

HWPI Functions in the Morphological Development of *Candida albicans* Downstream of *EFG1*, *TUP1*, and *RBF1*

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The morphological plasticity of *Candida albicans* is an important determinant of pathogenicity, and non-filamentous mutants are avirulent. *HWPI*, a hypha-specific gene, was identified in a genetic screen for developmentally regulated genes and encodes a cell surface protein of unknown function. Heterozygous and homozygous deletions of *HWPI* resulted in a medium-conditional defect in hyphal development. *HWPI* expression was blocked in a Δ *efg1* mutant, reduced in an Δ *rbf1* mutant, and derepressed in a Δ *tup1* mutant. Therefore, *HWPI* functions downstream of the developmental regulators *EFG1*, *TUP1*, and *RBF1*. Mutation of *CPH1* had no effect on *HWPI* expression, suggesting that the positive regulators of hyphal development, *CPH1* and *EFG1*, are components of separate pathways with different target genes. The expression of a second developmentally regulated gene, *ECE1*, was similarly regulated by *EFG1*. Since *ECE1* is not required for hyphal development, the regulatory role of *EFG1* apparently extends beyond the control of cell shape determinants. However, expression of *ECE1* was not influenced by *TUP1*, suggesting that there may be some specificity in the regulation of morphogenic elements during hyphal development.

Candida albicans is a significant opportunistic fungal pathogen and causes superficial mucosal disease as well as life-threatening systemic infections in immunocompromised patients. *C. albicans* is described as a dimorphic fungus that can grow as a budding yeast or a true hyphal form but can also adopt a range of intermediate pseudohyphal morphologies. *C. albicans* strains defective in the ability to form hyphae are avirulent in a mouse model of systemic infection (22). Thus, hyphal development is essential to pathogenicity, but its precise role in this process is as yet undefined.

Hyphal development is under both positive and negative control. *TUP1* is a general transcriptional repressor in *Saccharomyces cerevisiae* (40). Deletion of the *C. albicans* homolog of *TUP1* results in a constitutive pseudohyphal phenotype under all growth conditions, suggesting that *TUP1* is responsible for maintenance of the yeast morphology through repression of genes required for filamentous growth (6). *RBF1* (for “RPG binding factor 1”) encodes a putative transcription factor of *C. albicans* that binds to the same consensus sequence as the *S. cerevisiae* transcription factor encoded by *RAP1* (14). Mutation of *RBF1* results in a stimulation of filamentous growth, suggesting that *RBF1* also plays a negative regulatory role in the yeast-to-hyphal-form transition (15).

Positive control of hyphal development is effected in part by *EFG1*, which encodes a basic helix-loop-helix-type transcription factor (36). Reduced expression or deletion of *EFG1* results in rod-like, elongated cells under the conditions tested and in the inability to form true hyphae (22, 36). Overexpression results in enhanced pseudohyphal growth (36). Additional positive control is signaled via a mitogen-activated protein (MAP) kinase cascade analogous to that which controls pseudohyphal development in *S. cerevisiae* (21, 28). Mutation in the kinase components encoded by *HST7*, *CST20*, or *CEK1*,

or the terminal transcription factor encoded by *CPH1*, results in a medium-conditional defect in hyphal development (8, 17, 18, 20). Conversely, mutation of the MAP kinase phosphatase *CPP1* results in a hyperhyphal phenotype (7). This kinase cascade cooperatively controls hyphal development in conjunction with *EFG1*. Mutation of either *EFG1* or *CPH1* alone partially compromises filamentation, while a *cph1/cph1 efg1/efg1* double mutant is restricted entirely to the yeast growth form under standard growth conditions (22).

The molecular interactions between these various regulators leads to the coordinate control of hyphal development, but these interactions remain incompletely defined since no downstream target genes have been identified. Three developmentally expressed genes, *ECE1*, *HYR1*, and *HWPI*, have been reported, but neither *ECE1* nor *HYR1* is required for hyphal morphogenesis (2, 5, 35). Here we report the morphogenic role of *HWPI*. Expression of *HWPI* is dependent upon *EFG1* but does not require *CPH1*. *TUP1* represses its expression, while *RBF1* appears to act as an inducer. Examination of *ECE1* expression demonstrated that this nonmorphogenic function is similarly regulated by *EFG1* but is not affected by *TUP1*. These results suggest that *EFG1* and *CPH1* function within largely independent control pathways and regulate distinct sets of morphology-related functions. In addition, the regulatory function of *EFG1* extends beyond the control of cell shape.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. They were routinely cultured on YPD medium (33) or YNB medium (2% glucose, 0.17% Difco yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate) at 30°C. Medium 199 (Gibco BRL, Gaithersburg, Md.) containing Earle’s salts and glutamine but lacking sodium bicarbonate was buffered with 150 mM Tris (pH 7). Spider medium was prepared as described previously (20). The medium of Lee et al. was prepared as described previously (19). *N*-Acetylglucosamine induction medium was prepared as described by Shepherd et al. (32). Media were solidified with 1.5% agar and supplemented with 25 μ g of uridine per ml for growth of Urd3⁻ strains. Germ tube induction was assessed at 37°C following inoculation of stationary-phase cells into prewarmed medium at a density of 5×10^6 cells/ml. Negative controls were incubated at 25°C. Filamentation on agar-solidified media was assessed by

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TABLE 1. *C. albicans* strains used in this study

