Roles of the *Candida albicans* Mitogen-Activated Protein Kinase Homolog, Cek1p, in Hyphal Development and Systemic Candidiasis†

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Extracellular signal-regulated protein kinase (ERK, or mitogen-activated protein kinase [MAPK]) regulatory cascades in fungi turn on transcription factors that control developmental processes, stress responses, and cell wall integrity. *CEK1* encodes a *Candida albicans* MAPK homolog (Cek1p), isolated by its ability to interfere with the *Saccharomyces cerevisiae* MAPK mating pathway. *C. albicans* cells with a deletion of the *CEK1* gene are defective in shifting from a unicellular budding colonial growth mode to an agar-invasive hyphal growth mode when nutrients become limiting on solid medium with mannitol as a carbon source or on glucose when nitrogen is severely limited. The same phenotype is seen in *C. albicans* mutants in which the homologs (*CST20*, *HST7*, and *CPH1*) of the *S. cerevisiae STE20*, *STE7*, and *STE12* genes are disrupted. In *S. cerevisiae*, the products of these genes function as part of a MAPK cascade required for mating and invasiveness of haploid cells and for pseudohyphal development of diploid cells. Epistasis studies revealed that the *C. albicans CST20*, *HST7*, *CEK1*, and *CPH1* gene products lie in an equivalent, canonical, MAPK cascade. While Cek1p acts as part of the MAPK cascade involved in starvation-specific hyphal development, it may also play independent roles in *C. albicans*. In contrast to disruptions of the *HST7* and *CPH1* genes, disruption of the *CEK1* gene adversely affects the growth of serum-induced mycelial colonies and attenuates virulence in a mouse model for systemic candidiasis.

Candida albicans, an opportunistic fungal pathogen, is the major causative agent of thrush and other forms of candidiasis. Diploid *C. albicans* alternates between a yeast form and mycelial and pseudomycelial forms but does not have a sexual cycle. Physiological temperatures, pH, and serum can promote the emergence of true hyphae from yeast cells in vitro, yet both these forms, as well as pseudohyphae, may be found in infected tissues (for a review, see reference 32). The roles of these different morphologies in pathogenesis have been controversial, but recently, hyphal differentiation has been found to be linked to systemic virulence (22, 26) and the ability of *C. albicans* cells to evade macrophages (26). Filamentous forms are also better than yeast forms at invading epithelial cells (7) and agar surfaces in vitro (5, 12, 34). This may be the result of both the mechanical advantages of hyphal forms in the penetration of solid substrates (11) and the production of hypha-specific hydrolytic enzymes such as some of the secreted aspartyl proteinases which also appear to contribute to virulence (14, 40).

Baker's yeast, *Saccharomyces cerevisiae*, is also able to switch to a pseudohyphal growth mode, as a possible nutrient foraging mechanism (10). *S. cerevisiae* diploids, when exposed to severe nitrogen limitation, start to grow as chains of attached elongated cells which invade solid surfaces, rather than growing as individual budding cells. In response to nutrient starvation, *S. cerevisiae* haploid cells also start to grow invasively, but in a more random direction than diploid cells (35). Nutritional limitation of *C. albicans* (24) triggers the development of true parallel-sided hyphae as well as pseudohyphae; the latter are distinguished by conspicuous constrictions present at pseudohyphal cell-cell junctions (31). Nutrient starvation is a potential environmental signal for *C. albicans* in different microenvironments including the spleen and liver (41), but serum is the best inducer of the true parallel-sided hyphal form (12).

In both *S. cerevisiae* and *C. albicans*, environmental changes induce filamentous growth through at least two parallel signal transduction pathways (17, 26). Cells with homozygous deletions of both of two independently regulated putative transcriptional activators (called Ste12p and Phd1p in *S. cerevisiae* and Cph1p and Efg1p in *C. albicans*) are locked in the yeast phase and cannot grow filamentously (26), but cells with deletions of either of these transcriptional regulators demonstrate variable defects in filament development (24, 26, 42). In addition, *C. albicans cph1 efg1* double null mutants are avirulent, but mutants with a deletion of either gene alone are virulent (26).

Little is known about the regulation of Phd1p in *S. cerevisiae*, but a regulatory kinase cascade which activates the Ste12p transcription factor functions not only during pseudohyphal

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TABLE 1. Yeast strains used in this study

C. albicans strain	Relevant genotype	Source or reference
SC5314	Clinical isolate; Ura⁺ parent of CAI4	9
CAI4	ura3/ura3	8
CK43A	ura3/ura3 CEK1/cek1Δ::hisG-URA3-hisG	This study
CK43AL	ura3/ura3 CEK1/cek1Δ::hisG	This study
CK43A-RI	ura3/ura3 CEK1/cek1Δ::hisG::CEK1-URA3	This study
CK43B-16	ura3/ura3 cek1Δ::hisG-URA3-hisG/cek1Δ::hisG	This study
CK43B-16L	ura3/ura3 cek1Δ::hisG/cek1Δ::hisG	This study
CK43B-RI	ura3/ura3 cek1Δ::hisG/cek1Δ::hisG::CEK1-URA3	This study
CK43B-4	ura3/ura3 cek1Δ::hisG-URA3-hisG/cek1Δ::hisG	This study
CK43B-4L	ura3/ura3 cek1Δ::hisG/cek1Δ::hisG	This study
CP29-1-7	ura3/ura3 cpp1Δ::hisG-URA3-hisG/cpp1Δ::hisG	5
CP29-1-7L4	ura3/ura3 cpp1Δ::hisG/cpp1Δ::hisG	5
CP29-1-7CK14	ura3/ura3 cpp1Δ::hisG/cpp1Δ::hisG; cek1Δ::hisG-URA3-hisG/cek1Δ::hisG-URA3-hisG	5
CDH22	ura3/ura3 cst20Δ::hisG-URA3-hisG/cst20Δ::hisG	19
CDH9	ura3/ura3 hst7Δ::hisG-URA3-hisG/hst7Δ::hisG	19
JKC19	ura3/ura3 cph1Δ::hisG-URA3-hisG/cph1Δ::hisG	16
CDH25	ura3/ura3 cst20Δ::hisG/cst20Δ::hisG	19
CDH12	ura3/ura3 hst7Δ::hisG/hst7Δ::hisG	19
CDH72	ura3/ura3 cph1Δ::hisG/cph1Δ::hisG	19

development but also during the mating response of haploid cells (25, 35). These sequentially acting kinases from *S. cerevisiae* have been studied as a paradigm for eukaryotic mitogen-activated protein kinase (MAPK) modules which transduce signals from the cell surface to the nucleus for a wide variety of responses in eukaryotic cells (20, 37). In response to mating pheromone, the MAPK Fus3p is activated on tyrosine and threonine residues by the MAPK kinase (MAPKK, or MEK) Ste7p. Ste7p is activated by the serine/threonine MAPKK kinase (MAPKKK) Ste11p, which may itself be activated by the serine/threonine MAPKKK kinase (MAPKKKK) Ste20p. All of these MAPK cascade components, with the replacement of Fus3p with the homologous MAPK Kss1p, are also involved in activating the Ste12p transcription factor to drive filamentous growth (4, 25, 28, 35). While most of the MAPK cascade components are shared for the different developmental pathways, process-specific factors help to guide the specificity of response (27).

