INFECTION AND IMMUNITY, Oct. 1999, p. 5514–5517 Vol. 67, No. 10
0019-9567/99/$04.00+0

Chlamydospore Formation in *Candida albicans* Requires the Efg1p Morphogenetic Regulator

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Received 29 March 1999/Returned for modification 10 June 1999/Accepted 30 June 1999

Chlamydospore formation of the fungal pathogen *Candida albicans* was found to depend on the Efg1 protein, which regulates the yeast-hyphal transition. Isogenic mutants lacking *EFG1* or encoding T206A and T206E variants did not differentiate chlamydospores, while *cek1*, *cph1*, or *tpk2* mutations had no effect. Furthermore, filamentation of *efg1 cph1* double mutants in microaerophilic conditions suggests a novel Efg1p/Cph1p-independent filamentation pathway in *C. albicans*.

Among *Candida* species, the human fungal pathogen *Candida albicans* has the special ability to form thick-walled cells, termed chlamydospores, in certain environmental conditions (18). Chlamydospores arise on elongated suspensor cells situated on pseudohyphae or hyphae. To efficiently induce chlamydospores, cells are distributed at low cell concentrations on nutrient-poor media, which optimally is supplemented with detergents, such as Tween 80, and incubated at a low temperature (25 to 30°C) for several days (12, 17). Chlamydospore formation is especially abundant on solid media under glass coverslips providing microaerophilic conditions (Dalmau inoculation technique). The presence of glucose inhibits chlamydospore formation, while nitrogen levels have no major influence (5). Light inhibits chlamydospore formation (6). Although chlamydospore formation is used routinely to differentiate *C. albicans* from other *Candida* species (only rare isolates of *Candida tropicalis* also form chlamydospores [11]), the molecular mechanisms leading to this form of cellular differentiation are unknown. Also, the functions of chlamydospores for the biology and possibly for the virulence of *C. albicans* are unclear. Although chlamydospores can be efficiently induced in vitro, they are rarely detected in vivo (3). Chlamydospores are considered dormant cellular forms, but because they rapidly loose viability, they do not appear to allow long-term survival (19).

Another striking feature of *C. albicans* is its ability to switch growth forms in certain environments, between a unicellular yeast and a filamentous multicellular form (dimorphism). Among *Candida* species, *C. albicans* has the strongest tendency to form hyphae, which parallels its role as the most virulent *Candida* species (18). *C. albicans* mutants defective in hyphal formation have almost completely lost their virulence (16). In recent years, two major signalling pathways leading to hyphal growth have been defined. One pathway leads via a conserved mitogen-activated protein (MAP) kinase cascade to phosphorylation and activation of the transcription factor Cph1p (13, 14). A second pathway includes the transcription factor Efg1p (23); recent evidence suggests that protein kinase A (PKA) is situated functionally upstream of Efg1p (22). The Efg1p pathway appears more important than the Cph1p pathway for hyphal morphogenesis, because *efg1* mutants are unable to form hyphae in most inducing conditions, e.g., in the presence of serum or GlcNAc as well as during growth on Spider medium (8, 16, 23), while *cph1* mutants show only a morphogenetic block on Spider medium (13, 14). *efg1* mutants have a strongly reduced virulence in the mouse model of infection (16), while *cph1* mutants essentially retain virulence (16); nevertheless, *efg1 cph1* double mutants show even a further attenuation in virulence than the *efg1* single mutant (16), suggesting a definite but minor contribution of Cph1p to virulence. The ability to undergo GlcNAc-induced hyphal formation is not a prerequisite for chlamydospore formation, since monomorphic yeast mutants can still differentiate chlamydospores (24).

