DNA Array Studies Demonstrate Convergent Regulation of Virulence Factors by Cph1, Cph2, and Efg1 in Candida albicans*

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Candida albicans, normally a human commensal, can cause fatal systemic infections under certain circumstances. Its unique ability to switch from yeast to hyphal growth in response to various environmental signals is inherent to its pathogenicity. Filamentation is regulated by multiple pathways including a Cph1-mediated mitogen-activated protein kinase pathway, an Efg1-mediated CAMPPKA pathway, and a Cph2 pathway. To gain a general picture of how these various signaling pathways regulate differential gene expression during filamentation, we have constructed a partial C. albicans DNA array of 7,000 genes and used it to study the gene expression profiles using various mutants and growth conditions. By combining this novel technology with a new liquid medium in which cph1cph1 is defective in filamentation, previously identified differentially expressed genes (ECE1, HWP1, HYR1, RBT1, SAP65–6, and RBT4) are found to be regulated by all three pathways. In addition, two novel genes, DDR48 and YPL184, have been found to be differentially regulated during hyphal development and by all three pathways. This suggests that distinct filamentation signaling pathways converge to regulate a common set of differentially expressed genes. As one of the mechanisms for the observed convergence, we find that the transcription of a key regulator, TEC1, is regulated by Efg1 and Cph2. Importantly, most of the genes regulated by multiple filamentation pathways encode known virulence factors. Perhaps, C. albicans utilizes converging pathways to regulate its vital virulence factors to ensure its survival and pathogenicity in various host environments.

Candida albicans is a common human commensal often associated with superficial colonization of the mucous epithelium. However, the incidence of fatal C. albicans infections, as well as drug-resistant strains, have increased dramatically in recent years in patients undergoing chemotherapy, transplantation, and in particular, in immunocompromised patients suffering from AIDS (1).

C. albicans exhibits the ability to grow in a variety of reversible morphological forms (yeast, pseudohyphal, and hyphal) in response to various environmental signals (2). The ability of C. albicans to switch its mode of growth has been shown to be required for the pathogenicity of this fungus (3–5). Clearly a better understanding of the differentiation pathways which permit C. albicans to switch between the different morphological forms will reveal more about C. albicans pathogenesis.

Multiple signaling pathways have been found to regulate filamentation. Cph1, a transcription factor homologous to Saccharomyces cerevisiae, Ste12, plays a role in hyphal development on certain solid media in C. albicans (6). As in S. cerevisiae, Cph1 in C. albicans is regulated by a mitogen-activated protein (MAP)1 kinase cascade that includes Csk20, Hst7, and Cek1 (7–9). Mutants with homozygous deletions of the genes encoding these proteins all display a medium-specific defect in hyphal development on certain solid media. Efg1, a basic helix-loop-helix protein similar to Phd1 of S. cerevisiae and StuA of Aspergillus nidulans, plays a major role in regulating hyphal development in C. albicans (3, 10). efg1 efg1 null mutant strains fail to produce hyphae under many conditions, including the presence of serum, which is one of the strongest inducers of hyphal formation (3, 10). Efg1 acts downstream of the CAMPPKA protein kinase A (PKA) signaling pathway (11, 12). The Efg1-mediated CAMPPKA pathway is thought to be distinct from the Cph1-mediated MAP kinase pathway, because the cph1cph1 efg1 efg1 double mutant has a greater defect in hyphal development and virulence than either single mutant (3). Cph1 and Efg1 are the first identified regulators of hyphal development. 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 (13). More recently, Cph2, a basic helix loop helix protein of the Myc subfamily, is found to regulate hyphal development in a medium-specific manner (34). Its activity is mediated, in part, through regulating TEC1 transcriptional induction. Condition-specific hyphal regulators such as Czf1 and Rim101/Prr2 have also been identified in C. albicans (14, 15). Czf1, a potential transcription factor with a zinc finger motif, regulates filamentous growth in response to embedded conditions. Rim101, a transcription factor with a zinc-finger domain similar to PacC of A. nidulans and Rim101 of S. cerevisiae, is involved in a pH-responsive pathway (15–18). Rim101-activated hyphal development requires Efg1 (15). C. albicans also has negative regulators of hyphal development. Tup1, a global transcriptional co-repressor, is required to maintain the organism in yeast form, as the disruption of TUP1 causes the organism to filament under conditions that normally induce it to grow as yeast (19). Tup1-mediated repression

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1 The abbreviations used are: MAP, mitogen-activated protein; SS, synthetic succinate; PCR, polymerase chain reaction; ORF, open reading frame.
of hyphal development is mediated through Rfg1 (20, 21) and Nrg1 (22), but not Cph1 or Efg1 (23).

