Calcineurin is required for hyphal elongation
during mating and haploid fruiting in
*Cryptococcus neoformans*

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*Cryptococcus neoformans* is a fungal pathogen that
causes meningitis in immunocompromised patients.
Its growth is sensitive to the immunosuppressants
FK506 and cyclosporin, which inhibit the Ca²⁺–
calmodulin-activated protein phosphatase calcineurin.
Calcineurin is required for growth at 37°C and virulence
of *C.neoformans*. We found that calcineurin is also
required for mating. FK506 blocks mating of
*C.neoformans* via FKBP12-dependent inhibition of
calcineurin, and mutants lacking calcineurin are bilaterally
sterile. Calcineurin is not essential for the initial
fusion event, but is required for hyphal elongation and
survival of the heterokaryon produced by cell fusion.
It is also required for hyphal elongation in diploid
strains and during asexual haploid fruiting of MATα
cells in response to nitrogen limitation. Because
mating and haploid fruiting produce infectious basidiospores,
our studies suggest a second link between calcineurin and virulence of *C.neoformans*. Calcineurin
regulates filamentation and 37°C growth via distinct pathways. Together with studies revealing
that calcineurin mediates neurite extension and
neutrophil migration in mammals, our findings indicate
that calcineurin plays a conserved role in the control
of cell morphology.

Keywords: cyclosporin/FK506/FKBP12/fungi/
phosphatase

Introduction

Cells sense and respond to their environment and other
cells via signal transduction cascades, which transfer
information from outside to inside the cell. Intracellular
signaling events include increases in intracellular Ca²⁺
ions, which bind to and activate calmodulin. The Ca²⁺–
calmodulin complex then binds to and activates calmodulin-dependent enzymes, including protein kinases
and the protein phosphatase calcineurin. Our studies
focus on the roles of calcineurin in regulating differentiation and virulence in the pathogenic fungus *Cryptococcus
neoformans*.

*Cryptococcus neoformans* is an opportunistic fungal
pathogen that causes fatal meningoencephalitis in
individuals with compromised immune function (Casadavall
and Perfect, 1998). Virulence is associated with the
α mating type, production of melanin, a polysaccharide
capsule and growth at 37°C (Kwon-Chung and Rhodes,
1986; Chang and Kwon-Chung, 1994, 1998; Chang et al.,
1996; Salas et al., 1996; Odom et al., 1997a; Cruz et al.,
2000b).

*Cryptococcus neoformans* has a defined sexual cycle
involving mating between haploid cells of MATa and
MATα mating types (Kwon-Chung, 1975, 1976; Alspaugh et al., 2000). In response to mating pheromone and
nutrient deprivation conditions, cells of the opposite
mating type produce filament-like structures called
conjugation tubes that project toward the mating partner in
response to mating pheromones (Moore and Edman, 1993;
Davidson et al., 2000b). The MATa and MATα cells fuse
and form dikaryotic filaments characterized by the presence
of unfused parental nuclei and fused clamp
connections that promote proper nuclear migration. The tips
of the mating filaments differentiate to form enlarged structures called basidia, where karyogamy and meiosis occur,
generating four recombinant haploid nuclei. These nuclei
then divide mitotically and bud off from the basidium,
generating four long chains of basidiospores. These spores
germinate to produce vegetative yeast cells. In addition,
in response to desiccation and nitrogen limitation, MATα
cells differentiate to form filaments, basidia and spores in
the absence of a mating partner, in a morphogenic event
termed haploid fruiting (Wickes et al., 1996). Haploid
fruiting of MATα cells is also dramatically stimulated by
confrontation with MATa cells (Wang et al., 2000).
Haploid fruiting is distinguished from mating filaments
by the presence of monokaryotic filaments, unfused clamp
connections and the exclusive production of MATα
basidiospores.

Calcineurin is a Ca²⁺–calmodulin-activated protein
phosphatase composed of a heterodimer of a catalytic
(Cna) and a regulatory subunit (Cnb), which is conserved
from yeast and fungi to humans (reviewed in Klee et al.,
1998; Hemenway and Heitman, 1999; Aramburu et al.,
2000). Both the calcineurin A and B subunits are required
for calcineurin activity. Calcineurin is the target of the
immunosuppressive drugs cyclosporin A (CsA) and
FK506, which suppress the immune system by inhibiting
calcineurin (Liu et al., 1991). The mechanism of action of
CsA and FK506 has been extensively studied in the
ascomycete Saccharomyces cerevisiae as well as in T cells
(for reviews see Heitman et al., 1992; Schreiber and
Crabtree, 1992; Cardenas et al., 1995, 1998, 1999). CsA
and FK506 diffuse into the cell and bind to intracellular
receptors known as immunophilins. CsA binds to
cyclophilin A, whereas FK506 binds to the protein
FKBP12. The resulting FKBP12–FK506 and cyclophilin
A–CsA protein–drug complexes bind to and inhibit
calcineurin activity (Liu et al., 1992). Calcineurin mediates
T-cell activation by dephosphorylating the transcrip-
tion factor NF-AT, unmasking its nuclear localization signal and promoting translocation to the nucleus where NF-AT induces genes required for T-cell activation (Jain et al., 1993; Northrop et al., 1993; Shaw et al., 1995; Luo et al., 1996; Shibasaki et al., 1996). Thus, CsA and FK506 inhibit T-cell activation by inhibiting calcineurin and preventing gene activation by NF-AT.

