Elongation factor 3 (EF-3) is an essential requirement of the fungi for translational elongation. EF-3 is an ATPase, and the hydrolytic activity is stimulated 2 orders of magnitude by yeast ribosomes. Limited trypsinolysis of EF-3 results in the cleavage of a single peptide bond between residues 774 (Arg) and 775 (Gln), generating polypeptides of approximate molecular mass 90 and 30 kDa. The 90-kDa fragment is relatively resistant to proteolysis and retains ribosome-independent ATPase activity. The 30-kDa fragment is further proteolyzed into smaller fragments and retains the specificity for binding to yeast ribosomes. Both the intact EF-3 and the 30-kDa fragment are protected from proteolysis by yeast ribosomes. EF-3 is NH$_2$-terminally blocked, and so is the 90-kDa fragment. The COOH terminally derived 30-kDa fragment contains glutamine (residue 775) at the NH$_2$-terminal end. A construct was designed representing the COOH-terminal domain of EF-3 (30-kDa fragment), subcloned, and expressed as a glutathione S-transferase fusion in yeast. The glutathione S-transferase-30-kDa peptide remains stringently associated with ribosomes. Isolated fusion peptide rebinds to yeast ribosomes with high affinity. Based on these results, we propose that at least one of the ribosome-binding sites of EF-3 resides at the COOH-terminal end of the protein.

Eukaryotic elongation factor 1 (EF-1a) and factor 2 (EF-2) are functional homologs of bacterial elongation factors EF-Tu and EF-G (1) and are essential requirements for translational elongation in all organisms (2). Fungal ribosomes uniquely require an additional protein, elongation factor 3 (EF-3), for peptidyl chain elongation reaction (3–5). Biochemical and immunological analysis of non-fungal eukaryotes show the absence of EF-3 (5, 6). EF-3 stimulates EF-1a-dependent binding of aminoaoyl-tRNA to the ribosomal A-site when the E-site is occupied by deacylated tRNA (7). EF-3 induces conformational change of ribosomes from the post- to the pretranslocational state, facilitating release of deacylated tRNA from the E-site with concomitant binding of aminoaoyl-tRNA to the ribosomal A-site (7–9).

EF-3 is an ATPase. The hydrolytic activity of EF-3 is enhanced 2 orders of magnitude by yeast ribosomes due to the increase in the turnover rate of EF-3-ATPase (10–12). The ATP hydrolytic activity of EF-3 is essential for its function in translation (7–12). The amino acid sequence of EF-3, derived from the DNA sequence data, reveals the presence of duplicated nucleotide-binding motifs referred to as ABC cassettes or Walker boxes (13–15). EF-3 binds 2 mol of ATP per mol of protein (16). The two ATP-binding sites of EF-3 display a positive co-operativity (11). By directed mutagenesis, we have identified a critical lysine residue in the second ABC cassette that is indispensable for the biological function of EF-3.

The structural gene for EF-3 has been cloned and sequenced from four fungal species (14–20). Gene disruption analysis (14) combined with the existence of conditionally defective EF-3 mutants (21, 22) confirm indispensability of the EF3 gene in yeast.

Several structural features of the EF-3 protein are noteworthy. Besides the duplicated nucleotide-binding motifs, EF-3 contains three blocks of lysine residues at the COOH-terminal end (23). Of the last 57 residues of EF-3, 50% are charged. Of these, 40% are basic (24). The conserved lysine and the arginine residues are implicated in ribosomal protein/rRNA interaction (25). EF-3 binds to the guanine-rich sequences of 18 S and 26 S rRNA (26). In this communication, we report the results of our studies defining the ribosome-binding domain of EF-3 using biochemical and molecular biological tools.

**EXPERIMENTAL PROCEDURES**

All biochemical materials were obtained from standard sources as described in previous communications (11). Radiolabeled ATP was obtained from Amersham Corp. TPCK-treated trypsin was obtained from Worthington; soybean trypsin inhibitor was from Sigma.