C. albicans homologs of these *S. cerevisiae* MAPK cascade elements include Cst20p (an Ste20p homolog) (16, 19), Hst7p (an Ste7p homolog) (3, 16, 19), Cek1p (a homolog of the Fus3p and Kss1p MAPKs) (5, 44), Cph1p (Ste12p transcription factor homolog) (24, 29), and also a MAPK phosphatase, Cpp1p (5). Cpp1p is a homolog of the *S. cerevisiae* MAPK phosphatase, Msg5p, which plays minor roles in the regulation of Fus3p (6, 46), and possibly the Mpk1p (Slt2p) MAPK (43) involved in cell wall integrity, bud emergence, and polarized cell growth (13, 45). Cst20p, Hst7p, and Cph1p are required for temperature-induced *C. albicans* hyphal formation under certain nutritional conditions on solid surfaces in vitro (16, 19, 24), and Cpp1p is a repressor of hyphal differentiation at ambient temperatures (5).

Here we show that the Cek1p MAPK, which was isolated by its ability to interfere with *S. cerevisiae* pheromone-induced cell cycle arrest when expressed on a high-copy-number plasmid (44), is part of the signal transducing machinery that coordinates hypha formation in *C. albicans*. Our studies suggest that Cst20p, Hst7p, Cek1p, and Cph1p act sequentially in the order of a canonical MAPK cascade to induce the *C. albicans* yeast-to-hypha transition in response to nutritional starvation. We also show that Cek1p fulfills an additional function during the growth of serum-induced mycelial colonies and that it is required for full pathogenicity during experimental systemic candidiasis.

MATERIALS AND METHODS

DNA manipulations and analysis. DNA manipulations were performed by standard procedures (39). Southern blot analysis was performed with a nonradioactive labeling and detection kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's recommendations.

Plasmid constructions. For gene disruptions, pMO3 was created in several steps. First, a 5.2-kb *Xba*I/*Kpn*I genomic DNA fragment containing the *CEK1* gene was excised from plasmid M161p11 (44) and ligated to *Xba*I/*Kpn*I-digested Bluescript KS(+) vector (Stratagene) to create pMO1. Oligodeoxynucleotide primers OT1 (5′GAAGATCTTATTCCATATGCTCCTTCTCC3′) and OT2 (5′GAAGATCTGTAAACATGTGTGAAGAATAG3′) were then used to delete a 1.2-kb region of the *CEK1* gene containing most of the open reading frame from pMO1 by divergent PCR while *Bgl*II sites were added (underlined). This fragment was digested with *Bgl*II and self-ligated to create pMO2. pMO2 was digested with *Bgl*II and joined to a 4-kb *Bam*HI/*Bgl*II *hisG-URA3-hisG* fragment from the plasmid p5921 (8) to create pMO3. Plasmids pCCa3 and pCCa4 were constructed by isolating the 5.2-kb *Xba*I/*Kpn*I insert from pMO1 and joining this fragment to *Xba*I/*Kpn*I-digested pBS-cURA3 (19) [pBluescript KS(+) containing the *C. albicans* URA3 gene between the *Not*I and *Xba*I polylinker—kindly provided by J. Douglas, I. D. Broadbent, and A. J. P. Brown, University of Aberdeen] for pCCa3 or the vector pVEC which contains *URA3* as a selectable marker and a *C. albicans* autonomously replicating sequence (kindly provided by C. Nombela, University of Madrid, and B. Magee, University of Minnesota) for pCCa4.

Construction of pYPB1-ADHpt-HST7, containing *HST7* driven by the *ADH1* promoter, has been previously described (19). To construct plasmid pLJ19, carrying *CPH1* under the control of the *ADH1* promoter, the coding region of *CPH1* flanked by *Bam*HI sites with the 5′ oligonucleotide CGCGGATCCACTCTTTCGCCATGTCAATTAC and the 3′ oligonucleotide CGCGGATCCTATTCATCTATGTTTGTGACTG was amplified with plasmid pKB83.1 containing the *CPH1* gene as a template (provided by K. Clark). The fragment was inserted into the *Bgl*II site of the plasmid YPB1-ADH1pl containing the *ADH1* promoter (1), *C. albicans* URA3 as a selectable marker, and an autonomously replicating sequence (kindly provided by G. Bertram, I. D. Broadbent, P. J. F. Feldman, and A. J. P. Brown) (see reference 19).

Chromosomal disruption of the *CEK1* gene. A sequential gene disruption strategy (8) was used to replace both chromosomal copies of the *CEK1* gene with an 8-kb *Kpn*I/*Not*I exogenously provided DNA fragment, from the plasmid pMO3, in which most (1.2 kb) of the 1.6-kb open reading frame of *CEK1* was replaced by a 4.0-kb fragment containing the selectable marker *URA3* flanked by *hisG* direct repeats. Spheroplasts from the Ura3 auxotrophic strain, CAI4, were transformed (18) with the exogenously provided fragment. Mutations were verified by Southern analysis with a 3.2-kb *Kpn*I/*Sac*I fragment from pMO1 containing the *CEK1* gene as a probe. Of 10 transformants analyzed, 9 had a disruption of one allele and 1 appeared to have a disruption of both alleles of the *CEK1* gene. Two transformants (including CK43A [Table 1]) with a disruption of one allele of *CEK1* (CEK1/cek1Δ::hisG-URA3-hisG) were chosen. Loss of the *URA3* gene by recombination between repeats (leaving behind one copy of the

hisG gene) was selected for on medium containing 5-fluoroorotic acid and uridine as described elsewhere (8) to create *CEK1/cek1Δ::hisG* strains (Table 1). These steps were repeated to obtain independent transformants with disruptions in both alleles of *CEK1*. CK43B-16 and CK43B-4 (*cek1Δ::hisG-URA3-hisG/ cek1Δ::hisG* [Table 1]) were chosen for further analysis.

To target reintegration of *CEK1* into the genome, the *C. albicans* reintegration plasmid containing the *CEK1* gene and flanking sequences, pCCa3, was linearized with *Pst*I and transformed into Ura⁻ *C. albicans* containing the double or single deletion of the *CEK1* gene. Strains were also transformed with the expression plasmid pCCa4.

