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Nonfilamentous C. albicans Mutants Are Avirulent

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Summary

Candida albicans and Saccharomyces cerevisiae switch from a yeast to a filamentous form. In Saccharomyces, this switch is controlled by two regulatory proteins, Ste12p and Phd1p. Single-mutant strains, *ste12/ste12* or *phd1/phd1*, are partially defective, whereas the *ste12/ste12 phd1/phd1* double mutant is completely defective in filamentous growth and is noninvasive. The equivalent *cph1/cph1 efg1/efg1* double mutant in Candida (Cph1p is the Ste12p homolog and Efg1p is the Phd1p homolog) is also defective in filamentous growth, unable to form hyphae or pseudohyphae in response to many stimuli, including serum or macrophages. This Candida *cph1/cph1 efg1/efg1* double mutant, locked in the yeast form, is avirulent in a mouse model.

Introduction

Candida albicans, the most frequently isolated fungal pathogen in humans (Edwards, 1990), can switch from a unicellular yeast form into two distinct filamentous forms: pseudohyphae (chains of distinct cells formed by the failure of mother and daughter to separate after each cell division) and hyphae (long continuous tubes with septae separating each of the nuclei but no distinct indentation at the site of septation). This switch is induced by many environmental cues, but perhaps the most critical for pathogenicity is the induction by serum or by macrophages (Shepherd et al., 1980; Dabrowa and Howard, 1981). The ability to switch between these forms is thought to be important for Candida's virulence (Cutler, 1991). However, this conjecture has not been tested by a comparison between wild-type strains and isogenic mutant strains that are unable to switch. The direct isolation of nonfilamentous mutants is difficult because Candida is diploid and has no known sexual cycle (Edwards, 1990; Diener and Fink, 1996).

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An alternative approach is based on the assumption that the yeast to filament switch is controlled by the same morphogenetic pathways in all fungi despite differences in the inductive signals and the shape and form of the filaments. If this assumption is correct, then one could identify genes required for morphogenesis in a genetically tractable system, clone and knockout the homologous genes from Candida, and determine whether these Candida mutants are defective in morphogenesis and/or pathogenesis. This approach became feasible when a filamentous cycle was discovered in a laboratory strain of Saccharomyces cerevisiae, which is amenable to the complete repertoire of modern molecular genetic techniques. Diploid strains of Saccharomyces switch from a unicellular yeast form to a pseudohyphal form upon starvation for nitrogen (Gimeno et al., 1992). This switch depends upon Ste20p, Ste11p, Ste7p, and Ste12p, elements of a conserved mitogen-activated protein kinase (MAPK) pathway. Null mutations in the genes encoding these proteins are defective in both mating and filamentous growth (Liu et al., 1993; Mösch et al., 1996).

The role of MAPK genes in Candida morphogenesis was explored using the information from Saccharomyces as a guide. To this end, Candida homologs of the *STE20*, *STE7*, and *STE12* genes were cloned and used to construct null mutations in Candida by transformation-mediated gene replacement. Strains of Candida homozygous for mutations in the *STE20*, *STE7*, and *STE12* homologs are partially defective in hyphal development on solid media (Liu et al., 1994; Köhler and Fink, 1996; Leberer et al., 1996). However, these MAPK pathway mutants still form hyphae in liquid culture and in response to serum. One interpretation of this result (Köhler and Fink, 1996) is that there is a second, Ste12p-independent pathway by which serum could induce filaments in Candida.

In this report, we again used Saccharomyces to identify the Ste12p-independent pathway in Candida. In Saccharomyces null Ste$^-$ mutants are only partially defective for their pseudohyphal development. The gene responsible for this residual activity is *PHD1*, whose overexpression was shown previously to enhance pseudohyphal growth (Gimeno and Fink, 1994). Double-mutant strains that contain deletions of both *STE12* and *PHD1* (e.g., *ste12/ste12 phd1/phd1*) are completely defective in pseudohyphal growth. Recently, *EFG1*, the Candida homolog of the Saccharomyces *PHD1* gene, was cloned. The study of the *EFG1* gene showed that a reduced *EFG1* expression level suppresses formation of true hyphae (Stoldt et al., 1997), though pseudohyphal filaments were still formed in response to serum. We constructed Candida double mutants lacking both *CPH1* and *EFG1* function. These Candida *cph1/cph1 efg1/efg1* strains fail to form filaments in response to serum or other known inducers of filamentous growth and are avirulent in a mouse infection model.

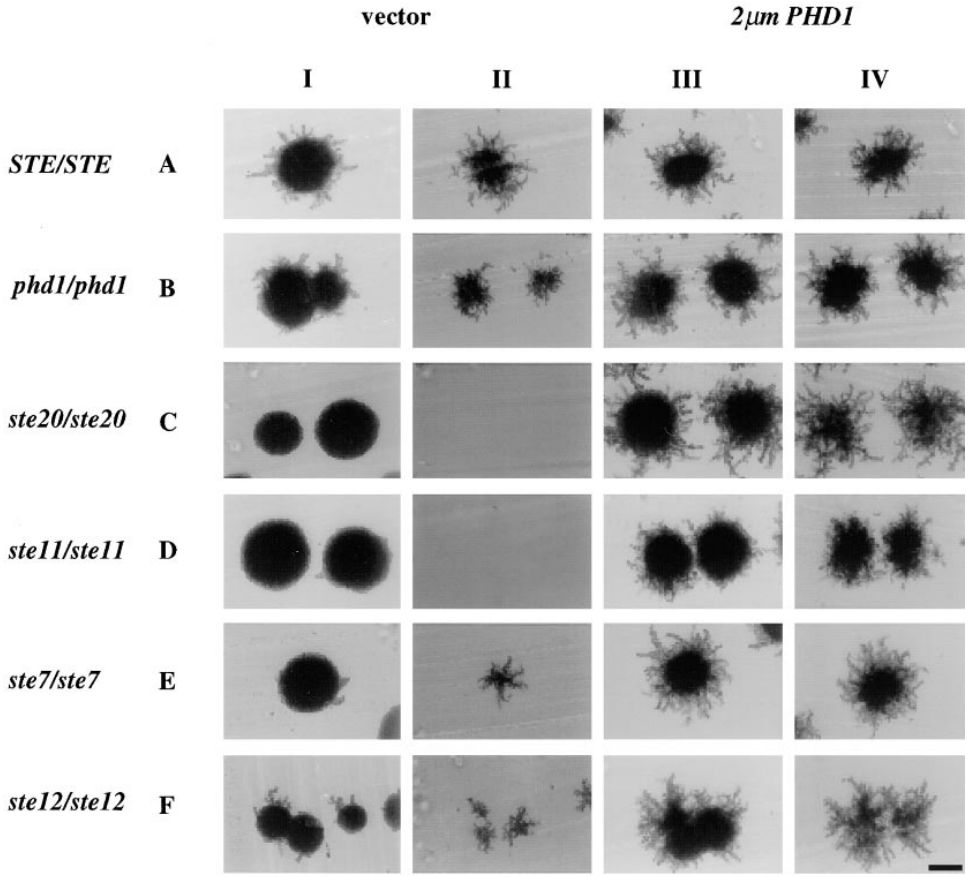

Figure 1. The Effect of Overexpression of *PHD1* on Filamentous and Invasive Growth in *ste* Mutants of Saccharomyces

Wild-type *STE/STE* and *ste/ste* diploids were streaked on synthetic low ammonia dextrose (SLAD) medium, and the resulting colonies were photographed after 5 days of growth at 30°C. The vertical columns I and II were strains transformed with high copy vector without an insert (vector) and the vertical columns III and IV were strains transformed with a high copy vector containing *PHD1* (2 μm *PHD1*). Columns I and III were photographed before washing; Columns II and IV were columns I and III, respectively, after washing. (A), STE/*STE* (L6219, L6220); (B), *phd1/phd1* (L6229, L6230); (C), *ste20/ste20* (L6221, L6222); (D), *ste11/ste11* (L6231, L6232); (E), *ste7/ste7* (L6225, L6226); and (F), *ste12/ste12* (L6227, L6228). Scale bar, 20 μm.