**Buffers**—The following buffers were used. Buffer A: 0.1 M Tris/HCl, pH 8.0; Buffer B: 25 mM Tris/Cl, pH 7.5, 10 mM Mg(OAc)$_2$, 50 mM NH$_4$Cl, 1 mM dithiothreitol, 3% (v/v) glycerol; Buffer C: 60 mM Tris acetate, pH 8.0, 5.0 mM Mg(OAc)$_2$, 20 mM NH$_4$Cl, 10 mM dithiothreitol; Buffer D: same as buffer B plus 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride; Buffer E: 60 mM Tris acetate, pH 7.0, 50 mM NH$_4$Cl, 5 mM Mg(OAc)$_2$, 20 mM spermidine, 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol, 0.008 trypsin inhibitory units/ml; Buffer F: 20 mM Tris/HCl, pH 7.6, 3.5 mM MgCl$_2$, 100 mM NH$_4$Cl, 6 mM 2-mercaptoethanol, 0.05 mM spermidine; Buffer G: 20 mM Tris/HCl, pH 7.5, 3 mM MgCl$_2$, 600 mM KCl, 6 mM 2-mercaptoethanol; Buffer H: 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol; Buffer J: 50 mM Tris/HCl, pH 7.5, 50 mM NH$_4$Cl, 7 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 25% (v/v) glycerol.

**Preparation of Ribosomes and Ribosomal Subunits**—Cell-free extracts (S-30), postribosomal supernatants (S-100), ribosomes, and ribosomal subunits from *Saccharomyces cerevisiae* were prepared essentially as described (11). *Escherichia coli* ribosomes were prepared according to the procedures of Minks et al. (27). The concentrations of ribosomes and subunits were determined taking 1 A$_{260}$ unit to be equivalent to 18.5 pmol of 80 S ribosomes, 24 pmol of 70 S ribosomes, 56 pmol of 40 S subunits, and 27 pmol of 60 S subunits. SDS-polyacrylamide gel electrophoresis was performed on 10% gels as described (28). For immunoblotting, proteins were electrophoretically transferred to polyvinylidene difluoride membrane at 4°C for 4 h at 100 V using a Bio-Rad mini transblot unit. Proteins transferred to polyvinylidene difluoride membranes were developed with polyclonal antibody to EF-3.

1 The abbreviations used are: EF, elongation factor; TPCK, L-1-tosylamide–2-phenylethyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

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Isolation of EF-3—EF-3 was purified to homogeneity from an overexpressing yeast strain containing plasmid-borne YEF3 gene by a modified protocol of Kamath and Chakraburtty (22). The detailed purification protocol will be published elsewhere.

Proteolytic Cleavage of EF-3—EF-3 (0.6 mg/ml) in Buffer A was digested at 0°C with TPCK-treated trypsin at an EF-3/trypsin ratio of 125:1 (w/w). In experiments in which EF-3 was treated with trypsin in the presence of ribosomes or ribosomal subunits, EF-3 was preincubated at 30°C for 10 min in Buffer B with appropriate ribosomal preparations at a ribosome:EF-3 molar ratio of 2:1. The reaction mixtures were digested at 0°C with TPCK-treated trypsin at an EF-3/trypsin ratio of 10:1. Aliquots were removed at various time intervals as indicated in the figure legends, and reactions were terminated with 5-fold excess of soybean trypsin inhibitor (w/w) over trypsin. Samples were analyzed for ATPase activity in the presence and the absence of ribosomes according to our previously published protocol (11). All samples were analyzed by SDS-PAGE (28).

Isolation of Fusion Peptide—GST-EF-3 fusion constructs were expressed in yeasts according to the protocols described elsewhere (30).3 Yeast vectors and the appropriate yeast strains for the expression of GST fusion protein were obtained from Dr. Robert Deschenes (University of Iowa, Iowa City). Truncated EF-3 protein was expressed as a GST fusion to facilitate separation from the wild type EF-3. Preparation of cell-free extracts and isolation of ribosomes were carried out as described.3 The GST-30-kDa fusion peptide was found to be stringently associated with the ribosomal fraction. Therefore, for the isolation of the fusion peptide, ribosomes (100 A260 units/ml) were incubated with 90 ml puromycin in Buffer F for 5 min at 30°C, and solid KCl was added to a final concentration of 0.5 M, layered on top of a 33-ml linear sucrose gradient (15–33%) in Buffer G. Gradients were centrifuged for 12 h at 131,000 × g in a SW 28 rotor. The fusion peptide was recovered from the top of the gradient, concentrated, and dialyzed four times for 1 h each against 250 ml of Buffer H. The dialyzed protein was applied to a glutathione-Sepharose column (bed volume, 10 ml) preequilibrated with Buffer H. After washing the column with 250 ml of Buffer H containing 150 mM NaCl, proteins bound to glutathione-Sepharose beads were eluted with Buffer H containing 20 mM glutathione. The peak fractions were pooled, concentrated, and dialyzed four times for 1 h each against 250 ml of Buffer J.