***Candida* strains and growth conditions.** All strains are listed in Table 1. Standard growth of the yeast form in liquid culture was performed in YPD medium at 30°C (38). To induce germ tube formation in liquid culture, cells were diluted 10-fold from overnight cultures grown in Lee's medium (23) into fresh Lee's medium or from YPD into 10% fetal bovine serum (Intergen Inc., Purchase, N.Y.) and incubated for 3 h at 37°C.

To induce hyphal development on solid medium, budding *C. albicans* cells were grown overnight at 30°C with vigorous shaking in YPD medium and washed once with sterile water prior to being plated. Petri plates were divided into six sectors, and 5 to 25 cells per sector were then incubated for the indicated times at 30 or 37°C on different media. Solid Spider medium contains 1% nutrient broth, 0.2% K$_2$HPO$_4$, 1.4% agar, and 1% either glucose or mannitol (24). Lee M medium is a modification of the synthetic medium described by Lee et al. (23) with the substitution of mannitol for glucose. Synthetic low-ammonium–dextrose (SLAD) medium, containing 50 μM ammonium sulfate as sole nitrogen source, and synthetic ammonium-dextrose (SAD), containing 50 mM ammonium sulfate, were prepared as described elsewhere by omitting L-histidine from SLAHD and SAHD (10); the pH of these media was 4.0.

For serum plates, 10% fetal bovine serum was added to 1.4% agar at 50°C after autoclaving. Statistical analysis of the differences between mean mycelial colony diameters was performed with Student's *t* test. Colony diameters obtained from 10 colonies of each strain grown on the same agar plate 18 h after incubation were measured in Adobe Photoshop (Adobe Systems, Inc.) after scanning photographs taken with a 2× objective. For comparative purposes, estimated mycelial colony growth rates were calculated from the mean colony diameters. To approximate growth rates, we assumed a direct relationship between mycelial colony diameter and growth rate (μ). We plotted the growth rates, obtained by counting hyphal tips for the wild-type SC5314 and the *cpp1* null mutant (5), versus the mean mycelial colony diameters and used this as a standard curve to obtain growth rates for the other strains presented in this study.

Photomicroscopy of colonies and invasive growth was performed with a Nikon TMS inverted microscope, and plates were photographed with Kodak TMAX film.

For epistasis analysis, Ura3⁻ derivatives of strains containing deletions of *CST20* (CDH25), *HST7* (CDH12), *CPH1* (CDH72), and *CEK1* (CK43B-16L) were transformed with plasmids YPB1-ADHpt, pYPB1-ADHpt-HST7, and pLJ19. The *CPP1* null mutant strain (CP29-1-7L) was transformed with plasmids YPB1-ADHpt and pLJ19. These were plated on SLAD solid medium for the times indicated and observed by microscopy.

Diagnosis of pseudohyphal and true hyphal forms. Filaments were classified as pseudohyphal if a constriction was apparent at junctions between two compartments (31). True hyphae were diagnosed if filaments were composed of parallel-sided compartments with no constrictions at compartment junctions and also by the presence of septa perpendicular to cell walls. To visualize these traits, photographs taken at a magnification of ×40 were scanned into the computer and images were magnified in Adobe Photoshop (Adobe Systems, Inc.). Filament compartments were traced, and the angles between walls and septa were examined.

Virulence studies. Inbred female BALB/c mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany) and used for infection at 8 to 10 weeks of age. *C. albicans* in vivo virulence testing was performed as described previously (5). Briefly, strains were grown to stationary phase in YPD. Cells were then harvested, washed, and adjusted to the desired density in phosphate-buffered saline and were injected intravenously into the tail vein in a final volume of 200 μl. Statistical analysis of the differences in survival between paired groups was performed with the Mantel-Haenszel log rank test with the GraphPad Prism 2.01 software package.

RESULTS

Disruption of *CEK1* causes defects in the shift from a unicellular colonial mode of growth to an invasive hyphal mode of growth. Cek1p belongs to the ERK family of MAP kinases and is closely related to the *S. cerevisiae* kinases Kss1 and Fus3 (44). Although *CEK1* overexpression does not reestablish mating in a *fus3 kss1* strain, disruption of the kinase domain of *CEK1* blocks its ability to interfere with pheromone arrest (44). Because of previous reports of the roles of *C. albicans* *CST20*, *HST7*, and *CPH1* genes in *C. albicans* hyphal development on
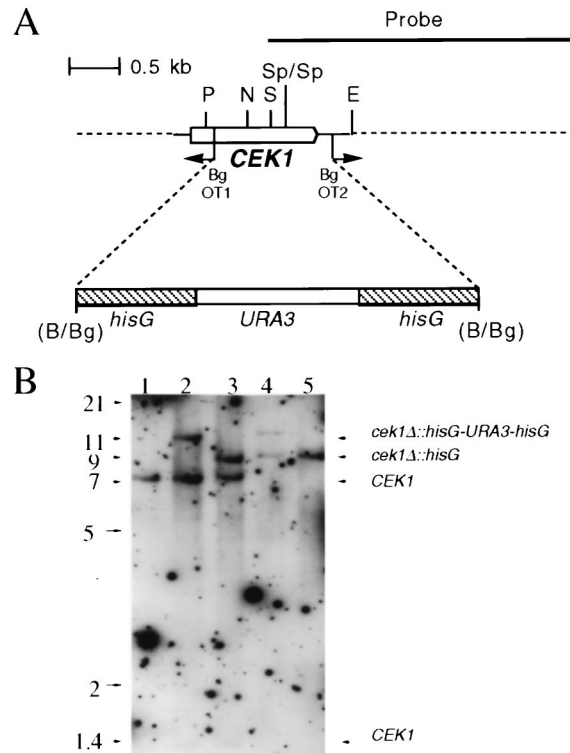

FIG. 1. (A) Deletion of *CEK1* in *C. albicans*; restriction map of and deletion strategy for the *CEK1* gene. PCR with the divergent oligodeoxynucleotides OT1 and OT2 was used to delete a 1.2-kb fragment of the *CEK1* gene. A 4.0-kb *hisG-URA3-hisG* cassette was then inserted. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; S, *Sac*I; N, *Nsi*I; E, *Eco*RI; Sp, *Spe*I. (B) Southern blot analysis of *CEK1* disruptions with a 3.2-kb *Kpn*I-*Sac*I fragment containing the *CEK1* gene as a probe. Genomic DNA samples from the following strains were digested with *Spe*I (absent from the *hisG-URA3-hisG* cassette): *CEK1/CEK1* (CAI4) (lane 1), *CEK1/cek1Δ::hisG-URA3-hisG* (CK43A) (lane 2), *CEK1/ cek1Δ::hisG* (CK43AL) (lane 3), *cek1Δ::hisG-URA3-hisG/cek1Δ::hisG* (CK43B-16) (lane 4), and *cek1Δ::hisG/cek1Δ::hisG* (CK43B-16L) (lane 5). Strains CK43B-4 and CK43B-4L gave results identical to the example shown here. Hybridization of a very small part (between the *Sac*I and *Spe*I sites) of the probe to a 1.4-kb *Spe*I fragment, present only in the wild-type *CEK1* gene, was barely detectable in these Southern blots and was not used for diagnostic purposes. The figure was assembled with Adobe Photoshop 3.0. Numbers on the left indicate sizes in kilobases.

solid medium containing mannitol as a carbon source (16, 19, 24), we disrupted *CEK1* in order to explore whether Cek1p assists in hyphal development.