Strain	Parent	Genotype	Source or reference
SGY243		$\Delta ura3::ADE2/\Delta ura3::ADE2$	16
SC5314		Clinical isolate	12
CAI4	CAF2-1	$\Delta ura3::imm434/\Delta ura3::imm434$	10
CAL1	CAI4	$\Delta hwp1::hisG-URA3-hisG/HWP1 \Delta ura3::imm434/\Delta ura3::imm434$	This work
CAL2	CAL1	$\Delta hwp1::hisG/HWP1 \Delta ura3::imm434/\Delta ura3::imm434$	This work
CAL3	CAL2	$\Delta hwp1::hisG/\Delta hwp1::hisG-URA3-hisG \Delta ura3::imm434/\Delta ura3::imm434$	This work
CAL4	CAL3	$\Delta hwp1::hisG/\Delta hwp1::hisG \Delta ura3::imm434/\Delta ura3::imm434$	This work
CAL5	CAL4	$\Delta hwp1::hisG/\Delta hwp1::hisG HWP1::URA3 \Delta ura3::imm434/\Delta ura3::imm434$	This work
CAL6	CAL4	$\Delta hwp1::hisG/\Delta hwp1::hisG EF1\alpha::HWP1::URA3 \Delta ura3::imm434/\Delta ura3::imm434$	This work
BCa2-10		$\Delta tup1::hisG/\Delta tup1::hisG-URA3-hisG$	6
JKC19		$\Delta cph1::hisG/\Delta cph1::hisG \Delta ura3::imm434/\Delta ura3::imm434$	22
HLC52		$\Delta def1::hisG/\Delta def1::hisG \Delta ura3::imm434/\Delta ura3::imm434$	22
HLC54		$\Delta cph1::hisG/\Delta cph1::hisG \Delta def1::hisG/\Delta def1::hisG \Delta ura3::imm434/\Delta ura3::imm434$	22
1161KR		$LYS/lys1 ARG4/arg4 ura3/ura3 ser57/ser57 gal1/gal1 MPA^r$	14
1161K1R13		$lys1/lys1 arg4/arg4 ura3/ura3 ser57/ser57 gal1/gal1 rbf1::LYS1/rbf1::ARG4 MPA^r$	14

diluting stationary-phase cells to 2×10^8 cells/ml in water, spotting 1×10^6 cells (5 μ l) onto the plate, and incubating them at 37°C. Invasive hyphal growth was assessed after washing the agar plates with sterile water to remove surface growth (28).

Gene isolation. cDNA clones of hypha-expressed genes and their corresponding genomic clones were isolated as previously described (5). A 4.3-kb *EcoRI* genomic fragment corresponding to cDNA 8 was subcloned from plasmid pSMS22 into pUC18. The gene was tentatively called *ECE2*.

DNA sequence analysis. Nucleotide sequences were determined by the dideoxy chain termination method with Sequenase 2.0 T7 DNA polymerase (United States Biochemical, Cleveland, Ohio). Reactions were primed with the T3 and T7 primers and custom-designed oligonucleotide primers. Sequence analysis was performed with DNA Strider (23). Homology searches were conducted with the BLAST algorithm (1) and SCAN (26). Sequence alignments were performed with MACAW (31) and ClustalW 1.6 (13).

Construction of mutant strains. The insert from plasmid pSMS22 was subcloned into the *EcoRI* site of a pBSK⁺ (Stratagene, La Jolla, Calif.) derivative in which the *Bam*HI site had been destroyed by filling in with Klenow DNA polymerase. The resulting plasmid, pELS-1, contained a unique *Bam*HI site within *HWP1*. A 434-bp *Bgl*II-*Bam*HI fragment in the 5' coding region was deleted and replaced with the 3.8-kb *Hind*III-*Bgl*II *hisG-URA3-hisG* fragment from plasmid pMB7 (10), generating plasmid pELS-2. The *Bam*HI and *Hind*III ends were blunt-end ligated following a filling-in reaction. pELS-2 was digested with *Sma*I and *Hind*III, releasing a 5.4-kb fragment containing the *hisG-URA3-hisG* fragment flanked by *HWP1* sequences. This DNA was used for the sequential disruption of both *HWP1* alleles in strain CAI4 by previously published methods (10).

To reintroduce a functional copy of *HWP1*, plasmid pELS-1 was digested with *Psh*AI and *Xho*I, filled in, and ligated to create plasmid pELS-5. This removed a 2.0-kb fragment of 3'-flanking sequence containing two *Hind*III restriction sites. A 1.4-kb *Xba*I-*Pst*I fragment containing *URA3* (30) was added to create pELS-6. pELS-6 was linearized at the unique *Hind*III site located 3' of the *Bam*HI site within *HWP1* to target integration to the *HWP1* locus.

Constitutive expression of *HWP1* was effected with the *TEF2* promoter (37). A *Cla*I site was introduced 5 bp upstream of the *HWP1* open reading frame in plasmid pELS-5 by QuikChange mutagenesis (Stratagene). The *HWP1* promoter was removed by *EcoRI-Cla*I digestion and replaced with the 0.75-kb *EcoRI-Cla*I fragment of the *TEF2* promoter from plasmid pEF1-Fow (30). The *Xba*I-*Pst*I fragment of *URA3* was added, resulting in plasmid pELS-10. Plasmid DNA was digested with *Bsp*EI prior to transformation to target integration to the *HWP1* locus. The integration events in all the transformations were verified by Southern blot analysis.

