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 Ca^{2+} -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 $\text{MAT}\alpha$ 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 Ca^{2+} ions, which bind to and activate calmodulin. The Ca^{2+} -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 (Casadevall 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 $\text{MAT}\alpha$ and $\text{MAT}\alpha$ 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 $\text{MAT}\alpha$ and $\text{MAT}\alpha$ 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, $\text{MAT}\alpha$ 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 $\text{MAT}\alpha$ cells is also dramatically stimulated by confrontation with $\text{MAT}\alpha$ 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 $\text{MAT}\alpha$ basidiospores.

Calcineurin is a Ca^{2+} -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 *S.cerevisiae*, 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; Farcasanu *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 Cyert, 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; Lautermilch 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 *MATa 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 *MATa frr1-1* and *MATα 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 *MATa* and *MATα* serotype D strains. *MATa cnal* and *MATα cnal* mutant strains were isolated by transformation and homologous recombination with a *cnal::ADE2* allele (Cruz *et al.*, 2000b; Davidson *et al.*, 2000a). When *MATa cnal* or *MATα cnal* mutant strains were mixed with wild-type *MATα* (JEC21) or *MATa* (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,

Fig. 1. The calcineurin inhibitors FK506 and CsA prevent mating of *C. neoformans*. Isogenic wild-type *MATa* and *MATα* strains were co-incubated on V8 mating medium with or without 1 μg/ml FK506 or 100 μg/ml CsA. In the middle and lower panels, isogenic *MATa* and *MATα* strains lacking FKBP12 (*frr1*) were co-incubated with wild-type and *frr1* mutant mating partners in the absence or presence of FK506 or CsA. After 7 days at 24°C, the edges of the mating mixtures were photographed (100×). Mating between *MATα* and *MATa* wild-type cells results in the production of abundant filaments bearing basidia and basidiospores, as shown in the upper left panel. When no mating occurs, a smooth border of only yeast cells is observed (upper middle panel).

when *MATa cna1* and *MATα cna1* 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 cna1* 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 cna1* 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 *cna1*

Fig. 2. Calcineurin mutants are bilaterally sterile. Isogenic wild-type, *MATa cna1*, *MATα cna1*, *MATa cnb1* and *MATα cnb1* mutant strains, the serotype D *MATa cna1* strain with the wild-type *CNA1* gene on the linear episomal plasmid pPM8 and the serotype A *MATα 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×).

calcineurin A mutant strains, the *MATα cnb1* and *MATa cnb1* mutant strains mated normally with *CNB1* wild-type strains, but failed to mate with each other, indicating that the *cnb1* mutation also confers a bilateral mating defect (Figure 2B).

Interestingly, a *MATa cna1* mutant lacking the calcineurin A catalytic subunit mated normally with a *MATα cnb1* 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 *cnb1* mutations by the wild-type genes present in the opposite mating partner. Similar findings were observed in a cross of *MATa cnb1* and *MATα 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 *MATα 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

Fig. 3. Calcineurin is required for conjugation tube formation in response to MF α 1 pheromone. (A) Isogenic *MATa CNA1* wild-type and *MATa cna1* mutant strains were transformed with a linear telomeric plasmid expressing the MF α 1 pheromone gene (pRCD3; see Materials and methods) (Davidson *et al.*, 2000b). Cells were grown on filament agar medium for 2 days at 24°C and photographed (200 $\times$). (B) *MATa* wild-type and *MATa frr1-1* mutant strains were transformed with a linear telomeric plasmid (pRCD3) expressing the MF α 1 pheromone gene. Cells were grown on V8 agar medium containing 1 μ g/ml FK506 or 100 μ g/ml CsA for 2 days at 24°C and photographed (200 $\times$).

findings provide additional evidence that calcineurin is not required for cell fusion, but rather functions during hyphal elongation of the *MATa/MAT α* 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 α 1 pheromone (see Materials and methods). Wild-type *MATa CNA1* cells expressing the MF α 1 pheromone responded in an auto-crine 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 α 1 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 α 1 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 α 1 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 *MF α 1* pheromone gene, suggesting that calcineurin is required for directed morphological responses to pheromone.

