The Ess1 Prolyl Isomerase Is Required for Growth and Morphogenetic Switching in Candida albicans

Gina Devasahayam,*† Vishnu Chaturvedi,*†‡ and Steven D. Hanes*‡†

*Molecular Genetics Program, †Mycology Laboratory, Wadsworth Center, New York State Department of Health, Albany, New York 12208 and ‡Department of Biomedical Sciences, School of Public Health, State University of New York, Albany, New York 12208

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ABSTRACT

Prolyl-isomerases (PPIases) are found in all organisms and are important for the folding and activity of many proteins. Of the 13 PPIases in Saccharomyces cerevisiae only Ess1, a parvulin-class PPIase, is essential for growth. Ess1 is required to complete mitosis, and Ess1 and its mammalian homolog, Pin1, interact directly with RNA polymerase II. Here, we isolate the ESSI gene from the pathogenic fungus Candida albicans and show that it is functionally homologous to the S. cerevisiae ESSI. We generate conditional-lethal (ts) alleles of C. albicans ESSI and use these mutations to demonstrate that ESSI is essential for growth in C. albicans. We also show that reducing the dosage or activity of ESSI blocks morphogenetic switching from the yeast to the hyphal and pseudohyphal forms under certain conditions. Analysis of double mutants of ESSI and TUP1 or CPH1, two genes known to be involved in morphogenetic switching, suggests that ESSI functions in the same pathway as CPH1 and upstream of or in parallel to TUP1. Given that switching is important for virulence of C. albicans, inhibitors of Ess1 might be useful as antifungal agents.

Pep-tidyl-prolyl cis-/trans-isomerases (PPIases) are enzymes that catalyze the cis-/trans-isomerization of the peptide bond preceding the amino acid proline. During translation, the ribosome is thought to synthesize all peptide bonds in the trans-isomer form. However, structural studies show that ~10% of peptidyl-prolyl bonds are in the cis-isomer form (Stewart et al. 1990). Although isomerization of the X-Pro bonds from trans- to cis- can occur spontaneously in nascent proteins, it is thought to be facilitated by the action of PPIases. PPIases also bind to mature proteins in different cellular compartments to control their activity, subunit assembly, and transport (Rutherford and Zuker 1994; Hunter 1998; Gotthel and Marahiel 1999).

There are three families of PPIases: cyclophilins, FK506-binding proteins (FKBPs), and parovulins; members of each family are conserved in both prokaryotes and eukaryotes (Dolinski and Heitman 1997). Cyclophilins and FKBPs are best known because they bind the immunosuppressive drugs cyclosporin A and FK506, respectively (Kunz and Hall 1993; Fruman et al. 1994). FKBPs also bind rapamycin, and all three compounds exhibit antifungal activity (Hemenway and Heitman 1993). Parvulins are structurally distinct from both the cyclophilins and FKBPs and do not bind these compounds (Raible et al. 1994a,b).

None of the eight cyclophilins or four FKBPs is essential for growth in the budding yeast Saccharomyces cerevisiae (Dolinski et al. 1997). However, the single parvulin-class PPIase in S. cerevisiae, Ess1, is essential (Hanes 1988; Hanes et al. 1989). Yeast cells depleted of Ess1 arrest late in mitosis and undergo nuclear fragmentation (Lv et al. 1996; Wu et al. 2000). Homologs of the ESSI gene have been isolated from a variety of organisms, including Aspergillus nidulans (Crenshaw et al. 1998), Neurospora crassa (Kops et al. 1998), Drosophila melanogaster (Maleszka et al. 1996), Xenopus laevis (Winkler et al. 2000), and humans (Lv et al. 1996). The fly homolog dodo and the human homolog PIN1 both complement ess1 mutations in budding yeast (Lv et al. 1996; Maleszka et al. 1996), indicating a highly conserved mechanism of action. However, dodo is not essential in flies; nor is PIN1 essential in mice, suggesting the presence of redundant or alternative pathways in metazoans (Maleszka et al. 1996, 1997; Fujimori et al. 1999). In Xenopus extracts, Pin1 has been shown to be important for the DNA replication checkpoint (Winkler et al. 2000).

Among the eukaryotic parvulins, only the Ess1 homologs have an amino-terminal WW domain, in addition to their carboxy-terminal PPIase domain. The WW domain is a protein-protein interaction module that recognizes proline-rich sequences (Einbond and Sudol 1996; Sudol 1996). The WW domains of Pin1 and Ess1 bind with high affinity to phospho-serine-proline motifs (Yaffe et al. 1997; Lu et al. 1999a). Human Pin1 has been shown to bind mitotic phosphoproteins that contain this motif (Shen et al. 1998) and to hyperphosphorylated tau protein, which is a component of fibroid tangles found...
in the brains of Alzheimer’s disease patients (LU et al.
1999b).

