Elongation factor-3 (EF-3) is an essential fungal-specific translation factor which exhibits a strong ribosome-dependent ATPase activity and has sequence homologies that may predict domains critical for its role in protein synthesis, including a domain at the N terminus, which exhibits sequence homology with Escherichia coli ribosomal protein S5. A portion of the N terminus of Saccharomyces cerevisiae EF-3 (spanning the S5 homology region) has been cloned, expressed, and purified from E. coli. UV cross-linking and filter-binding experiments revealed that the N-terminal EF-3 protein (N-term EF-3) can be specifically cross-linked to 18 S rRNA. Filter-binding assays confirmed these data, and also established that the interaction has a $K_d \sim 238$ nm. Additional evidence shows that N-term EF-3 is able to associate with yeast ribosomes and inhibit the ribosome-dependent ATPase activity of native EF-3. These data taken together suggest that at least one of the ribosome-binding sites of EF-3 may be at the N terminus, and also raises the possibility that the association of EF-3 with the fungal ribosome may be dependent upon RNA/protein interactions.

**EXPERIMENTAL PROCEDURES**

**Cloning and Plasmid Constructions**—A plasmid containing the S. cerevisiae RNA operon was digested with XbaI and EcoRI to yield a 1414-bp fragment corresponding to nt 160–1573 of S. cerevisiae 18 S rRNA. This fragment was cloned downstream of a T7 promoter in XbaI/EcoRI-digested pBSK+ (Stratagene) to yield pKS-18 S. A fragment containing nt 542–577 of E. coli 16 S rRNA was constructed by annealing the following two oligos: (sense) 5′-AATCTCGTAAAACA-GTCTACATTGCGGAAGCTTACCTGAGGCGTAAAGC-3′; (antisense) 5′-GGCTTAAACCCGATTTCCGATTAAC-GCTGACATATGCTAGTCGATCA-3′. The annealed DNA, containing EcoRI and KpnI sites at the 5′ and 3′ ends respectively, was cloned into pUC18 to yield pUC-16SF. A plasmid encoding a subfragment of 18 S rRNA (nt 588–625) downstream of a T7 promoter (pUC-18 S) was a gift from Ian Jeffrey (St. George’s Hospital, London), and pECM1RNA, containing the E. coli RNase P (M1) RNA sequence downstream of a T7 promoter, was a generous gift from Lisa Hegg (SB Pharmaceuticals).

**Cloning and Expression of N-term EF-3**—DNA corresponding to nucleotides 202–1168, encoding the S5 homology domain of EF-3 (amino acids 98–388), was amplified directly from yeast genomic DNA by a polymerase chain reaction using the following primers: (sense) 5′-CGG-GATCCAACGCAGGTAACAAGGAC-3′; (antisense) 5′-GGGTTACCAGTCCGATTAAC-GCTGACATATGCTAGTCGATCA-3′. The polymerase chain reaction fragment was cloned into the BamHI/KpnI sites of the 6 × His-expression vector pQE-30 (Qiagen). The resulting plasmid, pQE-NtermEF3, was transformed into competent E. coli M15 (pREP4) cells according to the directions of the QiAexpress Kit (Qiagen). 1 liter of bacterial cells containing the plasmid was induced with isopropyl-$\beta$-D-galactopyranoside, and the overexpressed protein was purified under denaturing conditions and then subsequently renatured following the manufacturer’s instructions.

**Purification of Native EF-3**—EF-3 was purified from S. cerevisiae cells (strain ABYS1) essentially as described previously (13). Cells were grown in YEPD media overnight, harvested, and lysed by passing through a French Press (SLM Instruments, Inc.) five times at 1100 psi. The supernatant was separated from cell debris by centrifuging at 15,000 rpm for 15 min. The supernatant was loaded onto an ATP-agarose column (Sigma). The column was washed sequentially with Buffer C (20 mM HEPES, pH 7.0, 25 mM KC1 2 mM Mg(OAc)$_2$, 1 mM (NH$_4$)$_2$SO$_4$, 0.8 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine), buffer C + 2 mM NAD, and buffer C (100 mM KC1) + 2 mM NAD. A gradient of buffer C...
containing from 200 to 500 mM KCl was used to elute EF-3. Pure EF-3 eluted at approximately 200 mM KCl. Fractions containing pure EF-3 were used without further treatment in ATPase assays.

