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Domain and Nucleotide Dependence of the Interaction between *Saccharomyces cerevisiae* Translation Elongation Factors 3 and 1A*

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Eukaryotic translation elongation factor 3 (eEF3) is a fungal-specific ATPase proposed to catalyze the release of deacylated-tRNA from the ribosomal E-site. In addition, it has been shown to interact with the aminoacyl-tRNA binding GTPase elongation factor 1A (eEF1A), perhaps linking the E and A sites. Domain mapping demonstrates that amino acids 775–980 contain the eEF1A binding sites. Domain III of eEF1A, which is also involved in actin-related functions, is the site of eEF3 binding. The binding of eEF3 to eEF1A is enhanced by ADP, indicating the interaction is favored post-ATP hydrolysis but is not dependent on the eEF1A-bound nucleotide. A temperature-sensitive P915L mutant in the eEF1A binding site of eEF3 has reduced ATPase activity and affinity for eEF1A. These results support the model that upon ATP hydrolysis, eEF3 interacts with eEF1A to help catalyze the delivery of aminoacyl-tRNA at the A-site of the ribosome. The dynamics of when eEF3 interacts with eEF1A may be part of the signal for transition of the post to pre-translocational ribosomal state in yeast.

The protein synthetic machinery is characterized by the interplay of different soluble factors in conjunction with ribosomes to translate the mRNA into the correct sequence of amino acids. The three phases of translation, initiation, elongation, and termination, are driven by factors that are highly conserved between yeast and metazoans (1). However, a major difference in elongation is the indispensability of eukaryotic elongation factor 3 (eEF3)[3] with yeast ribosomes (2, 3). eEF3 catalyzes an essential step in each elongation cycle by virtue of its ATPase activity. It has been proposed to act as an Exit-site (E-site) factor, facilitating the release of deacylated-tRNA and simultaneously impacting on the delivery of aminoacyl-tRNA (aa-tRNA) at the aminoacyl site (A-site) (4). Metazoan ribosomes have been reported to possess a compensatory intrinsic ATPase activity, although they differ kinetically from the fungal eEF3 (5). *Escherichia coli*, on the other hand, expresses the 911 amino acid RbbA protein that exhibits ATPase activity and is tightly associated with ribosomes (6, 7). Both pathogenic and non-pathogenic fungi have been reported to contain eEF3 (8–10). In *Saccharomyces cerevisiae*, eEF3 is encoded by a single copy essential *YEF3* gene. A paralog of the *YEF3* gene, designated *HEF3* or *YEF3B*, encodes an 84% identical protein but is not expressed during vegetative growth (11). However, expression of the *HEF3* coding sequence under the *YEF3* promoter produces a protein that has similar ATPase activity and ribosome binding properties to *YEF3*-encoded eEF3.

eEF3 is a class 1 member of the ATP binding cassette (ABC) family of proteins. eEF3 possesses distinct motifs including the HEAT repeats on the N terminus, two nucleotide binding domains with tandemly arranged bipartite (ABC) cassettes in the middle, a conserved insertion in the intervening region of the Walker A and B motifs of ABC2, and a highly basic C terminus. HEAT (Huntington elongation factor 3, A subunit of protein phosphatase 2A and TOR1) repeats correspond to a tandem α-helical structure that appears to serve as flexible scaffolding on which other proteins can assemble. Amino acids 98–388 within the eEF3 N-terminal HEAT domain have also been shown to interact with the 18 S rRNA (12). Within the Walker A and B motifs, the nucleotide binding stretch of seven amino acids in ABC1 and -2 are 100% conserved among the ATP-binding proteins (13). The Walker C motif is the conserved LSGGQ sequence, the presence of which distinguishes the ABC proteins from other ATPases (14). Alterations of the conserved glycine and lysine residues within the Walker A of either ABC1 or -2 abolish the ATP hydrolytic activity of eEF3 *in vitro* and are lethal for growth *in vivo* (15). Interestingly, a temperature-sensitive (Ts^-) F650S point mutant in the intervening region of the two ABC cassettes also affects the catalytic ATPase activity of the protein, indicating that the linker region affects either ATP binding or its hydrolysis (16). Crystal structures of the *E. coli* transporter system ABC proteins HisP (17) and MalK (18) as well as the human Rad50 ATPase (19) and cystic fibrosis transmembrane conductance regulator (20) demonstrate that all possess two associated monomers. Each monomer harbors a single ABC cassette and forms a homodimer in the presence of ATP to carry out hydrolysis, although there is variation in the manner by which each nucleotide binding domains from the two mono-

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[3] The abbreviations used are: eEF, eukaryotic elongation factor; GST, glutathione *S*-transferase; Ni^{2+}-NTA, nickel nitrilotriacetic acid; aa-tRNA, aminoacyl-tRNA; E-site, exit site; A-site, aminoacyl site; ABC, ATP binding cassette; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

TABLE 1
***S. cerevisiae* strains used in this study**

Strains	Genotype	Source
TKY554	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243 met2-1 his4-713 yef3::LEU2 2μ pYEF3 URA3*	Ref. 16
TKY555	*MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2μ HIS3)*	This study
TKY597	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243 met2-1 his4-713 yef3::LEU2 CEN pYEF3 TRP1*	This study
TKY616	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ CEN His_6 pTEF1 TRP1*	This study
TKY676	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243 met2-1 his4-713 yef3::LEU2 CEN His_6 pYEF3 TRP1*	This study
TKY702	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243 met2-1 his4-713 yef3::LEU2 2μ His_6 pYEF3 TRP1*	Ref. 16
TKY800	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243 met2-1 his4-713 yef3::LEU2 CEN pyef3 TRP1*(P915L)	This study
TKY805	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243met2-1 his4-713 yef3::LEU2 2μ His_6 pyef3 TRP1* (980eEF3)	This study
TKY819	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243 met2-1 his4-713 yef3::LEU2 CEN His_6 pyef3 TRP1* (P915L)	This study
TKY822	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243 MET2 his4-713 yef3::LEU2 CEN His_6 pYEF3 TRP1*	This study
TKY824	*MATα ura3-52 leu2-3, 112 trp1-7 lys2-1243 MET2 his4-713 yef3::LEU2 CEN pyef3 TRP1* (P915L)	This study

mers collaborate to bind ATP molecules (21). The lysine-rich C terminus of eEF3 (amino acids 980–1044) has previously been implicated as required for binding to the ribosome (22, 23).