In yeast, genetic studies have shown that Ess1 is important for transcription by RNA polymerase II (RNA pol II) and that the cell-cycle defect in ess1 mutants is probably an indirect effect of misregulation of genes required for mitosis (Wu et al. 2000). Biochemical and structural studies have shown that Ess1 and Pin1 bind directly to the carboxy-terminal domain (CTD) of RNA pol II, which contains multiple phospho-Ser-Pro motifs, and to components of the Sin3/Rpd3 histone deacetylase complex (Morris et al. 1999; Arevalo-Rodriguez et al. 2000; Verdecia et al. 2000; Wu et al. 2000). Our current model is that Ess1 isomerizes the CTD and regulates the sequential binding of proteins to the RNA pol II complex that are required for transcription initiation, elongation, termination, and mRNA processing (Wu et al. 2000). In addition, Ess1 negatively regulates the Sin3/Rpd3 histone deacetylase complex (Arevalo-Rodriguez et al. 2000).

To further understand the function of Ess1 and to assess its potential as an antifungal drug target, we are studying homologs from human fungal pathogens, including C. albicans. C. albicans exists as either a commensal or an opportunistic pathogen in humans and causes life-threatening systemic infections in immunocompromised individuals (Oddss 1988; Kao et al. 2000). Systemic C. albicans infections are also a serious problem for patients undergoing cancer chemotherapy or organ transplantation (Anaissie et al. 1998). C. albicans is dimorphic in that it undergoes morphogenetic switching between a yeast form and a hyphal (and pseudohyphal) form (Oddss 1988; Brown and Gow 1999). This switching is important for virulence (Oddss 1988; Cutler 1991; Madhani and Fink 1998) and is initiated by external signals that are transduced to transcriptional regulatory proteins, such as Cph1 and Tup1 that activate or repress downstream target genes, respectively (Liu et al. 1994; Braun and Johnson 1997). Given the role of Ess1 in transcription and its requirement for growth in budding yeast, we examined its importance for the hyphal transition in C. albicans.

Here, we describe the isolation of the C. albicans homolog of Ess1 and demonstrate that it is essential for growth in this organism. This was done by traditional gene knockout experiments and by generating a C. albicans strain bearing a conditional-lethal ess1Δ mutation. Work described here suggests a method by which conditional alleles of C. albicans genes can be generated using S. cerevisiae as a surrogate host and that such alleles can be used to prove essentiality and to study gene function in C. albicans. We also show that C. albicans Ess1 is important for morphogenetic switching. Our results suggest that some, but not all, of the functions of Ess1 in budding yeast are conserved in C. albicans and that the Ess1 prolyl-isomerase might be a useful target for the development of antifungal drugs.

MATERIALS AND METHODS

Media and culture conditions: C. albicans strain SC5314 was grown in rich medium (YPD) and the ura3Δ derivative strain CAH was grown in YEPD + 25 μg/ml uridine. Filamentation-inducing media were prepared as described: Spider medium (Liu et al. 1994), liquid Lee’s medium (Lee et al. 1975), solid modified Lee’s (Liu et al. 1994), and milk-tween agar and cornmeal-tween agar (Jitsurong et al. 1993). For serum-containing media, the liquid contained YEPD + 10% fetal bovine serum (FBS; Sigma, St. Louis), and the solid contained 4% FBS and 2% agar. For growth rate experiments, cells were grown to midlog phase and were used to inoculate cultures to a starting OD540 of 0.1. For plating efficiency experiments, cells were grown in liquid YEPD at 30°C to midlog phase prior to plating on solid YEPD medium. C. albicans cells were made competent and transformed by a standard lithium acetate procedure (Ito et al. 1983).

Cloning of C. albicans ESS1 by complementation: Temperature-sensitive S. cerevisiae strain ess1Δ was transformed with a C. albicans genomic DNA library and ura3Δ protoplasts were selected. The library was made in a high-copy episomal plasmid, YEp352 (2μ; URA3; Navarro-Garcia et al. 1995). The transformed cells were incubated at 30°C overnight in liquid synthetic complete medium lacking uracil before being plated and incubated at 37°C for 4–5 days. Of an estimated 2 × 108 transformants plated, 28 grew repeatedly at 37°C upon sequential passaging. Plasmids were rescued from all 28 transformants and retransformed into S. cerevisiae ess1Δ. Of the 28, 5 were again able to rescue the no-growth phenotype of ess1Δ at 37°C. These five were also 5-fluoroorotic acid (5-FOA) sensitive, suggesting that the complementation was plasmid linked. On the basis of restriction mapping and size of insert, the clones were divided into two classes. The first class (insert size ~3.5 kb) contained RPB7 (GenBank accession no. AF224269; Wu et al. 2000) and the second class (insert size ~8 kb) contained ESS1 (GenBank accession no. AF224270), with the putative start codon being 253 bp from one end of the insert. A 1-kb fragment containing ESS1 was subcloned, and the resulting plasmid (pGD-CaESS1) also complemented the S. cerevisiae ess1Δ mutant at 37°C.