Preparation of Yeast Ribosomes—An overnight culture of yeast cells was washed with buffer A (20 mM HEPES, pH 7.5, 10.5 mM Mg(OAc)_2, 0.5 mM EDTA, 60 mM NH_4Cl, 100 mM β-mercaptoethanol). 100 μl of a 10 mg/ml solution of lyticase were added to 30 g of cell paste and left on ice for 3.5 h. 40 ml of buffer A were then added, and the cells were lysed by passage through a French press. The lysate was then centrifuged at 17,000 rpm for 40 min in an SS-34 rotor to remove the cell debris. The resulting supernatant was then layered onto a sucrose cushion containing buffer B (20 mM HEPES, pH 7.5, 10.5 mM Mg(OAc)_2, 0.5 mM EDTA, 0.5 mM NH_4Cl, 10 mM β-mercaptoethanol, and 1.1 M sucrose) and centrifuged at 40,000 rpm in a Beckman 60Ti rotor at 4 °C for 16 h. The ribosome pellet was rinsed with 2× buffer B and then resuspended in 1× buffer B containing 50% glycerol. Ribosomes were stored at −20 °C.

Synthesis of Labeled RNA—Radiolabeled RNAs were synthesized by in vitro transcription of pKS-18 S rRNA linearized with HindIII using T7 RNA polymerase and [α-32P]UTP. Nonspecific control RNAs were synthesized from pECM1RNA linearized with KpnI and pUC18-18 S, linearized with HindIII. Unlabeled RNAs were synthesized using MEGAshortscript kit (Ambion Inc., Austin, TX).

UV Cross-linking and Filter-binding Assays—In vitro binding reactions were carried out using 200 ng of purified N-term EF-3 in a reaction volume of 30 μl containing 50 mM Tris-Cl, pH 7.5, 2 mM MgCl_2, and 100 mM KCl. Reaction mixtures containing 5 fmol of radiolabeled RNA (with or without unlabeled competitor RNAs) were incubated at 30 °C for 20 min, then transferred to Parafilm on ice and exposed to UV light (254 nm) at a distance of 4 cm for 10 min. Following treatment with RNase A (final concentration 1 mg/ml) at 37 °C for 15 min, the samples were resolved by SDS-PAGE on 10% gels. For filter-binding experiments, binding reactions were incubated for the indicated times at 30 °C and then filtered immediately on 0.45 μm nitrocellulose filters (Whatman), washed with 1.0 ml of binding buffer, and then counted in a scintillation counter.

ATPase Activity Assays—A colorimetric assay adapted from Chan et al. (14) was used to assess ribosome-dependent EF-3 ATPase activity with the following modifications. Each 40 μl of assay contained 2.4 pmol of purified EF-3, 18 pmol of 80 S ribosomes, 12 nmol of ATP, and different amounts of N-term EF-3 in 1× assay buffer (50 mM Tris-Cl, pH 8.5, 100 mM KCl, 10 mM MgCl_2). After a 30-min incubation at 30 °C, the reaction was terminated by the addition of 75 μl of malachite green mixed reagent, and incubation was continued at room temperature for 5 min. The plates were then read at 655 nm in an enzyme-linked immunosorbent assay plate reader.

Binding of N-term EF-3 to Yeast 80 S Ribosomes—5 pmol of yeast 80 S ribosomes were incubated with native or N-term EF-3 in buffer B (25 mM Tris-Cl, pH 7.5, 10 mM Mg(OAc)_2, 50 mM NH_4Cl, 1 mM dithiothreitol, 3% glycerol) in a total volume of 30 μl for 10 min at 30 °C. To detect proteins that bind to ribosomes, reaction mixtures were layered onto 5% sucrose cushion at the N terminus of EF-3 with B. stearothermophilus and E. coli. An alignment of the N terminus of EF-3 with B. stearothermophilus S5 revealed 26% identity and 55% similarity between the two proteins (9), including a region of S5 predicted to interact with rRNA (15). Based upon these sequence similarities, as well as studies on the interaction between S5 and 16 S rRNA (10–12), we were interested to determine if this region within the N-terminal domain of EF-3 could interact with 18 S rRNA in an analogous manner. We cloned the N terminus of EF-3 (amino acids 98–388) as a His-tagged fusion protein, and expressed the resultant protein in E. coli. Fig. 1 shows the purification of the protein (referred to here as N-term EF-3) under denaturing conditions. By SDS-PAGE and Coomassie blue staining, we judge the protein preparation to be highly enriched for N-term EF-3, with only minor smaller molecular weight contaminants.

