Note

Invasive Filamentous Growth of *Candida albicans* Is Promoted by Czf1p-Dependent Relief of Efg1p-Mediated Repression

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ABSTRACT

Filamentation of *Candida albicans* occurs in response to many environmental cues. During growth within matrix, Efg1p represses filamentation and Czf1p relieves this repression. We propose that Czf1p interacts with Efg1p, altering its function. The complex regulation of filamentation may reflect the versatility of *C. albicans* as a pathogen.

THE opportunistic fungal pathogen, *Candida albicans*, grows invasively in tissues of candidiasis patients by converting from budding yeast form cells to filamentous forms. The ability to convert from one morphology to another is important for virulence (Sobel *et al.* 1984; Shepherd 1985; Ryley and Ryley 1990; Leberer *et al.* 1997; Lo *et al.* 1997). To understand the mechanisms by which filamentous growth is stimulated during infection, regulation of hyphal development has been studied extensively (for review, see Gow 1997). Conditions that promote hyphal growth in the laboratory include growth at elevated temperature in medium containing special components. In the absence of these conditions, growth within a matrix also promotes hyphal growth (Brown *et al.* 1999). The embedded condition may simulate conditions encountered by the pathogen during growth in human tissue.

Several genes whose products regulate filamentous growth have been identified (for review, see Ernst 2000), including *CPH1* (Liu *et al.* 1994), *EFG1* (Stoldt *et al.* 1997), and *CZF1* (Brown *et al.* 1999). Efg1p regulates the expression of several genes (Sharkey *et al.* 1999; Braun *et al.* 2000; Schroppel *et al.* 2000; Leng *et al.* 2001) and plays a major role in promoting filamentous growth (Lo *et al.* 1997; Stoldt *et al.* 1997). *C. albicans* Cph1p, a homolog of *Saccharomyces cerevisiae STE12*, is important for hyphal development in some media (Liu *et al.* 1994; Lane *et al.* 2001) and nutritional signals are thought to be important in stimulation of the mitogen-activated protein kinase cascade that regulates Cph1p (Köhler and Fink 1996; Leberer *et al.* 1996; Csank *et al.* 1998). *CZF1*, a zinc-finger-containing protein that has no homolog in the *S. cerevisiae* genome, is important for regulation of hyphal development when *C. albicans* cells are grown within matrix (Brown *et al.* 1999).

Although Efg1p has been previously shown to promote filamentous growth (Lo *et al.* 1997; Stoldt *et al.* 1997), this study demonstrates that Efg1p acts as a repressor of filamentous growth when cells are grown within matrix at low temperature. Genetic analysis indicates that Czf1p antagonizes the function of Efg1p. In addition, a two-hybrid interaction between Czf1p and Efg1p was observed, suggesting that Czf1p relieves repression of filamentation by binding to Efg1p.

Efg1p represses filamentous growth under low-temperature embedded conditions: To determine the role of Efg1p in controlling morphology in response to growth within agar matrix at 25°, the phenotype of an *efg1/efg1* null mutant, CKY136, was analyzed. Colonies of the *efg1* null strain produced filaments much earlier than colonies of the wild-type strain CKY101 or the complemented mutant strain HLC74 (*efg1/efg1/EFG1*; Figure 1, A–C; Table 1). To demonstrate that the differences in filamentation were not caused by differences in growth rates, individual cells growing in agarose matrix at room temperature were observed. The average times required to complete one cell cycle were very similar for all three strains, ~91 min for wild-type cells, 121 min for CKY136, and 114 min for HLC74. In contrast to these results, the *efg1* null mutant exhibited defective filamentous growth when grown on the surface of several media at 37° (data not shown), consistent with previous reports (Lo *et al.* 1997; Stoldt *et al.* 1997).