Plasmid construction: pGD-CaESS1 was constructed in pRS426 (Skorski and Hieter 1989) by insertion of an EcoRI-BamHI fragment from the original library clone containing ESS1. The EcoRI site is in the polylinker of YEp352 and BamHI is 200 bp downstream of the stop codon. pGD-CaRPB7 was also constructed in pRS426, except that the 1-kb fragment containing RPB7 was amplified from the original library clone using primers with EcoRI and BamHI sites, with the putative start codon being 253 bp from one end of the insert. A 1-kb fragment containing ESS1 was subcloned, and the resulting plasmid (pGD-CaESS1) was constructed in pUC19 in several steps. First, the 253-bp region upstream of ESS1 was amplified using primers with KpnI and BamHI sites. This fragment was cloned into KpnI and BamHI sites of pUC19 to give pGD-1. Next, the ~4.4-kb hisG-CaURA3-hisG cassette (BigE-BamHI) from pCUB-6 (Fonzi and Irwin 1993) was cloned into the BamHI site of pGD-1 to give pGD-2. Finally, the ~3.2-kb BamHI fragment that begins 200 bp downstream of the ESS1 stop codon was cloned into the correct orientation into the BamHI site of pGD-2 to generate pGD-CaESS1. Mutant alleles of C. albicans ESS1 were generated by site-directed PCR mutagenesis (Horton et al. 1990): CAT > AGA for H171R and TCA > CCA for S129P. The PCR fragments containing the mutant alleles were cloned as EcoRI-BamHI fragments into pRS426, yielding pGD-ess1H171R and pGD-ess1S129P. Replacement constructs pGD-3 and pGD-4, designed to introduce the ts alleles into C. albicans, were similar to the deletion construct, pGD-CaESS1Δ, except that ESS1 sequences were derived from pGD-ess1H171R and pGD-ess1S129P, the mutant alleles being the left flanking region.
Strain construction: To generate a heterozygous \( \text{ess1}:\text{hisG-URA3-hisG/ESS1} \) (CaGD1) mutant strain, a ~7-kb insert (Sad-SphI) was excised from pGD-Caes\( \Delta \) and used to transform \( S. \) *cerevisiae* CAI4 and uracil prototrophs were selected. To select for \( \text{ura3} \) derivatives (\( \text{ess1}:\text{hisG/ESS1} \)), in which the URA3 gene is looped out by recombination between the \( \text{hisG} \) repeats, the mutant strain CaGD1 was grown in rich medium (YEpd) for 1–2 days, plated on 5-FOA-containing medium, and incubated at 30\( ^\circ \). The resulting strain was CaGD2. To attempt to generate homozygous \( \text{ess1} \) deletion mutants, the mutant strain CaGD2 was transformed again with Sad-SphI-digested pGD-Caes\( \Delta \).

Temperature sensitivity of the *C. albicans* mutant alleles \( \text{ess1}^{1989} \) and \( \text{ess1}^{37} \) was tested using *S. cerevisiae* \( \text{ess1} \) strains. For \( \text{ess1}^{1989} \), diploid strain YSH-55 (\( \text{ess1}:\text{hisG/ESS1} \)) was transformed with pGD-\( \text{ess1}^{1989} \), cells were induced to sporulate, and tetrads were dissected. Segregants that were HIS\( ^+ \) and URA\( ^+ \) were tested for temperature sensitivity by replica plating to 30\( ^\circ \) and 37\( ^\circ \). For \( \text{ess1}^{37} \), haploid strain YXW 2.1 (\( \text{ess1}:\text{TRP1} \)) containing human PIN1 on a 2-\( \mu \)m LEU2 plasmid was transformed with pGD-\( \text{ess1}^{37} \) and grown in the absence of the selection for the PIN1 plasmid in liquid medium containing leucine for 2–3 days at 25\( ^\circ \). A total of 0.2% of colonies analyzed lost PIN1. They were tested for temperature sensitivity by replica plating at 25\( ^\circ \) and 37\( ^\circ \), and all were found to be \( \text{ess1}^{37} \). To construct *C. albicans* \( \text{ess1} \) strains, the replacement constructs pGD-3 and pGD-4 were digested with Sad-XhoI and transformed into the heterozygous \( \text{ess1}:\text{hisG/ESS1} \) mutant (CaGD2) and uracil prototrophs were selected. They were tested for temperature sensitivity by replica plating at 25\( ^\circ \), 30\( ^\circ \), 37\( ^\circ \), and 42\( ^\circ \). Multiple isolates of the heterozygous strain (CaGD2) were used to generate the \( \text{ess1}:\text{hisG/\text{ess1}^{1989}:\text{hisG-URA3-hisG} \text{(CaGD5) mutant strains}. \)

**CPh1** was disrupted in three strain backgrounds: CAI4, two isolates of CaGD2, and two isolates of CaGD4. The strains were transformed with a Xho-Sad digest of the disruption construct (pHL156) containing the \( \text{hisG-URA3-hisG} \) cassette inserted at the \( \text{hnr} \) site of the open reading frame (Lu et al. 1994). To generate homozygous CPh1 deletions, the transformation was repeated after reversion of the URA3 marker to ura-minus using 5-FOA. TUP1 was also deleted in CAI4, two isolates of CaGD2, and two isolates of CaGD4. The strains were transformed with an SphI digest of the deletion construct (p3883c) containing the \( \text{hisG-URA3-hisG} \) cassette that replaces the TUP1 open reading frame and 390 bp of upstream sequence (Braun and Johnson 1997). Homozygous CPh1 deletions were generated as described above for CPH1.