To examine whether N-term EF-3 was capable of interacting with 18 S rRNA, a UV cross-linking assay was used (Fig. 2). This analysis revealed an RNA/protein cross-link which migrated at ~35 kDa on the gel, in accordance with the size of the recombinant protein (Fig. 3, lane 1). Addition of 5-, 10-, and 250-fold molar excess of unlabeled competitor RNAs indicated that fragments of E. coli 16 S rRNA (nt 542–577, lanes 8–10) and S. cerevisiae 18 S rRNA (nt 588–625, lanes 5–7) were unable to compete for the cross-link, while addition of unlabeled specific competitor 18 S rRNA (lanes 2–4) did effectively compete. These results indicate that N-term EF-3 is able to form a cross-link with 18 S rRNA, and that this interaction is specific.

In an attempt to further characterize the interaction between N-term EF-3 and 18 S rRNA, a filter-binding assay was established. Binding reactions were performed as described under “Materials and Methods.” These results, shown in Fig. 3, demonstrate that the addition of 125-fold molar excess of unlabeled specific competitor 18 S rRNA results in a 66% decrease in binding of N-term EF-3 to labeled 18 S rRNA, while the
addition of other nonspecific RNAs does not appreciably compete binding. These results are in good general agreement with the UV cross-linking data shown in Fig. 2, and further supports the notion that the interaction between N-term EF-3 and 18 S rRNA is specific. An additional filter-binding experiment was done using a range of purified N-term EF-3 in molar excess of 18 S rRNA (Fig. 4). The results indicate the apparent binding site(s) on ribosomes or rRNA.

The results described above suggest that the N terminus of EF-3 interacts specifically with 18 S rRNA, suggesting that native EF-3 may interact with ribosomal RNA via its N-terminal domain. To directly address the question whether N-term EF-3 could interact with ribosomes, purified native and N-term EF-3 were incubated with yeast 80 S ribosomes. Factors associated with ribosomes were sedimented through a glycerol cushion, and resolved via SDS-PAGE. An immunoblot analysis showed that native EF-3 (Fig. 5A, lane 2) and N-term EF-3 (Fig. 5B, lane 5) do not pellet through the glycerol cushion in the absence of 80 S ribosomes. As expected, native EF-3 associates with yeast 80 S ribosomes (Fig. 5A, lane 4). A similar experiment was done using increasing amounts of N-term EF-3 bound to ribosomes. Fig. 5B shows that N-term EF-3 is able to specifically associate with yeast 80 S ribosomes (lanes 3 and 4), further supporting the hypothesis that the N terminus of EF-3 can interact with ribosomes.

Because the ATPase activity of EF-3 is enhanced by two orders of magnitude in the presence of yeast ribosomes (3, 6), it is likely that this activity requires a direct contact between EF-3 and ribosomal proteins and/or 18 S rRNA. We next asked whether the ribosome-dependent ATPase activity of native EF-3 could be inhibited by the addition of excess N-term EF-3. When increasing amounts of N-term EF-3 were added to the reaction, a concomitant decrease in the ribosome-dependent ATPase activity of native EF-3 was observed (Fig. 6); For example, at a 25-fold molar excess of N-term EF-3 to native EF-3, we observed almost a 50% decrease in the ATPase activity of native EF-3 stimulated by yeast ribosomes. This dramatic inhibition of ATPase activity was not seen in control experiments when excess bovine serum albumin was added to the reaction (Fig. 6). The results of these experiments suggest that N-term EF-3 is able to inhibit the ribosome-dependent ATPase activity of native EF-3, presumably by competing for the EF-3 binding site(s) on ribosomes or rRNA.

Fig. 3. Filter-binding assays also reveal that N-term EF-3 binds specifically to 18 S rRNA. Radiolabeled 18 S rRNA was incubated with purified N-term EF-3 for 10 min at 30 °C in the absence or presence of unlabeled nonspecific RNAs (18 S, RNA, nt 588–624, or E. coli M1 RNA) or specific competitor (18 S rRNA). Binding reactions were filtered on 0.45 μm nitrocellulose filters and washed with binding buffer prior to scintillation counting.

Fig. 4. Filter-binding assay reveals that N-term EF-3 binds to 18 S rRNA with an apparent Kd of 238 nM. 8.0 pmol of radiolabeled 18 S rRNA was incubated with increasing amounts of purified N-term EF-3 for 10 min at 30 °C, and then filtered and washed as described under “Experimental Procedures.” The maximal binding activity was normalized to 1.0 in this experiment.