Several differentially expressed genes have been identified by screening C. albicans libraries with cDNA probes derived from yeast or hyphal cultures (24, 25). These include ECE1, HW1, HYR1, ALS3, RB17, RB47, and ALS8 (24–29). Many of them encode either cell wall or secreted proteins and some have been shown to be important virulence factors for systemic infection. HW1 encodes a glycosylphosphatidylinositol-modified cell wall protein that can serve as a target for mammalian transglutaminases to form covalent attachments between C. albicans and host epithelial cells (30). Rbt1 and Rbt4, a glycosylphosphatidylinositol-modified cell wall protein and a secreted protein similar to plant pathogenesis-related protein (PRY-1), are also necessary for the full virulence of C. albicans in a systemic mouse model. Als3 and Als8 belong to a family of components and mechanism of fungal pathogenesis.

### 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 hyphal inducing media were used: Lee’s medium (36) with 1% mannitol as the carbon source, YPD + 10% serum, SS (synthetic succinate) medium (0.0425% YNB without amino acids and ammonium sulfate (Difco), 0.125% ammonium sulfate, 2% succinic acid, pH 6.5), and SSA (SS with amino acids) medium. A modified lithium acetate method was used for C. albicans transformation (34). Strains HLY3223 were obtained by transforming an ura− strain of HLC52 with the PCK1p-TEC1 plasmid BP2-TEC1 (34). The strain HLY3134 was obtained by transforming an ura− strain of JKCl9 with the PCK1p-CPH1 plasmid BP1-CPH1 (34). The C. albicans strains used in this study are listed in Table I.

#### Generation of a Partial C. albicans Array—We used the Primer 3 program (Whitehead Institute for Biochemical Research) to design PCR primers based on sequence information from the Candida Genome Data base at the Stanford Genome Center. The primers were designed to generate DNA fragments 200–400 base pairs in length with the following settings in Primer 3: mispriming library (human), product size range (200–400), GC clamp (2), optimum primer size (22), minimum primer size (18), maximum primer size (27), optimum T° (65), minimum T° (62), maximum T° (68), maximum complementarity (4), maximum 3° complementarity (2), and maximum poly-X (3). The primer information was collated in a Candida Primer Pairs Data base using the Filemaker Pro software program and sequence information exported directly to the Stanford PAN Facility for the synthesis of primers in a 96-well format (20 nmol scale).
1 µg of *C. albicans* SC5314 genomic DNA was used for each 100-µl PCR reaction with 2.5 units of Biolase (ISC) in 96-well PCR plates (Greiner). The PCR conditions were as follows: 94 °C for 3 min, 8 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, 5 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s. An aliquot of each PCR reaction was run on a Visigel Separation Matrix (Stratagene) to determine the yield of the PCR reaction and to confirm that the product was of the expected size. Approximately 70% of the PCR reactions (from a total of 1,000 primer pairs) were successful on the first attempted amplification. As the *C. albicans* genome is estimated to contain 7,000 open reading frames (ORFs), the PCR products represent roughly 10% of these ORFs. Without additional purification, 20 µl of each PCR product was transferred to 384-well plates and directly glued onto a positively charged nylon membrane in triplicate (Fig. 1), using a Biomek 2000 robotic gliding machine. The gliding of each filter was repeated for a total of three to five overprints, and the membranes were subsequently fixed by UV cross-linking. In addition to the randomly selected ORFs, several controls were also included on the membrane. The previously reported differentially expressed genes, such as *ECE1, HWP1*, and *HYR1*, were included to serve as positive controls. As shown in Fig. 1, many of these are shown to be up-regulated in hyphal versus yeast form, a positive indication of the success of the array. *C. albicans* genomic DNA was also spotted at multiple locations throughout each filter to control for the evenness of hybridization across the filter. Furthermore, pBluescript DNA was used to control for nonspecific hybridization. Finally, a series of PCR products that spanned *TDH3* and *TEF1* (two relatively long, highly expressed *C. albicans* genes) from the 5′ to 3′ end were included to control for transcriptase processivity.

Two sets of array filters were constructed during the course of this study. Differences in the intensity of hybridization for some genes were observed between filters A and B. This was likely due to a difference in the concentration of the PCR reactions used for gliding. Some PCR products were re-made for filter set A and the PCR reactions of several positive controls were diluted in the set A.

**Array Hybridization**—The array filters were pretreated, prehybridized, and washed using the protocol for the Yeast Index GENEFILTERS Microarrays (Research Genetics). They were probed with 32P-labeled cDNA reverse transcribed with oligo(dT) from 1 µg of total RNA extracted using a hot acidic phenol method (Current Protocols in Molecular Biology, 13.12) from different *C. albicans* morphological forms. The arrays were scanned with a PhosphorImager (Molecular Dynamics) (Fig. 1).