Null mutations of one or both alleles of *CEK1* were made by sequential gene disruption and confirmed by Southern blot analysis (Fig. 1). The development of hyphae emanating from colonies grown for 4 days at 30°C on Lee's (Fig. 2) or Spider (data not shown) medium containing mannitol as a carbon source was partially blocked when one allele of *CEK1* was deleted from cells (*cek1* heterozygous mutants) and was completely blocked when both *CEK1* alleles were deleted (*cek1* homozygous null mutants). Strains carrying null mutations of the *CST20*, *HST7*, and *CPH1* genes were compared in parallel as controls and gave similar results (data not shown). The strain with the homozygous null mutation of the *CEK1* gene regained the ability to make hyphae when wild-type *CEK1* was reintroduced by the *CEK1* expression plasmid pCCa4 (Fig. 2), and partial ability was regained by reintegration of the *CEK1* gene at its chromosomal locus (data not shown). Like strains with homozygous *CST20*, *HST7*, and *CPH1* null mutations, strains with homozygous *CEK1* null mutations formed hyphae

SC5314 CEK1/cek1Δ cek1Δ/cek1Δ cek1Δ/cek1Δ pVEC cek1Δ/cek1Δ pVEC-CEK1
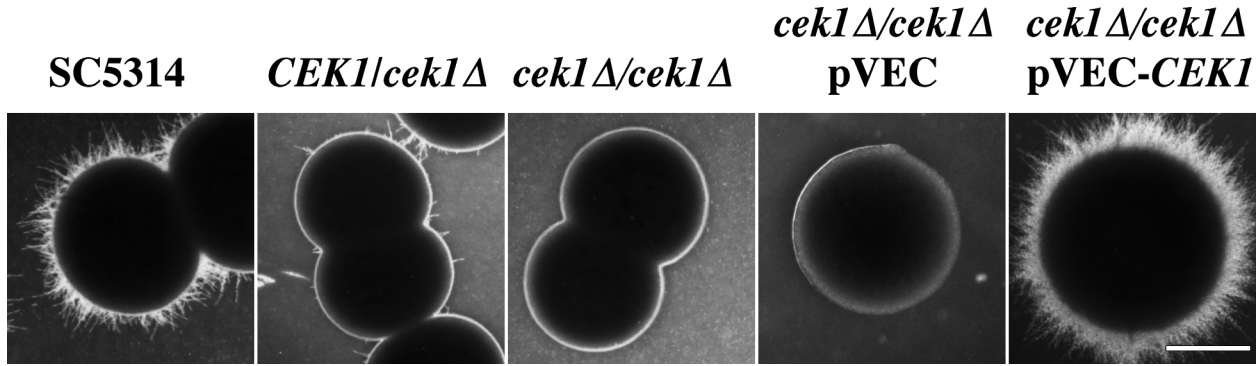
FIG. 2. Growth of heterozygous and homozygous *CEK1* deletion mutants on medium containing mannitol as a carbon source. Shown are uracil prototrophic strains: SC5314 (parental strain), *CEK1/cek1Δ* (CK43A), *cek1Δ/cek1Δ* (CK43B-16, identical to CK43B-4), *cek1Δ/cek1Δ* pVEC (plasmid vector pVEC in CK43B-4L), and *cek1Δ/cek1Δ* pVEC-CEK1 (plasmid pCCa4 containing the *CEK1* gene in CK43B-4L). Cells were grown for 4 days at 30°C. Lee M medium (shown) and Spider medium gave similar results. Bar = 1.5 mm. The figure was assembled with Adobe Photoshop 4.0.

on solidified Lee's or Spider medium containing glucose or on serum medium and in all hypha-inducing liquid media investigated (data not shown). These results indicated that Cek1p, like Cst20p, Hst7p, and Cph1p, is required for agar-invasive hypha formation from mature colonies under some conditions and suggested that they may act in the same regulatory cascade.

Because we found that agar-invasive growth of *C. albicans* hyphae was stimulated by severe nitrogen starvation (50 μM ammonium sulfate) on solid medium (SLAD) at a pH of 4.0 (Fig. 3), the condition that also stimulates pseudohyphal development in *S. cerevisiae* (10), we decided to explore whether the *C. albicans* MAPK cascade deletion mutants affected this process. Strains with deletions of both alleles of *CST20*, *CPH1*, *HST7*, or *CEK1* were all defective in true hyphal outgrowth on solid SLAD at 30°C and made mainly large agar-invasive pseudohypha-type structures with constrictions between cells (Fig. 3). Strains with deletions of both alleles of the *CPP1* gene, which encodes a tyrosine phosphatase that blocks hyphal development potentially by acting on the Cek1p MAPK (5), also

developed parallel-sided hyphae on SLAD (Fig. 3). *C. albicans* wild type and null mutants grew only in the yeast form when the defined solid medium used was supplemented with 50 mM ammonium sulfate (SAD medium) as a nitrogen source (data not shown). In liquid culture, however, neither SLAD nor SAD medium (at 37°C) promoted *C. albicans* hyphal development (data not shown), suggesting that nitrogen limitation and contact with a solid surface, or an additional factor associated with colonial growth, act together to promote hyphal formation.

Suppression of the *cek1* null mutant hyphal defect by *CPH1* under the control of the strong *ADH1* promoter but not by *ADH1*-driven *HST7* during nitrogen limitation. *HST7* linked to the *ADH1* promoter (1) on the plasmid pYPB-ADHpt-HST7 has been reported to be expressed 180-fold above wild-type *HST7* levels (19). We examined whether *HST7* and *CPH1* under the control of the *ADH1* promoter were able to overcome the hyphal growth defects of strains with homozygous deletions of the different MAPK cascade components. ADH-HST7 (pYPB-ADHpt-HST7) and ADH-CPH1 (pLJ19) bypassed the phenotypes of mutations in the *C. albicans* genes