**Southern hybridization:** Genomic DNA from *C. albicans* was prepared as described (Adams et al. 1997). High-stringency Southern hybridization was done at 65\( ^\circ \). The probes used were ESS1 (a 539-bp PCR fragment containing 255 bp upstream of the start codon and 284 bp of ORF sequence), \( \text{hisG} \), and URA3. Labeling of the probes was done by random priming (Boehringer Mannheim, Indianapolis). Genescreen Plus hybridization transfer membranes (New England Nuclear Life Science, Boston) were used. Filters were stripped in boiling 1% SDS and 0.1% SSC (0.015 M sodium chloride and 0.0015 M sodium citrate) prior to reuse in subsequent hybridizations.

**Microscopy:** For 4',6-diamidino-2-phenylindole (DAPI) staining, cells were grown at 30\( ^\circ \) and 40\( ^\circ \), sonicated, fixed in 70% ethanol, resuspended in mounting medium (1 mg/ml phenylendiamine in 2\% PBS/80% glycerol) containing 50 mg/ml DAPI (Sigma), and examined and photographed on a Nikon Optiphot equipped with a Quad Fluor epifluorescence attachment and a 60\( \times \)1.4 NA Planapo objective. Filamentous cells were treated with Triton X-100 (2\%) and briefly sonicated and fixed in 3.7% formaldehyde before being visualized. Cells with morphologies other than the yeast form (rounded) were considered filamentous and included cells with both hyphal and pseudohyphal. To view colony morphology, a Zeiss SV6 stereo microscope was used at 20\( \times \) magnification.

**RESULTS**

**Isolation of the *C. albicans* homolog of ESS1:** We used the *S. cerevisiae* ESS1 gene as a probe in low-stringency Southern hybridization against *C. albicans* genomic DNA. The results suggested the existence of a single-copy homolog of ESS1 (data not shown). To clone the gene, we took advantage of a temperature-sensitive strain of *S. cerevisiae* (strain \( \text{ess1}^{37} \), Wu et al. 2000). We transformed a *C. albicans* genomic DNA library into this strain to search for *C. albicans* genes capable of complementing the no-growth phenotype at the restrictive temperature (37\( ^\circ \)). Of the 2\( \times \)10\( ^6 \) transformants screened, we obtained five complementing clones representing two different genes (Figure 1).

Three of these clones contained a high-copy suppressor called RB7 that is the *C. albicans* homolog of the RNA polymerase II seventh subunit (McKune et al. 1993; Khazak et al. 1995). We think that this gene suppresses by a mechanism that involves rescue of an RNA pol II transcription deficiency (Wu et al. 2000). The two other clones contained a gene that encodes a predicted 177-amino-acid protein with 42% identity to *S. cerevisiae* Ess1, 47% to *A. nidulans* Pin1, 41% to Drosophila Dodo, and 43% to human Pin1 (Figure 2). The overall structure of the encoded protein is conserved with that of all
known Ess1/Pin1 homologs. It has an N-terminal WW domain and a C-terminal PPlase domain, and the four active-site residues (H157, C113, H59, and S154 in Pin1) that are predicted on the basis of the crystal structure of Pin1 (Ranganathan et al. 1997) are conserved. The C. albicans gene complemented a complete deletion of the ESS1 gene (ess1Δ) in S. cerevisiae (data not shown). These results show that the C. albicans gene is the functional homolog of budding yeast ESS1.

The ESS1 gene is essential in C. albicans: C. albicans is a diploid organism. Therefore, to determine whether ESS1 is essential in C. albicans, we attempted to delete both alleles by sequential gene deletion. To do this we used the modified Ura-blast approach (Fonzi and Irwin 1993; Figure 3A). The first allele was deleted successfully in strain CAI4, as demonstrated by Southern hybridization (Figure 4, lanes 2 and 3) and PCR (data not shown). In the resulting heterozygous strains (CaGD1/ΔH9004; Table 1) no obvious growth defect was observed. In these strains the URA3 marker was excised, and the resulting strains were transformed with the same deletion construct, in an attempt to delete the second allele. Of the 60 transformants analyzed, none showed a deletion of the remaining wild-type allele, as determined by PCR and Southern hybridization (data not shown). The inability to recover homozygous deletions (Δ/Δ) suggested that ESS1 is an essential gene. However, it is also possible that deletion of the second allele did not occur due to heterozygosity at the second locus, which might have inhibited homologous recombination (Yesland and Fonzi 2000). Also, the deletion construct shared more homology with the deleted allele than with the wild type, and it therefore may have been a favored target.