Southern and Northern blot analyses. Southern and Northern blotting were conducted as described previously (25). Blots were hybridized with the *HWP1* 4.3-kb *EcoRI* insert from plasmid pSMS22, the 1.6-kb *Bam*HI-*EcoRV ECE1* fragment from plasmid pCAN4, or *ACT1* DNA as a control. Northern blots were quantitated with a Molecular Dynamics 445SI PhosphorImager and associated software.

Nucleotide sequence accession number. The sequence of *ECE2* was entered into GenBank under accession no. AF001978.

RESULTS

Isolation and identification of *HWP1*. Hyphal development is presumably effected by differential expression of morphogenic functions in response to external signals. Previously, we

isolated several cDNA clones of genes differentially expressed during the yeast-to-hyphal-form transition (5). One of these clones, cDNA 8, hybridized to a 2.4-kb mRNA expressed only in pseudohyphae and hyphae (Fig. 1). The expression pattern was similar to that previously described for *ECE1* (5); thus, the gene was tentatively designated *ECE2*. Expression was independent of the medium used to induce filamentation, and the expression pattern in the medium-conditional strain SGY243 (16) demonstrated that expression was morphologic specific rather than being a response to the inducing environment.

Sequence analysis of the genomic clone identified an open reading frame of 1,902 bp encoding a 634-residue protein with four distinct repeat domains (Fig. 2). The amino terminus contained a potential 27-residue secretory leader sequence (27), and a second hydrophobic region was located at the carboxy terminus. The latter region was preceded by residues G613, A614, and G615, which forms a potential glycosylphosphatidylinositol (GPI) attachment site according to the ω , $\omega+1$, and $\omega+2$ rule (39). The protein also contained three consensus sites for N-linked glycosylation (3).

A BLAST (1) comparison revealed that *ECE2* was >99% identical to the independently isolated and characterized *HWP1* (for "hyphal wall protein") (35). No obvious homologs were identified. However, a region of the third repeat domain of Hwp1p was conserved in *CHT2*, encoding a *C. albicans* chitinase (24), the *S. cerevisiae* flocculation protein Flo1p (4), the α -agglutinin subunit encoded by *AGA1* (29), the pheromone-regulated protein Fig2p (9), and the *SED1*-encoded cell

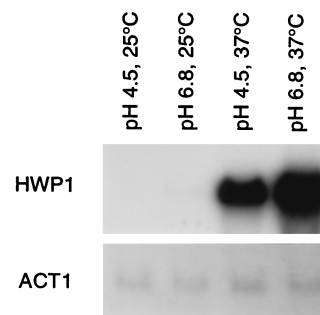


FIG. 1. Differential expression of cDNA 8. RNA was isolated from strain SC5314 grown in medium 199 at the indicated pH and temperature. A Northern blot of the sample was hybridized with cDNA 8 (*HWP1*) or *ACT1*.

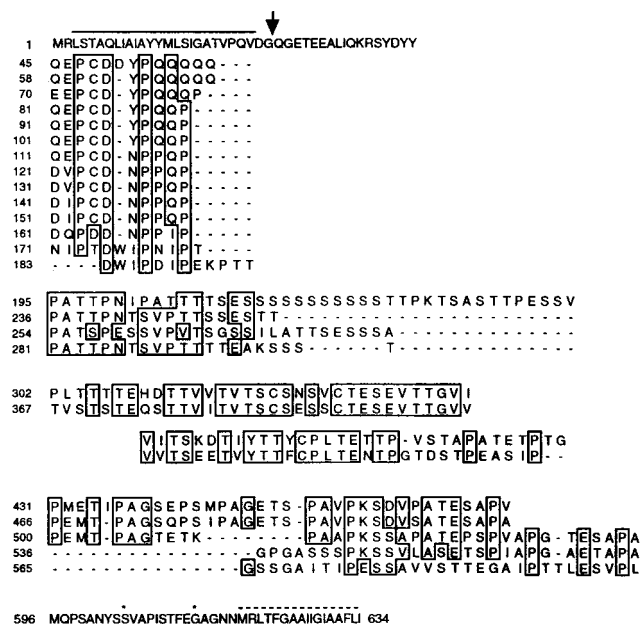


FIG. 2. Predicted amino acid sequence encoded by *HWP1*. Identical residues are boxed. Dashes indicate gaps introduced to maximize the alignment. The line above the sequence indicates the predicted leader. The arrow indicates the potential cleavage site. The broken line indicates the C-terminal hydrophobic region. Potential GPI anchor sites are marked with stars.

wall protein (11) (Fig. 3). All of these proteins are either known or predicted to be GPI-anchored cell surface proteins. This conserved domain was characterized by the consensus motif YTTWCPL, present in one to five copies.

Construction of deletion mutants. Expression of *HWP1* correlated with cell elongation and filamentation, and this suggested a potential role in morphogenesis. Deletion mutants were constructed to test this possibility. A 434-bp segment of the *HWP1* open reading frame was replaced with the *hisG-URA3-hisG* cassette (10) in the Urd⁻ strain CAI4, as illustrated in Fig. 4A. Integration at the *HWP1* locus occurred in 22 of 23 Urd⁺ transformants. The wild-type allele yields a 4.3-kb *EcoRI* band in Southern blots (Fig. 4B, lane CAI4). The disrupted allele contains an *EcoRI* restriction site located in *URA3* and yields a hybridization band of 5.6 kb, as seen in the representative heterozygous mutant CAL1 (Fig. 4B). Loss of the *URA3* gene and one of the *hisG* repeats in the Urd⁻ segregant strain CAL2 generated a 5.0-kb band (Fig. 4B).

