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Evolutionary Divergence of an Elongation Factor 3 from *Cryptococcus neoformans*

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Elongation factor 3 (EF3) is considered a promising drug target for the control of fungal diseases because of its requirement for protein synthesis and survival of fungi and a lack of EF3 in the mammalian host. However, EF3 has been characterized only in ascomycete yeast. In order to understand the role of EF3 in a basidiomycete yeast, we cloned the gene encoding EF3 from *Cryptococcus neoformans* (*CnEF3*), an important fungal pathogen in immunocompromised patients, including those infected with human immunodeficiency virus. *CnEF3* was found to encode a 1,055-amino-acid protein and has 44% identity with EF3 from *Saccharomyces cerevisiae* (*YEF3*). Expressed CnEF3 exhibited ATPase activity that was only modestly stimulated by ribosomes from *S. cerevisiae*. In contrast, CnEF3 showed tight binding to cryptococcal ribosomes, as shown by an inability to be removed under conditions which successfully remove *Saccharomyces* EF3 from ribosomes (0.5 M KCl or 2 M LiCl). *CnEF3* also poorly complemented a *YEF3* defect in a diploid null mutant and two temperature-sensitive mutants which have been shown previously to be complemented well by *EF3* from other ascomycetes, such as *Candida albicans*. These data clearly identify the presence of a functioning *EF3* in the basidiomycete yeast *C. neoformans*, which demonstrates an evolutionary divergence from *EF3* of ascomycete yeast.

Cryptococcus neoformans is an important fungal pathogen which causes a lethal meningoencephalitis in a significant number of persons with AIDS and afflicts an increasing number of immunocompromised patients on steroids, chemotherapy, or posttransplant immunosuppressives (18). Therapy of cryptococcosis is limited by toxicity of such agents as amphotericin B (1); newer agents such as the azole inhibitors are less toxic, but increasing reports of resistance may limit their eventual usefulness (3, 15, 17). Echinocandins and pneumocandins are important new antifungal agents which are inhibitors of 1,3-β-glucan synthetases and show excellent activity against ascomycete pathogens such as *Candida albicans* and *Aspergillus fumigatus*, but alterations in this enzyme from basidiomycetes make this important class of agents ineffective against *C. neoformans* (27). The latter example shows the potential gap in antifungal coverage which may occur when inhibitors are chosen without consideration of possible evolutionary differences in drug targets within various fungal pathogens.

Elongation factor 3 (EF3) has been shown to be a required translation cofactor in the ascomycete *Saccharomyces cerevisiae* (7). The factor is also present in a variety of pathogenic ascomycete fungi, including *Candida albicans* (8) and the pathogen *Pneumocystis carinii* (34), which has been shown to be closely related to ascomycete yeasts based on analysis of its rRNA gene as well as genes encoding dihydrofolate reductase, thymidylate synthetase, β-tubulin, and ATP (for a review, see reference 31). While an anti-EF3 antibody has been shown to react with basidiomycete yeasts (4), EF3 has not been characterized from this class of fungi. When present, EF3 is a required translational cofactor essential for growth of the organism. EF3 is believed to modulate the inverse relationship between protein translation rate and amino acid fidelity by altering the binding affinities of the ternary complex to the ribosomal A site and that of the deacylated tRNA to the E site (29). It is unique among the translational factors in that it is not present or required in mammalian translational systems (4). This makes EF3 a putative drug target for a wide variety of fungal pathogens while offering the possibility of a favorable side effect profile in the mammalian host. Many valuable antibacterial agents such as the macrolides and aminoglycosides are inhibitors of the prokaryotic translational apparatus. These antibiotics have had a profound and long-lasting impact on the outcome of bacterial infections and show the extensive precedent for the role of translational inhibitors in the chemotherapy of infectious agents.

While studies in model yeasts such as the ascomycete *Saccharomyces* may yield important information about essential biological systems, recent advances in the molecular biology of *C. neoformans* allow the study of drug targets such as EF3 in the pathogen itself, allowing direct application of findings to the rational design of antifungal agents. The present study seeks to identify and characterize EF3 from *C. neoformans* in order to extend the role for this factor to basidiomycete fungi. This will allow further study of its properties in protein translation and may enable the design of antifungal compounds directed against recombinant cryptococcal EF3.

MATERIALS AND METHODS

Strains. *C. neoformans* ATCC 34873 was a generous gift of K. J. Kwon-Chung. *S. cerevisiae* strain BJ3505/G was from Eastman Kodak (New Haven, Conn.). *Escherichia coli* SURE (Stratagene, La Jolla, Calif.) was the host strain used for screening the cDNA library after mass excision of the Uni-Zap cDNA library. *E. coli* XL1-Blue (Stratagene) was the recipient strain of the Bluescript phagemid

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following in vivo excision for the Uni-Zap XR vector (Stratagene)-containing cDNA clones. *E. coli* DH10B (Life Technologies, Bethesda, Md.) was the host strain for recovery of ligated plasmids.

Enzyme assay. The pyruvate kinase/lactate dehydrogenase-coupled ADP assay for EF3 ATPase activity was performed according to the method of Sarthy et al.(25). Activity was expressed in nanomoles of ADP produced per minute at 30°C.

Nested PCR amplification and screening a cryptococcal cDNA library. A stationary-phase cryptococcal cDNA library in Uni-Zap described previously (32) was mass excised and inserted into *E. coli* SURE according to the manufacturer's directions (Stratagene). Library plasmid was prepared from cells (Qiagen, Valencia, Calif.) and subjected to endonuclease digestion with *Not*I. Linearized library plasmids were used as the template (50 ng) and subjected to 25 cycles of amplification by PCR using *Taq* polymerase (Life Technologies, Bethesda, Md.), an annealing temperature of 40°C, and degenerate primers constructed from amino acid sequence contained in two of the ATP-binding regions of *S. cerevisiae* EF3 (20) (primer 1360S, CCNAAYGGNTGYGGNAAA, and primer 2760A, RTARTTNGTNGGYTCRTC). Products of 1,200 to 1,800 bp were gel purified and subjected to a second round of 25 cycles of PCR amplification using an annealing temperature of 48°C and internal degenerate primers from *S. cerevisiae* EF3 (primer 1690S, GAYCCNACNAAYCAT, and primer 2100A, YTTNCCNGCNCCRTTNGG). A 420-bp single band was gel purified, labeled with [α-^{32}P]ATP, and used to screen the cryptococcal stationary-phase cDNA library by standard techniques (23). Clone p5a was selected based on the size of its insert and its ability to bind the PCR probe described above, and both strands were sequenced by automated methods (CRC-DNA Sequencing Facility, University of Chicago).