***efg1* mutants are defective in chlamydospore formation.** This study was performed to determine if members of signal transduction pathways leading to hyphal development also have a function in the formation of chlamydospores. Therefore, a wild-type strain, SC5314 (9), as well as an isogenic series of *C. albicans* strains derived from CAI4 (9) (containing a plasmid- or genomically borne *URA3* gene to complement the *ura3* mutation), was streaked out lightly on cornmeal agar (Difco)–0.33% Tween 80, covered by coverslips, and incubated at 25°C for 5 to 7 days. The strains and plasmids used in this study are listed in Table 1. Figure 1 shows that strain CAI4 (9) transformed with control plasmid pBI-1 (23) developed chlamydospores; the wild-type strain SC5314 had an identical phenotype (data not shown). Chlamydospores formed as terminal thick-walled cells on short side branches (suspensor cells) or as terminal cells on filaments. Similar to wild-type cells, a *cph1* mutant (strain JKC19 [15]) lacking the Cph1p transcription factor activated by the Cek1p MAP kinase, as well as a *tpk2* strain (22), which lacks an isoform of PKA, was competently able to form chlamydospores (data not shown). On the other hand, *efg1*/*efg1* mutants, such as strain HLC67 (16) containing the control plasmid pBI-1 (Fig. 1) or strain HLC52 (16) (data not shown), were completely deficient for chlamydospore formation, although filamentous growth and occasionally short side branches resembling suspensor cells were detected. The double knockout strain HLC54 (*efg1*/*efg1 cph1*/*cph1*) (16) was as defective as strain HLC52 for chlamydospore formation. These results demonstrate that Efg1p is needed for chlamydospore formation. Because an *efg1* mutant reconstituted with a single wild-type *EFG1* gene (HLC74 [16]) was able to develop chlamydospores (data not shown), it appears that one functional allele for the *EFG1* gene is sufficient for this morphogenetic process.

Efg1p residue T206 is essential for chlamydospore formation. We sought to determine if the lack of chlamydospore formation in *efg1* mutants could be suppressed by expression of

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
C. albicans		
SC5314	Prototrophic	9
CAI4	Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	9
HLC52	Same as CAI4 but *efg1*::*hisG*/*efg1*::*hisG-URA3-hisG*	16
HLC67	Same as CAI4 but *efg1*::*hisG*/*efg1*::*hisG*	16
HLC54	Same as CAI4 but *cph1*::*hisG*/*cph1*::*hisG efg1*::*hisG*/*efg1*::*hisG-URA3-hisG*	16
HLC74	HLC67 (*EFG1*)	16
JKC19	Same as CAI4 but *cph1*::*hisG*/*cph1*::*hisG-URA3-hisG*	16
Plasmids		
pBI-1	*URA3*-marked control vector	23
pLJ19	*URA3*-marked vector containing *CEK1*	4
pCCa4	*URA3*-marked vector containing *CPH1*	4
pBI-HAHYD	*URA3*-marked vector containing *PCK1p-EFG1*	A. Sonneborn
pDB1	Same as pBI-HAHYD but encoding Efg1p(T206A) variant	D. P. Bockmühl
pDB2	Same as pBI-HAHYD but encoding Efg1p(T206E) variant	D. P. Bockmühl

several plasmid-borne genes. To perform this analysis, strain HLC67 (*efg1*/*efg1 ura3*/*ura3* [16]) was used as the host for plasmids derived from pRC2312 (2). While strain HLC67 transformed with basic plasmid pBI-1 (23) did not develop chlamydospores, a transformant carrying the *EFG1* expression plasmid pBI-HAHYD (23) was complemented (Fig. 2). In contrast, neither high expression of *CPH1* carried by plasmid pLJ19 (4) nor high expression of the *CEK1* gene encoding the MAP kinase Cek1p present on plasmid pCCa4 (4) was able to restore the formation of chlamydospores. Thus, a functional Efg1 protein cannot be substituted by extraneous levels of components of the parallel MAP kinase pathway. Efg1p contains a single potential site for PKA phosphorylation at T206 (sequence I R P R V $\underline{T}^{206}$ T T), which we had determined previously to be essential for hyphal development (22). To determine the effect of this residue on chlamydospore formation, we used expression plasmids encoding a T206A or T206E substitution (plasmid pDB1 or pDB2, respectively [22]) and tested transformants containing these plasmids. Transformants carrying pDB1 or pDB2 were completely deficient in chlamydospore formation, similar to the *efg1* host strain (Fig. 2). These results demonstrate that the status of the single PKA phosphorylation site in Efg1p is crucial for chlamydospore differentiation to occur. In parallel experiments, we have determined that hyphal formation on Spider medium is blocked in strains producing the T206A but not the T206E mutant version of Efg1p (22). Since both Efg1p variants do not support chlamydospore formation, it appears that structural requirements for the Efg1 protein are different for its functions in hyphal development and in chlamydospore formation. Also, because *tpk2* mutants are not affected in chlamydospore formation, it appears that the PKA isoform encoded by *CaTPK2* is not involved in this process, unlike its important function in hyphal development (22). Possibly, a yet undefined isoform of A-type kinases has an essential function in chlamydospore formation.