Each filter could be stripped in boiling 0.5% SDS (Research Genetics) and re-probed up to 7 times without a significant loss of signal. As shown in Table 1, filter A1 was hybridized, sequentially, with wild type, *cph1/cph1, cph2/cph2*, and *efg1/efg1* strains that were grown in YPD at 25 °C. Similarly, filter A2 was probed in succession with the above four strains grown in Lee’s medium at 25 °C. Likewise, filter A3 was prehybridized with cells from YPD filter at 37 °C, and filter A4 with cells in Lee’s medium at 37 °C. Additional hybridizations were performed on filters A2 and A4 with the *cph1/cph1* and *cph1/cph1* + *PCR1* strains grown in SS medium at 25 and 37 °C, respectively. Filters B5 and B6 were sequentially hybridized with the wild type strain in yeast, hyphal, and the *cph1/cph1 efg1/efg1* strain in hyphal growth media (Table I). The same filter was used for each of the set of compared experiments to avoid any variation among filters.

**Array Data Analysis**—The images from the PhosphorImager for each array experiment were analyzed for initial data quantification using ArrayVision software (Imaging Research, Inc.). The background subtracted intensity of each spot on the array was calculated by taking the average intensity of the area surrounding each of 9 spots and subtracting it from the intensity value of each spot within the group. The data were subsequently exported to Excel where the values for each of the three spots corresponding to a particular gene were averaged and divided by the sum of the values for every gene spotted on the array (not including controls) to get an average % intensity value for each gene. Lowly expressed genes, with an average % intensity value less than or close to that of pBluescript in all hybridizations, were eliminated from further data analysis. Also, mRNA levels of the control gene DNA, *TDH3*, *TEF1*, *rDNA*, (pBluescript). The ratio value using the average % intensity values for each gene from a pair of hybridizations was calculated. As a further measure, we visually checked the array images to eliminate false positives caused by a flaw on the array filters. The remaining gene ratio values from 16 pairs of comparisons were used for the clustering analysis (using software available at ranastanford.edu). The results were viewed using the TreeView program. Several genes were clustered with the positive controls of differentially regulated genes, but were found to be false positives by Northern analyses (data not shown). These false positive genes usually appeared in only one batch of the arrays (either A or B), and were all in close proximity to the highly expressed control genes in a 384-well plate; and were removed from the final data set. The remaining 657 genes were used in the clustering analysis (Fig. 2).

**Northern Analysis**—Methods for RNA isolation and Northern blot hybridization were as described (Current Protocols in Molecular Biology, 13.12). A ClaI-SalI ACT1 fragment from the plasmid p15953 (37) was used as the probe for ACT1 for Northern blots. PCR products of ECE1, HWP1, HYR1, SAP4-6, ALS3, RBT4, EBP1, WH11, ECE99, HSP12, TEC1, and DDR48 were used for probing Northern blots. The oligos used for PCR are listed in Table III. The sizes of the mRNAs on the Northern blots were compared with the expected lengths based on information from the Stanford *C. albicans* Genome Data Base.

**Promoter Sequence Analysis**—1-Kilobase upstream sequences were used for computational search of conserved binding sequences as described (34).

## RESULTS

**Cph1, Cph2, and Efg1 Regulate the Same Set of Differentially Expressed Genes**—A *cph1/cph1 efg1/efg1* double mutant is avirulent in a mouse systemic infection model, whereas the single mutants, *cph1/cph1* and *efg1/efg1*, are virulent (5). Two models can explain how Cph1 and Efg1 can exhibit this synergy in regulating virulence genes. One is a divergence model, which proposes that the two pathways have their own downstream genes. In the divergence model, Cph1 and Efg1 are predicted to regulate different virulence genes. Another is a convergence model, which proposes that a common set of virulence genes are regulated coordinately by both Cph1 and Efg1 pathways. Therefore, the deletion of one pathway is not sufficient to block the expression of virulence genes in *vivo*. It is also possible that the above two models exist. In this case, these pathways regulate the transcription of an overlapping set of genes, while each pathway can also activate its pathway-specific targets.

