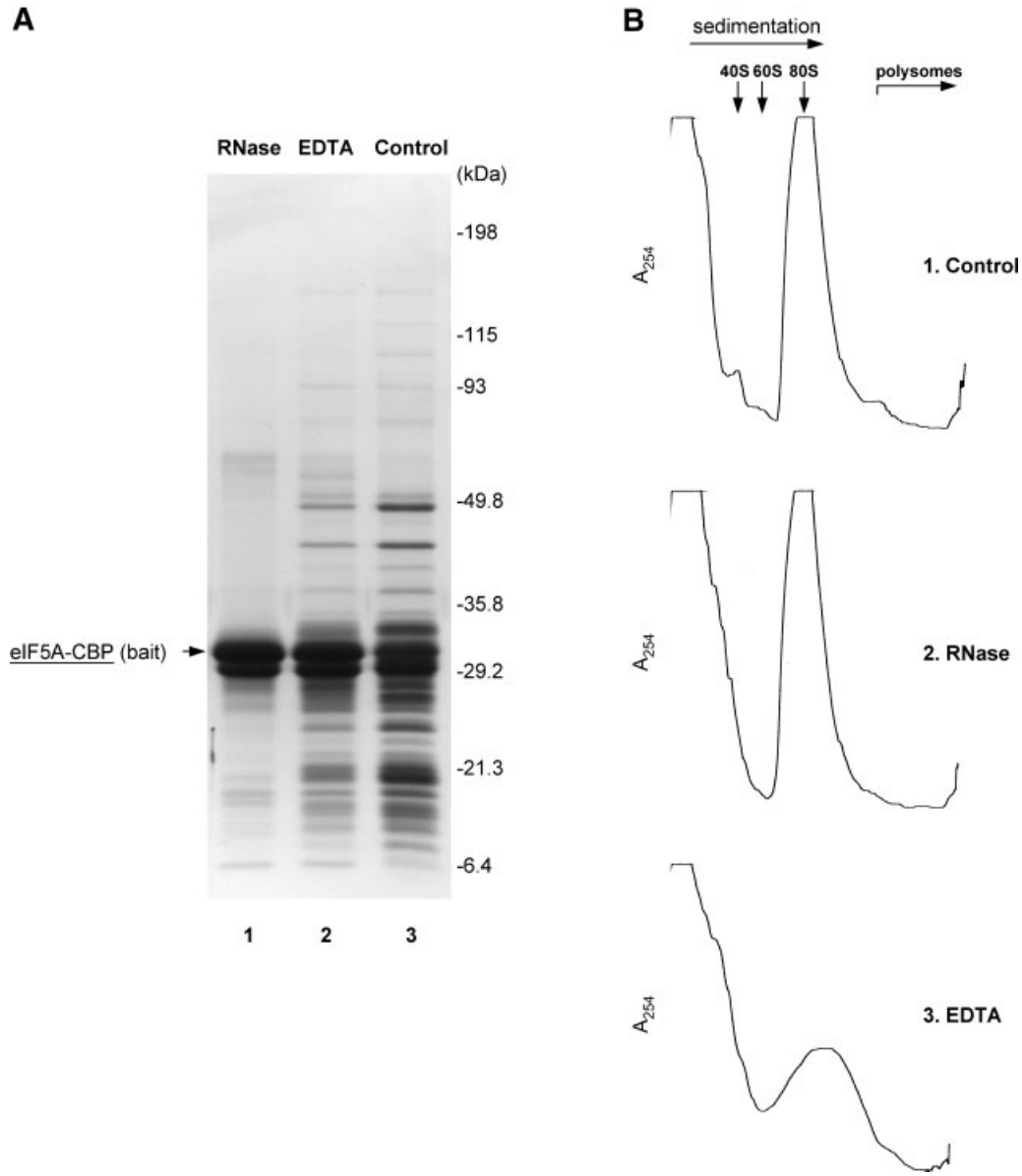

Fig. 3. eIF5A–ribosome interaction is sensitive to RNase and EDTA treatments. **A**: Cell extracts prepared from strain KCY195 were treated with 100 μg/ml of RNase A (lane 1), 40 mM EDTA (lane 2), or with no additional treatment (lane 3) in the IgG binding step of the TAP procedure under 50 mM salt concentrations. Fractions eluted from the calmodulin affinity resin were analyzed on 4%–16% SDS–PAGE followed by Coomassie Blue staining. The eIF5A-CBP (bait) was underlined. **B**: A portion of the flow-through extracts after the IgG binding step was layered over a continuous 7%–47% sucrose gradient and subjected to ultracentrifugation. Gradients were fractionated from the top of the tube while the UV absorbance at 254 nm was monitored to produce the absorbance profiles. (1) Control: The UV absorbance profile of the fractionated flow-through extract with no additional treatment. (2) RNase: The UV absorbance profile of the fractionated flow-through extract treated with 100 μg/ml of RNase A in the IgG binding step. (3) EDTA: The UV absorbance profile of the fractionated flow-through extract treated with 40 mM EDTA in the IgG binding step. Sedimentation was from left to right. The positions of free 40S and 60S subunits, 80S ribosomes, and polyribosomes were indicated. Note that no cycloheximide was added so that the polyribosomes were not preserved well. In (3), EDTA was only added in the IgG binding step, not in the sucrose gradients.

