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The Basic Helix-Loop-Helix Transcription Factor Cph2 Regulates Hyphal Development in *Candida albicans* Partly via Tec1

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Candida albicans **undergoes a morphogenetic switch from budding yeast to hyphal growth form in response to a variety of stimuli and growth conditions. Multiple signaling pathways, including a Cph1-mediated mitogen-activated protein kinase pathway and an Efg1-mediated cyclic AMP/protein kinase A pathway, regulate the transition. Here we report the identification of a basic helix-loop-helix transcription factor of the Myc subfamily (Cph2) by its ability to promote pseudohyphal growth in *Saccharomyces cerevisiae*. Like sterol response element binding protein 1, Cph2 has a Tyr instead of a conserved Arg in the basic DNA binding region. Cph2 regulates hyphal development in *C. albicans*, as *cph2*/*cph2* mutant strains show medium-specific impairment in hyphal development and in the induction of hypha-specific genes. However, many hypha-specific genes do not have potential Cph2 binding sites in their upstream regions. Interestingly, upstream sequences of all known hypha-specific genes are found to contain potential binding sites for Tec1, a regulator of hyphal development. Northern analysis shows that *TEC1* transcription is highest in the medium in which *cph2*/*cph2* displays a defect in hyphal development, and Cph2 is necessary for this transcriptional induction of *TEC1*. In vitro gel mobility shift experiments show that Cph2 directly binds to the two sterol regulatory element 1-like elements upstream of *TEC1*. Furthermore, the ectopic expression of *TEC1* suppresses the defect of *cph2*/*cph2* in hyphal development. Therefore, the function of Cph2 in hyphal transcription is mediated, in part, through Tec1. We further show that this function of Cph2 is independent of the Cph1- and Efg1-mediated pathways.**

Candida albicans is one of the most frequently isolated fungal pathogens of humans. It is capable of causing superficial mucosal infections as well as life-threatening systemic infections in immunocompromised individuals. *C. albicans* can undergo reversible morphogenetic transitions among budding yeast, pseudohyphal, and hyphal growth forms. The pathogenicity of this fungus is linked to its capacity to switch among different growth forms (27).

A wide range of signals or culture conditions can trigger the yeast-to-hypha transition in *C. albicans*. These include serum, *N*-acetylglucosamine, proline, a temperature of 37°C, neutral pH, and microaerophilic conditions (8). Levels of expression of several genes have been shown to be associated with hyphal morphogenesis (hypha-specific genes), rather than with a specific hypha-inducing condition. Hypha-specific genes identified so far include *ECE1*, *HWP1*, *HYR1*, *ALS3*, *RBT1*, and *RBT4* (2, 4, 7, 18, 43). Some of them, such as *HWP1* (42), *RBT1*, and *RBT4* (7), encode important virulence factors.

Molecular cloning and characterization of hyphal regulators or signaling pathways have been based largely on the strong conservation between *C. albicans* and other genetically tractable fungi. Cph1 is homologous to *Saccharomyces cerevisiae* Ste12, which encodes a transcription factor required for mating and filamentous growth (26). As in *S. cerevisiae*, Cph1 is regulated by a mitogen-activated protein (MAP) kinase cascade that includes Cst20, Hst7, and Cek1. Homozygous mutations in these genes of the MAP kinase pathway all display a medium-specific defect in hyphal development (9, 21, 24). Efg1, a basic helix-loop-helix (bHLH) protein similar to Phd1 of *S. cerevisiae* and StuA of *Aspergillus nidulans*, plays a major role in regulating hyphal development in *C. albicans* (44). *efg1*/*efg1* null mutant strains are severely blocked in hyphal development under many conditions, including serum (27, 44). Efg1 may be regulated by the cyclic AMP/protein kinase A signaling pathway (6, 41). The Efg1-mediated pathway is distinct from that of Cph1 because *cph1*/*cph1 efg1*/*efg1* double mutants are more defective than *cph1*/*cph1* or *efg1*/*efg1* single mutants in hyphal development under most conditions examined and are avirulent (27). Recently, a new member of the TEA/ATTS family of transcription factors, Tec1, has been shown to regulate hyphal development and virulence in *C. albicans* (40). TEA/ATTS family members AbaA in *A. nidulans* and Tec1 in *S. cerevisiae* are involved in the regulation of conidiophore formation and filamentous growth, respectively (1, 15, 34). Considering that *C. albicans* can respond to a large number of extracellular signals and growth conditions in controlling hyphal development, *C. albicans* cells are likely to utilize multiple signal transduction pathways to integrate these signals.

Here we report the identification of a novel hyphal regulator of *C. albicans* identified by using *S. cerevisiae*. The hyphal regulator is a bHLH protein of the Myc subfamily. It is important for hyphal development and the transcription of hypha-specific genes in a medium-specific manner. The functional relationship of the bHLH protein with Cph1, Efg1, and Tec1 in hyphal regulation is addressed.

MATERIALS AND METHODS

Media and *C. albicans* manipulation. *S. cerevisiae* media were used for routine culturing of *C. albicans*, except that uridine instead of uracil was used for growing Ura$^-$ *C. albicans* strains. Several hypha-inducing media were used: Lee's medium (25) with either 2% glucose, 1% mannitol, or 2% succinic acid as the

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
Library	*C. albicans* genomic library in an *S. cerevisiae* 2μm *URA3* vector	26
pHL17	Clone from the library with a 4-kb insertion carrying *CPH2*	This study
pHL541	*CPH2* in BES116	This study
BP1	*PCK1p* in BES116	This study
BP2	*PCK1p* in BES116-MluI	This study
BP1-*CPH2*	*PCK1p-CPH2* in BES116	This study
BP1-*CPH1*	*PCK1p-CPH1* in BES116	This study
BP2-*TEC1*	*PCK1p-TEC1* in BES116	This study
CAHUH	*PCK1p-EFG1*	44
pGEX-2T-*CPH2* C′	GST-CPH2 C′ (aa 190–290)	This study

carbon source; yeast extract-peptone-dextrose (YPD)–serum; RPMI medium (40); synthetic succinate (SS) medium (0.0425% yeast nitrogen base without amino acids or ammonium sulfate [Difco], 0.125% ammonium sulfate, 2% succinic acid [pH 6.5]); and SSA medium (SS medium plus amino acids at the concentrations used for *S. cerevisiae* media). The lithium acetate method of Ito et al. (19) was used for *C. albicans* transformation, except that fewer cells were used (10^7 cells/ml), 1 M Tris (pH 7.4) (10 μl) was added to 50 μl of cells, more transforming DNA was used (~2 μg), and heat shock was done for 22 min at 42°C. Photographs of cell and colony morphologies were obtained as described by Loeb et al. (28).

***CPH2* cloning and plasmid construction.** Plasmids pHL17, pHL33, and pHL34 were isolated from a *C. albicans* genomic library based on their ability to promote invasive growth and cell elongation in diploid *S. cerevisiae* on SC-Ura–1 M sorbitol medium (26). Inserts from all three plasmids gave similar patterns of restriction digestion. pHL17 contained a 4-kb insert. Deletions from both directions narrowed down a region responsible for invasive-pseudohyphal growth. The DNA sequence of the region was determined. Half of the *CPH2* coding sequence is not in the current *C. albicans* sequence database.