Calcineurin is not required for transcription of the *MF α 1* pheromone gene

To test whether calcineurin regulates the expression of the *MF α 1* pheromone gene, *MAT α CNA1* wild-type and *MAT α cna1* mutant strains were transformed with a plasmid containing the promoter of the *C.neoformans* pheromone *MF α 1* gene fused to the open reading frame of the *Escherichia coli lacZ* gene (Davidson *et al.*, 2000b). The *MAT α CNA1* + *MF α 1-lacZ* and *MAT α cna1* + *MF α 1-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 *MAT α cna1* mutant or *MAT α CNA1* wild-type cells. The cells were then collected and β -galactosidase assays were performed. The *MF α 1* gene was induced to similar extents in both *MAT α CNA1* wild-type and *MAT α cna1* mutant strains in response to both nutrients and a *MAT α CNA1* wild-type or a *MAT α cna1* mutant mating partner (Figure 4), indicating that calcineurin is not required to sense nutrients or mating pheromone, or for expression of the *MF α 1* pheromone gene.

Calcineurin is not required for fusion but is required for the production of recombinant basidiospores

The role of calcineurin in cell fusion and production of heterokaryons and recombinant basidiospores was directly assessed with quantitative mating assays. For this purpose, *MAT α CNA1 ura5* (JEC43) and *MAT α 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 basidiospores 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 basidiospores. 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. Calcineurin does not regulate expression of the *MF α 1* pheromone gene. (A) The wild-type *MAT α CNA1* + *MF α 1-lacZ* (RDC10) and (B) *MAT α cna1* + *MF α 1-lacZ* (MCC82) calcineurin mutant strains were grown on YPD medium (white bars) and V8 mating medium (grey bars) in the presence or absence of *MAT α cna1* (MCC10) calcineurin mutant or *MAT α CNA1* (JEC20) calcineurin wild-type cells for 24 h, harvested, and β -galactosidase was assayed with the chromogenic substrate CPRG and expressed in Miller units as previously described (Davidson *et al.*, 2000b). Assays were conducted in triplicate, and the mean values are presented with the SEM as error bars.

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, *MAT α* cells differentiate by a process called haploid fruiting, in

Fig. 5. Calcineurin is required for hyphal elongation of diploid strains and haploid fruiting. (A) A suspension of cells of the *MATα/MATa* diploid strain RAS011 was spotted on V8 medium with or without 1 μ g/ml FK506 or 100 μ g/ml CsA, and incubated for 14 days at 24°C. The edges of the confluent patch of colonies exhibiting filamentous growth (left panel) or lacking filamentous growth (middle and right panels) were photographed (100 $\times$). (B) To induce haploid fruiting, the wild-type *MATα* strain JEC21 was incubated on filament agar that also lacked or contained 1 μ g/ml FK506 or 100 μ g/ml CsA. After 14 days at 24°C, the edges of the colonies were photographed (100 $\times$). (C) Isogenic *MATα* wild-type and *MATα cna1* and *MATα cnb1* strains were grown on filament agar to induce haploid fruiting. After 14 days at 24°C, the edges of the colonies were photographed (100 $\times$).

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 *MATa* 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* Ste12 α 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 *MATa* 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 *MATa* cells, whereas *MATa* 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 *MATa* cells (Figure 6). *MATa cna1* (Figure 6A) and *MATa cnb1* (Figure 6B) mutant strains stimulated haploid fruiting of confronting *MATα* wild-type cells, indicating that calcineurin is not required for *MATa* cells to express or export MFa 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 *MATa* 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 *MATa CNA1* wild-type and *MATα cna1* and *MATa cna1* mutant strains, and isogenic *MATα CNB1* and *MATa CNB1* wild-type and *MATα cnb1* and *MATa 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 *MATa 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 *MATa 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 *MATa 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 (Görlach *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 *cnal* mutant strain MCC3 (data not shown). Moreover, a serotype A *cbp1* mutant strain exhibited no mating defect with serotype D *cnal* 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 *cnal* 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, Ste12 α , MF α 1), 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 MF α 1 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 *MAT α* cells. These observations indicate that calcineurin is not required for nutrient or pheromone sensing, or for pheromone expression, production or secretion. Mutation (*MAT α cnal*) or inhibition of calcineurin (with FK506 or CsA) did prevent conjugation tube formation in response to the MF α 1 mating phero-