Calcineurin homologs have been identified and characterized from several fungi. Calcineurin is essential and regulates cell cycle progression in Aspergillus nidulans (Rasmussen et al., 1994) and hyphal elongation and vegetative growth in Neurospora crassa (Prokisch et al., 1997; Kothe and Free, 1998). In the fission yeast Schizosaccharomyces pombe, calcineurin mutants are sterile and have defects in cytokinesis, cell polarity and spindle body positioning (Yoshida et al., 1994; Plochocka-Zulinska et al., 1995). In the budding yeast Saccharomyces, calcineurin activity is required for recovery from pheromone-induced cell cycle arrest (Cyert and Thorner, 1992; Foor et al., 1992; Moser et al., 1996; Withee et al., 1997) and cation homeostasis (Nakamura et al., 1993; Breuder et al., 1994; Cunningham and Fink, 1994, 1996; Mendoza et al., 1994; Faracasou et al., 1995; Pozos et al., 1996). In S. cerevisiae, calcineurin regulates ion homeostasis and cell wall synthesis by activating nuclear import of the transcription factor Tcn1/Crz1, which regulates genes encoding ion pumps and cell wall biosynthetic enzymes (including Fks2, Pmr2, Pmc1 and Pmr1) (Matheos et al., 1997; Stathopoulos and Eyert, 1997; Stathopoulos-Gerontides et al., 1999).

The genes encoding calcineurin A and B homologs from C. neoformans have been identified, sequenced, and disrupted by homologous recombination (Odom et al., 1997a; Cruz et al., 2000b; Fox et al., 2001). Earlier studies demonstrated that calcineurin is essential for the growth of C. neoformans at 37°C, in 5% CO₂, or in alkaline pH—all conditions that mimic the host environment. Calcineurin is also required for Na⁺ and Li⁺ cation homeostasis in serotype A strains (Odom et al., 1997a; Cruz et al., 2000b). Cryptococcus neoformans growth is sensitive to CsA, FK506 and non-immunosuppressive analogs, and the antifungal activity of these agents is mediated via inhibition of calcineurin by the cyclophilin A–CsA and FKBP12–FK506 complexes (Odom et al., 1997a,b; Cruz et al., 1999, 2000a,b; Fox et al., 2001). Accordingly, mutant strains that lack FKBP12 (frr1) are resistant to FK506.

Here we report that calcineurin plays an essential role during mating and haploid fruiting of C. neoformans. Inhibition of calcineurin with FK506 or CsA, or mutation of the genes encoding the calcineurin A or B subunits, prevents mating, haploid fruiting and basidiospore production. Because infection with C. neoformans is thought to occur by inhalation of basidiospores into the alveoli of the lung (Zimmer et al., 1984; Sukroongreung et al., 1998), our findings suggest that calcineurin is involved in both the production of the putative infectious propagule and subsequent survival in the harsh conditions found in the host. Calcineurin regulates hyphal elongation in both C. neoformans and N. crassa, and also plays a conserved and related role in regulating morphological changes in mammalian cells, including the extension of neurites by neurons and migration of neutrophils (Ferreira et al., 1993; Lyons et al., 1994; Chang et al., 1995; Lawson and Maxfield, 1995; Lauterhilm and Spitzer, 2000). These studies illustrate the central role that calcineurin plays in the life cycle of a human fungal pathogen, and the potential of studies in model organisms to reveal molecular mechanisms by which calcineurin regulates cellular morphogenesis.

**Results**

**CsA and FK506 inhibit C. neoformans mating**

Calcineurin is required for C. neoformans growth at 37°C, and the calcineurin inhibitors CsA and FK506 inhibit C. neoformans growth at 37 but not 24°C (Odom et al., 1997a). To determine whether calcineurin also regulates mating of C. neoformans, we tested the effects of FK506 and CsA under conditions where calcineurin is not required for growth (24°C). The calcineurin inhibitors FK506 and CsA both inhibited mating and prevented filamentation and later steps, including nuclear fusion, meiosis and sporulation (Figure 1, upper panels), suggesting that calcineurin is required for mating in C. neoformans.

To test whether FK506 exerts its action via FKBP12-dependent inhibition of calcineurin, matings were performed with strains lacking the FK506 target protein FKBP12 (MATa frr1-1 and MATα frr1-3), which are resistant to calcineurin inhibition by FK506 (Cruz et al., 1999). FK506 still inhibited mating when a MATα frr1-1 mutant lacking FKBP12 was mated with a MATα FRR1 wild-type strain (Figure 1, middle panels), or when a MATα frr1-3 mutant strain was mated with a MATa FRR1 wild-type strain (data not shown). In these cases, although the frr1 mating partner is FK506 resistant, FK506 still inhibits calcineurin in the wild-type mating partner prior to cell fusion, and also in the FRR1/frr1 heterokaryon in which the recessive frr1 mutation is complemented following cell fusion. In contrast, when MATα frr1-1 and MATa frr1-3 mutant strains, which both lack FKBP12, were co-cultured, mating was completely resistant to FK506, but not to CsA (Figure 1, lower panels). Thus, FK506 blocked mating when either or both mating partners expressed FKBP12, allowing FKBP12–FK506 complexes to form and inhibit calcineurin, whereas FK506 was unable to inhibit calcineurin and mating occurred normally when both mating partners lacked FKBP12. These findings suggest that calcineurin activity is either required in both mating partners for cell fusion, or for hyphal elongation of the heterokaryon following cell fusion.

