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CAP1, an Adenylate Cyclase-Associated Protein Gene, Regulates Bud-Hypha Transitions, Filamentous Growth, and Cyclic AMP Levels and Is Required for Virulence of *Candida albicans*

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In response to a wide variety of environmental stimuli, the opportunistic fungal pathogen *Candida albicans* exits the budding cycle, producing germ tubes and hyphae concomitant with expression of virulence genes, such as that encoding hyphal wall protein 1 (*HWP1*). Biochemical studies implicate cyclic AMP (cAMP) increases in promoting bud-hypha transitions, but genetic evidence relating genes that control cAMP levels to bud-hypha transitions has not been reported. Adenylate cyclase-associated proteins (CAPs) of nonpathogenic fungi interact with Ras and adenylate cyclase to increase cAMP levels under specific environmental conditions. To initiate studies on the relationship between cAMP signaling and bud-hypha transitions in *C. albicans*, we identified, cloned, characterized, and disrupted the *C. albicans CAP1* gene. *C. albicans* strains with inactivated *CAP1* budded in conditions that led to germ tube formation in isogenic strains with *CAP1*. The addition of 10 mM cAMP and dibutyryl cAMP promoted bud-hypha transitions and filamentous growth in the *cap1*/*cap1* mutant in liquid and solid media, respectively, showing clearly that cAMP promotes hypha formation in *C. albicans*. Increases in cytoplasmic cAMP preceding germ tube emergence in strains having *CAP1* were markedly diminished in the budding *cap1*/*cap1* mutant. *C. albicans* strains with deletions of both alleles of *CAP1* were avirulent in a mouse model of systemic candidiasis. The avirulence of a germ tube-deficient *cap1*/*cap1* mutant coupled with the role of Cap1 in regulating cAMP levels shows that the Cap1-mediated cAMP signaling pathway is required for bud-hypha transitions, filamentous growth, and the pathogenesis of candidiasis.

For many pathogenic fungi, interconversions between morphological growth forms, particularly between yeast growth and filamentous growth, coincide with adaptation to a host environment followed by tissue destruction. Morphological interconversions in fungi are dependent upon signal transduction pathways, including the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway (8, 10, 28, 40, 46). For the plant pathogens *Ustilago maydis* and *Magnaporthe grisea*, cAMP signaling is important for the establishment of filamentous growth in the former and for formation of the infecting appressorium structure of the latter (40, 46). Knowledge about how cAMP signaling mediates morphological interconversion is best understood for *Saccharomyces cerevisiae*, a budding yeast that produces elongated pseudohyphal cells and forms filamentous colonies in the presence of limiting nitrogen (28, 46). Pseudohyphae exhibit unipolar budding, do not separate, and invade agar (31). Recent experiments involving gene disruption and epistasis analyses have elucidated both upstream and downstream elements of the cAMP-dependent pseudohyphal growth pathway in *S. cerevisiae* (28, 40, 46). Adenylate cyclase is activated either through a receptor (Gpr1) that is coupled to a G protein (Gpa2) or by Ras2 (31, 41, 52, 53, 58, 80). The subsequent activation of PKA then results in activation of the Flo8 transcription factor to produce a mucin-like protein, Flo11, that is localized to the cell surface and is required for pseudohyphal growth (44, 50, 63, 67). Although cross-talk between mitogen-activated protein kinase (MAPK) and cAMP signaling pathways is evident (58), transcription factor targets important for filamentous growth appear not to be shared by the two pathways (28, 46). Pseudohyphal defects caused by mutations in *STE12* of the MAPK pathway and *PHD1* are suppressed by the constitutive activation of PKA through deletion of the regulatory subunit gene (*BCY1*) (63).

Candida albicans is a common, opportunistic fungal pathogen that exhibits both budding and filamentous growth when proliferating in host tissues. Filamentous growth of *C. albicans* includes not only the pseudohyphal, elongated yeast-like forms described for *S. cerevisiae* but true hyphae as well. Compared to that of most pathogenic fungi, the morphological response of *C. albicans* to environmental conditions is rapid. Germ tubes are produced within 1 h of placing cells in appropriate conditions. The mechanisms employed by *C. albicans* to quickly achieve this apparently advantageous spectrum of growth morphologies along with optimized metabolic activities are poorly understood.

The relative contribution of yeast and filamentous forms to the pathogenesis of candidiasis is an unresolved issue. However, mutants that do not produce hyphae in vitro have reduced virulence in animal models (29, 49, 73). Expression of hypha-specific virulence factors, such as the hyphal wall protein (*HWP1*) adhesin gene (75, 76) and secreted aspartyl proteinase (*SAP*) genes (68, 77), are correlated with the virulence of hyphal forms. Research into the mechanisms that lead to the production of these virulence factors is important for develop-

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

Strain	Genotype	Parent strain	Reference
SC5314	Wild type		30
CAI4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	SC5314	25
UnoPP-1[a]	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*eno1*::*URA3*/*ENO1*	CAI4	65
CAC1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 CAP1*/*cap1*::*hisG-URA3-hisG*	CAI4	This study
CAC1-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 CAP1*/*cap1*::*hisG*	CAC1	This study
CAC1-1A	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 cap1*::*hisG*/*cap1*::*hisG-URA3-hisG*	CAC1-1	This study
CAC1-1A1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 cap1*::*hisG*/*cap1*::*hisG*	CAC1-1A	This study
CACRE1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 CAP1*/*cap1*::*hisG ENO1*/*eno1*::*URA3*	CAC1-1A1	This study

[a] A CAI4 derivative made Ura$^+$ by disruption of an enolase gene with *URA3* (65).

ing strategies to interfere with candidiasis. Studies of the role of cAMP-dependent signaling in morphogenesis may also bring to light common virulence pathways for distantly related fungal pathogens.

Biochemical studies implicate cAMP increases in promoting bud-hypha transitions of *C. albicans*. Intracellular levels of cAMP increase and, under nutrient limitation, exogenous cAMP or dibutyryl cAMP (dbcAMP) increases the frequency of bud-hypha transitions (14, 61, 62, 86). Inhibitors of cAMP phosphodiesterase or cAMP-dependent protein kinase induce or block germ tube formation, respectively (13, 14). However, genetic studies involving mutational analysis of genes that control cAMP levels and assessment of their role(s) in regulating bud-hypha transitions and filamentous growth have not been reported.

In *S. cerevisiae*, Ras activation of adenylate cyclase also involves the adenylate cyclase-associated protein (CAP, also known as Srv2p) (20, 24, 72). The *CAP* gene was identified in a genetic screen for mutants that suppressed defective growth of a strain carrying an inducible hyperactive *RAS2*^{val19} gene (20). The *CAP* gene was also isolated by screening a yeast cDNA expression library with antisera to a 70-kDa protein that copurified with adenylate cyclase (24). CAP is required for normal budding morphology and growth rates in nutrient-rich media (20, 24). Interestingly, the *S. cerevisiae CAP* gene has been shown to be involved in pseudohyphal differentiation using transposon mutagenesis to screen for mutant strains defective for filamentous growth (57). CAPs of mice (82) and humans (55) are 34% identical and 35% similar to *S. cerevisiae* CAP, showing that *CAP* genes are conserved throughout evolution. Although CAPs from different organisms have similar primary and secondary structures, the function of CAPs in developmental programs has diverged among fungi. CAP mutants of *Schizosaccharomyces pombe* but not *S. cerevisiae* conjugate and sporulate in inappropriate conditions (35).

Modulation of adenylate cyclase activity by CAP in *S. cerevisiae* (24, 85) suggested that the *CAP* gene of *C. albicans* might affect intracellular cAMP levels, allowing assessment of the role of cAMP in the filamentous growth and virulence of *C. albicans*. The *C. albicans CAP1* gene was cloned, and its identity was established by the presence of sequence similarities to *CAP* gene products of other organisms, by the reduction in cAMP levels in *cap1*/*cap1* mutants, and by the ability of exogenous cAMP or dbcAMP to promote bud-hypha transitions and filamentous growth in *cap1*/*cap1* mutants. *cap1*/*cap1* mutants were unconditionally deficient in forming bud-hypha transitions and filamentous growth in rich and minimal, liquid and agar-based culture media as well as in serum and saliva at 37°C. Predictably, *cap1*/*cap1* mutants also showed reduced virulence in a systemic model of candidiasis. This is the first report to provide genetic evidence showing that increases in cAMP promote true hypha formation in *C. albicans*. Interference with *CAP1* function has potential for providing novel strategies for interfering with candidiasis.

MATERIALS AND METHODS

***C. albicans* strains and growth conditions.** *C. albicans* strains are listed in Table 1. Yeast forms were grown in yeast extract peptone dextrose (YPD) or a yeast nitrogen base containing 50 mM glucose (YNB) (66). Mass conversion of stationary-phase yeasts (grown at 30°C for 48 h) to germ tubes was induced at 37°C in the following prewarmed media: Lee's (pH 6.8) (45), medium 199 (Gibco-BRL) with 150 mM HEPES (pH 7.0) (M199), M199 containing 5% bovine calf serum (Sigma) (M199+serum), 50 mM potassium phosphate (pH 6.0) plus 10% bovine calf serum (23), and 10 mM imidazole-HCl buffer (pH 7.0) containing 0.2 mM $MnCl_2$ (with the following inducing agents: 4 mM *N*-acetylglucosamine, 10 mM L-proline plus 10 mM glucose, or 2.5 mM glutamine plus 2.5 mM glucose) (18, 49, 71). Whole human saliva was collected on ice and clarified by centrifugation at 10,000 × *g* for 15 min at 4°C (37). Tetracycline was added to clarified saliva at a concentration of 50 μg/ml to inhibit bacterial growth.

