Calcium- and Calcineurin-Independent Roles for Calmodulin in Cryptococcus neoformans Morphogenesis and High-Temperature Growth†

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The function of calcium as a signaling molecule is conserved in eukaryotes from fungi to humans. Previous studies have identified the calcium-activated phosphatase calcineurin as a critical factor in governing growth of the human pathogenic fungus Cryptococcus neoformans at mammalian body temperature. Here, we employed insertional mutagenesis to identify new genes required for growth at 37°C. One insertion mutant, cam1-ts, that displayed a growth defect at 37°C and hypersensitivity to the calcineurin inhibitor FK506 at 25°C was isolated. Both phenotypes were linked to the dominant marker in genetic crosses, and molecular analysis revealed that the insertion occurred in the 3′ untranslated region of the gene encoding the calcineurin activator calmodulin (CAM1) and impairs growth at 37°C by significantly reducing calmodulin mRNA abundance. The CAM1 gene was demonstrated to be essential using genetic analysis of a CAM1/cam1Δ diploid strain. In the absence of calmodulin function, the cam1-ts mutant displayed a severe morphological defect with impaired bud formation. Expression of a calmodulin-independent calcineurin mutant did not suppress the growth defect of the cam1-ts mutant at 37°C, indicating that calmodulin promotes growth at high temperature via calcineurin-dependent and -independent pathways. In addition, a Ca2+-binding-defective allele of CAM1 complemented the 37°C growth defect, FK506 hypersensitivity, and morphogenesis defect of the cam1-ts mutant. Our findings reveal that calmodulin performs Ca2+- and calcineurin-independent and -dependent roles in controlling C. neoformans morphogenesis and high-temperature growth.

Calcium is a ubiquitous second messenger that functions in signal transduction pathways in eukaryotic organisms. Ca2+-signaling has been implicated in governing myriad biological processes as broad as fertilization and development, exocytosis and muscle contraction, and transcription and chromatin remodeling in multicellular eukaryotes (5). In the fungal kingdom, key features of the Ca2+-signaling machinery are conserved with multicellular eukaryotes, and Ca2+-signaling underlies diverse fungal physiological processes. In the budding yeast Saccharomyces cerevisiae, Ca2+-signaling is an essential component of cell cycle regulation, mating, and stress responses (11). In filamentous fungi, the role of Ca2+ extends into the regulation of hyphal morphogenesis, including hyphal tip growth events involving branching and orientation (22). The recent availability of fungal genome sequences has allowed a comparative analysis of fungal Ca2+-signaling components, and it is clear that fungi share key regulators of Ca2+-signaling (45). These include Ca2+-permeable channels, pumps, and transporters; calmodulin; and calmodulin-regulated proteins including calmodulin-dependent kinases and the protein phosphatase calcineurin.

Calmodulin is a small Ca2+-binding protein that is conserved from fungi to humans and contributes to the regulation of mitosis, transcription, cytoskeletal rearrangements, and stress responses (8). Calmodulin functions as a critical Ca2+-sensor, and yet calmodulin has both Ca2+-dependent and Ca2+-independent binding partners, highlighting the versatility of calmodulin as a signaling molecule. Calmodulin acts as a Ca2+-sensor by binding Ca2+-ions via four EF hands, which each contain a Ca2+-binding loop in which conserved aspartate and glutamate residues bind the Ca2+-ions (4). Ca2+-binding induces a conformational change in calmodulin that results in the release of free energy, which is the basis of its ability to act as a Ca2+-sensor (28). Studies of genetically tractable organisms have shown that calmodulin is essential and that its essential functions require the ability to bind Ca2+ in most organisms (13, 30, 42). Saccharomyces cerevisiae is the sole known exception, where only the Ca2+-independent functions of calmodulin are essential for cell viability (21).