To develop an overall picture of how genes are regulated during hyphal development, we used the partial *C. albicans* DNA array to study differences in gene expression between wild type and the signaling mutants *cph1/cph1, cph2/cph2*, and *efg1/efg1* strains that were grown in YPD at 25 °C. Similarly, filter A2 was probed in succession with the above four strains grown in Lee’s medium at 25 °C. Likewise, filter A3 was prehybridized with cells from YPD filter at 37 °C, and filter A4 with cells in Lee’s medium at 37 °C. Additional hybridizations were performed on filters A2 and A4 with the *cph1/cph1* and *cph1/cph1 + PCR1* strains grown in SS medium at 25 and 37 °C, respectively. Filters B5 and B6 were sequentially hybridized with the wild type strain in yeast, hyphal, and the *cph1/cph1 efg1/efg1* strain in hyphal growth media (Table I). The same filter was used for each of the set of compared experiments to avoid any variation among filters.

| Gene | Oligo |
|------|-------|
| ECE1 | 5′-TGGCAACTTCCACAAATGCC |
| HWP1 | 5′-TGCTCAGGCTGACTGGCC |
| HYR1 | 5′-CTTACAAGGGTGCTGACCC |
| SAP4 | 5′-GACGGGAGGAAATACGAC |
| SAP5 | 5′-TTAAGAAAGGAAATACGAC |
| ALS3 | 5′-ATGTTCTCGGCGGTATCGCC |
| YJL79 | 5′-AAGTGGAGAAGATCTGAG |
| EBP1 | 5′-ATGCATGAGAAGATCTGAG |
| ECE99 | 5′-TCCCAAGAAGAATACGAC |
| HSP12 | 5′-GACGGGAGGAAATACGAC |
| TEC1 | 5′-ACATGAGAAGATCTGAG |
| DDR48 | 5′-AACCTGAGAAGATCTGAG |
| YPL184 | 5′-CCCCAAATAACATGTCGAC |
| SAP6 | 5′-ATTCCGGAGGAAATACGAC |
| SAP6 | 5′-ATTCCGGAGGAAATACGAC |
| SAP6 | 5′-ATTCCGGAGGAAATACGAC |
| SAP6 | 5′-ATTCCGGAGGAAATACGAC |
Gene Expression during Hyphal Development in C. albicans

4 and 5 are comparisons between wild type and cph1/cph1 or cph2/cph2 strains in YPD + serum at 37 °C, in which the mutants formed hyphal filaments indistinguishable from those of wild type. Interestingly, they were grouped among comparisons between yeast-form cells by the program. This indicates that Cph1 and Cph2 do not play any roles in gene transcription under this condition, which is consistent with their lack of a morphological defect. Columns 9 through 16 are comparisons of wild type with single or double mutants under hyphal growth conditions, or a comparison of wild type cells in hyphal versus yeast growth conditions. Except column 10 where cph1/cph1 cells formed filaments, all other columns are comparison of gene expression between hyphal and yeast cells. It is intriguing that the comparison between wild type and cph1/cph1 was grouped among comparisons of hyphal versus yeast, rather than grouped with columns 4 and 5. Overall, the expression profiles for most genes did not show dramatic changes during filamentation under the two hyphal inducing conditions. Only three small clusters of genes showed significant changes in expression level.

The first cluster represents genes whose expression was highly induced in filamentous cells (Fig. 2B, a, columns 9–16). They were induced in both types of hyphal inducing media. Their induction was blocked in mutants that did not form hyphae such as efg1/efg1 mutants in both YPD + serum and Lee’s media and cph2/cph2 mutants in Lee’s medium. This group of genes includes all known differentially expressed genes seeded on the array (ECE1, HWP1, and HYR1). In addition, two new genes, YJL79 and ECE99, clustered together with the known genes. Recently, YJL79 and ECE99 have been shown to be RBT4 and RBT1, respectively (25). The expression patterns of these genes were further confirmed by Northern analysis (Fig. 3), and are consistent with the published results (23).

The second group includes SAP5 and SAP6. Although they were also highly induced under both hyphal inducing conditions, and the induction required Efg1 and Cph2 (Fig. 2B, panel b, and Fig. 3), they were not on the same branch of the dendrogram generated during the data clustering. This may be due to the fact that their expression was repressed by Cph2 in YPD + serum at 37 °C.

The third cluster includes two genes, WH11 and HSP12 (a small heat shock protein) (Fig. 2B, panel c). WH11 is a white-specific gene identified previously by using a strain capable of white-opaque switching (39). It encodes a protein with 48% identity to Hsp12, and therefore, is likely to have a function that is similar to Hsp12. Both genes were expressed only in Lee’s medium at 25 °C, and the expression requires Efg1. This is consistent with the finding that Efg1 was involved in the regulation of phase switching in C. albicans (40). Interestingly, Cph1 and Cph2 showed some repressive effects on their expression in Lee’s medium at 37 °C (Fig. 3).

Although EBP1 (originally isolated as an estrogen-binding protein (41)) also clustered with hypha-specific genes in the array experiments, further analysis of its expression by Northern blotting showed that it was induced only in YPD + serum at 37 °C, and its induction was dependent on Efg1 (Fig. 3). We suspect that the signal intensity of the EBP1 spots on our array was falsely elevated due to the proximity of the sample to that of the neighboring gene tag for HWP1 on the 384-well plate used for constructing the array (Fig. 1).