Results

Pseudohyphal Growth in Saccharomyces Is Controlled by Both a Ste12p-Dependent and -Independent Pathway

Pseudohyphal growth in Saccharomyces is controlled by elements of the mating MAPK pathway; however, previous work had shown that null mutants in that pathway are only partially defective (Liu et al., 1993; Mösch et al., 1996). For example, strains homozygous for a deletion of *STE12* (*ste12/ste12*) and *STE7* (*ste7/ste7*) form filaments and invade the agar, albeit at a considerably reduced frequency as compared with *STE/STE* strains (compare Figures 1AI and 1AII with Figures 1EI, 1EII, 1FI, and 1FII). This partial defect suggests that there are other genes responsible for the residual pseudohyphal growth. Since overexpression of the *PHD1* gene suppresses the filamentation and invasion defect of these *ste/ste* mutants (Figure 1), we posited that *PHD1* function was responsible for the residual activity of these mutants. Diploid strains containing a deletion of the *PHD1* gene (*phd1/phd1*) show only slight diminuation of pseudohyphal growth (Figures 1BI and 1BII). Furthermore, *ste12/ste12* mutants have reduced expression of the filamentous growth reporter *FG(Ty)::lacZ* (Mösch et al., 1996), but the *phd1/phd1*-null mutants do not. The β-galactosidase activity of *FG(Ty)::lacZ* with respect to *PHD1 STE12* (100%) is: *STE12 phd1*, 100%; *PHD1 ste12*, 5%; and *phd1 ste12*, 5%.

To test the hypothesis that *PHD1* is responsible for the residual pseudohyphal growth of the *ste/ste* mutants, we constructed a *ste12/ste12 phd1/phd1* double-mutant strain. This double mutant forms no visible filaments and fails to invade the agar medium (Figures 2D and 2H). Furthermore, this *ste12/ste12 phd1/phd1* double mutant does not form long pseudohyphal cells in the colony (see Experimental Procedures). Transformation of the *ste12/ste12 phd1/phd1* double mutant with either the functional *PHD1* or *STE12* gene restores pseudohyphal growth and invasion to the level shown by the single-mutant strains (compare Figures 2B, 2F and 2C, 2G with Figure 1BI, 1BII and 1FI, 1FII). The

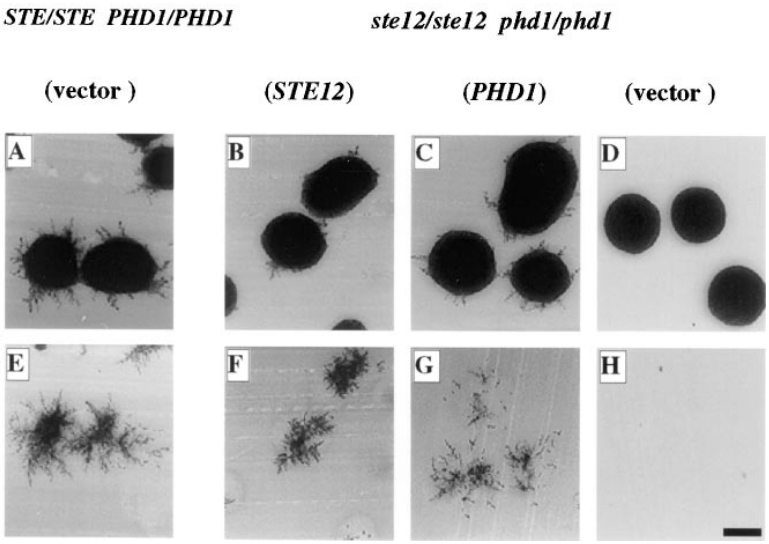

Figure 2. The *ste12/ste12 phd1/phd1* Double Mutant Is Completely Defective in Pseudohyphal Growth

Wild-type and mutant diploids were streaked on SLAD medium, and the resulting colonies were photographed after 5 days of growth at 30°C. (A)–(D) were photographed before washing; (E)–(H) were (A)–(D), respectively, photographed after washing. (A) and (E) are *STE/STE* (L6220); (B) and (F) are *ste12/ste12 phd1/phd1* containing a p650 (Kirkman-Correia et al., 1993) *CEN-STE12* plasmid (L6235); (C) and (G) are *ste12/ste12 phd1/phd1* containing an integrated copy of *PHD1* (L6236); (D) and (H) are *ste12/ste12 phd1/phd1* (L6234) containing a vector plasmid. The letters in parentheses denote a gene introduced into the strain by transformation. Scale bar, 20 μm.

ste7/ste7 phd1/phd1 double mutant also fails to form filaments and is less invasive than either of the single mutants (data not shown). Thus, both the morphological and reporter analysis suggest that pseudohyphal growth is controlled by two distinct inputs, a Ste12p-dependent pathway and a Phd1p-dependent pathway, both of which can signal pseudohyphal and invasive growth under conditions of nitrogen starvation.

Construction of an *efg1/efg1* Mutant in Candida

Previous work had shown that Candida strains containing null mutations of the *CPH1* gene, the Candida homolog of Saccharomyces *STE12* (Liu et al., 1994), are, like their Saccharomyces counterparts, only partially defective in their ability to switch from the yeast to filamentous forms. Candida is much more versatile than Saccharomyces and can switch from the yeast form to pseudohyphae as well as hyphae. This switch occurs on agar plates in response to many external signals. The most dramatic switch is observed in response to serum: on plates containing serum and no other nutrient, Candida yeast form cells switch to form abundant hyphae. The Candida *cph1/cph1* strains show reduced hyphal formation on solid media but with time form both hyphae and pseudohyphae. *cph1/cph1* strains respond to serum: they form germ tubes when suspended in serum and hyphae on solid media containing serum (Liu et al., 1994; Köhler and Fink, 1996; Leberer et al., 1996). Based on our results with Saccharomyces, we surmised that serum might induce Candida hyphae via two pathways: a Cph1p- (Ste12p-) dependent pathway and an Efg1p- (Phd1p-) dependent pathway.

To determine whether our results from Saccharomyces were relevant to Candida morphogenesis, we cloned the Candida homolog of the Saccharomyces *PHD1* gene called *EFG1* (Stoldt et al., 1997) and constructed disruptions of this *EFG1* gene. The predicted amino acids 188 to 319 of the putative Candida *EFG1* gene are 68% identical (81% similar) to residues 170 to 301 of the *PHD1* gene. There appears to be only a single *EFG1* sequence in the Candida genome, as no other hybridizing bands are observed by Southern analysis (Figure 3B). An *efg1::hisG-URA3-hisG* disruption construct was used to transform a Ura3$^-$ Candida strain in order to replace the chromosomal *EFG1* gene. As Candida is diploid, there are two copies of the *EFG1* gene, which had to be sequentially deleted to construct the null mutant (see Experimental Procedures). As shown in Figure 3B, this procedure resulted in Candida strains lacking an intact copy of the *EFG1* gene.