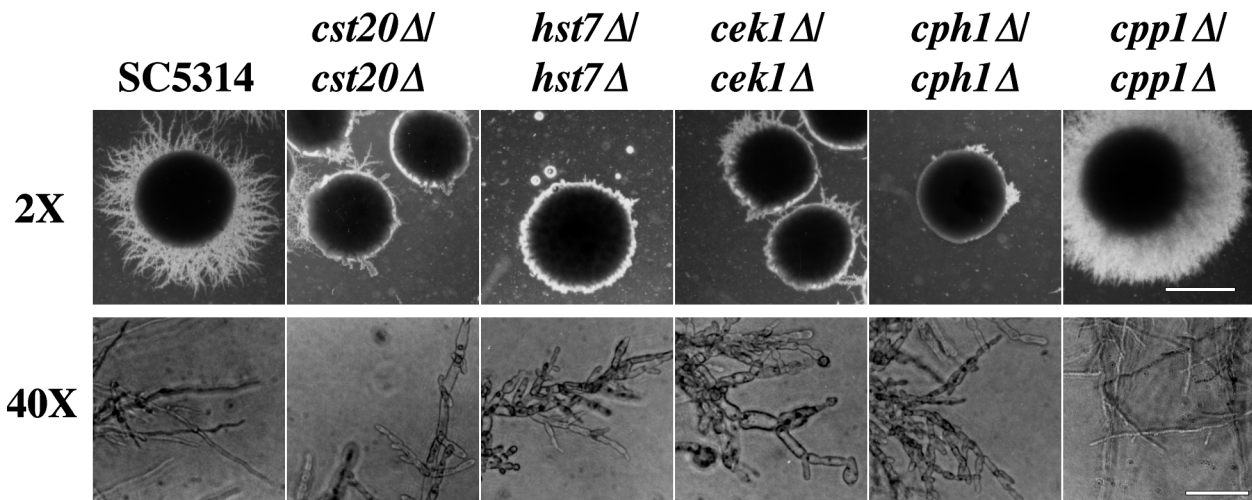SC5314 cst20Δ/cst20Δ hst7Δ/hst7Δ cek1Δ/cek1Δ cph1Δ/cph1Δ cpp1Δ/cpp1Δ

2X

40X

FIG. 3. Defective formation of parallel-sided hyphae from uracil prototrophic MAPK cascade null mutants grown on low-ammonia–dextrose medium (SLAD). Shown are SC5314 (wild-type parent), *cst20Δ/cst20Δ* (CDH22), *hst7Δ/hst7Δ* (CDH9), *cek1Δ/cek1Δ* (CK43B-16), *cph1Δ/cph1Δ* (JKC19), and *cpp1Δ/cpp1Δ* (CP29-1-7). Cells were grown for 7 days at 30°C. (2× objective, bar = 1.5 mm; 40× objective, bar = 90 μm.) Constrictions around septa were examined. Parallel-sided hyphae with no constrictions at septa are seen emanating from the borders of SC5314 and *cpp1Δ/cpp1Δ* colonies. Pseudohyphal filaments of *cst20Δ/cst20Δ*, *hst7Δ/hst7Δ*, *cek1Δ/cek1Δ*, and *cph1Δ/cph1Δ* strains are aberrant in shape and have constrictions between cells. Noninvasive cells from colonies scraped off plates were composed entirely of blastospores (data not shown). The figure was assembled with Adobe Photoshop 4.0.
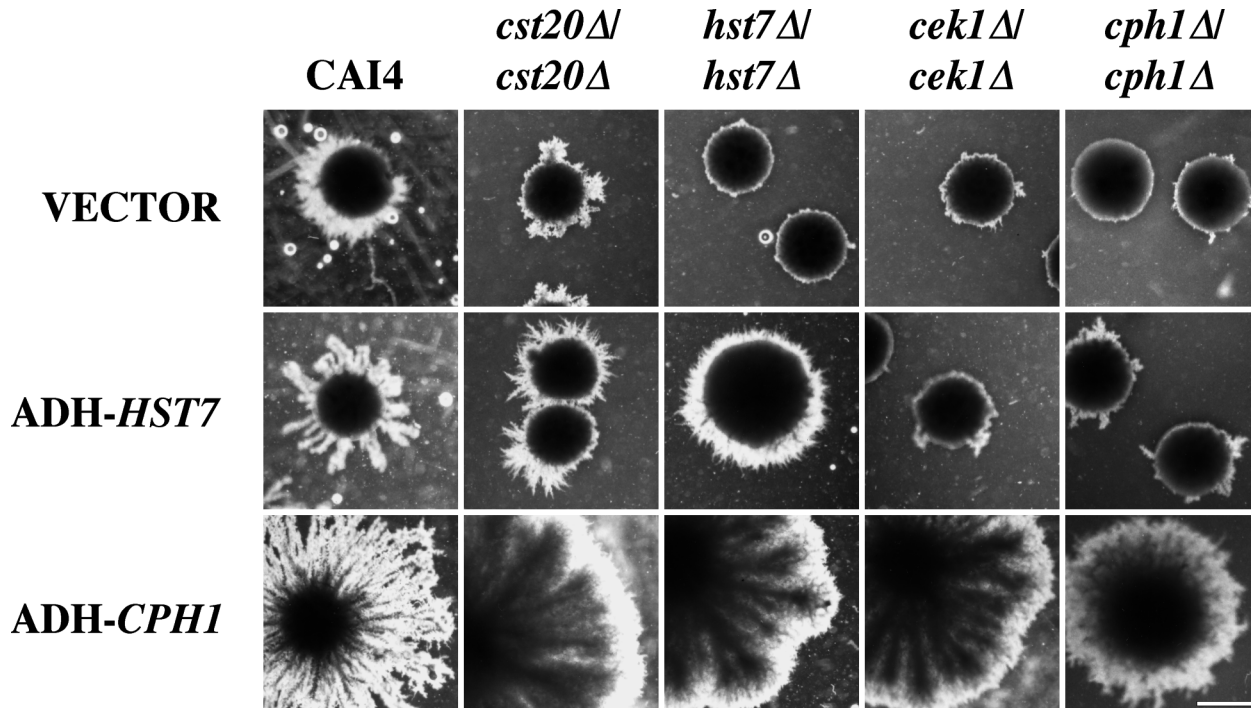
FIG. 4. Complementation analysis of null mutants grown in SLAD medium. Shown are the uracil auxotrophic strains CAI4 (parent), *cst20Δ/cst20Δ* (CDH25), *hst7Δ/hst7Δ* (CDH12), *cek1Δ/cek1Δ* (CK43B-16L), and *cph1Δ/cph1Δ* (CDH72) transformed with pADH (YPB1-ADHpl), pADH-HST7 (pYPB1-ADHpt-HST7), or pADH-CPH1 (pLJ19). Cells were grown for 10 days at 30°C. As described above, filaments were examined for constrictions and for the radial penetration of filamentous growth beyond colony borders. The zones of filamentous growth surrounding pADH vector-transformed *cst20/cst20* null mutants were composed of constricted pseudohyphae (as seen above), as were the less extensive filaments emanating from pADH vector-transformed *hst7Δ/hst7Δ*, *cek1Δ/cek1Δ*, and *cph1Δ/cph1Δ* null mutants. Bar = 1.5 mm. The figure was assembled with Adobe Photoshop 4.0.