Binding of GST Fusion Peptide to 80 S Ribosomes—Yeast ribosomes were incubated with increasing concentrations of the purified GST-30-kDa peptide in Buffer B at 30°C for 10 min. To detect ribosome-bound proteins, reaction mixtures were layered on top of 1-ml cushions of Buffer D containing 10% (v/v) glycerol and centrifuged for 1 h at 50,000 rpm in a Beckman Instruments TLA 100.2 rotor. Pelleted ribosomes were rinsed with the same buffer in order to remove loosely associated proteins. Ribosomal pellets were resuspended in SDS sample buffer and subjected to electrophoresis on SDS-PAGE. Separated proteins were electrophorized onto a polyvinylidene difluoride membrane for Western blot analysis.

ATPase Activity Assays—The nucleotide hydrolytic activity of EF-3 was measured in standard assay conditions (12). The reactions were carried out in a 50-μl volume of Buffer C for 5 min at 30°C. For the ribosome-stimulated EF-3 ATPase activity assays, 6.3 pmol of yeast 80 S ribosomes were included in the reaction mixtures in addition to the other components. The amount of 32P released from [γ-32P]ATP was measured according to standard protocol (11).

RESULTS

In an attempt to define the functional domains of EF-3, highly purified protein was subjected to mild proteolysis. EF-3 was treated with TPCK-trypsin at 0°C and at a trypsin/EF-3 ratio of 1:125. Data presented in Fig. 1 show the time course of EF-3 fragmentation. The Coomassie Blue-stained gel (Fig. 1A) shows that EF-3 was degraded into two major fragments of approximate molecular mass 90 and 30 kDa. The 90-kDa fragment, designated as N peptide, appeared within 5 min of digestion and remained stable to further proteolysis up to 60 min. The 30-kDa fragment, designated as C1 peptide, was further degraded into smaller fragments during the course of digestion.

Proteolytic products generated were also visualized by immunoblotting with polyclonal antibodies raised against intact EF-3 protein (Fig. 1B). The two low molecular weight fragments having estimated molecular masses of 23 and 15 kDa (designated as C2 and C3 peptides, respectively) were generated from further degradation of the C1 peptide. Close examination of Fig. 1B revealed that the C1 peptide appeared at 5 min, after which the intensity of the fragment continued to diminish and was completely degraded after 20 min of digestion. The disappearance of fragment C1 followed the appearance of fragments C2 and C3. All three peptides disappeared by the end of 60 min of proteolysis.

The effect of trypsinicalavage on the activity of EF-3 was followed by ATPase activity assays (Fig. 2). The time course of ATP hydrolysis revealed that about 75% of the intrinsic ATPase activity (ribosome-independent) of EF-3 was retained at the end of digestion (60 min). Ribosome-stimulated ATPase activity of EF-3 was rapidly inactivated within the first 10 min of reaction. The Western blot and the Coomassie Blue-stained gels show complete disappearance of intact EF-3 by 20 min of trypsin digestion (Fig. 1A and B, lanes 4–6). Therefore, the intrinsic ATPase activity observed at the end of digestion (60 min) is attributed to the N peptide and is not due to the presence of a residual amount of intact EF-3.

In an attempt to identify the subdomain of EF-3 involved in direct interaction with ribosomes, we asked the question whether ribosomes will protect EF-3 from proteolytic cleavage and, if so, whether the protective effect is specific for yeast ribosomes. When EF-3 was treated with trypsin in the presence of ribosomes, the trypsin/EF-3 ratio was 1:10 compared with the ratio of 1:125 in the absence of ribosomes. The proteolytic products of EF-3 were subjected to SDS-PAGE and were analyzed by immunoblotting rather than by Coomassie Blue staining. This is to avoid interference by the ribosomal proteins in visualizing the tryptic fragments of EF-3. The concentration of trypsin used in these analyses has been shown to have minimal effect on the overall ribosomal conformation (32). The results from the experiments on the protective effect of yeast ribo-

\(^3\) O. Kovalchuke, E. Pladies, and K. Chakraburtty, submitted for publication.
somes on the trypsinolysis of EF-3 are presented in Figs. 3–5. Data presented in Fig. 3 show the comparative proteolysis of EF-3 with trypsin in the absence and the presence of yeast 80 S ribosomes. The digestion pattern in the absence of ribosomes (Fig. 3A, lanes 1–5) is identical to the one shown in Fig. 1. It is evident that the rate of EF-3 proteolysis was significantly slower in the presence of ribosomes. Much of EF-3 remained intact as judged from the intensities of the EF-3 bands (Fig. 3A, lanes 1–5). The two primary proteolytic products of EF-3 (N and C1 peptides) were protected from further degradation in the presence of ribosomes (Fig. 3B, lanes 1–5). The protective effect was best seen for the C1 peptide. In the presence of ribosomes, C1 peptide was protected from further degradation, and hence the peptide accumulated during the time course of digestion (Fig. 3B, lanes 1–5). In the absence of ribosomes, C1 peptide was further degraded into C2 and C3 peptides (Fig. 3A, lanes 1–5). The molecular mass of the N peptide (90 kDa) remained unaltered either in the absence or in the presence of ribosomes (Fig. 3, A and B).

