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

Received for publication, February 28, 2006, and in revised form, August 30, 2006. Published, JBC Papers in Press, September 5, 2006, DOI 10.1074/jbc.M601895200

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Eukaryotic translation elongation factor 3 (eEF3) is a fungal-specific ATPase proposed to catalyze the release of decacylated-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)2 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 catalyzes an essential step in each elongation cycle by virtue of pre-translocational ribosomal state in yeast.

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3 The abbreviations used are: eEF, eukaryotic elongation factor; GST, glutathione S-transferase; Ni2+-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.
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 decylated-tRNA is a prerequisite for corequisite 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 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 binding 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.**

**TABLE 1**

| Strains | Genotype | Source |
|---------|----------|--------|
| TKY555 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY597 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY616 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY676 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY702 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY800 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY805 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY819 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY822 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |
| TKY824 | MATα ura3-3 leu2-2 trp1-1 his3-3 pMA210 (GAL4 2p HIS3) | This study |

**Cloning, Expression, and Purification of GST and His₆-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 into TKY555 and maintained on C-Ura-His+galactose media for protein expression. Yeast cultures expressing the GST-eEF3 fusions were harvested at an A₆₀₀ 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$_{980}$ 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 *TEFI* 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 pTKB875. Plasmids pTKB852 and pTKB875 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*-³²P]ATP. Hydrolysis was allowed to proceed for 5 min at 30 °C, and ³²P 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 Ni²⁺-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 Ni²⁺-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 Ni²⁺-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).

**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$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$)/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$_2$CO$_3$ and 50 µM MgCl$_2$/well. The extent of *p*-nitrophenyl phosphate hydrolysis represents the antigen-antibody binding measured by *A$_{415}$*.
eEF3 and eEF1A Interaction

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. 1C). The same experiment was also performed for co-elution of ribosomes with the eEF3-eEF1A complex. As shown in Fig. 1C, bottom panel, RPL10e, a ribosomal protein, is absent in the bound fractions. The middle panel, Fig. 1C, 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. 1D). 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. 1E 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.

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. 1A). These include full-length eEF3 (amino acids 1–1044), 85NT, (1–775), HEAT (100–367), 15CT (910–1044), 30CT (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. 1B). The GST-tagged fusion proteins migrate at 140 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 fragments were cloned into a 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 pull-down assay with purified yeast eEF1A (100 µg) and GST-tagged eEF3, 30CT, 15CT, and 85NT fragments (20 µg). E, the yeast strains from B were grown to mid-log phase at 30 °C, diluted to equal A400, spotted as 10-fold serial dilutions, and grown at 13 or 24 °C for 2–7 days.

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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. 3A). 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. 3B). 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. 3C). Furthermore, when ATP was added
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 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$_6$eEF3 was subjected to gel filtration analysis by fast protein liquid chromatography on a Superdex 200 column (Amersham Biosciences). The elution profile of His$_6$eEF3 was determined by SDS-PAGE and Western blot with an anti-eEF3 antibody.

**eEF3 and eEF1A Interaction**

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. 4A). 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. 4B). To determine the eEF3 defect causing this effect, the ATPase activity of purified His$_6$P915L eEF3 was determined. The mutant lacks both intrinsic and ribosome-stimulated ATPase activity (Fig. 4C). 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$_6$P915L eEF3 pulls down reduced levels of eEF1A as compared with wild type His$_6$eEF3 in both cell extracts (Fig. 4D) 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 eEF1B. Domain III has been shown to interact with actin and is responsible for the non-canonical functions of eEF1A in actin binding and bundling. 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-

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**FIGURE 3.** ADP stimulates eEF1A binding to eEF3. A, 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 with 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$_6$eEF3 was subjected to gel filtration analysis by fast protein liquid chromatography on a Superdex 200 column (Amersham Biosciences). The elution profile of His$_6$eEF3 was determined by SDS-PAGE and Western blot with an anti-eEF3 antibody.


**eEF3 and eEF1A Interaction**

**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 A600, spotted as 10-fold serial dilutions, and grown at 30 or 37 °C for 2–7 days. B, strains expressing His<sub>6</sub>P915LeEF3 (TKY824) or His<sub>6</sub>eEF3 (TKY822) were monitored for total translation in [35S]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/A600 unit. Wt, wild type. C, intrinsically and ribosome (Rbs)-stimulated ATP hydrolytic activities of purified His<sub>6</sub>P915L and His<sub>6</sub>eEF3 were measured. The pII<sub>F</sub> released from [γ-<sup>32</sup>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<sub>6</sub>eEF3 (TKY702), and His<sub>6</sub>P915L (TKY819), and equal amounts of total protein were incubated with Ni<sup>2+</sup>-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, His<sub>6</sub>eEF3, and His<sub>6</sub>P915LeEF3 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<sub>6</sub>eEF3 or His<sub>6</sub>P915L proteins, were incubated with Ni<sup>2+</sup>-NTA beads. Supernatant (S, 5%) and pellet (P, 100%) were separated by SDS-PAGE and analyzed by Western blot with an anti-eEF1A antibody.

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**DISCUSSION**

Protein synthesis in yeast relies not only on the availability of the eEF1A<sub>βγ</sub> 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<sub>98</sub>eEF3. 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...
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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.

Acknowledgment—We acknowledge the assistance of Robert Wood Johnson Medical School DNA core facility.

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