RNase treatment did not grossly change the global structure of the 80S ribosome.

We then examined whether the endogenous eIF5A would co-sediment with the dissociated ribosomal subunits upon depletion of Mg^{2+} [Zinker and Warner, 1976; Ramirez et al., 1991]. The distribution of L3 and the rRNAs confirmed the location of 60S and 40S subunits but showed that both of the subunits moved at position corresponding to 50S and 30S,

respectively (Fig. 4C vs. 4A), presumably due to the loss of certain ribosome-associated factors [Zinker and Warner, 1976]. Under the Mg^{2+}-free condition, eIF5A did not co-sediment with the 60S subunit, indicating a lack of binding between them. The apparent co-sedimentation of eIF5A with the 40S subunit in Figure 4C is most likely fortuitous because (i) we found that eIF5A alone was able to sediment to the same position (fractions 6 and 7) (data not shown) due to its propensity for self-polymerization as previously reported [Kemper et al., 1976; Chung et al., 1991; Lee et al., 1999] and (ii) as shown above, no binding protein was observed using the RNase-treated extract in the TAP experiment (Fig. 3A, lane 1), even though endogenous eIF5A could be detected in the 40S fractions (Fig. 4B). Together, the results from the TAP procedure and the sucrose gradient analysis indicate that eIF5A interacts specifically with the intact 80S ribosome, but not the individual ribosomal subunits, and that the interaction involves intact RNA species.

eIF5A Prefers to Bind the Translating Ribosomes

We next asked whether eIF5A has any binding preference toward different populations of 80S ribosomes. Specifically, does it preferentially bind to the free 80S ribosome monomers (i.e., monosomes), which are not engaged in active translation, or prefer to bind to the actively translating ribosomes and polyribosomes? Or does it bind to these two populations with equal affinity? To address these questions, we have examined the co-sedimentation profile of eIF5A in two experimental systems. We first compared the distribution of endogenous eIF5A in the sucrose gradient containing the extracts prepared from either the log or stationary phase cells. Since protein synthesis is greatly reduced in the stationary phase cells, the inactive 80S monosomes are expected to accumulate in the stationary phase cells but not in the log phase cells. This difference is clearly revealed by the UV profiles as shown in Figure 5 (upper panels, B vs. A). However, despite of the large increase in the 80S peak in Figure 5B, the amount of eIF5A found in the 80S fractions (fractions 12 and 13) did not show any proportional increase. On the contrary, less eIF5A was present with the 80S fractions in the stationary phase cells than that in the log phase cells (Fig. 5B vs. 5A, fractions 12 and 13). These results suggest that eIF5A has low affinity toward the 80S ribosomes that are not engaged in active translation.

The second system we used is a yeast mutant strain harboring *prt1-1*, a temperature-sensitive allele of the gene encoding a subunit of eIF3 [Foiani et al., 1991; Marton et al., 1997]. Extracts prepared from the mutant cells grown at the restrictive temperature show a large accumulation of the 80S monosomes [Foiani et al., 1991]. Most of the 80S monosomes that accumulate under these conditions are inactive because functional eIF3 is not there to prevent subunit association in the absence of mRNA [Foiani et al., 1991; Kainuma and Hershey, 2001]. We grew the *prt1-1* mutant cells either at 23°C or shifted the cells to the restrictive temperature (37°C) for 30 min before harvesting and prepared the cell extracts for sucrose gradient sedimentation analysis. The UV profiles show a significant difference in the size of the 80S peak between these two samples. The large increase in the 80S peak at the restrictive temperature (Fig. 6B) did not result in a proportional increase of the eIF5A protein in the 80S fractions (Fig. 6B vs. 6A, fractions 12 and 13), suggesting that eIF5A has low affinity toward inactive 80S ribosomes. We therefore concluded that yeast eIF5A prefers to bind to the 80S ribosomes engaged in active translation.