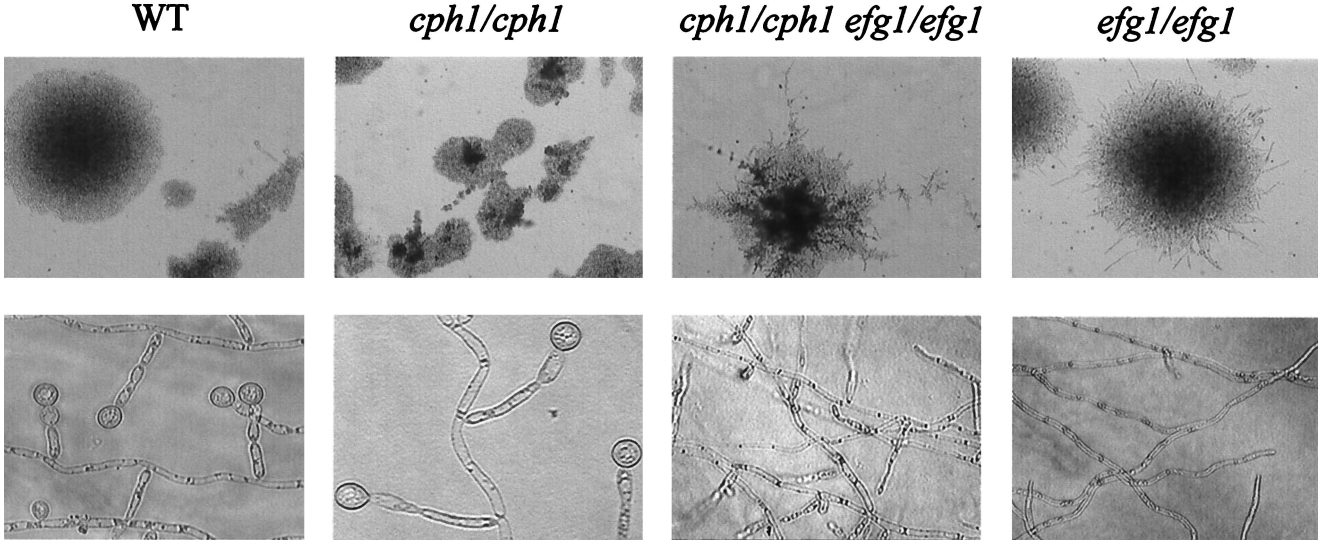

FIG. 1. Chlamydospore formation of *C. albicans* is defective in *efg1* strains. All strains were streaked out lightly on chlamydospore induction medium (cornmeal agar [Difco]–0.33% Tween 80), covered by a coverslip, and incubated for 5 days at 20°C. Strains used were CAI4 (WT, wild type; *EFG1*/*EFG1*) complemented with empty plasmid pBI-1 (23), JKC19 (*cph1*/*cph1*) (15), and HLC67 (*efg1*/*efg1*) (16) complemented with pBI-1 and HLC54 (*efg1*/*efg1 cph1*/*cph1*) (16). Colonies were visualized by phase-contrast microscopy after 2 days of growth at 100-fold magnification (top row). Photographs of chlamydospores and filaments magnified 400-fold (across coverslips on plates) were obtained after 5 days of growth (bottom row).