none, 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 *MAT α* cells in response to nitrogen starvation or *MATa* cells. A similar role in hyphal elongation has previously been ascribed to calcineurin in *N.crassa* (Prokisch *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 *MAT α* cells produce elongated conjugation tubes in response to pheromone produced by the mating partner cell (Alspaugh *et al.*, 2000). This is most prominent in *MAT α* 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, *MATa* cells produce fewer conjugation tubes, but instead produce a large number of enlarged, round cells in confrontation with *MAT α* cells, or when transformed with the MF α 1 pheromone or the STE12 α gene (Moore and Edman, 1993; Wickes *et al.*, 1997; Davidson *et al.*, 2000b). In the studies reported here, we found that both *MAT α cnal* and *MATa cnal* mutant strains failed to form elongated conjugation tubes in response to mating pheromones. In addition, *MAT α cnal* mutant strains produced large, round cells in response to MFa 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 *MAT α* cells, and that in the absence of calcineurin, *MAT α* 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 *MAT α* 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 morphogenic 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*.

Our previous studies revealed an important role for calcineurin in regulating growth at 37°C and virulence of *C. neoformans* (Odom *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 multicopy suppressor gene, *CTSI*, 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 (Görlach *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 multicopy 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 Ca^{2+} 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 (Cyert 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 $>3\times$ 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 mutants are sterile, but the calcineurin-dependent step in mating has not yet been defined

(Yoshida *et al.*, 1994). In *S. cerevisiae*, both calcineurin and calmodulin-dependent protein kinase are required for physiological responses during late phases of pheromone signaling (Cyert and Thorner, 1992; Foor *et al.*, 1992; Moser *et al.*, 1996; Withee *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 Ca^{2+} 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 Ca^{2+} 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 (Ferreira *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 *MATa* and *MAT α* serotype D strains, respectively. JEC43 (*MAT α ura5*) and JEC171 (*MATa lys2 ade2*) are serotype D strains derived from strains JEC20 and JEC21 (Moore and Edman, 1993). MCC5 (*MAT α cna1::ADE2 ade2*) and MCC10 (*MATa 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 (*MATa fir1-1*) and C21F3 (*MAT α fir1-3*) have been described previously (Odom *et al.*, 1997a; Cruz *et al.*, 1999) and are isogenic FKBP12 mutants derived from strains JEC20 and JEC21. Strain RAS011 (*MATa/MAT α ade2/+ ura5/ura5 lys1/+ lys2/+*) is a diploid strain (Sia *et al.*, 2000).

Plasmid pRCD3 contains the wild-type *MF α 1* 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 pRCD41 containing the promoter of the *MF α 1* pheromone gene fused to the *E. coli lacZ* gene was constructed by cloning the *MF α 1-lacZ* gene fusion into the *C. neoformans*/*E. coli* shuttle vector pJMM97-3 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 Alsbaugh *et al.* (1997). FK506 was from Fujisawa. CsA was from Alexis Biochemicals. Drug stock solutions were prepared in 90% ethanol, 10% 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. Haploid 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 frl1-1 ura5*) and MCC3 (*MATa cna1::ADE2 ade2 ura5*) were transformed by electroporation with plasmid pRCD3, which is an episomal linear telomeric plasmid derived from plasmid pCnTel1 (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 CsA for 2 days at 24°C, and conjugation tubes were observed by microscopy at 200× magnification with a Nikon Eclipsed E400 microscope and photographed.

Fusion/basidiospore 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 basidiospores 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 CNA1 ura5*) and MCC27 (*MATa cna1::ADE2 ura5*), and transformants were selected on synthetic medium lacking uracil and containing 1 M sorbitol, resulting in strains RDC10 (*MATa CNA1+ MFα1-lacZ*) and MCC82 (*MATa cna1+ 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 × 10⁷ cells were grown on solid YPD rich medium, and 1.5 × 10⁸ cells on V8 mating medium alone or in the presence of an equal number of JEC20 (*MATa CNA1*) or MCC10 (*MATa cna1*) cells for 24 h. The cells were then collected and β-galactosidase assays were performed with the chromogenic substrate CPRG, 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 pCnTel1-based plasmids, which were cleaved with the rare cutting enzyme *I-SceI* to reveal the telomeric ends before transformation. Biolistic transformations were performed by the protocol previously described by Toffaletti and Perfect (1994) and Davidson *et al.* (2000a) using a Bio-Rad Model PDS-1000/He biolistic particle delivery system.

Multicopy suppressor library

The multicopy suppressor library was constructed with genomic DNA from the serotype D strain C21F2, 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 cna1::ADE2 ura5 ade2* calcineurin 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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