BES116-*CPH2* (pHL541), a 3-kb *Kpn*I-*Hin*dIII *CPH2* fragment from pHL17, was subcloned into the *Hin*dIII-*Kpn*I site of plasmid BES116, a *C. albicans ADE2*-integrating vector (13). BES116-*PCK1p* (BP1), a 1.4-kb *Not*I-*Bgl*II (blunt-ended) *PCK1p* fragment from plasmid CA01 (44), was subcloned into the *Not*I-*Eco*RV site of plasmid BES116. BP1-*CPH1*, a 2-kb *CPH1* PCR fragment with *Hin*dIII at both ends, was subcloned into the *Hin*dIII site of plasmid BP1. The primers used for PCR were P303 (5′CCCAAGCTTGCCTAATACACTCTTTC GCC) and P304 (5′CCCAAGCTTACAAGTCCATAAACATAATGC). BP1-*CPH2*, a 1.1-kb *Kpn*I-*Bsp*LU11I (blunt-ended) *CPH2* fragment from plasmid BES116-*CPH2*, was subcloned into the *Kpn*I-*Hin*dIII (blunt-ended) site of plasmid BP1. BES116-*PCK1p*-MluI (BP2), an *Mlu*I linker with *Cla*I ends, was subcloned into the *Cla*I site of plasmid BP1. The oligonucleotide annealed to make the linker was P 342 (5′ P-CGATGACGCGTCAT). BP2-*TEC1*, a 2.2-kb *TEC1* PCR fragment with *Mlu*I at both ends, was subcloned into the *Mlu*I site of plasmid BP2. pGEX-2T-*CPH2* C′, a 300-bp *CPH2* PCR fragment with nucleotides corresponding to amino acids (aa) 190 to 290 of Cph2 and with *Bam*HI at both ends, was subcloned into the *Bam*HI site of plasmid pGEX-2T (Pharmacia Biotech). The primers used for PCR were P353 (5′CGGGATCCAACACCAC TAAAAAACCGGCC) and P354 (5′CGGGATCCGCCTATGCAACTCAATA TTG). All constructs were confirmed by DNA sequence analysis. The plasmids used in this study are listed in Table 1.

***C. albicans* strain construction.** The *C. albicans* strains used in this study are listed in Table 2. *CPH2* was deleted based on the method of Wilson et al. (46). Primers P273 (5′TTGATATATTCTGTAGCTTTGGTTAAAACACTAGCTT TGTTCAATTTAGATGCTGGTGTTTGTGGAATTGTGAGCGGATA) and P272 (5′GCATCTTTATATTCGTTTGATTTT GTTGATGCCGACGATTTC

TABLE 2. *C. albicans* strains used in this study

Strain	Genotype	Reference or source
SC5314	Wild type	14
BWP17	*ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*	46
HLY1907	*cph2*::*ARG4*/*CPH2 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*	This study
HLY1909	*cph2*::*HIS1*/*CPH2 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*hisI*::*hisG arg4*::*hisG*/*arg4*::*hisG*	This study
HLY1906	*cph2*::*URA3*/*CPH2 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*hisl*::*hisG arg4*::*hisG*/*arg4*::*hisG*	This study
HLY1921	*cph2*::*ARG4*/*cph2*::*URA3 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*	This study
HLY1927	*cph2*::*ARG4*/*cph2*::*HIS1 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*	This study
HLY1928	*cph2*::*ARG4*/*cph2*::*HIS1 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG ADE2*/*ade2*::*URA3*	This study
HLY1929	*cph2*::*ARG4*/*cph2*::*HIS1 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG ADE2*/*ade2*::*CPH2-URA3*	This study
JKC19	*cph1*::*hisG*/*cph1*::*hisG-URA3-hisG ura3*::*1 imm434*/*ura3*::*1 imm434*	26
HLC52	*efg1*::*hisG*/*efg1*::*hisG-URA3-hisG ura3*::*1 imm434*/*ura3*::*1 imm434*	44
CAI4	*ura3*::*1 imm434*/*ura3*::*1 imm434*	14
HLY3119	*ura3*::*1 imm434*/*ura3*::*1 imm434 ADE2*/*ade2*::*PCK1p-CPH1-URA3*	This study
HLY3120	*ura3*::*1 imm434*/*ura3*::*1 imm434 ADE2*/*ade2*::*PCK1p-CPH2-URA3*	This study
HLY3125	*ura3*::*1 imm434*/*ura3*::*1 imm434 ADE2*/*ade2*::*PCK1p-EFG1-URA3*	This study
HLY3214	*ura3*::*1 imm434*/*ura3*::*1 imm434 ADE2*/*ade2*::*PCK1p-TEC1-URA3*	This study
HLY3183	*efg1*::*hisG*/*efg1*::*hisG ura3*::*1 imm434*/*ura3*::*1 imm434 ADE2*/*ade2*::*PCK1p-CPH2-URA3*	This study
HLY3139	*cph1*::*hisG*/*cph1*::*hisG ura3*::*1 imm434*/*ura3*::*1 imm434 ADE2*/*ade2*::*PCK1p-CPH2-URA3*	This study
HLY3220	*cph2*::*ARG4*/*cph2*::*HIS1 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG arg4*::*hisG ADE2*/*ade2*::*PCK1p-TEC1-URA3*	This study
HLY3156	*cph2*::*ARG4*/*cph2*::*HIS1 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG ADE2*/*ade2*::*PCK1p-CPH1-URA3*	This study
HLY3164	*cph2*::*ARG4*/*cph2*::*HIS1 ura3*::*1 imm434*/*ura3*::*1 imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG efg1*::*URA3-PCK1p-EFG1*	This study

TTAGATTCGATATCTGGCGTTTTCCCAGTCACGACGTT) were used to amplify *C. albicans HIS1*, *URA3*, and *ARG4* from plasmids pGEM-*HIS1*, pGEM-*URA3*, and pRS-*ARG4*Δ*Spe*I (46), respectively; the underlined sequences in the primers are the segments that annealed to the plasmids. Each primer also has 60 bp of sequence from the *CPH2* coding region. The PCR products were used to transform *C. albicans* strain BWP17 (46), yielding *cph2*/*CPH2* heterozygous strains HLY1909, HLY1906, and HLY1907 (Table 2). The replacement of one copy of the *CPH2* genes by a selectable marker was detected by PCR with primers P277 (5′CCCATAACAGCAGCCATACATCCCAAC) and P278 (5′ATAACCAAGTGAAGGAAGAATACCC), which are located about 500 bp outside of the *CPH2* coding region. P277 and P278 were also used to amplify DNA from *cph2*/*CPH2* heterozygous strains to produce *cph2* deletion fragments with 500-bp homologies on each end of the selectable markers. *Xcm*I-digested genomic DNA from HLY1907 was used to generate *cph2*::*ARG4*. *Hpa*I-digested genomic DNA from HLY1909 and HLY1906 was used for *cph2*::*HIS1* and *cph2*::*URA3*, respectively. Both enzymes had a restriction site in *CPH2*, but not in the selectable markers, thus eliminating the PCR product from *CPH2*. *cph2*::*URA3* and *cph2*::*ARG4* PCR products were used to transform HLY1907 and HLY1909, respectively, generating *cph2*/*cph2* homozygous deletion strains HLY1921 and HLY1927 (Table 2).

Expression and purification of GST fusion proteins. Glutathione *S*-transferase (GST) protein and a GST-Cph2 C′ (bHLH region) fusion protein were expressed from plasmid pGEX-2T transformed into *Escherichia coli* strain BL21 as described by Dooley et al. (11). Crude lysates (10 ml) were incubated with 3 ml of glutathione-agarose beads (Sigma) on a rotating wheel at 4°C for 4 h. The mixture was loaded onto a column, the flowthrough was collected, and the beads were washed five times with 4 column volumes (3 ml) of HEGN solution (50 mM HEPES [pH 7.6], 0.1 mM EDTA [pH 8.0], 10% glycerol, 0.1% Nonidet P-40) containing 0.1 M KCl, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin/ml, and 0.7 μg of pepstatin A/ml. The purified protein was eluted with 10 mM glutathione. The expression and purification of the proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis followed by Coomassie blue staining. The most concentrated fractions were pooled and dialyzed (molecular weight cutoff, 12,000 to 14,000) in Z 0.1 solution (25 mM HEPES [pH 7.6], 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol) at 4°C overnight.

Gel mobility shift experiments. Gel mobility shift experiments were performed using 1 ng of labeled DNA probe and 0.33, 1, or 3.3 μg of GST-Cph2 C′ fusion protein in a 20-μl reaction mixture (39). For competition experiments, 200 ng of nonlabeled DNA was incubated with 1 μg of GST or GST-Cph2 C′ fusion protein before the addition of 1 ng of labeled DNA probe (45). Double-stranded DNA for probes or competitor were generated by annealing complementary oligonucleotides containing the wild-type −316 to −297 *TEC1* upstream sequence as well as sequences with the first sterol regulatory element 1 (SRE-1) site or both SRE-1 sites mutated. All DNAs have 5′ GATC overhangs for polynucleotide kinase end labeling. See Fig. 8C for wild-type and mutant oligonucleotide probe sequences.