that would be expected to act earlier in the pathway by analogy with the *S. cerevisiae* pheromone response pathway. Colonies of *cst20*, *hst7*, *cek1*, and *cph1* null mutant strains, containing ADH-*CPH1*, developed large zones of radial agar-penetrating hyphae, unlike colonies of null mutants containing the *ADH1* vector (YPB1-ADHpt) alone (Fig. 4). The extensive mycelial networks seen were composed mainly of parallel-sided hyphae (data not shown) resembling those seen in Fig. 3 for SC5314. ADH-*HST7* restored the ability of *cst20* null mutant colonies to make parallel-sided hyphae (data not shown), resulting in more extensive agar-invasive radial hyphal growth (Fig. 4) than that for null mutants containing the vector alone, which produced pseudohyphae as shown in Fig. 3 (also see reference 19). The ADH-*HST7* plasmid also restored radial hyphal growth to the *hst7* null mutant, but not to the *cek1* null or *cph1* null strain, as assessed by examining the zones of radial mycelial growth extending beyond colony borders (Fig. 4) for several transformants.

In addition, hyperhyphal phenotypes were observed for CAI4 transformants containing the ADH-*HST7* and ADH-*CPH1* plasmids. This is consistent with the ability of *HST7* and *CPH1* to hyperactivate pseudohyphal growth when expressed in *S. cerevisiae* (3, 24). The abundant hyphal growth of strains containing the ADH-*CPH1* plasmid resembled that seen for the *cpp1* phosphatase null mutant, and the presence of ADH-*CPH1* in the *cpp1* null mutant strain did not enhance the hyper-radial-hyphal phenotype of *cpp1* null mutants on SLAD (data not shown).

Disruption of *CEK1* causes a mycelial colony radial growth defect on serum. *C. albicans* cells plated on serum and incubated at 37°C display growth that is considered "truly mycelial" (12). All individual cells plated on solid medium containing agar and 10% serum make germ tubes within hours, and the growth kinetics of the entirely mycelial colony which penetrates and assimilates the agar has been well characterized elsewhere (12). Like strains with mutations of both alleles of the *CST20*, *CPH1*, and *HST7* genes (16, 19, 24), *cek1* homozygous null mutants make morphologically normal hyphae on serum (Fig. 5A), in contrast to strains with null mutations of *EFG1*, which make only pseudohyphae (26, 42), and *cph1 efg1* double null mutants, which make only yeast cells (26). These results suggest that the MAPK cascade is at least partially active on serum. Despite the normal appearance of hyphae on serum, we observed significant differences ($P < 0.05$) in colony diameters and thus also in growth rate (μ) by paired comparisons of the wild-type (SC5314; $\mu = 0.12$ h^{-1}) and the heterozygous (*CEK1/cek1*; $\mu = 0.092$ h^{-1}) and homozygous (*cek1/cek1*; $\mu = 0.063$ h^{-1}) null strains (Table 2 and Fig. 5). The growth defect of the heterozygous mutants was reversed by two independent transformants containing reintegrations of the *CEK1* gene (Fig. 5 and data not shown), and that of the homozygous *cek1* null mutant was reversed by transformants containing the *CEK1* gene on the plasmid pCCa4 (Fig. 5A).

Intriguingly, a mutant containing a double deletion of the *CPP1* MAPK phosphatase and *CEK1* together significantly ($P < 0.05$) suppresses defects in radial hyphal growth on serum of the independent *cek1* and *cpp1* null mutants (Fig. 5B; Table 2) (5). The estimated growth rate of the double mutant was 0.10 h^{-1}, compared to similar rates of 0.062 and 0.063 h^{-1} for the individual *cpp1* (5) and *cek1* null mutants, respectively. Previously, we reported that the *cek1 cpp1* double null mutant resembled the wild-type parent when grown on serum (5); however, further analysis demonstrated that the double null
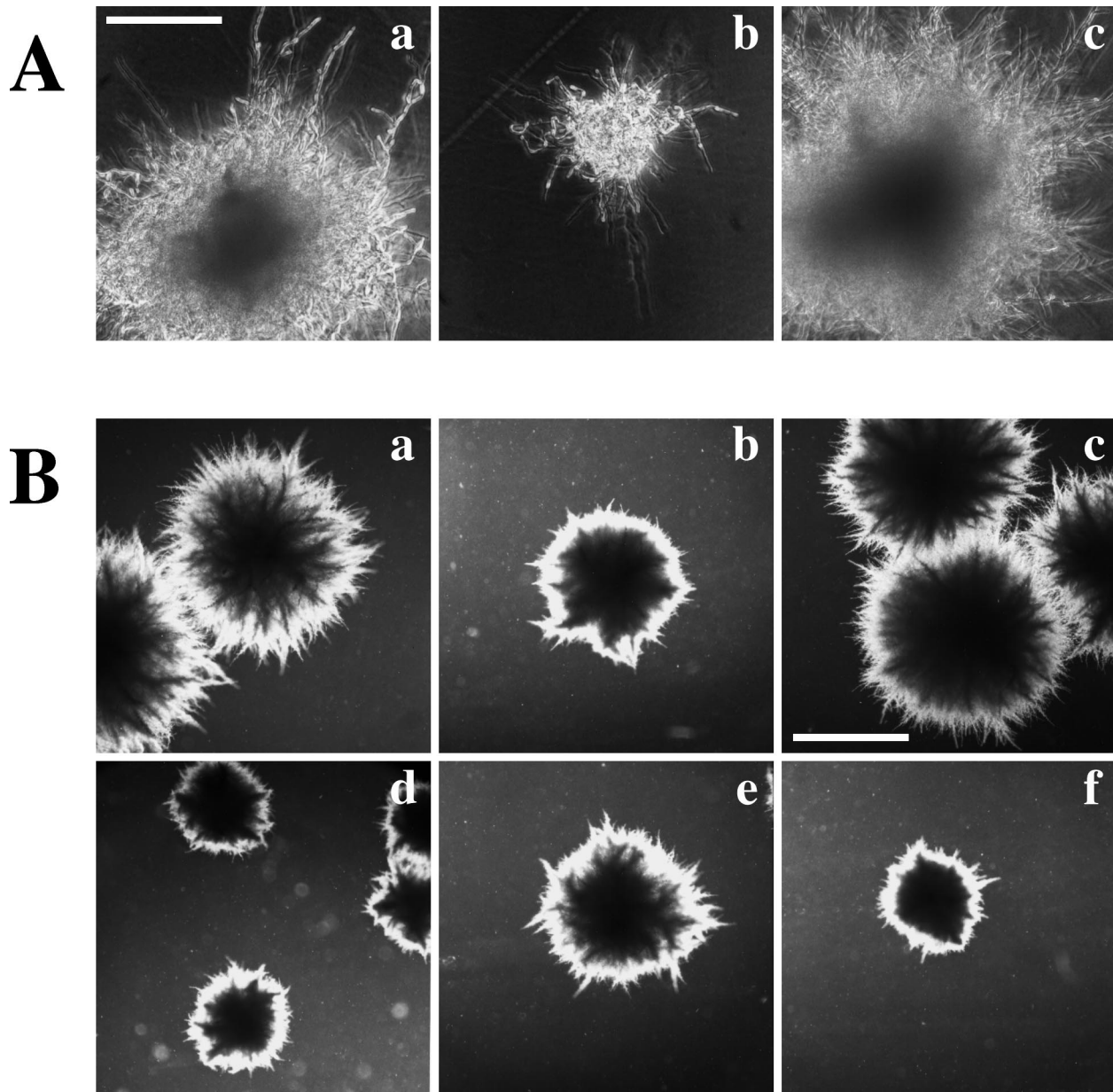
FIG. 5. Growth of invasive mycelial colonies on solidified serum. (A) Cells grown for 24 h on serum at 37°C (magnification, ×20; bar = 0.75 mm). Shown are uracil prototrophic strains SC5314 (parental strain) (a), *cek1Δ/cek1Δ* (CK43B-4) (b), and *cek1Δ/cek1Δ* (CK43B-4L) (c) transformed with the pVEC-*CEK1* plasmid pCCa4. (B) Cells grown for 3 days on serum at 37°C (magnification, ×2; bar = 3 mm). Shown are uracil prototrophic strains SC5314 (parental strain) (a), *CEK1/cek1Δ* (CK43A) (b), *CEK1/cek1Δ::CEK1-URA3* (CK43A-RI) (c), *cek1Δ/cek1Δ* (CK43B-4) (d), *cek1Δ/cek1Δ cpp1Δ/cpp1Δ* (CP29-1-7CK14) (e), and *cpp1Δ/cpp1Δ* (CP29-1-7) (f). The figure was assembled with Adobe Photoshop 4.0. Panels A and B are from independent experiments.