Hypusine Residue is Required for the Binding of eIF5A with Ribosomes

Since hypusine is essential for the biological activities of eIF5A [Schnier et al., 1991; Sasaki et al., 1996; Park et al., 1998], we wish to know whether hypusine is required for the eIF5A–ribosome interaction. Because of the essential nature of hypusine, we expressed the plasmid-borne hypusine-mutant eIF5A-TAP fusion protein, eIF5A-TAP (K51R), in a wild-type yeast strain. To ensure that the plasmid-borne eIF5A-TAP fusion will be expressed at its natural level, the promoter region (about 1 kilobase upstream of the *TIF51A* coding sequence), along with the coding regions of the *TIF51A* gene and TAP tag, was also included in the plasmid. Plasmids carrying the wild-type and the K51R mutant eIF5A-TAP construct were introduced into the wild-type strain (KCY307) to generate strains KCY210 and KCY211, respectively. To validate this "plasmid-borne TAP" approach, we first tested whether the plasmid-borne eIF5A-TAP fusion protein can serve as the effective substrate for hypusination in the presence of

the endogenous eIF5A. Figure 7 shows that the plasmid-borne wild-type eIF5A-TAP was labeled by the radioactive spermidine as equally well as the genomic eIF5A-TAP or endogenous eIF5A (Fig. 7, lane 3 vs. lanes 1 and 2), indicating that the plasmid-borne eIF5A-TAP did contain hypusine. As expected, the K51R mutant could not be labeled by [^{14}C]spermidine

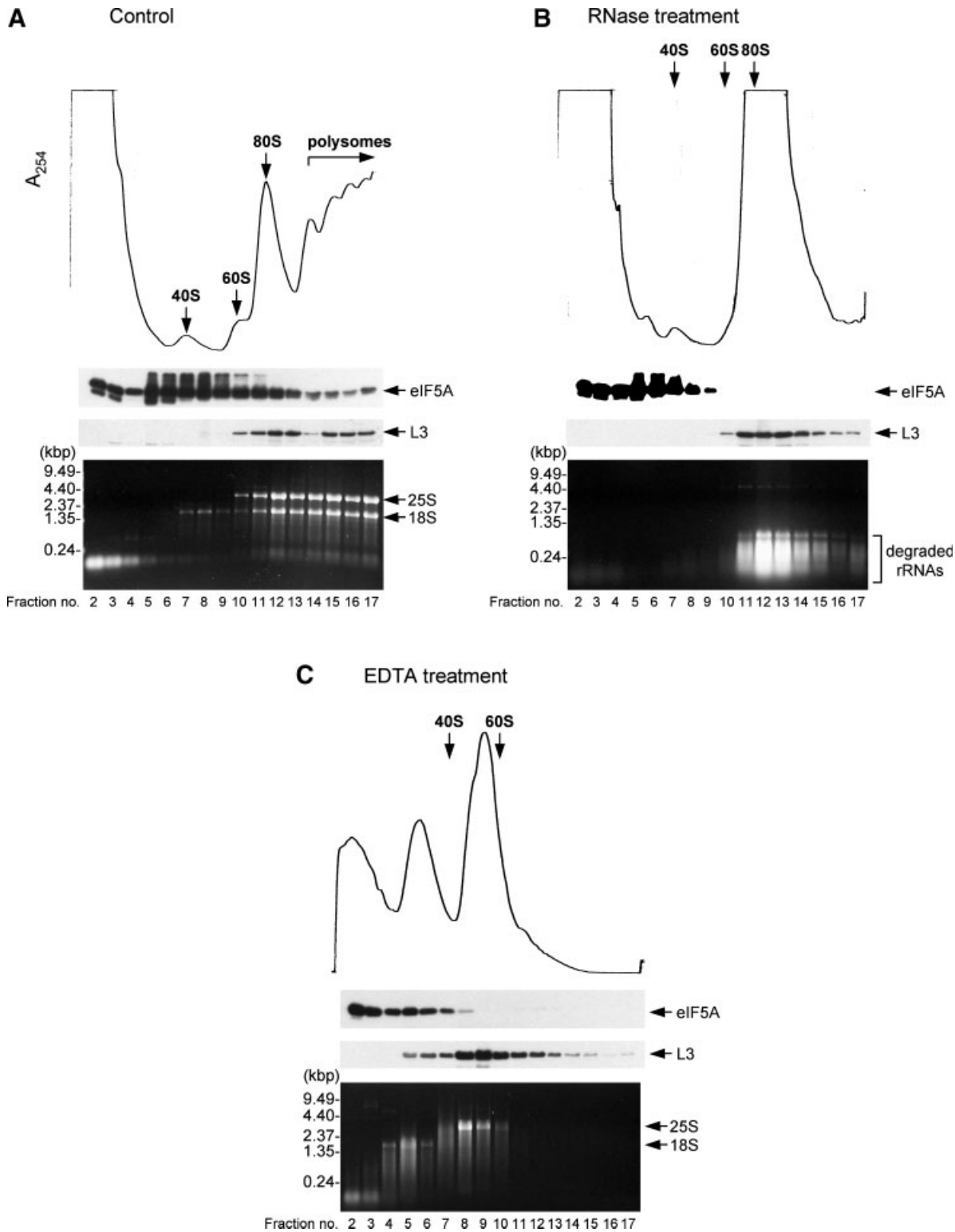

Fig. 4.