The comparisons of wild type to the cph1/cph1 mutant in Lee’s medium at 37 °C (Fig. 2B, column 10) clustered together with other comparisons of hyphal versus yeast cells, no obvious reduction in the level of differentially expressed genes was observed in the cph1/cph1 strains under both induc-
ing conditions by Northern blotting (Fig. 3), which is consistent with its lack of a defect in hyphal development in these liquid media. One explanation for this lack of a phenotype could be that Cph1 was not activated under the in vitro hyphal inducing conditions examined. However, this pathway is active under certain in vivo conditions in mice, as the cph1/cph1 efg1/efg1 mutant is much less virulent than the efg1/efg1 mutant in a systemic infection model (3). To address this possibility, we searched for liquid media in which cph1/cph1 mutant strains exhibited a defect in filamentation. In particular, SS medium...

**Fig. 2.** Cluster analysis reveals groups of highly regulated genes during hyphal development. 637 genes (see “Materials and Methods”) on the partial C. albicans array were clustered based on their expression patterns in 16 comparisons that followed the yeast-to-hyphal transition in wild type and mutants of three filamentation signaling pathways, as indicated in panel B. Columns 1–3 are comparisons of gene expression between wild type (SC5314) and cph1/cph1 (JKC19), cph2/cph2 (HLY1921), or efg1/efg1 (HLC52) strains grown in Lee’s medium at 25 °C, obtained from sequential hybridizations on filter A2 (Table II). Similarly, columns 4, 5, and 12 are comparisons of the same strains grown in YPD + serum at 37 °C (Filter A3). Likewise, columns 6–8 are comparisons of the strains grown in YPD at 25 °C (Filter A1), and columns 9–11 are comparisons of the same strains grown in Lee’s medium at 37 °C (Filter A2), respectively. Columns 13 and 14 are comparisons of wild type (SC5314) with the cph1/cph1 efg1/efg1 (HLC52) mutant grown in YPD + serum (column 13) and Lee’s medium at 37 °C (column 14) in the same conditions, respectively. Columns 15 and 16 are comparisons of wild type (SC5314) grown in YPD + serum at 37 °C versus YPD at 25 °C, and Lee’s medium at 37 °C versus at 25 °C. Each column of colored boxes represents the variation in transcript level of every gene in a given pair of RNA samples on the array, and each row represents the variation in transcript abundance for each gene. The variations in transcript abundance are depicted by means of a color scale, in which shades of red represent increases and shades of green represent decreases in transcript level (a color scale is indicated). A black color indicates an undetectable change in transcript level. Based on the dendrogram constructed during the clustering program (not shown), three different clusters of genes, as indicated in panel A, were found to be co-regulated by multiple mutants. The patterns of gene expression in the 16 pairs of compared experiments are shown in panel B, a-c. a, hypha-specific cluster. Gene expression was highly induced in hyphal versus yeast form, and in wild type versus mutant strains in YPD + serum and Lee’s medium at 37 °C. Genes encoding virulence factors are marked with an asterisk. b, SAP cluster. Genes in this cluster were not only up-regulated in hyphal versus yeast form, and in wild type versus mutant strains, but also were repressed by Cph2 in YPD + serum at 37 °C. SAP4 also showed a similar pattern of gene expression as SAPs 5,6, although it was not clustered into the same group as SAP 5.6, c, WH11 cluster. Genes in this cluster were repressed in Lee’s medium, and their transcription required Efg1. Differential gene expression was also observed with ALS genes, as indicated in panel A. However, our PCR tags do not distinguish between different ALS genes. By Northern analysis, the differential expression of CTR1 was found to be regulated in response to extracellular copper concentrations (data not shown), but not by filamentation pathways. Growth media are indicated as YPD (Y), YPD + serum (S), and Lee’s medium (L).
allowed the hyphal development of wild type cells, while it did not induce filamentation in the cph1/cph1 mutant cells in liquid medium (Fig. 4A). The carbon source in the media was varied between the different genes. For example, CPH1 overexpression activated the expression of YJL79 dramatically at 25°C, but did not affect the expression of ECE1 or HYR1 (Fig. 4B). This may be a reflection of whether Cph1 has direct interaction with the Dig1 and Dig2 proteins that interact with the Dig1 and Dig2 proteins.