**Calcineurin A mutants are bilaterally sterile**

To elucidate further the role of calcineurin in mating, the CNA1 gene encoding the calcineurin A catalytic subunit was disrupted in congenic MATα and MATα serotype D strains. MATα cna1 and MATα cna1 mutant strains were isolated by transformation and homologous recombination with a cna1::ADE2 allele (Cruz et al., 2000b; Davidson et al., 2000a). When MATα cna1 or MATα cna1 mutant strains were mixed with wild-type MATα (JEC21) or MATα (JEC20) mating type tester strains on V8 medium, mating occurred and abundant filaments were produced within 7 days of incubation at 24°C (Figure 2A). However,
when MATa cnal and MATα cnal mutant strains were co-cultured, no filamentation, basidia or basidiospores were observed (Figure 2A). These findings demonstrate that calcineurin A mutations confer a bilateral mating defect in C. neoformans. This mating defect was complemented when the wild-type CNA1 gene was reintroduced into the serotype D MATa cnal mutant strain on a linear episomal telomeric plasmid (Figure 2A) (see Materials and methods), or when the wild-type CNA1 gene was ectopically integrated into the genome of a serotype A MATα cnal mutant strain (Figure 2A), confirming that calcineurin is required for mating. In summary, calcineurin A mutations confer a bilateral mating defect either because calcineurin activity in only one parent is sufficient for cell fusion or because calcineurin is required for hyphal elongation of the fused heterokaryon.

**Calcineurin B is also required for mating: calcineurin regulates filamentation of the heterokaryon and is not required for cell fusion**

Calcineurin is a heterodimer composed of a catalytic (CnA) and a regulatory subunit (CnB). We, therefore, tested whether the calcineurin B regulatory subunit is also required for mating. The CNB1 gene encoding the calcineurin B subunit has been disrupted by homologous recombination (Fox et al., 2001). Similarly to the cnal...
Calcineurin mutants are bilaterally sterile. Isogenic wild-type, MATa cna1, MATa cna1, MATa cnbl and MATa cnbl mutant strains, the serotype D MATa cna1 strain with the wild-type CNA1 gene on the linear episomal plasmid pPM8 and the serotype A MATa cna1 mutant with the CNA1 gene re-introduced ectopically in the genome, as previously described (Odom et al., 1997a), were co-incubated on mating medium (V8) to assay the roles of calcineurin A (A) and calcineurin B (B) in mating. After 7 days at 24°C, the edges of the mating mixtures were photographed (100×).

Interestingly, a MATa cna1 mutant lacking the calcineurin A catalytic subunit mated normally with a MATa cnbl mutant lacking the calcineurin B regulatory subunit (Figure 2B). In this case, both mating partners lack calcineurin activity prior to cell fusion, but following cell fusion calcineurin activity is restored by complementation of the cna1 and cnbl mutations by the wild-type genes present in the opposite mating partner. Similar findings were observed in a cross of MATa cnbl and MATa cna1 mutant strains (Figure 2B). These findings suggest that calcineurin is not absolutely required for cell fusion in either mating partner, but is required for the production of the filamentous state by the heterokaryon produced by cell fusion.

The conclusion that calcineurin is necessary for filamentation of the heterokaryon is further supported by genetic crosses with a strain expressing a dominant FK506-resistant calcineurin B mutant subunit (CNB1-1) (Odom et al., 1997a; Fox et al., 2001). Mating of a MATa CNB1-1 mutant strain with a wild-type MATa strain was partially restored in the presence of FK506 but not CsA (data not shown). In this case, only the CNB1-1 mutant strain has calcineurin activity in the presence of FK506 prior to cell fusion. Following cell fusion, the CNB1-1 dominant mutation confers FK506 resistance in the CNB1-1/CNB1 heterokaryon, and renders cell filamentation largely FK506 resistant. Taken together, these
findings provide additional evidence that calcineurin is not required for cell fusion, but rather functions during hyphal elongation of the $MATa/MAT\alpha$ heterokaryon.

**Calcineurin regulates conjugation tube formation in response to pheromone**

To establish further which steps in mating are regulated by calcineurin, we analyzed cellular responses to mating pheromone. For this purpose, $MATa$ $CNA1$ wild-type and $MATa$ $cna1$ mutant cells were transformed with a linear episomal plasmid expressing the $MF\alpha1$ pheromone (see Materials and methods). Wild-type $MATa$ $CNA1$ cells expressing the $MF\alpha1$ pheromone responded in an autocrine fashion and produced conjugation tubes, as previously described (Moore and Edman, 1993; Davidson et al., 2000b) (Figure 3A). On the other hand, $MATa$ $cna1$ mutant strains failed to produce conjugation tubes in response to $MF\alpha1$ pheromone (Figure 3A). Similarly, $MATa$ wild-type cells exposed to the calcineurin inhibitors FK506 or CsA also failed to produce conjugation tubes in response to $MF\alpha1$ pheromone (Figure 3B). In a $MATa$ $frr1-1$ mutant strain lacking FKBP12, conjugation tube formation was restored in the presence of FK506, but not CsA, indicating that the effects of FK506 are mediated via FKBP12-dependent inhibition of calcineurin (Figure 3B). These findings indicate that calcineurin mutants have a defect in either producing or responding to pheromone. We note that $MATa$ cells expressing $MF\alpha1$ pheromone make both conjugation tubes and enlarged round cells. $MATa$ $cna1$ mutant cells still produced some enlarged
round cells, but no conjugation tubes, in response to overexpression of the MFα1 pheromone gene, suggesting that calcineurin is required for directed morphological responses to pheromone.