One well-defined target of calmodulin is calcineurin, a serine/threonine-specific protein phosphatase whose mechanisms of activation and inhibition are conserved throughout eukaryotic organisms (3, 24). Calcineurin exists as a heterodimer consisting of a catalytic A subunit and a regulatory B subunit. Association of the two subunits is necessary but not sufficient for calcineurin function. Activation of calcineurin occurs when the Ca2+/calmodulin complex binds to the calmodulin-binding domain in the C-terminal regulatory region of the A subunit, eliciting conformational changes that free the calcineurin active site from occlusion by an autoinhibitory domain (43). Calcineurin function is inhibited by the immuno-
suppressive antifungal drugs cyclosporine A and FK506 (tacrolimus) in complex with the peptidyl-prolyl isomerases cyclophilin A and FKBP12, respectively. The drug-protein complexes inhibit calcineurin function by binding to the hydrophobic interface between the A and B subunits and, by steric hindrance, preventing large substrates from docking into the active site.

In the yeast *S. cerevisiae* calcineurin serves at least three functional roles: regulating stress-activated transcription, Ca\(^{2+}\) homeostasis, and morphogenesis (11, 17). The role of calcineurin in stress responses is conserved between model and pathogenic fungi, but the specific functions of calcineurin are unique (32). Calcineurin is critical for virulence in two pathogenic fungi that infect humans, *Cryptococcus neoformans* and *Candida albicans*, yet the precise roles of calcineurin in promoting virulence differ between the two. Calcineurin is required for growth at mammalian body temperature of *C. neoformans* but not of *C. albicans*, whereas calcineurin is required for *C. albicans* to survive in serum and disseminate in the host (6, 38). The ability to grow at 37°C is a prerequisite for pathogenesis, and *C. neoformans* strains that lack either the calcineurin A or B subunit are avirulent in animal models of cryptococcosis (10, 16).

Here, we have analyzed the role of calmodulin as a Ca\(^{2+}\) sensor that activates Ca\(^{2+}\)\-signaling in *C. neoformans*. By using gene disruption approaches and diploid strains, calmodulin was found to be essential, as in other fungi. A unique temperature-sensitive allele of the *CAM1* gene encoding calmodulin was isolated via insertional mutagenesis and found to confer temperature-sensitive growth by reducing but not abolishing calmodulin expression. The cam1-ts allele, a calcium-independent calmodulin mutant in which the four Ca\(^{2+}\)-binding EF hands are mutated (cam1-4DA), and a truncated CNA1 allele encoding a calmodulin-independent calmodulin A allele (CNA1-AIΔ) were employed to probe Ca\(^{2+}\), calmodulin, and calcineurin signaling. Our studies reveal that calmodulin plays Ca\(^{2+}\)-independent and -dependent roles, and also calcineurin-dependent and independent roles, to govern morphogenesis and growth at 37°C in *C. neoformans*.

### MATERIALS AND METHODS

**Strains and media.** Strains used are listed in Table 1. Strains were grown in the standard yeast growth media YPD, SD, and YNB with appropriate supplements (2). For growth with FK506, strains were grown on YPD medium or synthetic proline (SP) medium (2% dextrose, 1 mM proline, and YNB without amino acids). Genetic crosses were conducted on V8 medium (19). Where indicated, FK506 was added to growth medium at a concentration of 1 μg/ml.

**Insertional mutagenesis.** The serotype D strain JEC43 (MATα *ura5*) was transformed by biolistic transformation with plasmid pCH233, which contains the nourseothricin (NEO) dominant marker as previously described (12, 19). The diploid strain RA9009 (41) was transformed with the cam1Δ::NEO allele, and G418-resistant transformants were isolated. Heterozygous cam1/ cam1Δ::NEO transformants with one allele of the *CAM1* gene disrupted were identified by Southern analysis and diagnostic PCR. Transformants were incubated on V8 medium for 7 days to obtain haploid basidiospores. Haploid progeny were isolated either by plating on YPD medium at 25°C and choosing adenine auxotrophs (based on red colony color) that were no longer self-filamentous and therefore a or α but not a/α or by micromanipulation as described above. All progeny were scored for resistance to G418.