Transformation of CAL2 resulted in replacement of the remaining wild-type allele in 8 of 36 transformants to generate strain CAL3. In these transformants, the 4.3-kb band of the wild-type allele was replaced by a 5.6-kb band representing the newly disrupted allele (Fig. 4B). Northern analysis confirmed the absence of *HWP1* mRNA in the null mutant.

Phenotype of the mutants. *HWP1* was nonessential as evidenced by the viability of the null mutant. No differences in the rate or frequency of germ tube formation were observed when the heterozygous or homozygous mutants were inoculated into Spider medium, the medium of Lee et al., 10% serum, or 25 mM *N*-acetylglucosamine. In medium 199, the mutant exhibited normal germ tube induction, but in approximately half the trials the germ tubes failed to extend into true hyphae and instead developed a pseudohyphal morphology. We were unable to identify the source of this variability. No differences were noted in the heterozygote.

The mutations had a more significant consequence for hyphal development on solid media. Strain SC5314 showed prolific hyphal growth, with lateral extension of the hyphae beyond the border of the colony (Fig. 5A). The heterozygous mutant, CAL1, exhibited greatly reduced hyphal growth, (Fig. 5B), and the null mutant, CAL3, exhibited a nearly complete loss of hyphal growth (Fig. 5C). The same results were seen on solid Spider medium and the medium of Lee et al. for seven independent heterozygotes and eight null mutants. Although the lateral extension of hyphae was compromised, all mutants maintained the ability to invade the agar directly beneath the colony. Microscopic examination revealed that these invasive filaments had a hyphal morphology.

When plated on 10% serum, a strong inducer of hyphal formation, all strains produced peripheral hyphae within 24 h. However, the hyphae extending from the null mutant colony were significantly reduced in both number and length compared to those in the wild type (Fig. 5E and F). By 48 h, the null mutant exhibited extensive filamentation around the colony, although the hyphae extended only half the distance from the colony as did those of the wild type. This is in contrast to incubation on medium 199, where the mutant failed to develop hyphae even with extended incubation.

To confirm that the mutant phenotype was directly associated with the loss of *HWP1*, the wild-type allele was introduced into the Urd⁻ homozygous null mutant CAL4 (Fig. 4B) to produce CAL5. Southern blot analyses confirmed proper integration of the functional allele in 8 of 15 transformants (Fig. 4B). Introduction of *HWP1* restored hyphal development in all eight of the revertants (Fig. 5D), verifying the correspondence between phenotype and genotype. However, the revertants,

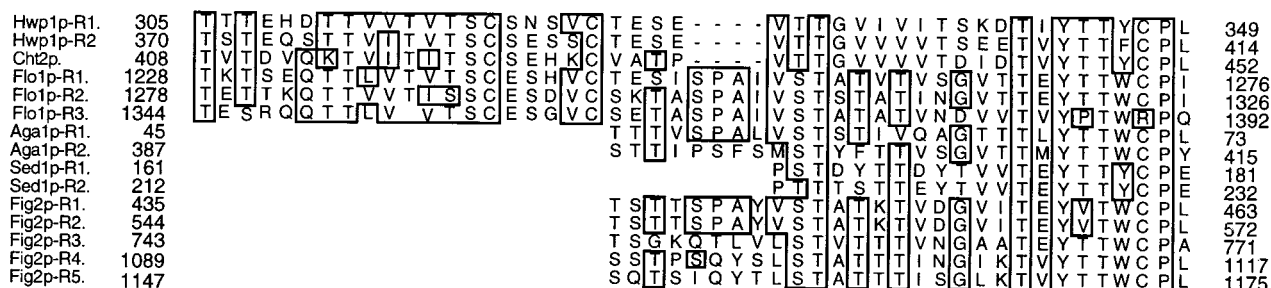


FIG. 3. Alignment of sequences with the conserved WCPL motif. Numbers to the left and right indicate the location within the amino acid sequence. R1, R2, etc., indicate consecutive repeats within the same protein. Residues conserved in at least 60% of the sequences within the region of overlap are boxed. The proteins and gene accession numbers are as follows: Ch2p, chitinase 2, U15800; Flo1p, flocculation protein, X78160; Aga1p, a-agglutinin core subunit, M60590; Sed1p, suppressor of *erd2*, X66838; Fig2p, factor induced gene, YCR089W.

