

Calcineurin is essential for survival during membrane stress in *Candida albicans*

M.Cristina Cruz¹, Alan L.Goldstein²,
Jill R.Blankenship¹, Maurizio Del Poeta³,
Dana Davis⁴, Maria E.Cardenas¹,
John R.Perfect^{2,5}, John H.McCusker^{1,2} and
Joseph Heitman^{1,2,5,6,7,8}

¹Departments of Genetics, ⁶Pharmacology and Cancer Biology,

²Microbiology and ⁵Medicine, and the ⁷Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710,

³Departments of Biochemistry and Microbiology and Immunology, Medical University of South Carolina, Charleston, SC and

⁴Department of Microbiology, University of Minnesota, Minneapolis, MN, USA

⁸Corresponding author at: Department of Genetics, Duke University Medical Center, Durham, NC 27710, USA
e-mail: heitm001@duke.edu

The immunosuppressants cyclosporin A (CsA) and FK506 inhibit the protein phosphatase calcineurin and block T-cell activation and transplant rejection. Calcineurin is conserved in microorganisms and plays a general role in stress survival. CsA and FK506 are toxic to several fungi, but the common human fungal pathogen *Candida albicans* is resistant. However, combination of either CsA or FK506 with the antifungal drug fluconazole that perturbs synthesis of the membrane lipid ergosterol results in potent, synergistic fungicidal activity. Here we show that the *C.albicans* FK506 binding protein FKBP12 homolog is required for FK506 synergistic action with fluconazole. A mutation in the calcineurin B regulatory subunit that confers dominant FK506 resistance (*CNB1-1/CNB1*) abolished FK506–fluconazole synergism. *Candida albicans* mutants lacking calcineurin B (*cnb1/cnb1*) were found to be viable and markedly hypersensitive to fluconazole or membrane perturbation with SDS. FK506 was synergistic with fluconazole against azole-resistant *C.albicans* mutants, against other *Candida* species, or when combined with different azoles. We propose that calcineurin is part of a membrane stress survival pathway that could be targeted for therapy.

Keywords: calcineurin/*Candida albicans*/cyclosporin A/fluconazole/antifungal drugs

Introduction

The immunosuppressants cyclosporin A (CsA) and FK506 block rejection of transplanted organs by inhibiting signaling pathways required for T-cell activation (Schreiber and Crabtree, 1992). Both drugs are in widespread clinical use and have had a dramatic impact on transplant therapy. Interestingly, CsA and FK506 are natural products of soil-dwelling bacteria or fungi (reviewed in Cardenas *et al.*, 1994). Both drugs are toxic to a variety of fungi, suggesting one natural role might be

to inhibit competing microorganisms in the soil (Tropschug *et al.*, 1989; Breuder *et al.*, 1994; Odom *et al.*, 1997a; Arndt *et al.*, 1999).

CsA and FK506 exert immunosuppressive and antifungal effects by inhibiting calcineurin (Liu *et al.*, 1991; Foor *et al.*, 1992; Nakamura *et al.*, 1993; Breuder *et al.*, 1994; Odom *et al.*, 1997a; Fox *et al.*, 2001), a conserved Ca²⁺-calmodulin activated protein phosphatase (reviewed in Klee *et al.*, 1998; Hemenway and Heitman, 1999; Aramburu *et al.*, 2000). Calcineurin is a heterodimer comprised of a catalytic A and a regulatory B subunit (Hubbard and Klee, 1989; Anglister *et al.*, 1994; Watanabe *et al.*, 1995). CsA and FK506 do not inhibit calcineurin on their own, but first bind to small, abundant, conserved binding proteins (immunophilins). CsA associates with cyclophilin A, and FK506 with FKBP12, to form protein–drug complexes that inhibit calcineurin by binding to the interface between the A and B subunits (Haddy *et al.*, 1992; Li and Handschumacher, 1993; Milan *et al.*, 1994; Cardenas *et al.*, 1995b; Griffith *et al.*, 1995; Kawamura and Su, 1995; Kissinger *et al.*, 1995). CsA and FK506 target this unique region of calcineurin and do not inhibit other phosphatases.

The mechanisms of action of CsA and FK506 are conserved from fungi to humans. Calcineurin, calmodulin and the CsA and FK506 binding proteins cyclophilin A and FKBP12 are all conserved in the yeast *Saccharomyces cerevisiae* (Tropschug *et al.*, 1989; Cyert *et al.*, 1991; Heitman *et al.*, 1991). Calcineurin is required for yeast to survive cation stress or recover from pheromone arrest, and CsA and FK506 are toxic under these conditions (Cyert and Thorner, 1992; Nakamura *et al.*, 1993; Breuder *et al.*, 1994; Cunningham and Fink, 1994, 1996; Moser *et al.*, 1996; Withee *et al.*, 1997). Yeast strains lacking cyclophilin A or FKBP12 are viable and CsA- or FK506-resistant, respectively (Tropschug *et al.*, 1989; Foor *et al.*, 1992; Breuder *et al.*, 1994; Cardenas *et al.*, 1995a). Moreover, the isolation of dominant drug resistant mutants revealed calcineurin residues dispensable for activity but necessary for inhibitor binding (Cardenas *et al.*, 1995b). These genetic studies support the model that CsA and FK506 actions are mediated via toxic protein–drug complexes that inhibit calcineurin.