During translation elongation, delivery of aa-tRNA to the A-site by eEF1A and the translocation of the ribosome by eukaryotic elongation factor 2 (eEF2) require GTP hydrolysis (1). The unique role of eEF3 may be part of the transition of the post-translocational to the pre-translocational state via its ATP hydrolytic activity in yeast. The allosteric three-site model suggests that only two tRNAs can occupy the ribosome at one time, and thus, the exit of deacylated-tRNA is a prerequisite or coreq-uisite for the delivery of aa-tRNA to the A-site (24). eEF3 has been proposed to aid this removal and help promote the delivery of only cognate aa-tRNA by eEF1A to the A-site (4). It remains unclear how and when eEF3 utilizes its ATP hydrolytic activity to carry out these functions.

To address this question the present study analyzed the regions involved in, and the nucleotide-bound state that favors eEF3 binding to eEF1A. Our results point toward an enhanced eEF3 and eEF1A association in the presence of ADP, suggesting that ATP hydrolysis likely precedes eEF3 binding to eEF1A. The eEF1A binding region of eEF3 has been mapped to 2 regions near the C terminus. A genetic screen conducted in the current study resulted in a point mutation in one of the regions. A strain expressing the P915L eEF3 exhibits a temperature-sensitive (Ts^-) growth defect and reduction in total translation. Additionally, the protein has negligible intrinsic and ribosome-stimulated ATPase activity and shows reduced affinity for eEF1A.

EXPERIMENTAL PROCEDURES

Yeast and Bacterial Strains, Growth, Drug Sensitivity, and Translation Assays—S. cerevisiae strains and their genotypes are listed in Table 1. *E. coli* DH5α was used for plasmid preparation. Procedures for cell growth and genetic manipulations were according to standard protocols (27). Yeast cells were grown in either YEPD (1% Bacto-yeast extract, 2% peptone, 2% dextrose) or in defined synthetic complete medium (C or C−) supplemented with 2% dextrose as the carbon source unless noted. Yeast were transformed by the lithium acetate method (28). Temperature sensitivity was assayed by growing strains to an A_{600} of 1.0. Serial 10-fold dilutions (5 μl each) were spotted on appropriate medium followed by incubation at 13, 24, 30, and 37 °C for 3–7 days. Phenotypic suppression of a non-programmed +1 frameshift allele (met2-1 and his4-713) was determined by spotting 10 μl of the same dilutions onto complete medium lacking methionine or histidine, respectively, and incubating for 5 days at 30 °C. Halo assays for sensitivity to cycloheximide, paromomycin, and hygromycin B were performed as previously described (29). Total yeast translation was monitored by *in vivo* [^{35}S]methionine incorporation as previously described at both 30 and 37 °C (30) using the indicated MET2 strains.

Isolation of the P915L eEF3 Mutant by Hydroxylamine Mutagenesis—Ten μg of plasmid DNA (pTKB594) harboring *YEF3* on a *CEN TRP1* plasmid was added to 500 μl of 1 M hydroxylamine, pH 7.0. The reaction was incubated at 37 °C for 20 h and stopped by adding 100 mM NaCl and 0.1 μg/μl bovine serum albumin. DNA was ethanol-precipitated, transformed into TKY554, and plated on C-Trp to select for the mutated *yef3 TRP1* plasmid at a density of ~150–300 cells/plate. Cells able to lose the wild type *YEF3 URA3* plasmid were identified by growth on 5-fluoroorotic acid-containing media. The resulting strains expressing the *yef3 TRP1* plasmid as the only form were analyzed for growth at 13, 30, or 37 °C. A colony unable to grow at 37 °C was recovered from the 30 °C plate, and the plasmid was extracted, transformed in *E. coli*, recovered, and retransformed into TKY554. Loss of the wild type *YEF3* plasmid was repeated to confirm the phenotype. The P915L eEF3 mutant plasmid pTKB753 isolated in this screen was also constructed with a His_6 tag on the N terminus by site-directed mutagenesis of pTKB602 by the QuikChange method (Stratagene), producing pTKB777.

Cloning, Expression, and Purification of GST and His_6-tagged eEF3, eEF1A, and Truncations—Full-length eEF3 and fragments containing amino acids 1–775 (85NT), 100–367 (HEAT), 775–910 (I), 910–1044 (15CT), and 775–1044 (30CT) were PCR-amplified using pTKB594 as the template. Fragments were cloned into pTKB544 for expression with a galactose-inducible promoter (*GAL1-10*) and an N-terminal GST tag, resulting in plasmids pTKB705, pTKB706, pTKB707, pTKB708, pTKB709, and pTKB710, respectively. The plasmids expressing the GST-tagged eEF3 fragments were transformed in TKY555 and maintained on C-Ura-His+galactose media for protein expression. Yeast cultures expressing the GSTeEF3 fusions were harvested at an A_{600} of 1.0–2.0, and total yeast extracts were clarified and loaded on the GST Trap column (Amersham Biosciences) in buffer A (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM DTT, and 0.2 mM PMSF). The protein was eluted with buffer A plus 20 mM

reduced glutathione (Sigma). The protein peak was dialyzed into buffer B (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.2 mM PMSF, and 100 mM KCl).

To facilitate eEF3 purification from yeast, a His_6 tag was added to the N terminus of *S. cerevisiae* eEF3 under the control of its own promoter on a *CEN TRP1* plasmid producing pTKB602 (31). A yeast plasmid expressing $His_6$980 eEF3 was produced by introduction of a stop codon at amino acid 981 by QuikChange, producing pTKB724 (31). The plasmids were introduced into *S. cerevisiae* strain TKY554, and loss of the wild type eEF3 on a *URA3* plasmid was monitored by growth on 5-fluoroorotic acid, producing TKY702 and TKY805, respectively.

His_6-tagged wild type eEF1A, eEF3, 980eEF3, and P915L eEF3 proteins were purified from strains TKY616, TKY702, TKY805, and TKY819, respectively, on a Ni^{2+} Hi Trap chelating column (Amersham Biosciences). Total yeast extracts were clarified and loaded on the column in buffer C (50 mM KPO_4, pH 7.6, 300 mM KCl, 1 mM DTT, and 0.2 mM PMSF) with 20 mM imidazole. The protein was eluted with buffer C plus 400 mM imidazole. The protein peak was dialyzed into buffer B.

BspEI restriction sites were introduced upstream of the ATG initiation codon and downstream of the TAA stop codon using the QuikChange protocol in *TEF1* on pTKB731 as template, producing pTKB740. His_6-tagged eEF1A with BspEI restriction sites upstream and downstream of the open reading frame was constructed by PCR and cloning into pTKB740, resulting in plasmid pTKB779. Domain I (amino acids 1–221) was constructed by QuikChange mutagenesis of the Lys-222 and Lys-224 codons to TAA using pTKB779, producing pTKB852. His_6-tagged domain III (amino acids 333–458) was obtained by looping out domains I and II using site-directed mutagenesis protocol of template pTKB779, producing pTKB785. Plasmids pTKB852 and pTKB785 were used as templates for PCR amplification of His_6-domain I and His_6-domain III fragments to clone into the pET11a vector, resulting in plasmids pTKB863 and pTKB851, respectively. His_6-tagged domain II (amino acids 222–316) in pET11a was constructed by QuikChange mutagenesis of the Glu-316 and Arg-318 codons to TAA and TGA, respectively, using pTKB864 as the template to produce pTKB920.