Strains lacking the *EFG1* gene are markedly affected in their ability to switch forms on solid medium containing serum (Figure 4C). Serum induces *efg1/efg1* strains to form long cells and filaments that are morphologically different from those found in *EFG1/EFG1* strains. *efg1/efg1* strains appear to make many pseudohyphae, whereas *EFG1/EFG1* strains make predominantly hyphae (compare Figure 4A with Figure 4C). The morphological abnormalities of the *efg1/efg1* strains make it difficult to determine whether there are any true hyphae, although the pseudohyphae appear to emanate from an initial hyphal-like structure. Despite the fact that the *efg1/efg1* mutant forms abundant pseudohyphae on solid agar/serum medium (Figure 4C), this strain fails to form germ tubes and hyphae in the presence of serum (Figures 4C and 5C). Germ tubes are the initial projections observed when Candida yeast form cells switch to hyphal growth.

Candida *cph1/cph1 efg1/efg1* Mutants Fail to Switch from Yeast to Filamentous Forms

Since the *efg1/efg1* mutants could still switch from the yeast form to make a recognizable filament, we made *cph1/cph1 efg1/efg1* double mutants, which were formally equivalent to the *ste12/ste12 phd1/phd1* strains of Saccharomyces. The *cph1/cph1 efg1/efg1* strain was constructed by transforming a *cph1/cph1 ura3/ura3* Candida strain (see Experimental Procedures). The genotypes of all transformants were verified by Southern analysis (Figure 3B). A comparison of the growth rates

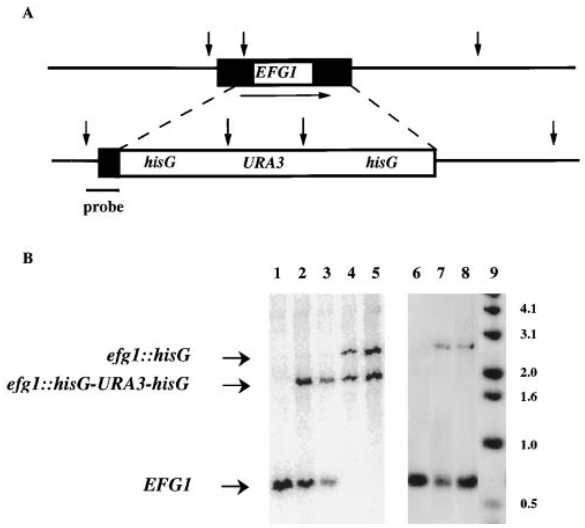

Figure 3. Disruption of the *EFG1* Gene in Candida

(A) The *EFG1* open reading frame (solid bar) was replaced by the *hisG-URA3-hisG* cassette (open bar). The EFG-AB fragment was used as the probe for Southern blot analysis. The AvaII sites were indicated by arrows.

(B) Southern blots of various strains used in this study. Genomic DNA digested with AvaII was from the following strains. Lane 1, *CPH1/CPH1 EFG1/EFG1* (SC5314); lane 2, *CPH1/CPH1 EFG1/efg1* (HLC37); lane 3, *cph1/cph1 EFG1/efg1* (HLC17); lane 4, *CPH1/CPH1 efg1/efg1* (HLC52); lane 5, *cph1/cph1 efg1/efg1* (HLC54); lane 6, *CPH1/CPH1 EFG1/EFG1* (SC5314); lane 7, *CPH1/CPH1 efg1/efg1* containing an integrated copy of *EFG1* (HLC74); lane 8, *cph1/cph1 efg1/efg1* containing an integrated copy of *EFG1* (HLC84); lane 9, molecular weight standards of which sizes (in kb) are labeled on the side. (*EFG1*): a functional *EFG1* gene that was introduced into the strain by transformation.

of wild type with all the mutant derivatives in 2× YPD medium showed that they were all roughly comparable (Table 1).

The double mutant had a striking phenotype: *cph1/cph1 efg1/efg1* strains fail to form germ tubes and hyphae under any conditions we have tested. On agar

Table 1. Doubling Time

Strain	Doubling Time[a] (hr)
SC5314 (*CPH1/CPH1 EFG1/EFG1*)	0.96 ± 0.054
HLC37 (*CPH1/CPH1 EFG1/efg1*)	1.18 ± 0.074
HLC52 (*CPH1/CPH1 efg1/efg1*)	1.15 ± 0.074
HLC74 [*CPH1/CPH1 efg1/efg1* (*EFG1*)]	1.11 ± 0.131
JKC19 (*cph1/cph1 EFG1/EFG1*)	1.05 ± 0.062
HLC17 (*cph1/cph1 EFG1/efg1*)	1.05 ± 0.087
HLC54 (*cph1/cph1 efg1/efg1*)	1.03 ± 0.100
HLC84 [*cph1/cph1 efg1/efg1* (*EFG1*)]	0.99 ± 0.085

[a]Average of three independent experiments with standard deviation.

containing serum plates they form colonies of yeast form cells (Figure 4G) and in liquid serum they remain as slightly elongated yeast form cells without any visible indication of germ tube formation (Figure 5G). Furthermore, other treatments that induce *CPH1/CPH1 EFG1/EFG1* strains to form germ tubes and hyphae, such as high temperature, neutral pH, 2.5 mM N-acetylglucosamine, 10 mM L-proline plus 2.5 mM glucose, 2.5 mM glutamine plus 2.5 mM glucose (Shepherd et al., 1980; Dabrowa and Howard, 1981; Birse et al., 1993), spider medium (Liu et al., 1994), and medium 199 (Saporito-Irwin et al., 1995), fail to induce *cph1/cph1 efg1/efg1* cells, which remain in the yeast form (data not shown). A functional *EFG1* gene was integrated into *efg1/efg1* strains by transformation. Filamentation was restored when a functional *EFG1* gene was introduced into both *efg1/efg1* and *cph1/cph1 efg1/efg1* strains by transformation, showing that the phenotypes of these strains are a consequence of the *efg1* mutation (Figures 4D, 4H, 5D, and 5H).