**Calcineurin is not required for transcription of the MFα1 pheromone gene**

To test whether calcineurin regulates the expression of the MFα1 pheromone gene, MATα CNA1 wild-type and MATα cnal mutant strains were transformed with a plasmid containing the promoter of the *C. neoformans* pheromone MFα1 gene fused to the open reading frame of the *Escherichia coli* lacZ gene (Davidson et al., 2000b). The MATα CNA1 + MFα1–lacZ and MATα cnal + MFα1–lacZ strains were grown for 24 h on solid yeast extract/peptone/dextrose (YPD) rich medium or V8 mating medium, either alone or in the presence of MATα cnal mutant or MATα CNA1 wild-type cells. The cells were then collected and β-galactosidase assays were performed. The MFα1 gene was induced to similar extents in both MATα CNA1 wild-type and MATα cnal mutant strains in response to both nutrients and a MATα CNA1 wild-type or a MATα cnal mutant mating partner (Figure 4), indicating that calcineurin is not required to sense nutrients or mating pheromone, or for expression of the MFα1 pheromone gene.

**Calcineurin is not required for fusion but is required for the production of recombinant basidiaospores**

The role of calcineurin in cell fusion and production of heterokaryons and recombinant basidiaospores was directly assessed with quantitative mating assays. For this purpose, MATα CNA1 *ura5* (JEC43) and MATα CNA1 *lys2 ade2* (JEC171) strains with complementary auxotrophic mutations were co-incubated on V8 agar with or without FK506. After 48 h or 6 days, mating patches were resuspended in phosphate-buffered saline (PBS) and plated on YNB minimal medium with or without FK506. After 48 h, filamentous prototrophic heterokaryons were detected from mating crosses on V8 medium with or without FK506 when plated on YNB medium alone, but no heterokaryons could be recovered on YNB medium containing FK506. After 6 days, no recombinant basidiaospores were detected from mating patches on V8 medium with FK506 in which calcineurin was inhibited.

These findings suggest that strains lacking calcineurin activity can initially undergo cell fusion and produce heterokaryons, but that the viability of the heterokaryons is inhibited by FK506. We tested this by assessing the fate of heterokaryons when calcineurin is inhibited. When heterokaryons were transferred from a wild-type mating to fresh low nitrogen agar medium, the hyphae continued elongating and gave rise to basidia and basidiaospores. In contrast, when dikaryotic hyphae were transferred to a medium containing either FK506 or CsA, the filaments stopped elongating and eventually disintegrated (data not shown). We conclude that calcineurin is essential for filamentation and viability of heterokaryons.

![Fig. 4](image.png)

**Calcineurin is required for hyphal elongation of diploid strains**

Recent studies reveal that *C. neoformans* can also exist in a diploid state. Diploid strains grow as yeast cells with single nuclei at 37°C on YNB medium, and are self-fertile and spontaneously filament and sporulate at 24°C (Sia et al., 2000). Because our findings suggest that calcineurin is required for heterokaryon viability and hyphal elongation, we tested whether calcineurin is also required for hyphal elongation and viability of diploid strains. When a self-fertile diploid strain was incubated on V8 mating medium at 24°C in the presence of FK506 or CsA, self- filamentation was inhibited (Figure 5A), providing further evidence that calcineurin is required for hyphal elongation. In addition, diploid cells were viable but grew more slowly when plated on either V8 medium or YPD with FK506 or CsA at 24°C (data not shown).

**Calcineurin is required for haploid fruiting**

In response to nitrogen limitation and desiccation, MATα cells differentiate by a process called haploid fruiting, in
which monokaryotic filaments are formed that produce basidia and MATα basidiospores (Wickes et al., 1996). Haploid fruiting can occur in response to nitrogen starvation, but is markedly stimulated by confrontation with MATα cells and likely plays a role during mating (Wang et al., 2000). In addition, haploid fruiting is stimulated in cells overexpressing the STE12α gene (Wickes et al., 1997) and haploid fruiting fails to occur in ste12α mutant strains (Yue et al., 1999; Chang et al., 2000). The C. neoformans Ste12α protein is a homolog of the Ste12 transcription factor, which regulates mating, invasive growth and filamentation in S. cerevisiae (Liu et al., 1993).