A 1-liter culture of *E. coli* BL21 with each plasmid was grown to an A_{600} of 0.6 in LB with 100 μg/ml ampicillin medium. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 3–4 h. Cells were harvested by centrifugation and lysed by sonication, and the recombinant protein was purified in accordance with the QIAexpressionist protocol for His_6-tagged proteins under native conditions. Protein-containing fractions were dialyzed into 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM EDTA, pH 8.0, 100 mM KCl, and 20% glycerol.

ATP Hydrolysis—ATP hydrolysis was performed using purified proteins as previously described (32). Briefly, the assay mixture contained 24 pM protein, 50 pM yeast ribosomes, and 150 μM [γ-^{32}P]ATP. Hydrolysis was allowed to proceed for 5 min at 30 °C, and $^{32}P_i$ release was determined. ATP hydrolysis levels were calculated after subtracting the background for buffer alone.

GST and His_6 Pulldowns of eEF1A and eEF3—Yeast extracts for *in vivo* binding assays were prepared by glass bead lysis in TEDG buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM DTT, 50 mM KCl, and 1 mM PMSF) from TKY555 with the empty plasmid pTKB544, GSTeEF3 (pTKB705), or the GST-eEF3 fragments (pTKB706, pTKB707, pTKB708, pTKB709, pTKB710). For GST and Ni^{2+}-NTA pulldown assays, 200-μl reactions containing 50 μg of total protein (determined by Bradford reagent; Bio-Rad) and 40 μl of either 50% glutathione-Sepharose 4B slurry (Sigma) in KETN 150 buffer (150 mM KCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% Nonidet, and 1 mM PMSF) or Ni^{2+}-NTA slurry (Amersham Biosciences, GE Healthcare) in buffer C were mixed at 4 °C for 1 h. Beads were washed 3 times with either KETN buffer with 150 or 300 mM KCl for GST pulldown or buffer C with 100 mM imidazole for Ni^{2+}-NTA pulldown. Samples were resolved by SDS-PAGE, and were proteins were detected with a polyclonal antibody to yeast eEF1A and ECL (Amersham Biosciences) and quantitated with the ImageQuant program (GE Healthcare). Ni^{2+}-NTA pulldown of purified untagged eEF1A with His_6-tagged eEF3 or untagged eEF3 with His_6-tagged eEF1A were performed with 2 μg of eEF3 and 3 μg of eEF1A as previously described (33).

Ribosome Binding Assay—The ribosome binding assay was performed as described previously (34) with minor modifications. Fifty-μl reactions containing 24 pM purified proteins and 24 pM 80 S ribosomes in binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM ammonium acetate, 10 mM magnesium acetate, and 2 mM DTT) were incubated for 5 min at room temperature, layered on top of a 200-μl sucrose cushion (10% sucrose in binding buffer), and centrifuged at 74,000 rpm for 20 min at 4 °C in S80-AT2 (Sorvall) rotor. The pellet (bound fraction) was resuspended in Laemmli loading buffer and subjected to SDS-PAGE and Western blot analysis using the ECL method (Amersham Biosciences).

Enzyme-linked Immunosorbent Assays—*In vitro* binding was measured by an indirect enzyme-linked immunosorbent assay. Purified GSTeEF3 (0.25 μg) in 50 μl of PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4)/well was coated overnight at room temperature in a 96-well ultrahigh binding polystyrene microtiter plate (Thermo-Labsystem). After blocking with 300 μl of 0.1% bovine serum albumin in PBST for 1 h at room temperature and washing 3 times with 300 μl of PBST, 50 μl of 5000-fold-diluted affinity-purified polyclonal anti-eEF3 antibody was added to each well and incubated at room temperature for 2 h. Varying amounts of eEF1A along with varying amounts of ATP, ADP, GTP, or GDP were added to the eEF3 antibody. After washing 3 times with 300 μl with PBST, 50 μl of 2500-fold-diluted secondary goat anti-rabbit antibody conjugated with alkaline phosphatase was added per well (Jackson ImmunoResearch). Unbound antibody was removed by three washes of 300 μl of PBST followed by the addition of 50 μl of 3 mM *p*-nitrophenyl phosphate (Sigma) in 50 mM Na_2CO_3 and 50 μM $MgCl_2$/well. The extent of *p*-nitrophenyl phosphate hydrolysis represents the antigen-antibody binding measured by A_{415}.