Northern analysis. Methods for RNA isolation and Northern blot hybridization were as previously described (28). A *Cla*I-*Sal*I *ACT1* fragment from plasmid p1595/3 (10) was used as a probe for Northern analysis. A 1.5-kb *CPH2* PCR product (primers P277 and P278) was used as a probe for Northern hybridization. PCR products of *ECE1*, *HWP1*, *HYR1*, and *RBT4* were used for probing Northern blots. The images were scanned with a PhosphorImager (Molecular Dynamics) and quantified using ImageQuant (Molecular Dynamics) and Quantity One (Bio-Rad) software. The gene expression signal intensities were first normalized to those of actin before fold change values were calculated. The sizes of the mRNAs on the Northern blots correlated with the expected lengths based on information from the *C. albicans* genome database.

Sequence analysis. The 800-bp upstream regions of hypha-specific genes and of 1,000 randomly chosen control genes were extracted from the Stanford *Candida* Genome Center (http://www-sequence.stanford.edu/group/candida/search.html). Weight matrices for SRE-1 and E-box motifs were entered into *S. cerevisiae* promoter database (SCPD) matrix search program (http://cgsigma.cshl.org/cgi-bin/jz/searchmatrix) to identify potential SRE-1-like or E-box motifs in the extracted upstream sequences. Motifs that were at least 80% identical to the matrices and contained the conserved core binding motif were considered potential SRE-1-like or E-box binding sites (see Table 3). The upstream sequences of *ECE1*, *HWP1*, *HYR1* and *ALS3* were also used to search for over-represented motifs using the GibbsDNA program (23) (available at the SCPD site). Then, the new motifs were used to find potential matches to known transcription factor binding motifs using the SCPD and TFSearch (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). A Tec1 site (AbaA) was revealed in our search, and the string CATTCY was used to search for this site in all hypha-specific genes.

Nucleotide sequence accession number. The GenBank accession number for the *CPH2* nucleotide sequence is AF349507.

RESULTS

Cloning of a novel *C. albicans* bHLH gene that can promote pseudohyphal growth in *S. cerevisiae*. The absence of a sexual cycle and the diploid nature of the *C. albicans* genome prohibit the direct isolation of nonfilamentous mutants of *C. albicans*. Therefore, we chose to clone *C. albicans* genes that enabled *S. cerevisiae* cells to undergo filamentous growth on medium repressive for invasive and pseudohyphal growth. A *C. albicans* genomic library constructed in a high-copy-number *S. cerevisiae* vector (26) was transformed into diploid *S. cerevisiae* cells, and transformants were grown on SC-Ura medium. From 100,000 transformed colonies, about 200 transformed colonies were invasive and remained on the agar plates after the surface cells were washed off (Fig. 1A). Nine of the invasive colonies displayed elongated cell morphology (Fig. 1B). These nine clones represented two *C. albicans* genes, *CPH1* and *CPH2* (*Candida* pseudohyphal regulator).

CPH2 encodes a protein with a bHLH domain in the Myc/Max subfamily (Fig. 2). The bHLH region of Cph2 is most similar to two *Schizosaccharomyces pombe* proteins of unknown function. It is also very similar to the *S. cerevisiae* bHLH proteins Hms1 and Tye7. *HMS1* has been cloned as one of the multicopy suppressors of pseudohyphal growth in an ammonium permease mutant strain (30). Like that of *CPH2*, the overexpression of *HMS1* enhances pseudohyphal growth in diploids. However, *hms1*/*hms1* mutant strains have no detectable defects in pseudohyphal growth (30). The overexpression of *TYE7* does not promote filamentous growth (unpublished data). The bHLH regions of Cph2, Hms1, and Tye7 share striking similarities with human SREBP1 (sterol response element binding protein 1), Max, and c-Myc proteins (Fig. 2). bHLH proteins of the Myc/Max subfamily bind to E-box motifs. Unlike Max and c-Myc proteins, SREBP1 binds to an E-box motif, as well as a non-E-box sequence, sterol regulatory element 1 (SRE-1) (20). The dual DNA binding specificity of SREBP1 is the result of an atypical Tyr residue in the conserved basic domain (20) (Fig. 2). Substitution of the atypical Tyr in the basic region with the Arg found in most bHLH proteins causes a restriction of only E-box binding. Cph2 has a Tyr residue at the position that correlates with the atypical Tyr in SREBP1 (Fig. 2) and is expected to have the same dual binding capacity as SREBP1. In addition, based on what is known about SREBPs, the Cph2 bHLH region predicts the formation of Cph2 homodimers (37).

***cph2*/*cph2* mutants show a medium-specific impairment in hyphal development.** To elucidate the function of *CPH2* in *C. albicans*, we deleted both copies of *CPH2* by homologous recombination as described by Wilson et al. (46). PCR fragments of *C. albicans URA3*, *HIS1*, or *ARG4*, flanked by 60 bp of sequence homologous to the *CPH2* coding region, were used in a *C. albicans* transformation to delete the first copy of *CPH2* (Fig. 3). To improve the efficiency of deleting the second *CPH2* gene, a pair of outside primers from various *cph2*/*CPH2* heterozygous strains obtained from the first round of transformation (Fig. 3) was used for PCR, generating *cph2*::*URA3*,

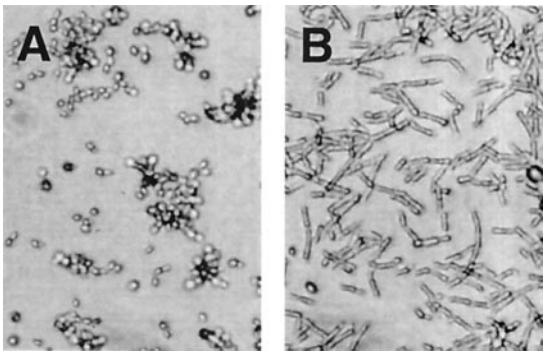

FIG. 1. Functional cloning of *CPH2* in *S. cerevisiae*. Morphologies of *S. cerevisiae* cells remaining on SC-Ura–sorbitol plates after noninvasive colonies and surface cells of invasive colonies were washed away with water. (A) Round cells from an invasive colony. (B) Elongated cells from one of the positive colonies carrying *CPH2*.

cph2::*HIS1*, and *cph2*::*ARG4* PCR fragments with 500 bp of sequence homologous to *CPH2* flanking each selectable marker. These PCR fragments were then used for the second round of transformation.

We examined the ability of *cph2*/*cph2* strains to undergo hyphal development in several liquid hypha-inducing media. The homozygous mutant strains exhibited no discernible defect in germ tube or hyphal development in many liquid hypha-inducing media, including serum (Fig. 4, first row), *N*-acetylglucosamine, and proline (data not shown). However, the *cph2*/*cph2* strains showed much less filamentation in Lee's medium (Fig. 4, second row). Changing the carbon source (mannitol, sucrose, or glucose) in Lee's medium did not seem to affect the level of filamentation of the *cph2*/*cph2* strains. The fact that *cph2*/*cph2* strains showed a defect in only certain hypha-inducing media suggested that Cph2 might be responsible for mediating medium-specific signals in hyphal development. The defect of *cph2*/*cph2* in hyphal development was exacerbated on solid hypha-inducing media. The homozygous *cph2*/*cph2* mutant strains exhibited a defect in hyphal colony formation on both serum-containing and solid Lee's media (Fig. 4). The defect in hyphal growth was directly linked to the *CPH2* deletion, because reintroducing a wild-type *CPH2* gene into *cph2*/*cph2* strain HLY1927 rescued the defect in hyphal development, while the same *cph2*/*cph2* strain with the vector alone was still defective in hyphal growth (Fig. 4).