The repressive effect of Efg1p on filamentation during growth within matrix was also observed in strains lacking Czf1p. *czf1* null mutants and *czf1 cph1* double null mu-

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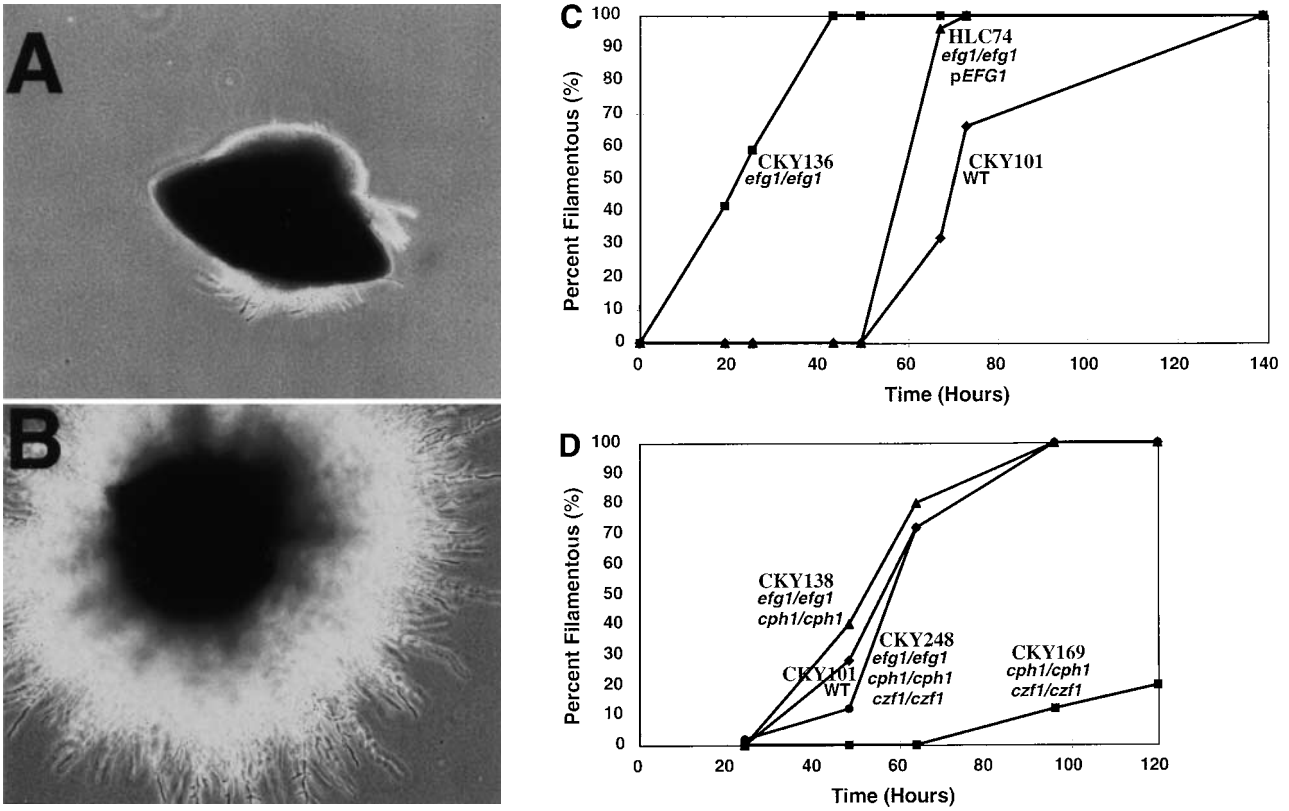

FIGURE 1.—Repressive effects of *EFG1* on filamentous growth within agar matrix. Strains (Table 1) were grown in YPD (AUSUBEL 1989, no. 20) overnight, diluted and grown in YPD at 30° for 4 hr, mixed with molten YPS (yeast extract, peptone, 2% sucrose) agar medium, and plated. Plates were incubated at either 25° (A, B, and D) or room temperature (C). At various times, 100–200 colonies were examined microscopically as described previously (BROWN 1999, no. 45), and the percentage of filamentous colonies was plotted as a function of time. To measure the doubling times of cells embedded in agar, cells were mixed with molten YPS containing 0.7% low-melt agarose and poured onto a glass microscope slide. During growth at room temperature, individual cells on the glass slide were observed and the time required for emergence of a bud was measured for two cell cycles. Emergence of 14 buds was monitored for strains CKY101 (*EFG1/EFG1*) and CKY136 (*efg1/efg1*) and emergence of 7 buds was monitored for strain HLC74 (*efg1/efg1/EFG1*). (A) Colony of CKY101 (*EFG1/EFG1*) grown for 43 hr at 25°. (B) Colony of CKY136 (*efg1/efg1*) grown for 43 hr at 25°. (C) Formation of filamentous colonies by strains CKY101 (*EFG1/EFG1*; diamonds), CKY136 (*efg1/efg1*; squares) and HLC74 (*efg1/efg1/EFG1*; triangles). (D) Formation of filamentous colonies by strains CKY101 (*EFG1/EFG1*; diamonds), CKY138 (*efg1/efg1 cph1/cph1*; triangles), CKY169 (*cph1/cph1 czf1/czf1*; squares), and CKY248 (*efg1/efg1 cph1/cph1 czf1/czf1*; circles).