For growth analysis in agar-containing media, stationary-phase yeasts were mixed (100 cells/20 ml of medium) with liquefied agar containing M199 adjusted to a neutral pH with 7.5% sodium bicarbonate, Spider medium (48), 2% agar containing 4% bovine calf serum (49), and synthetic low ammonium dextrose (SLAD) containing 50 μM ammonium sulfate (17). Filamentous growth on YPD agar was assessed by streaking strains on YPD plates followed by incubation at room temperature for 2 weeks. Each plate was examined daily for the presence of filamentous growth.

To determine the effect of exogenous cAMP on bud-hypha transitions and filamentous growth of *cap1*/*cap1* mutants, stationary-phase yeasts were induced to form germ tubes and hyphae in liquid M199+serum (10^6 cells/ml) or in SLAD agar plates containing 10 mM cAMP or dbcAMP (Sigma). M199+serum containing cAMP or dbcAMP was incubated at 37°C for 20 h, and the frequency of germ tube formation was measured at various time points. SLAD plates containing cAMP or dbcAMP were incubated at 37°C for 5 days, and filamentous growth was monitored daily.

Isolation and DNA sequencing of cDNA and genomic clones for *CAP1*. *CAP1* cDNA clones were found while attempting to identify germ tube-specific surface antigens by screening a *C. albicans* germ tube cDNA library (78), but cDNAs encoding cell wall surface proteins were not found. Five of the thirteen cDNA clones isolated encoded proteins with homology to adenylate cyclase-associated proteins. pBluescript SK(−) phagemids of the five clones were rescued by in vivo excision (Stratagene) according to the manufacturer's directions. pCAP1, with a 1,655-bp *CAP1* cDNA insert, was analyzed further.

Three λ genomic *CAP1* clones (CAP2, CAP3, and CAP5) were isolated by screening a λGEM12 genomic library of *C. albicans* SC5314 (6) with *CAP1* cDNA excised from pCAP1 with *Xba*I and *Xho*I. pGHCP17 was constructed by subcloning the 3.7-kbp *CAP1* genomic *Hin*dIII fragment of CAP5 into pBluescript SK(−) and transforming *Escherichia coli* HB 101 (9). DNA sequences of cDNA and genomic clones were determined by automated cycle sequencing using an automated DNA sequencer (ABI Prism, model 377 and 373; Perkin-Elmer Co.).

The complete genomic DNA sequence of *CAP1* was compared with the se-

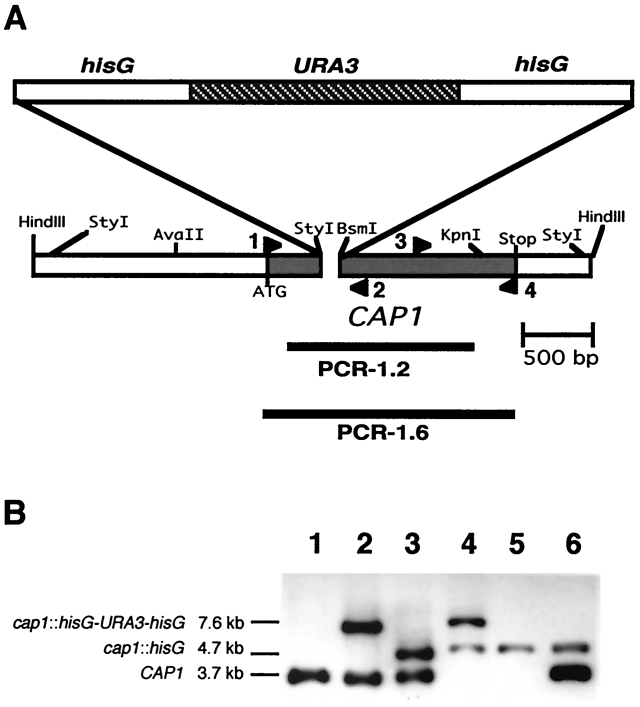

FIG. 1. Disruption of *C. albicans CAP1*. (A) Genetic organization of the *CAP1* locus. The *CAP1* open reading frame (shaded bar) and PCR products (solid line) (PCR-1.2 and PCR-1.6) are indicated. Each arrowhead indicates primers used for RT-PCR to confirm the disruption of *CAP1* (1, CAP-NRT1; 2, CAP-F1; 3, CAP-R3; 4, CAP-3F1). (B) Southern blot analysis of *Hind*III-digested *C. albicans* genomic DNA probed with PCR-1.2 as described in Materials and Methods. Lanes: 1, parental strain CAI4; 2 and 3, *CAP1*/*cap1* strains CAC1 and CAC1-1, Ura^+ and Ura^-, respectively; 4 and 5, homozygous *cap1*/*cap1* strains CAC1-1A and CAC1-1A1, Ura^+ and Ura^-, respectively; 6, *CAP1*-complemented strain CACRE1.

quence of *SRV2* in the current assembly 6 of the *C. albicans* genomic sequences from the Stanford DNA Sequencing and Technology Center website (http://www-sequence.stanford.edu/group/candida).

Disruption of *CAP1*. To disrupt *CAP1* in *C. albicans*, plasmid pCAPURA3 was constructed by replacing a 132-bp *Sty*I-*Bsm*I segment of *CAP1* cDNA in pCAP1 with the 4.0-kbp *Bam*HI-*Bgl*II *hisG-URA3-hisG* cassette from p5921 (25) after generating blunt ends using T4 DNA polymerase (Gibco-BRL) and the Klenow fragment of *E. coli* DNA polymerase I. *E. coli* HB101 served as the host strain for transformation and propagation of pCAPURA3.

CAI4 (*CAP1*/*CAP1 ura3*/*ura3*) was transformed using spheroplast transformation (42) with 10 μg of pCAPURA3 digested with *Pst*I to release the *CAP1* disruption cassette. Ura^+ transformants with a *CAP1*/*cap1*::*hisG-URA3-hisG* genotype were identified by Southern blotting using *Hin*dIII-digested genomic DNA prepared by the method of Scherer and Stevens (69). Southern blots were probed with *hisG-URA3-hisG* from p5921 and PCR-1.2 (Fig. 1A). PCR-1.2 (nucleotides 98 to 1318) was generated by PCR using pGHCP17 as a template and oligonucleotides CAP-R4 (5′-CCATTTTCCAAGAGGAAGCA-3′) and CAP-F4 (5′-CCGACACTGCATTTGCTTTA-3′). Probes were labeled using the enhanced chemiluminescence (ECL) Direct Nucleic Acid Labeling and Detection System (Amersham). CAC1-1 (*ura3*/*ura3 CAP1*/*cap1*::*hisG*) was selected on YNB media (0.002% uridine) containing 0.05% 5-fluoroorotic acid (7) and used in a second round of transformation to disrupt the remaining copy of *CAP1*. Colony PCR (81), using the *Taq*Plus Long PCR system (Stratagene) with primers CAP-R4 and CAP-F4, and Southern blotting were used to determine genotypes. Gene inactivation was confirmed by Northern blot analysis and reverse transcription-PCR (RT-PCR).

Complementation of *cap1*/*cap1* mutants at the *CAP1* genomic locus was accomplished by cotransformation of a *ura3* homozygous *cap1*/*cap1* mutant strain, CAC1-1A1, with *eno*::*URA3* (75) and PCR-1.2, creating CACRE1. DNA sequencing of genomic DNA clones from CACRE1 confirmed that mutations were not inadvertently introduced from PCR-1.2 into the *CAP1* locus in the revertant (data not shown).

Cell morphologies were examined using a 40× or 20× objective and differential interference contrast microscopy (OLYMPUS B×60) and photographed (OLYMPUS Magnafire, model S99806). Colonial morphologies were examined using a stereomicroscope (OLYMPUS SZX12) (1.6× objective) with a transmitted light console base or OLYMPUS BX60 microscope (4× objective), and cellular morphologies at colony rims were examined with bright-field illumination using a light microscope (LABOPHOT-2; Nikon) (10× objective) equipped with a charge-coupled device video camera system (OPTRONICS). Photographed images were processed using Adobe PhotoShop 2.5.

Northern blot analysis. Total RNA was isolated (76) from middle-logarithmic-phase yeasts cultured in 250 ml of YNB at 27°C or in germ tubes (yeasts for the *cap1*/*cap1* mutant) cultured for 3 h in M199 at 37°C and treated with RNase-free DNase I (Gibco-BRL). Probes were PCR-1.2 (Fig. 1A) and a 687-bp PCR product amplified from the 18S rRNA gene of *C. albicans* SC5314 using primers (5′-ACTTTCGATGGTAGGATAG-3′ and 5′-TGATCATCTTCGATCCCCTA-3′) (54). Electrophoresis, radiolabeling of probes using the random primer method (21, 22), hybridization, and molecular size determination were performed as previously described (76), except that blots were hybridized first with the *CAP1* probe (10^7 cpm), autoradiographed, and then hybridized with the 18S rRNA probe (10^6 cpm).