CsA and FK506 also exhibit antifungal activity against the human fungal pathogen *Cryptococcus neoformans* (Odom *et al.*, 1997a). CsA and FK506 have no antifungal activity against *C.neoformans* at 24°C, but become potently toxic at 37°C by associating with cyclophilin A and FKBP12 homologs that target the fungal calcineurin homolog (Odom *et al.*, 1997a; Cruz *et al.*, 1999; Fox *et al.*, 2001; Wang *et al.*, 2001). Mutants lacking calcineurin A or B are viable at 24°C, but inviable at 37°C and avirulent in animal models of cryptococcosis (Odom *et al.*, 1997a; Cruz *et al.*, 2000b; Fox *et al.*, 2001). CsA can protect

Table I. FKBP12 homolog Rbp1 and calcineurin are targets of FK506 synergistic activity with fluconazole

<i>C.albicans</i> strains	MIC alone ($\mu\text{g/ml}$)			MIC combined		FIC index	
	Flu	FK506	CsA	Flu + FK506	Flu + CsA	Flu + FK506	Flu + CsA
Wild-type (SC5314)	>16	>3.12	>12.5	0.5/ \leq 0.04	0.5/0.78	0.018	0.032
Wild-type (CAI4)	>16	>3.12	>12.5	4/ \leq 0.04	4/0.78	0.13	0.16
<i>rbp1</i> Δ / <i>RBP1</i> (YAG116)	>16	>3.12	>12.5	1/ \leq 0.04	2/1.56	0.034	0.12
<i>rbp1</i> Δ / <i>rbp1</i> Δ (YAG171)	>16	>3.12	>12.5	>16/>3.12	4/0.78	2	0.16
<i>CNB1-1</i> / <i>CNB1</i> (YAG237)	>16	>12.5	>12.5	>16/>12.5	2/3.12	2	0.19
<i>erg11</i> / <i>ERG11</i> (4A)	>16	>12.5	>12.5	2/ \leq 0.19	2/1.56	0.066	0.12
<i>erg6</i> / <i>ERG6</i> (KPC1)	>16	>12.5	>12.5	2/0.39	2/3.12	0.078	0.19
<i>erg6</i> / <i>erg6</i> (KPC8)	>16	\leq 0.19	1.56	\leq 0.01/ \leq 0.19	\leq 0.01/1.56	1.0001	1.0001
Wild-type (BWP17)	>16	>12.5	>12.5	2/0.39	4/1.56	0.078	0.19
<i>cnb1</i> / <i>CNB1</i> (DAY345)	>16	>12.5	>12.5	0.5/ \leq 0.19	0.5/1.56	0.019	0.078
<i>cnb1</i> / <i>cnb1</i> (DAY364)	0.5	>12.5	>12.5	0.5/ \leq 0.19	0.5/ \leq 0.19	1.003	1.003

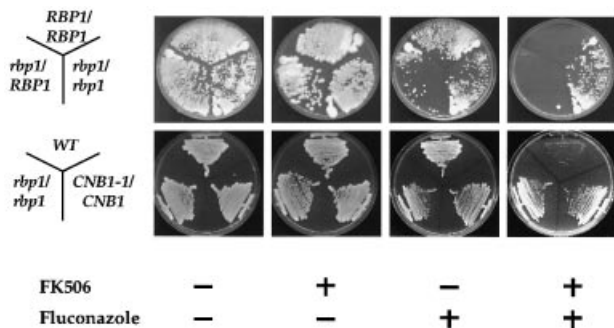


Fig. 1. FKBP12 and calcineurin are the targets of FK506–fluconazole synergism. The *RBP1/RBP1* wild-type (SC5314), *rbp1/RBP1* heterozygous mutant (YAG116), *rbp1/rbp1* homozygous mutant (YAG171) and *CNB1-1/CNB1* heterozygous mutant strains (YAG237) were grown on YPD medium alone or with 1 $\mu\text{g/ml}$ FK506, 50 $\mu\text{g/ml}$ fluconazole, or 1 $\mu\text{g/ml}$ FK506 and 50 $\mu\text{g/ml}$ fluconazole for 72 h at 37°C.

animals from pulmonary cryptococcal infection (Mody *et al.*, 1988, 1989), but does not cross the blood–brain barrier and exacerbates cryptococcal meningitis by suppressing helper T-cell function (Perfect and Durack, 1985). Non-immunosuppressive CsA and FK506 analogs that retain antifungal activity have been identified and hold promise as novel antifungal agents (Odom *et al.*, 1997b; Cruz *et al.*, 2000a). These analogs are impaired in their ability to inhibit mammalian calcineurin, yet inhibit fungal calcineurins in an immunophilin-dependent fashion by exploiting structural differences between the human and fungal targets.

