A Potential Phosphorylation Site for an A-Type Kinase in the Efg1 Regulator Protein Contributes to Hyphal Morphogenesis of Candida albicans

Dirk P. Bockmühl and Joachim F. Ernst
Institut für Mikrobiologie, Heinrich-Heine-Universität, 40225 Düsseldorf, Germany

MANUSCRIPT received July 20, 2000
Accepted for publication January 9, 2001

ABSTRACT

Efg1p in the human fungal pathogen Candida albicans is a member of the conserved APSES class of proteins regulating morphogenetic processes in fungi. We have analyzed the importance for hyphal morphogenesis of a putative phosphorylation site for protein kinase A (PKA), threonine-206, within an Efg1p domain highly conserved among APSES proteins. Alanine substitution of T206, but not of the adjacent T207 and T208 residues, led to a block of hypha formation on solid and in liquid media, while a T206E exchange caused hyperfilamentation. The extent of the morphogenetic defect caused by the T206A mutation depended on hypha-induction conditions. Exogenous suppression of mutations in signaling components, including tpk2 and cek1 mutations, was achieved by wild-type- and T206E-, but not by the T206A-variant-encoding allele of EFG1. All muteins tested were produced at equal levels and at high production levels supported pseudohyphal formation. The results are consistent with a role of Efg1p as a central downstream component of a PKA-signaling pathway including Tpk2p or other PKA isoforms. Threonine-206 of Efg1p is essential as a putative phosphorylation target to promote hyphal induction by a subset of environmental cues.

ENVIRONMENTAL cues are known to trigger different cellular morphologies of the fungal pathogen Candida albicans (reviewed in Odins 1988; Ernst 2000). Elevated temperatures and inducing substances including serum components and N-acetylglucosamine lead to the conversion of yeast to hyphal cells; other conditions induce pseudohyphae or chlamydospores. Environmentally regulated filamentation is instrumental in the attachment and penetration of human host cells and thereby contributes to fungal virulence. Some signaling pathways and components, which are essential for cellular differentiation of C. albicans, have been uncovered in recent years.

A cAMP-dependent signaling pathway leading to hyphal growth has been established, in which the Ras1 protein and the protein kinase A (PKA) isofrom Tpk2p are defined components (Feng et al. 1999; Sonneborn et al. 2000). Mutants deleted in RAS1 or in TPK2 are severely compromised in their ability to form true hyphae and a dominant-active variant of Ras1p or overproduction of Tpk2p lead to enhanced filamentation. A cAMP-dependent pathway triggering morphogenesis (pseudohyphal growth) has also been discovered in Saccharomyces cerevisiae (Gimeno et al. 1992; Mösch et al. 1996), in which the three PKA isoforms contribute differently to cellular morphogenesis (Robertson and Fink 1998; Pan and Heitman 1999). A signaling pathway parallel to the cAMP pathway, which includes a MAP kinase and the Ste12 transcription factor, has a major influence on filamentous growth in S. cerevisiae, while the role of a similar pathway in C. albicans appears less important for morphogenesis (reviewed in Ernst 2000).

Efg1p as a key regulator of C. albicans morphogenesis influences yeast-hypha interconversion, as well as chlamydospore formation and spontaneous “phenotypic switching” (Lo et al. 1997; Stoldt et al. 1997; Sonneborn et al. 1999a,b). Strains deleted in EFG1 are defective in hypha formation under most standard inducing conditions. Nevertheless, in special environments even efg1 mutants are able to develop true hyphae, suggesting that Efg1p is part of a signaling pathway, but not of the cellular machinery involved in hypha construction (Brown et al. 1999; Riggle et al. 1999; Sonneborn et al. 1999b). Overexpression of EFG1 leads to strong filamentation in the form of pseudohyphae (elongated yeast cells that remain attached; Stoldt et al. 1997). Efg1p is a member of the APSES protein group of fungal transcription factors involved in morphogenetic processes leading to the interconversion between spherical growth forms (yeast, conidiospore, chlamydospore) and tubular forms (hyphae, pseudohyphae, conidiophores; Miller et al. 1992; Aramayo et al. 1996). In S. cerevisiae the Efg1p APSES homologs are Phd1p and Sok2p, which regulate pseudohyphal growth (Gimeno and Fink 1994; Ward et al. 1995). APSES proteins share 80–90% identical residues in a domain encompassing ~100 amino acids. The central portion of this domain is homologous.
to the basic helix-loop-helix (bHLH) motif, which is known to be required for dimerization and DNA binding of transcription factors (DUTTON et al. 1997; STOLDT et al. 1997).