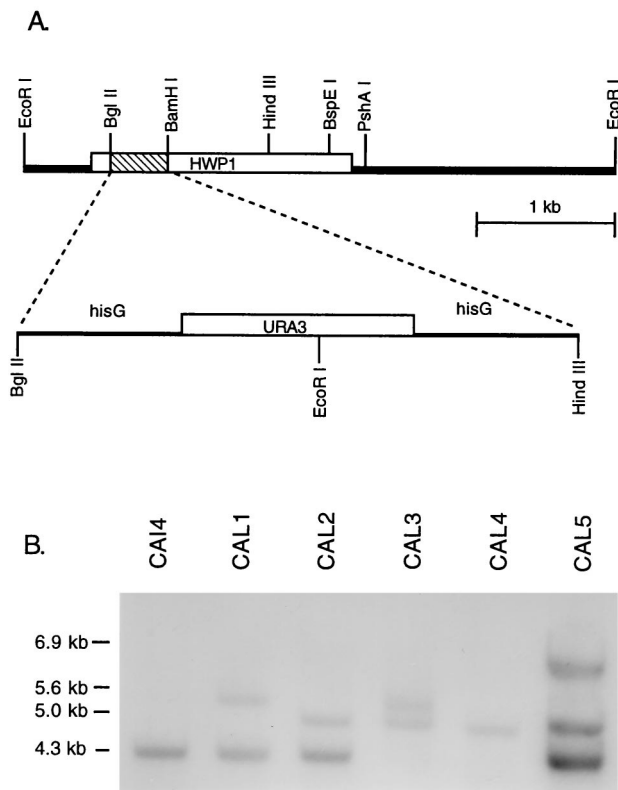


FIG. 4. Construction of *HWP1* null mutants. (A) Schematic representation of the *HWP1* disruption scheme. (B) Southern blot analysis of representative *HWP1* disrupted strains. Genomic DNA was digested with *EcoRI* and hybridized with labeled *HWP1* DNA.

which contained only one functional allele, did not exhibit the partial defects evident in the heterozygous parent of the null mutant. Instead, they resembled the homozygous wild type. This phenotype was evident in several other independently constructed revertants and may indicate that the fragment of the promoter driving expression of the reintroduced allele is inadequate for complete regulation.

To test whether *HWP1* was simply necessary or both necessary and sufficient for hypha production, the *HWP1* promoter was replaced with the constitutive promoter from *TEF2* (37) and integrated into the null mutant strain CAL4. Constitutive expression of *HWP1* was confirmed by Northern analysis. Constitutive expression restored the ability of the mutant to form hyphae on medium 199 plates. However, when cells were incubated under conditions that repress filamentation (medium 199 plates at pH 4.5 and 25°C), no hyphae were formed. Thus, *HWP1* is insufficient to initiate or promote hyphal development.

Regulation of *HWP1* and *ECE1*. Because of the developmental expression pattern of *HWP1* and its involvement in filamentation, it was potentially under the control of one or more of the known regulators of this process. Their influence on the expression of *HWP1* was assessed by examining *HWP1* induction in the corresponding null mutants. Induction of *HWP1* was completely blocked by deletion of *EFG1*, either alone or in combination with *CPH1* (Fig. 6A). Deletion of *CPH1* alone had no effect. Thus, *HWP1* expression is under the positive regulatory control of *EFG1* but is independent of the MAP kinase cascade.

The *EFG1* dependence of *HWP1* expression suggested the

possibility that the defective hyphal development of $\Delta efg1$ mutants was due to the absence of *HWP1*p. To determine if forced expression of *HWP1* could suppress the $\Delta efg1$ mutant phenotype, *HWP1*, under the control of the constitutive promoter from the *TEF2* gene, was transformed into the $\Delta efg1$ background. No changes in the phenotype were observed.

The repressors encoded by both *TUP1* (6) and *RBF1* (15) modulated *HWP1* expression. In a *TUP1* null mutant, *HWP1* was expressed even under noninducing conditions (Fig. 6B). However, expression was lower than the induced level. Alternatively, deletion of *RBF1* resulted in a 70% reduction of *HWP1* expression under inducing conditions (Fig. 6C).

ECE1 exhibits a developmental expression pattern similar to *HWP1*, but *Dece1* mutants are not affected in filamentation ability. However, as with *HWP1*, expression of *ECE1* was abrogated in an *EFG1* null mutant (Fig. 7) but occurred normally in a $\Delta cph1$ background. *TUP1* also had no effect. In contrast, *ECE1* was not expressed in the 1161KR strain background in the presence or absence of the *RBF1* null mutation.

DISCUSSION

HWP1 was identified in a genetic screen for developmentally regulated genes. This same gene was previously identified in an immunological screen for hypha-specific proteins (35). Deletion analysis revealed that *HWP1* is conditionally required for

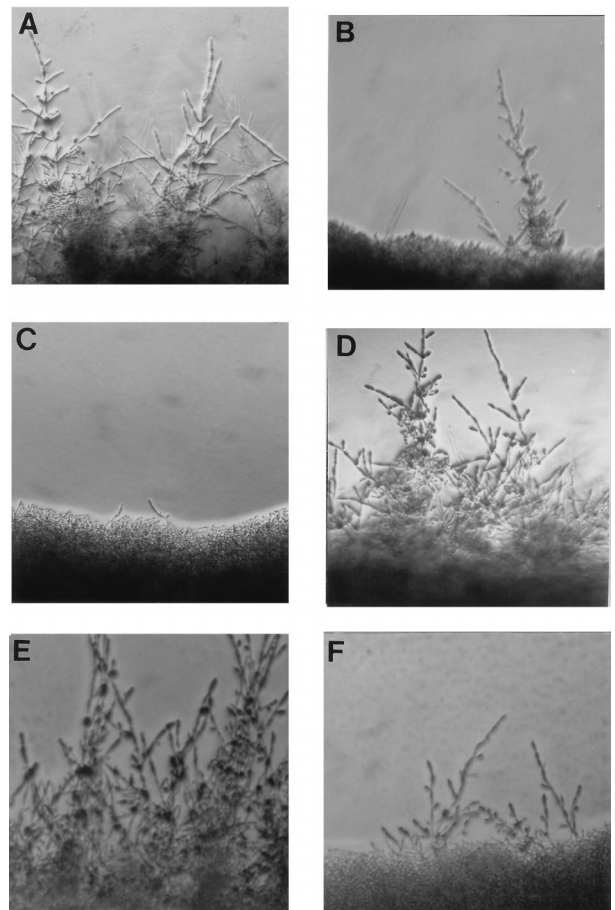


FIG. 5. Effect of *HWP1* mutation on hyphal formation. Strains were cultured for 48 h on medium 199 (pH 7) at 37°C (A to D) or for 24 h on 10% serum (E and F). The strains used are SC5314 (A and E), CAL1 (B), CAL3 (C and F), and CAL5 (D). Magnification, $\times 10$.