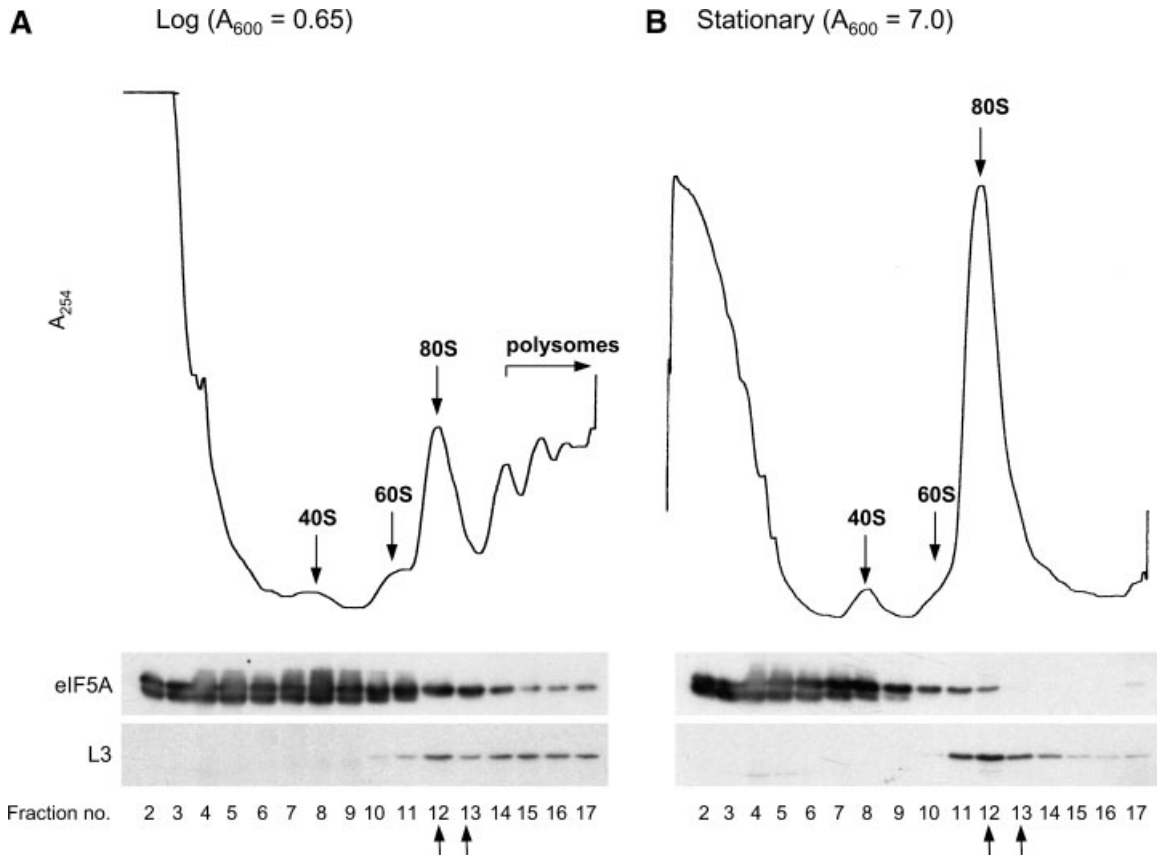

Fig. 5. Comparison of the distribution of endogenous eIF5A proteins in sucrose gradient fractions of wild-type cell extracts from logarithmic or stationary phase cells. Cell extracts prepared from the logarithmic phase (**A**, $OD_{600} = 0.65$) or stationary phase (**B**, $OD_{600} = 7.0$) KCY307 cells were layered over a continuous 7%–47% sucrose gradient and subjected to ultracentrifugation. Gradients were fractionated from the top of the tube while the UV absorbance at 254 nm was monitored to produce the absorbance profile (the top panel of each figure). Totally 17 fractions were collected from each tube. Fractions 2–17 were subjected to anti-eIF5A and anti-L3 Western blot analysis (the bottom panels of each figure). In all Western blots, half of samples from fractions 5–17 were electrophoresed, but only one-twentieth of samples from fractions 2–4 were loaded to prevent overloading the gel. Cycloheximide and Mg^{2+} ions were both added in the lysis buffer and sucrose gradients to preserve the polyribosomes. Sedimentation was from **left** to **right**. The positions of free 40S and 60S subunits, 80S ribosomes, and polyribosomes were indicated in the UV profiles. The fractions containing the majority of 80S ribosomes (i.e., fractions 12 and 13) were marked by arrows.