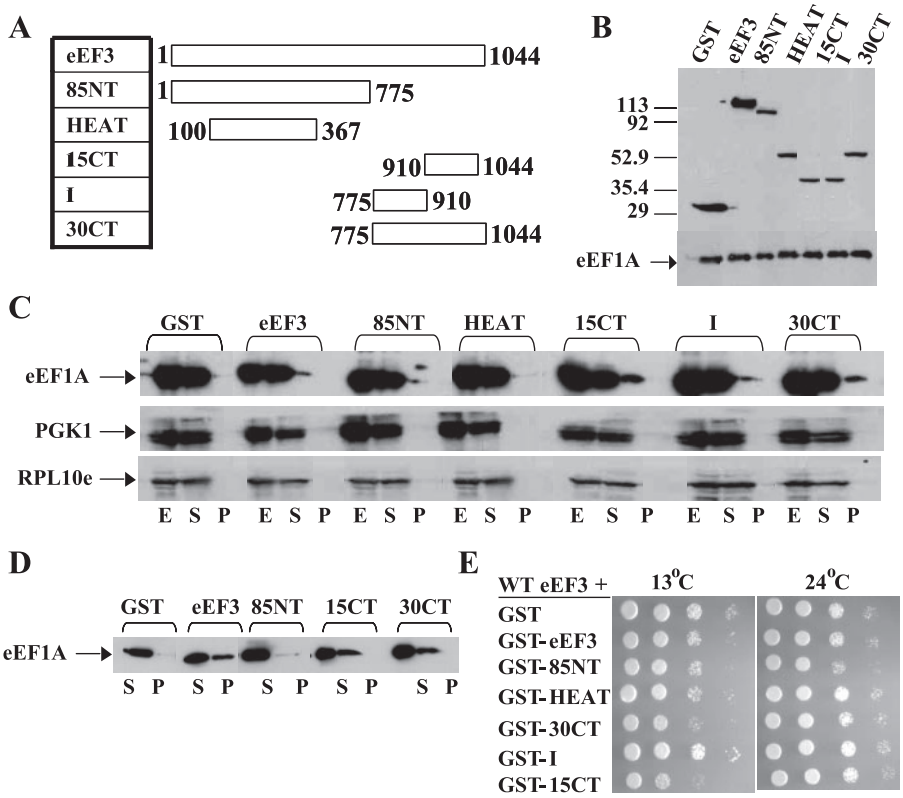

FIGURE 1. **C-terminal regions of eEF3 bind eEF1A.** *A*, eEF3 fragments cloned as GST fusions under the *GAL1-10* promoter in pTKB544. *B*, plasmids expressing the GST fusion fragments from A were transformed in TKY555 and maintained in C-Ura-His+galactose. Strains were grown to mid-log phase at 30 °C, yeast extracts were prepared, and equal amounts of protein (5 μg) were separated by SDS-PAGE and subjected to Western blotting with an anti-GST monoclonal antibody. The *lower panel* shows eEF1A as the internal loading control. *C*, a GST pulldown assay was performed with the extracts (50 μg) from the same strains as in *B*, and the Western blot was developed with an anti-eEF1A antibody (*top panel*), anti-phosphoglycerate kinase (*PGK1*) antibody representing internal loading control (*middle panel*) and anti-RPL10e antibody to detect co elution ribosomes with the eEF3·eEF1A complex (*lower panel*). *E*, extract (10% input); *S*, supernatant (5%); *P*, pellet (100%). *D*, a GST pulldown assay with purified yeast eEF1A (100 pM) and GST-tagged eEF3, 30CT, 15CT, and 85NT fragments (20 pM). *E*, the yeast strains from B were grown to mid-log phase at 30 °C, diluted to equal A_{600}, spotted as 10-fold serial dilutions, and grown at 13 or 24 °C for 2–7 days.

RESULTS

eEF3 Interacts with eEF1A through Its C-terminal Region—Prior studies have demonstrated that eEF3 and eEF1A interact, as monitored by both genetic and physical assays *in vivo* and *in vitro* (16). To map the site of interaction, five fragments of eEF3 corresponding approximately to natural proteolytic sites were cloned into a *GAL1-10*-inducible expression vector with a GST tag at the N terminus (Fig. 1*A*). These include full-length eEF3 (amino acids 1–1044), 85NT, (1–775), HEAT (100–367), 15CT (910–1044), I (775–910), and 30CT (775–1044). All the fragments are expressed in yeast although at different levels, as monitored by Western blot with anti-GST antibody (Fig. 1*B*). The GST-tagged fusion proteins migrate at 140 (eEF3), 105 (85NT), 57 (HEAT), 42 (15CT), 58 (30CT), and 42 (I) kDa, with 29 kDa contributed by the GST tag. The same gel is also probed with anti-eEF1A antibody as the internal loading control.

Because none of the eEF3 fragments can replace wild type eEF3 *in vivo* (data not shown), all were co-expressed with an untagged wild type copy of eEF3 to support growth. A GST pulldown assay was performed to determine the binding of eEF3 to eEF1A in total cell extracts. The 15CT, I, and 30CT GST fusion fragments co-purified eEF1A at levels similar to or above that of full-length eEF3. The 85NT, GST alone, and HEAT fragments co-purified less eEF1A, although some background level of binding was observed (Fig. 1*C*). The same experiment was also probed for co-elution of ribosomes with the eEF3·eEF1A complex. As shown in Fig. 1*C*, *bottom panel*, RPL10e, a ribosomal protein, is absent in the bound fractions. The *middle panel*, Fig. 1*C*, shows probing for phosphoglycerate kinase (*PGK1*) as the internal loading control. Because the fusion truncations are expressed at different levels *in vivo*, the 85NT, 30CT, and 15CT GST-tagged fragments were purified from yeast, and GST pulldown experiments were performed with purified untagged yeast eEF1A. The GST-HEAT and GST-I fusion were not stably expressed at sufficient levels for purification. GST-15CT and GST-30CT co-purified with eEF1A at levels comparable with wild type GSTeEF3, whereas the GST-85NT was at background levels (*GST*, Fig. 1*D*). The results in Fig. 1, *C* and *D*, demonstrate that the eEF3·eEF1A interaction occurs in the absence of any cellular factors via the C-terminal region of eEF3.

Dominant growth phenotypes conferred by the truncations were monitored on C-Ura-His+galactose medium at different temperatures. The 30CT and 15CT fragments confer a dominant slow growth phenotype at 13 °C, whereas no effects were seen at 30 or 37 °C (Fig. 1*E* and data not shown). Because there appear to be two eEF1A binding sites, one within amino acids 775–910 and one within 910–1044, fragments of eEF3 containing these amino acids may exhibit a dominant slow growth phenotype due to the formation of inactive complexes with eEF1A. The I fragment (775–910) does not show this growth phenotype, indicating the site from 910 to 1044 may have a larger effect *in vivo*.

$His_6$980eEF3 Is Functional in Vivo and Retains Binding to Ribosomes and eEF1A—Prior work proposed that the C-terminal 64 amino acids (980–1044), containing 40% basic residues, is the primary ribosome binding region of eEF3 (22). Other work suggests the N-terminal 98–388 amino acids binds to 18 S rRNA *in vitro* and inhibit the ribosome-dependent ATPase activity of eEF3 (12). To determine the function of the basic C terminus of eEF3, His_6-tagged eEF3 1–980 was expressed from a 2μ *TRP1* plasmid. This construct was able to function as the only form of eEF3 (Fig. 2*A*). Cells expressing $His_6$980eEF3 as the only form of eEF3 have a slight slow growth phenotype (Fig.