Candida Double Mutants Are Defective in Their Interaction with Macrophages

To test the interaction of our mutants with host cells, we observed the interaction of Candida with a macrophage-like cell line, IC-21. Approximately 40 to 60 min after ingestion by a macrophage, wild-type Candida cells

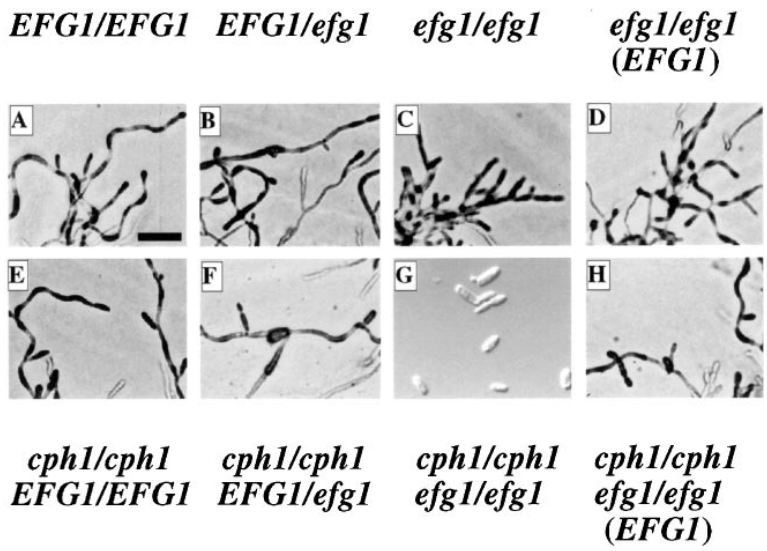

Figure 4. Filamentous Growth Defect of *cph1/cph1 efg1/efg1* Strains

Cells were grown on 2% agar plus 4% bovine calf serum medium for 16 hr at 37°C. (A) *CPH1/CPH1 EFG1/EFG1* (SC5314); (B) *CPH1/CPH1 EFG1/efg1* (HLC37); (C) *CPH1/CPH1 efg1/efg1* (HLC52); (D) *CPH1/CPH1 efg1/efg1* containing an integrated copy of *EFG1* (HLC74); (E) *cph1/cph1 EFG1/EFG1* (JKC19); (F) *cph1/cph1 EFG1/efg1* (HLC17); (G) *cph1/cph1 efg1/efg1* (HLC54); (H) *cph1/cph1 efg1/efg1* containing an integrated copy of *EFG1* (HLC84). (*EFG1*): a functional *EFG1* gene that was introduced into the strain by transformation. Scale bar, 20 μm.

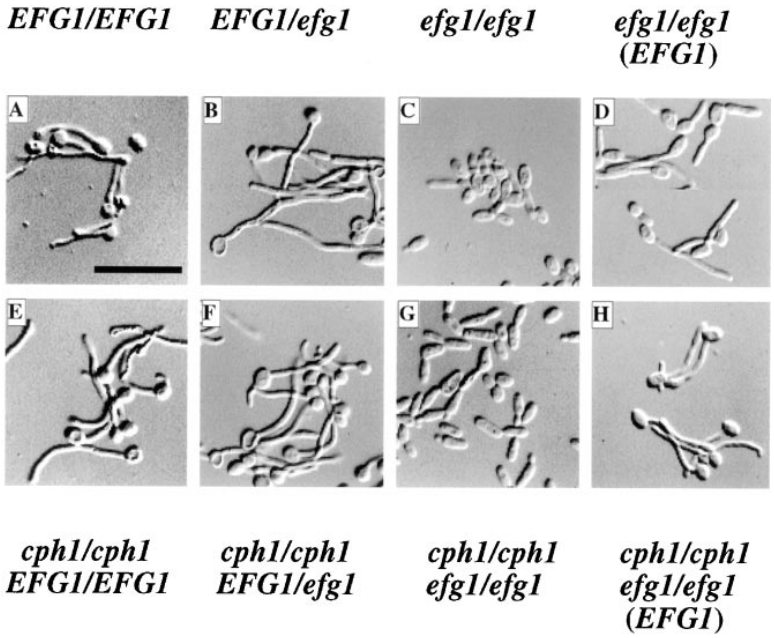

Figure 5. Germ Tube Formation Defect of *efg1/efg1* and *cph1/cph1 efg1/efg1* Strains

Cells were incubated in YPD liquid medium containing 20% bovine calf serum (Sigma S-6648) for 2 hr at 37°C. (A) *CPH1/CPH1 EFG1/EFG1* (SC5314); (B) *CPH1/CPH1 EFG1/efg1* (HLC37); (C) *CPH1/CPH1 efg1/efg1* (HLC52); (D) *CPH1/CPH1 efg1/efg1* containing an integrated copy of *EFG1* (HLC74); (E) *cph1/cph1 EFG1/EFG1* (JKC19); (F) *cph1/cph1 EFG1/efg1* (HLC17); (G) *cph1/cph1 efg1/efg1* (HLC54); (H) *cph1/cph1 efg1/efg1* containing an integrated copy of *EFG1* (HLC84). (*EFG1*): a functional *EFG1* gene that was introduced into the strain by transformation. Scale bar, 20 μm.

form germ tubes. Despite vigorous activity of the macrophage, the majority of hyphae continue growing, ultimately piercing the macrophage from within (Figures 6Ac–6Ae). The dynamics vary with the ratio of Candida cells to macrophages. Those hyphae that escape the macrophages continue to grow and to produce fresh yeast form cells, which are eventually ingested and in turn germinate into hyphae. The culture is transformed into a dense hyphal mat with macrophages impaled on the hyphae.

Ingestion of the *cph1/cph1* Candida mutant by macrophages results in induction of hyphae and destruction of the macrophages (Figures 6Ba–6Bc). The course of events is similar to that when the isogenic *CPH1/CPH1* strain is ingested. The scenario is quite different with the double mutant. Although the *cph1/cph1 efg1/efg1* strain is ingested normally by the macrophage, it does not form either germ tubes or hyphae once inside. While ingesting the *cph1/cph1 efg1/efg1* Candida cells, the movement and morphology of the macrophage remain normal. Macrophages that have internalized as many as 80 mutant Candida cells remain flat and adhere to the coverslip even 20 hr after they were infected with Candida (Figure 6Bh). The *efg1/efg1* single-mutant Candida strain has a distinct phenotype: though most of the Candida cells inside macrophages do not form hyphae and their host macrophage retains its morphology (Figures 6Be and 6Bf), a few ingested cells divide to form filaments and rupture the membrane of the macrophage (Figure 6Bd).

The *cph1/cph1 efg1/efg1* Double Mutant Is Not Virulent in a Mouse

The virulence of the various mutants was tested in the CF-1 mouse model (see Experimental Procedures). Injection of mice with wild-type Candida is lethal: a high inoculum of yeast form cells (1×10^7 cells) kills 90% of the mice 2 days after injection, and even a low inoculum (1×10^5 cells) kills 50% of the mice 6 days after injection (Figure 7A). All single-mutant Candida strains are still virulent, although the virulence patterns do not completely parallel the morphology of the strains: the *EFG1/efg1* heterozygote, which appears to form hyphae and pseudohyphae normally, is less virulent than the *EFG1/EFG1* strain (compare Figure 7A with Figure 7B). The *cph1/cph1* strain has a virulence pattern that is similar to that of the wild-type strain (Figure 7D), a result that is consistent with its interaction with macrophages. The *efg1/efg1* strain, which is defective in forming germ tubes and hyphae in the presence of serum and in its interaction with macrophages, shows reduced virulence: wild type kills half of the mice upon inoculation with 10^5 cells, whereas the *efg1/efg1* strain achieves the same level of killing only with an inoculum of 10^7 cells (compare Figure 7A with Figure 7C). By contrast with the single mutants, the *cph1/cph1 efg1/efg1* double mutant is nearly avirulent: with inocula of 10^6 cells or less the double mutant fails to kill in this mouse model. Even with an inoculum of 1×10^7 cells, half of the mice survive after 15 days (Figure 7F).