Northern and Southern blot analysis of *CnEF3*. Cryptococcal cells were grown to mid-log phase ($A_{600} < 1.0$), and total RNA was obtained as described (32). DNA was obtained from cryptococcal cells as described (30). Northern blots were performed by standard methods (23) using *CnEF3* from plasmid p5a.

Construction of expression plasmid pmyep.NS-CnEF3. A double-stranded oligonucleotide (sense, TCGACCACCACCACCACCACCACTAGGCTAGC; antisense, TAGCCTAGTGGTGGTGGTGGTGGTGG) was inserted into *S. cerevisiae* expression plasmid YEpFLAG-1 (IBI/Kodak, Rochester, N.Y.) between restriction sites *Apa*I and *Sac*II to produce the plasmid pmyep (pmyep was kindly obtained from K. Williamson). The Flag affinity tag and hydrophobic leader sequence were removed from pmyep by divergent PCR using primers YEP-1496S (GCCGCCGAATTCCTCGAGCCCGGG) and YEP-1217A (GCCGCCGAATTCTGAAGGAAATCTCATCGC) to create plasmid myep.NS. Plasmid myep.NS was selected for its correct size, ability to transform *S. cerevisiae* strain BJ3505/G by a polyethylene glycol-lithium acetate (PEG-LiAc) protocol (Stratagene), and the presence of an a *Eco*RI site. The *CnEF3* open reading frame (ORF) was PCR amplified using *Pfu* polymerase (Stratagene, La Jolla, Calif.) using primers CnEF-5Ba (GCCGCCGGATCCCTCCTGCTGCTACCGCTGCTG) and CnEF3Apa1 (GCCGCCGGGCCCAAGCTCTTCATCACTGAAGAC). The amplified product was restriction endonuclease digested with *Bam*HI and *Apa*I and inserted into the respective sites of myep.NS to produce plasmid myns.CnEF3.1. Use of the multiple cloning site of myep.NS resulted in a 43-nucleotide addition to the 5′ region of the *CnEF3* ORF, resulting in a putative polypeptide having 14 additional amino acids at the N terminus with the sequence MRFPSEFREIYRWIP. Integrity of sequence was verified by automated sequencing of the CnEF3 ORF (CRC-DNA Sequencing Facility).

Expression and purification of recombinant CnEF3. *S. cerevisiae* strain BJ 3505/G was transformed with myns.CnEF3.1 by means of a PEG-LiAc protocol (Stratagene). Transformants containing the myns.CnEF3.1 plasmid were selected by Southern blot and for ability to overexpress a 116-kDa protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (approximately 20-fold that of untransformed BJ3505/G). To induce recombinant protein, a selected transformant was grown in yeast expression medium (3% glycerol, 1% glucose, 20 mM $CaCl_2$, 1% yeast extract, 8% peptone) according to the manufacturer's instructions (Invitrogen). Cells were isolated by centrifugation and subjected to glass bead (0.45 μm) breakage in a Braun homogenizer (Germany) for 1.5 min at 4°C in buffer A containing 10 mM Tris-HCl(pH 7.5), 10 mM $MgSO_4$, 0.2 mM EDTA, and 10% glycerol. Cell debris was removed by centrifugation, and the supernatant was adsorbed on a 20-ml column of carboxymethyl (CM)-Sepharose equilibrated in buffer A, washed, and eluted with buffer A containing 100 mM KCl. Fractions were assayed by SDS-PAGE, and fractions containing the predominant 116-kDa band were pooled, dialyzed in buffer B (20 mM Tris-HCl pH[7.0], 50 mM KCl, 10 mM magnesium acetate, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT], 10% [vol/vol] PMSF, glycerol) and adsorbed on a 1-ml ATP-agarose column (Sigma) equilibrated in buffer C, and then washed and eluted with buffer C containing 2 mM ATP. Fractions were again assayed on SDS-PAGE, and suitable fractions were pooled, tested for ATPase activity, and subjected to N-terminal sequencing.

Cryptococcal ribosome isolation. The method used was based on that by Otaka and Kobata (16). Briefly, cryptococcal cells were grown in 2 liters of YPD (2% glucose, 1% yeast extract, 2% Bactopeptone) to an A_{600} of 0.8, followed by homogenization in a Braun homogenizer using 0.46-μm glass beads for 2 min at 4°C. Two milliliters of cold buffer A (50 mM Tris-HCl [pH 7.0], 50 mM NH_4Cl, 10 mM magnesium acetate, 5 mM DTT, 0.1 M EDTA, 0.2 mM PMSF, 10% glycerol) was added, and the mixture was centrifuged at 5,000 × *g* for 10 min. The supernatant was clarified by centrifuging twice at 35,000 × *g* for 15 min, and the supernatant was then centrifuged for 3 h at 4°C at 150,000 × *g*. The pellet from the 150,000 × *g* centrifugation was resuspended in buffer B (buffer A plus 0.5 M KCl) and centrifuged at 10,000 × *g* for 10 min to remove aggregates, and the supernatant was overlaid on a cushion of buffer C (buffer B plus 25% glycerol) and centrifuged at 150,000 × *g* at 4°C for 3 h, and the pellet was recovered. These two sets of low-speed and high-speed centrifugations were repeated once again in the same way, and the pellet was resuspended in buffer A containing 25% glycerol. Cryptococcal ribosomes were also prepared in the presence of buffers excluding 0.5 M KCl. Cryptococcal ribosomes were assayed by ultracentrifugation using a 5 to 30% sucrose gradient (110,000 × *g* for 10 h) and showed a protein profile similar to that of *S. cerevisiae* ribosomes prepared as described (16).

Antibody production to recombinant CnEF3. Purified recombinant cryptococcal EF3 was subjected to SDS-PAGE, and the 116-kDa band was excised, homogenized in the presence of Freund's adjuvant, and administered to 6-week-old CAF1/J mice (20 μg of protein/mouse) by intraperitoneal injection on days 0, 21, 49 and 74. Serum was obtained from the mice prior to the first injection and 1 week after the fourth injection. Western blots and dot blots were performed using the indicated amount of protein and primary antibody and a 1:1,000 dilution of horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin (Ig) antibody (Accurate Antibodies, Westbury, N.Y.).

ELISA. For the enzyme-linked immunosorbant assay (ELISA), 96-well plates were incubated with antigen (10 mg/mlμ) overnight at 4°C, washed three times with phosphate-buffered saline (PBS), and blocked with 5% milk for 1 h. The serum was then diluted as indicated with PBS and added to the wells. Following an overnight incubation at 4°C, the wells were washed PBS, and then alkaline phosphatase-labeled goat anti-mouse Ig antibody (1:2,000 dilution) was added. After incubation at room temperature for 1 h, the wells were washed with PBS, 3 mM *p*-nitrophenyl phosphate in 0.05 M $NaCO^3$–0.05 mM $MgCl_2$ was added, and the optical density of 405 nm of the wells was read on a microtiter plate reader (Titertek, Huntsville, Ala.).