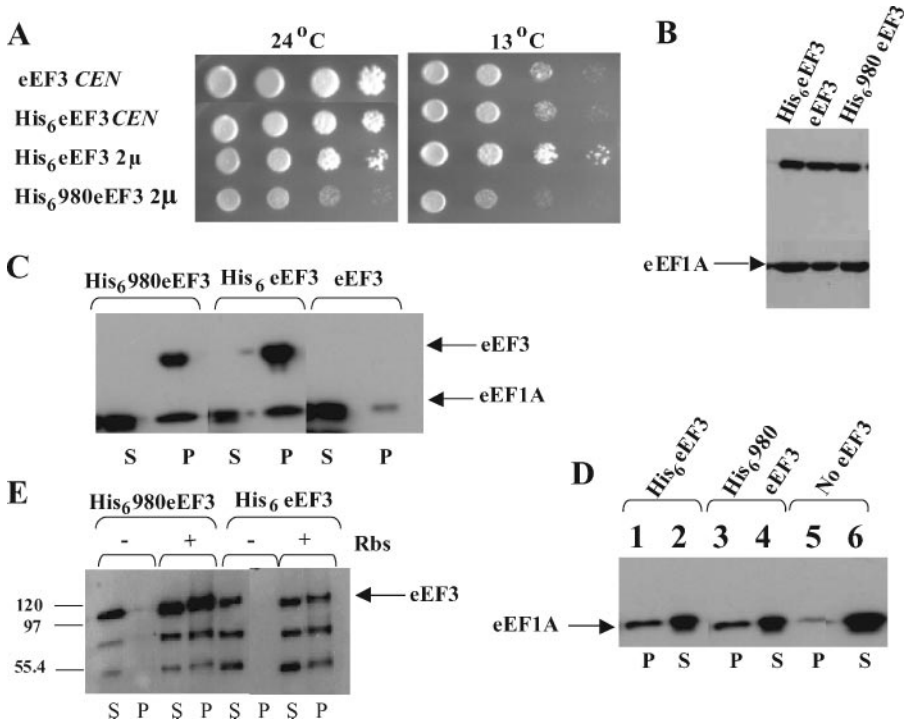

FIGURE 2. **His$_6$980eEF3 is functional *in vivo* and retains binding to eEF1A and ribosomes.** *A*, strains containing plasmid-borne untagged eEF3 (2 μm, TKY554), His$_6$-tagged eEF3 (*CEN*, TKY676), His$_6$eEF3 (2μ, TKY702), and His$_6$980eEF3 (2μ, TKY805) were grown to mid-log phase at 30 °C, diluted to equal A_{600}, spotted as 10-fold serial dilutions, and grown at 24 or 13 °C for 2–7 days. *B*, yeast extracts (2 μg) were prepared from strains expressing His$_6$eEF3 (TKY702), eEF3 (TKY554), and His$_6$980eEF3 (TKY805) and analyzed for the expression of His$_6$980eEF3 by Western blotting with an anti-eEF3 antibody. The *lower panel* shows equal loading of eEF1A as internal control. *C*, *in vivo* eEF1A binding to His$_6$eEF3 and His$_6$980eEF3 was analyzed by Ni^{2+}-NTA pulldown of yeast extracts (50 μg) from strains as in A. *S*, supernatant (5%) and *P*, pellet (100%) were subjected to SDS-PAGE and Western blot with anti-eEF1A and anti-His$_6$ antibodies. *D*, *in vitro* binding of purified His$_6$eEF3 and His$_6$980eEF3 in a 5-fold molar excess of untagged eEF1A was assessed by Ni^{2+}-NTA pulldown and analyzed by SDS-PAGE and Western blot with anti-eEF1A antibody. *E*, association of eEF3 with purified 80 S ribosomes through a 10% sucrose cushion is shown for His$_6$980eEF3 and His$_6$eEF3 and analyzed as in *D* with an anti-eEF3 antibody. *P*, pellet (100%); *S*, supernatant (20%).

2*A*). This effect is most noticeable at 13 °C. Western blot analysis of His$_6$eEF3, wild type eEF3, and His$_6$980eEF3 from strains TKY702, TKY554, and TKY805 with anti-eEF3 antibody (Fig. 2*B*) shows that His$_6$980eEF3 protein is expressed at similar levels as full-length-tagged and untagged eEF3. The same gel was also probed with anti-eEF1A antibody as internal loading control. Therefore, although His$_6$980eEF3 is stably expressed, its function *in vivo* is likely partially compromised.

The role of amino acids 981–1044 in binding eEF1A was determined by Ni^{2+}-NTA pulldown of extracts from strains expressing His$_6$980eEF3, His$_6$eEF3, or untagged eEF3. Supernatant and pellet fractions were resolved by SDS-PAGE, and the Western blot was probed with anti-eEF1A and anti-eEF3 antibodies. eEF1A associates with His$_6$980eEF3 at levels comparable with full-length His$_6$eEF3 (Fig. 2*C*). In the negative control with untagged eEF3, minimal background eEF1A was present in the pellet. The Ni^{2+}-NTA pulldown was also performed with purified proteins, confirming that eEF1A binds to both His$_6$eEF3 and His$_6$980eEF3 directly (Fig. 2*D*). This indicates that residues 981–1044 are not required for eEF1A binding. Taken together with the truncation data (Fig. 1, *C* and *D*), it appears one eEF1A binding site is located within the 205-amino acid stretch from 775 to 910 and a second within amino acids 910–980.

To determine whether the C terminus of eEF3 is dispensable for ribosome binding, purified His$_6$eEF3 and His$_6$980eEF3 were assayed for co-association with ribosomes through a sucrose cushion (Fig. 2*E*). The slowest migrating bands corresponding to the full-length and 1–980 proteins pellet with ribosomes. Interestingly, the same degradation products were observed for both eEF3 and 980eEF3. All three bands reacted with the anti-His$_6$ antibody (data not shown), and because the His tag was located at the N terminus, this indicates the N terminus is intact. Thus, these fragments represent C-terminal truncations and imply ribosome binding occurs near the N terminus. This is consistent with work showing an N-terminal fragment binds 18 S rRNA (12). The negative control bovine serum albumin stayed in the supernatant both in the presence and absence of ribosomes (data not shown).

ADP Enhances the Association of eEF3 with eEF1A—Because both eEF3 and eEF1A bind nucleotides, an enzyme-linked immunosorbent assay-based binding assay was developed to look at the effect of these molecules on the eEF1A-eEF3 interaction. Subsequent to coating the wells with purified eEF3, eEF1A was added to compete with an anti-eEF3 antibody. Because eEF1A binding competes with antibody binding, the absorbance value is reduced in the presence of eEF1A. Concentration-dependent eEF1A binding to eEF3 was observed (Fig. 3*A*). A 10-fold molar excess of eEF1A to eEF3 was used for all further assays. A series of controls was included in this assay to validate these results. These included demonstrating that the anti-eEF3 antibody does not show any affinity for eEF1A, the addition of nucleotide alone in the absence of anti-eEF3 antibody exhibits negligible absorbance, and the addition of nucleotides alone (in the absence of competing factor eEF1A) along with anti-eEF3 antibody does not affect absorbance (data not shown).