(Fig. 7, lane 4). We then compared the compositions of eIF5A co-purified proteins using strain KCY195 (genomic eIF5A-TAP) and strain KCY210 (plasmid-borne wild-type eIF5A-TAP). As shown in Figure 8, the SDS–PAGE gel patterns of eIF5A-TAP co-purified proteins isolated from these two strains were almost identical (lane 2 vs. lane 1), indicating that the

Fig. 4. Distribution of endogenous eIF5A proteins in sucrose gradient fractions of wild-type cell extracts. Cell extracts prepared from the logarithmic phase KCY307 cells (OD_{600} around 1.0) were layered over a continuous 7%–47% sucrose gradient and subjected to ultracentrifugation. Gradients were fractionated from the top of the tube while the UV absorbance at 254 nm was monitored to produce the absorbance profile (the top panel of each figure). Totally 17 fractions were collected from each tube. Fractions 2–17 were subjected to anti-eIF5A and anti-L3 Western blot analysis (the middle panels of each figure) or the total RNA was extracted from each fraction and analyzed by formaldehyde-agarose gel electrophoresis (the bottom panel of each figure). In all Western blots, one-third of samples from fractions 5–17 were electrophoresed, but only one-twentieth of samples from fractions 2–4 were loaded to prevent overloading the gel. The additions of cycloheximide and Mg^{2+} ions to the lysis buffer and sucrose gradients were varied as follows: **(A)** and **(B)**, cycloheximide and Mg^{2+} ions were both added; **(C)**, cycloheximide and Mg^{2+} ions were both omitted but 10 mM and 40 mM of EDTA were added in the lysis buffer and sucrose gradients, respectively. In **(B)**, the experimental conditions were identical to those in **(A)** except that the cell extract was treated with 100 μg/ml of RNase A on ice for 15 min prior to ultracentrifugation. Sedimentation was from left to right. The positions of free 40S and 60S subunits, 80S ribosomes, and polyribosomes were indicated in the UV profiles. The positions of 25S and 18S ribosomal RNA were also indicated in all RNA gels.