Construction of complementation plasmid Y24EF-CnEF3 7. Divergent PCR in the direction away from the plasmid insert was used to remove the *S. cerevisiae EF3* ORF and insert a *Not*I site into plasmid YEpEF3 using primers YE24-EF5 (GCCGCCGCGGCCGCATCTTTTAATGTTATCGATGGATT) and YE24-EF3 (GCCGCCGCGGCCGCATCACTGCTTTCACAGTTTTCTT). The modified YepEF3 plasmid retained sufficient 5′ untranslated region and 3′ termination sequence necessary for complementation and expression in *S. cerevisiae* (24). Plasmid YEpEF3ϕ-4 was selected for its correct size, ability to transform *S. cerevisiae* strain BJ3505/G by a PEG–LiAc protocol (Stratagene), and presence of a *Not*I site. The *CnEF3* ORF was PCR amplified in from *Not*I endonuclease-treated p5a using *Pfu* polymerase (Stratagene) and primers Crypto 5 (GCCGCCGCGGCCGCCTGCTACCGCTGCTGCCTC) and Crypto 3 (GCCGCCGCGGCCGCTTAAAGCTCTTCATCACTGAAGA). The amplified fragment was ligated to *Not*I digested plasmid YEpEF3ϕ-4. Insertion of the *Not*I site resulted in a nine-nucleotide substitution after the ATG codon of the ORF which would be expected to lead to a two-amino-acid alteration of the *CnEF3* ORF at the N terminus, resulting in an expected N-terminal sequence of MRPPATA in the recombinant protein. Suitable clones were screened for the presence of a suitably sized insert in the correct orientation by means of PCR using appropriate primers. Clone Y24EF-CnEF3#7 was further characterized by automated sequencing (CRC-DNA Sequencing Facility) to establish fidelity of sequence.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned accession number AF316889.

RESULTS

Isolation and sequence of *C. neoformans* EF3 gene. Initial unsuccessful cloning attempts using full-length *S. cerevisiae* EF3 cDNA clones suggested poor conservation of nucleotide sequence of this factor between ascomycetes and basidiomycetes. Thus, a nested PCR approach was used to produce a

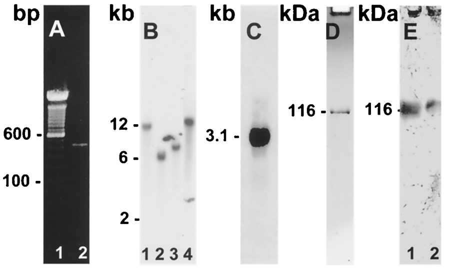

FIG. 1. Cloning and characterization of cryptococcal EF3. (A) Lane 1, 100-bp ladder; lane 2, PCR-amplified product from cryptococcal cDNA library. (B): Southern blot of *CnEF3*. Digestions: lane 1, *Spe*I and *Bam*HI; lane 2, *Spe*I and *Apa*I; lane 3, *Spe*I and *Xba*I; lane 4, *Spe*I alone. (C) Northern blot of *CnEF3*. (D) SDS-PAGE of 1 μg of recombinant cryptococcal EF3. (E) Western blot of whole-cell extract of *C. neoformans* (lane 1) and 1 μg of purified recombinant CnEF3 (lane 2).

suitable probe for cloning using fully degenerate oligonucleotide primers derived from conserved *S. cerevisiae* sequence within the ATP-binding cassettes and use of a cryptococcal cDNA template from a mass-excised stationary-phase library of *C. neoformans* to avoid untranscribed sequences. This approach yielded a single 420-bp amplified fragment of the expected size (Fig. 1A), which was then radiolabeled and used to screen a *C. neoformans* cDNA library (a gift from J. Edman). Two cDNA clones of appropriate size were obtained; p5a was selected, and both strands were sequenced. Southern blots of cryptococcal DNA showed the presence of a single-copy gene (Fig. 1B) using restriction enzymes predicted to cut outside the *CnEF3* ORF according to sequence generated from clone p5a. Northern blot analysis of *CnEF3* showed the presence of a 3.1-kb transcript which was present in cells obtained in log-phase growth (Fig. 1C).

Analysis of *CnEF3* suggests evolutionary sequence divergence from other fungi. Clone p5a was found to contain a 3,447 bp insert containing a 3,165-bp. ORF which encoded a putative 1,055 amino acid polypeptide having a calculated molecular mass of 116.4 kDa (Fig. 2). Amino acid sequence was used to query the nonredundant combined nucleotide databases of GenBank, EMBL, DDBI, and PDB using MacVector software and the Blast search engine (1). Comparison of amino acid sequence showed significant amino acid homology to EF3 of *S. cerevisiae*, especially within the two ATP-binding "cassettes" (motifs A and B). Overall conservation of amino acid sequence between *C. neoformans* and *S. cerevisiae* (44% identity) is lower than that reported between *S. cerevisiae* and *Candida* (78% identity) (8) and between *S. cerevisiae* and *Pneumocystis* (57% identity) (34). In addition, there is no serine at position 277, a proposed phosphorylation site for EF3 in *S. cerevisiae* (20). However, there were three putative phosphorylation sites that were conserved between ascomycetes and *C. neoformans*, T_{244}, T_{680}, and T_{772}. There were also observed regions of homology between ribosome-binding sites within the N terminus and C terminus of the CnEF3 protein.