RT-PCR. The first-strand cDNA was synthesized using 1 μg of total RNA according to the manufacturer's directions (Reverse Transcription System; Promega) and was diluted in a final 100-μl volume of nuclease-free water. Two PCR products, representing the 5′ (1 to 605) and 3′ (922 to 1634) portions of *CAP1* message (Fig. 1A), were amplified from the first-strand cDNA (10 μl) using oligonucleotides CAP-NRT1 (5′-ATGTCAACCGAGGAGAGTCA-3′) and CAP-F1 (5′-ATGTACGAGATTGGTGTAGG-3′) and CAP-R3 (5′-AGTGAAAATCCATCTCCAGC-3′) and CAP-3F1 (5′-CCAGCATGTTCAACAATTTGAG-3′), respectively. *ACT1* cDNA (304 bp), amplified using two *ACT1*-specific primers, ACT-3R (5′-GGAGTTGAAAGTGGTTTGGTCAATAC-3′) and ACT-5L (5′-GGCTGGTAGAGACTTGACCAACCATTTG-3′) (59), served as a control. PCR products were detected by Southern blotting using PCR-1.6, which spanned the entire *CAP1* coding region, as a probe (Fig. 1A). PCR-1.6 (nucleotides 1 to 1634) was generated by PCR using pGHCP17 and oligonucleotides CAP-NRT1 and CAP-3F1. Probe PCR-1.6 was labeled with [α-^{32}P]dCTP (Amersham) as for the Northern blot except that 2×10^6 cpm was added to the membrane.

cAMP assay. Intracellular cAMP in M199 was extracted as previously described (20) and measured using the cAMP enzyme immunoassay (Amersham). Strains (UnoPP-1, CAC1, CAC1-1A, and CACRE1) were grown to middle-logarithmic phase (optical density at 600 nm [OD_{600}] = 0.6 to 0.7) in M199 at 27°C and then inoculated (4×10^6 cells/ml) into M199 prewarmed to 37°C to induce germ tubes or fresh M199 at 27°C for budding growth. At each time point during germ tube formation (or budding in the case of the *cap1*/*cap1* mutant), 27- and 1.5-ml portions were withdrawn for measurement of cAMP levels and protein concentrations, respectively.

Protein concentrations (Coomassie protein assay; Pierce) were determined on cell extracts from 1.5 ml of culture lysed by boiling for 5 min in 50 μl of 2 N NaOH. Bovine serum albumin (5 to 25 μg/ml) was used to generate a standard curve.

Virulence studies. The role of the *CAP1* gene in the pathogenesis of systemic candidiasis was investigated using male CBA/J mice (5 to 6 weeks old) as previously described (75). *C. albicans* strains (SC5314 [*CAP1*/*CAP1*], CAC1 [*CAP1*/*cap1*], CAC1-1A [*cap1*/*cap1*] and CACRE1 [*CAP1*/*cap1*, revertant]) were grown to stationary phase in peptone-dextrose media. Cells were then harvested, washed, and resuspended in pyrogen-free 0.9% NaCl at a concentration of 10^6 cells/ml. Four groups of mice (six per group) were injected via the lateral tail vein with 2×10^5 cells in a final volume of 200 μl in two independent studies. Survival was monitored daily. Kidney tissues were cultured on YPD plates to determine the numbers of CFU per gram of tissue and to verify germ tube formation phenotypes. Survival curves were illustrated by the Kaplan-Meier method using the PRISM program 2.0b (GraphPad Software, San Diego, Calif.), and statistical differences between paired groups were compared using the log-rank test.

Nucleotide sequence accession number. The *C. albicans CAP1* genomic sequence has been submitted to GenBank under accession no. AF163838.

RESULTS

Screening and DNA sequence analysis of genomic *CAP1*.

C. albicans cDNAs homologous to *CAP* (also called *SRV2*)

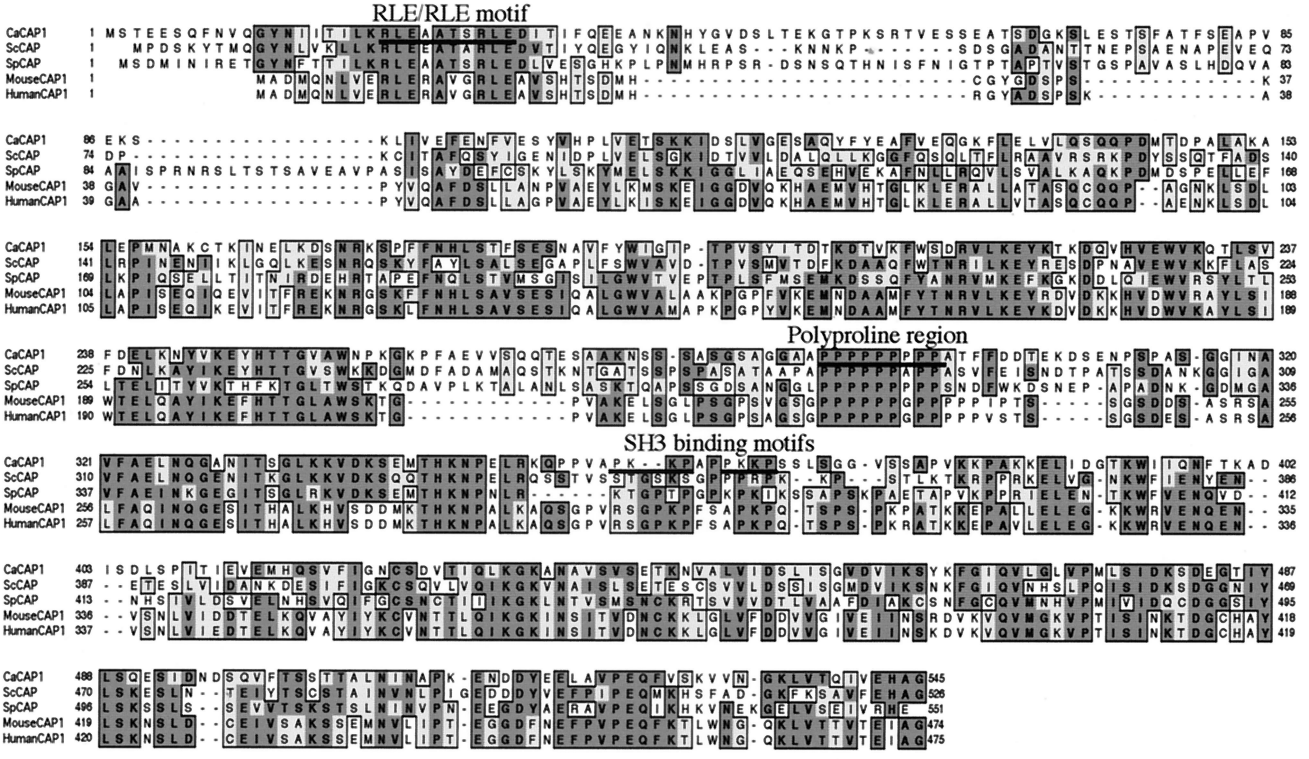

FIG. 2. Primary structure alignment of *C. albicans* Cap1 with CAPs of other organisms. Multiple sequence alignments of CAPs from *C. albicans* (CaCAP1), *S. cerevisiae* (ScCAP), *S. pombe* (SpCAP), mouse (MouseCAP1), and human (HumanCAP1) were performed with ClustalW (79) and illustrated with MacVector 6.5.3 (Oxford Molecular Company). Solid lines indicate residues for the conserved RLE/RLE motif (21 to 30), the polyproline region (289 to 297), and two consensus SH3-binding motifs (358 to 361 and 364 to 367) in *C. albicans*.

genes (see Materials and Methods) were used to isolate three independent genomic clones, each containing a 3.7-kb *Hin*dIII fragment (diagrammed in Fig. 1A) found in *C. albicans* genomic DNA (Fig. 1B, lane 1). A gene encoding an open reading frame identical to that found in the cDNA was named *CAP1* because of similarities to *CAP* genes from other organisms, as described below. The protein product of *CAP1* was designated Cap1. Two silent nucleotide differences were found between *C. albicans CAP1* and *C. albicans SRV2* (reported by the Stanford DNA Sequencing and Technology Center) (assembly 6).

The predicted *C. albicans* Cap1 protein was 28 to 44% identical in overall primary amino acid sequence to CAPs from other organisms. The conserved RLE/RLE motif important for monomer association, protein localization, and Ras/cAMP-dependent signaling (72, 85, 87), the universally conserved and centrally located stretch of proline residues of unknown function, and two consensus SH3-binding motifs (PXXP) were found in *C. albicans* Cap1 (Fig. 2). Interestingly, the first 100 amino acids of *C. albicans* Cap1 showed more dissimilarities to CAPs from other organisms than did the remainder of the protein. The first 100 amino acids of *C. albicans* Cap1 showed only 28.2 and 26.5% identity to the corresponding regions of *S. cerevisiae* and *S. pombe* CAPs, respectively, compared with 45.1 and 41.1% identity in carboxy-terminal regions, respectively.