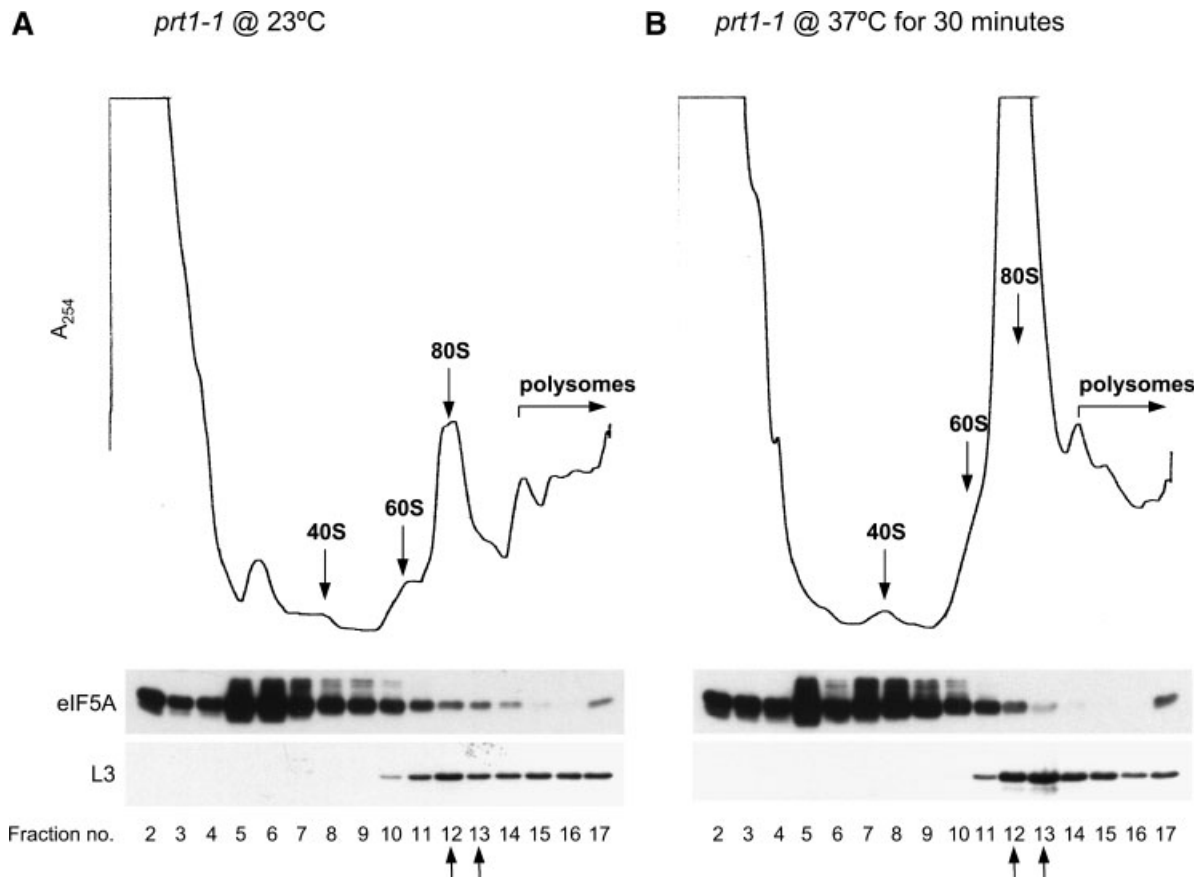

Fig. 6. Comparison of the distribution of endogenous eIF5A proteins in sucrose gradient fractions of extracts from *prt1-1* mutant cells grown at permissive or restrictive temperature. Cell extracts prepared from *prt1-1* mutant strain TP11B-4-1 grown at the permissive temperature (23°C; **A**) or shifted to the restrictive temperature (37°C; **B**) for 30 min were layered over a continuous 7%–47% sucrose gradient and subjected to ultracentrifugation. Gradients were fractionated from the top of the tube while the UV absorbance at 254 nm was monitored to produce the absorbance profile (the top panel of each figure). Totally 17 fractions were collected from each tube. Fractions 2–17 were subjected to anti-eIF5A and anti-L3 Western blot analysis (the bottom panels of each figure). In all Western blots, half of samples from fractions 5 to 17 were electrophoresed, but only one-twentieth of samples from fractions 2 to 4 were loaded to prevent overloading the gel. Cycloheximide and Mg^{2+} ions were both added in the lysis buffer and sucrose gradients to preserve the polyribosomes. Sedimentation was from **left** to **right**. The positions of free 40S and 60S subunits, 80S ribosomes, and polyribosomes were indicated in the UV profiles. The fractions containing the majority of 80S ribosomes (i.e., fractions 12 and 13) were marked by arrows.

plasmid-borne approach is equivalent to the original genomic approach. We then performed the TAP affinity purification using strain KCY211, which expresses the K51R-mutant eIF5A-TAP in addition to the endogenous wild-type eIF5A. As shown in Figure 8, although the K51R-mutant eIF5A-CBP was purified as efficiently as its wild-type counterpart, ribosomes were no longer co-purified with it (Fig. 8, lane 3 vs. lane 2). These results clearly demonstrate that hypusine is required for the binding of eIF5A to the 80S ribosome. The fact that a single amino acid change completely abolished the binding between the eIF5A-TAP and the ribosome is striking. It underscores the high specificity of this interaction.

DISCUSSION

To understand the physiological function of eIF5A, it is necessary to identify the eIF5A interacting partners that can explain the essential and conserved nature of eIF5A and its hypusine modification. Using yeast two-hybrid or traditional biochemical methods, a number of putative eIF5A binding proteins as listed in Table II have been reported in the literature. Among these proteins, only exportin 4 appears to interact with eIF5A in a hypusine-dependent manner. Intriguingly, exportin 4 or its orthologue does not exist in yeast [Lipowsky et al., 2000]. Thus, the contribution of these putative binding proteins, including exportin 4, to the