**Identification of the marker insertion site.** The insertion site of the NAT dominant marker was determined using a modified version of the vectorette PCR approach (40). Genomic DNA from insertion mutant JEC7 was digested with EcoRV, and anchor-bubble linkers were ligated onto the blunt ends. PCR primers specific for the NAT dominant marker and the complementary strand of the anchor-bubble were used to amplify a DNA fragment containing the genomic DNA that flanks the marker insertion site. Conditions used were as follows: 94°C for 20 s, 60°C for 30 s, and 72°C for 3 min. The anchor-bubble primer is designed such that it can prime synthesis only on newly synthesized DNA primed by the NAT dominant marker primer, conferring specificity upon the PCR. PCR products were cloned and sequenced, and the *C. neoformans* genomic DNA portion of the PCR product was used for BLAST analysis of the *C. neoformans* JEC21 genome and the nonredundant NCBI database.

**Northern analysis, RT-PCR, RACE, and real-time PCR analysis.** Northern analyses were performed as previously described (31). Probes for the *CAM1* and *ACT1* genes were generated by PCR and labeled with [α-32P]dCTP using the Redi-Prime kit (Amersham Biosciences). Reverse transcription (RT)-PCR and rapid amplification of cDNA ends (RACE) analysis were performed using the SMART Race cDNA kit (Clontech). First-strand synthesis was primed with oligo(dT), and amplification of calmodulin cDNA was accomplished using primers that can prime synthesis only if introns have been spliced from the template to allow discrimination of the desired PCR products from those that resulted from spurious amplification of contaminating genomic DNA (JOHE 9363 plus JOHE 9364 [see Table S1 in the supplemental material]). cDNA from wild-type and cam1-ts strains (JEC21 and PK7, respectively) was used as a template for quantitative real-time PCR using iQ SYBR Green Supermix (Bio-Rad) and a Bio-Rad iCycler iQ Multicolor real-time detection system. The iCycler iQ Multicolor real-time detection system was used as the fluorescence detector under

### TABLE 1. *C. neoformans* strains

| Strain | Genotype | Source |
|--------|----------|--------|
| JEC20  | MATα ade2 2 lys2 2 | 36 |
| JEC21  | MATα ade2 2 lys2 2 | 36 |
| JEC43  | MATα ura5 | 36 |
| JEC171 | MATα ade2 2 lys2 2 | 36 |
| MCC16  | MATα cam1Δ::ADE2 ade2 ura5 | 9 |
| MCC10  | MATα cam1Δ::ADE2 ade2 | 9 |
| RA9009 | MATα cam1Δ::ADE2 ade2 LYS2/lys2 ura5/ura5 | 41 |
| CSB56  | MATα cam1-ts | This study |
| CSB57  | MATα cam1-ts | This study |
| PK7    | MATα ura5 cam1-ts | This study |
| PK14   | MATα/MATαADE2 ade2 LYS2/lys2 ura5/ura5 | This study |
| PK15   | PK7 [p535] | This study |
| PK42   | MCC16 [p533] | This study |
| PK43   | MCC16 [p533-CAM1-AIΔ] | This study |
| PK45   | PK7 [p533-CAM1-AIΔ] | This study |
| PK47   | PK7 [p535-CAM1] | This study |
| PK50   | PK7 [p535-cam1-4DA] | This study |

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An insertional mutagenesis screen for high-temperature growth-defective mutants. To identify other components of the calcineurin signaling cascade that governs growth at 37°C, we implemented an insertional mutagenesis approach. Approximately 3,000 nourseothricin-resistant transformants were obtained after biolistic transformation of either circular or linear DNA of plasmid pH233 into the C. neoformans serotype D strain JEC43 (MATα ura5). Transformants were tested for stability of the NAT dominant marker insertion, and 430 transformants exhibited stable nourseothricin resistance after passage on nonselective medium (14.3% stable). Stable transformants were tested for a growth defect at 37°C, and 12 isolates were unable to grow or grew poorly at 37°C compared to the parental strain JEC43.