tants are defective in production of filamentous colonies under low-temperature embedded conditions (Figure 1D; BROWN *et al.* 1999). However, a triple null mutant lacking Efg1p, Cph1p, and Czf1p exhibited close to wild-type production of filamentous colonies under low-temperature embedded conditions (Figure 1D).

In the absence of Efg1p, changes in the expression of Czf1p had no effect on development of filamentous colonies: In wild-type cells incubated under low-temperature embedded conditions, changing the expression of Czf1p altered the kinetics of filamentous colony production (BROWN *et al.* 1999). When Czf1p was ectopically expressed using the maltase promoter, filamentous colonies were observed precociously. When Czf1p was absent, production of filamentous colonies was delayed. We propose that ectopically expressed Czf1p relieves repression by Efg1p precociously and that, in the absence of Czf1p, repression by Efg1p is not relieved normally. A prediction of this model is that in the absence of Efg1p, changes in the expression of Czf1p will have no effect.

To test this model, we first showed that double null mutants, lacking Efg1p and Czf1p, exhibited precocious filamentation indistinguishable from the *efg1* null mutant (data not shown). This observation is consistent with the hypothesis that Czf1p acts on Efg1p-dependent repression.

Second, we ectopically expressed Czf1p in strains lacking Efg1p. An *efg1* null strain ectopically expressing Czf1p produced filamentous colonies with the same kinetics as an *efg1* null mutant alone (data not shown). However, due to the precociously filamentous phenotype of this mutant strain, it might be difficult to detect an effect of Czf1p ectopic expression. During growth within matrix at low temperature, the *efg1 cph1* double null mutant is precociously filamentous in many media

TABLE 1

Strains used in this study

Name	Genotype	Source
	C. albicans	
CAI-4	SC5314 Δ*ura3::imm434*/Δ*ura3::imm434*	Fonzi and Irwin (1993)
CKY101	CAI-4 (pDBI52)	Brown *et al.* (1999)
CKY136	CAI-4 *efg1::hisG/efg1::hisG* (pDBI52)	This study
HLC74	CAI-4 *efg1::hisG/efg1::hisG* (p*EFG1*)	Lo *et al.* (1997)
CKY138	CAI-4 *efg1::hisG/efg1::hisG cph1::hisG/cph1::hisG* (pDBI52)	Riggle *et al.* (1999)
CKY139	CAI-4 *efg1::hisG/efg1::hisG cph1::hisG/cph1::hisG* (pP_{MAL}-*CZF1*)	This study
CKY169	CAI-4 *cph1::hisG/cph1::hisG czf1::hisG/czf1::hisG* (pDBI52)	Brown *et al.* (1999)
CKY196	CAI-4 *cph1::hisG/cph1::hisG* (pDBI52)	This study
CKY197	CAI-4 *cph1::hisG/cph1::hisG* (pP_{MAL}-*CZF1*)	This study
CKY248	CAI-4 *efg1::hisG/efg1::hisG cph1::hisG/cph1::hisG czf1::hisG/czf1::hisG-URA3-hisG*	This study
	S. cerevisiae	
EGY40	*MAT*α *ura3-1 his3-11 trp1-1 leu2-3,112*	Golemis *et al.* (1996)

but produces filamentous colonies with close to wild-type kinetics in YPS media (Riggle *et al.* 1999). Therefore, we examined the effect of ectopic expression of Czf1p in the *efg1 cph1* double null mutant. While ectopic expression of Czf1p in a *cph1* single null mutant did accelerate production of filamentous colonies (Figure 2), ectopic expression of Czf1p in the *efg1 cph1* double null mutant did not accelerate production of filamentous colonies (Figure 2). These results demonstrate that in the absence of Efg1p ectopic expression of Czf1p did not alter the rate of production of filamentous colonies and are consistent with the model that Czf1p promotes filamentous growth by relieving repression due to Efg1p.