Production and analysis of recombinant cryptococcal EF3. To assess the ability of cryptococcal EF3 to function with *S. cerevisiae* ribosomes, recombinant CnEF3 was heterologously expressed in *S. cerevisiae* using a new expression plasmid, pmyep.NS, designed for intracellular expression of proteins using the *ADH2*-promoter. Plasmid pmyep was constructed from the yeast expression plasmid FLAG1 by addition of a terminal histidine affinity tag, divergent PCR was used to remove the pro-α-leader sequence, and Flag affinity sequence was used to construct plasmid pmyep.NS. The *CnEF3* ORF was inserted into pmyep.NS and used to transform *S. cerevisiae*. Transformed yeast cells expressed 20-fold-higher levels of CnEF3 over background YEF3. Recombinant CnEF3 was successfully purified from native YEF3 because CnEF3 did not bind to negatively charged affinity matrices and showed only minimal binding to positively charged matrices such as DEAE-Sepharose (eluted with 0.1 M KCl) in contrast to YEF3, which binds more strongly to both (25). As shown in Fig. 1D, recombinant cryptococcal EF3 purified as a 116 kDa protein, consistent with its predicted size based on cDNA sequence. Western blot analysis of cryptococcal cell extract using antibody prepared from recombinant CnEF3 showed a single band at 116 kDa (Fig. 1E) which showed no reactivity against *Saccharomyces* cell extracts, ribosomes, or EF3 (data not shown). N-terminal sequencing of the purified factor showed exclusively the expected CnEF3 fusion protein sequence MRFPS. Steady-state kinetics of ATP hydrolysis by CnEF3 using heterologous *S. cerevisiae* ribosomes showed a ribosome-dependent alteration in K_m (709 μM with versus 318 μM without ribosome), whereas there was no significant ribosome-dependent alteration in $V_{\max}$ (11 versus 13 nM/min). This is in contrast to previous work showing a 100-fold increase in $V_{\max}$ of the *S. cerevisiae* factor with the same species ribosomes (9). The stimulation in K_m observed in the present case was also not as marked as that reported previously for *Saccharomyces* EF3 and may be due to a suboptimal interaction between CnEF3 and ribosomes from *S. cerevisiae*. It is possible that the suboptimal interaction between recombinant CnEF3 and ribosomes could have been due to the additional 14 amino acids at the N terminus of the expressed protein construct, although a lack of significant homology between the elongation factors within the first 100 amino acids makes this less likely to be a region of critical importance. Stimulation of CnEF3 by cryptococcal ribosomes could not be assessed because of an inability to remove ATPase activity from cryptococcal ribosomes, as described in the next section. Western blot of whole cryptococcal extract using an anti-CnEF3 antibody showed a single 116-kDa band, consistent with the size of the recombinant protein and the expected size based on the cDNA sequence (Fig. 1E). Anti-CnEF3 antibody did not cross-react with either cell wall extract or ribosomes from *S. cerevisiae* (data not shown).

Analysis of CnEF3 binding to cryptococcal ribosomes. Typically, extraction with 0.5 M KCl removes essentially all ATPase activity from *S. cerevisiae* ribosomes and allows the ready separation of elongation factors from ribosomal proteins in *S. cerevisiae* systems. In contrast, we found that extraction of *C. neoformans* ribosomes yielded a preparation with a large amount of residual ATPase activity which prevented assay of ribosome-dependent ATPase activity of the cryptococcal EF3. For example, ribosomes prepared in the absence of 0.5 M KCl exhibited ATPase activity of 78 nmol min^{-1} $A_{260\text{-rib}}{}^{-1}$ which, after washing three times with 0.5 M KCl, continued to show an ATPase activity of 200 nmol min^{-1} $\mathrm{A}_{260\text{-rib}}{}^{-1}$. (An increase in ATPase activity with washing may have been due to the