Some evidence has suggested that APSES proteins function in the cAMP signaling pathways. First, epistasis analyses are consistent with Efg1p being situated downstream of the Tpk2 PKA isoform (SONNEBORN et al. 2000). Second, an apparent inhibitor of S. cerevisiae PKA activity suppressed filamentation induced by PKA and the APSES proteins Efg1p or Phd1p, but had no effect on the parallel mitogen-activated protein (MAP) kinase pathway (RADMACHER et al. 1998). Here we provide a third argument by showing that the state of a putative PKA phosphorylation site in the conserved APSES domain of Efg1p is directly related to morphogenetic properties. In particular, a T206E variant protein, in which a glutamic acid mimics a phosphoryl group, leads to hyperfilamentation, while a T206A variant is inactive. Thus, we suggest that Tpk2p or yet unknown A-type kinases regulate the activity of the downstream Efg1 regulator protein.

MATERIALS AND METHODS

Strains and culture conditions: Strains and plasmids are listed in Table 1. Strain CDB1 (efg1Δ/efg1Δ phd1Δ/phd1Δ) is the equivalent of strain HLC69 and was constructed from strain HLC54 (Lo et al. 1997) by flooreosotic acid (FOA)-selection. C. albicans strains were transformed by the spheroplast method and were grown in YPD or on supplemented SD minimal medium (SHERMAN et al. 1986) or S4D medium (as SD, but containing 4% glucose). Modified Lee’s medium (Spider) has been described (LIU et al. 1994). The PKC1 promoter was induced in 0.67% yeast nitrogen base, 2% casamino acids (SCAA) medium (SONNEBORN et al. 2000). Hyphae were induced at 37°C using 5% horse serum, Spider medium, or 2.5 mM N-acetylglucosamine (GlcNAc; SONNEBORN et al. 2000).

Efg1p variants: Mutations in EFG1 were generated in the expression plasmid pBI-HAHYD by a commercial protocol (Stratagene, La Jolla, CA). The primers EFG-T/A1 (5′-CAGAGTGCGGACTGACATTGATGTTG) and EFG-T/A2 (complementary to EFG-T/A1) were used to generate the A206-encoding sequence. The presence of the mutation in the resulting plasmid pDB1 was verified by demonstration of a novel NruI site (underlined) and by sequencing. The primers EFG-T/E1 (5′-CAGAGTGCGGACTGACATTGATGTTG) and EFG-T/E2 (complementary to EFG-T/E2) were used to generate the A206-encoding sequence. In this case the mutation in the resulting plasmid pDB2 was verified by showing a novel Tag sequence (underlined) and by sequencing. To obtain the A207-encoding sequence primers T207A-1 (5′-CAGAGTGCGGACTGACATTGATGTTG) and its complementary T207A-2 (complementary to T207A-1) were used. The resulting plasmid pDB21 was sequenced to verify the mutation. The primer T208A-1 (5′-CAGAGTGCGGACTGACATTGATGTTG) and its complementary T208A-2 were used to generate the sequence encoding A208, while primer T208E-1 (5′-CAGAGTGCGGACTGACATTGATGTTG) and its complementary T208E-2 were used to obtain the A208-encoding sequence. The mutations in the resulting plasmids pDB3 (T208A) and pDB6 (T208E) were verified by introducing a novel AclI or DdeI restriction site, respectively, (underlined) and by sequencing.

Wild-type and mutated plasmids were linearized by KpnI to direct integration into the chromosomal LEU2 gene and were transformed into the efg1-null mutant HLC67, selecting for uridine prototrophy. Integration of the plasmids into a chromosomal location was shown by Southern blotting using a URA3 probe; signals were obtained only at a position corresponding to high-molecular-weight DNA (chromosomes); in addition, transformants remained uridine prototroph after extended nonselective growth (data not shown).