Discussion

Dual Control of Morphogenesis in Saccharomyces and Candida

Complete abolition of filamentation in both Saccharomyces and Candida requires mutations in two different functions: one in a component of the MAPK cascade and another in the *PHD1/EFG1* gene. Both the Candida *cph1/cph1 efg1/efg1* and the Saccharomyces *ste12/ste12 phd1/phd1* mutants fail to filament under any condition tested, whereas neither single mutation is sufficient to block this morphogenetic pathway completely. One explanation for this dual requirement is that morphogenesis is controlled by two parallel signaling pathways, each with a different and additive input. For example, Ste12p and Phd1p, both likely transcriptional

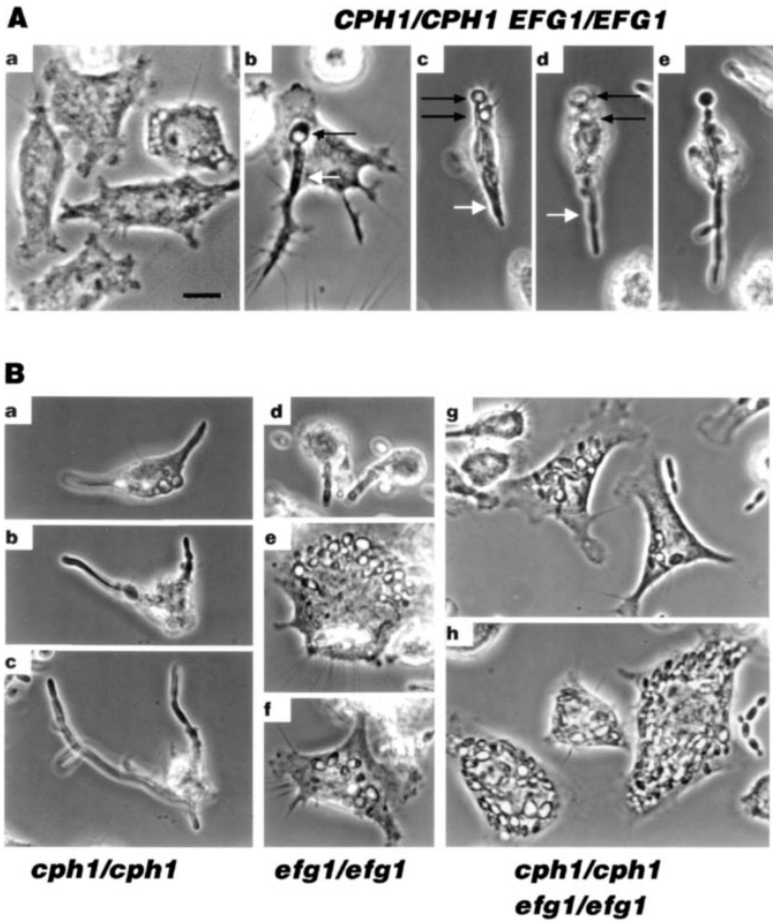

Figure 6. Candida and Macrophage-like Cultured Cell Interaction

(A) (a) Uninfected macrophages in culture medium. Scale bar, 10 μm. (b–e) Wild-type Candida (SC5314). Black arrows point to yeast form cells; white arrows point to germ tubes. (b) Macrophage containing Candida that is forming a germ tube, 1 hr after infection. (c)–(e) are the same macrophage photographed at 3, 4, and 6 hr after infection, respectively. (d) One Candida filament has pierced through the macrophage's cell membrane; (e) the macrophage has moved down along the Candida filament, so that the original yeast form cell is protruding outside the macrophage's cytoplasm.

(B) Mutant strains of Candida in macrophages. (a)–(c) are the same macrophage photographed at 4, 6, and 9 hr, respectively, after infection with *cph1/cph1* (JKC19). (b) The hyphae have ruptured the cell membrane at two points. (c) The macrophage has rounded up completely and one of the original yeast form Candida cells is sprouting a second germ tube. (d) shows two macrophages each containing one cell of the *efg1/efg1* mutant (HLC52), with a germ tube protruding 3 hr after infection. The macrophages have rounded up. (e and f) Yeast form *efg1/efg1* cells in macrophages 17 hr after infection. The Candida vacuoles are the white circles and ovals within the dark fungal cell outline. The macrophages are flat and adherent to the coverslip. (g) Yeast form *cph1/cph1 efg1/efg1* cells (HLC54) in macrophages 3 hr after infection. (h) *cph1/cph1 efg1/efg1* in three macrophages 20 hr after infection, containing up to 80 yeast cells.

activators, could independently activate a gene(s) required for filamentation. Although the gene(s) could be activated by either protein, maximum expression would require the binding of both proteins. The bypass of the Ste12 defect in filamentation by overexpression of Phd1p is consistent with this model. Ste12p has been shown to bind at a filamentation response element (FRE) (Madhani and Fink, 1997). However, Ste12p and Phd1p do not act together at the FRE element because expression of the *FRE:lacZ* reporter is reduced only in *ste12* and not in *phd1* mutants (data not shown).

Of course, the exact nature of the defects of the single and double mutants must be understood within the context of the biology of each organism. In Candida, the *efg1/efg1* mutant is more severe than the *cph1/cph1* mutant. The *efg1/efg1* mutant appears to make very few hyphae or germ tubes, yet it still makes pseudohyphae. Our results with the *efg1/efg1* single mutant are similar to those reported by another group (Stoldt et al., 1997) that recently cloned the same gene. Their results on the morphology of strains with reduced *EFG1* function is similar to ours: namely, a defect in hyphal formation. They were unable to obtain the homozygous *efg1/efg1* disruption strain and therefore presumed that *EFG1* is essential for viability. These authors reduced *EFG1* expression using a repressible promoter construction. Using a different disruption construct and a different strain, we have been able to obtain the *efg1/efg1* homozygous disruptant.

One interpretation of the differences between the *efg1/efg1* and *cph1/cph1* phenotypes is that germ tube and hyphal formation are the same developmental event, one that is distinct from pseudohyphal formation. According to this interpretation, *efg1/efg1* strains form pseudohyphae because they are defective in the hyphal pathway and not in the pseudohyphal pathway. Another interpretation is that pseudohyphal and hyphal formation represent a continuum of stages in a single pathway of filamentous growth and that a quantitatively stronger regulatory input is needed to produce hyphae than to produce pseudohyphae. By contrast, in Saccharomyces, which does not make hyphae, the *ste12/ste12* mutant has a much more dramatic defect in pseudohyphal formation than that found in the *phd1/phd1* mutant. What is striking is that despite differences in the ecology, developmental options, and evolutionary history, the double mutants in both species are restricted to growth in the yeast form.