Predicted secondary structures of *C. albicans* Cap1 and CAP of *S. cerevisiae* were strikingly conserved, with amino-terminal halves consisting of α-helices separated by loops with small regions of β-sheet, and carboxy-terminal thirds consisting of β-sheets and loops (not shown). The central domain containing prolines was predicted to be a loop in both proteins. Hydrophobicity profiles (43) of the two proteins were also similar.

Expression of the *CAP1* gene. *CAP1* was neither a highly expressed nor a developmentally regulated gene (Fig. 3). Detection of the 1.7-kb *CAP1* transcript in yeast (Fig. 3A) and germ tube RNA (Fig. 3B) by Northern blotting required long exposure times. Low mRNA levels were consistent with unbiased codon usage in that the effective number of codons (83), 43.1, was typical of genes that are expressed at low levels, such as those for protein kinase C (*PKC1*) (64) and MAPK (*MKC1*) (60), with values of 45 and 54.8, respectively.

Construction of the *cap1*/*cap1* mutant and *CAP1*-complemented strains of *C. albicans*. Reiterative site-specific disruption of genomic *CAP1* DNA sequences with *hisG-URA3-hisG* or *hisG* produced *Hin*dIII fragments of 7.6 and 4.7 kb in size, respectively, that hybridized to probes for *CAP1* (Fig. 1B) and *hisG-URA3-hisG* DNA (not shown). To verify that phenotypes of the *cap1*/*cap1* mutant were caused by disruption of *CAP1* genes, a complemented strain, CACRE1, was constructed by reintroducing the wild-type *CAP1* DNA into one of the *cap1*::*hisG* loci of the Ura$^-$ *cap1*/*cap1* mutant using cotransformation (75). *CAP1* disruption was confirmed by the absence of *CAP1* mRNA in the *cap1*/*cap1* mutant CAC1-1A in Northern blot analysis (Fig. 3A and B). To show that read-through or truncated *CAP1* mRNA was not present in the *cap1*/*cap1* mutant, RT-PCRs were performed using *CAP1*-specific primers.

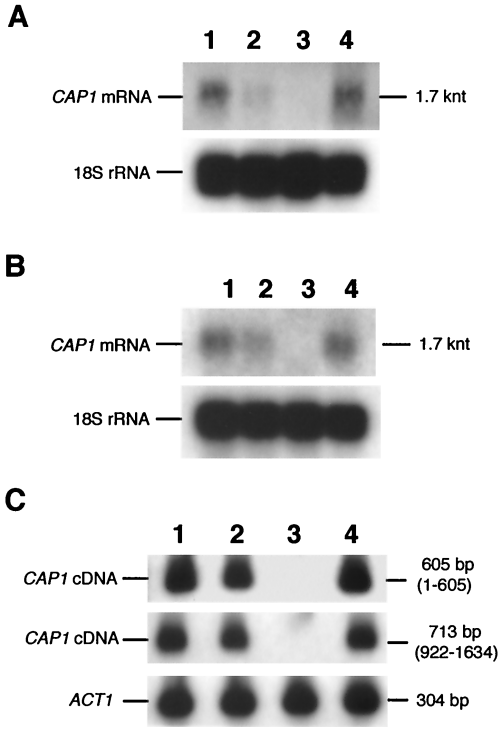

FIG. 3. Northern blot and RT-PCR analysis of *cap1*/*cap1* mutants. *CAP1* mRNA is absent in the *cap1*/*cap1* strain and present at equivalent low levels in other strains during yeast growth (A) or germ tube induction (B). Total RNA (7 μg/lane), isolated as described in Materials and Methods, was separated in a formaldehyde agarose gel transferred to a nitrocellulose membrane and probed with radiolabeled PCR-1.2 to detect *CAP1* mRNA and 18S rRNA as a control. The membrane was exposed to X-ray film for 7 days for detection of *CAP1* mRNA and for 4 h for detection of 18S rRNA. (C) Amplification of 5′ (605 bp, 1 to 605) and 3′ (713 bp, 922 to 1634) portions of *CAP1* mRNA using RT-PCR followed by Southern blotting using radiolabeled PCR-1.6 as probe. *ACT1* mRNA (304 bp) was amplified as a positive control. Lanes 1 to 4, strains UnoPP-1, CAC1, CAC1-1A, and CACRE1.

CAP1 mRNA could not be detected using a probe (PCR-1.6) which spans the entire coding region of *CAP1* (Fig. 3C). Equivalent levels of *ACT1* cDNA (304 bp) were present in all strains (Fig. 3C). The *cap1*/*cap1* mutant does not have *CAP1* mRNA and cannot produce full or truncated Cap1 proteins.

Analysis of *cap1*/*cap1* mutants. Growth rates of the *cap1*/*cap1* mutant were equivalent to that of the other strains in rich medium (YPD) but were reduced in minimal medium (YNB) (Table 2). Budding appeared morphologically normal in both media (not shown).

Mass conversion of yeasts to germ tubes (bud-hypha transitions) was induced in liquid media. *cap1*/*cap1* mutants were unconditionally deficient in producing germ tubes in liquid suspension compared to *CAP1*/*cap1* and *CAP1*/*CAP1* strains. For the latter strains, the percentages of yeasts with germ tubes approached 100% in Lee's medium (pH 6.8), M199, M199 with 5% bovine serum albumin, and saliva (Fig. 4). Media containing simple inducers also did not support germ tube production by *cap1*/*cap1* yeasts (not shown). *cap1*/*cap1* yeast cells in M199 with or without serum appeared elongated or pseudohyphal, but germ tubes were not seen. *cap1*/*cap1* mutant cells budded in all conditions, as determined by cell counting and differential labeling of parent yeasts with anti-*C. albicans* antiserum, permitting unlabeled nascent buds and yeasts produced during the incubation period to be distinguished from inoculum yeasts (not shown).

Upon prolonged incubation, germ tubes were found at low frequencies in cultures of the *cap1*/*cap1* mutant (Fig. 4B). After 20 h of incubation in M199 and in saliva, a few (<10%) *cap1*/*cap1* yeast cells had germ tubes. In M199 containing 5% serum, the percentage was higher (approximately 20 to 30%), resembling cultures of wild-type strains inoculated at cell concentrations that exceed the threshold for germ tube formation (34). Reducing the inoculum led to the emergence of germ tubes in approximately 40% of the cells after 5 h of incubation in M199+serum. By 9 h, most *cap1*/*cap1* mutant cells (>80%) had formed germ tubes (not shown). Germ tubes of *cap1*/*cap1* mutant cells were shorter in length than wild-type germ tubes at 20 h. Further reductions in inoculum concentration did not lead to a higher frequency of germ tube formation. Germ tube formation in the *cap1*/*cap1* mutant in the presence of serum was deficient in that the time required to form germ tubes averaged four to five times longer and average frequencies of germ tube-forming cells were reduced for *cap1*/*cap1* mutant cells compared to strains with *CAP1*. Similar results were found in 10% serum with 50 mM potassium phosphate buffer (pH 6.0) (not shown).

The ability of *cap1*/*cap1* mutant cells to form germ tubes upon prolonged incubation was limited to media containing serum. Lowering the cell concentration did not enhance germ tube formation in any of the other media tested, including saliva or M199 without serum.

The *cap1*/*cap1* mutant was also unconditionally deficient in producing filamentous growth on agar-containing media (Fig. 5). *CAP1* strains grew predominantly as hyphae, but in some cases, pseudohyphae were also seen. The term "filamentous growth" refers collectively to the production of pseudohyphae as well as true hyphae. The periphery of colonies with circular symmetry of *CAP1* strains in Spider or M199 medium consisted of extended hyphae with short branches, whereas hyphae in SLAD were septate, with numerous buds and thick-walled terminal buds resembling chlamydospores at hyphal tips. Characteristics of *CAP1* strains in asymmetric colonies in serum media were mixed, consisting primarily of numerous branched hyphae bereft of buds and infrequent filaments coated with buds. The spectrum of morphological responses exhibited by strains with *CAP1* was absent in colonies produced by *cap1*/*cap1* mutant cells that consisted of budding yeasts independent

TABLE 2. Doubling times

Strain	Doubling time (h)[a] in:					
	Rich medium (YPD)			Minimal medium (YNB)		
	27°C	30°C	37°C	27°C	30°C	37°C
UnoPP-1	2.2	1.6	2.0	2.9	2.9	3.0
CAC1	2.2	1.7	2.0	2.9	2.9	3.0
CAC1-1A	2.3	1.7	2.2	3.9	3.7	3.8
CACRE1	2.2	1.7	2.1	2.8	2.9	2.9

[a] Mean value from two independent experiments whose results differed by less than 20%.