To demonstrate that the protective effect of yeast ribosomes on the C1 peptide is due to a direct physical association, ribosomes from the above reactions were isolated immediately after the termination of tryptic digestion. Immunoblot analyses of the ribosomal pellets showed that most of the C1 peptide remained associated with the 80 S ribosomes along with the undigested EF-3 (Fig. 3C, lanes 1–5). In contrast, the N peptide showed minimal binding and retention in the ribosomal fractions (compare the banding pattern in Fig. 3, B and C, lanes 1–5).

We have previously demonstrated that, in heterologous translational systems, the requirement for EF-3 depends on the source of the 40 S subparticles (6). Based on this observation, we analyzed the protective effects of the 40 S and the 60 S subunits on the proteolysis of EF-3. The Western blot data presented in Fig. 4 indicate that both 40 S and 60 S subunits protected EF-3 from trypsin to the same extent (Fig. 4, B and C, lanes 2–5) but showed differences in the protection of C1 peptide. In spite of the functional specificity of EF-3 for the 40 S subunits (6), the C1 peptide appeared to be better protected by 60 S subunits (Fig. 4C, lanes 2–5). This, we assume, could be due to the higher binding affinity of EF-3 for the 60 S subparticles. Experimental data from the direct binding studies of EF-3 with ribosomes and subunits support this conclusion.3 In any case, intact 80 S ribosomes provided the maximum protection to EF-3 and to C1 peptide against trypsinolysis (Fig. 4D, lanes 2–5).

The specificity of EF-3/ribosome interaction was further analyzed by comparing the tryptic digestion pattern of EF-3 in the presence of yeast 80 S and E. coli 70 S ribosomes. Data presented in Fig. 5A demonstrate that neither the yeast 80 S nor the E. coli 70 S ribosomal proteins were degraded significantly under the reaction conditions used in these analyses. EF-3 was extensively degraded in the presence of E. coli ribosomes (Fig. 5A, lanes 10–13, boxed area), whereas yeast 80 S ribosomes, as expected, protected EF-3 from proteolysis. Results of the immunoblot analyses presented in Fig. 5B substantiate the same
conclusion. The C1 peptide appears to be somewhat protected by E. coli ribosomes (Fig. 5B, lanes 10–12). However, compared with 80 S ribosomes, the protective effect of E. coli 70 S ribosomes is insignificant.

A molecular biological approach was taken to substantiate the experimental data obtained from the proteolytic cleavage analyses. For this, a construct was designed representing the C1 peptide containing all of the COOH-terminal basic amino acid residues of EF-3 including the three polylysine blocks (23). This construct was designated as 30-kDa YEF3. The subcloned EF-3 fragment was expressed as a GST fusion in yeast in order to facilitate separation of the truncated protein from the chromosomally expressed wild type EF-3. Subcloning, overexpression, and purification of the GST-30-kDa fusion peptide will be described elsewhere.

The localization of GST-C1 peptide in the cell-free extracts prepared from yeast overexpressing the fusion protein is shown in Fig. 6. Western blot analysis revealed the presence of two immunoreactive bands in the S-30 supernatant fraction. One of these bands has the molecular mass of 116 kDa, representing the wild type EF-3 protein. The second immunoreactive band has an approximate molecular mass of 52 kDa (22-kDa mass contributed by the GST peptide), representing the GST-C1 peptide (Fig. 6B, lane 2). The fusion peptide, like the wild type EF-3, remained associated with the ribosomal fraction (Fig. 6B, lane 4). The complete absence of fusion peptide in the postribosomal supernatant fraction (S-100) indicates that, in comparison with intact EF-3, GST-C1 peptide has high affinity for 80 S ribosomes (Fig. 6B, compare lanes 3 and 4). The stringent association of the fusion peptide with 80 S ribosomes can also be seen in the Coomassie Blue-stained gel (Fig. 6A, compare lanes 3 and 4, boxed areas). The GST protein expressed from the vector lacking the C1 peptide did not bind to yeast ribosomes (data not shown). Yeast cells overexpressing the GST-C1 peptide exhibited a slow growth phenotype (data not shown). We presume that a pool of ribosomes becomes unavailable for translation due to irreversible association of the fusion peptide to yeast ribosomes.