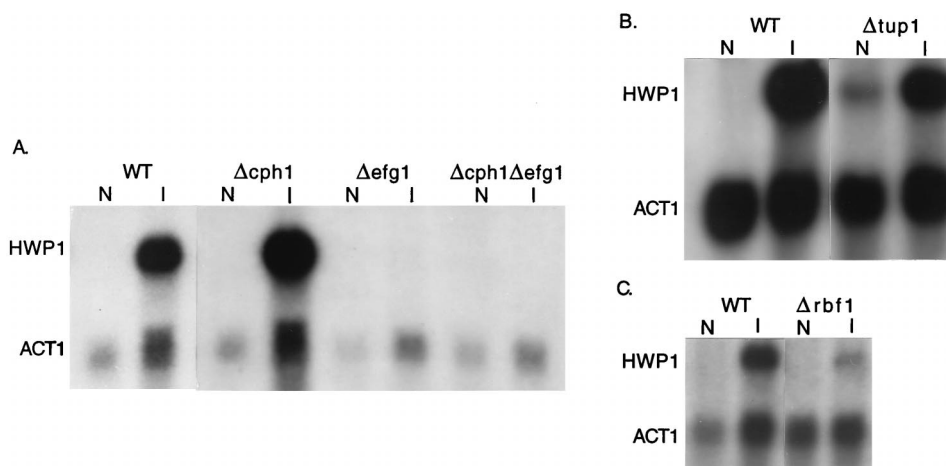


FIG. 6. Effect of regulatory mutations on *HWPI* expression. RNA was prepared from cells grown under noninducing conditions (N) or hypha-inducing conditions (I), and the blots were probed with *HWPI* and *ACT1*. The relevant genotypes are indicated above the lanes. (A and B) SC5314 was the wild-type strain (WT). (C) The parental strain of the $\Delta rbf1$ mutant, 1161KR, was used as the control.

hyphal formation. The ability to form hyphae on solid media was severely reduced in the *HWPI* heterozygous mutant and essentially eliminated in the null mutant. In the presence of serum, colonies of the null mutant were able to produce peripheral hyphae, but at reduced levels compared to the wild type. Reintroduction of a functional allele of *HWPI* resulted in a return to the wild-type phenotype, confirming that the loss of *HWPI* expression was responsible for the defects in hyphal development in the mutant strains. All mutants maintained the ability to invade the agar directly beneath the colony and to form germ tubes in liquid suspension cultures.

Presently, no specific role in hyphal development can be assigned to *HWPI*. No homologs or functional motifs were identified. *HWPI* encodes a putative GPI-linked surface protein and has been localized to the cell surface by immunofluorescence labeling (35). Staab et al. reported that Hwp1p can serve as a substrate for mammalian transglutaminase and can mediate the stable attachment of *C. albicans* hyphae to human buccal epithelial cells (34). They also reported that *HWPI* is required for virulence in a mouse model of systemic infection, a result corroborated by Tsuchimori et al. (34, 38).

Hwp1p may belong to a unique subset of GPI-anchored proteins characterized by the presence of a conserved structural motif, YTTWCPL. The diverse functions of the proteins that contain this motif suggest that it imparts a general property, e.g., interaction with specific surface proteins or wall polysaccharides. The surface localization of Hwp1p is compat-

ible with diverse functions, from cell wall assembly to cell signaling. However, Hwp1p is unlikely to participate in the reception of the initial developmental signal since it is not expressed in the yeast form and is downstream of the developmental regulator *EFG1*. It is also unlikely to function directly in the formation of the filamentous wall structure. The heterozygous null mutant exhibited a reduction in the frequency of hyphal elements but no alteration in their morphology. Similarly, hyphae formed by the null mutant in the presence of serum, while shorter, appeared otherwise normal. Thus, Hwp1p may be required for hyphal development to proceed or be sustained once the signal has been received. Notably, there are no apparent homologs in the *S. cerevisiae* genome, and so *HWPI* may be one of the developmental components that allows true filamentation in *C. albicans* versus the pseudohyphal morphology seen in baker's yeast. Whatever its function, the conditional requirement for *HWPI* on solid medium indicates that the function is either unnecessary or redundant in liquid culture.

HWPI is the first morphogenic target identified that is downstream of the signaling components and regulators of hyphal development. Examining the influence of these regulators on *HWPI* expression has elucidated several facets of hyphal development. Previous studies had demonstrated that mutation of either *CPH1* or *EFG1* alone resulted in partial suppression of development but that deletion of both genes caused complete suppression (22). The simplest interpretation of these results is that *CPH1* and *EFG1* provide additive inputs in regulating a set of genes required for filamentation. However, expression of *HWPI* was entirely *EFG1* dependent and was not influenced by *CPH1*. This is consistent with the phenotype of the *HWPI* null mutant, which is compromised in hyphal development on several media on which the $\Delta cph1$ mutant is not compromised. These results indicate that *CPH1* and *EFG1* are components of separate pathways with different target genes. The $\Delta hwp1$ and $\Delta efg1$ mutants differ in that the $\Delta hwp1$ mutant is able to form true hyphae in the presence of serum while the $\Delta efg1$ mutant is not. This difference and the inability of constitutive expression of *HWPI* to suppress the $\Delta efg1$ mutation indicate that *EFG1* regulates additional genes required for hyphal development.