***cph2*/*cph2* strains are impaired in the induction of hypha-specific transcripts.** Since Cph2 encodes a Myc type of bHLH protein and *cph2*/*cph2* mutants exhibit a medium-specific defect in hyphal development, we suspected that Cph2 might play a role in regulating the hyphal transcriptional program. We therefore examined the levels of expression of hypha-specific genes in the *cph2*/*cph2* strains by Northern hybridization. The levels of *ECE1*, *HWP1*, *HYR1*, *RBT4*, and *SAP5* expression in the *cph2*/*cph2* strains were similar to those in the wild-type strains in YPD, YPD-serum, Lee's medium-serum, or serum alone but were reduced by about 20-fold in the *cph2*/*cph2* strains in Lee's medium at 37°C (Fig. 5). The expression of *ALS3* was similar to that of other hypha-specific genes (data not shown). Therefore, the *cph2*/*cph2* strains exhibited a specific defect in the induction of hypha-specific genes in Lee's medium (Fig. 5, eighth lane from left) consistent with the medium-specific morphological defect shown in Fig. 4. Interestingly, we also observed that in some serum-containing media, such as YPD-serum, Cph2 appeared to have repressive activity for a group of hypha-specific genes, including *RBT4* and *SAP5* (Fig. 5, fourth lane from left).

Cph2 regulates *TEC1* transcription. Since Cph2 is responsible for the transcriptional induction of several hypha-specific genes in certain media, this activity of Cph2 may be mediated through the direct binding of Cph2 to SRE-1 or E-box motifs in the promoters of these hypha-specific genes. To address this possibility, we analyzed the upstream sequences of Cph2-regulated hypha-specific genes (Table 3). Surprisingly, the majority of the Cph2-regulated genes did not contain a potential Cph2 binding motif in their upstream regions. Of six genes, only *ECE1* and *RBT4* had a potential SRE-1-like site (Table 3). *HWP1* and *ECE1* were the only two genes found to have a potential E-box motif (Table 3). Moreover, many of these putative sites may not be actual Cph2 binding sites if one considers the possibility of random occurrences of these motifs in the genome (Table 3). In addition, SREBP1-regulated genes usually contain multiple binding sites in their promoters. Collectively, sequence analysis suggests that most of the hypha-specific genes may not be regulated directly by Cph2 but are regulated through a mediator(s).

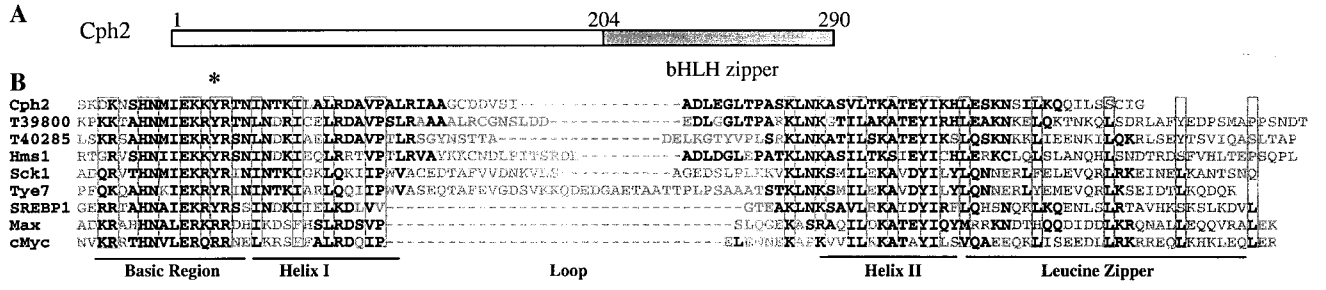

FIG. 2. *CPH2* encodes a bHLH protein. (A) A schematic diagram illustrating the location of the bHLH domain in the deduced Cph2 protein. (B) Protein sequence alignment of the bHLH domain (aa 204 to 290) of Cph2 with two *S. pombe* proteins of unknown function (accession numbers T39800 and T40285 in the EMBO data library), *S. cerevisiae* Hms1 (30) and Tye7 (29), *Kluyveromyces lactis* Sck1 (KLSCK1 in the EMBO data library), and human SREBP1a (47), Max (5), and c-Myc (38). Darker (black) letters show identical residues or conserved changes between the Cph2 protein and other bHLH proteins. Conserved residues for the basic region, helix I, loop, helix II, and leucine zipper are indicated by boxes. The asterisk denotes Tyr residues of SREBPs and Cph2.

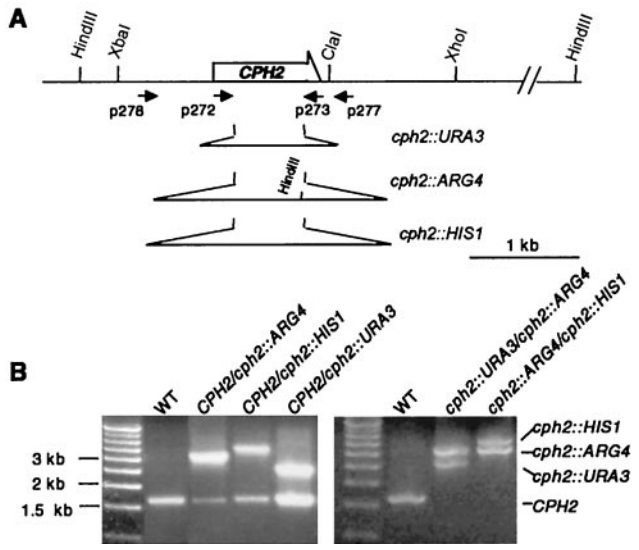

FIG. 3. Disruption of *CPH2* in *C. albicans*. (A) Restriction map and disruption strategy for *CPH2*. Positions of PCR primers P272, P273, P278, and P277 are marked. The positions and relative lengths of the *cph2* deletions are also marked. (B) Analysis of *CPH2* deletions by PCR. PCRs with outside primers P278 and P277 for *C. albicans* strains SC5314 (wild type [WT]), HLY1907 (*CPH2*/*cph2*::*ARG4*), HLY1909 (*CPH2*/*cph2*::*HIS1*), HLY1906 (*CPH2*/*cph2*::*URA3*), HLY1921 (*cph2*::*ARG4*/*cph2*::*URA3*), and HLY1927 (*cph2*::*HIS1*/*cph2*::*ARG4*) are shown. The genomic DNA was treated with *Xcm*I for HLY1901 and *Hpa*I for HLY1909 and HLY1906 to reduce the amount of PCR product from the wild-type copy of *CPH2*. The deletion of one copy of *CPH2* is based on the appearance of a larger PCR fragment (left panel). The deletion of the second copy is evident from the disappearance of the wild-type *CPH2* PCR product and the existence of two larger deletion fragments (right panel).

To identify potential Cph2-regulated mediators of hyphal development, we used the GibbsDNA sampling program (23) to analyze the 800-bp upstream sequences of four previously published hypha-specific genes: *ECE1*, *HWP1*, *HYR1*, and *ALS3*. Several motifs were found to be overrepresented in the upstream sequences of these four genes when compared to the occurrences in those of 1,000 randomly chosen *C. albicans* genes (unpublished data). In a search against known transcription factor binding sites, one of the motifs, TCATTCY, turned out to be very close to the binding site for *A. nidulans* AbaA and *S. cerevisiae* Tec1 (Table 3). AbaA binds to the sequence TTCATTCYTT (1), of which CATTCY is the core sequence for the TEA/ATTS family of transcription factors. The extracted motif is closer to the AbaA binding site than to other family members. The actual occurrence of the TCATTCY sequence in *RBT4* and *SAP5* upstream sequences was also much higher than what was expected to occur randomly (Table 3). The finding of Tec1 (AbaA) binding sites in hypha-specific genes was significant because Tec1 has recently been shown to regulate hyphal development in *C. albicans* (40). In addition, the *TEC1* upstream region contains two highly conserved SRE-1-like sequences adjacent to each other (Fig. 6B). The proximity of the two SRE-1-like sites in the *TEC1* upstream region is significant because two or more consecutive SRE-1 sites are a common feature for many SREBP1-regulated genes in mammalian cells (32). Therefore, we suspected that Cph2 may directly regulate the transcription of *TEC1*.