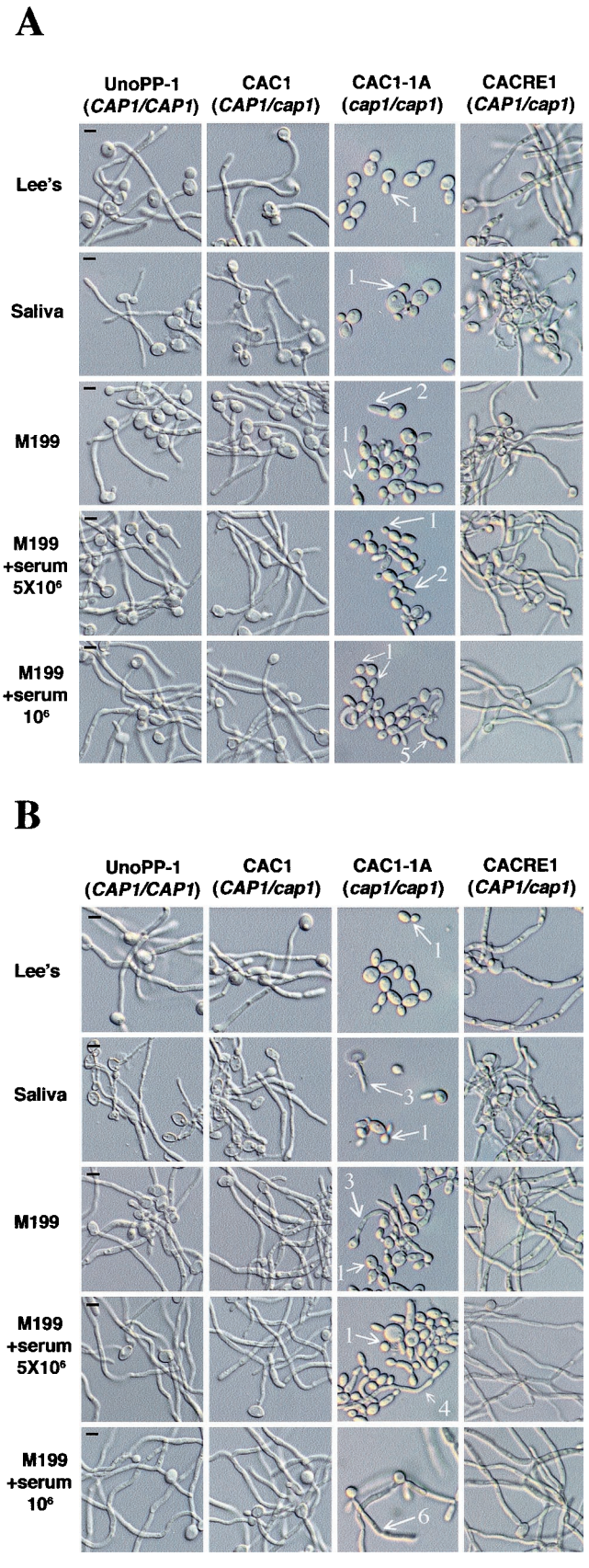

FIG. 4. *cap1*/*cap1* strains are defective in bud-hypha transitions. Germ tubes were induced at cell concentrations of 5×10^6 cells/ml (A and B, first four rows) or 10^6 cells/ml (A and B, bottom rows) in prewarmed Lee's medium, saliva, M199, or M199+serum for 5 h (A) and 20 h (B). *cap1*/*cap1* mutant cells formed buds (arrows 1) or pseudohyphae at low frequency (arrows 2), whereas strains having *CAP1* (UnoPP-1, CAC1, and CACRE1) produced typical germ tubes (A and B, first two and fourth columns). At 20 h a few *cap1*/*cap1* mutant yeasts (<10%) produced germ tubes in saliva or M199 (arrows 3). In the presence of serum the frequency of germ tube formation was higher (20 to 30%) (arrow 4). Reducing the inoculum concentration in the presence of serum led to production of germ tubes by 40% of *cap1*/*cap1* mutant yeasts at 5 h (arrow 5), and at 20 h the majority of yeasts had formed germ tubes that were shorter than those of the other strains (arrow 6). Bars, 5 μm.

of medium composition. Strains with *CAP1* formed filamentous growth on YPD agar at as early as 1 week, but *cap1*/*cap1* mutant colonies were devoid of filamentous growth even after 2 weeks of culture (not shown).

A single allele of *CAP1* was sufficient for normal bud-hypha transitions and filamentous growth of *C. albicans*. Differences in the timing of germ tube emergence, in the length of hyphae in liquid media, or in colonial morphologies in agar media between strains with one or two copies of the *CAP1* gene were not observed.

Measurement of intracellular cAMP levels during germ tube induction. Cytoplasmic cAMP levels were measured under conditions that induce germ tubes (M199 at 37°C) or lead to budding (M199 at 27°C) in wild-type strains. Yeasts grown to middle-logarithmic phase in M199 at 27°C were used as the inoculum. Under germ tube-inducing conditions, the majority of the cells (>95%) in strains with *CAP1* had germ tubes by 3 h, whereas *cap1*/*cap1* cells produced buds (Fig. 6C).

Intracellular cAMP levels of strains with *CAP1* increased sharply after placement in induction conditions, peaking at levels that were 2- to 2.5-fold higher than initial concentrations at 1 h (Fig. 6A). After a small decrease at 2 h, cAMP levels gradually increased over the 5-h incubation period. Consistent with the results for germ tube induction described above, copy number effects were not seen for *CAP1* in regulating cAMP levels prior to germ tube emergence. Significant differences in cAMP levels between *CAP1*/*CAP1* and *CAP1*/*cap1* strains were not observed. The *cap1*/*cap1* mutant exhibited a small increase in cAMP at 30 min that plateaued and achieved only a 1.5-fold increase over the 5-h period.

The increase in the cAMP level for *CAP1* strains was not seen under conditions where germ tubes were not induced (Fig. 6B).

The effect of cAMP or dbcAMP on colonial morphologies and bud-hypha transitions of the *cap1*/*cap1* mutant. If the reduced cAMP levels were responsible for the defective bud-hypha transitions and colonial morphologies of the *cap1*/*cap1* mutant, then exogenous addition of cAMP should reverse the defects. Both cAMP and dbcAMP dramatically altered the colony morphology of the *cap1*/*cap1* mutant (Fig. 7A). Filamentous growth that closely resembled that of the positive control *CAP1* strain was induced. The timing of the onset of filamentous growth for *CAP1* strains and for the *cap1*/*cap1* mutant induced by cAMP and dbcAMP was the same, 2 days. dbcAMP was more dramatic in restoring filamentous growth to the *cap1*/*cap1* mutant strain than cAMP (Fig. 7A), indicating

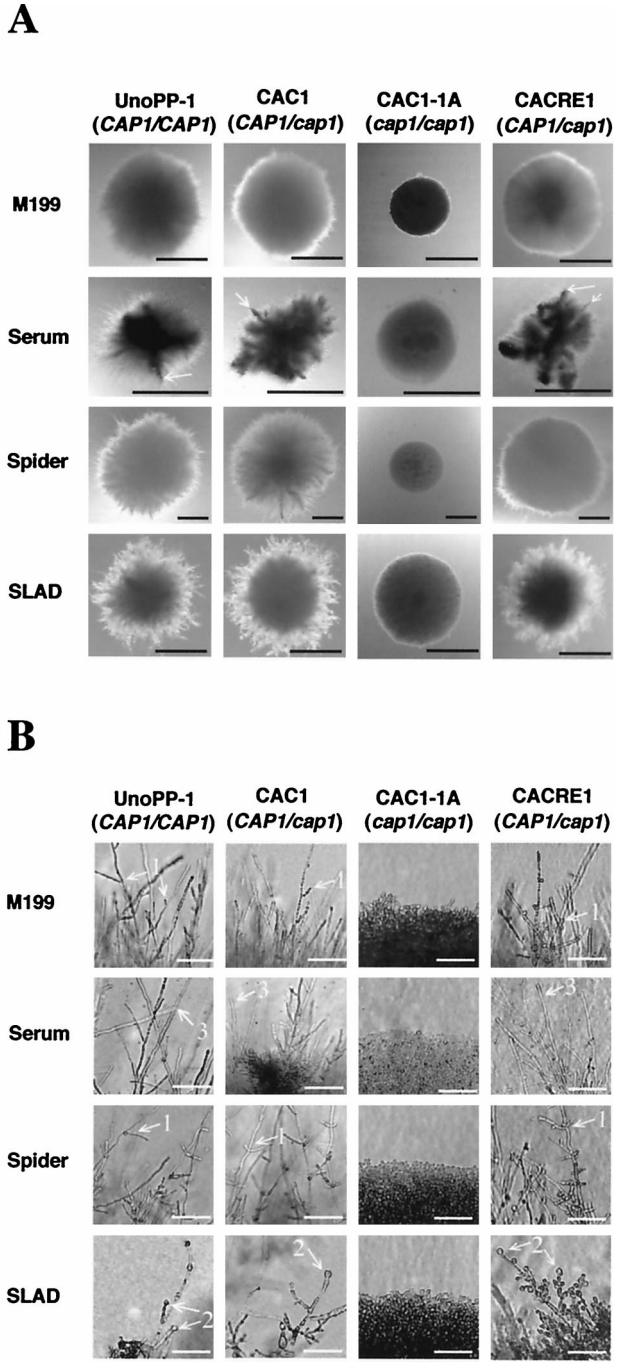

FIG. 5. *cap1*/*cap1* strains are defective in filamentous growth. Colonial appearances (A) and cellular morphologies at colony rims (B), respectively, in each agar medium condition are shown. (A) Colonies of the *cap1*/*cap1* mutant consisted of budding yeasts (A and B, third columns), whereas strains with *CAP1* (UnoPP-1, CAC1, and CACRE1) produced filamentous growths of differing characteristics depending on the media. The asymmetric colonies formed by strains with *CAP1* in serum contained infrequent thick plumes composed of filaments covered with buds radiating from the colony center (arrow). (B) Strains with *CAP1* produced uniform hyphae with short branches in M199 and Spider plates (arrows 1) or hyphae with thick-walled terminal buds in SLAD medium (arrow 2). In media with serum, colonies of strains with *CAP1* were composed primarily of hyphae bereft of buds (arrow 3). M199 plates were incubated first at 30°C for 48 h and transferred to 37°C for another 48 h, whereas the other plates were incubated for 6 days at 37°C. Black (A) and white (B) bars, 1 mm and 50 μm, respectively.

that dbcAMP may be taken up by cells more efficiently than cAMP. Filamentous growth of the wild-type strain also appeared to be slightly enhanced in the presence of cAMP and dbcAMP (Fig. 7A).