The empty control vector pRC2312 (CANNON et al. 1992) has been described.

Detection of hemagglutinin-tagged proteins: Hemagglutinin (HA)-tagged variants of Efg1p were detected in cell extracts by growing HLC67 transformants carrying pBI-HAHYD, pDB1, pDB2, pDB5, pDB21, or the control vector pRC2312 in 30 ml SCAA medium to an OD600 nm of 1.0. Cells were washed in ice-cold breaking buffer (50 mM HEPES, pH 7.5; 10 mM Trition X-100; 150 mM NaCl; 5 mM EDTA) and then disrupted by shaking with glass beads in 500 µl breaking buffer containing protease inhibitors (1 µg/ml antipain, pepstatin, leupeptin). Cell extracts were cleared by centrifugation in a microfuge (13,000 rpm; 5 min); to 200 µl of extract 100 µl Laemmli loading buffer (threefold concentrated) was added and pro-

---

### TABLE 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAI4</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>FONZI AND IRWIN (1993)</td>
</tr>
<tr>
<td>HLC67</td>
<td>As CAI4, but efg1::hisG/efg1::hisG</td>
<td>LO ET AL. (1997)</td>
</tr>
<tr>
<td>CK43B-16L</td>
<td>As CAI4, but eck1Δ::hisG/eck1Δ::hisG</td>
<td>CSANK ET AL. (1998)</td>
</tr>
<tr>
<td>AS1</td>
<td>As CAI4, but tpk2Δ::hisG/tpk2Δ::hisG</td>
<td>SONNEBORN ET AL. (2000)</td>
</tr>
<tr>
<td>CDB1</td>
<td>As CAI4, but efg1::hisG/efg1::hisG/phd1::hisG</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Transformation vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRC2312</td>
<td>URA3, LEU2-marked CaARS vector</td>
<td>CANNON ET AL. (1992)</td>
</tr>
<tr>
<td>pBI-HAHYD</td>
<td>PCK1p-HA-EFG1 fusion in pRC2312</td>
<td>SONNEBORN ET AL. (2000)</td>
</tr>
<tr>
<td>pDB1</td>
<td>As pBI-HAHYD, but encoding T206A-Efg1p variant</td>
<td>This work</td>
</tr>
<tr>
<td>pDB2</td>
<td>As pBI-HAHYD, but encoding T206E-Efg1p variant</td>
<td>This work</td>
</tr>
<tr>
<td>pDB5</td>
<td>As pBI-HAHYD, but encoding T208A-Efg1p variant</td>
<td>This work</td>
</tr>
<tr>
<td>pDB6</td>
<td>As pBI-HAHYD, but encoding T208E-Efg1p variant</td>
<td>This work</td>
</tr>
<tr>
<td>pDB21</td>
<td>As pBI-HAHYD, but encoding T207A-Efg1p variant</td>
<td>This work</td>
</tr>
</tbody>
</table>
teins were denatured at 95° for 5 min. A total of 30 μl of treated extracts was separated by SDS-PAGE (7.5% polyacrylamide). Following migration, proteins were transferred to nitrocellulose membranes by elect blotting. Proteins were detected using rat anti-HA high affinity antibody (dilution 1:1000; Roche), followed by peroxidase-coupled anti-rat IgG (1:1000; Roche) as secondary antibody. HA-tagged proteins were detected using the Super-Signal chemiluminescent assay (Pierce Chemical, Rockford, IL).

**Microscopy:** Hyphal development on solid media was observed using a phase contrast microscope [Zeiss (Göttingen, Germany) Axioskop] and a video imaging system (Sony 3CCD Color Video Camera; AppleVideo). Colonies were observed at 35-fold magnification, while single cells or pseudohyphae were observed at 400-fold magnification. Following inoculation of induction media hyphal development was quantitated by counting cells developing hyphae among a total of 200 cells; cells with a germ tube of at least blastospore-length were considered as hyphae.