Northern analysis of the *TEC1* transcription level showed that *TEC1* expression was medium dependent and was highest in Lee's medium (Fig. 6C). Interestingly, *TEC1* expression was decreased in *cph2*/*cph2* strains in Lee's medium (Fig. 6C) but was unchanged compared to that of the wild-type parent strains in other hypha-inducing media (Fig. 6C). Therefore, Cph2 was necessary for the transcriptional induction of *TEC1* in Lee's medium. Additional regulators may control the basal expression of *TEC1*. The medium-dependent requirement of Cph2 for *TEC1* transcriptional induction is consistent with the medium-specific impairment of *cph2*/*cph2* in hyphal development.

Cph2 directly binds to the two SRE-1-like elements upstream of *TEC1* as well as to an E-box sequence. To address whether Cph2 binds directly to the SRE-1-like elements upstream of *TEC1*, we performed gel mobility shift experiments with a Cph2 recombinant protein. The Cph2 recombinant protein (including aa 190 to 290 of Cph2) contains all of the protein information necessary for DNA binding, protein dimerization, and transcriptional activation of this family of bHLH proteins (45). A DNA fragment corresponding to the

TABLE 3. Computational search of potential Cph2 and Tec1 binding sites[a]

Gene	Cph2 sites		Tec1 sites (AbaA)
	SRE-1	E-box	
ECE1	GTCACCTCAC (−724, −715)	TATCACCTGAT (−316, −306)	TCATTCT (−371, −365)
HWP1		TATCACCTGTA (−222, −212)	TCATTCT (−153, −147)
HYR1			TCATTCT (−513, −519), TCATTCC (−270, −264), TCATTCT (−32, −26)
RBT4	GTCATACCAC (−618, −609)		GCATTCT (−532, −526)
SAP5			TCATTCC (−116, −110), TCATTCC (−91, −85), TCATTCC (−66, −60)
ALS3			TCATTCT (−808, −802)
Total no. of potential sites	2	2	10
Expected random occurrence	0.356 × 6 = 2.14	0.235 × 6 = 1.41	0.728 × 6 = 4.37

[a] SRE-1 and E-box weight matrices (see Fig. 6A) and the Tec1 consensus sequence were used to search for potential Cph2 and Tec1 binding sites in the upstream sequences of hypha-specific genes. The sequences and positions of putative binding sites found from the search are shown. The expected random occurrence of a motif in this set of hypha-specific genes is calculated by multiplying the frequency of its occurrence in the control set by the number of hypha-specific genes.

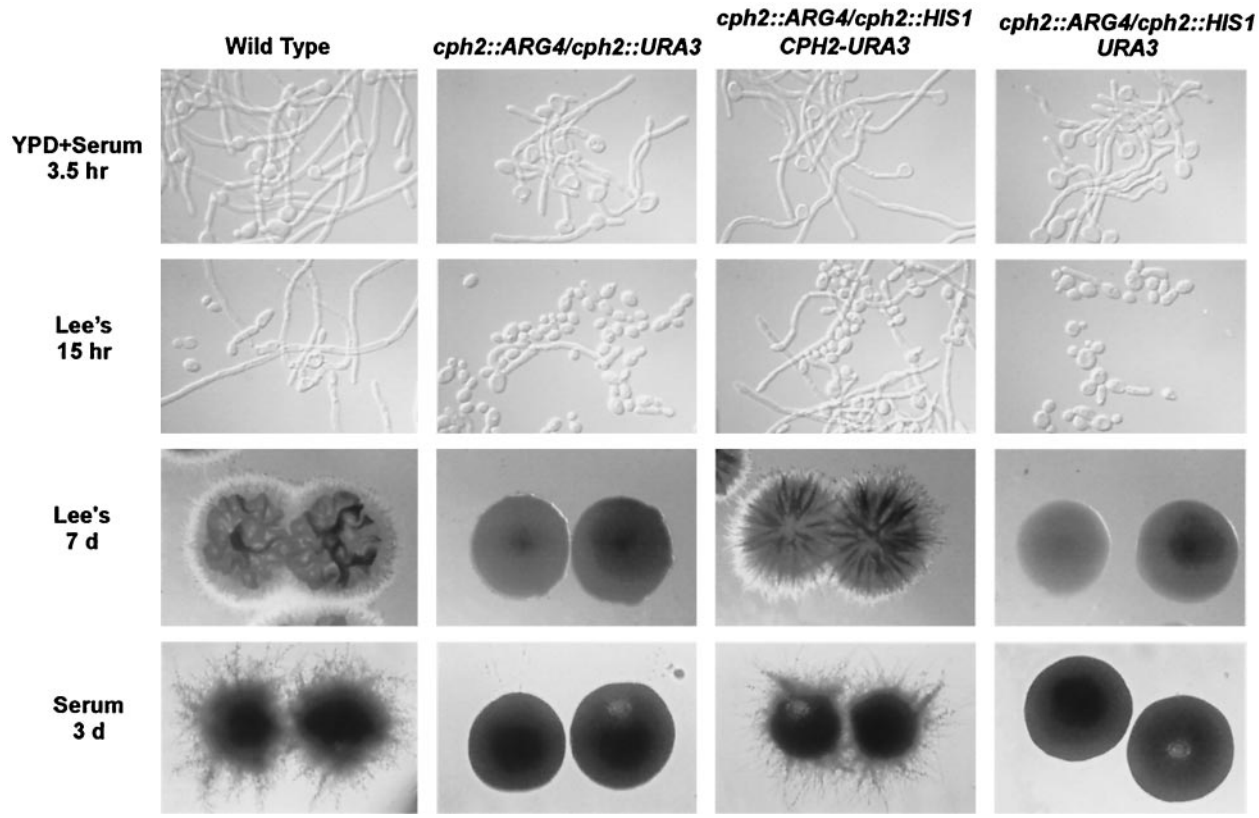

FIG. 4. Mutations in *CPH2* suppress hyphal development. Cell and colony morphologies of wild-type and *cph2*/*cph2* mutant strains after growth either in liquid (top two rows) or on solid (bottom two rows) Lee's or serum medium for the times (days [d]) indicated are shown. Strains: SC5314 (wild type), HLY1921 (*cph2*::*ARG4*/*cph2*::*URA3*), HLY1929 (*cph2*::*ARG4*/*cph2*::*HIS1 ADE2*/*ade2*::*CPH2-URA3*), and HLY1928 (*cph2*::*ARG4*/*cph2*::*HIS1 ADE2*/*ade2*::*URA3*).

two SRE-1-like elements from −316 to −297 of the *TEC1* upstream sequence was used as a probe in the gel mobility shift experiments. The binding specificity was evaluated by gel mobility shift experiments using DNA probes for the *TEC1* upstream sequence with mutations in one or both of the SRE-1-like elements (Fig. 7C). Mutating one of the two SRE-1-like elements did not abolish the binding of recombinant Cph2. However, mutating both of the SRE-1-like elements completely abolished the binding (Fig. 7A). Furthermore, competition with the same DNA mutated in both SRE-1 elements of the *TEC1* upstream sequence failed to compete for Cph2 binding, while DNA with either one or both SRE-1-like elements intact efficiently competed with the probe for Cph2 binding (Fig. 7B). Therefore, the Cph2 recombinant protein bound specifically to the SRE-1-like elements (Fig. 7).

To examine whether Cph2 is able to bind to an E-box se-

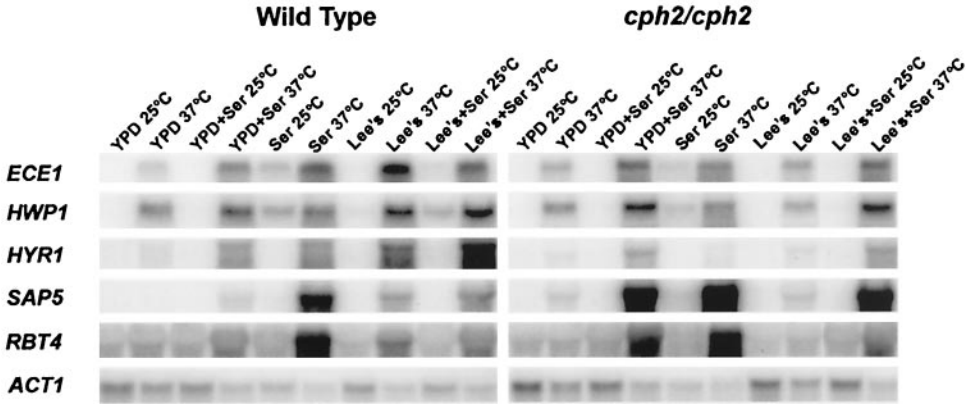

FIG. 5. Impairment in the transcription of hypha-specific genes in the *cph2*/*cph2* mutant. Northern analysis of hypha-specific genes in wild-type (SC5314) and *cph2*/*cph2* (HLY1921) strains is shown. Cells were diluted from overnight cultures into the media indicated and grown for 3 h in the conditions indicated, except for growth in Lee's medium for 6 h. Ser, serum.