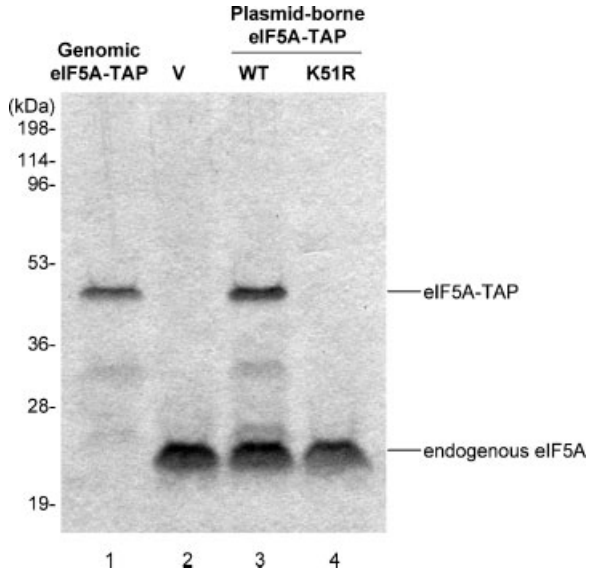

Fig. 7. In vivo labeling of eIF5A with [^{14}C]spermidine. Four yeast strains, KCY195 (Genomic eIF5A-TAP, lane 1), KCY209 (V, lane 2), KCY210 (WT, lane 3), and KCY211 (K51R, lane 4) were grown at 30°C in the presence of 0.2 μCi/ml of [^{14}C]spermidine to late log phase. The cell extracts were prepared and separated by 15% SDS–PAGE followed by autoradiography. Note that the wild-type eIF5A-TAP, but not the K51R-mutant eIF5A-TAP, can serve as an effective substrate for hypusination.

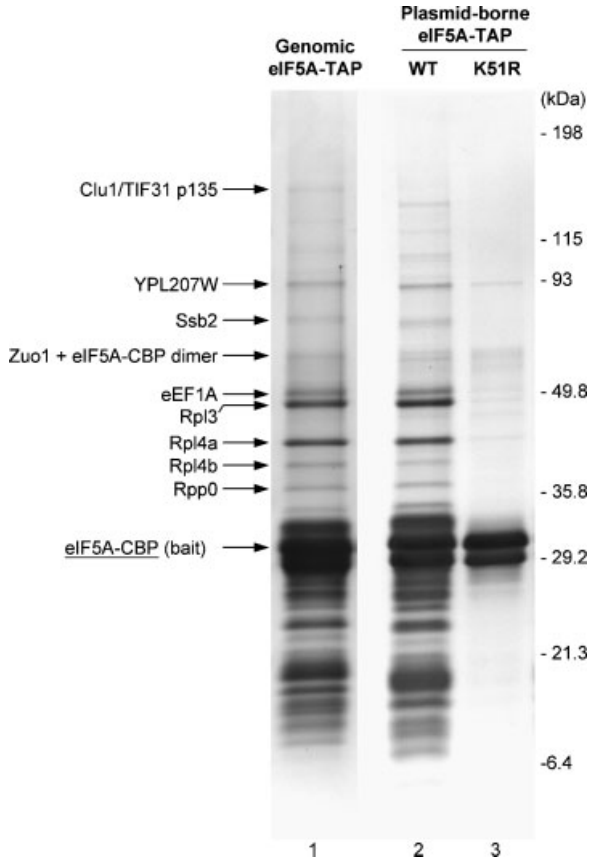

Fig. 8. eIF5A–ribosome interaction requires hypusine modification. Two different strategies of eIF5A-TAP procedures were performed: the original ''genomic eIF5A-TAP,'' where the genomic *TIF51A* gene was TAP-tagged and the eIF5A-TAP fusion proteins were expressed from the genomic sequence; the ''plasmid-borne eIF5A-TAP,'' where the genomic *TIF51A* gene was untagged while the eIF5A-TAP fusion proteins were expressed from the transformed plasmids, which harbor either the wild-type or the K51R mutant *TIF51A* gene. The fractions eluted from the calmodulin affinity resin of the TAP procedures under 50 mM salt concentrations were analyzed on 4%–16% SDS–PAGE followed by Coomassie Blue staining. Note that the protein compositions of the eluted fractions from the genomic eIF5A-TAP (lane 1) and the plasmid-borne eIF5A-TAP (WT, lane 2) are very similar and those eIF5A-associated proteins were not recovered in the eluted fraction from the plasmid-borne K51R mutant eIF5A-TAP (K51R, lane 3). Some of the eIF5A-associated proteins identified by mass spectrometry were indicated on the left of the gel. The eIF5A-CBP (bait) was underlined.

essential and conserved function of eIF5A is still unclear.

In this study, we used the TAP method to identify yeast eIF5A-interacting proteins. At 150 mM salt concentrations, deoxyhypusine synthase is the only protein that co-purifies with eIF5A (Fig. 1A), but as the salt concentrations decrease, the 80S ribosome and its associated proteins become the major eIF5A binding partners (Figs. 1B and 2). It is interesting to note that as the binding of the ribosome to eIF5A increases, there is a concomitant decrease in the binding of deoxyhypusine synthase to eIF5A (Fig. 1B), suggesting that both the ribosome and deoxyhypusine synthase share the same binding site on eIF5A and that this site should be close to the hypusine residue because this is where the deoxyhypusine synthase acts. Indeed, we found that the binding of the ribosome to eIF5A requires the presence of hypusine (Fig. 8). The requirement of hypusine for the eIF5A–ribosome interaction was also observed in mammalian cells [Jao and Chen, unpublished data], suggesting that the hypusine-dependent eIF5A–ribosome interaction is universally conserved in eukaryotes. Thus, the stringent hypusine-dependency of the eIF5A–ribosome interaction, from yeast to mammalian cells, not only indicates that the interaction is highly specific but also suggests that the interaction is physiological relevant.