Several findings demonstrate that calcineurin is required for haploid fruiting. First, haploid fruiting of the wild-type MATα strain JEC21 in response to nitrogen limitation was inhibited by the calcineurin inhibitors FK506 or CsA (Figure 5B). Secondly, overexpression of the STE12α gene stimulated haploid fruiting of wild-type MATα cells, but failed to stimulate haploid fruiting in cells in which calcineurin was inhibited by FK506 or CsA (data not shown). Thirdly, whereas the wild-type MATα strain JEC21 underwent haploid fruiting in response to nitrogen limitation, isogenic MATα cna1 and MATα cnb1 mutant strains lacking calcineurin A or calcineurin B, respectively, failed to haploid fruit under these conditions (Figure 5C). Taken together, these findings indicate that calcineurin is required for haploid fruiting.

The role of calcineurin in mating and filamentous growth was further analyzed in confrontation assays in which MATα and MATα cells are streaked on the surface of filament agar as lines 2 mm apart. In this assay, wild-type MATα cells produce conjugation tubes and haploid fruit in response to MATα cells, whereas MATα cells produce enlarged round cells. In contrast, MATα cna1 and MATα cnb1 mutant strains failed to haploid fruit in a setting in which MATα haploid cells are normally stimulated and respond to factors secreted by MATα cells (Figure 6). MATα cna1 (Figure 6A) and MATα cnb1 (Figure 6B) mutant strains stimulated haploid fruiting of confronting MATα wild-type cells, indicating that calcineurin is not required for MATα cells to express or export MFA1 pheromone (Figure 6). In these confrontation assays, both the MATα cna1 and MATα cnb1 mutant strains produced few or no conjugation tubes or haploid filaments, but both strains produced some enlarged, round cells that normally only wild-type MATα cells produce in response to pheromone (Figure 6B). This observation suggests that calcineurin is not required to produce or sense pheromone, but is required for the formation of elongated conjugation tubes and filaments. Taken together, these findings reveal that calcineurin is required for hyphal elongation during mating, haploid fruiting, growth of diploid strains and the filamentous response to pheromone.

**Calcineurin regulates mating and growth at 37°C via distinct pathways**

To understand further the role of calcineurin in mating, we sought to identify targets of calcineurin required for mating. We took three approaches: the isolation of
Fig. 6. Calcineurin is required for haploid fruiting in response to pheromone. Isogenic MATα CNA1 and MATα CNA1 wild-type and MATα cna1 and MATα cna1 mutant strains, and isogenic MATα CNB1 and MATα CNB1 wild-type and MATα cnb1 and MATα cnb1 mutant strains, were grown in confrontation on filament agar to evaluate the role of calcineurin A (A) or calcineurin B (B). After 4–7 days at 24°C, the edges of the confronting cells were photographed (200×).

classical genetic suppressors, the isolation of multicopy genetic suppressors and the analysis of candidate genes. First, classical genetic suppressors were isolated that restored growth at 37°C in MATα cna1 and MATα cna1 calcineurin mutants. These extragenic suppressors restored growth at 37°C and conferred resistance to FK506 or CsA, indicating that the requirement for calcineurin for 37°C growth has been bypassed. However, although growth at 37°C was restored, mating was not. All of the suppressed mutant strains failed to mate with either MATα cna1 or MATα cna1 calcineurin mutant strains, or with cna1 mutant strains bearing a suppressor mutation that restores growth at 37°C (R.Sia and J.Heitman, unpublished results). These findings suggest that calcineurin regulates mating and growth at 37°C via independent pathways.

In the second approach, we performed multicopy suppressor screens to identify genes that would restore growth at 37°C or mating of calcineurin mutant strains. For this purpose, the MATα cna1::ADE2 ura5 ade2 calcineurin A mutant strain (MCC3) was transformed with a genomic library (8–12 kb inserts, Sau3A partial digest, >99% inserts) constructed in the episomal linear shuttle plasmid pPM8 (see Materials and methods) (Mondon et al., 2000). Ura+ transformants (1700) were colony purified and individually tested for restoration of mating when crossed to a MATα cna1::ADE2 ade2 calcineurin A mutant strain (MCC2), and examined for the production of mating filaments. No multicopy suppressor clones were identified that restored mating. However, a similar screen with the same library identified two clones that restored growth of the cna1 calcineurin A
mutant strain at 37°C. One of these clones also restored mating in calcineurin mutant strains in a bilateral mutant cross and was subsequently found to be the calcineurin A gene CNA1 (see Figure 2A). The second multicopy suppressor defines a novel gene, CTS1 (calcineurin temperature suppressor 1), which restores growth at 37°C but not mating of calcineurin mutant strains (D. Fox and J. Heitman, unpublished results). This finding provides further evidence that calcineurin regulates growth at 37°C and mating via different targets.

In recent studies, a highly conserved calcineurin binding protein, Cbp1, was identified, which physically interacts with calcineurin and functions as a regulator of calcineurin, a downstream effector, or both (Görlich et al., 2000; Kingsbury and Cunningham, 2000; Rothermel et al., 2000). When the C. neoformans Cbp1 protein was overexpressed by introducing the CBP1 gene in multiple copies on the episomal plasmid pPM8 (see Materials and methods), neither growth at 37°C nor mating was restored in the calcineurin A cna1 mutant strain MCC3 (data not shown). Moreover, a serotype A cbp1 mutant strain exhibited no mating defect with serotype D cna1 or cnb1 calcineurin mutant strains (data not shown). These findings suggest that the conserved calcineurin binding protein Cbp1 is not the target of calcineurin required for mating.