To ascertain the effect of the nucleotide-bound state on the binding of the two proteins, ATP, ADP, GTP, or GDP was added with the anti-eEF3 antibody and eEF1A. Whether GTP or GDP was incubated with eEF1A and eEF3, the signal remained constant, and thus, binding was unaffected (Fig. 3*B*). On the other hand, there is a concentration-dependent reduction in signal, and hence, stimulation of eEF1A binding when ADP was added. This is shown as binding normalized to absorbance in the presence of nucleotide alone and in the absence of eEF1A (Fig. 3*C*). Furthermore, when ATP was added

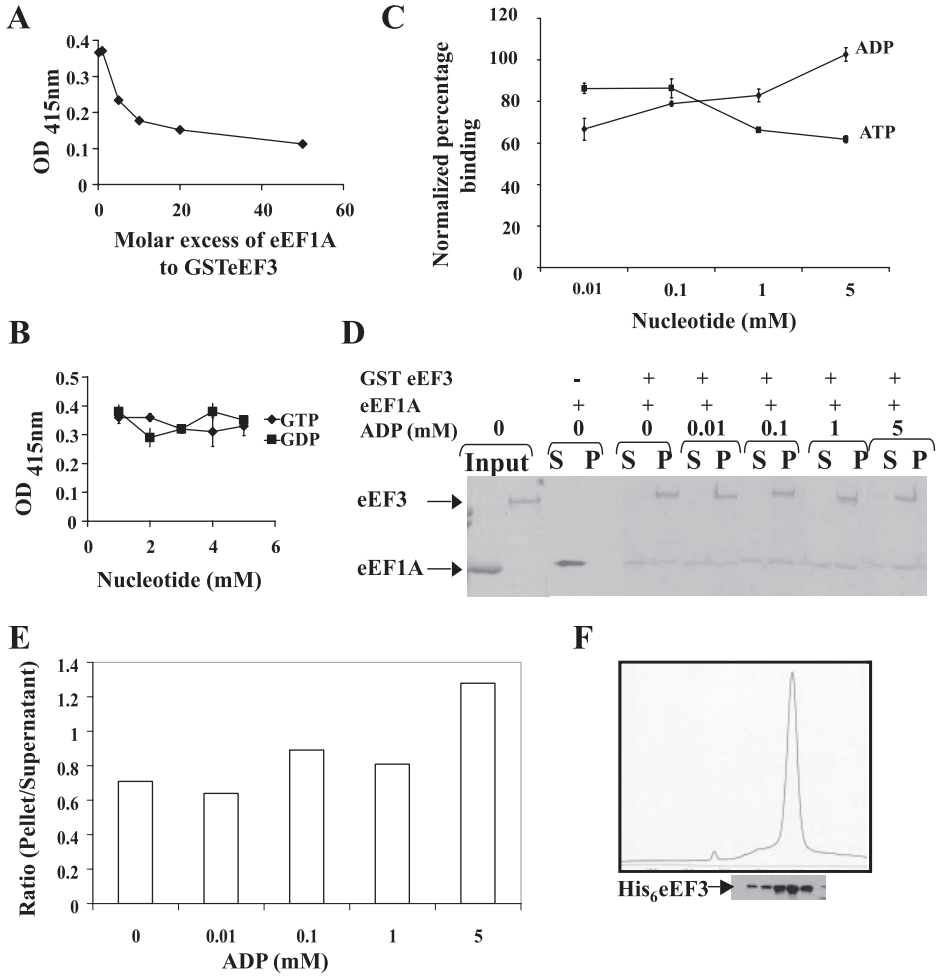

FIGURE 3. **ADP stimulates eEF1A binding to eEF3.** *A*, microtiter 96-well plates (Falcon) were coated with purified GSTeEF3 (0.25 μg), and an affinity-purified anti-eEF3 antibody was added with or without increasing amounts of eEF1A. eEF1A (1.25 μg) was incubated in the GSTeEF3-coated microtiter plate as in *A*, with different concentrations of nucleotides, GTP (*diamonds*) or GDP (*squares*) in *B* expressed as A_{415} or ATP (*diamonds*), or ADP (*squares*) in *C* expressed as percentage bound normalized to the presence of nucleotide alone in the absence of eEF1A. *D*, eEF1A bound to GSTeEF3 after GST pulldown in the presence of varying amounts of ADP were analyzed by SDS-PAGE and stained with gel code blue (Pierce). *S*, supernatant (5%); *P*, pellet (100%). *E*, the results of GST pulldown experiments as in *D* was analyzed with the ImageQuant program (GE Healthcare), and the ratio of pellet to supernatant was plotted. *F*, purified His_6eEF3 was subjected to gel filtration analysis by fast protein liquid chromatography on a Superdex 200 column (Amersham Biosciences). The elution profile of His_6eEF3 was determined by SDS-PAGE and Western blot with an anti-eEF3 antibody.

there was a concentration-dependent increase in signal, and hence, reduction in binding was observed. The experiment was done multiple times to confirm a reproducible trend.

To confirm the enzyme-linked immunosorbent assay-based assay, a GST pulldown of purified untagged eEF1A with GSTeEF3 was performed in the presence of different concentrations of nucleotide and analyzed by SDS-PAGE followed by gel code blue staining. The amount of eEF1A bound to GSTeEF3 in the pellet increases in the presence of increasing amounts of ADP (Fig. 3, *D* and *E*). Thus, results from two independent methods indicate that binding of eEF1A to eEF3 is likely stimulated after ATP hydrolysis.

Proteins belonging to the ABC superfamily are inter- or intramolecular dimers, and the presence of two ATPase domains is required for function (35). To confirm His_6eEF3 is a monomer, purified protein was subjected to analysis by gel filtration chromatography on a Superdex 200 column (Amersham Biosciences) (Fig. 3*F*). His_6eEF3 eluted as a single sharp peak with a retention time of 29.24 min corresponding to a molecular mass of 140 kDa. The migration remains unchanged in 1 M KCl, 1 mM ATP, 1 mM ADP, or 50 mM ethylene glycol (data not shown), showing that eEF3 exists as a monomer in its purified form.

A P915L Mutation in an eEF1A Binding Site of eEF3 Alters ATPase Activity and eEF1A Binding—A genetic screen for conditional mutants in eEF3 was conducted using unbiased *in vitro* mutagenesis of a *YEF3* plasmid. A pool of hydroxylamine-treated plasmids was transformed into yeast, and plasmids able to replace the wild type *YEF3 URA3* plasmid were determined by growth on 5-fluoroorotic acid. Approximately 7000 colonies were screened for temperature-sensitive growth yielding a strain expressing a single eEF3 point mutation, P915L, in the C-terminal region (Fig. 4*A*). The doubling time of the P915L mutant strain was 5.5 h compared with 3.5 h for the wild type strain. Total protein synthesis monitored by measuring [^{35}S]methionine in the P915L strain was 20% less than a wild type strain at permissive temperatures and 22% less than wild type when cells were shifted to 37 °C (Fig. 4*B*). To determine the eEF3 defect causing this effect, the ATPase activity of purified His_6P915L eEF3 was determined. The mutant lacks both intrinsic and ribosome-stimulated ATPase activity (Fig. 4*C*). To assess if this loss of catalytic activity affects eEF1A binding to the P915L eEF3 mutant, association was assessed by Ni^{2+}-NTA pulldown assay. His_6P915L eEF3 pulls down reduced levels of eEF1A as compared with wild type His_6eEF3 in both cell extracts (Fig. 4*D*) and with purified proteins (Fig. 4, *E* and *F*). A small amount of eEF1A is nonspecifically pulled down by untagged eEF3 using Ni^{2+}-NTA beads. This implies that binding of eEF3 to eEF1A is sensitive to structural and functional alterations caused by a point mutation in a region proposed to bind eEF1A.