All of the temperature-sensitive isolates were crossed with strain JEC171 (MATα ade2 lys2) to determine if the 37°C growth defect was linked to the NAT insertion. Crosses were analyzed by both using random spore analysis and isolating individual basidiospores by micromanipulation and determining the phenotypes of meiotic progeny produced by germination. For 1 of the 12 isolates, 3E7, all meiotic segregants that displayed the phenotypes of meiotic progeny produced by germination were analyzed by both using random spore analysis and isolating individual basidiospores by micromanipulation and determining the phenotypes of meiotic progeny produced by germination. For 1 of the 12 isolates, 3E7, all meiotic segregants that displayed a growth defect at 37°C were also nourseothricin resistant, and nourseothricin-sensitive segregants grew normally at 37°C, indicating that the NAT dominant marker and the growth defect are genetically linked. In addition to the growth defect at 37°C, isolate 3E7 displayed hypersensitivity to the calcineurin inhibitor FK506 at the permissive growth temperature of 25°C (Fig. 1A). This phenotype was the result of calcineurin inhibition by the FK506-FKBP12 drug-protein complex, as an frr1-3 cam1-ts mutant isolated following a genetic cross, in which FKBP12 contains an active-site point mutation, was resistant to FK506 (data not shown). Thus, cal-
Calmodulin cDNA was not detected after 20 PCR cycles determine if any calmodulin mRNA is present in the wild-type strain, whereas any message in the cam1-ts mutant was still below the limit of detection. RT-PCR was then used to wild-type backgrounds.

**Insertion in CAM1 reduces CAM1 mRNA abundance.** Recovery of the genomic DNA adjacent to the NAT dominant marker insertion in isolate 3E7 using a modified version of the vectorette PCR technique revealed an insertion in the CAM1 gene encoding calmodulin (40). Sequence analysis of the genomic DNA adjacent to the NAT dominant marker insertion in isolate 3E7 revealed that the insertion occurred in the 3' UTR of the CAM1 gene. The insertion occurred 49 bp downstream from the stop codon, and its location was confirmed by Southern analysis of genomic DNA and RACE (Fig. 1A and data not shown). Introduction of the wild-type CAM1 gene on an episomal plasmid restored growth at 37°C (see Fig. 4C). Based on the insertion location, the phenotype of the 3E7 isolate, and complementation by the wild-type CAM1 gene, the mutant allele was designated cam1-ts.

The NAT dominant marker insertion does not disrupt the CAM1 coding region, yet the cam1-ts allele confers strong growth defects both at 37°C and in the absence of calcineurin function. We hypothesized that the insertion in the CAM1 3' UTR might disrupt mRNA stability and reduce CAM1 message abundance and thereby confer a temperature-sensitive growth defect. Therefore, calmodulin mRNA abundance was assessed by Northern analysis, RT-PCR, and real-time PCR. In the cam1-ts mutant, the mRNA level was significantly reduced compared to that of the wild-type strain (Fig. 2A). Furthermore, when Ca²⁺ or Na⁺ ions were added to the culture medium, the levels of calmodulin mRNA increased in the wild-type strain, whereas any message in the cam1-ts mutant was still below the limit of detection. RT-PCR was then used to determine if any calmodulin mRNA is present in the cam1-ts cells. Calmodulin cDNA was not detected after 20 PCR cycles using untreated or Ca²⁺-treated cam1-ts cells; however, it was detected after 25 cycles (data not shown). Real-time PCR analysis of wild-type and cam1-ts strains revealed an approximately 60-fold reduction in the calmodulin transcript in the cam1-ts strain compared to that of the wild type (less than 2% of the wild type), whereas the abundance of the GPD1 control transcript was the same in wild-type and cam1-ts mutant cells. (Fig. 2B).

Calmodulin transcripts in the cam1-ts mutant were also apparent during RACE analysis. Based on RACE analysis, the 3' untranslated region of the cam1-ts mRNA is 162 nucleotides (nt) long [49 nt from the CAM1 gene and 113 nt from the NAT plasmid, excluding the poly(A) tail], while the 3' untranslated region of CAM1 mRNA is 147 nt. Because the size of the message is similar, we attribute the decrease in the calmodulin cDNA level of cam1-ts cells compared to that of the wild type to a decrease in mRNA abundance rather than a decrease in first-strand synthesis efficiency due to a large mRNA size difference. This decrease in mRNA abundance may be due to decreased message stability caused by the insertion or a disruption of 3' regulation of expression.