**RESULTS**

**Construction of strains producing Efg1p variants:**
Within the conserved domain among APSES proteins Efg1p contains the sequence R-P-R-V-T-T-T (residues 202–208). The threonine residues 206 and 207 are potential sites for phosphorylation by an A-type kinase (PKA), because an arginine residue is located at position −2 or −3, respectively, relative to the presumed phosphorylation site (Pearson and Kemp 1991). Furthermore, computer predictions assign threonine 207 as the phosphorylation site of a calmodulin-dependent protein kinase II and threonine 208 as a possible site for casein kinase II (Kreiegipuu et al. 1999). To explore if any of the threonine 206–208 residues have a relevant role in C. albicans biology we constructed expression plasmids encoding corresponding Efg1p variants (Figure 1A). The parental plasmid pBI-HAHYD contains the EFG1 gene under transcriptional control of the PKC1 promotor, which is repressed by glucose (Leuken et al. 1997); furthermore, a sequence specifying a HA epitope is present at the beginning of the EFG1 coding region (Sonnenborn et al. 2000). The HA-Efg1p fusion protein is fully functional in complementing the morphogenetic defect of an efg1 mutant strain (Sonnenborn et al. 2000) and overexpression of the HA-EFG1 gene, as overexpression of unmodified EFG1 (Stoldt et al. 1997), leads to extensive pseudohyphal filaments (see below). To construct plasmids specifying Efg1p variants the EFG1 fusion gene was altered by site-specific mutagenesis. The resulting plasmids were integrated into the C. albicans genome to prevent plasmid loss and to avoid altering phenotypes due to copy number effects.

Transformants produced the different variants of Efg1p at equal levels, comparable to strains producing the unaltered tagged Efg1 protein, as shown by immunoblotting using an anti-HA antibody (Figure 1B); thus, the changes in protein structure did not affect protein levels (Figure 1B). In SDS-PAGE all Efg1p versions migrated anomalously as a doublet of proteins at ~90 kD, instead of the 65 kD expected from the sequence data; we assume that this behavior is in part due to its unusual composition containing 9% each of proline, threonine, serine, and glycine residues, as well as 19% of glutamine residues. Because no differences in electrophoretic migration among the Efg1p variants were observed, we conclude that the two Efg1p forms separated by SDS-PAGE are not due to modifications at T206, T207, or T208.

**Overproduction of all Efg1p variants induces pseudohyphae:** We had reported previously that massive overexpression of EFG1 leads to the formation of extensive filaments, which by the presence of constrictions at cell separations and of buds at filament tips were characterized as pseudohyphae, which are still competent to form true hyphae, e.g., upon the addition of serum (Stoldt et al. 1997). To explore if this function of Efg1p was maintained by the Efg1p variants we examined their overproduction phenotypes. For this purpose the transformants described above were pregrown in PKC1-promoter-inducing SCAA medium, by which any residual glucose was consumed, and were then regrown in SCAA medium for 20 hr. By this procedure maximal expression of the PKC1p-EFG1 fusion gene was obtained, which resulted in typical pseudohyphal growth (Figure 2).

All transformants generated pseudohyphae at identical rates and with identical cellular morphologies, irrespective of the Efg1p variant, which was overproduced.
The lack of \( EFG1 \) expression in control cells resulted in rod-like cell shape, which is typical for \( efg1 \) mutants in SCAA medium (Stoldt et al. 1997; Sonneborn et al. 1999b). Thus, the state of Efg1p residues 206–208 not only had no effect on intracellular Efg1p levels, but also did not influence the ability of Efg1p to support pseudohyphal growth at high production levels.

**Alanine replacement of threonine-206, but not threonine-207 or -208, blocks hyphal formation:** To investigate the function of threonine residues 206–208 on hypha formation we tested transformant strains producing Efg1p variants, in which these residues were replaced by alanine (plasmids pDB1, pDB21, and pDB6; Figure 1). Transformants were grown on solid modified Lee’s (Spider) medium or were induced in various liquid media and morphogenetic phenotypes were recorded.