eEF1A Binds eEF3 via Domain III—The co-crystal structure of eEF1A with its guanine nucleotide exchange factor eEF1Bα shows the G-protein has three domains. Domain I contains the GTP binding motifs, and domains I and II contact eEF1Bα (33, 36). Domain III has been shown to interact with actin and is responsible for the non-canonical functions of eEF1A in actin binding and bundling (25) and the slow growth phenotype asso-

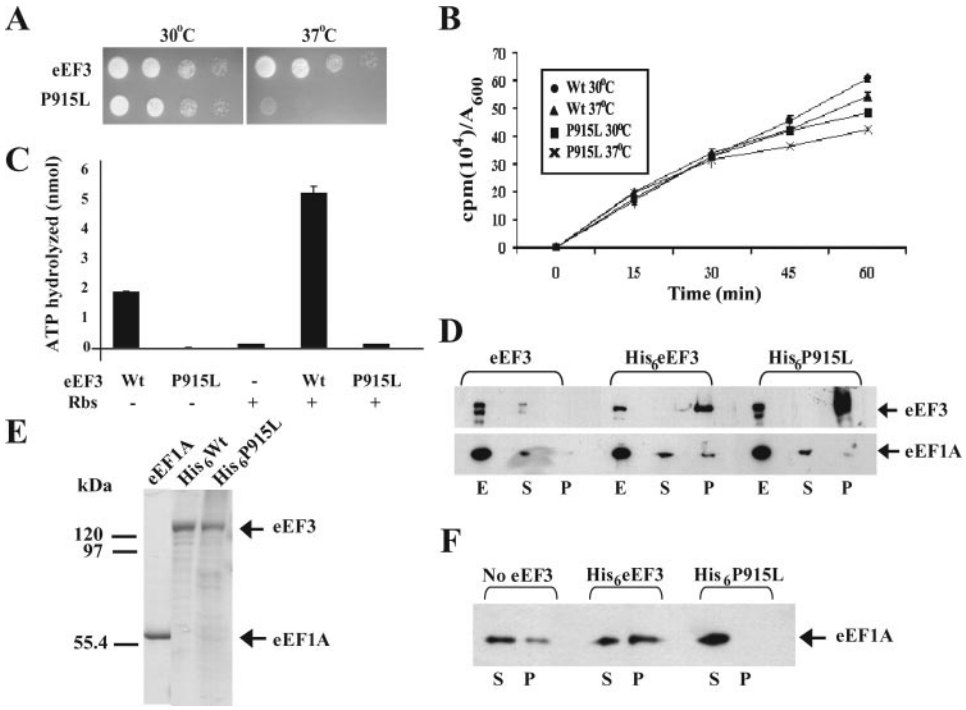

FIGURE 4. **The ATP hydrolysis deficient P915LeEF3 mutant shows reduced affinity for eEF1A.** *A*, strains containing wild type eEF3 (TKY597) or P915LeEF3 (TKY800) were grown to mid-log phase at 30 °C, diluted to equal A_{600}, spotted as 10-fold serial dilutions, and grown at 30 or 37 °C for 2–7 days. *B*, strains expressing His_6P915LeEF3 (TKY824) or His_6eEF3 (TKY822) were monitored for total translation by [^{35}S]methionine incorporation after growth to mid-log phase in C-Met and labeled for varying times at both 30 and 37 °C. Total translation is expressed as cpm/A_{600} unit. *Wt*, wild type. *C*, intrinsic and ribosome (*Rbs*)-stimulated ATP hydrolytic activities of purified His_6P915L and His_6eEF3 were measured. The pM P_i released from [γ-^{32}P]ATP are shown after subtracting the hydrolysis in the presence of buffer alone. The results are an average of three experiments and the S.D. shown. *D*, yeast extracts were prepared from strains containing eEF3 (TKY597), His_6eEF3 (TKY702), and His_6P915L (TKY819), and equal amounts of total protein were incubated with Ni^{2+}-NTA beads. Extract (*E*, 5%), supernatant (*S*, 5%), and pellet (*P*, 100%) were separated by SDS-PAGE and analyzed by Western blot. The blot was probed with both anti-eEF3 and anti-eEF1A antibodies. *E*, eEF1A, His_6eEF3, and His_6P915LeEF3 proteins were purified and ran on a SDS-PAGE gel and stained with GelCode Blue (Pierce). *F*, a 5-fold molar excess of purified eEF1A, either alone or with purified His_6eEF3 or His_6P915L proteins, were incubated with Ni^{2+}-NTA beads. Supernatant (*S*, 5%) and pellet (*P*, 100%) were separated by SDS-PAGE and analyzed by Western blot with an anti-eEF1A antibody.

ciated with eEF1A overexpression *in vivo* (26). To identify the eEF1A region involved in binding to eEF3, purified wild type His_6eEF1A from yeast and His_6 fusions of domain I (1–221, 22 kDa,), II (222–332, 11 kDa), or III (333–458, 33 kDa) purified from *E. coli* (Fig. 5*A*) was used to determine GSTeEF3 binding by Ni^{2+}-NTA pulldowns. GSTeEF3 was pulled down only by wild type His_6eEF1A and His_6-domain III (Fig. 5*B*). No GSTeEF3 binding was seen by either domains I or II.

DISCUSSION

Protein synthesis in yeast relies not only on the availability of the eEF1AB$\alpha\gamma$ complex and eEF2 but also another unique factor, eEF3. The absolute dependence of the pathogenic fungal translation machinery on the presence of eEF3 can be exploited as a fungal-specific drug target (37). To achieve this long-term goal, our primary aim is to understand the role of eEF3 in protein synthesis. Previously published work has assigned eEF3 the dual roles of removing the deacylated-tRNA from the E-site of the ribosome and aiding eEF1A in the delivery of the correct aa-tRNA to the A-site. eEF3 has been shown to interact physically with both eEF1A and ribosomes. The mystery of how and when eEF3 collaborates with its interacting partners to carry out its essential steps in translation elongation is still not well understood. Recent work in bacteria confirms the allosteric link between the A and E sites (38). This supports the hypothesis that a general ribosome function is the release of deacylated tRNA from the E-site preceding the GTP hydrolysis required to deposit aa-tRNA at the A-site. This step likely involves a conformational change in the 70 S ribosome. Because bacteria lack eEF3, although the ribosome-associated ATPase RbbA has been implicated as a bacterial counterpart of eEF3 (39), the binding of the ternary complex of aa-tRNA-EF-Tu-GTP has been suggested to induce the required conformational change in the ribosome to catalyze the release of deacylated-tRNA from the E-site (38). In mammals, the ribosome-associated ATPase activity from pig liver differs from the yeast eEF3 ATPase activity in its sensitivity to translation inhibitors and nucleotide dependence (40).