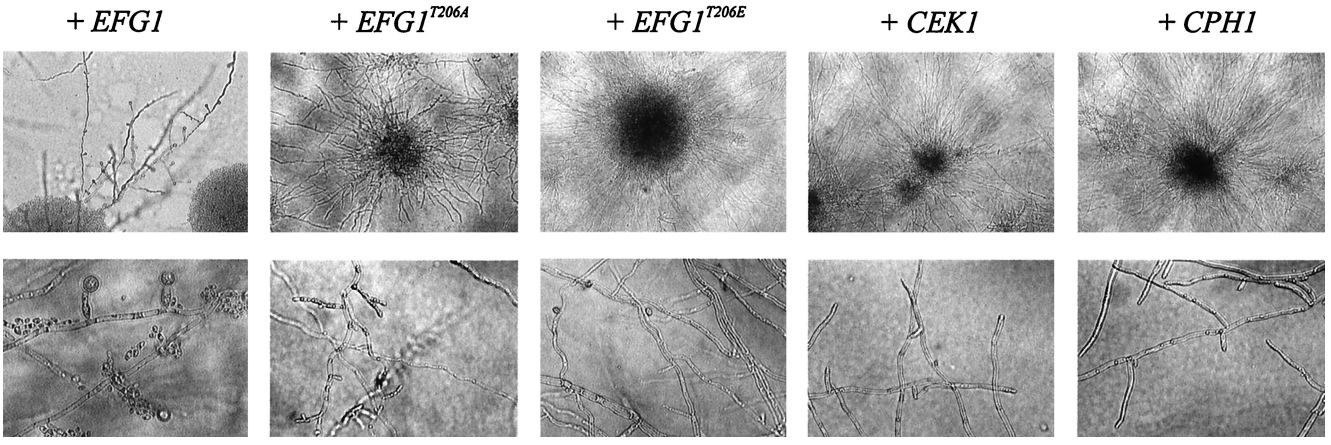

FIG. 2. Attempts to suppress the block of chlamydospore formation in *efg1* mutants. The *efg1*/*efg1* mutant strain HLC67 was transformed with an *EFG1* expression vector (pBI-HAHYD) (+ *EFG1*), expression vectors for the T206A (pDB1) and T206E (pDB2) variants of Efg1p (+ $EFG1^{T206A}$ and + $EFG1^{T206E}$) (22), the expression vector pLJ19 carrying *CEK1* (pLJ19) (+ *CEK1*) (4), and the expression vector pCCa4 carrying *CPH1* (+ *CPH1*) (4). Phase-contrast microscopy on colonies and on chlamydospores and filaments was performed after 5 days of growth at 100-fold (top row) or 400-fold (bottom row) magnification.

Filamentous growth of *efg1* strains. Deletions in *EFG1* are known to block hyphal formation in a variety of standard inducing conditions, e.g., in the presence of serum or GlcNAc or during growth on Spider medium (16, 23); an *efg1 cph1* double mutant was unable to form residual pseudohyphae as *efg1* strains in some conditions (16). Therefore, our result demonstrating that the *efg1* and *cph1* single mutants, as well as the *efg1 cph1* double mutant, were not blocked in filament formation under conditions of chlamydospore formation (Fig. 1 and 2) appeared surprising. Even more surprisingly, the *efg1* mutant (but not the *cph1* mutant) appeared to form even more filaments, which were more elongated than those of the *EFG1* wild-type strains. The more extensive filamentation of the *efg1* strains than of the *EFG1* strains was most obvious after about 2 days of incubation on cornmeal agar–Tween 80 (Fig. 1). Filaments developed by the *EFG1* wild-type strains can be qualified as pseudohyphae, since they carry constrictions at the site of cell separations and buds are observed occasionally at the tips of filaments. In the case of the *efg1* mutant strains, many filaments appeared to be extremely elongated and it cannot be decided with certainty if such filaments are to be classified as abnormally elongated pseudohyphae or true hyphae. Regardless of the nature of the filaments as pseudohyphae or hyphae, the results demonstrate that in the conditions of chlamydospore induction, filaments are formed by hitherto unknown signal transduction pathways not including known components of hyphal induction pathways. The determining factor for activation of this novel pathway appears to be anaerobiosis, since growth of colonies on cornmeal agar (Difco) or on Spider medium in an anaerobic jar also induced filaments in an *efg1* mutant strain (data not shown).

Efg1p belongs to a group of bHLH proteins termed APSES-proteins (1), which contain a highly homologous region of about 100 amino acids and which regulate morphogenetic processes in fungi. Specifically, all of these proteins are required for the interconversion between a spherical, yeast-like cell and an elongated filamentous cell. In *asm-1* mutants of *Neurospora crassa*, the transition from filamentous to spherical growth is blocked and mutants are unable to form ascospores; similarly, in *Aspergillus nidulans stuA* mutants, the formation of conidiophores is blocked (7). In *sok2* mutants of *Saccharomyces cerevisiae* (25) and in transformants overexpressing *PHD1* (10), pseudohyphal growth is induced. Hyphal development in *C. albicans efg1* mutants is blocked in standard induction conditions, e.g., in the presence of serum and other inducers (however, as discussed above, not in anaerobic conditions). Here we have demonstrated that the transition from a filamentous growth form to a spherical cell type, chlamydospores, also is blocked in *efg1* mutants. Efg1p is the first defined component known to regulate chlamydospore formation. The results demonstrate that Efg1p, possibly in association with auxiliary proteins, has a dual role in *C. albicans* cell differentiation in that it regulates the spherical cell-filament interconversion in both directions. It has recently been uncovered (21) that *EFG1* also regulates the spontaneous opaque white phenotypic switching in strain WO-1 (20). We found that overexpression of *EFG1* forces the opaque white switch and that lowered expression of *EFG1* induces an opaque-like state in cells of strain CAI8 (9). Thus, Efg1p emerges as a central regulator of morphogenetic processes in *C. albicans*, regulating dimorphism, phenotypic switching, and chlamydospore formation.

We thank C. Csank, G. Fink, and H. Liu for strains and plasmids. We acknowledge the excellent technical assistance of M. Gerads.

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Editor: T. R. Kozel