**CAM1 is essential.** Calmodulin is encoded by essential genes in Drosophila melanogaster, Aspergillus nidulans, Saccharomyces cerevisiae, and Schizosaccharomyces pombe (13, 23, 30, 42). Given that the cam1-ts mutant cells express less than 2% of the wild-type level of calmodulin mRNA, we sought to test if CAM1 is an essential gene and if this reduced level is sufficient to maintain cell viability or alternatively whether the CAM1 gene is dispensable for viability in C. neoformans at 25°C but required at 37°C. To distinguish between these models, a cam1Δ::URA5 deletion allele was constructed using PCR overlap. A total of 125 Ura⁺ transformants were isolated at 25°C following biolistic transformation of the ura5 mutant strain JEC43 with the cam1Δ::URA5 allele. PCR screening and Southern analysis of genomic DNA revealed that none of the 125 transformants contained a disrupted CAM1 gene. These findings provided presumptive evidence that the CAM1 gene is essential.

To definitively establish whether or not the CAM1 gene is essential, a heterozygous cam1Δ/CAM1 diploid strain was constructed and analyzed. The diploid strain RAS009 that is heterozygous for the ade2 marker (41) was transformed with the

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**FIG. 2.** Reduction in CAM1 mRNA abundance in the cam1-ts mutant. (A) Northern analysis of total RNA from the wild-type and cam1-ts mutant strains. Cells were untreated or treated with either 50 mM CaCl₂ or 0.4 mM NaCl for 3 hours prior to RNA extraction. (B) Real-time PCR analysis of wild-type (JEC21 [solid lines]) and cam1-ts (PK7 [dashed lines]) strains. ΔCt (where Ct denotes threshold cycle) values shown represent the difference in average cycle threshold values in two independent experiments determined at a value of 25 relative fluorescence units (RFU). One ΔCt corresponds to an approximately twofold difference in template abundance. The GPD1 gene was included as a standard.
CAM1 promotes high-temperature growth in a calcineurin-dependent and -independent manner. Calcineurin is essential for *C. neoformans* high-temperature growth (16, 38). The *cam1-ts* mutant grows very slowly at 37°C, and this defect may result from the inability to activate calcineurin, the loss of a different function of calmodulin, or both. To determine this, we constructed a calcineurin A truncated allele (*CNA1-AIΔ*) that lacks the coding region for the C-terminal calmodulin-binding site and the autoinhibitory domain. Calcineurin lacking these domains is catalytically active and constitutive as the requirement for Ca2+/calmodulin binding for activation has been relieved (26, 44). The *CNA1-AIΔ* allele was subcloned into the multicycoplasmid pPM8 and transformed into *cna1* and *cam1-ts* mutants. Growth of the *cna1* mutant strain harboring the *CNA1-AIΔ* allele was restored at 37°C, while the *cna1* mutant harboring only the control plasmid failed to grow at 37°C (Fig. 4C). In contrast, the *cam1-ts* mutant strain was not capable of growing at 37°C when transformed with the calmodulin-independent *CNA1-AIΔ* allele (Fig. 4C), demonstrating that calmodulin has at least a second function in promoting high-temperature growth in addition to its well-established role in activating calcineurin.

CAM1 promotes high-temperature growth independently of calcium ions. Calmodulin has both Ca2+-dependent and Ca2+-independent functions. To test if the calcineurin-independent function of calmodulin in promoting high-temperature growth depends on Ca2+ binding, a calmodulin mutant allele (*cam1-4DA*) that contains a substitution in each of the four Ca2+ binding loops of the EF hand domains was constructed. Aspartic–acid-to-alanine substitutions in the EF hands of *S. cerevisiae* calmodulin completely abolish Ca2+ binding ability (21), and the corresponding mutations were engineered in codons 21, 57, 94, and 130 of *C. neoformans* calmodulin.

cDNA versions of the *CAM1* gene and the *cam1-4DA* allele were subcloned into the *E. coli* expression plasmid pRSET-A, and the Ca2+ binding ability of the expressed proteins was assessed by a 45Ca2+ overlay assay. The wild-type Cam1 protein bound 45Ca2+, while no 45Ca2+ binding to the Cam1-4DA mutant protein was detected, indicating that the engineered mutations in the EF hands of the Cam1-4DA protein abolish Ca2+-binding activity (Fig. 4B). The *cam1-4DA* allele and wild-type *CAM1* were then subcloned into plasmid pPM8 and transformed into the *cam1-ts* strain. Growth of the *cam1-ts* strain at 37°C was restored by either the *cam1-4DA* allele or wild-type *CAM1* (Fig. 4C). These observations indicate that Ca2+ binding is not required for the calcineurin-independent function of calmodulin in promoting high-temperature growth.