Since C. albicans is diploid and asexual, proving the essentiality of genes is problematic, and the results described above suggest that ESS1 is essential, they are not conclusive. We therefore adopted an independent approach. We replaced the remaining wild-type allele, as determined by PCR and Southern hybridization (data not shown). In the resulting heterozygous strains (CaGD1/ΔH9004; Table 1) no obvious growth defect was observed. In these strains the URA3 marker was excised, and the resulting strains were transformed with the same deletion construct, in an attempt to delete the second allele. Of the 60 transformants analyzed, none showed a deletion of the remaining wild-type allele, as determined by PCR and Southern hybridization (data not shown). The inability to recover homozygous deletions (Δ/Δ) suggested that ESS1 is an essential gene. However, it is also possible that deletion of the second allele did not occur due to heterozygosity at the second locus, which might have inhibited homologous recombination (Yesland and Fonzi 2000). Also, the deletion construct shared more homology with the deleted allele than with the wild type, and it therefore may have been a favored target.

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ess1<sup>ts</sup> alleles are temperature sensitive in an <em>S. cerevisiae</em> host, suggesting that they will be temperature sensitive in <em>C. albicans</em>. Moreover, these results suggest that <em>S. cerevisiae</em> can be used as a surrogate host to generate new <em>ts</em> alleles in <em>C. albicans</em> genes or genes of other pathogenic fungi (see Discussion).

Next, we used the <em>ess1</em><sup>S129P</sup> and <em>ess1</em><sup>H171R</sup> alleles to replace wild-type <em>ESS1</em> in a <em>C. albicans</em> heterozygous strain (<em>Δ</em>/<em>+</em>). The replacement strategy is depicted in Figure 3B. Linearized replacement constructs containing each of the mutant alleles were transformed into three independent heterozygous strains. Of 295 transformants obtained for the H171R transformation, 18 were temperature sensitive. For 3 of these, the chromosomal locus was amplified and the DNA sequence determined; all contained the H171R mutation. In addition, Southern analysis demonstrated that the integrations occurred at the correct locus and yielded the expected recombinants (Figure 4, lanes 6–8). In contrast, we were unable to generate an <em>ess1</em><sup>S129P/ess1</sup><sup>Δ</sup> strain. Of 958 transformants obtained for the S129P transformation (at 25° and 30°), none were temperature sensitive, suggesting that the mutation was lethal over a deletion (<em>ts</em>/Δ).

To test whether <em>ESS1</em> is essential in <em>C. albicans</em>, growth
of the \( \text{ess}^{H78R}/\text{ess}^{\Delta} \) strain (\( \text{ess}^{P} \)) was compared at permissive and nonpermissive temperatures. Mutant cells streaked on solid media grew normally at 30\(^\circ\) but not at 37\(^{\circ}\) or 42\(^{\circ}\), whereas wild-type and heterozygous cells grew at all temperatures (Figure 6A). In liquid media, \( \text{ess}^{P} \) cells grew well at 30\(^{\circ}\), albeit slightly slower than wild type. However, at 37\(^{\circ}\), \( \text{ess}^{P} \) cells grew very poorly (data not shown), and at 40\(^{\circ}\) growth was arrested (Figure 6B). To further demonstrate that \( \text{ESSI} \) is required for growth, we tested the plating efficiency of wild-type vs. \( \text{ess}^{P} \) cells at different temperatures. As shown in Figure 6C, \( \text{ess}^{P} \) cells form colonies at 30\(^{\circ}\) but are unable to form colonies at 40\(^{\circ}\), whereas wild-type cells form equivalent numbers of colonies at both temperatures. At 40\(^{\circ}\), the plating efficiency of wild-type cells was \(~75\%\) vs. \(0\%\) for the mutant cells. Note that the size of the \( \text{ess}^{P} \) colonies is smaller than wild type, indicating a slight growth defect even at permissive temperature.

Microscopic examination of mutant cells shows that, as with \( S.\ ceriseae \) \( \text{ess}^{P} \) mutants, the \( C.\ albicans \) \( \text{ess}^{P} \) mutant cells undergo uniform arrest late in mitosis (Figure 6D). DAPI staining indicates that the nuclear division is completed prior to arrest (Figure 6D). However, we did not observe nuclear fragmentation as in \( S.\ ceriseae \) (Lu et al. 1996; Wu et al. 2000). Mutant cells that were growth arrested at high temperature (40\(^{\circ}\) and 42\(^{\circ}\)) retained viability for up to 18 hr (data not shown), perhaps because they activated a checkpoint control. At low temperature (25\(^{\circ}\)) in YEPD, we observed that some cells (\(\sim20-40\%\)) displayed a filamentous pheno-

### TABLE 1

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**S. cerevisiae**

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**C. albicans**

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**CPH1 mutants**

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<td>ESS1::hisG ura3::imm434/ura3::imm434 TUP1::tup1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>CaGD13</td>
<td>CaGD12</td>
<td>ESS1::hisG ura3::imm434/ura3::imm434 tup1::hisG-URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>CaGD14</td>
<td>CaGD13</td>
<td>ess1H171R::hisG/ess1::hisG ura3::imm434/ura3::imm434 TUP1::tup1::hisG-URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>CaGD15</td>
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<td>This study</td>
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<tr>
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<td>This study</td>
</tr>
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</table>
We tested the ability of _S. cerevisiae_ STE12 homologs to induce filamentation in culture using various media and methods. We generated double-mutant combinations between _C. albicans_ wild-type and _S. cerevisiae_ STE12 deletion strains (YSH-55 haploids or YXW21; see MATERIALS AND METHODS) carrying _C. albicans_ ess1 (wild-type or ess1H171R alleles) were spotted onto plates as serial 1:5 dilutions from an original concentration of ~10^6 cells/ml and incubated for 3–4 days at the indicated temperatures.