The Relationship between Morphogenesis and Virulence in Candida

The Candida *cph1/cph1 efg1/efg1* mutant shows a dramatic reduction in pathogenicity in the mouse. This strain is remarkably avirulent, even when 10^7 cells are injected into the tail vein of a mouse. By contrast, the *efg1/efg1* and *cph1/cph1* single mutants are still virulent, even though the *efg1/efg1* mutant shows a pattern that is discernibly different from that of a wild-type

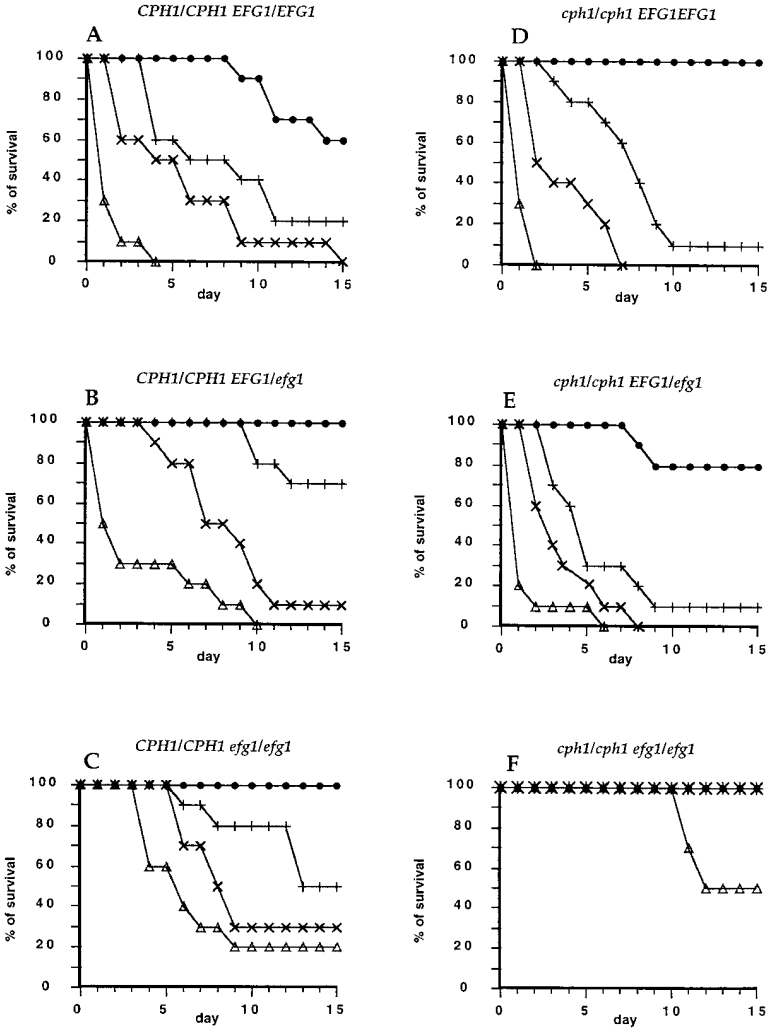

Figure 7. Virulence Assay

CF1 mice were injected with the following strains: (A), *CPH1/CPH1 EFG1/EFG1* (SC5314); (B), *CPH1/CPH1 EFG1/efg1* (HLC37); (C), *CPH1/CPH1 efg1/efg1* (HLC52); (D), *cph1/cph1 EFG1/EFG1* (JKC19); (E), *cph1/cph1 EFG1/efg1* (HLC17); (F), *cph1/cph1 efg1/efg1* (HLC54). Four concentrations of Candida were injected into mice: open triangles, 1×10^7; x, 1×10^6; +, 1×10^5; and closed circles, 1×10^4 cells. In (F), all the mice injected with 1×10^4, 1×10^5, and 1×10^6 *cph1/cph1 efg1/efg1* cells survived.

strain. It is noteworthy that the *efg1/efg1* strain is still virulent despite the fact that it is unable to form abundant hyphae.

The interaction between macrophages and the various Candida strains provides an interesting insight into the functions necessary for virulence. All the strains appear to be recognized to an equal degree by the macrophage. However, after being ingested, the wild type is induced to form a hypha that ultimately kills the macrophage. By contrast, the *cph1/cph1 efg1/efg1* mutant fails to form filaments. In fact, after the macrophage ingests the *cph1/cph1 efg1/efg1* mutant, it continues to scavenge for more Candida cells and, after 20 hr, can be filled with as many as 80 yeast cells. Even when engorged with this internal population of yeast, the macrophage appears to retain its adherence, shape, and motility.

Although we have shown that the ability to switch from yeast to the filamentous form is required for virulence, our experimental design still leaves open the question of whether it is the yeast or the hyphal form that is responsible for pathogenicity. It is also possible that genes unrelated to cell shape but regulated by the transcription factors *CPH1* and *EFG1* are required for virulence. Other pathogenic fungi such as Cryptococcus neoformans, Histoplasma capsulatum, and Blastomyces dermatitidis appear to proliferate in the host exclusively as yeast form cells (Bullock, 1990; Chapman, 1990; Diamond, 1990). One possibility is that the virulence of Candida is due to its ability to switch between growth forms in the host. The filamentous growth form may be required to evade the cells of the immune system, whereas the yeast form may be the mode of proliferation once Candida reaches a target tissue.

Induction of Filamentation in Candida

Our data show that the *cph1/cph1 efg1/efg1* mutant is defective in filament formation under many different growth conditions. These varied growth conditions are a broad sample of those known to induce morphogenesis in Candida (for a review, see Odds, 1985), suggesting that the double mutant may be unable to switch in response to any external signal. Knowledge of all the genes and proteins involved in this dual pathway could reveal important therapeutic targets.

Experimental Procedures

Strains and Media

Saccharomyces cerevisiae strains, Candida albicans strains, and transforming plasmids are listed in Table 2, Table 3, and Table 4,

Table 2. Saccharomyces cerevisiae Strains

Strain	Genotype	Source
10560-4A	*MAT***a** *his3::hisG leu2::hisG trp1::hisG ura3-52*	Fink laboratory collection
10560-6B	*MAT*α *his3::hisG leu2::hisG trp1::hisG ura3-52*	Fink laboratory collection
L6211	*MAT***a** *his3::hisG leu2::hisG trp1::hisG ura3-52 ste11::URA3*	This study
L6212	*MAT*α *his3::hisG leu2::hisG trp1::hisG ura3-52 ste11::URA3*	This study
L6213	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 phd1::hisG/phd1::hisG*	This study
L6214	*MAT***a** *his3::hisG leu2::hisG trp1::hisG ura3-52 ste11::ura3-LEU2-ura3*	This study
L6215	*MAT*α *his3::hisG leu2::hisG trp1::hisG ura3-52 ste11::ura3-LEU2-ura3*	This study
L6216	*MAT***a** *his3::hisG leu2::hisG trp1::hisG ura3-52 ste11::ura3-LEU2-ura3 (B2068)*	This study
L6217	*MAT*α *his3::hisG leu2::hisG trp1::hisG ura3-52 ste11::ura3-LEU2-ura3 (B2068)*	This study
L6218	*MAT***a**/*MAT*α *his3::hisG/his3::hisGleu2::hisG/leu2::hisG trp1::hisG/trp1::hisG ura3-52/ura3-52 ste11::ura3-LEU2-ura3/ste11::ura3-LEU2-ura3*	This study
L6219	*MAT***a**/*MAT*α *his3::hisG/his3::hisG ura3-52/ura3-52 (pCG38, B3161)*	This study
L6220	*MAT***a**/*MAT*α *his3::hisG/his3::hisG ura3-52/ura3-52 (pRS202, B3161)*	This study
L6221	*MAT***a**/*MAT*α *his3::hisG/his3::hisG ura3-52/ura3-52 trp1::hisG/trp1::hisG ste20::TRP1/ste20::TRP1 (pCG38, B3161)*	This study
L6222	*MAT***a**/*MAT*α *his3::hisG/his3::hisG ura3-52/ura3-52 trp1::hisG/trp1::hisG ste20::TRP1/ste20::TRP1 (pRS202, B3161)*	This study
L6223	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG ura3-52/ura3-52 ste11::ura3-LEU2-ura3/ste11::ura3-LEU2-ura3 (pCG38 B3161)*	This Study
L6224	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG ura3-52/ura3-52 ste11::ura3-LEU2-ura3/ste11::ura3-LEU2-ura3 (pRS202 B3161)*	This study
L6225	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 ste7::LEU2/ste7::LEU2 (pCG38 B3161)*	This study
L6226	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 ste7::LEU2/ste7::LEU2 (pRS202 B3161)*	This study
L6227	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-62/ura3-52 ste12::LEU2/ste12::LEU2 (pCG38 B3161)*	This study
L6228	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 ste12::LEU2/ste12::LEU2 (pRS202 B3161)*	This study
L6229	*MAT***a**/*MAT*α *his3::hisG/his3::hisG ura3-52/ura3-52 phd1::hisG/phd1::hisG (pCG38, B3161)*	This study
L6230	*MAT***a**/*MAT*α *his3::hisG/his3::hisG ura3-52/ura3-52 phd1::hisG/phd1::hisG (pRS202, B3161)*	This study
L6231	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 ste11::ura3-LEU2-ura3/ste11::ura3-LEU2-ura3 (pCG38 B3161)*	This study
L6232	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 ste11::ura3-LEU2-ura3/ste11::ura3-LEU2-ura3 (pRS202 B3161)*	This study
L6234	*MAT***a***MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 phd1::hisG-URA3-hisG/ phd1::hisG-URA3-hisG ste12::LEU2/ste12::LEU2 (pRS314, pRS316)*	This study
L6235	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 phd1::hisG-URA3-hisG/ phd1::hisG-URA3-hisG ste12::LEU2/ste12::LEU2 (pRS314, B2552)*	This study
L6236	*MAT***a**/*MAT*α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 phd1::hisG-URA3-hisG/ phd1::hisG-URA3-hisG ste12::LEU2/ste12::LEU2 (pRS314, HLB6)*	This study