Hypha formation of the *cap1*/*cap1* mutant in liquid media (M199+serum) was also enhanced by the addition of dbcAMP (10 mM). Hyphae of the *cap1*/*cap1* mutant were much longer, and more hyphae and pseudohyphae were seen, if the media contained dbcAMP. The results appeared most dramatic at 13 h (Fig. 7B). At 3 h twice as many pseudohyphae were detected and the pseudohyphae were longer in the presence of dbcAMP (not shown). Thus, dbcAMP decreased the time required for the emergence of filamentous structures. It was difficult to estimate the effect of exogenous dbcAMP on enhancement of hyphal formation of the wild-type strain because of extensive hypha formation produced independently of the presence of dbcAMP (Fig. 7B). Exogenous cAMP (10 mM) produced similar but less dramatic effects on hyphal formation of the *cap1*/*cap1* mutant (not shown).

These results are consistent with *CAP1* regulation of bud-hypha transitions of *C. albicans* by modulation of cAMP levels.

Virulence studies. Mice injected with the wild-type *C. albicans* strain (SC5314) expired within 10 days of injection (Fig. 8). *C. albicans* strains with a single copy of the *CAP1* gene (CAC1 and CACRE1) showed reduced virulence compared with the parental *CAP1*/*CAP1* strain ($P = 0.0006$); however, 80% of the mice became ill and were sacrificed by 35 days (Fig. 8). In contrast, six mice given the *cap1*/*cap1* mutant survived and behaved normally during the entire period of observation. The survival rate of mice injected with the *cap1*/*cap1* mutant was significantly different from that of control strains (SC5314 versus CAC1-1A, $P = 0.0006$; CAC1 versus CAC1-1A, $P = 0.0007$; CACRE1 versus CAC1-1A, $P = 0.0069$). No statistically significant difference was found between results for the heterozygous *CAP1*/*cap1* mutant (CAC1) and the revertant (CACRE1) ($P = 0.3661$). CFU of *C. albicans* were detected in sacrificed mice injected with *CAP1* strains (10^7 CFU per g of kidney). Of the six mice injected with the *cap1*/*cap1* mutant, three had infected kidneys (1.9×10^8 CFU per g of kidney) and three cleared the infection. Yeasts isolated from kidneys of mice that received the *cap1*/*cap1* mutant showed the same defects in forming germ tubes as those used for intravenous injection, verifying the authenticity of strains and the importance of normal kinetics of germ tube formation in virulence.

DISCUSSION

Identification and molecular cloning of the *CAP1* gene of *C. albicans*. Structural features of *C. albicans* Cap1 conformed closely to those of adenylate cyclase-associated proteins from other organisms (24, 35, 55, 82, 87). Amino- and carboxy-terminal halves rich in alpha-helices and beta-sheets, respectively, separated by a central loop containing a stretch of prolines, are typical of CAPs that have two domains with separable functions. The SH3 binding motifs and the conserved actin-binding region at the carboxy terminus may interact with an Abp1 homologue and actin monomers in *C. albicans*, as has been shown for similar regions of *S. cerevisiae* CAP (26, 27, 47, 85). An Abpl homologue was found in the *C. albicans* genome. Differences in cAMP responses of the *cap1*/*cap1* mutant from

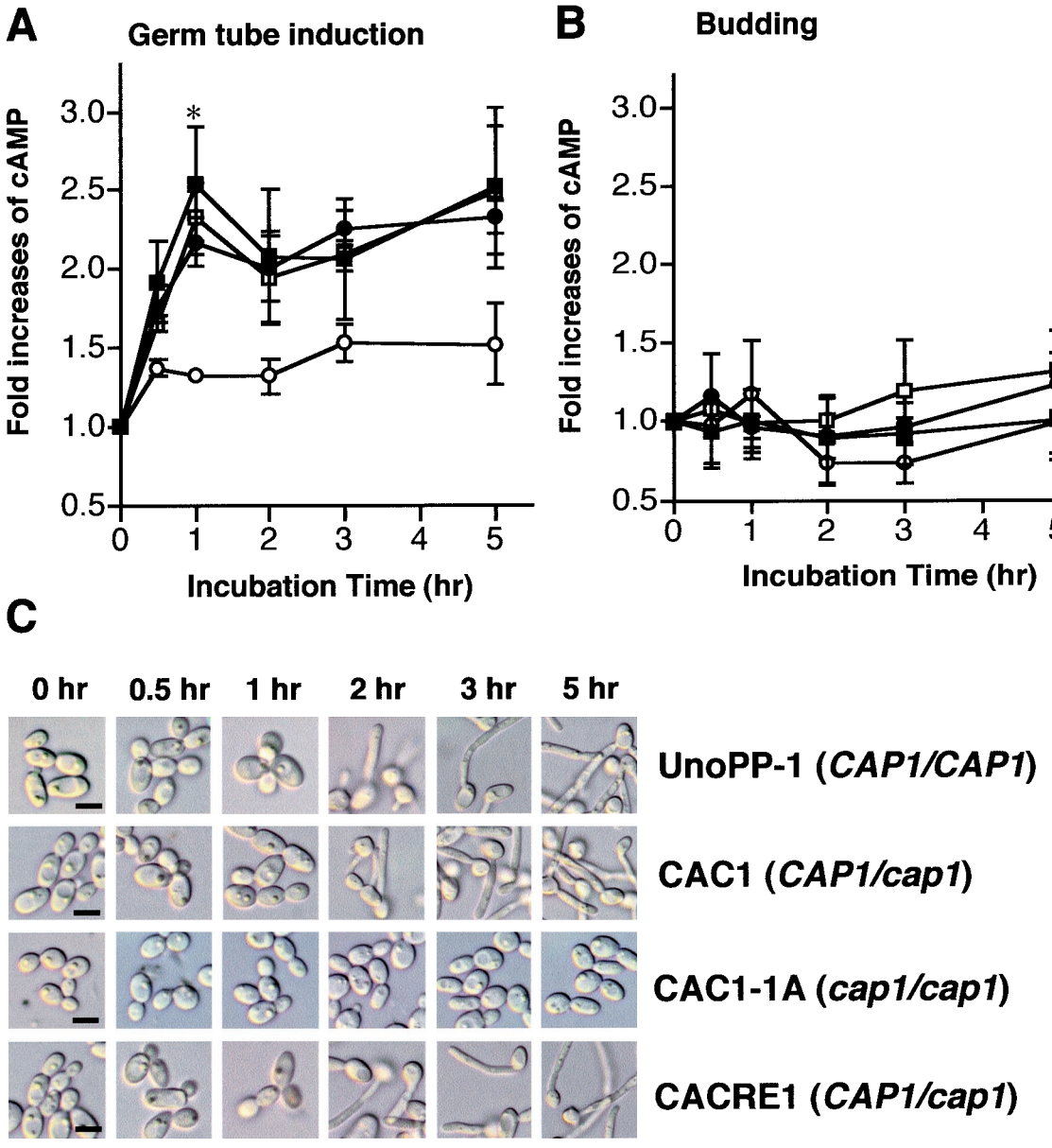

FIG. 6. Reduced cAMP levels of the *C. albicans cap1*/*cap1* mutant in germ tube-inducing conditions compared to those of strains with *CAP1*. Intracellular cAMP levels for each strain (UnoPP-1 [*CAP1*/*CAP1*] [□], CAC1 [*CAP1*/*cap1*] [■], CACRE1 [*CAP1*/*cap1*] [●], and CAC1-1A [*cap1*/*cap1*] [○]) were measured as described in Materials and Methods. Each value in the *y* axis indicates the fold increase in cAMP over the basal level in each strain at time zero. Error bars indicate the standard deviation of each value from three independent experiments performed in triplicate. (A) Germ tube-inducing conditions (M199 at 37°C). cAMP levels (picomoles per milligram of protein) at time zero for UnoPP-1, CAC1, CAC1-1A, and CACRE1 were 45.3 ± 4.6, 55.1 ± 6.9, 61.8 ± 6.5, and 51.4 ± 6.7 (mean value ± standard deviation), respectively. The decreased cAMP level in the *CAP1*/*cap1* mutant compared to results for strains with *CAP1* at 1 h was statistically significant (asterisk, $P < 0.01$ [UnoPP-1 or CAC1 versus CAC1-1A] and $P < 0.05$ [CACRE1 versus CAC1-1A] using Bonferroni's multiple comparison test performed with Prism 2.0b [GraphPad Software]). (B) Budding growth in M199 at 27°C. cAMP levels (picomoles per milligram of protein) at time zero for UnoPP-1, CAC1, CAC1-1A, and CACRE1 were 50.9 ± 22.4, 58.1 ± 8.4, 37.4 ± 2.9, and 52.6 ± 6.6, respectively. (C) Morphological changes of UnoPP-1 (*CAP1*/*CAP1*), *CAP1*/*cap1* strain (CAC1 and CACRE1), and *cap1*/*cap1* strain (CAC1-1A) were monitored during germ tube induction. Bars, 5 μm.

those of isogenic *CAP1* strains indicate that Cap1 regulates adenylate cyclase activity. cAMP or its membrane-permeable derivative, dbcAMP, partially restored filamentation and enhanced hypha production of the *cap1*/*cap1* mutant strain, further confirming that Cap1 acts through regulation of cAMP levels. *CAP1* encodes the adenylate cyclase-associated protein of *C. albicans*.