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C. neo   MAPAATAAAS SGKGSFDLAT LFVADKAARD EAGLALADAV KKSGVEFFTQ IGFNDAIVKA LNDKKSQSAR EGACEVISTL CENGAAQLLE PHVISSAENT
PCP      _____________________________________________________________________esKdtlAR EqAlkal-lL tldatnkrvE Pylv-rl--l
Calb     ___________________Lsk LqVADn--kD EAasnistfl nsSiVEhdvp veFfedlkKq iqsKdakvsl aaldaykhia stNGlspsvE PyVvdlvsev
Sacc     ___________________fqk LsVAtadnRh EiasevAsfl ngniiEhdvp ehFfgelaKg ikDKKt-aA- nam-qavahi -aN-qsn-Ls PsVepyivql
Vir      ___________________________________________________________fiKe viseetllkR detyrg-kym ydrlmnifLk rqfyShimNm
        101
C. neo   PFPALLEAFA DKVAAVKTAA IAAVKAIVQS MNPWASFVLL PALLNLIRTS GKWQIKAGSL EILQQLITSA PYQMGEAMPD LVPVLAGAVW DTKSDVKKAA
PCP      P-rvLkqvgl eKVAAVrTqA stvaedIikt MNPyAvktiL shvtNsIkTS GKWmeKmcaf rlLdmLvekA PcQMsyrlPe LiPiLsesmW DTrtDiKnqA
Calb     ---A-vkA-g DKnkdVqTAA sdAllAIasa itPtAvkaiL PkLidnltnt nKWteKvaiL ravsQLvdtA kaQialrMPe LiPVLsesmW DTKkeVKeAA
Sacc     -vPAictnag nKdkeiqsvA setlisIVna vNPvAikaLL PhLtNaIvet nKWQeKiaiL aafsamvdaA kdQvalrMPe LiPVLsetmW DTKkeVKaAA
Vir      sFvpmtihin eliyli-lrc InhlrkmakS fNssln-eLf sgvsNkdkvr deW-IskGS- sfinsm---A Pysvkmvlpk vfsamkdpkW qTK-ef--Ac
        201                                                                         ▼
C. neo   KATLEKAVSL VENKDIEKFV PALVKSLLNP IEEVPKTISL LSATTFVSEV TAPTISLIAP LLIRGLDERP TATKRKVCVI ADNMSKLVDS EYTVRPFLPQ
PCP      rkTmtsvctL isNpDIdKFi PvLidciaqP -EkVPeTIht LgATTFVqEV hAsTlSimvP LLyRGLnERe TtiKRKsaVI iDNMcKLVed pYiiaPFLPk
Calb     tATmtKstet idNKDIEKFi PqLisciakP -tEVPeTvhL LgATTFVSEV TmaTlSimAP LLsRGLaERd TAiKRKaaVI vDNMcKLVed pqiVaPFmdk
Sacc     tAamtKAtet VdNKDIErFi PsLiqciadP -tEVPeTvhL LgATTFVaEV TpaTlSimvP LLsRGLnERe TgiKRKsaVI iDNMcKLVed pqviaPFLgk
Vir      -gfLEn-laL -thKnvcvyl PeiVpvvtdc mldlkqvvkd natkaliaqi dnkdIepfiP hLvksig-sP qdv-pe-CVh nlsattfVqS daktlsiLtp
        301
C. neo   LLPRLIKTAE TIADPEARSV ANRAIVTLRR IGKVPVESDG SDLPPLPVAE GPHLATNFVA LVKKHGGVSV EQTNPGLAYA GVLAASLVNH HNFDQKTWES
PCP      LiPtLehikE TIgDPEcRSV vNRslaTLiR vGnVk-Egki pevlniakpE n-cmeT-lls ilKgqelVpV sdvy--LnYi sciAsqLide kNnevvdWdv
Calb     LLPgLknnfa nmADPEAReV tqRAlnTLRR vGaV-gEnD- t-iPevstAg didvtneFnk LVadk-kia- krfdvaLnYi aaiAgdLVde reiqpeaWlq
Sacc     LLPgLksnfa TIADPEAReV tlRAlkTLRR vGnV-gE-Dd a-iPeLshAg dvsttlqvVn ellKdetVa- prfkivveYi aaigAdLide riiDQqaWft
Vir      LLvRLadrtt pvrrktcvii rNmAvddpsd aaKfaVdkvk SaaegmsnpE arkvAeecld ilstidtsfV ntTent-dit sVv-kkytdd yeyvsgivEi
        401                                                                                  A
C. neo   TLPPYLKLAL PSYDSLPAVR ELLQKKADEA ETDDAKFPDE EEGEDLCNIE QFNLAYGAKI LLHHANMRLK RGHRYGLCGR NGSGKSTLMN AIINNQVEGF
PCP      nisPYLqpii lkaD-incii dqfrKrsisg fhsssaeseE EEGEDLCNcE -FsLAYGAKI LLnrtslnLK RGyRYGLCGp NGSGKSTLlr sIfNgQlEGF
Calb     nvlPfatifL hekea-keii EefrKrAidn ipqppsFeDE EEGEDLCNcE -FsLAYGAKI LLnktqfRLK RnrRYGLCGp NGaGKSTLMr AIaNgQVEGF
Sacc     hitPYmtifL he-kkakdil defrKrAvdn ipvgpnFdDE EEGEDLCNcE -FsLAYGAKI LLnktqlRLK RarRYGiCGp NGcGKSTLMr AIaNgQVdGF
Vir      dLkefnvdiw kSaidvedfs gLydtcfeEv kkknvtndDd EEGEDLCdcE -FsLAYGgKI LLnntrfnLK RGnRYGLCGp NGaGKSTLMr AIvNgQlEGF
        501                                                            B                           B          B
C. neo   PPPTEVRTFY VQHDIDGSEA EISILDWVLS DKRLLATPEE IKSTLESVGF DEVKQKNSIG SLSGGWKMKL ALARAILFKA DILLLDEPTN HLDVLNVDWL
PCP      --PTElkTaY VeHDIDdtEs ktSvfDfian DpsvvvnkqE viSsLlehsF tEdmlsipIs nLSGGWKMKL ALvRAmLrqv DILLLDEPTN HLDVkNVaWL
Calb     PtqdEckTvY VeHDIDGthA dttvvefVie DgevglTkdv vvdkLrefnF sdeminmpIq SLSGGWKMKL ALARAvLknA DILLLDEPTN HLDtvNVaWL
Sacc     PtqeEcRTvY VeHDIDGths dtSvLDfVf- esg-vgTkEa IKdkLiefGF tdemiampIs aLSGGWKMKL ALARAvLrnA DILLLDEPTN HLDtvNVaWL
Vir      PsadvlkTaY VeHDlDGSds ntaIiDfian DeqvkentEk IvtTLESVGF DierQsapIn tLSGGWKMKL ALARAmLldA kLLLLDEPTN HIsVsNVrWL
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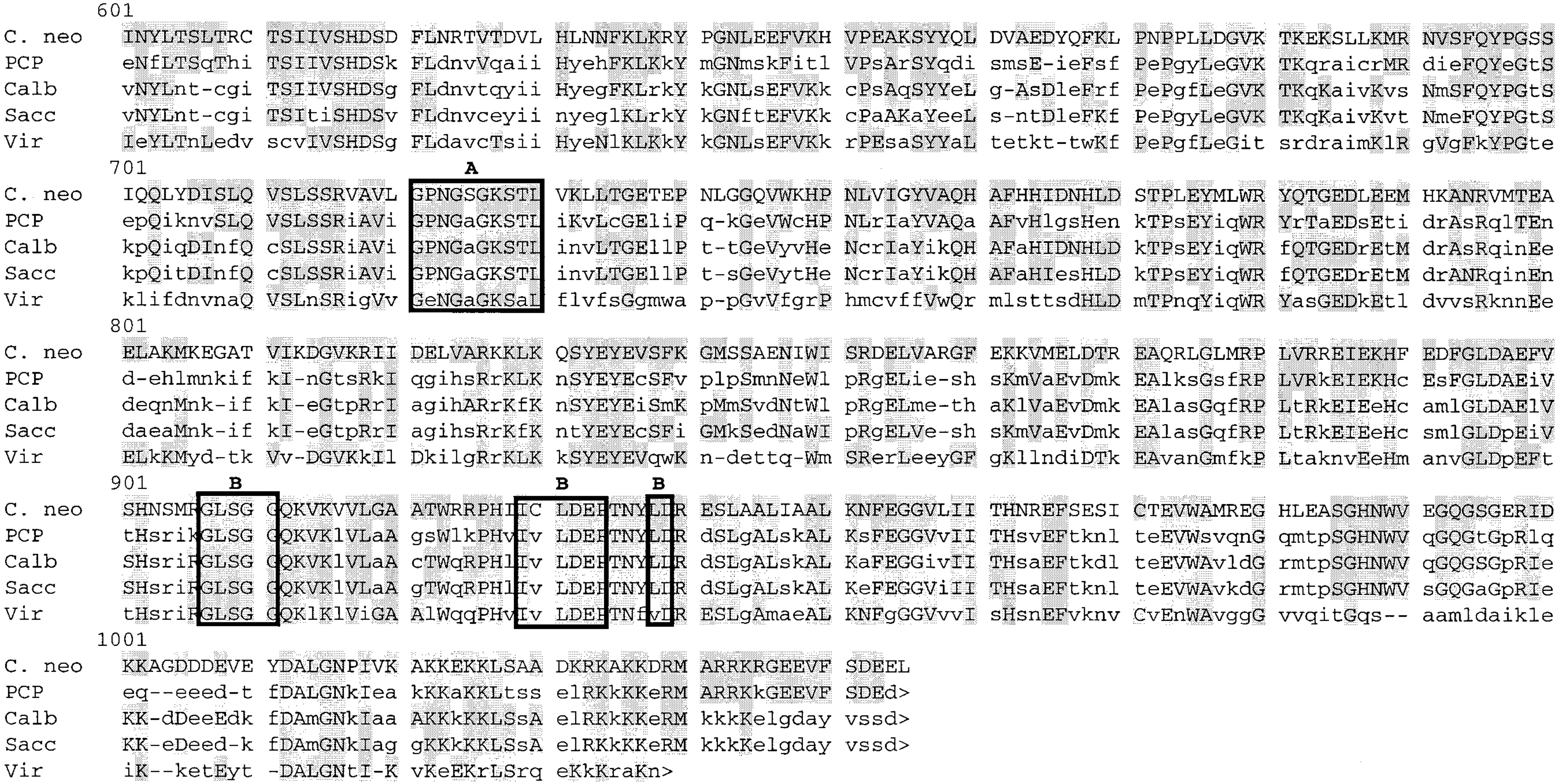