Interaction between Czf1p and Efg1p: Czf1p might antagonize repression by Efg1p through physical interaction. To test this possibility, a two-hybrid experiment was performed. As shown in Table 2, the ability of *CZF1-lexA* or *GAL4*AD-*EFG1* to activate *lacZ* reporter gene expression in the presence of unfused *GAL4*AD or unfused *lexA*, respectively, or in the presence of irrelevant protein fusions was poor. However, when *CZF1-lexA* and *GAL4*AD-*EFG1* were both present in the strain, *lacZ* reporter gene expression was observed. These results indicate that Czf1p and Efg1p are capable of interaction and suggest that relief of Efg1p-mediated repression by Czf1p occurs via protein-protein interaction.

Discussion: Efg1p regulates filamentous growth under several conditions but performs different functions. At 37° in many media, strains lacking Efg1p are defective in filamentation. In contrast, during growth within matrix at low temperature, Efg1p acts as a repressor of filamentous growth. The negative effects of Efg1p on filamentous growth under these conditions are observed in strains containing or lacking Czf1p and Cph1p, indicating that the repressive effects do not require either protein. However, the *efg1* null mutant is not hyperfilamentous when grown on the surface of agar media. Therefore, cells also possess an Efg1p-independent mechanism for repression of filamentous growth under non-embedded conditions.

Ectopic expression of Czf1p accelerates production of filamentous colonies under low-temperature embedded conditions and ectopic expression of Cph1p has a similar, though weaker, effect (D. H. Brown, Jr. and C. Kumamoto, unpublished observations). Ectopic expression of Czf1p also accelerates production of filamentous colonies in the absence of Cph1p. Similarly, deletion of *czf1* results in defective production of filamentous colonies during growth within matrix at low temperature in both *CPH1* and *cph1* strains. These results support the model that Czf1p and Cph1p have independent functions. In contrast, when *efg1* is deleted, the effects of changes in *CZF1* expression are eliminated. This epi-

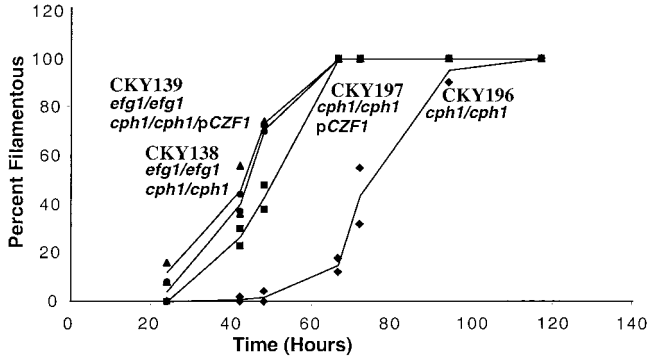

Figure 2.—Filamentous growth in strains lacking Efg1p is not affected by changing the expression of Czf1p. Cells were grown and plated as in Figure 1 and the percentage of filamentous colonies was plotted as a function of time. Results of duplicate samples are shown and the average value is plotted. Strains were as follows: CKY138 (*efg1/efg1 cph1/cph1*; circles) and CKY139 (*efg1/efg1 cph1/cph1* P_{MAL}-*CZF1*; triangles), CKY196 (*cph1/cph1*; diamonds), and CKY197 (*cph1/cph1* P_{MAL}-*CZF1*; squares).