Finally, the mating defect of cna1 mutant strains lacking calcineurin A was not suppressed by overexpression or mutational activation of components of the pheromone-responsive mitogen-activated protein kinase signaling pathway (Gpb1, Ras1, Cpk1, Ste12α, MFα1), or by the addition of exogenous cAMP, providing evidence that calcineurin regulates mating via an independent pathway.

**Discussion**

We have discovered and analyzed a novel role for calcineurin in regulating mating and haploid fruiting of *C. neoformans*. These two processes both involve cell adaptation and responses to nutritional limitation and mating pheromones. Moreover, both mating and haploid fruiting result in the production of basidiospores, which are thought to be the infectious propagule of *C. neoformans*. Taken together with our previous findings that calcineurin is required for growth at 37°C and hence for virulence of *C. neoformans* (Odom et al., 1997a; Cruz et al., 2000b), these findings suggest that calcineurin could play a role in both the generation of the infectious particle and survival in the infected host.

Our studies have dissected the contribution of calcineurin to the different stages of mating using mutations in the genes encoding the calcineurin A and B subunits and the calcineurin inhibitors FK506 and CsA. Calcineurin is not required for expression of the MFα1 pheromone gene in response to either nutritional signals or mating partner cells. In addition, *MATa* cells lacking calcineurin still secreted mating pheromone and induced haploid fruiting of confronting *MATa* cells. These observations indicate that calcineurin is not required for nutrient or pheromone sensing, or for pheromone expression, production or secretion. Mutation (*MATa cna1*) or inhibition of calcineurin (with FK506 or CsA) did prevent conjugation tube formation in response to the MFα1 mating pheromone, suggesting that calcineurin is required for directed morphological responses to pheromone.

Calcineurin is required for mating, and no recombinant basidiospores were produced when calcineurin mutants were mated or when wild-type strains were mated in the presence of FK506 or CsA. Our studies indicate that calcineurin is not required for cell fusion, but is required for viability and hyphal elongation of the heterokaryon produced by cell fusion. In the absence of calcineurin, fused heterokaryons degenerate and failed to differentiate into basidia, where nuclear fusion, meiosis and mitosis would normally occur to generate chains of basidiospores. In addition, self-fertile diploid strains failed to filament in the presence of FK506 and CsA, further supporting the hypothesis that calcineurin is required for morphogenesis. Finally, calcineurin was also found to be absolutely required for haploid fruiting in *MATa* cells in response to nitrogen starvation or *MATa* cells. A similar role in hyphal elongation has previously been ascribed to calcineurin in *N. crassa* (Proksch et al., 1997), and Ca²⁺ fluxes across the tips of elongating hyphae that have been reported in a variety of fungi may similarly implicate calcineurin and other calcium-activated enzymes in hyphal growth (Levina et al., 1995; Lew, 1999).

Our studies revealed that calcineurin is required for directed morphogenesis in response to mating pheromone. Calcineurin mutant strains exhibit a bilateral mating defect in which the initial cell fusion event appears to occur normally, but subsequent filamentous growth of the heterokaryon fails to occur and viability of the heterokaryon is reduced. Interestingly, during the earliest steps in mating, both *MATa* and *MATα* cells produce elongated conjugation tubes in response to pheromone produced by the mating partner cell (Alspaugh et al., 2000). This is most prominent in *MATα* cells, which form conjugation tubes and then undergo robust haploid fruiting in response to confrontation with *MATa* cells (Davidson et al., 2000b; Wang et al., 2000). In contrast, *MATα* cells produce fewer conjugation tubes, but instead produce a large number of enlarged, round cells in confrontation with *MATa* cells, or when transformed with the *MFα1* pheromone or the STE12α gene (Moore and Edman, 1993; Wickes et al., 1997; Davidson et al., 2000b). In the studies reported here, we found that both *MATα cna1* and *MATα cna1* mutant strains failed to form elongated conjugation tubes in response to mating pheromones. In addition, *MATa cna1* mutant strains produced large, round cells in response to MFα1 pheromone, which are normally prominently produced by only wild-type *MATa* cells. These findings suggest that calcineurin normally functions to promote directed, elongated conjugation tube formation in *MATα* cells, and that in the absence of calcineurin, *MATα* cells adopt a default fate and respond to pheromone in a manner like that of *MATa* cells. Because calcineurin is not required for cell fusion during mating, the ability of *MATα* cells to produce elongated conjugation tubes and haploid fruit in response to pheromone may not be required for mating under conditions of high cell density. Directed morphogenetic responses to pheromone may only play a role under conditions in which mating partners are limiting or at a distance, similar to the courtship stages of mating in *S. cerevisiae*. 