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          601
C. neo    INYLTSLTRC TSIIVSHDSD FLNRTVTDVL HLNNFKLKRY PGNLEEFVKH VPEAKSYYQL DVAEDYQFKL PNPPLLDGVK TKEKSLLKMR NVSFQYPGSS
PCP       eNfLTSqThi TSIIVSHDSk FLdnvVqaii HyehFKLKkY mGNmskFitl VPsArSYqdi smsE-ieFsf PePgyLeGVK TKqraicrMR dieFQYeGtS
Calb      vNYLnt-cgi TSIIVSHDSg FLdnvtqyii HyegFKLrkY kGNLsEFVKk cPsAqSYYeL g-AsDleFrf PePgfLeGVK TKqKaivKvs NmSFQYPGtS
Sacc      vNYLnt-cgi TSItiSHDSv FLdnvceyii nyeglKLrkY kGNftEFVKk cPaAKaYeeL s-ntDleFKf PePgyLeGVK TKqKaivKvt NmeFQYPGtS
Vir       IeYLTnLedv scvIVSHDSg FLdavcTsii HyeNlKLKkY kGNLsEFVKk rPEsaSYYaL tetkt-twKf PePgfLeGit srdraimKlR gVgFkYPGte
          701                    A
C. neo    IQQLYDISLQ VSLSSRVAVL GPNGSGKSTL VKLLTGETEP NLGGQVWKHP NLVIGYVAQH AFHHIDNHLD STPLEYMLWR YQTGEDLEEM HKANRVMTEA
PCP       epQiknvSLQ VSLSSRiAVi GPNGaGKSTL iKvLcGEliP q-kGeVWcHP NLrIaYVAQa AFvHlgsHen kTPsEYiqWR YrTaEDsEti drAsRqlTEn
Calb      kpQiqDInfQ cSLSSRiAVi GPNGaGKSTL invLTGEllP t-tGeVyvHe NcrIaYikQH AFaHIDNHLD kTPsEYiqWR fQTGEDrEtM drAsRqinEe
Sacc      kpQitDInfQ cSLSSRiAVi GPNGaGKSTL invLTGEllP t-sGeVytHe NcrIaYikQH AFaHIesHLD kTPsEYiqWR fQTGEDrEtM drANRqinEn
Vir       klifdnvnaQ VSLnSRigVv GeNGaGKSaL flvfsGgmwa p-pGvVfgrP hmcvffVwQr mlsttsdHLD mTPnqYiqWR YasGEDkEtl dvvsRknnEe
          801
C. neo    ELAKMKEGAT VIKDGVKRII DELVARKKLK QSYEYEVSFK GMSSAENIWI SRDELVARGF EKKVMELDTR EAQRLGLMRP LVRREIEKHF EDFGLDAEFV
PCP       d-ehlmnkif kI-nGtsRkI qgihsRrKLK nSYEYEcSFv plpSmnNeWl pRgELie-sh sKmVaEvDmk EAlksGsfRP LVRkEIEKHc EsFGLDAEiV
Calb      deqnMnk-if kI-eGtpRrI agihARrKfK nSYEYEiSmK pMmSvdNtWl pRgELme-th aKlVaEvDmk EAlasGqfRP LtRkEIEeHc amlGLDAElV
Sacc      daeaMnk-if kI-eGtpRrI agihsRrKfK ntYEYEcSFi GMkSedNaWI pRgELVe-sh sKmVaEvDmk EAlasGqfRP LtRkEIEeHc smlGLDpEiV
Vir       ELkKMyd-tk Vv-DGVKkIl DkilgRrKLK kSYEYEVqwK n-dettq-Wm SRerLeeyGF gKllndiDTk EAvanGmfkP LtaknvEeHm anvGLDpEFt
          901   B                                B     B
C. neo    SHNSMRGLSG GQKVKVVLGA ATWRRPHIIC LDEPTNYLDR ESLAALIAAL KNFEGGVLII THNREFSESI CTEVWAMREG HLEASGHNWV EGQGSGERID
PCP       tHsrikGLSG GQKVKlVLaA gsWlkPHvIv LDEPTNYLDR dSLgALskAL KsFEGGVvII THsvEFtknl teEVWsvqnG qmtpSGHNWV qGQGtGpRlq
Calb      SHsriRGLSG GQKVKlVLaA cTWqRPHlIv LDEPTNYLDR dSLgALskAL KaFEGGivII THsaEFtkdl teEVWAvldG rmtpSGHNWV qGQGSGpRIe
Sacc      SHsriRGLSG GQKVKlVLaA gTWqRPHlIv LDEPTNYLDR dSLgALskAL KeFEGGViII THsaEFtknl teEVWAvkdG rmtpSGHNWV sGQGaGpRIe
Vir       tHsriRGLSG GQKlKlViGA AlWqqPHvIv LDEPTNfvDR ESLgAmaeAL KNFgGGVvvI sHsnEFvknv CvEnWAvggG vvqitGqs-- aamldaikle
         1001
C. neo    KKAGDDDEVE YDALGNPIVK AKKEKKLSAA DKRKAKKDRM ARRKRGEEVF SDEEL
PCP       eq--eeed-t fDALGNkIea kKKaKKLtss elRKkKKeRM ARRKkGEEVF SDEd>
Calb      KK-dDeeEdk fDAmGNkIaa AKKkKKLSsA elRKkKKeRM kkkKelgday vssd>
Sacc      KK-eDeed-k fDAmGNkIag gKKkKKLSsA elRKkKKeRM kkkKelgday vssd>
Vir       iK--ketEyt -DALGNtI-K vKeEKrLSrq eKkKraKn>
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FIG. 2. Predicted amino acid sequence of *CnEF3* product compared to EF3 from other organisms. Shaded boxes indicate regions of identity, and the arrow indicates the previously proposed serine phosphorylation site. amino acid sequences encoding ABC cassettes A and B and associated hydrophobic elements are indicated by boxes. Putative phosphorylation sites proposed by Qin et al. (25) are shown by arrows. Sequences shown are form *C. neoformans* (C. neo) this study), *P. carinii* (PCP) (34), *C. albicans* (Calb) (8), *S. carevisiae* (Sacc) (19), and *Chlorella* virus CVK2 (Vir) (33).