**Figure 5.** _C. albicans_ ess1^H171R_ mutant alleles are temperature sensitive in _S. cerevisiae_. _S. cerevisiae_ deletion strains (YSH-55 haploids or YXW21; see MATERIALS AND METHODS) carrying _C. albicans_ ess1 (wild-type or ess1H171R alleles) were spotted onto plates at 25°C and 30°C. Comparison of hyphal induction in liquid medium at permissive temperature (30°C) was not feasible, since even the wild type failed to form filaments with high frequency. The mutant strains were also tested in milk-tween agar, cornmeal agar, and Medium 199 (pH 7.0), and similar results were obtained; filamentation was reduced in the heterozygote and the ts mutant (data not shown). These results suggest that, at least in certain inducing media, Ess1 is required for efficient morphogenetic switching in _C. albicans_.

**Epistasis analysis between ESS1 and TUP1 and CPH1 mutations:** Several distinct genetic pathways have been shown to control morphogenetic switching in _C. albicans_ (reviewed in Ernst 2000; Lengeler et al. 2000). To determine whether ESS1 could be placed within one of the known pathways, we carried out epistasis analysis with two genes, TUP1 and CPH1, known to be involved in switching. TUP1 is a homolog of _S. cerevisiae_ TUP1 and in _C. albicans_ is a repressor of genes required for filamentation (Braun and Johnson 1997). CPH1 is a homolog of the _S. cerevisiae_ STE12 transcription activator (Liu et al. 1994). In both organisms, CPH1/STE12 are targets of mitogen-activated protein (MAP) kinase pathways (Liu et al. 1994; Kohler and Fink 1996; Leberer et al. 1996; Madhani and Fink 1997).

We generated double-mutant combinations between _ESS1_ and _TUP1_ and between _ESS1_ and _CPH1_. We also examined filamentation in liquid-inducing media. At 37°C, germ-tube formation (the initial stage of hypha formation) occurs rapidly, within the first few hours of induction. We compared the ability of wild-type, heterozygous (ess1^Δ/ESS1), and ess1^H171R/ess1^Δ mutants to form filaments in Lee’s and Spider media, although they did form some in serum-containing medium (data not shown). However, at 37°C the ts mutant grows slowly, and the resulting colonies were much smaller than wild type.

We also examined filamentation in liquid-inducing media. At 37°C, germ-tube formation (the initial stage of hypha formation) occurs rapidly, within the first few hours of induction. We compared the ability of wild-type, heterozygous (ess1^Δ/ESS1), and ess1^H171R/ess1^Δ mutant strains to form germ tubes. As expected, wild-type cells formed abundant germ tubes in all three inducing media (Figure 9). In contrast, the ess1^Δ/ESS1 strain was severely impaired for germ-tube formation in Lee’s medium and to a lesser extent in Spider medium. A minor reduction was observed in serum-inducing medium. The ess1^H171R mutant showed similar defects; germ-tube formation was severely reduced in Lee’s medium and to a lesser extent in Spider and serum-inducing media. At this temperature, however, growth of the ts strain slows (but does not stop) and cells begin to accumulate in mitosis; thus it is not clear whether the failure to form germ tubes in Lee’s medium is a direct effect or is due to the growth impairment. Comparison of hyphal induction in liquid medium at permissive temperature (30°C) was not feasible, since even the wild type failed to form filaments with high frequency. The mutant strains were also tested in milk-tween agar, cornmeal agar, and Medium 199 (pH 7.0), and similar results were obtained; filamentation was reduced in the heterozygote and the ts mutant (data not shown). These results suggest that, at least in certain inducing media, Ess1 is required for efficient morphogenetic switching in _C. albicans_.
(Figure 10A). Filamentation was also observed under inducing conditions using Lee’s, serum-containing, and Spider media (Figure 10A, Table 2). As expected, control heterozygous tup1 mutants (tup1Δ/+) did not form filaments in rich medium (YEPD), regardless of the ESS1 genotype (Table 2). Thus, in all cases the tup1 ess1 double mutants displayed the phenotype of the tup1 single mutant, suggesting that ESSI acts genetically upstream of, or in a different pathway from, TUP1.