mutant has a minor but significant ($P < 0.05$) reduction in growth rate as assessed by differences in colony diameters (Table 2). Previous reports (16, 19) describe no influence of the *cst20* or *hst7* null mutant on hyphal growth on serum agar.

Virulence studies. Mice were injected intravenously with the following uracil prototrophic strains: the parental strain (SC5314), *cek1* null mutants (*cek1Δ/cek1Δ*), or null mutants containing the plasmid pCCa4 (*cek1Δ/cek1Δ* pVEC-CEK1). pCCa4 contains the *CEK1* gene on the replicative vector pVEC, and pCCa4 suppressed the in vitro phenotypes of the *cek1* null mutant (Fig. 2 and 5). Doubling times of the yeast form of these strains grown in YPD medium at 30°C were

essentially the same. Two days after infection, mice injected with strains containing a wild-type copy of the *CEK1* gene (*cek1Δ/cek1Δ* pVEC-CEK1) or the parental *CEK1/CEK1* strain (SC5314) showed signs of severe systemic disease, including weight loss, while the *cek1* null mutant-infected animals behaved normally. As illustrated in Fig. 6, infection with *cek1* null mutants resulted in 50% mortality, while 100% mortality was observed when mice were infected with the control strains (*cek1Δ/cek1Δ* pVEC-CEK1 and SC5314) for the observed period of 35 days. These differences were significant ($P < 0.05$). Similar results were obtained with the null mutant strains CK43B-4 and CK43B-16 as well as with an additional independent null mutant.

TABLE 2. Mean mycelial colony diameters and estimated growth
rates (μ) of strains grown on serum agar for 18 h[a]

Ura3 prototrophic strain	Mean diam (mm) ($n = 10$)	SEM	Estimated growth rate (h^{-1})
SC5314 parent	0.41	0.024	0.12
CEK1/cek1 (CK43A)	0.30	0.017	0.092
cek1/cek1 (CK43B-4)	0.19	0.025	0.063
cpp1/cpp1 (CP29-1-7)	0.18	0.011	0.062
cpp1/cpp1 cek1/cek1 (CP29-1-7CK14)	0.34	0.022	0.10

[a] See Materials and Methods for details. Results presented here are for an
experiment different from that for which the results are presented in Fig. 5.

DISCUSSION

C. albicans harbors both structural and functional homologs
of a starvation-activated MAPK cascade required for filamen-
tation of the budding yeast *S. cerevisiae*. Our results show that
the Cek1p MAPK plays a positive role in *C. albicans* hyphal
development in response to nutritional starvation. We found
that homozygous *cek1Δ/cek1Δ* null mutants, and to a lesser
extent *CEK1/cek1Δ* heterozygous mutants, are defective in
agar-invasive growth of hyphae in response to nutritional lim-
itation, as are cells containing heterozygous and homozygous
deletions of the *C. albicans CST20*, *HST7*, and *CPH1* genes
(16, 19, 24). Although Cek1p appears to function in this
MAPK cascade, it also plays a role in modulating radial hyphal
growth on serum and cells from which *CEK1* has been deleted
are less virulent when injected into mice, suggesting that
Cek1p may be required for additional pathways in *C. albicans.*

Like pseudohyphal formation in *S. cerevisiae*, the develop-
ment of agar-invasive parallel-sided *Candida* hyphae can be
triggered from stationary colonies of yeast cells grown on me-
dium severely limited for nitrogen. In *S. cerevisiae*, the starva-
tion message is relayed to the MAPKKKK Ste20p via the GTP
binding proteins Ras2p and Cdc42p (21, 30, 33) and two 14-3-3
homologs (36). We found that null mutation of MAPK cascade
elements did not block *C. albicans* pseudohyphal development
but did block the formation of parallel-sided hyphae instead. *S.
cerevisiae* pseudohyphal formation is suppressed by deletion of
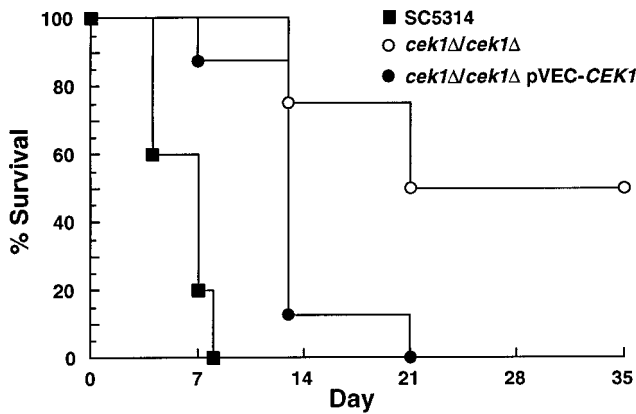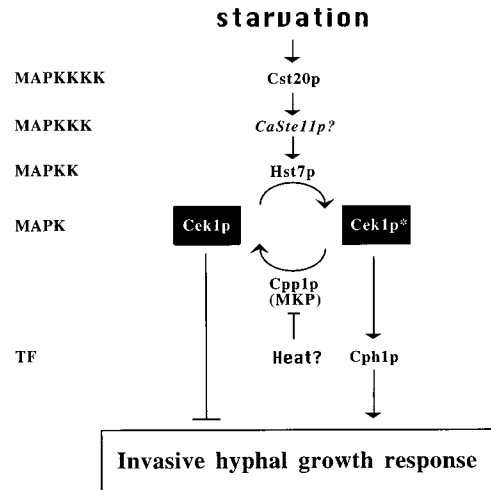elements of its cognate MAPK cascade, but a few pseudohy-