The partial C. albicans array was then used to compare the gene expression profiles of the cph1/cph1 mutant strain with a cph1/cph1 strain carrying the CPH1 overexpression construct, both grown in SS medium. Analysis of the array data indicated that most of the changes in gene expression were limited to the same set of differentially expressed genes identified by clustering analysis in Fig. 2. Northern blotting of their expression in cph1/cph1, wild type, and CPH1 overexpression strains is shown in Fig. 4B. All of the genes had reduced expression in the cph1/cph1 mutant versus wild type. Therefore, Cph1 regulates the expression of the same set of genes as Cph2 and Efg1. The extent of the transcriptional activation with CPH1 overexpression varied between the different genes. For example, CPH1 overexpression activated the expression of YJL79 dramatically at 25°C, but did not affect the expression of ECE1 or HYR1 (Fig. 4B). This may be a reflection of whether Cph1 has direct binding sites upstream of the hypha-specific genes, as conserved Ste12 binding motifs are found upstream of HYR1 and ECE1, but not upstream of HYR1 and ECE1 by DNA sequence analysis (Table IV).

Two novel genes, DDR48 and YPL184, were identified as being regulated by Cph1 from the array experiments by comparing the cph1/cph1 strain with the strain carrying a CPH1 overexpression construct (Fig. 4C). DDR48 of S. cerevisiae encodes a stress protein of unknown function. The YPL184 protein product is similar to Nrd1 (negative regulator of differentiation-conjugation) of S. pombe. The expression level of YPL184 is much lower than that of previously identified differentially regulated genes, and would not have been identified with the traditional method of screening cDNA libraries. Interestingly, as with other differentially expressed genes, DDR48 and YPL184 were also regulated by Efg1 and Cph2 (Fig. 4C).

From array studies and Northern analyses, we have found differences in filamentation between wild type and cph1/cph1 strains, but succinate produced a visible difference. The addition of amino acids to SS medium increased filamentation in both wild type and cph1/cph1 strains. In addition to testing different media, we also created C. albicans strains with an activated Cph1 pathway. This was achieved by overexpressing CPH1 from the PCK1 promoter (34). The CPH1 transcript was detectable by Northern blotting only in strains carrying PCK1p-CPH1, not in wild type (data not shown). CPH1 overexpression led to pseudohyphal growth (filamentation) under conditions that favor yeast growth (34). The mechanism for Cph1 activation by overexpression is likely to be analogous to Ste12 regulation in S. cerevisiae (42, 43). During pseudohyphal growth activation of the filamentation MAP kinase pathway, Kss1 releases the inhibition of Dig1 and -2 on Ste12. A similar effect can be obtained by overproducing the Ste12 protein, or overproducing the Ste2 mutant domain with the Dig1 and Dig2 proteins (44, 45).

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From array studies and Northern analyses, we have found
that Cph1, Cph2, and Efg1 regulate the expression of a common set of differentially expressed genes, including YJL79, HYR1, ECE1, HWP1, ECE99. SAPs5,6, WH11, HSP12, DDR48, and YPL184. The expression patterns of most of them are associated with hyphal morphogenesis, rather than with growth conditions (therefore, defined hypha-specific genes). The expression of some of them, however, did not show a strict correlation with cell morphogenesis. For example, ECE99 was expressed in Lee’s medium at 25 °C. Similarly, DDR48 also showed some expression under the same condition. Interestingly, ECE99 expression was derepressed in cph1/cph1 mutants. It is possible that a combination of repression and activation from different pathways can lead to a medium-dependent pattern of expression. The expression of WH11 and HSP12 can be viewed as two such examples to an extreme (Fig. 3). They were best expressed in Lee’s medium at room temperature, and Cph1 and Cph2 were inhibitory to their expression in Lee’s medium at 37 °C. In addition to genes affected by all pathways, we also identified a pathway-specific gene, 

| TABLE IV |
| --- |
| Computational search of potential Cph1-, Efg1-, and Tec1-binding sites |
| Ste12 and AbaA consensus sequences and E-box sequence were used to search for potential Cph1-, Tec1-, and Efg1-binding sites in the 1-kb 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 1-kb upstream sequences of 1000 randomly chosen genes by the number of hypha-specific genes. |
| **Cph1 sites (Ste2)** | **Efg1 sites CANNTG** | **Tec1 sites (AbA)** |
| **ECE1** | CAATTG (−764, −759) | TCATTCT (−371, −365) |
| TGAACA (−271, −265) | CATCTG (−902, −897) | TCATTCT (−153, −147) |
| CAACTG (−569, −584) | CAGCTG (−309, −304) | |
| CACCTG (−219, −214) | |
| **HWP1** | CAAATG (−455, −450) | GCATTCT (−840, −830) |
| TGAACA (−971, −965) | CATTG (−314, −309) | TCATTCT (−513, −519) |
| CAGTTG (−237, −232) | CATTG (−53, −48) | TCATTCT (−32, −26) |
| CAAATG (−53, −48) | |
| **HYR1** | |
| CAAATG (−53, −48) | |
| **RBT4** | CAAATG (−53, −48) | |
| TGAACA (−971, −965) | CAACTG (−341, −336) | |
| CAACTG (−218, −213) | |
| CAACTG (−156, −151) | |
| **SAP5** | CAAATG (−537, −532) | TCATTCC (−91, −85) |
| TGAACA (−971, −965) | CAAATG (−253, −248) | TCATTCC (−66, −60) |
| CAAATG (−253, −248) | |
| **SAP6** | |
| TGAACA (−822, −816) | CAAATG (−252, −247) | |
| TGAACA (−753, −746) | |
| **ALS3** | CAAATG (−951, −946) | ACATTCC (−860, −850) |
| CATTG (−123, −118) | TCATTCT (−808, −802) |
| CAACTG (−16, −11) | |
| Total no. of potential sites | 5 | 25 | 17 |
| Expected random occurrence | 0.46 × 7 = 3.22 | 3.65 × 7 = 25.6 | 0.91 × 7 = 6.37 |