Results with CPH1 suggest that ESSI and CPH1 might function in a common pathway. Mutations in CPH1 block morphogenetic switching in Spider medium, but they do not block switching in response to serum-containing media (Liu et al. 1994). In our experiments, cph1 mutants were also reduced for filamentation in Lee’s medium. Mutations in ESS1 behaved similarly, with a switching defect most apparent in Lee’s and Spider, but less so in serum-containing media (Figure 9). Results with cph1 ess1 double-mutants are shown in Figure 10B. In Lee’s medium, double-mutant cells were completely defective in filamentation. This phenotype is similar to that seen in the individual cph1 or ess1 single mutants, although the penetrance appears to be higher, with more cells remaining exclusively in the yeast form. In serum-containing medium, cells appear partially competent to form filaments, similar to ess1 single mutants. In Spider medium, double-mutant cells were unable to form filaments, similar to cph1 single mutants. Thus, the induction response of ess1 cph1 double-mutant cells was, in general, similar to that of each of the single mutants, perhaps with some degree of increased pene-
Inducing magnification (20×/H1001, solid serum-containing, and solid Spider media at 30° for 4 hr at 37° in liquid hyphal-inducing media. Cell morphologies of wild-type and loss-of-function phenotypes and provide a way to search for second-site suppressors. In our case, these cells do not undergo nuclear fragmentation as in budding yeast, loss-of-function mutants arrest in mitosis. However, these cells do not undergo nuclear fragmentation as in C. albicans, suggesting that the mitotic arrest and nuclear fragmentation are genetically separable, at least in C. albicans.

S. cerevisiae as a surrogate host for generating ts alleles of C. albicans genes: Demonstrating that genes are essential in an obligate diploid such as C. albicans has been problematic. Although there are some new approaches (e.g., Enloe et al. 2000; Lee et al. 2001), the use of conditional alleles would, as we have shown, offer major advantages. Not only do they provide a way to prove essentiality of a gene, but they also allow the study of terminal loss-of-function phenotypes and provide a way to search for second-site suppressors. In our case, the ts alleles previously identified in S. cerevisiae ESS1 provided the basis for engineering equivalent mutations in C. albicans.

**DISCUSSION**

The results presented here show that the Ess1 prolyl-isomerase is conserved in C. albicans and suggest that its function is similar to that in budding yeast. Using a novel ts-mutant approach, we showed that ESS1 is essential for growth in C. albicans and that, as in budding yeast, loss-of-function mutants arrest in mitosis. However, these cells do not undergo nuclear fragmentation as in S. cerevisiae, suggesting that the mitotic arrest and nuclear fragmentation are genetically separable, at least in C. albicans.

**TABLE 2**

Summary of filamentation phenotypes of *tup1 ess1* and *cph1 ess1* double mutants

<table>
<thead>
<tr>
<th>Inducing medium</th>
<th>ESS1 genotype</th>
<th>TUP1 genotype</th>
<th>CPH1 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>+/+</td>
<td>Δ/Δ</td>
</tr>
<tr>
<td>Lee’s</td>
<td>+/+</td>
<td>+/+</td>
<td>Δ/Δ</td>
</tr>
<tr>
<td>Spider</td>
<td>+/+</td>
<td>–</td>
<td>+/+</td>
</tr>
<tr>
<td>Serum</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Noninducing</td>
<td>+/+</td>
<td>–</td>
<td>+/+</td>
</tr>
<tr>
<td></td>
<td>ts/Δ</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>ts/Δ</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>ts/Δ</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Cells were grown at 30° in YEPD and then shifted to the indicated liquid-inducing medium at 37° for 4 hr. +++, a population in which almost all cells are hyphal and/or pseudohyphal; +++, a population in which >50% are hyphal and/or pseudohyphal; +, a population in which <50% are hyphal and/or pseudohyphal; ±, a population in which there are few hyphal and/or pseudohyphal; –, a population in which no filamentous cells were observed; ND, not determined.

**Figure 8.** ESS1 mutants are defective in filamentation on solid hyphal-inducing media. Colony morphologies of wild-type SC5314, ess1+/+, and ess1Δ/Δ mutants grown on solid Lee’s, solid serum-containing, and solid Spider media at 30° for 7 days are shown. Colonies were photographed at the same magnification (20×).

**Figure 9.** ESS1 mutants are defective in filamentation in liquid hyphal-inducing media. Cell morphologies of wild-type SC5314, ess1+/+, and ess1Δ/Δ mutants in liquid Lee’s, serum-containing, and Spider media are shown. Cells were grown in YEPD media at 30° and then shifted to the indicated inducing medium for 4 hr at 37°. Bar, 10 μm.
the C. albicans gene. However, this site-directed strategy might not succeed in all cases. For example, a mutation generated in C. albicans NMT1 based on a ts allele in S. cerevisiae showed a loss-of-function phenotype, but this phenotype was not temperature sensitive in C. albicans, although the enzyme activity did show a ts effect in vitro (Weinberg et al. 1995).

Our use of S. cerevisiae as a surrogate host to prescreen for a conditional-mutant phenotype suggests it should be possible to generate de novo conditional alleles in any C. albicans gene that has a phenotype when expressed in S. cerevisiae. This could be accomplished using gapped-plasmid mutagenesis methods (Muhlrad et al. 1992). The generation and use of such conditional alleles will be an important addition to the repertoire of genetic techniques available for pathogenic fungi.