TABLE 2

Two-hybrid interaction between Czf1p and Efg1p

DNA-binding domain fusion	Gal4p-activation domain fusion	β-Galactosidase activity[a]
CZF1-lexA[b]	*GAL4*AD (unfused)	1.9 ± 1.4
lexA (unfused)	*GAL4*AD-*EFG1*[c]	4.4 ± 0.9
CZF1-lexA	*GAL4*AD-*EFG1*	59.4 ± 10.4
CZF1-lexA	*GAL4*AD-*SLK19*[d]	0.35 ± 0.12
lexA-Fos[d]	*GAL4*AD-*EFG1*	12.8 ± 1.3
lexA-Fos	*GAL4*AD (unfused)	10.5 ± 0.9

[a] Strain EGY40 carrying the *lacZ* reporter plasmid pSH18-34 (Golemis *et al.* 1996) was transformed with the *lexA* plasmids (pNLexA, pBOM1a, and *plexA-Fos*) and *GAL4*AD plasmids (pGAD-C2, pKLEF4, and pC1.1.2) using standard methods (Ausubel *et al.* 1989). For quantitative analyses, cells were grown overnight in synthetic complete medium lacking uracil, histidine, and leucine. Cells were diluted into fresh medium and grown to exponential phase for 3–5 hr to an OD_{600} of 0.4–0.9. Cells were harvested and β-galactosidase activity was determined using *o*-nitrophenyl β-D-galactopyranoside as a substrate as described in the *Clontech Yeast Protocols Handbook*. Analyses were done in quadruplicate.

[b] *CZF1-lexA* was constructed by PCR amplification of the *CZF1* gene with primers 5′-GGTGAATTCCATT CAAACGAAAACTATCTGGT-3′ and 5′-GGTGGATCCTACTTC TGTATTCAACAATACCT-3′ and cloning of the PCR product into vector pNLexA (Golemis *et al.* 1996).

[c] *GAL4*AD-*EFG1* was constructed by PCR amplification of *EFG1* with primers 5′-CGGAATTCTCAACGTATTC TATACCCTATTAC-3′ and 5′-CGGGATCCGATGTACACGAATGATATTTATTA C-3′ and cloning of the PCR product into vector pGAD-C2 (James *et al.* 1996).

[d] Control fusion proteins that are not expected to interact with either Efg1p or Czf1p. Plasmids *plexA-Fos* and pC1.1.2 encoding *GAL4*AD-*SLK19* were generously provided by R. Brent (Massachusetts General Hospital, Boston) and R. Kamieniecki (Tufts University, Boston).

static effect is inconsistent with a model in which Czf1p and Efg1p function independently and supports the model that Czf1p acts on Efg1p. We propose that Czf1p acts to antagonize repression mediated by Efg1p, by binding and altering the function of Efg1p. As a result, expression of critical genes needed for formation of highly elongated, filamentous cells occurs, leading to filamentous growth.

At least two mechanisms for repression of transcription by Efg1p could be imagined. As a bHLH protein with homology to the Myc family of transcription factors, Efg1p may bind a partner that mediates transcriptional repression (Facchini and Penn 1998), analogous to Mad. A second possibility is that Efg1p may interact with components of the basal transcription machinery or with other transcription factors. Myc represses expression of its own gene in the absence of DNA binding (Facchini *et al.* 1997), possibly through interaction with the basal transcription machinery. v- and c-Myc also interact with and repress the transcription factors C/EBPβ and MIZ-1 (Mink *et al.* 1996; Peukert *et al.* 1997).

Several studies, including this one, demonstrate that numerous mechanisms for promotion of filamentous growth exist in *C. albicans* (Riggle *et al.* 1999; Braun and Johnson 2000). The presence of these multiple pathways presumably allows the cell to produce hyphae in a variety of environments and in the presence of different stimuli. For example, the mutant lacking Efg1p and Cph1p fails to form hyphae in serum and other media at 37° but forms filaments well under low-temperature matrix embedded conditions (Riggle *et al.* 1999). This mutant forms filaments in the tongue during experimental infection (Riggle *et al.* 1999) but fails to develop filaments following ingestion by macrophages (Lo *et al.* 1997). The complex regulation of hyphal growth in *C. albicans* may reflect the high versatility of *C. albicans* as a pathogen and the wide variety of environmental cues that may be encountered by the organism during infection in different tissue sites.

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