Gene Expression during Hyphal Development in C. albicans

TEC1 Expression Is Regulated by Efg1 and Cph2—Previously, we have shown that Cph2 directly regulates the induction of TEC1 transcription in Lee’s medium and that the function of Cph2 in filamentation is mediated, in part, through Tc1 (34). However, TEC1 expression is not abolished in the cph2/cph2 mutant, suggesting additional regulation of TEC1. Therefore, TEC1 transcription represents a potential convergence point for regulating the expression of differentially expressed genes.

The fact that AbaA expression is under the direct control of StuA in A. nidulans (36), and that AbaA/Tec1-binding sites are found in the upstream regions of all known hypha-specific and serum-induced genes (Table IV), led us to investigate the possibility that Efg1 may regulate TEC1 transcription. We examined TEC1 expression in efg1/efg1 mutants by Northern analysis. As shown in Fig. 5A, the TEC1 transcript level was diminished in the efg1/efg1 mutant under all growth conditions examined. In contrast, the cph1/cph1 mutant did not affect TEC1 expression in SS medium (data not shown). This, together with the fact that EFG1 expression was unchanged in

that Cph1, Cph2, and Efg1 regulate the expression of a common set of differentially expressed genes, including YJL79, HYR1, ECE1, HWP1, ECE99. SAPs5,6, WH11, HSP12, DDR48, and YPL184. The expression patterns of most of them are associated with hyphal morphogenesis, rather than with growth conditions (therefore, defined hypha-specific genes). The expression of some of them, however, did not show a strict correlation with cell morphogenesis. For example, ECE99 was expressed in Lee’s medium at 25 °C. Similarly, DDR48 also showed some expression under the same condition. Interestingly, ECE99 expression was derepressed in cph1/cph1 mutants. It is possible that a combination of repression and activation from different pathways can lead to a medium-dependent pattern of expression. The expression of WH11 and HSP12 can be viewed as two such examples to an extreme (Fig. 3). They were best expressed in Lee’s medium at room temperature, and Cph1 and Cph2 were inhibitory to their expression in Lee’s medium at 37 °C. In addition to genes affected by all pathways, we also identified a pathway-specific gene, 

| TABLE IV |
| --- |
| Computational search of potential Cph1-, Efg1-, and Tec1-binding sites |
| Ste12 and AbaA consensus sequences and E-box sequence were used to search for potential Cph1-, Tec1-, and Efg1-binding sites in the 1-kb 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 1-kb upstream sequences of 1000 randomly chosen genes by the number of hypha-specific genes. |
| **Cph1 sites (Ste2)** | **Efg1 sites CANNTG** | **Tec1 sites (AbA)** |
| **ECE1** | CAATTG (−764, −759) | TCATTCT (−371, −365) |
| TGAACA (−271, −265) | CATCTG (−902, −897) | TCATTCT (−153, −147) |
| CAACTG (−569, −584) | CAGCTG (−309, −304) | |
| CACCTG (−219, −214) | |
| **HWP1** | CAAATG (−455, −450) | GCATTCT (−840, −830) |
| TGAACA (−971, −965) | CATTG (−314, −309) | TCATTCT (−513, −519) |
| CAGTTG (−237, −232) | CATTG (−53, −48) | TCATTCT (−32, −26) |
| CAAATG (−53, −48) | |
| **HYR1** | |
| CAAATG (−53, −48) | |
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Convergent Regulation of Virulence Genes by Multiple Filamentation Signaling Pathways: An in Vitro Model for Candidal Virulence in Vivo—The virulence factors examined so far are all regulated by multiple filamentation signaling pathways. We have discovered that Efg1, Cph2, and Cph1 all regulate the expression of hypha-specific genes in C. albicans. Combinatorial control not only allows cells to integrate signals from two pathways, but can also increase the specificity of the target genes. This may explain why the DNA binding sequences for many of the hyphal regulators are not highly overrepresented in the promoters of hypha-specific genes. As shown in Table IV, the frequencies of E-box sequences, which have been shown to be recognized by Efg1 (29), are similar between upstream regions of hypha-specific genes and 1000 randomly chosen genes. Another potential level of convergence is through complicated pathway cross-talk. The fact that more genes regulated by multiple pathways have been identified than pathway-specific genes implies the existence of cross-talk between different pathways.