After pregrowth in liquid SD medium, which was used to avoid the development of pseudohyphae, transformants were streaked on solid modified Lee’s medium, which contains mannitol instead of glucose as a carbon source and thereby should allow moderate expression of the fusion genes. After 3 days of growth at \( 37^\circ \)C lateral hyphae emerged from colonies of the strain producing unmodified Efg1p. Likewise, the cells producing the T207A and T208A variants were not affected in the development of hypha and their phenotypes were indistinguishable from wild-type cells. In contrast, a striking defect of the T206A variant to support hyphal formation was observed (Figure 3). Transformants carrying pDB1 formed colonies with drastically reduced lateral hyphae compared to wild-type cells.

Because of the clear effect of the T206A mutation on solid medium, we sought to investigate if a strain producing this Efg1p variant was also defective in hyphal morphogenesis induced in liquid medium. Previous results had suggested that hypha induction in liquid media reveals major defects in morphogenesis, while relatively minor defects become apparent on inducing solid media such as modified Lee’s medium (discussed in Ernst 2000). In this experiment cells were pregrown in SD medium, in which no hyphae or pseudohyphae were induced, and were then diluted into the different liquid induction media. In liquid Lee’s medium the strain producing the T206A Efg1p variant was unable to form hyphae, while 50% of the cells expressing the wild-type \( EFG1 \) gene had formed hyphae within 2 hr of induction (Figure 4). In a second induction medium, containing 2.5 mM GlcNAc as the inducer, hypha formation was also not as efficiently supported by the T206A variant.
Efg1p in *C. albicans* Morphogenesis

because it was phosphorylated *in vivo*. To obtain support for this notion we also analyzed an Efg1p variant, in which T206 was exchanged by a glutamate residue. In some cases the acidic side chain of glutamate is able to mimic a phosphoryl group, such that a variant protein containing a glutamate exchange functions similarly as the phosphorylated original protein. Transformants carrying genomically integrated plasmid pDB2 were tested on solid and in liquid media.

On solid modified Lee’s medium the T206E exchange led to cells able to filament vigorously (Figure 3). Daily inspections of colony appearances even suggested that the rate at which the T206E exchange supported the development of lateral hyphae was even greater compared to the wild-type Efg1p sequence in several independent transformants. Because gradual differences in hypha formation are difficult to quantify on solid plates, we also tested hypha formation of cells producing the T206E variant in liquid modified Lee’s medium. Note that the values shown in Figure 4 are the means of three independent transformants, which were assayed. The results show that in this condition a significantly accelerated filamentation of T206E-variant-containing cells was observed compared to cells carrying the wild-type Efg1p variant (Figure 4). In the other two liquid induction media, in the presence of GlcNAc or serum, the T206E exchange did not increase hypha formation, but led even to lower rates of filamentation. This was especially notable in GlcNAc medium, while in the presence of serum filamentation proceeded similar to wild-type cells. In all experiments, however, the rates of filamentation of cells producing the T206E variant were significantly higher compared to T206A variant-containing cells.

Thus, these results are compatible with the notion that phosphorylation at residue T206 (as mimicked by a glutamate residue) causes hyperfilamentation in some media, while this residue is of lesser importance in other induction conditions.

**Suppression activity of EFG1 depends on the T206 residue**: We had reported previously that overexpression of EFG1 is able to rescue the morphogenetic defect of *tpk2* and *cek1* mutants (Sonneborn et al. 2000). The availability of the *EFG1*<sup>T206A</sup> and *EFG1*<sup>T206E</sup> alleles allowed the test, if the suppression of the *tpk2* phenotype was simply due to an elevated level of the Efg1 protein, at low specific activity, or depended critically on residue T206 and thereby most likely on its phosphorylation status. Therefore, we transformed plasmids pDB1, pDB2, or control plasmids into defined *efg1*, *tpk2*, *cek1*, or *efg1 ceh1* mutants and examined hyphal morphogenesis on modified Lee’s medium.