HWPI expression is also influenced by *TUP1* and *RBF1*, both negative regulators of hyphal development (6, 15). *HWPI*

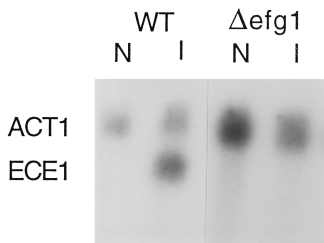


FIG. 7. Effect of regulatory mutations on *ECE1* expression. RNA was prepared from cells grown under noninducing conditions (N) or hypha-inducing conditions (I), and the blots were probed with *ECE1* and *ACT1*. The relevant genotypes are indicated above the lanes. SC5314 was the wild-type strain (WT).

was partially derepressed under noninducing conditions in a *TUP1* null mutant. The lack of complete derepression indicates that expression of *HWPI* is under multiple controls and probably requires an additional positive signal(s). This may be supplied via the *EFG1*-dependent pathway. The data do not allow us to distinguish whether *TUP1* acts through *EFG1* or independently. However, it is clear from the results that *TUP1* acts independently of *CPHI*. Previous work had shown that *TUP1* mutations are epistatic to a Δ *cph1* mutation. From this, it could be inferred that *TUP1* functions downstream of *CPHI*, assuming that they act within the same pathway. Since *TUP1* influences *HWPI* expression but *CPHI* does not, *CPHI* cannot be acting upstream of *TUP1*. Unexpectedly, deletion of *RBF1* resulted in reduced expression of *HWPI* under inducing conditions, indicating that *RBF1* is a positive regulator of *HWPI*.

HWPI is distinguished from other developmentally regulated genes of *C. albicans* by its role in filamentation. *ECE1* and *HYR1* exhibit a similar patterns of expression, but null mutations in these genes do not affect morphogenesis (2, 5). This distinction made it possible to determine whether the similar developmental expression patterns of morphogenic and nonmorphogenic genes reflect a common regulation. This question is relevant to the role of hyphal development in pathogenesis. As demonstrated by Lo et al. (22), nonfilamentous mutants are avirulent. However, it is not known if this reflects the inability of the organism to adopt the filamentous morphology and/or the effect of the mutations on coregulated virulence determinants. Comparison of *HWPI* and *ECE1* expression demonstrated common and unique regulatory interactions. Expression of both genes was similarly affected by mutation of *EFG1* and unaffected by *CPHI*. However, *ECE1* was not affected by mutation of *TUP1* and was not induced in strain 1161KR. The lack of *ECE1* expression in 1161KR is probably a consequence of the highly mutagenized genetic background of this strain (15). Nonetheless, it demonstrates that there are distinct regulatory features in the developmental expression of *HWPI* and *ECE1*. Although it is not known if *ECE1* encodes a virulence attribute, the data demonstrate that the regulatory role of *EFG1* extends beyond the control of cell shape determinants. Furthermore, the observed differences in *HWPI* and *ECE1* regulation offers the possibility that expression of morphogenic and nonmorphogenic functions can be dissociated and their relative contributions to the virulence process can be independently assessed.