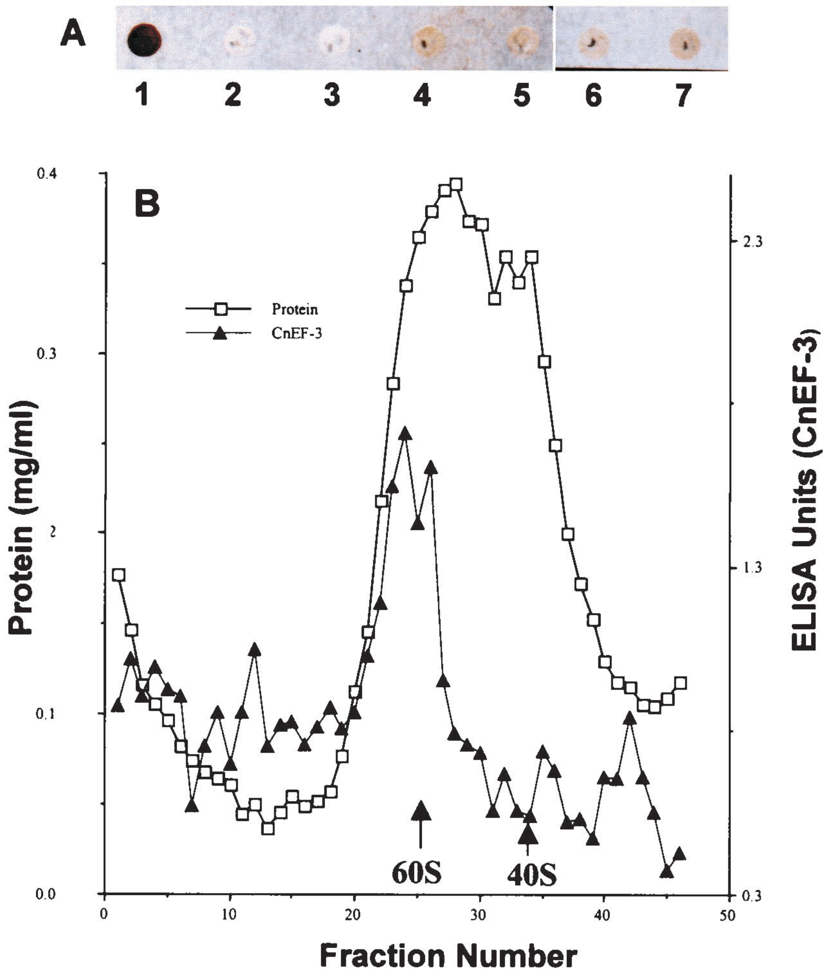

FIG. 3. Extraction of cryptococcal ribosomes with KCl and LiCl. (A) Dot blots of ribosomes from *S. cerevisiae* and *C. neoformans*. Lane 1, 0.6 μg of recombinant CnEF-2; lane 2, 0.5 μg of unwashed *S. cerevisiae* ribosomes; lane 3, 0.5 μg of *S. cerevisiae* ribosomes washed with 0.5 M KCl; lane 4, 0.5 μg of unwashed ribosomes from *C. neoformans*; lanes 5 to 7, 0.5 μg of *C. neoformans* ribosomes washed with 0.5 M KCl (lane 5), 1 M LiCl (lane 6), or 2 M LiCl (lane 7). Blots were treated with anti-CnEF3 antibody (1:1,000) and HRP-conjugated anti-mouse Ig as described in Materials and Methods. (B): Cryptococcal ribosomes were washed three times with 0.5 M KCl and subjected to sucrose density centrifugation as described in Material and Methods. Fractions were assayed for protein and CnEF3 protein by ELISA. 40S and 60S refer to expected positions of cryptococcal ribosomes based on centrifugation of purified *S. cerevisiae* ribosome subunits.

removal of ATPase inhibitors.) Since one source of ATPase activity could be cryptococcal EF3, we investigated the binding properties of the cryptococcal factor for its homologous ribosome. Dot-blot analysis of nitrocellulose-absorbed cryptococcal ribosome preparations showed that extraction of ribosomes with 0.5 M KCl, 1 M LiCl, or 2 M LiCl did not remove significant amounts of immunoreactive EF3 from the ribosome preparation (Fig. 3A). Western blot of 0.5 M KCl-washed ribosomes using anti-CnEF3 antibody showed an immunoreactive band at 116 kDa, consistent with the presence of CnEF3 (data not shown). These conditions have been shown previously to remove *S. cerevisiae* but not mammalian ATPase activity from ribosomes (13).

Since aggregation of CnEF3 under these extraction conditions could also result in coprecipitation of CnEF3 with ribosomes, additional experiments were performed to assess the molecular mass of the precipitated CnEF3-containing ribosomes after extraction with salt. Purified cryptococcal ribosomes were extracted with 0.5 M KCl, subjected to 5 to 30% sucrose gradient ultracentrifugation in buffer containing 5 mM DTT and 0.1 M EDTA, and assayed for fractions containing CnEF3 by ELISA. As shown in Fig. 3B, CnEF3 comigrates with ribosomal subunits (fractions 20 to 30) even after extraction with 0.5 M KCl, whereas fractions either at the top of the gradient (fraction 47) or in the pellet (fraction 1) did not contain significant amounts of CnEF3 reactivity. The magnesium-free conditions of the gradient were severe enough to dissociate the cryptococcal ribosome, as has been reported previously for *S. cerevisiae* (16), but still did not dissociate CnEF3 from cryptococcal ribosomes, as shown by lack of soluble CnEF3 at the top of the gradent (fraction 47), and did not cause aggregation, as shown by lack of CnEF3 reactivity in the pellet (fraction 1). These data suggest that cryptococcal EF3 remains associated with the ribosome even after 0.5 M KCl extraction.

Complementation between *YEF3* of *S. cerevisiae* and *CnEF3* of *C. neoformans*. Diploid strain KC44 of *S. cerevisiae*, bearing one wild-type and one disrupted *YEF3* gene (*MAT***a**/ *MAT*α Δ*yef3*::*LEU2*/*leu2-3,112*/*leu2-3,112 lys2*/+ *met2-1*/+*trp1-7*/+*ura3-52*/*ura3-52*) (24) was transformed with plasmid-borne *CnEF3* driven by the *YEF3* promoter in a 2μm, Y24 vector (y24-CnEF3.7). The diploid yeasts were sporulated and dissected by standard protocols (26). The viable spores showed a segregation pattern of 2:2, 3:1, and 4:0. In all dissected spores, two of the spores grew equally to the wild-type strain, while the rest grew at a much slower rate (data not shown). Complementation of two strains of haploid *S. cerevisiae* bearing a temperature-sensitive mutation in the *YEF3* gene (strains ts22 and ts27) revealed the appearance of significantly slower-growing colonies at the nonpermissive temperature of 34°C. Similar complementation with wild-type *YEF3* genes from *S. cerevisiae* and *C. albicans* gave normal-sized colonies, as reported previously (6, 8). From these results, we conclude that *CnEF3* complements the function of *YEF3* poorly, although it does show that *CnEF3* has functional capability. To verify that the cryptococcal factor was expressed from y24-CnEF3.7 under the *YEF3* promoter, CnEF3 protein was purified from ts22 complemented with y24-CnEF3.7 using the purification procedure which resolves CnEF3 from YEF3 (see Materials and Methods). The protein was readily expressed at the permissive temperature as a protein having the predicted 116-kDa size on SDS-PAGE, intact ATPase activity ($V_{\max}$ = 90 mol/min/mg), and the expected unique N-terminal sequence of the cryptococcal fusion protein from Y24EF-CnEF3#7, MRPPA. It is possible that poor complementation was due to the two-amino-acid substitution in the N terminus of the cryptococcal fusion protein, although this is unlikely, as very little homology exists between the cryptococcal factor and the *Saccharomyces* factor in the first 100 amino acids of the N terminus.