The *EFG1*<sup>T206A</sup> allele, which was defective in complementation of the *efg1* mutation, was unable to restore effective hyphal formation in any of the signaling mutants tested, including the *efg1 ceh1* double mutant, while the *EFG1*<sup>T206E</sup> allele was at least as effective as the

**Figure 4.**—Hypha formation of transformants in liquid medium. HLC67 transformants carrying genomically integrated plasmids pBI-HAHYD (T206) (▽), pDB1 (T206A) (■), pDB2 (T206E) (▲), or the control vector pRC2312 (◆) were pregrown in SCAA medium, to induce *PCK1*-*EFG1* expression, and then shifted to modified Lee’s medium (Spider), 2.5 mM GlcNAc, or 5% serum at 37°C. Hyphae (germ tubes) developing on yeast cells were monitored microscopically and expressed as percentage of yeast cells forming hyphae. The means and standard deviations of values obtained from three independent transformants are shown. Note the complete absence of hypha formation in transformants carrying pDB1 (T206A) or pRC2312 in modified Lee’s medium.

compared to wild-type Efg1p, although in this case hypha formation occurred at significant rates. The least effect of the T206A mutation was observed in medium containing 5% serum as the inducer, where hypha formation was reduced only slightly. Thus, these results demonstrate that the importance of the T206 residue in Efg1p varies on the induction condition used: in modified Lee’s medium, which is considered a relatively weak induction medium, T206 must be present to allow maximal rates of morphogenesis, while in serum, considered as the strongest hypha-inducing condition, the role of T206 is minimal.

The T206E exchange causes hyperfilamentation: The above results suggested that residue T206 was essential,
wild-type EFG1 gene (Figure 5). The clearest results were obtained for suppression of the efg1, efg1 cph1, and tpk2 mutants, which with an empty control vector showed no or few lateral hyphae, while the EFG1 wild-type and EFG1 alleles, but not the EFG1T206A allele, were able to induce strong filamentation. The partial defects of a cek1 mutant on modified Lee’s medium also could not be suppressed by Efg1pT206A. Thus, these results demonstrate that the activity of Efg1p as a suppressor does not reflect merely a nonspecific elevation of Efg1p levels, but depends specifically on the state of its T206 residue.

**DISCUSSION**

The C. albicans Efg1 protein is a member of a conserved class of proteins in fungi, whose apparent function is to regulate the switch between a spherical cell type, such as yeast cells or spores, and a tubular hyphal growth form. Among such APSES proteins there is a stretch of ~100 amino acids is conserved, whose function is yet unknown. We had proposed that the central portion of the conserved domain represents a bHLH domain (Stoldt et al. 1997), a theory that was supported subsequently by molecular modeling (Dutton et al. 1997). Within the conserved APSES domain of Efg1p, but outside of the putative bHLH region, is a stretch of threonine residues (T206–T208), which constitute possible phosphorylation sites for protein kinases, specifically for PKA (T206 or T207). Here we describe the construction of strains that contain versions of Efg1p with altered residues T206–T208. In the case of residues T207 and T208, which represent putative phosphorylation sites for PKA, calmodulin-dependent protein kinase II, and casein kinase II, respectively (Kreegipuu et al. 1999), no effect of alanine exchanges on hyphal morphogenesis was observed. Furthermore, a T208E exchange, which potentially could mimic an Efg1p version, which is phosphorylated at T208 by a casein kinase II homolog, had no effect on Efg1p function.