The role of cAMP in hypha production and filamentous growth of *C. albicans*. Increases in cAMP levels under conditions used in this study were directly correlated with bud-hypha transitions and were not simply a response to the presence of fresh media. Comparable cultures placed under conditions supporting budding growth did not show increases in the cAMP level. These results agree with earlier reports of in-

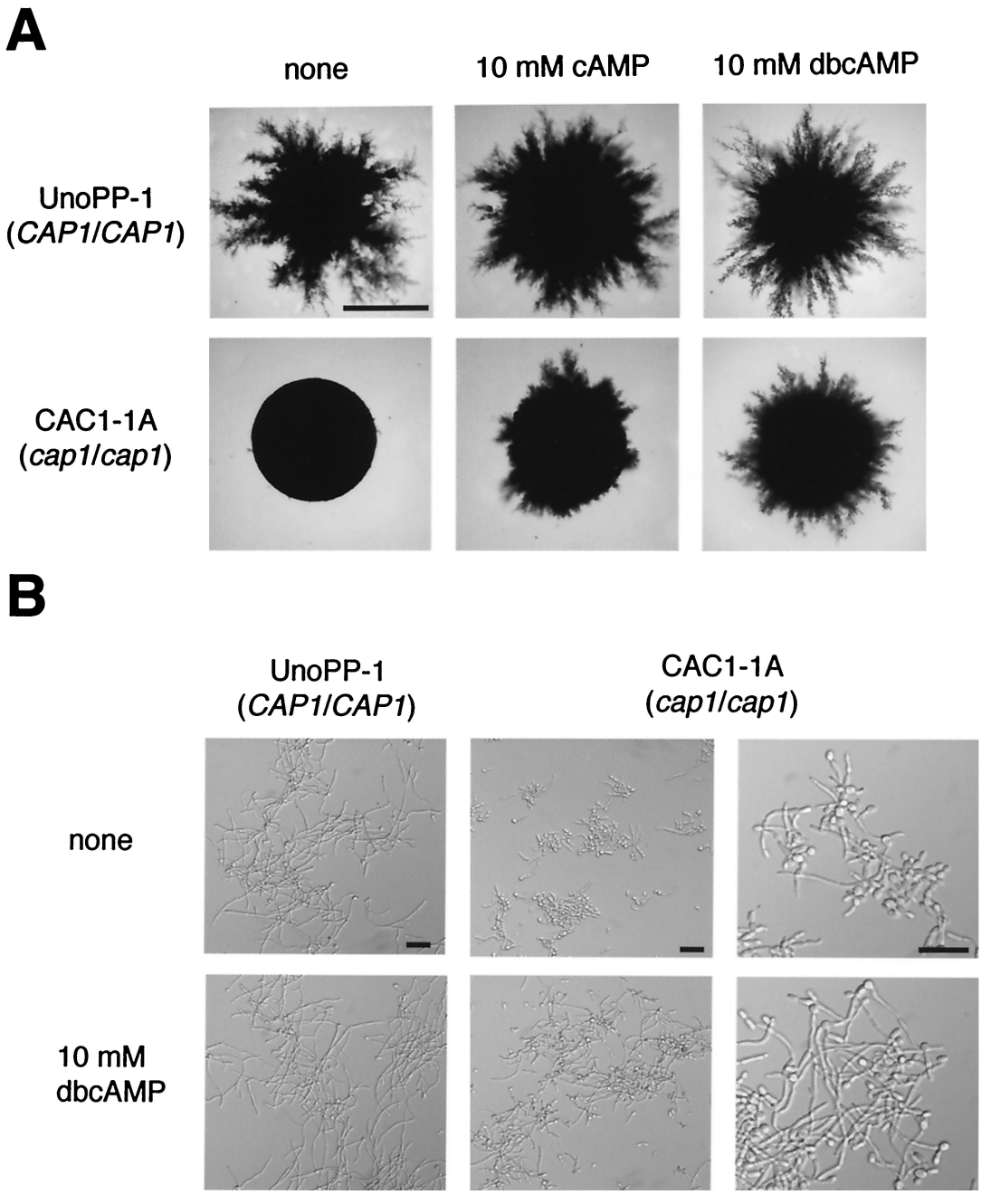

FIG. 7. Suppression of defective bud-hypha transitions and filamentous growth in the *cap1*/*cap1* mutant by exogenous cAMP or its derivative, dbcAMP. (A) The wild-type *CAP1*/*CAP1* strain, UnoPP-1, and the *cap1*/*cap1* mutant strain, CAC1-1A, were grown in SLAD medium with or without 10 mM cAMP or dbcAMP for 5 days at 37°C. Bars, 1 mm. (B) Bud-hypha transitions were induced at cell concentrations of 10^6 cells/ml in prewarmed M199+serum with or without 10 mM dbcAMP for 13 h (first [UnoPP-1] and second [CAC1-1A] columns, 20× objective; third [CAC1-1A] column, 40× objective). Bars, 30 μm.

creases in cAMP levels prior to and accompanying germ tube formation (14, 16, 62). In accord with our findings, cAMP levels are generally found to be low in budding yeasts that are used to induce germ tubes, except in one study (19), which reported basal cAMP levels to be threefold higher than in the other studies at time zero. But cAMP levels dropped within 15 min to levels that were consistent with the time zero values of the other studies, prior to rising. Reasons for the differences are unknown, but the use of late-stationary-phase yeasts (96 h) to induce germ tubes might have contributed to the high cAMP levels at time zero.

The ability of the majority of *cap1*/*cap1* mutant cells to produce hyphae upon prolonged incubation in serum is consistent with a role for cAMP in germ tube formation. An increased length of time may be required for accumulation of threshold levels of cAMP in *cap1*/*cap1* mutant cells that are unable to generate pulses of cAMP but are able to generate cAMP at reduced rates independently of Cap1. The presence

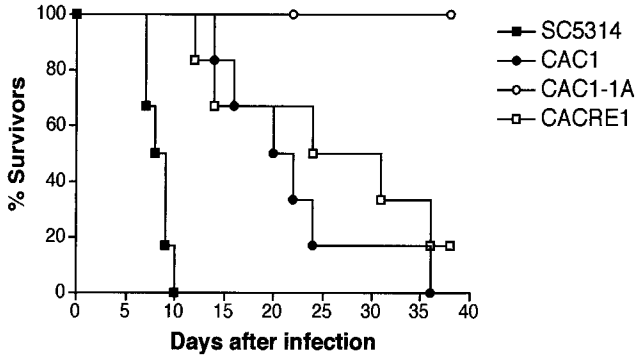

FIG. 8. Survival curves of mice (CBA/J, 5 to 6 weeks old, six mice per group) infected with 2×10^5 cells of *C. albicans* strains SC5314 (*CAP1*/*CAP1*), CAC1 (*CAP1*/*cap1*), CAC1-1A (*CAP1*/*cap1*), and CACRE1 (*CAP1*/*cap1*, revertant). Similar results were obtained in two independent experiments. Survival curves were illustrated according to the Kaplan-Meier method using the PRISM program and compared using the log-rank test. A P value of <0.05 was considered significant.

of mechanisms independent of Cap1 with lesser effects on cAMP levels is shown by the small increase in cAMP in the *cap1*/*cap1* mutant under germ tube induction conditions. Also, cAMP levels in middle-logarithmic-phase cultures of *cap1*/*cap1* and *CAP1* strains were similar, indicating that, as is found for *S. cerevisiae* (20), basal levels of cAMP are not under Cap1 control in *C. albicans*. Steroid hormones (38) and unidentified factors of low molecular weight in serum and seminal fluid that promote hyphal formation (4, 23) may interact with *C. albicans* G protein-coupled receptors, leading to Cap1-independent cAMP responses in *C. albicans*. Even cAMP itself, which is present in serum at low levels (36), may work in combination with other factors to promote delayed hypha formation in serum in *cap1*/*cap1* mutant cells. Superior hypha-inducing properties of serum relative to other conditions have been noted by others (13, 23, 49). Reasons for the formation of hyphae, albeit at low frequencies, upon prolonged incubation in saliva and M199 without serum are also unknown but may reflect cell cycle influences on the bud-hypha transition (51).