All these strains are congenic to the Σ1278b genetic background.

respectively. Standard media were prepared as described in Sherman et al. (1986). SLAD medium was prepared as described in Gimeno et al. (1992); Spider medium was prepared as described in Liu et al. (1994); medium 199 powder was obtained from GIBCO BRL (31100-084) and used at pH 7 to make 2% agar plates (Saporito-Irwin et al., 1995). Solid agar/serum medium was 2% agar containing 4% bovine calf serum. Bovine calf serum was obtained from Sigma (Sigma S-6648).

Saccharomyces Strains Containing a Disrupted *ste11* Gene

Saccharomyces strains, L6211 and L6212 (*ste11::URA3*) were made by transforming 10560-4A and 10560-6B strains with a *ste11::URA3* disruption plasmid pNC202 (Rhodes et al., 1990). Plasmid B2387 contains a *ura3-LEU2-ura3* cassette that was used to convert URA3 to LEU2 (a gift from Yona Kassir, Technion, Haifa, Israel). L6211 and L6212 (*ste11::URA3*) were transformed with the HindIII fragment of B2387 containing the *ura3-LEU2-ura3* cassette. Leu^+ transformants were obtained that had the *URA3* gene replaced by the *LEU2* gene. L6214 and L6215 were Leu^+, Ura^- transformants from L6211 and L6212, respectively. To generate a *ste11/ste11* diploid, we constructed L6216 and L6217 from L6214 and L6215, respectively, by transformation with B2068 containing *CEN-STE11-TRP1*. L6219 was constructed by crossing L6216 and L6217 and obtaining segregants of the resulting diploid that lack the *CEN-STE11-TRP1* plasmid.

Determination of Cell Shape

The shape of cells was analyzed by measuring the dimensions of cells growing in colonies after 3 days of growth at 30°C on SLAD medium. Because the *ste12/ste12 phd1/phd1* double mutant fails to invade the agar and is completely washed off the plate, we could not measure the dimension of cells in the agar as described by Mösch and Fink (1997). The colonies were removed with a toothpick, suspended in 15 μl of water, and the cells were analyzed for cell shape by light microscopy. Cell shape patterns of different strains were quantified by determining the length to width (l/w) ratio of 1000 cells from six independent colonies. As in our earlier study, cells were designated "long pseudohyphal cells" if the ratio length/width was greater than 2.

***EFG1* Disruption Construct**

The disruption construct was made by first synthesizing short fragments from the 5′(EFG-AB) and 3′(EFG-CD) ends of *EFG1* and inserting the *hisG-URA3-hisG* in between these fragments. Oligonucleotides (oligo) EFG-A 5′d(gcctcccacattagttgctcag)3′ complementary to the positions −155 to −130 nucleotides (nt) (A of the translation initiation codon ATG is designated as +1) of the *EFG1* gene and oligo EFG-B 5′d(GAAGATCTgtccttgttgttgatagaacatgtag)3′ complementary to the positions +213 to +187 nt in the *EFG1* coding region were used to amplify an EFG-AB fragment. The 3′ end of this

Table 3. Candida albicans Strains

Strain	Genotype	Sources
SC5314	Wild type	(Gillum et al., 1984)
CAI4	*ura3::1 imm434/ura3::1 imm434*	(Fonzi and Irwin, 1993)
JKC18	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG*	(Liu et al., 1994)
JKC19	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG-URA3-hisG*	(Liu et al., 1994)
HLC17	*ura3::1 imm434/ura3::1 imm434 EFG1/efg1::hisG-URA3-hisG*	This study
HLC46	*ura3::1 imm434/ura3::1 imm434 EFG1/efg1::hisG*	This study
HLC37	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG EFG1/efg1::hisG-URA3-hisG*	This study
HLC44	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG EFG1/efg1::hisG*	This study
HLC52	*ura3::1 imm434/ura3::1 imm434 efg1::hisG/efg1::hisG-URA3-hisG*	This study
HLC54	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG-URA3-hisG*	This study
HLC67	*ura3::1 imm434/ura3::1 imm434 efg1::hisG/efg1::hisG*	This study
HLC69	*ura3::1 imm434/ura3;;1 imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG*	This study
HLC74	*ura3::1 imm434/ura3::1 imm434 efg1::hisG/efg1::hisG (EFG1)*	This study
HLC84	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG (EFG1)*	This study

EFG-AB fragment contains a BglII site within the EFG-B oligo. Oligo EFG-C 5′d(GAAGATCTcattcgtgtacatcaccttctgc) complementary to the positions 6 to 29 nt after the translation stop codon of the *EFG1* gene and oligo EFG-D 5′d(gtaccttccgcattagacgcttac)3′ complementary to the positions 570 to 547 nt after the translation stop codon of the *EFG1* gene were used to generate an EFG-CD fragment. The 5′ end of this EFG-CD fragment contains a BglII site within the EFG-C oligo. HLB61 and HLB62 are the pGEM-T vectors (Promega, CA 3600) containing the EFG-AB and EFG-CD fragment, respectively. The orientation of each insert was determined by restriction mapping. The EFG-AB fragment was inserted in the same direction as *lacZ* and the EFG-CD was inserted in the opposite orientation from *lacZ*. A 0.6 kb BglII-NcoI fragment containing EFG-CD fragment from HLB62 was inserted into HLB61 digested with BglII and NcoI. HLB64 is a plasmid containing both the EFG-AB and the EFG-CD fragments with a BglII site in between. An approximately 4 kb BglII-BamHI fragment containing the *hisG-URA3-hisG* cassette from pUCB-6 (Fonzi and Irwin, 1993) was inserted into HLB64 digested with BglII to generate HLB67. This *EFG1* disruption plasmid contains EFG-AB-BglII/BglII-hisG-URA3-hisG BamHI/BglII-EFG-CD. The EFG-AB-BglII/BglII-hisG-URA3-hisG BamHI/BglII-EFG-CD fragment is released when HLB67 is digested with both SacI and SacII.