Significant phenotypes were obtained, however, when the directly neighboring residue T206 of Efg1p was exchanged. In all APSES proteins there is a threonine residue in this position, except for the Phd1 protein in S. cerevisiae, which contains an isoleucine (Sok2p, the second APSES protein in S. cerevisiae, however, contains a threonine). The context of this threonine is similar to sites phosphorylated by PKA (Pearson and Kemp 1991). We had already reported that strains containing the T206A and T206E variants of Efg1p did not support chlamydospore formation (Sonnenborn et al. 1999b). With respect to hypha formation we report here that the phenotypes of cells containing the T206A or T206E variants are opposite. The T206A exchange in Efg1p leads to an inability of cells containing this variant to form hyphal and overexpression of an EFG1T206A allele is not able to suppress the phenotypes of tpk2, cek1, and cph1 mutations. On the other hand, a T206E version of Efg1p is hyperactive in stimulating filamentation. We propose that Efg1p is able to adopt two activity states: an unphosphorylated inactive version, represented by the T206A variant, and an active phosphorylated version, which is mimicked by the T206E variant. The state of residue 206 was not equally important in all hypha-induction conditions, but was especially critical in modified Lee’s medium; in the presence of GlcNAc hyphal development of cells containing the T206A variant was moderately decreased, while in the presence of serum only a minor effect of the T206 replacement was obtained. Thus, it appears that hypha induction in modified Lee’s medium is strongly dependent on residue T206, presumably due to phosphorylation, while in serum different parameters (possibly including other Efg1p modifications) may be essential. Wild-type Efg1p had identical electrophoretic mobility as the T206A and T206E derivatives in SDS-PAGE, and by phosphate-labeling experiments and phosphate-specific antibodies it was impossible to detect phosphorylation of T206 because of other, still undefined phosphorylation sites in Efg1p (D. Bockmühl and J. F. Ernst, unpublished results). Thus, we could not biochemically prove yet the position of a phosphate residue on T206, as suggested by our genetic results.

The PKA isoform, which directly phosphorylates Efg1p on T206, is unknown. It is possible that Tpk2p (Sonnenborn et al. 2000) directly phosphorylates Efg1p, although in two-hybrid analyses and by coprecipita-
tion experiments we were unable to prove a close association of Tpk2p with Efg1p (D. Bockmühl and J. F. Ernst, unpublished results). Genomic sequences in *C. albicans* (http://www-sequence.stanford.edu/group/candida/search.html) predict a second PKA isof orm, designated Tpk1p, which we found to be also involved in hyphal morphogenesis (D. P. Bockmühl and J. F. Ernst, unpublished results). The electrophoretic mobility of Efg1p was identical in homozygous tpk1 or tpk2 mutants and in the wild-type strain (D. P. Bockmühl and J. F. Ernst, unpublished results); this finding does not necessarily exclude phosphorylation of Efg1p by Tpk1/2 proteins, because we have shown here that the charge difference between the T206A and T206E variants also has no effect on electrophoretic mobility. Other kinases more distantly related to PKA, such as homologs of Sch9p in *S. cerevisiae* (Toda et al. 1988), could also be involved in the direct modification of Efg1p.

Current models of hyphal morphogenesis in *C. albicans* suggest multiple parallel signaling pathways, which are triggered by different environmental cues, and whose cumulative output results in hypha formation (Braun and Johnson 2000; Ernst 2000). Efg1p is essential for hyphal morphogenesis in most inducing conditions (Lo et al. 1997; Stoldt et al. 1997) and epistasis analyses described here and in a previous study (Son neborn et al. 2000) have shown that the defective phenotype of efg1 mutants cannot be suppressed by any of the known genes encoding signaling components. On the other hand, Efg1p overproduction was able to rescue the morphogenesis-defective phenotype of cek1 and tpk2 mutants. Interestingly, the T206A variant of Efg1p did not show such extravagant suppression activity, while the T206E variant was active, suggesting that T206 is required in its phosphorylated form. Although such suppression results could occur if Efg1p represented a third essential pathway parallel to the MAP kinase and cAMP pathways we favor a model in which Efg1p functions as a central regulator downstream of the components of both pathways. This model explains the importance of a potential PKA phosphorylation site in Efg1p and is also consistent with the findings that a suppressor of PKA activity also suppressed filamentation in *C. albicans* (Rademacher et al. 1998).

We thank C. Csank and G. R. Fink for plasmids and strains. We acknowledge the excellent technical assistance by M. Gerads. This study was supported by the Deutsche Forschungsgemeinschaft.

**LITERATURE CITED**


Rademacher, F., V. Kehren, V. R. Stoldt and J. F. Ernst, 1998 A *Candida albicans* chaperonin subunit (CaCct8p) as a suppressor of morphogenesis and Ras phenotypes in *C. albicans* and *Saccharomyces cerevisiae*. Microbiology 144: 2951–2960.


Communicating editor: J. J. Loros