The availability of the *cap1*/*cap1* mutant that grows in yeast forms under hypha-inducing conditions permitted us to clearly show for the first time that cAMP profoundly affects bud-hypha transitions and filamentous growth in *C. albicans*. For strains with *CAP1* genes, the role of cAMP was difficult to detect because of the filamentous appearance of wild-type colonies. Addition of cAMP or its membrane-permeable derivative, dbcAMP, to the *cap1*/*cap1* mutant in agar media promoted growth as filamentous rather than yeast colonies. Filamentous growth of the *cap1*/*cap1* mutant in the presence of dbcAMP was not quite as extensive as for *CAP1* strains. Insufficient uptake or rapid degradation of exogenous cAMP or dbcAMP of *cap1*/*cap1* cells might have led to an incomplete restoration of filamentous growth. For *S. cerevisiae*, the ability to take up cAMP is greatly enhanced by the presence of at least one *cam* mutation. Without at least one *cam* mutation, strains having mutations in the gene encoding adenylate cyclase, *CYR1*, cannot survive. One of the *cam* mutations causes a loss of *PDE* function, whereas the others are uncharacterized (32, 33; Warren Heideman, personal communication). By analogy with *S. cerevisiae*, disruption of the *C. albicans PDE2* gene would be predicted to generate strains with enhanced filamentous growth properties (41, 52, 63).

The cAMP-dependent signaling pathway in *C. albicans*. The positive correlation between addition of cAMP and filamentous growth in both *S. cerevisiae* and *C. albicans* (52, 53, 61, 86; this study) along with the requirement of CAP for filamentous growth of *S. cerevisiae* (57) suggest that the cAMP-dependent signaling pathway of *S. cerevisiae* during pseudohyphal growth is a good working model for the *C. albicans* cAMP-dependent signaling pathway during bud-hypha transitions. Gpr1-Gpa2 regulation of cAMP signaling may be also conserved in *C. albicans*. A Gpr1 homologue with 43% identity in the first five transmembrane regions and an overall identity of 19% to *S. cerevisiae* Gpr1 was found in the *C. albicans* genome, as was a Gpa2 homologue (CAG99) with an overall identity of 43% to *S. cerevisiae* Gpa2. *C. albicans* Ras1 is strongly implicated in cAMP signaling by its 50% identity to Ras2 of *S. cerevisiae*, which interacts with CAP and affects cAMP levels. Importantly, the phenotype of *ras1*/*ras1* null mutants of *C. albicans* is very similar to that of the *cap1*/*cap1* mutant, with defective bud-hypha transitions and filamentous growth in all hypha-inducing conditions investigated, including both liquid and solid media containing serum at 37°C. The similarity in phenotypes between *C. albicans ras1*/*ras1* mutants and *cap1*/*cap1* mutants strongly suggests that *C. albicans RAS1* acts in the same signal transduction pathway as *CAP1*, the cAMP-dependent signaling pathway (23). Phenotypic similarities also potentially connect a recently identified Cdc2-related kinase, *CRK1* (15), to *CAP1* and *RAS1*. *CRK1* gene null mutants have a profound defect in hyphal development in all media tested and express reduced amounts of hypha-specific genes under germ tube-inducing conditions. We have also found reduced amounts of *HWP1* expression in *cap1*/*cap1* mutants (not shown). Crk1 has been suggested to be one of the downstream targets of Ras1 in hyphal development of *C. albicans*. The transcription factors in *C. albicans* targeted by cAMP signaling are less clear. Crk1 and $Ras1^{V13}$ suppress the defects in hypha production of *C. albicans cph1*/*cph1 efg1*/*efg1*, pointing to the presence of an unknown transcription factor(s) that serves as a downstream target of cAMP signaling. Expression of the *C. albicans CRK1* gene in *S. cerevisiae* led to enhanced filamentous growth that was dependent on Flo8, a PKA-dependent transcription factor. But a homologue of Flo8 has not been found in the *C. albicans* genome. Another part of the cAMP signaling pathway that is poorly understood involves PKA. Unlike the case with *cap1*/*cap1* and *ras1*/*ras1* mutants, defective germ tube formation is not seen at 37°C on solid media in *C. albicans* strains lacking *TPK2*, which encodes a catalytic subunit of PKA (74). Whether additional *TPK* genes with differing effects on filamentous growth, as is found in *S. cerevisiae* (63), are present in *C. albicans* is unknown. A gene encoding the regulatory subunit of PKA has been identified in the *C. albicans* genome. The role of the PKA regulatory subunit gene in bud-hypha transitions and filamentous growth is currently under investigation in this laboratory.

Defects in hypha formation have been reported for a growing list of null mutants in signal transduction pathway genes; however, the media and temperatures that are required to detect the phenotype for most genes are limited compared to

the case with the *cap1*/*cap1* mutant. Null mutants devoid of any one of many other signal transduction pathway genes, such as *COS1, SSK1*, or MAPK cascade genes (*CST20, HST7, CEK1*, and *CPH1*), have medium-conditional deficiencies in filamentous growth (1, 11, 17, 39, 48). Strains with mutations of both alleles of the MAPK genes are unable to produce filamentous growth in solid Spider medium but make normal hyphae in all other solid or liquid media tested (17, 39, 48). The *COS1* and *SSK1* genes, encoding proteins involved in a two-component signaling pathway, are required for hyphal development in solid media but not in liquid media (1, 11). The phenotypes of *cap1*/*cap1, ras1*/*ras1*, and *crk1*/*crk1* mutants suggest that the presence of defective hypha formation in serum-containing medium at 37°C provides a means for identifying proteins involved in the cAMP-dependent pathway.

The role of Cap1 in hypha production. Cap1 is required for normal hyphal development under all conditions examined. The ability of *cap1*/*cap1* mutants to form germ tubes after a delay and correction of the phenotype by exogenous cAMP and dbcAMP indicate that modulation of cAMP levels, and not cytoskeletal interactions, is probably responsible for the hypha-promoting effect of Cap1 in *C. albicans*. This result is consistent with studies in *S. cerevisiae* showing that neither targeting of CAP to actin cortical patches through the SH3 binding domain nor interaction of CAP with actin monomers is necessary for CAP to transduce cAMP signals (85, 87).

The absence of the growth defects and aberrant budding phenotypes in *C. albicans cap1*/*cap1* mutants compared to results with *S. cerevisiae* and *S. pombe cap* null mutants points to possible differences in Cap protein-actin interactions that may relate to the capacity of *C. albicans*, but not the other yeasts, to form germ tubes and true hyphae (24, 35). Although related, pseudohyphal formation and true hyphal formation are distinct processes that are characterized both by morphological differences and by differences in gene expression patterns in *C. albicans*. Cap1 may be in part responsible for the morphological differences between germ tubes and pseudohyphae. In *S. cerevisiae*, the interaction of CAP with actin monomers through the 27 carboxy-terminal amino acids (87) may prevent the hyperpolarization and accentuated concentration of actin filaments seen in buds of *cap* null mutants (5). However, filamentous actin is highly concentrated at the hyphal tip in *C. albicans* germ tubes and true hyphae (3). Growth from hyphal tips may require weaker interactions between Cap1 and actin for *C. albicans* than for *S. cerevisiae* to facilitate polarized growth during germ tube and hypha formation. The results suggest that CAP function is not required for cytoskeletal organization in *C. albicans*, as it is in *S. cerevisiae*.

The mechanism of Cap1-mediated modulation of bud-hypha transitions and filamentous growth is unknown. Studies with *S. cerevisiae* suggest that the cAMP-dependent pathway causes cells to undergo unipolar budding, a process that, when coupled with elongated growth controlled by the MAPK pathway, produces pseudohyphal cells (63). Mösch and Fink reported that the *S. cerevisiae CAP*/*SRV2* mutant constructed by transposon mutagenesis is defective in pseudohyphal growth and undergoes random budding (57). These reports prompt the idea that *C. albicans* Cap1 may function to interrupt processes important for budding and that interruption of budding processes is required for bud-hypha transitions and filamentous growth.

Role of Cap1 in virulence. The avirulence of the *cap1*/*cap1* mutant extends the findings of other studies (11, 12, 49, 70, 84) in showing that the ability to produce hyphae with normal kinetics, as well as the absolute ability to produce hyphae, is important for candidiasis. The avirulence of *cap1*/*cap1* mutants is also supportive of an important role for the cAMP signaling pathway in the growth of *C. albicans* in host tissue. The rapid production of hypha-specific factors, such as Hwp1 adhesin (75) and others (68, 77), coincident with germ tube formation is likely to be important for systemic candidiasis in mice. The virulence study shows that *C. albicans* joins other pathogenic fungi in the involvement of the cAMP signaling pathway in pathogenesis. Disruption of the gene encoding the catalytic subunit of cAMP-dependent PKA and disruption of the *GPA1* gene affect the virulence of *M. grisea* (56) and *Cryptococcus neoformans* (2), respectively.

The divergent phenotypes of *cap* mutants in *S. cerevisiae* and *S. pombe* illustrate that *CAP* genes play a key role in the variable responses of different fungi to similar environmental conditions. The primary role of *CAP1* in *C. albicans* appears to be to mediate a rapid induction of bud-hypha transitions and filamentous growth in response to a variety of environmental conditions, a hallmark of *C. albicans* growth. The finding that *cap1*/*cap1* mutants are avirulent in a murine model of systemic candidiasis suggests that antifungal strategies interfering with *CAP1*-mediated signaling will be important for preventing or inhibiting candidiasis.

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