Deletion of *EFG1*

Sequential disruption of both alleles of the *EFG1* gene was performed using the strategy developed by Fonzi and Irwin (1993). The Candida strains *CPH1/CPH1 EFG1/EFG1* (CAI4) (Fonzi and Irwin, 1993) and *cph1/cph1 EFG1/EFG1* (JKC18) (Liu et al., 1994) were transformed with the *EFG1* disruption construct. Transformants were screened for the EFG1/*efg1::hisG-URA3-hisG* genotype by PCR using oligo EFG-E 5′d(gctgctgtttattgtttgttc)3′ complementary to the positions 617 to 600 nt after the translation stop codon of the *EFG1* gene and an oligo 5′d(ggcggaaccctgtgatggag)3′ complementary to the *hisG* sequence, which is 65 to 46 nt from the junction of *hisG* and EFG-CD fragment. HLC37 and HLC17 are *CPH1/CPH1 EFG1/efg1::hisG-URA3-hisG* and *cph1/cph1 EFG1/efg1::hisG-URA3-hisG*, respectively. To disrupt the second chromosomal copy of *EFG1*, we derived *ura3/ura3 EFG1/efg1* strains by streaking HLC17 and HLC37 on complete medium containing 5-FOA (which selects for recombinants that have lost *URA3*). The resulting strains HLC46 and HLC44, *CPH1/CPH1 EFG1/efg1::hisG* and *cph1/cph1 EFG1/efg1::hisG*, respectively, were transformed with the *EFG1* disruption construct. The status of the *EFG1* gene, either heterozygous or homozygous for the *EFG1* deletion allele, was confirmed by Southern blot analysis.

The *EFG1* Integrating Plasmid

The wild-type *EFG1* gene was used in transformation experiments to complement the *efg1* defect. Restoration of filamentation in the transformants provided evidence that the phenotypes of the *efg1/efg1* strains were a consequence of the *efg1* mutation. The full-length *EFG1* gene was identified by hybridizing a ^{32}P-labeled EFG-AB fragment to colonies of a Candida genomic library (Boone et al., 1991). HLB100 identified by this method contains the *EFG1* gene in a Saccharomyces high copy *URA3* vector. A 4.2 kb HindIII fragment with the *EFG1* gene from HLB100 was inserted into pRC2312, a vector containing Candida *LEU2* and *URA3* genes (Cannon et al., 1992), at the HindIII site to create plasmid HLB134. HLB134 digested

Table 4. Plasmids

Plasmid	Description	Sources
B2068	*STE11 TRP1 CEN* plasmid	Fink laboratory collection
B2387	*ura3-LEU2-ura3* cassette	Yona Kassir
B3161	*pFG(TYA)::lacZ-HIS3* plasmid	(Mosch et al., 1996)
HLB6	3.1 kb SacI-BamH1 fragment containing *EFG1* in pRS306	This study
HLB61	EFG-AB fragment in pGEM-T vector	This study
HLB62	EFG-CD fragment in pGEM-T vector	This study
HLB64	EFG-ABCD fragment in pGEM-T vector	This study
HLB67	EFG-AB-BglII/BglII-*hisG-URA3-hisG* BamHI/BglII-EFG-CD	This study
HLB100	*EFG1 URA3* 2 μm plasmid	This study
HLB134	4.2 kb HindIII fragment with *EFG1* in pRC2312	This study
p650	*STE12 CEN* plasmid	(Kirkman-Correia et al., 1993)
pCG38	*PHD1 URA3* 2 μm plasmid	(Gimeno and Fink, 1994)
pNC202	*ste11::URA3* disruption plasmid	(Rhodes et al., 1990)
pRC2312	*LEU2 URA3 ARS* C. albicans plasmid	(Cannon et al., 1992)
pRS202	*URA3* 2 μm plasmid	(Gimeno and Fink, 1994)
pRS306	*URA3* integrating plasmid	(Sikorski and Hieter, 1989)
pRS314	*TRP1 CEN* plasmid	(Sikorski and Hieter, 1989)
pRS316	*URA3 CEN* plasmid	(Sikorski and Hieter, 1989)
pUCB-6	*hisG-URA3-hisG* cassette for C. albicans	(Fonzi and Irwin, 1993)

with BstEII within the *LEU2* gene was used to integrate linearized HLB134 into the *LEU2* locus in Candida.

Growth Rate

The doubling times of Candida strains were determined in 2× YPD at 37°C, which is close to the human body temperature. Candida strains grown in 2× YPD at 37°C remain in the yeast form. An overnight culture in 2× YPD at 37°C was diluted 1 to 500 into fresh medium, grown at 37°C, and the density (OD_{600}) of each culture was determined every hour. The doubling times shown in Table 1 are the average of three independent experiments.

Interaction of Candida and Macrophages

The interaction between Candida and the macrophages was studied using the cell line IC-21 (ATCC TIB-186) derived from normal mouse peritoneal macrophages, transformed with SV40. The cells were maintained in RPMI1640 (GIBCO BRL) with 10% fetal bovine serum (GIBCO BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO_2. For microscopy, cells were grown on 25 mm diameter coverslips, No. 1 thickness, which were placed into a 37°C heated microscopy chamber and covered with ca. 6 ml medium and silicon oil. Macrophages retain their adherence to the coverslip, their flat spread out shape, and their motility for at least 20 hr under these conditions.

For these interaction experiments, Candida was grown to saturation in YPD (4% glucose) at 37°C. Greater than 95% of cells were in the yeast form. Before mixing the yeast with macrophages, the yeast cells were washed twice with PBS and counted with a hemocytometer. Candida were added to macrophages in a microscope chamber during time lapse microscopy or in the cell culture dish using RPMI 1640 medium without serum, with five washes to remove extracellular Candida 1 hr after infection and subsequent placement of the coverslip into the microscope chamber.

Phase contrast time lapse videomicroscopy was performed on a Nikon Diaphot 300 inverted microscope equipped with a 40× Fluor objective and 2× relay lens. Video frames were acquired with a Hamamatsu Newvicon C2400-07 camera, digitized, averaged (eight frames), and eventually stored on CD-ROM. Both image acquisition and subsequent analysis were performed on a Pentium PC computer with Metamorph imaging software (Universal Imaging Corporation, West Chester, PA). Light exposure was kept to a minimum by shuttering the light source.

The Mouse Model for Candida Virulence

CF1 mice (white, male) from Charles River Laboratories (Wilmington, MA) weighing between 18 and 20 g were used to test the virulence of different strains. An experiment was initiated by growing the Candida strains on YPD plates for 48 hr at 28°C, suspending the cells in saline, and adjusting them to the desired concentration after measurement of OD (Cacciapuoti et al., 1992). The actual concentration was then verified by counting the cells in a hemocytometer and by plating to determine the viable count. Each Candida strain was tested for virulence by injecting 0.1 ml of cells into the tail vein of a mouse. Ten mice were injected for each Candida strain at each concentration of cells. Survival was monitored daily. These studies were carried out in accordance with the NIH Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act in an AAALAC-accredited program.

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