The ability of the *cam1-4DA* allele to complement the *cam1-ts* growth defect when calcineurin function is impaired was assessed by a growth assay on medium containing FKS06. Wild-type *CAM1* complemented the FKS06 hypersensitivity of the *cam1-ts* mutant at 25°C, while the *cam1-4DA* allele only partially complemented this defect (Fig. 4D). These findings indicate that calmodulin promotes *C. neoformans* viability and high-temperature growth in a calcineurin-dependent and -independent manner and that both Ca2+-bound and Ca2+-free
calmodulin share critical functions with calcineurin in *C. neoformans* growth control.

**CAM1 promotes mating filament formation.** In addition to its role in growth at 37°C, calcineurin is essential for filamentous growth during *C. neoformans* mating (9). To examine if calmodulin plays a similar role, the ability of the *cam1-ts* mutant to produce mating filaments was assessed by a mating assay. Mating filament production was reduced but not abolished in a bilateral cross in which both parents harbored the *cam1-ts* mutation compared to a cross between wild-type strains (Fig. 5A and D). Mating filament production was restored to the wild-type level in crosses when one strain contained either the wild-type *CAM1* gene or the *cam1-4DA* allele (Fig. 5E and F). In contrast, crosses in which both parents contained calcineurin mutations failed to produce mating filaments, as expected (Fig. 5G). The mating defect of calcineurin mutant cells was complemented by the constitutive, calmodulin-independent *CNA1-AI/H9004* allele (Fig. 5I). However, the *CNA1-AI/H9004* allele failed to complement the mating defect of the *cam1-ts* mutant cells (Fig. 5C). These observations provide evidence that calmodulin plays a Ca\(^{2+}\)/calcineurin-independent role in mating in addition to its known role in activating calcineurin in a calcium-dependent fashion.

**CAM1 and calcineurin regulate cellular morphology.** Calcineurin is a known downstream target of Ca\(^{2+}\)-bound calmodulin, and in previous studies, we established that calcineurin A indeed binds calmodulin in *C. neoformans* (38). In the *cam1-ts* mutant, calcineurin becomes essential at 25°C as *cam1-ts* mutant cells were hypersensitive to the calcineurin inhibitor FK506 (Fig. 1C) and *cam1-ts cna1/H9004* double mutants could not be recovered following a genetic cross (data not shown). Because the phenotype of the *cam1-ts cna1Δ* double mutant is more severe than the *cna1Δ* single mutant, this synthetic lethal genetic interaction indicates that calmodulin and calcineurin

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**FIG. 4.** Calcineurin and calmodulin contribute a shared function in promoting high-temperature growth. (A) Schematic diagram of the calcineurin A subunit and the truncated, constitutively active calmodulin-independent Cna1-AI mutant. (B) \(^{45}\)Ca\(^{2+}\) overlay blot of *E. coli*-expressed Cam1 and Cam1-4DA, which contains mutations in the Ca\(^{2+}\)-binding loops of the four calmodulin EF hand domains. Western blot analysis using antibodies directed against the Xpress epitope (Invitrogen) contained in the expression plasmid confirmed that both the Cam1 and the Cam1-4DA proteins were expressed. (C) Fivefold serial dilutions of strains with the indicated genotype were spotted onto YNB medium and incubated at the indicated temperature for 3 days. Two independent transformants for each strain were tested. (D) Fivefold serial dilutions of the indicated strains were inoculated onto SP medium or SP medium containing 1 \(\mu\)g/ml FK506 and incubated for 3 days at 25°C.