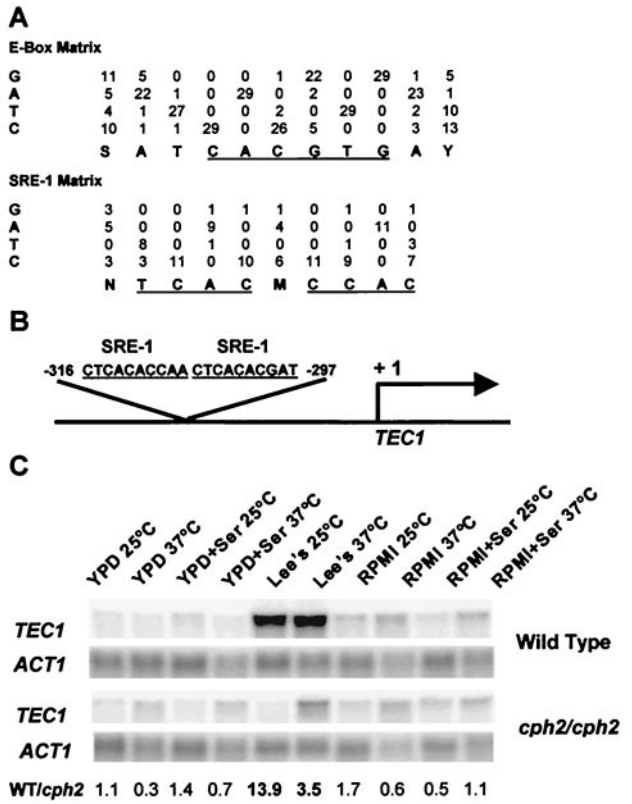

FIG. 6. Cph2 regulates the transcription of *TEC1*. (A) Weight matrices for E-box (20) and SRE-1 (32) motifs were constructed from previously published data. Conserved core sequences are underlined. (B) Positions and sequences of two SRE-1-like motifs in the *TEC1* upstream sequence. (C) Northern analysis of *TEC1* in wild-type (SC5314) and *cph2*/*cph2* (HLY1928) strains. Cells were grown in the conditions and media indicated for 3 h (YPD medium), 5 h (RPMI medium), and 6 h (Lee's medium with succinate). The fold change in *TEC1* expression between wild-type (WT) and *cph2*/*cph2* strains for each condition is indicated below each lane. Ser, serum.

quence, we also performed a gel mobility shift experiment using the Cph2 recombinant protein with a DNA fragment that contains an E-box sequence (20). As shown in Fig. 7A, Cph2 bound to the E-box-containing DNA fragment as well as or even better than it bound to the SRE-1-like elements from *TEC1*. Therefore, Cph2 can bind to both SRE-1 elements and E-box sequences and has the same dual binding capacity as SREBP1.

Ectopic expression of *TEC1* in *cph2*/*cph2* strains suppresses the defect in hyphal development. Although Cph2 is required for the transcriptional induction of *TEC1* in Lee's medium and Cph2 binds directly to the two SRE-1-like elements upstream of *TEC1*, it is not clear whether the function of Cph2 in hyphal development is mediated through the regulation of *TEC1* expression. To test this idea, we placed *TEC1* under the control of the *PCK1* promoter (44) and transformed the construct into wild-type and *cph2*/*cph2* strains. To induce the expression of *TEC1* from the *PCK1* promoter, cells were grown on SSA medium. Wild-type cells produced wrinkled colonies with some filaments around the colonies after 7 days at 37°C (Fig. 8a). *TEC1* overexpression in wild-type cells produced abundant and densely packed fine filaments in each colony (Fig.

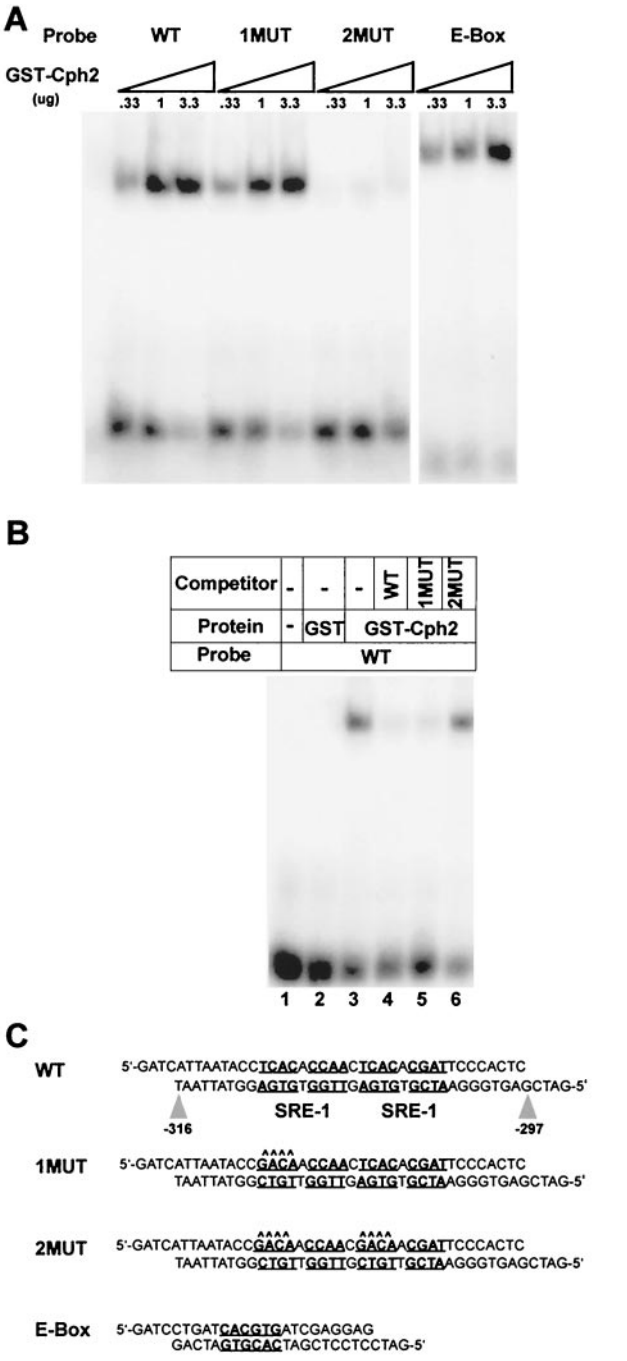

FIG. 7. The bHLH region of Cph2 binds to the SRE-1 site in the *TEC1* upstream sequence as well as an E-box sequence. (A) Analytical gel mobility shift analysis. Increasing concentrations of GST-Cph2 fusion protein were incubated with labeled DNA fragments containing SRE-1-like elements (wild type [WT]) and the corresponding DNAs with one (1MUT) or both (2MUT) SRE-1 sites mutated. Similarly, a labeled DNA probe corresponding to the E-box sequence was used in the titration experiments with recombinant GST-Cph2. (B) Competition binding reactions were carried out with the GST-Cph2 recombinant protein incubated first with unlabeled competitor DNA and then with a labeled SRE-1-containing (WT) probe (lanes 4 to 6). The labeled probe alone was loaded in lane 1. GST and the GST-Cph2 fusion protein were incubated with the probe in lanes 2 and 3, respectively. (C) DNA sequences of the probes and competitors used in the mobility shift analysis. The conserved SRE-1-like sites and E-box motifs are underlined and boldfaced. The mutated residues are indicated by carets.