Fig. 5. N-term EF-3 binds to yeast 80 S ribosomes. A, binding of native EF-3 to yeast 80 S ribosomes and analysis of ribosome-bound proteins was performed as described under “Experimental Procedures.” An immunoblot using a polyclonal antibody against native EF-3 revealed EF-3 to be associated with 80 S ribosomes. 5 pmol of EF-3 was run as a marker in lane 1; lane 2, EF-3 (in the absence of 80 S ribosomes) sedimented through the glycerol cushion; lane 3, 80 S ribosomes alone; lane 4, 5 pmol EF-3 plus 5 pmol of 80 S ribosomes. B, N-term EF-3 was incubated in the absence or presence of yeast 80 S ribosomes as described above, and an immunoblot of ribosome-associated proteins was performed using a monoclonal antibody against the His tag (Qiagen). Lane 1, 5 pmol of N-term EF-3 as a marker; lane 2, 80 S ribosomes alone; lane 3, 5 pmol of N-term EF-3 plus 5 pmol of 80 S ribosomes; lane 4, 15 pmol of N-term EF-3 plus 5 pmol of 80 S ribosomes (lanes 3 and 4), further supporting the hypothesis that the N terminus of EF-3 is able to specifically associate with 80 S ribosomes.

Fig. 6. N-term EF-3 inhibits ribosome-dependent ATPase activity of native EF-3. Purified, native EF-3 was incubated with S. cerevisiae 80 S ribosomes with increasing amounts of N-term EF-3 (□□□□ or bovine serum albumin (●●●●), and ATPase activity was determined by a colorimetric assay (see “Experimental Procedures.”).
DISCUSSION

Sequence comparisons between *E. coli* ribosomal protein S5 and *S. cerevisiae* EF-3 have revealed some degree of homology, particularly with the N-terminal domain of EF-3. Because S5 has been shown to interact with 16 S rRNA (10–12), we wished to test the possibility that EF-3 might make contact with 18 S rRNA via its N terminus. A 291-amino acid region of EF-3 (N-term EF-3) encompassing the S5 homology domain was expressed as a His-tagged protein in *E. coli*, and two independent biochemical assays were used to show a specific interaction between 18 S rRNA and N-term EF-3. That a specific RNA/protein cross-link could be induced by UV light is intriguing, particularly in light of the fact that this region of EF-3 lacks any of the canonical motifs common to many RNA-binding proteins (16). However, the failure to detect these motifs should not be taken to reflect an inability to interact with RNA. More work is needed to precisely identify and characterize the amino acid residues of N-term EF-3 involved in making contact with RNA.

Previously, Kovalchuke and Chakraburtty (17) reported an interaction between EF-3 and polynucleotides, with preferential binding to poly(G). While they showed that both 18 S and 26 S rRNA could effectively inhibit activation of EF-3 ATPase by yeast ribosomes, they did not examine whether EF-3 could interact directly with either of these RNAs in an isolated system. The results of our RNA-binding experiments demonstrate a physical interaction between N-term EF-3 and 18 S rRNA that is specific. While we did not examine the binding of N-term EF-3 to 26 S rRNA, it is possible that different domains of EF-3 are required for contacting different regions of the ribosome (see below).

Because the C terminus of EF-3 is very basic and contains three clusters of lysine residues, it has been speculated that EF-3 may interact with yeast ribosomes via this region. In support of this, Uritani *et al.* (18) reported that a monoclonal antibody specific for the C-terminal region of EF-3 inhibits its ribosome-dependent ATPase activity. Other work has reported that a GST-fusion protein containing the C-terminal domain of EF-3 remains associated with yeast ribosomes, suggesting that at least one of the ribosome-binding sites of EF-3 resides within this region (19). Our observations that the N-terminal domain of EF-3 binds 18 S rRNA and also associates with ribosomes do not necessarily conflict with the results described above with the C-terminal domain. The two results can be easily reconciled by taking into account the fact that multiple contacts may exist between EF-3 and ribosomes or rRNA. It may be possible that some regions of EF-3 (like the N terminus) may interact directly with rRNA, while other domains are responsible for making protein-protein contacts. Further dissection of the putative protein and RNA-binding domains of EF-3 will help to elucidate the complex nature of the interactions described above, and may provide additional information regarding the functions of EF-3 in fungal translation.

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