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**FIG. 5.** Calmodulin promotes mating filament formation. Strains with the indicated genotype were cocultured on V8 mating medium and photographed after incubation for 5 days at 25°C. Strain PK7 (MATa cam1-ts ura5) was used as the parental strain for the *cam1-ts* mutants harboring pPM8-derived plasmids which contain the wild-type *URA5* gene. Crosses performed were as follows: (A) CSB57 (MATa cam1-ts) × CSB56 (MATa cam1-ts); (B) PK15 (PK7 [pPM8]) × CSB56; (C) PK45 (PK7 [pPM8-CNA1-AIΔ]) × CSB56; (D) JEC20 (MATa) × JEC21 (MATa); (E) PK47 (PK7 [pPM8-CAM1]) × CSB56; (F) PK50 (PK7 [pPM8-cam1-4DA]) × CSB56; (G) MCC16 (MATa cam1Δ::ADE2 ade2 ura5) × MCC10 (MATa cam1Δ::ADE2 ade2); (H) PK42 (MCC16 [pPM8]) × MCC10; (I) PK45 (MCC16 [pPM8-CNA1-AIΔ]) × MCC10. Scale bar, 100 \(\mu\)M.
share a function that is independent of the activation of calcineurin by calmodulin. To gain insight into this shared function, the \textit{cam1-ts} mutant was examined by microscopy after treatment with FK506 to inactivate calcineurin. After 24 h of FK506 exposure at 25°C, \textit{cam1-ts} mutant cells uniformly exhibited an aberrant bud morphology, forming elongated cells, whereas wild-type cells exposed to FK506 exhibited a normal budding yeast morphology (Fig. 6). Nuclear staining revealed the presence of multiple nuclei, indicating that FK506-treated \textit{cam1-ts} cells are capable of continued nuclear division in the absence of cell division. Actin filaments were concentrated at the tip of the hyphal structures in FK506-treated \textit{cam1-ts} mutant cells, indicating that these cells are still capable of directing polarized growth via the actin cytoskeleton. Introduction of wild-type \textit{CAM1} or the \textit{cam1-4DA} allele restored the ability of \textit{cam1-ts} cells to produce a normal bud. These results indicate that calcineurin and Ca\textsuperscript{2+}-free calmodulin contribute a shared function in the regulation of cellular morphology.

**DISCUSSION**

The goal of this study was to determine the role of Ca\textsuperscript{2+} signaling in promoting \textit{C. neoformans} high-temperature growth, a crucial virulence attribute. Here, we isolated a mutant containing an insertion in the 3' UTR of the gene encoding calmodulin, \textit{CAM1}, and utilized the mutant strain to probe calmodulin and calcineurin functions in high-temperature growth and morphogenesis. Similar to calmodulin in other genetically tractable organisms, calmodulin was found to be essential for viability in \textit{C. neoformans}. In addition to activating calcineurin, we found that calmodulin plays calcineurin-independent roles in promoting \textit{C. neoformans} high-temperature growth and morphogenesis during mating and bud formation. These functions are also independent of Ca\textsuperscript{2+} binding. Taken together, our findings provide evidence for a bifurcated pathway whereby high-temperature growth and cellular morphogenesis are controlled by Ca\textsuperscript{2+}-free calmodulin in one branch and Ca\textsuperscript{2+}/calmodulin-activated calcineurin in another branch (Fig. 7).

The \textit{cam1-ts} allele was generated using insertional mutagenesis, capitalizing upon the observation that transforming DNA often integrates into the genome via nonhomologous recombination in \textit{C. neoformans} (12, 37). The disadvantages of this procedure are the relatively low frequency of integration (10 to 15% of transformants) compared to the maintenance of transforming DNA as an extrachromosomal episome. In addition, multiple integrations are possible, complicating the ability to demonstrate linkage between the inserted marker and associated phenotypes by a genetic cross. The finding that a low percentage of mutants isolated displayed linkage of the phenotype and the inserted marker was unexpected and may be a result of the low number of transformants analyzed, the possible mutagenic effects of the biolistic transformation procedure, or epigenetic events that limit growth at elevated temperatures. Importantly, a major advantage of using random insertional mutagenesis as a tool for genetic analysis is the ability to recover hypomorphic alleles in addition to null alleles, extending the analysis to include essential as well as nonessential genes. We note that some of the disadvantages encountered in this study have been addressed by the development of \textit{Agrobacterium}-mediated transformation as a transkingdom DNA delivery vehicle for mutagenesis of \textit{C. neoformans} (27).