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REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic Local Alignment Search Tool. *J. Mol. Biol.* **215**:403–410.
- Bailey, D. A., P. J. F. Feldmann, M. Bovey, N. A. R. Gow, and A. J. P. Brown. 1996. The *Candida albicans* *HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J. Bacteriol.* **178**:5353–5360.
- Bairoch, A. 1992. PROSITE: a dictionary of sites and patterns in proteins. *Nucleic Acids Res.* **20**:2013–2022.
- Bidard, F., M. Bony, B. Blondin, S. Dequin, and P. Barre. 1995. The *Saccharomyces cerevisiae* *FLO1* flocculation gene encodes for a cell surface protein. *Yeast*. **11**:809–822.
- Birse, C. E., M. Y. Irwin, W. A. Fonzi, and P. S. Sypherd. 1993. Cloning and characterization of *ECE1*, a gene expressed in association with cell elongation of the dimorphic pathogen *Candida albicans*. *Infect. Immun.* **61**:3648–3655.
- Braun, B. R., and A. D. Johnson. 1997. Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. *Science* **277**:105–109.
- Csank, C., C. Makris, S. Meloche, K. Schroppel, M. Rollinghoff, D. Dignard, D. Y. Thomas, and M. Whiteway. 1997. Derepressed hyphal growth and reduced virulence in a VH-1 family-related protein phosphatase mutant of the human pathogen *Candida albicans*. *Mol. Biol. Cell* **8**:2539–2551.
- Csank, C., K. Schroppel, E. Leberer, D. Harcus, O. Mohamed, S. Meloche, D. Y. Thomas, and M. Whiteway. 1998. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infect. Immun.* **66**:2713–2721.
- Erdman, S., L. Lin, M. Malczynski, and M. Snyder. 1998. Pheromone-regulated genes required for yeast mating differentiation. *J. Cell Biol.* **140**:461–483.
- Fonzi, W. A., and M. Y. Irwin. 1993. Isogenic Strain construction and gene mapping in *Candida albicans*. *Genetics* **134**:717–728.
- Hardwick, K. G., J. C. Boothroyd, A. D. Rudner, and H. R. B. Pelham. 1992. Genes that allow yeast cells to grow in the absence of the HDEL receptor. *EMBO J.* **11**:4187–4195.
- Hilenski, L. L., F. Naider, and J. M. Becker. 1986. Polyoxin D inhibits colloidal gold-wheat germ agglutinin labelling of chitin in dimorphic forms of *Candida albicans*. *J. Gen. Microbiol.* **132**:1441–1451.
- Hogging, D. G., and P. M. Sharp. 1988. CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. *Gene* **173**:237–244.
- Ishii, N., M. Yamamoto, H.-W. Lahm, S. Lizumi, F. Yoshihara, H. Nakayama, M. Arisawa, and Y. Aoki. 1997. A DNA-binding protein from *Candida albicans* that binds to the RPG box of *Saccharomyces cerevisiae* and the telomeric repeat sequence of *C. albicans*. *Microbiology* **143**:417–427.
- Ishii, N., M. Yamamoto, F. Yoshihara, M. Arisawa, and Y. Aoki. 1997. Biochemical and genetic characterization of Rbf1p, a putative transcription factor of *Candida albicans*. *Microbiology* **143**:429–435.
- Kelly, R., S. M. Miller, M. B. Kurtz, and D. R. Kirsch. 1987. Directed mutagenesis in *Candida albicans*: one-step gene disruption to isolate *ura3* mutants. *Mol. Cell. Biol.* **7**:199–207.
- Kohler, J. R., and G. R. Fink. 1996. *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. *Proc. Natl. Acad. Sci. USA* **93**:13223–13228.
- Leberer, E., D. Harcus, I. D. Broadbent, K. L. Clark, D. Dignard, K. Ziegelbauer, A. Schmidt, N. A. R. Gow, A. J. P. Brown, and D. Thomas. 1996. Signal transduction through homologs of the STE20p and STE7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **93**:13217–13222.
- Lee, K. L., H. R. Buckley, and C. C. Campbell. 1975. An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13**:148–153.
- Liu, H., J. Kohler, and G. R. Fink. 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. *Science* **266**:1723–1726.
- Liu, H., C. A. Styles, and G. R. Fink. 1993. Elements of the yeast pheromone response pathway required for filamentous growth of diploids. *Science* **262**:1741–1744.
- Lo, H., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink. 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**:939–949.
- Marck, C. 1988. "DNA Strider": a C program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic Acids Res.* **16**:1829–1836.
- McCreath, K. J., C. A. Specht, and P. W. Robbins. 1995. Molecular cloning and characterization of chitinase genes from *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **92**:2544–2548.
- Muhlschlegel, F. A., and W. A. Fonzi. 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. *Mol. Cell. Biol.* **17**:5960–5967.
- Nevill-Manning, C. G., T. D. Wu, and D. L. Brutlag. 1998. Highly specific protein sequence motifs for genome analysis. *Proc. Natl. Acad. Sci. USA* **95**:5865–5871.
- Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**:1–6.
- Roberts, R. L., and G. R. Fink. 1994. Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genet. Dev.* **8**:2974–2985.
- Roy, A., C. F. Lu, D. L. Marykwas, P. N. Lipke, and J. Kurjan. 1991. The *AGA1* product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein α -agglutinin. *Mol. Cell. Biol.* **11**:4196–4206.
- Saporito-Irwin, S. M., C. E. Birse, P. S. Sypherd, and W. A. Fonzi. 1995. *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. *Mol. Cell. Biol.* **15**:601–613.
- Schuler, G. D., S. F. Altschul, and D. J. Lipman. 1991. A workbench for multiple alignment construction and analysis. *Proteins Struct. Funct. Genet.* **9**:180–190.

32. **Shepherd, M. G., C. Y. Yin, S. P. Ram, and P. A. Sullivan.** 1980. Germ tube induction in *Candida albicans*. *Can. J. Microbiol.* **26**:21–26.
33. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. *Methods in yeast genetics.* Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
34. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283**:1535–1538.
35. **Staab, J. F., C. A. Ferrer, and P. Sundstrom.** 1996. Developmental expression of a tandemly repeated, proline- and glutamine-rich amino acid motif on hyphal surfaces of *Candida albicans*. *J. Biol. Chem.* **271**:6298–6305.
36. **Stoldt, V. R., A. Sonneborn, C. E. Leuker, and J. F. Ernst.** 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J.* **16**:1982–1991.
37. **Sundstrom, P., D. Smith, and P. S. Sypherd.** 1990. Sequence analysis and expression of the two genes for elongation factor 1 alpha from the dimorphic yeast *Candida albicans*. *J. Bacteriol.* **172**:2036–2045.
38. **Tsuchimori, N., L. L. Sharkey, W. A. Fonzi, S. W. French, J. E. Edwards, and S. G. Filler.** Interaction of HWP-1 deficient mutants of *Candida albicans* with host cells in vivo and in vitro. Submitted for publication.
39. **Udenfriend, S., and K. Kodukula.** 1995. Prediction of w site in nascent precursor of glycosylphosphatidylinositol protein. *Methods Enzymol.* **250**:571–582.
40. **Williams, F. E., and R. J. Trumbly.** 1990. Characterization of TUP1, a mediator of glucose repression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**:6500–6511.