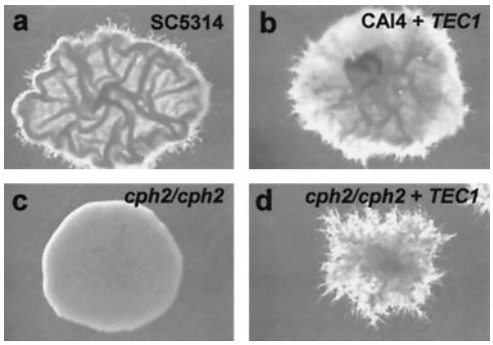

FIG. 8. Ectopic *TEC1* expression suppresses the *cph2*/*cph2* defect in hyphal development. *C. albicans* wild-type strain (SC5314), wild type strain (CAI4) transformed with *PCK1p-TEC1* (HLY3214), *cph2*/*cph2* strain (HLY1928), and *cph2*/*cph2* strain transformed with *PCK1p-TEC1* (HLY3220) (a to d, respectively) were grown on SSA medium for 7 days at 37°C.

8b). The *cph2*/*cph2* strain produced completely smooth yeast colonies (Fig. 8c), and the ectopic expression of *TEC1* in the *cph2*/*cph2* strain suppressed the defect and produced colonies with fine filaments (Fig. 8d). However, the number of filaments was smaller than that seen with the wild-type strain with ectopic *TEC1* expression (Fig. 8d versus Fig. 8b). *TEC1* ectopic expression in wild-type and *cph2*/*cph2* mutant strains showed similar phenotypes when the strains were grown on Lee's medium-succinate, but wild-type colonies were not as filamentous as those grown on SSA plates (data not shown). Our data suggest that *TEC1* ectopic expression can suppress the *cph2*/*cph2* defect in filamentation, a result which suggests that Cph2 regulates hyphal development through the regulation of *TEC1* expression. However, phenotypic differences between wild-type cells and *cph2*/*cph2* cells overexpressing *TEC1* were observed (Fig. 8d versus Fig. 8b). This result indicates that Cph2 may have additional functions in regulating hyphal development that are independent of the regulation of *TEC1* expression. Therefore, we suggest that Cph2 activity in regulating hypha-specific transcription is, in part, mediated through Tec1.

Overexpression of *CPH2* promotes filamentation. Since the overexpression of *CPH2* in *S. cerevisiae* promoted pseudohyphal growth, we expected that the overexpression of *CPH2* in *C. albicans* might also enhance hyphal formation. To test this idea, we placed *CPH2* under the control of the *PCK1* promoter (44) and transformed a *PCK1p-CPH2* construct into a wild-type *C. albicans* strain. As shown in Fig. 9l, the *CPH2* transcript (of the expected size of 0.9 kb) was readily detectable in strains carrying the *PCK1p-CPH2* construct under inducing conditions for the *PCK1* gene. However, *CPH2* expression was not detected by Northern analysis in wild-type cells on Lee's medium with succinate as the carbon source at either 25 or 37°C, nor was it detected under several other hypha-inducing conditions examined, including YPD–10% serum and Lee's medium with mannitol as the carbon source (data not shown). The overexpression of *CPH2* in *C. albicans* induced cell elongation under conditions usually favorable for yeast growth (Fig. 9c versus Fig. 9a). Therefore, Cph2 is a positive regulator of hyphal development in *C. albicans*.

Cph2 functions independently of the Cph1- and Efg1-mediated pathways in *C. albicans*. To address whether Cph2 function is associated with the Cph1- or Efg1-mediated signaling pathway for hyphal development, we performed epistasis studies with *C. albicans*. The overexpression of *CPH1*, *CPH2*, or *EFG1* from the *PCK1* promoter (44) in wild-type cells on SSA medium promoted filamentation with elongated pseudohyphal cells (Fig. 9a to d). We then introduced several of these overexpression constructs into the appropriate *C. albicans* strains mutated in *CPH1*, *CPH2*, or *EFG1* and compared the phenotypes of these strains with those of the original mutant strains. *cph1*/*cph1* mutant strains were unable to undergo hyphal development on solid SSA medium (Fig. 9e). Overexpression of *CPH2* in *cph1*/*cph1* mutant strains could suppress the defect in hyphal formation (Fig. 9f). Similarly, *efg1*/*efg1* single mutants were unable to undergo hyphal development on SS medium, and the defect was partially suppressed by introducing the *PCK1p-CPH2* construct (Fig. 9g and h). *cph2*/*cph2* mutant strains were defective in filamentation on SSA medium (Fig. 9i). The overexpression of either *CPH1* or *EFG1* from the *PCK1* promoter suppressed the defect in hyphal development in *cph2*/*cph2* strains on SSA medium (Fig. 9j and k). Based on the data that *EFG1* overexpression could suppress filamentation in *cph2*/*cph2* strains and, vice versa, that *CPH2* overexpression could promote filamentation in *efg1*/*efg1* strains, we suggest that Cph2 and Efg1 function in two different pathways. Similar reciprocal suppression was observed when *CPH1* was expressed in *cph2*/*cph2* strains and when *CPH2* was expressed in *cph1*/*cph1* strains, suggesting independent functions of Cph1 and Cph2 in regulating hyphal development.

DISCUSSION

In this study, we have identified a new Myc-type bHLH protein, Cph2, in *C. albicans*. Cph2 has a Tyr residue in the basic region at the position that correlates with the atypical Tyr in SREBP1 that is responsible for the dual DNA binding specificity of SREBP1. Like SREBP1, Cph2 also binds to both E-box motifs and non-E-box SRE-1-like elements. Therefore, Cph2 is the second naturally occurring bHLH protein with an atypical Tyr residue in the basic region that has been shown to bind to both classes of DNA motifs.

We have demonstrated that Cph2 plays an important role in hyphal development and in the induction of hypha-specific genes. Although Cph2 is necessary for the induction of all known hypha-specific genes examined, significant percentages of these genes do not have any potential Cph2 binding sites in their upstream sequences. Instead, potential Tec1 binding sites are present in the upstream regions of all known hypha-specific genes and even multiple times in some of them. Furthermore, *TEC1* expression is induced particularly in the medium in which *cph2*/*cph2* hyphal development is impaired. By gel mobility shift experiments with recombinant Cph2, we showed that Cph2 binds specifically to the two SRE-1-like elements upstream of *TEC1*. Therefore, Cph2 may regulate *TEC1* transcription directly by binding to the two SRE-1 elements. Not only does Cph2 regulate *TEC1* transcription but also the ectopic expression of *TEC1* suppresses the defect in *cph2*/*cph2* hyphal development. Together, our data suggest that the function of Cph2 in regulating hyphal development is likely mediated, in part, through Tec1 (Fig. 10). These data provide another example of a transcription factor cascade involved in