The discovery of the hypomorphic \textit{cam1-ts} allele of the es-
sential CAM1 gene in *C. neoformans* provided a unique opportunity to probe the mechanics of Ca\(^{2+}\) signaling in this ubiquitous human fungal pathogen. The insertion of the marker into the 3' UTR of the CAM1 gene significantly reduces calmodulin mRNA abundance; however, enough calmodulin gene expression remains to sustain cell viability at 25°C but not 37°C. We initially suspected that the high-temperature growth defect of the *cam1-ts* mutant might be attributable to an increased requirement for calmodulin to activate calcineurin, which itself is required for 37°C growth. However, the Ca\(^{2+}\)-binding-defective calmodulin mutant (Cam1-4DA) was sufficient to restore growth of the *cam1-ts* mutant at 37°C, illustrating a role independent of Ca\(^{2+}\) binding required for promoting 37°C growth beyond the known Ca\(^{2+}\)-dependent role of calmodulin inactivating calcineurin. The calmodulin function that remains in the *cam1-ts* mutant is sufficient to activate calcineurin, as evidenced by the strong growth defect at 25°C when calcineurin is inhibited by FK506. These findings further indicate that there is a synthetic lethal interaction between calcineurin inhibition (via FK506) and compromised calmodulin function (via the *cam1-ts* mutation), a conclusion that was reinforced by our inability to recover *cam1-ts cam1* double mutants following genetic crosses (data not shown).

The results reported here indicate that calmodulin and calcineurin share an essential function that governs cellular morphogenesis. *S. cerevisiae* calmodulin has diverse essential functions as revealed by a study of intragenic complementation of conditional mutants, and the defects observed in three of the complementation groups (*cmd1A, cmd1B*, and *cmd1D*) indicate that calmodulin may function at multiple steps of the pathway controlling morphogenesis in yeast (39). The regulation of actin organization is a common theme revealed by these previous studies in yeast; however, recent reports have also identified a role for calcium signaling in microtubule dynamics in other fungi (1, 15).

Here, we found that the execution of the shared function of calcineurin and calmodulin occurs via the Ca\(^{2+}\)-free form of calmodulin. Calmodulin has a panoply of Ca\(^{2+}\)-dependent and -independent binding partners, which are best understood in *S. cerevisiae* (11). Microscopic evaluation of *cam1-ts* cells in which calcineurin function has been inhibited suggests that a shared essential function of Ca\(^{2+}\)-free calmodulin and calcineurin is involved in cellular morphogenesis. Attractive candidates for the binding partner(s) of calmodulin that contribute to this essential cellular function include Myo2, which is required for polarized growth in *S. cerevisiae* and binds the Ca\(^{2+}\)-free form of calmodulin (7). In our studies, the Ca\(^{2+}\)-binding-defective allele of calmodulin complemented the morphological defect of FK506-treated *cam1-ts* mutant cells. Interestingly, the *C. neoformans* MYO2 gene lies in the MAT locus, is essential, and is a member of the most ancient class of MAT genes, which is indicative of a potential functional role in *C. neoformans* mating (18). Furthermore, calmodulin controls the actin cytoskeleton by regulating phosphatidylinositol (4, 5) bisphosphate synthesis via Ms54, although it has not yet been determined if this interaction is Ca\(^{2+}\) dependent or independent (14). An important consideration is that the phenotype of FK506-treated *cam1-ts* cells appears to be a hyperpolarization of growth rather than a loss of the ability to undergo polarized growth, as has been observed in *S. cerevisiae* calmodulin and calmodulin mutants (25, 29). Genetic interactions between calcineurin and type 2 myosin have been observed in *S. pombe*, suggesting that calcineurin functions in actin contractile ring formation and septum formation (20). The observation that calcineurin is required for the production of *C. neoformans* mating filaments implicates calcineurin in the regulation of polarized growth; however, a direct role for calcineurin in promoting actin polymerization or other processes important for cellular morphogenesis remains to be established in *C. neoformans*.

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