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Our previous studies revealed an important role for calcineurin in regulating growth at 37°C and virulence of *C. neoformans* (Odum et al., 1997a; Cruz et al., 2000b), whereas the studies described here illustrate an additional and central role for calcineurin in mating and filamentous differentiation. Several lines of evidence indicate that calcineurin regulates growth at 37°C and mating via different pathways. First, classical genetic suppressors that restore growth of calcineurin mutant strains at 37°C failed to restore mating. Secondly, we have identified a novel multicyclic suppressor gene, *CTS1*, which restores growth at 37°C but not mating of calcineurin mutant strains (D. Fox and J. Heitman, unpublished results). Finally, overexpression of the Cbp1 protein, which physically interacts with calcineurin and may play a role in some calcineurin-regulated pathways with respect to cation resistance (Görlich et al., 2000; Kingsbury and Cunningham, 2000), failed to restore growth at 37°C or mating of calcineurin mutant strains. These findings suggest that calcineurin regulates growth at 37°C, mating and filamentous differentiation, and cation homeostasis via distinct pathways, possibly involving different target proteins. In our studies, we have thus far been unable to identify any extragenic multicyclic suppressor genes that restore mating in calcineurin A mutant strains. While these screens may not yet be fully saturating, it is also possible that there is more than one target protein required for mating such that overexpression of only one gene at a time does not suffice. Alternatively, overexpression of the target might be deleterious to the cell or overexpression might not overcome the requirement for calcineurin. Finally, the levels of the relevant target protein might be regulated or the protein unstable, making overexpression difficult to achieve by these approaches. Further studies will be required to identify the target(s) of calcineurin that regulates mating and filamentous differentiation of *C. neoformans*.

The significant evolutionary conservation of calcineurin among eukaryotes supports a general and important role for calcineurin and calcineurin-regulated pathways in the normal physiology of eukaryotic cells (Crabtree, 2001). Calcineurin belongs to a superfamily of protein serine-threonine phosphatases, and is regulated by intracellular Ca²⁺ concentrations. The role of calcineurin in T-cell activation has been well studied and involves the regulation of nuclear transport of the transcription factor NF-AT, which induces T-cell activation genes. In fungi, calcineurin has been shown to play a role in cellular adaptations to stress. For example, in *S. cerevisiae*, calcineurin is required for cation homeostasis and regulates cell wall biosynthesis by the transcription factor Tcn1/Crz1, which is distantly related to NF-AT, promoting nuclear import and gene activation (Cyr and Thorner, 1992; Nakamura et al., 1993; Cunningham and Fink, 1994, 1996; Hemenway et al., 1995; Mazur et al., 1995; Moser et al., 1996). Although the *C. neoformans* genome sequencing project has resulted in >3X coverage of the entire cryptococcal genome, no homologs of NF-AT or Tcn1p/ Crz1p are as yet apparent.

Calcineurin plays a general role in regulating mating in *S. pombe*, *S. cerevisiae* and *C. neoformans*. In *S. pombe*, calcineurin-dependent mutations are sterile, but the calcineurin-dependent step in mating has not yet been defined (Yoshida et al., 1994). In *S. cerevisiae*, both calcineurin and calcmodulin-dependent protein kinase are required for physiologic responses during late phases of pheromone signaling (Cyr and Thorner, 1992; Fair et al., 1992; Moser et al., 1996; Witzee et al., 1997). Calcineurin mutation, or inhibition of calcineurin with FK506 or CSA, prevents recovery from pheromone-induced cell cycle arrest. When yeast cells are exposed to pheromone, intracellular Ca²⁺ levels transiently increase 45 min later (Iida et al., 1990, 1994; Nakajima-Shimada et al., 1991; Paidhungat and Garrett, 1997), activating calmodulin and, in turn, both calcineurin and CAM kinase to regulate cell responses required for recovery.

In *C. neoformans*, calcineurin is involved in hyphal elongation during mating and haploid fruiting, suggesting that calcineurin regulates cell morphogenesis during differentiation. Calcineurin may regulate filamentation of *C. neoformans* by either regulating transcription, as in *S. cerevisiae*, by locally responding to Ca²⁺ fluxes and post-translationally regulating target proteins that promote hyphal extension, as is thought to be the case in *N. crassa*, or by both mechanisms. Remarkably, calcineurin is involved in the regulation of neurite extension and directed filopodial motility in mammalian cells, indicating that calcineurin may have functions in regulating morphogenesis that are conserved from fungi to humans (Reirre et al., 1993; Lyons et al., 1994; Chang et al., 1995). Further studies will be required to identify the substrates of calcineurin that regulate morphogenesis, which, like NF-AT and Tcn1/Crz1, may share an evolutionary heritage from fungi to humans.

**Materials and methods**

**Strains, plasmids, media and compounds**

Strains JEC20 and JEC21 are congenic MATα and MATα serotype D strains, respectively. JEC43 (MATα ura5) and JEC171 (MATα lys2 ade2) are serotype D strains derived from strains JEC20 and JEC21 (Moore and Edman, 1993). MCC5 (MATα cna1::ADE2 ade2) and MCC10 (MATα cna1::ADE2 ade2) have been described previously (Cruz et al., 2000b) and are congenic calcineurin A mutants of the JEC20/JEC21 strain series produced by transformation and homologous recombination. Strains C20F1 (MATα frq1-1) and C21F3 (MATα frq1-3) have been described previously (Odum et al., 1997a; Cruz et al., 1999) and are isogenic FKBP12 mutants derived from strains JEC20 and JEC21. Strain RAS011 (MATα/MATα ade2+ ura3/lara5 lys2+ lys2+4) is a diploid strain (Sia et al., 2000).