Previous reports have proposed two different ribosome binding regions in eEF3, the 64 amino acids at the C terminus (22) and the N-terminal residues 98–388 (12). In the present study we report that yeast expressing eEF3 in the absence of its 64 amino acids at the C terminus are viable, and both the eEF1A and ribosome binding properties are retained by $His_6$980eEF3. Thus, the N-terminal region is likely the predominant ribosome binding site.

The family of ABC protein includes membrane-bound factors, which function in transporting solute molecules against a concentration gradient. However, the soluble members of this family, including Gcn20p, RL11 (41), eEF3, and the recently reported ARB1 (42) in yeast are also implicated in functions related to protein synthesis, ribosome biogenesis, and translation elongation. The crystal structure of several members of the class I ATPases clearly establish the phenomena of homodimerization of two ABC proteins to sandwich two ATP molecules utilizing the Walker A and B motifs of the one monomer (43, 44) and Walker C or the conserved LSGGQ motif, characteristic of only the ABC members of the ATPases superfamily, from the other monomer. It has been shown for cystic fibrosis transmembrane conductance regulator that upon ATP hydrolysis, the dimerized cassettes come apart, and this motor motion drives the transport across the membrane (45). Interestingly, the soluble members of the ABC family harbor both the cassettes in tandem in a single molecule. Our investigation

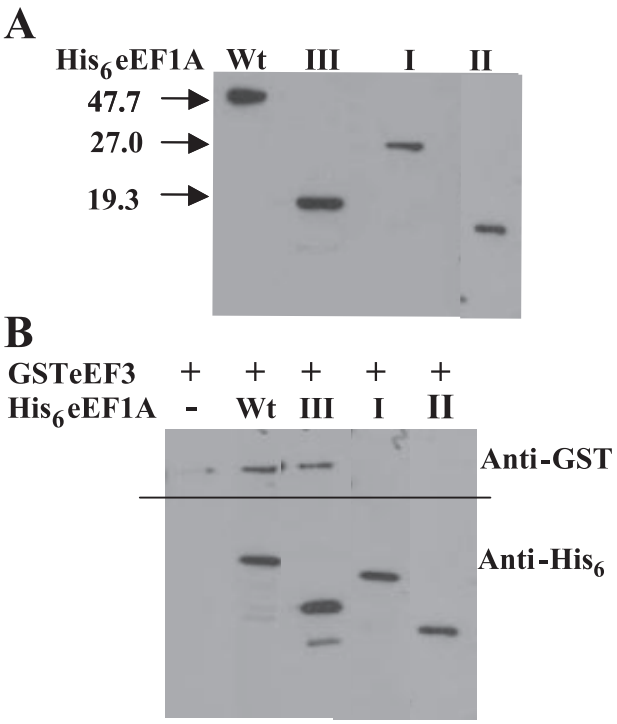

FIGURE 5. **Domain III of eEF1A binds eEF3.** *A*, His_6-tagged full-length eEF1A was purified from yeast, whereas the His_6-tagged eEF1A domains I, II, and III were expressed and purified from *E. coli* BL21 cells. Purified proteins separated by SDS-PAGE are shown by Coomassie Blue stain. *B*, Ni^{2+}-NTA pulldown was performed with 20 pM purified GSTeEF3 and 100 pM proteins from *A*. Shown is the pellet (100%) after pulldown. The *top half* of the blot was developed with anti-GST, and the *bottom half* was developed with anti-His_6 antibodies. *Wt*, wild type.

confirms the prediction that eEF3 remains a monomer under multiple conditions, including in the presence of ATP or ADP.

The binding of eEF3 to eEF1A occurs via amino acids 775–980 that can be separated into 775–910 and 910–980, the latter determined by analysis of the minimal overlapping binding sites in a series of truncation. An important determinant in the extent of the interaction of the two proteins is the nucleotide bound state of eEF3. The ATPase inactive eEF3 mutants F650S (16) and P915L (present study) are in different regions of the protein but have a similar effect on eEF1A binding. The F650S is in the region between the two ATP binding domains. The P915L mutant is in one of the eEF1A binding regions. Both lose the ability to interact with eEF1A, pointing toward hydrolysis of ATP as a critical event in eEF1A binding. Consistent with these findings, the presence of ADP stimulates eEF1A binding. This shows that a sequential order of events likely occurs during the steps catalyzed by eEF3.

EF-Tu, the bacterial homolog of eEF1A, has been shown to bind aa-tRNA predominantly via residues in domain II, whereas domain I contains the consensus GTP binding motif (46, 47). Domain III of eEF1A is responsible for the overexpression phenotype of actin cytoskeletal disorganization (26). This effect is lost for mutants located in domain III (26). This work shows that domain III (333–458) of eEF1A has another function, the interaction with eEF3. This is also consistent with the finding that neither GDP nor GTP affects eEF3 binding. Overexpression of eEF3 results in enhanced growth at all temperatures (16). This could be a result of its enhanced interaction with either eEF1A and/or ribosomes driving translation elongation forward, and hence, total translation is increased (16). If eEF3 competes with actin to bind eEF1A via domain III, then the increase in eEF3 may shift the balance of the cellular machinery in favor of protein synthesis rather than toward the function of eEF1A in cytoskeletal arrangements. This dynamic cross-talk between the two cellular processes of protein synthesis and cytoskeletal arrangement is likely mediated by the elongation factor eEF1A and may also be affected by the interaction of eEF3 *versus* actin with eEF1A.

This study supports the model that the ATP hydrolysis by eEF3 stimulates the interaction with eEF1A. This observation fits in nicely with the model of eEF3 function, where ribosome-stimulated nucleotide hydrolysis of the ATP-bound eEF3 precedes its interaction with eEF1A and the delivery of only cognate aa-tRNA at the A-site. It is still speculative if eEF1A binding occurs, whereas eEF3 is bound to or upon its release from the ribosome. The latter situation is more likely since upon ATP hydrolysis, eEF3 is likely released from the ribosome.

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Domain and Nucleotide Dependence of the Interaction between *Saccharomyces cerevisiae* Translation Elongation Factors 3 and 1A

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