Convergent Regulation of Virulence Genes by Multiple Filamentation Signaling Pathways: An in Vitro Model for Candidal Virulence in Vivo—The virulence factors examined so far are all regulated by multiple filamentation signaling pathways. We have discovered that Efg1, Cph2, and Cph1 all regulate the expression of SAP5 and SAP6, which may be important for the organism to invade and damage the oral epithelium, and are present at sites of oropharyngeal candidiasis in humans (49). The filamentation signaling pathways examined so far all regulate the expression of HWPI, whose product is important for specific genes, and their expression is likely medium-dependent. This model is in agreement with the “network control” model from Braun and Johnson (23), where they proposed that, rather than using a central processor, which integrates signals from different pathways to regulate all differentially expressed genes, C. albicans uses a network of connections between regulatory pathways and downstream genes. But unlike the network control model, which stresses the differences in genes regulated by each pathway and suggests that distinct types of filaments and genes are induced in response to different inducing conditions, our model emphasizes convergent regulation by multiple signaling pathways.

The discovery of a liquid medium in which the cph1/cph1 mutant displays a defect in hyphal formation is a key to establishing the convergence model. Several previous reports have failed to identify the effects of cph1/cph1 mutants on the transcription of the differentially regulated genes in other hyphal inducing media (23, 33), and therefore, have concluded that Cph1 regulates genes that are different from those regulated by Efg1. By using an appropriate medium for the cph1/cph1 and CPH1 overexpression strains, we have shown that Cph1 regulates the same differentially regulated genes that are under the control of Efg1.

Potential Mechanisms for the Observed Convergence in Transcriptional Regulation by Multiple Signaling Pathways—We show TEC1 expression is regulated by Efg1 and Cph2. Thus, the regulation of TEC1 transcription provides one example of how C. albicans cells can integrate two different upstream signals into a single downstream output. Another logical convergence point is at the promoters of the regulated genes, analogous to the convergent regulation of FLO11 transcription by the MAP kinase pathway and cAMP pathway in S. cerevisiae (46). In addition, synergistic activation by cooperative interaction between hyphal regulators of different pathways at the promoters of hypha-specific genes may happen during filamentation since many of the hypha-specific genes are induced over 100-fold during filamentation. Cooperative transcriptional regulation has been reported between Ste12 and Tec1 in regulating filamentation responsive elements in S. cerevisiae (47), as well as between Max and TEF-1, a mammalian member of the TEA/ATTS transcription family, in activating MCAT1 elements of muscle-specific genes (48). By homology, Cph1 and Tec1, or Cph2 and Tec1 may also act cooperatively to regulate hypha-specific genes in C. albicans. Combinatorial control not only allows cells to integrate signals from two pathways, but can also increase the specificity of the target genes. This may explain why the DNA binding sequences for many of the hyphal regulators are not highly overrepresented in the promoters of hypha-specific genes. As shown in Table IV, the frequencies of E-box sequences, which have been shown to be recognized by Efg1 (29), are similar between upstream regions of hypha-specific genes and 1000 randomly chosen genes. Another potential level of convergence is through complicated pathway cross-talk. The fact that more genes regulated by multiple pathways have been identified than pathway-specific genes implies the existence of cross-talk between different pathways.
the adherence of *C. albicans* cells to oral epithelial cells (30). All pathways that were analyzed also affected the transcription of *YJL79* and *ECE99* (also known as *RBT4* and *RBT1*), which are important for the virulence of *C. albicans* in two animal models examined (25). Interestingly, other Tup1-repressed genes are not regulated by Efg1 and Cph1, and they are not required for virulence either. Therefore, many genes that are regulated by multiple filamentation signaling pathways are likely to encode important virulence factors. This pattern of transcriptional regulation may have important implications for how *C. albicans* cells can thrive in the varied microenvironments of the host. They may have implemented a convergent regulatory system to ensure that the genes whose products enable the organism to invade and injure host cells in specific microenvironments of the host during specific phases of infection are expressed at appropriate levels. In bacterial pathogens, such as *Salmonella* and *Listeria*, the expression of many key virulence factors is known to be tightly regulated (50–52), and they are only expressed in certain host cells under specific conditions. It is possible that *C. albicans* controls the expression of its most important virulence factors with multiple signaling pathways to ensure its survival in a variety of host environments.

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DNA Array Studies Demonstrate Convergent Regulation of Virulence Factors by Cph1, Cph2, and Efg1 in *Candida albicans*

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