FIG. 7. Model of the *C. albicans* MAPK cascade for the yeast-to-hypha
transition. *CaSte11p* is an undescribed putative homolog of the *S. cerevisiae*
Ste11p MAPKKK whose role has not yet been examined for this pathway. TF,
transcription factor. The phosphorylated form of Cek1p is designated by an
asterisk.

phae are still observable (26). In both organisms, two pathways
involving the transcription factors Ste12p (Cph1p) and Phd1p
(Efg1p) function together and independently to promote fila-
ment development (26, 42), whereas commitment to a polar-
ized hyphal growth mode requires the action of the kinase
CaCla4p (22). In *C. albicans*, the nature and intensity of stimuli
combined with the differential activities of these signal trans-
duction pathways may help determine whether cells undergo
pseudohyphal or true hyphal differentiation.

The similar phenotypes of strains with null mutations of the
CEK1, *CST20*, *HST7*, and *CPH1* genes under starvation con-
ditions suggest that they function as part of the same regula-
tory cascade. We found that *HST7* and *CPH1* when expressed
from the strong *ADH1* promoter could complement deletions
of *CST20* and *HST7*, whereas expression of *CPH1* but not of
HST7 from the *ADH1* promoter could complement deletions
of *CEK1* and *CPH1*. These epistatic relationships suggest that
this filamentation MAPK cascade functions in an order typical
of other MAPK cascades (Fig. 7) (see also reference 19). The
Cek1p MAPK is thus required for the filamentation MAPK
cascade in *C. albicans* and probably holds functions analogous
to the Kss1p MAPK of *S. cerevisiae* (4, 28). Negative regulation
by MAPK phosphatases can shut down or control the intensity
of MAPK signaling (15, 46) (Fig. 7). Deletion of the *CEK1*
gene completely suppresses the extensive invasive hyphal
growth that occurs at room temperature when the MAPK
phosphatase gene *CPP1* is removed from cells (5), suggesting
that Cpp1p inhibits the hyphal-stimulating roles of Cek1p (Fig.
7). Higher temperatures probably lift the repression of hyphal
development mediated by the Cpp1p MAPK phosphatase (5).
Indeed, central control of hyphal formation through repression
mechanisms may be a theme in *C. albicans*, since a strain with
a deletion of the *TUP1* gene encoding a general transcriptional
repressor grew exclusively as filaments (2).

Like strains with null mutations of the *CST20*, *CPH1*, and
HST7 genes (16, 19, 24, 26), serum-induced *cek1* mutants grow
as invasive hyphal colonies on solidified serum medium. De-
spite the normal hyphal development of null mutants of the
MAPK cascade-regulated transcription factor Cph1p on se-
rum, Cph1p nevertheless contributes to the development of

FIG. 6. Survival curves for BALB/c mice injected intravenously with 5×10^5
cells of the uracil prototrophic strains SC5314 ($n = 5$ mice; clinical isolate),
cek1Δ/cek1Δ (CK43B-4; $n = 8$ mice), and *cek1Δ/cek1Δ* pVEC-*CEK1* (CK43B-4L
transformed with the plasmid pCCa4; $n = 8$ mice). Results were confirmed in
two independent experiments. The *cek1* null mutant strain CK43B-16 and an-
other independent null mutant gave similar results (data not shown). Mortality
data were quantified on days of sampling.

hyphae on serum (26). A strain with deletion mutations of the two filamentation transcription factor genes *CPH1* and *EFG1* makes only single cells on solid serum medium, whereas *efg1* null mutants make pseudohyphae (26, 42). This suggests that the MAPK cascade plays a role during serum-induced hyphal growth, which is usually masked by Efg1p. We found, however, that the zones of hyphal penetration of both heterozygous and homozygous *cek1* null mutants were reduced on serum. We had previously suggested that a similar mycelial colony growth rate defect of strains with null mutations of the MAPK phosphatase gene *CPP1* on serum was the result of detrimentally high levels of hypha-stimulating cellular activities because deletion of *CEK1* suppressed the phenotype of the *cpp1* null mutants (5). If Cek1p were the sole MAPK contributing to hyphal growth on serum, we would expect strains with double mutations of *CEK1* and the MAPK phosphatase gene *CPP1* to display the same phenotype as *cek1Δ/cek1Δ* single mutants, but the phenotype of the double null mutant is more similar to that of the wild type (5) (although they still have a minor defect in radial hyphal growth) than to those of the individual null mutants. A second MAPK, inappropriately active or hyperactive when the phosphatase is absent, could be partially compensating for loss of *CEK1*; this unknown MAPK could also, in its inactive form, inhibit hyphal growth when the phosphatase is present. Such a picture is plausible in view of the complexities of the roles of MAPKs during filamentous growth in *S. cerevisiae* (4, 28).

A role for the hyphal form of *C. albicans* in virulence is strongly supported by reports that a mutant carrying a deletion of the *CaCLA4* gene, a regulator of polarized growth of the fungal germ tube into a true hyphal compartment, is completely avirulent (22), as are *cph1 efg1* double null mutants which are locked in the yeast form (26). On the other hand, individual *cph1*, *efg1* (26), and *hst7* (19) null mutants are as pathogenic or almost as pathogenic as parental strains, suggesting that the starvation-induced MAPK pathway is not required alone for systemic virulence but can contribute to virulence. The *cst20* (19) and *cek1* null mutants are different. Injection of these strains into mice allows them to live longer on average than does injection of the parental strain, suggesting that they have overlapping or distinct pleiotropic functions in cells which affect virulence. Because *cek1 cpp1* double null mutants (unpublished results) suppress both the attenuated virulence and the serum-induced mycelial colony growth defect common to single *cek1* and *cpp1* null mutants, it is possible that these phenotypes are related. Future analysis of these mutants should lead to further insights into the functions of the wild-type regulatory enzymes deleted from these strains and may unmask novel genes and cellular functions involved in the pathogenicity of this important fungal pathogen.

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