Plasmid pRCD3 contains the wild-type MFA1 gene and was constructed by PCR amplification from genomic DNA of strain JEC21 with synthetic primers. The PCR fragment was then cloned into the *C. neoformans/E. coli* shuttle vector pCntel1, which contains the selectable marker gene URA5 (Edman, 1992; Davidson et al., 2000b). The plasmid pRCD4 containing the promoter of the MFA1 pheromone gene fused to the E. coli lacZ gene was constructed by cloning the MFA1–lacZ gene fusion into the *C. neoformans/E. coli* shuttle vector pMM97-3 containing the *C. neoformans* URA5 gene as a selectable marker, as previously described (Davidson et al., 2000b).

YPD synthetic medium, V8 agar and filament agar were described in Alsop et al. (1997). FK506 was from Fujisawa. CSA was from Alexis Biochemicals. Drug stock solutions were prepared in 90% ethanol, in Tween-20.

**Mating, haploid fruiting and confrontation assays**

For mating assays, strains of opposite mating types were grown on YPD agar for 48 h at 30°C, and then co-cultured on V8 agar at 24°C. Mating patches were scored microscopically for filamentation using a Nikon Eclipse E400 microscope. Haplod fruiting was assayed by incubating spotted suspensions of cells on filament agar at 24°C for up to 4 weeks. Confrontation assays were performed as follows: isolated colonies of
opposite mating types were streaked with sterile toothpicks on the surface of filament agar, in confronting lines ~2 mm from each other, and the cells were then incubated for 4–7 days at 24°C.

Conjugation tube formation assay

Strains JEC34 (MATa ura5), MCC83 (MATa tet1-1 ura5) and MCC3 (MATa can1::ADE2 ade2 ura5) were transformed by electroporation with plasmid pRCD3, which is an episomal linear telomeric plasmid derived from plasmid pCnTeII (Edman, 1992), which contains the wild-type MFα1 gene and URA5 as the selectable marker (Davidson et al., 2000b).

Transformants were selected on synthetic medium lacking uracil, grown on V8 agar medium containing 1 μg/ml FK506 or 100 μg/ml CanA for 2 days at 24°C, and conjugation tubes were observed by microscopy at 200× magnification with a Nikon Eclipse E400 microscope and photographed.

Fusion/basidiopore production assay

Strains JEC43 (MATa ura5) and JEC171 (MATa lys2 ade2) were grown in liquid YPD overnight, pelleted, and resuspended in water at 10⁶ cells/ml. Two sets of mating mixes were made by mixing 100 μl of each suspension. Ten microliters of this mixture as well as 10 μl of the individual suspensions (control) were spotted onto mating medium (V8) and V8 medium containing 1 μg/ml FK506, and were incubated at 24°C. At 48 h and 6 days, the mating reaction and the JEC43 and JEC171 individual patches were cut out and resuspended by vortexing in PBS. Dilutions of these suspensions were plated on minimal YNB medium and YNB containing 1 μg/ml FK506, and incubated at 30°C for 3 days to select for prototrophic basidiopores and heterokaryons. The parental strains are auxotrophic and hence cannot grow on minimal medium.

MFα1–lacZ reporter analysis

The promoter of the C. neoformans pheromone MFα1 gene was fused to the open reading frame of the E. coli lacZ gene resulting in plasmid pRCD41, which also contains the selectable marker gene URA5 (Davidson et al., 2000b). Circular DNA of plasmid pRCD41 (MFα1–lacZ) was then biolistically transformed into strains JEC43 (MATa CAN1 ura5) and MCC27 (MATa can1::ADE2 ura5), and transformants were selected on synthetic medium lacking uracil and containing 1 M sorbitol, resulting in strains RDC10 (MATa CAN1+ MFα1–lacZ) and MCC82 (MATa can1+ MFα1–lacZ), respectively, in which the reporter plasmid is stably integrated into the genome. Strains RDC10 and MCC82 were grown overnight in liquid YPD and, subsequently, 2 × 10⁶ cells were grown on solid YPD rich medium, and 1.5 × 10⁷ cells on V8 mating medium alone or in the presence of an equal number of JEC20 (MATa CAN1) or MCC10 (MATa can1) cells for 24 h. The cells were then collected and β-galactosidase assays were performed with the chromogenic substrate CTPR, as described (Cardenas et al., 1994), and corrected to adjust for the fact that only one half of the cells in the population contain the pRCD41 reporter plasmid.

Transformations

Transformations by electroporation were performed according to the method described by Edman and Kwon-Chung (1990) using a Bio-Rad gene pulser (480 mV, 25 μF, 600 Ω). This method was used for all pCnTeII-based plasmids, which were cleaved with the rare cutting enzyme I-SceI to reveal the telemistic ends before transformation. Biologic transformations were performed by the protocol previously described by Toffalletti and Perfect (1994) and Davidson et al. (2000a) using a Bio-Rad Model PDS-1000/He biologic particle delivery system.

Multicopy suppressor library

The multicopy suppressor library was constructed with genomic DNA from the serotype D strain C212F2, which was partially digested with Sau3AI and ligated into the BamHI restriction site of the plasmid pPM8 (Mondon et al., 2000). The genomic library was cut with I-SceI and electroporated into the MATa can1::ADE2 ade2 ura5 A mutant strain (MCC3). The transformation was performed according to the method described by Edman and Kwon-Chung (1990) using a Bio-Rad gene pulser (470 mV, 25 μF, resistance =).

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