DISCUSSION

While EF1 and EF2 are required for efficient protein translation for all eukaryotic organisms, EF3 is unique in its identification and requirement only within the kingdom Fungi. Since inhibition of protein synthesis by targeting EF3 might represent an effective drug strategy for the control of fungal diseases, identification and characterization of this factor among diverse fungi of medical importance are essential to allow design of pharmacological inhibitors having the broadest application. Thus far, EF3 has only been cloned and characterized from yeasts of the order Ascomycota as well as the fungus-like organism *Pneumocystis carinii* (6). Basidiomycete yeasts are distinct from ascomycete yeasts in both morphology (2) and 18S ribosomal DNA sequence criteria (21). Of the more than 20,000 species described (11), *C. neoformans* represents the most important human pathogen within this phylum. Various properties of this fungus, such as less efficient homologous recombination, complex transcriptional processes, and a promiscuous telomerase (2), suggest that essential cellular machinery may be distinct from that in the more highly studied ascomycetes such as *S. cerevisiae* and *Neurospora crassa*.

In order to compare the functional properties of EF3 among fungi, we undertook the characterization of EF3 from the basidiomycete *C. neoformans*. Consistent with its more distant relationship to the ascomycetes, *CnEF3* showed less homology to *EF3* from *S. cerevisiae* (46% identity) than to EF3 from ascomycetes such as *C. albicans* (78% identity to *S. cerevisiae*) and the ascomycete-like organism *P. carinii* (57% identity to *S. cerevisiae*) (5). An *EF3*-like gene cloned from the *Chlorella* virus shows a similar low identity (36%), but the functional significance of this latter gene has not been established (33). In contrast, the homology of *EF1* between the two classes is much higher (84.7% identity) (28) and suggests a greater evolutionary drift of the third translational cofactor within phyla of Fungi. Low identity between *CnEF3* and ascomycete fungi may also help in determining the functional significance of amino acids within the protein. For example, lack of a conserved serine at position 277, corresponding to a proposed phosphorylation site in *YEF3* (19), makes this amino acid unlikely to have a role in regulation by phosphorylation in *C. neoformans*. In contrast, important ATP-binding regions A and B that form the ATP-binding cassette are conserved in *CnEF3*, consistent with their role in ATP hydrolysis and protein translation in *Saccharomyces* (4).

Ribosome binding is believed to be an important function of EF3 and to be essential for the factor's translation-modulating effects (29). Analysis of peptide regions of EF3 believed to be involved in ribosome binding may shed light on possible structural differences in EF3-ribosome binding interactions between ascomycetes and basidiomycetes. Previous studies have shown that an acidic N-terminal region of *S. cerevisiae* (amino acids 98 to 388) (10) and a basic C-terminal region (775 to 1044) (12) of EF3 are both involved in ribosome binding. Comparison of the amino acid sequence of the homologous regions of CnEF3 (N-terminal residues 108 to 404 and C-terminal residues 796 to 1055) show that the cryptococcal N-terminal region is less acidic (12 versus 17 D and 16 versus 23 E) with a calculated pI of the cyptococal fragment of 8.98 versus 4.85 for the *S. cerevisiae* fragment. In addition, the cyptococal C-terminal fragment is much less basic (26 versus 31 K), yielding a calculated pI of 6.73, versus 8.82 for the *S. cerevisiae* fragment. Indeed, differences in surface charge of the cryptococcal factor were also suggested by its lack of binding to CM-cellulose and its relatively poor binding to DEAE-cellulose, in contrast to the stronger binding of the *S. cerevisiae* factor to both matrices. Such differences in amino acid sequence and CnEF3 binding characteristics suggest an evolutionary drift in the nature of CnEF3-ribosome binding from that of ascomycetes.

Recombinant CnEF3 showed significant ATPase activity, as has been shown for YEF3 (4). Since ribosome-dependent ATPase activity is a key characteristic of EF3, the ability of *S. cerevisiae* ribosomes to stimulate CnEF3 ATPase activity was assessed. While previous reports have shown a 50- to 100-fold increase in $V_{\max}$ of YEF3 ATPase activity in the presence of homologous ribosomes, we found no significant increase in ATPase $V_{\max}$ of the cryptococcal EF3 in the presence of *S. cerevisiae* ribosomes and only a small decrease in K_m. This suggests that evolutionary divergence from ascomycetes exhibited by the CnEF3 sequence results in alterations in properties leading to poor EF3-*S. cerevisiae* ribosome interactions.

In order to study the nature of the interaction of CnEF3 with cryptococcal ribosomes, attempts were made to remove

CnEF3 from a ribosome preparation with 0.5 M KCl, which typically removes all *S. cerevisiae* elongation factors. Even after extraction with potassium (0.5 M) or lithium (2 M) salts, significant ribosome-ATPase activity remained, preventing the measurement of CnEF3-cryptococcal ribosome-dependent ATPase activity. Both dot-blot and sucrose sedimentation experiments showed that CnEF3 exhibited an unusually strong binding to ribosomes, which may account for at least part of the observed residual ATPase activity of salt-extracted cryptococcal ribosomes. It is interesting that in higher eukaryotes such as mammals, 80S ribosomes are able to hydrolyze ATP without the addition of soluble protein factors. In addition, mammalian ribosomal ATPase activity cannot be removed with 0.5 M KCl and is only poorly removed with 2 M LiCl (14, 22). However, there has been no evidence of a third elongation factor in mammalian ribosomes, and the identity of the mammalian ribosomal ATPase activity remains unknown.

In order to further characterize the functional significance of EF3 amino acid differences between basidiomycetes and ascomycetes, *CnEF3* was tested for its ability to replace the function of *YEF3* in vivo. It was found that *CnEF3* only poorly complemented either of two temperature-sensitive mutants of *S. cerevisiae* (ts22 and ts27). In addition, the sizes of colonies of a KC44 Δ*yef3*::*LEU2* null mutant rescued by *CnEF3* were significantly smaller than those rescued by *YEF3*. These results are in contrast to those of Cholthurst et al., who showed good complementation and normal colony size after complementation of these same strains using *EF3* from the ascomycete *C. albicans* (6). The poor complementation by *CnEF3* in vivo may again be a reflection of structural or functional differences between the elongation factors of ascomycetes and basidiomycetes.

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