Combining both the TAP affinity isolation and the sucrose gradient sedimentation analysis, we also demonstrated that the eIF5A–ribosome interaction requires the joining of two ribosomal subunits as an intact 80S ribosome

TABLE II. Putative eIF5A-Interacting Proteins

eIF5A-binding proteins	Methods	Hypusine-dependent?	References
80S ribosome complex	TAP	Yes	This study
Deoxyhypusine synthase	TAP, yeast two-hybrid	—	This study, Chen and Liu [1997]; Thompson et al. [2003]
L5	Yeast two-hybrid	No	Schatz et al. [1998]
Lia1	Yeast two-hybrid	Unclear	Thompson et al. [2003]
Syntenin	Yeast two-hybrid	No	Li et al. [2004]
Tissue transglutaminase II	TGase column	Unclear	Singh et al. [1998]
Exportin 4	RanGTP column	Yes	Lipowsky et al. [2000]
CRM1	Far Western analysis	No	Rosorius et al. [1999]
CAN/nup214, nup153, nup98, nup62, and actin	Far Western analysis	No	Hofmann et al. [2001]

(Figs. 3 and 4C). Furthermore, the binding is sensitive to partial RNA degradation, suggesting that RNA plays a role in the eIF5A–80S ribosome interaction (Figs. 3 and 4B). Given that hypusine appears to be required for eIF5A to bind RNA in vitro [Liu et al., 1997; Xu and Chen, 2001] and that the eIF5A–ribosome interaction requires hypusine (Fig. 8), it is likely that the hypusine residue is involved in the RNA-mediated eIF5A–80S ribosome interaction. Although we do not yet know which RNA species is critical in the eIF5A-ribosome binding, the findings that eIF5A prefers to interact with the translating 80S ribosomes and polyribosomes but has little affinity toward the inactive 80S monosomes (Figs. 5 and 6) would suggest that mRNA plays a role in the eIF5A–ribosome interaction. This notion is consistent with an early proposal that eIF5A is responsible for the translation of a subset of mRNA [Kang and Hershey, 1994; Xu et al., 2004]. Thus, a possible scenario is that this subset of mRNA species may mediate the specific interaction between eIF5A and 80S ribosomes.

The sensitivity of the eIF5A–ribosome interaction to ionic strength suggests that the interaction is dynamic. In yeast, the copy numbers of the endogenous eIF5A proteins and the ribosomes are estimated to be 1.4×10^6 and 2.0×10^5 molecules per cell, respectively [our unpublished data and Warner, 1999]. Assuming that intracellular volume available to proteins is 25–30 μm^3 [Tyson et al., 1979], the concentration of ribosomes and eIF5A in yeast would be 11–13 μM and 77–91 μM, respectively. If 10%–12% of eIF5A proteins were ribosome-bound as estimated based on the sucrose gradient experiments (Figs. 5A and 6A), the dissociation constant of the eIF5A-ribosome complex would be around 15–22 μM. This number is close to the affinity suggested for the transient interactions between the protein factors and ribosomes during translation [von der Haar and McCarthy, 2002]. If we further consider the crowding effect [Hall and Minton, 2003], the binding of eIF5A to ribosomes in vivo could be even higher. Nevertheless, the relatively high concentrations of eIF5A proteins guarantee that every ribosome molecule is to have a chance to interact with eIF5A. This, together with the relatively weak interaction between eIF5A and 80S ribosomes, ensures that the binding is transient and dynamic, readily switching between a bound and an unbound state.

Since depletion of eIF5A in conditional yeast mutants only inhibits general protein synthesis up to 30% [Kang and Hershey, 1994; Zuk and Jacobson, 1998], eIF5A does not appear to be directly involved in translation initiation. However, the dynamic interaction between eIF5A and targeted ribosomes would suggest that eIF5A participates in ribosome-related events other than the synthesis of polypeptide chains per se. Assisting the folding of nascent polypeptide chains during translation and maintaining translation fidelity by preventing the formation of aberrant polypeptides are two possible functions within this category. Alternatively, eIF5A may be responsible for the translation of a subset of special mRNA required directly for cell growth [Kang and Hershey, 1994; Xu et al., 2004]. Given that identification of the interacting partners of eIF5A holds the key to understanding the function of eIF5A, our findings that eIF5A binds to the translating 80S ribosomes in a hypusine-dependent manner enable us to focus attention on the potential role of eIF5A in ribosome-related activities.

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