Ess1 is essential in some organisms but not others: While Ess1 is essential in both S. cerevisiae and C. albicans, its homologs (Pin1/Dodo) do not appear to be essential in Schizosaccharomyces pombe (H. Huang and T. Hunter, personal communication) or in metazoans (Maleszka et al. 1996; Fujimori et al. 1999). It is not known why Ess1 is essential in some organisms, but not others. This difference is not simply a matter of the presence of duplicated genes, as no closely related genes are found in these organisms. It is possible that there exist natural suppressor pathways, similar to those identified by Wu et al. (2000) that affect gene transcription. In fact, some ess1-deletion strains of S. cerevisiae are able to grow at reduced temperature (25°C), albeit very slowly (H. Huang and T. Hunter, personal communication). Whether Ess1 is essential in other human fungal pathogens remains to be determined.

Ess1 is required for hyphal induction: Under conditions in which Ess1 function is compromised, e.g., in the ess1<sup>H171R</sup> ts mutant at permissive temperature, C. albicans cells were defective in filamentation when shifted to inducing medium. Likewise, when the gene dosage was reduced, as in heterozygous mutant cells (ess1/ESS1), filamentation was severely compromised. Surprisingly, C. albicans ess1<sup>H171R</sup> seems to filament spontaneously at reduced temperature (25°C), even in the absence of inducing signals. This cold-sensitive phenotype might be due to an increase in inappropriate protein-protein interactions by the defective protein at low temperatures (Jarvick and Botstein 1973). Taken together, these data suggest that Ess1 function is involved in morphogenetic switching, perhaps via a role in transcription (see below). Since switching is important for virulence, it will be interesting to determine whether mutations in ESS1 also affect pathogenicity of C. albicans in animal models.

Morphogenetic switching, and in particular the transition from the yeast to hyphal forms, can be induced by different environmental stimuli ranging from starvation, to pH changes, to the presence of N-acetylglucosamine and proline in the medium (Ernst 2000). These signals are transduced by several independent signal-transduction pathways, including a MAP-kinase pathway and a cAMP-dependent pathway. As in S. cerevisiae, a common theme is that the downstream targets of these pathways appear to be transcription regulatory proteins. Ess1 has been linked to transcription regulation (Wu et al. 2000); thus, it is not surprising that Ess1 is important for the yeast-to-hyphal transition. It is possible that the expression of genes required for filamentation is altered in ess1 mutant cells.

Epistasis analysis with the transcription repressor TUP1 revealed that mutations in TUP1 are epistatic to those in ESS1. It is unclear from these experiments whether Ess1 functions upstream of Tup1 or in a parallel pathway. It is possible, for example, that Ess1 is required for the transcription of the TUP1 gene. In the absence of TUP1, filamentation genes are derepressed regardless of whether Ess1 is functional. Alternatively, Ess1 might function in a different pathway. Two findings favor this latter possibility. First, previous work has suggested that TUP1 is likely to have a function in the serum-induction
pathway (Braun and Johnson 2000). In contrast, ESSI does not appear to function in the serum-induction pathway, as suggested by the fact that ess1 mutants, despite having filamentation defects in Lee’s and Spider media, are capable of forming filaments in serum-containing medium (Figure 9).

Second, ess1 mutant strains show defects similar to eph1 mutants, suggesting that they are in the same pathway. CPH1 is a member of a MAP kinase cascade (Liu et al. 1994; Kohler and Fink 1996), which is serum independent and distinct from that of TUP1. That ESSI and CPH1 function in the same pathway is supported by the observation that, upon induction, eph1 ess1 double-mutant cells show neither enhanced nor decreased filamentation relative to the single mutants. Moreover, the phenotypes of ess1 tup1 (Figure 10) and eph1 tup1 double mutants (Braun and Johnson 1997) are similar. Thus, it is possible that ESSI functions in a MAP kinase pathway, perhaps by modulating transcription of downstream target genes. Interestingly, recent work in Drosophila indicates that Dodo functions as part of a MAP kinase pathway to control dorso-ventral patterning in the early embryo (Hsu et al. 2001). Finally, it is possible that Ess1 in C. albicans may function in multiple pathways, by isomerization of different prolyl-containing target proteins.

**Ess1 as an antifungal drug target:** Our results suggest that Ess1, a parvulin-class PPIase, might be a viable target for antifungal agents. Although the cyclophilin- and FKBP-class PPIases have been traditional targets for drug development, Ess1 offers several potential advantages. First, Ess1 inhibitors might be used to prevent cell growth. Or, at lower doses that reduce (but do not eliminate) Ess1 function, such inhibitors might prevent morphogenetic switching, which is required for virulence. Furthermore, since Ess1 is an essential gene, cells cannot become resistant, as they do against cyclosporin A or FK506, by mutations that abolish production of the respective PPIase. That is, a null mutation in ESSI would be lethal, and mutations that reduce Ess1 activity would be switching defective. Finally, since the mammalian homolog Pin1 does not appear to be essential, toxic side effects might be minimal. Further study will be needed to identify high-affinity Ess1 inhibitors and to test their efficacy in preventing fungal growth and pathogenicity.

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**LITERATURE CITED**


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