The binding of the purified GST fusion peptide to yeast ribosomes is shown in Fig. 7. As a control, the binding of EF-3 to 80 S ribosomes was also included in these analyses. Increasing amounts of purified fusion peptide were incubated with yeast ribosomes. The presence of the fusion peptide in the reconstituted ribosomal fractions was detected by immunoblot analysis. As can be seen, much like intact EF-3 (Fig. 7B, lane 4), isolated 30-kDa-GST fusion peptide remained bound to yeast 80 S ribosomes (Fig. 7B, lanes 5–8).

**DISCUSSION**

Biochemical and genetic investigations described in this communication provide evidence that the COOH-terminal end of EF-3 is involved in ribosome binding. Using proteolysis as a tool, we have demonstrated that the 116-kDa EF-3 protein is organized into two functional domains. The NH2 terminally derived large (90-kDa) subdomain of EF-3 containing the two ATP-binding cassettes is catalytically active in ribosome-independent functions. The COOH terminally derived small (30-kDa) domain retained the ribosome-binding activity. The NH2-terminal domain of EF-3 is relatively resistant to proteolysis, indicating that this part of the molecule may have a compact structural organization. On the other hand, the COOH terminally derived domain is relatively exposed and is further proteolized into smaller fragments.

The NH2-terminal end of the N peptide is blocked and, therefore, it was not possible to obtain the sequence information for this peptide. Amino acid glutamine was identified as the NH2-terminal residue of the C1 peptide. A cleavage between residues 774 (Arg) and 775 (Gln) is consistent with the approximate molecular masses of the two peptides. Analyses of the C2 and C3 peptides (Fig. 1) indicate that all three peptides (C1, C2, and C3) contain glutamine at the NH2-terminal end (data not shown). The proposed primary trypsin cleavage site of EF-3 is shown below.

\[
\text{(EF-3 \sim 116 kDa)}
\]

\[
\begin{align*}
\text{NH}_2 & \quad \text{-----} \quad \text{R} \quad \text{-----} \quad \text{COOH} \\
(90 \text{kDa}) & \quad (30 \text{kDa}) \\
\text{NH}_2 & \quad \text{-----} \quad \text{R} \quad \text{-----} \quad \text{COOH}
\end{align*}
\]

**Diagram 1**
The amino acid sequence derived from the DNA sequence data indicates that the COOH-terminal end of EF-3 is highly charged. Of the last 57 amino acid residues, 60% are charged. Of these, 40% are basic (23). The positively charged lysine and arginine residues are organized in four distinct blocks as follows: RIEKKEDDEKFDAMGKNIAGGKKKKLSSAE LRKKKERMKKKELGDAYVSSDEEF. The hydropathy test of this region of EF-3 suggests that the COOH-terminal end of the protein is exposed and, therefore, may interact with negatively charged molecules such as RNA and acidic ribosomal proteins (24). We have previously demonstrated a direct interaction between EF-3 and rRNA (26). Site-specific mutational analyses are planned to identify the amino acid residues of EF-3 involved in the direct interaction with rRNA. A number of ribosomal proteins and translational factors are known to contact ribosomes through the COOH-terminal end (31, 33, 34).

In that respect, yeast EF-3 seems to follow the same reaction mechanism. In the case of the bacterial elongation factor EF-G, the protein associates with ribosomes via the COOH-terminal Gln-495 and Gly-502 residues (33). Deletion or point mutations involving these amino acids severely affect the ribosome-dependent functions of bacterial EF-G (33). The basic amino acid residues in the COOH-terminal region of the yeast ribosomal protein L1 (34) and the bacterial ribosomal protein L6 (31) have been implicated as the binding sites for rRNA. In the case of yeast EF-3, the NTPase activity of the protein is enhanced 2 orders of magnitude by yeast ribosomes (6). It will be of interest to learn how information concerning the nucleotide-bound state of EF-3 is transmitted into its ribosome-binding domain (COOH-terminal domain) and its role in the conformational change of yeast ribosomes from the post- to the pretranslocational states during the peptide chain elongation reaction. An answer to this question is of fundamental importance since higher eukaryotes carry out this reaction in the absence of a physical homolog of yeast EF-3.

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