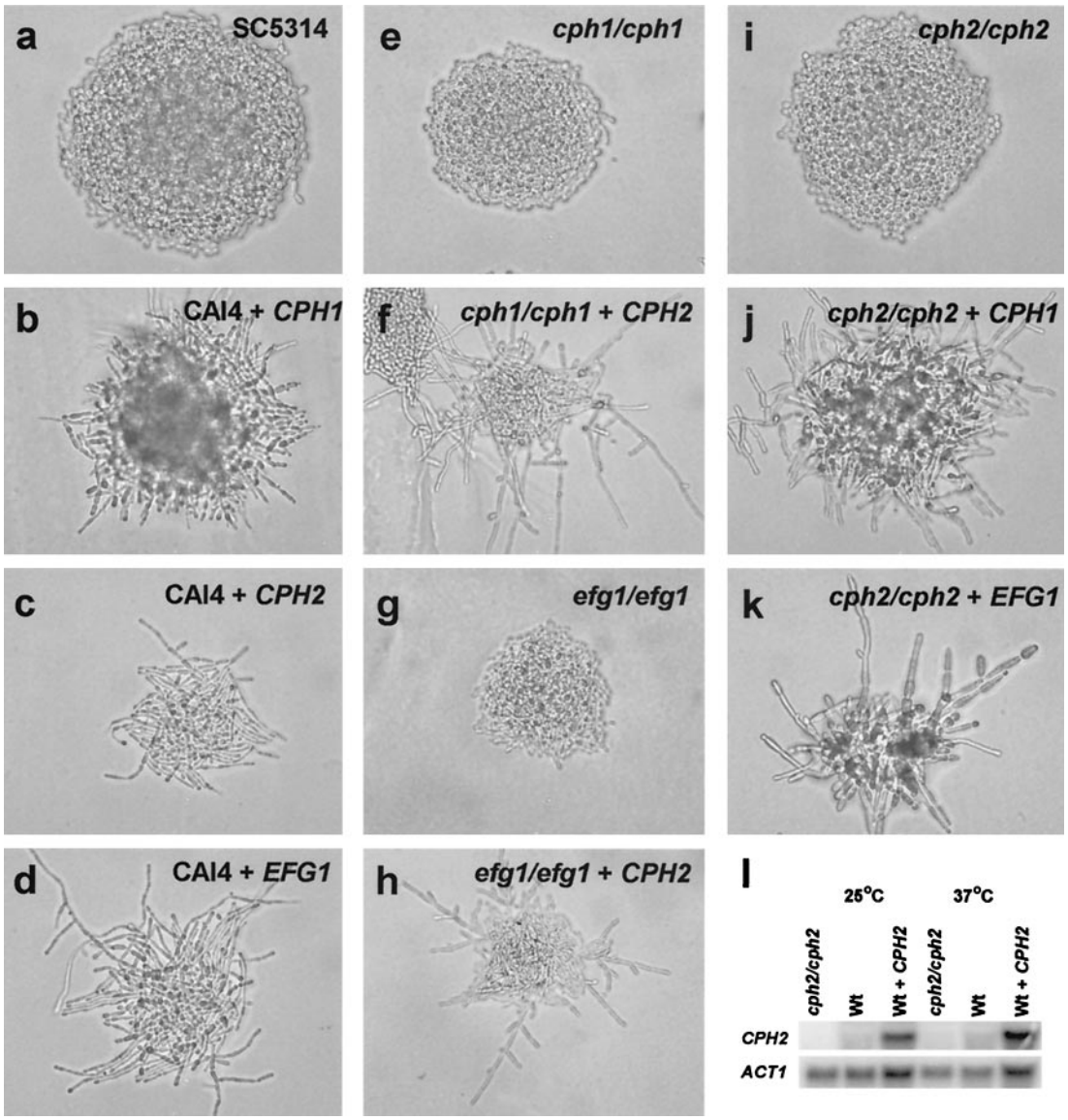

FIG. 9. Functional relationship of Cph2 with Cph1 and Efg1 in *C. albicans*. Colony morphologies of various single and double mutant strains grown on SSA or SS media for 18 h are shown. (a to d) Colonies of a wild-type strain (SC5134) and a wild-type strain (CAI4) transformed with PCK1p-*CPH1* (HLY3119), PCK1p-*CPH2* (HLY3120), and PCK1p-*EFG1* (HLY3125), respectively. (e and f) Colony morphologies of a *cph1*/*cph1* strain (JKC19) and a *cph1*/*cph1* strain transformed with PCK1p-*CPH2* (HLY3138), respectively. (g and h) Colony morphologies of an *efg1*/*efg1* strain (HLC52) and an *efg1*/*efg1* strain transformed with PCK1p-*CPH2* (HLY3183), respectively. (i to k) Colony morphologies of a *cph2*/*cph2* strain (HLY1928) and a *cph2*/*cph2* strain transformed with *PCK1p-CPH1* (HLY3156) and *PCK1p-EFG1* (HLY3164), respectively. Cells in panels g and h were grown on SS medium. Cells in all other panels were grown on SSA medium. (l) Northern blot analysis of *CPH2* overexpression. A *cph2*/*cph2* strain (HLY1928), a wild-type (Wt) strain (SC5314), and a wild-type strain transformed with *PCK1p-CPH2* (HLY3120) were grown on Lee's medium with succinate as the carbon source at 25 or 37°C for 6 h before being harvested for Northern analysis.

regulating cellular differentiation in fungi. Similar transcription factor cascades have been shown for pseudohyphal growth in *S. cerevisiae* (31, 36) and conidiophore formation in *A. nidulans* (33). Interestingly, members of the TEA/ATTS family are the ones being regulated in the transcription factor cascades in all three fungi. However, the upstream regulators are different in each species. The functional relationship between Cph2 and Tec1 that we have discovered in *C. albicans* could not have been deduced by analogy to the regulation of pseudohyphal growth in *S. cerevisiae* or conidiophore formation in *A. nidulans*.

Our data do not exclude the possibility that Cph2 may also play a direct role in the transcriptional activation of some hypha-specific genes that contain SRE-1-like or E-box motifs in their upstream sequences (Table 3). The observation that *TEC1* overexpression in *cph2*/*cph2* cells does not generate the same level of hyphal growth as that seen in wild-type cells indicates that Cph2 may have other functions in addition to regulation of *TEC1* expression. Because some of the hypha-specific genes contain SRE-1-like sequences or E-box motifs in their upstream sequences (Table 3) and we have shown that Cph2 binds to both types of DNA sequences, it is possible that

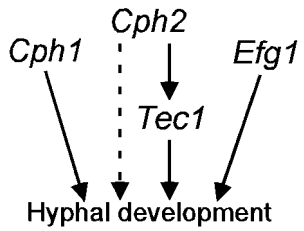

FIG. 10. Proposed functional relationship between Cph2 and other regulators in hyphal development.

Cph2 directly regulates the transcription of these genes. For mammalian cells, Max has been shown to act cooperatively with TEF-1, a mammalian member of the TEA/ATTS family, in regulating the expression of a cardiac α-myosin heavy-chain gene (16). The same cooperative transcriptional activation may exist between Cph2 and Tec1 in *C. albicans*. Interestingly, hypha-specific genes that have one SRE-1-like element or E-box motif in their upstream regions all have only one potential Tec1 binding site, while genes lacking potential Cph2 binding sites tend to have multiple potential Tec1 binding sites (Table 3). Therefore, the second group of genes may be induced by Tec1, which in turn is regulated by Cph2, while the first group of genes may be coordinately regulated by both Cph2 and Tec1 (Fig. 10).

How Cph2 activity is regulated during hyphal development in *C. albicans* is not clear. The Cph2 protein sequence predicts many potential phosphorylation sites for casein kinase II, protein kinase C, and cyclic AMP-dependent protein kinase. Therefore, phosphorylation may play a significant role in regulating its activity. Phosphorylation has been shown to lead to a change in the transcriptional activities of bHLH proteins, as in the case of Max, where the DNA binding activity of Max homodimers is inhibited by its phosphorylation by casein kinase II (3). The state of phosphorylation can also regulate the nuclear localization of bHLH proteins. For example, Pho4 is phosphorylated by the Pho80-Pho85 cyclin-dependent kinase complex and is subsequently exported to the cytoplasm when yeast cells are grown in phosphate-rich conditions (22, 35). Phosphorylation may also lead to a change in protein stability, as in the case of HLF in response to hypoxia (12). In some instances, the transcriptional activation of bHLH proteins is achieved by removal of their inhibitory interaction partners, which are modulated by phosphorylation (17). Determination of whether Cph2 is regulated by any of the known mechanisms requires further experiments.

We have data to suggest that Cph2 functions independently from the Cph1-mediated MAP kinase pathway and the Efg1-transmitted protein kinase A pathway. *CPH2* overexpression is able to partially suppress the defect in hyphal development in *efg1*/*efg1* mutant strains, and *EFG1* overexpression overcomes the defect of filamentation in *cph2*/*cph2* strains. Similarly, reciprocal suppression by *CPH2* and *CPH1* is observed in *cph1*/*cph1* and *cph2*/*cph2* strains, respectively. Furthermore, the medium-specific impairment in hyphal development displayed by each mutant also suggests that Cph1, Cph2, and Efg1 function in independent pathways. Therefore, different signaling pathways may respond to different growth and environmental signals in regulating filamentous growth (Fig. 10). Our data showing how Cph2 functions in hyphal development provide a clear molecular explanation for a mechanism by which *C. albicans* can integrate several different upstream signals into a common downstream output during hyphal development.

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