Fungal infections are an increasingly common threat to human health. The most prevalent human fungal pathogen, *C.albicans*, is part of the normal human flora and resides on mucosal surfaces. *Candida albicans* is capable of causing superficial mucosal infections (thrush, esophagitis, vaginitis) in immunocompetent hosts with transient waning of immunity or in immunocompromised hosts. Serious systemic infections also occur in neutropenic hosts and are frequently associated with indwelling catheters, mucositis, or high dose broad spectrum antibiotics.

Fungal infections are difficult to treat because, unlike bacteria or viruses, fungi are eukaryotic cells that are similar to host cells. Relatively few antifungal agents are in clinical use, these agents can possess toxic side effects and resistant isolates are emerging. The polyene antifungal drugs include amphotericin B and nystatin, which bind to the fungal membrane sterol ergosterol and compromise the cell membrane. The azole class of antifungal drugs, such as fluconazole, inhibits the enzyme lanosterol 14 α demethylase (Erg11) and exerts a fungistatic effect by perturbing membrane integrity.

The calcineurin inhibitors FK506 and CsA have no effect on vegetative growth of *C.albicans* under a variety of conditions, but Sanglard and colleagues recently reported that combining CsA with fluconazole results in dramatic synergistic antifungal activity (Marchetti *et al.*, 2000b). The synergistic action of CsA and fluconazole is potent, and can sterilize heart valves infected with *C.albicans* in a murine model of fungal endocarditis (Marchetti *et al.*, 2000a). One model that has been advanced for this observation is that the ability of CsA to inhibit multidrug resistance (mdr) pumps might increase intracellular fluconazole levels (Marchetti *et al.*, 2000b). Fluconazole resistance often involves increased mdr pump expression (White, 1997; White *et al.*, 1998; Calabrese *et al.*, 2000; Lyons and White, 2000), suggesting that this might be a plausible mechanism.

Instead, we demonstrate that the synergistic action of fluconazole with CsA and FK506 is mediated via inhibition of the *C.albicans* calcineurin homolog. We propose that azoles deplete ergosterol in the cell membrane and trigger a membrane stress response pathway in *Candida*. Recent whole genome expression has revealed that azole exposure globally induces the ergosterol biosynthetic pathway (Bammert and Fostel, 2000; De Backer *et al.*, 2001). Taken together, these studies suggest that an ergosterol sensing pathway maintains membrane integrity in yeast, analogous to the cholesterol sensing, homeostatic pathways that operate in animals, and to fungal cell-wall integrity pathways. Inhibitors of this pathway might be useful as antifungal agents.

Results

CsA and FK506 exhibit synergistic toxicity with fluconazole against *C.albicans*

CsA and FK506 exhibit no activity against *C.albicans* when grown under a variety of conditions, including the presence of 100 mM LiCl or growth at 37°C. Sanglard and colleagues recently reported that CsA and FK506 exhibit a potent synergistic action with fluconazole (Maesaki *et al.*, 1998; Marchetti *et al.*, 2000a,b) and we have confirmed these observations (Table I).

Using standard NCCLS criteria for antifungal drug activity, the minimum inhibitory concentration at which growth of *C.albicans* strain SC5314 was completely inhibited (MIC₁₀₀) was >16 µg/ml for fluconazole and >12.5 µg/ml for CsA. In contrast, for the drug combination the MIC was 0.5 µg/ml for fluconazole and 0.78 µg/ml for CsA (Table I). The resulting FIC index was 0.032, indicating that the two drugs are potentially synergistic. Similar findings were obtained with FK506 (Figure 1, Table I). The ability of CsA and FK506 to enhance sensitivity of *C.albicans* to fluconazole was comparable, and the MIC of FK506 was ~10-fold lower than CsA, as in other cells. In these studies the drug concentration was measured at which growth was completely inhibited (MIC₁₀₀) rather than the MIC₈₀ (80% inhibition) since the fungistatic agent fluconazole has a 'trailing' endpoint. The combination of CsA or FK506 with fluconazole is fungicidal and abolishes the trailing endpoint for fluconazole alone.

FK506 receptor protein FKBP12 is required for FK506–fluconazole synergism

Both CsA and FK506 inhibit not only calcineurin but also mdr pumps (Arceci *et al.*, 1992; Saeki *et al.*, 1993). Because azoles are exported from *C.albicans* cells by mdr pumps (White *et al.*, 1998), it has been proposed that CsA and FK506 might increase intracellular fluconazole levels by blocking mdr pumps (Maesaki *et al.*, 1998; Marchetti *et al.*, 2000a,b). To address these mechanisms, we tested if the intracellular protein required to bind FK506 and inhibit calcineurin plays a role in synergistic drug action. In previous studies, the gene encoding the *C.albicans* FKBP12 homolog Rbp1 was identified (Ferrara *et al.*, 1992). We recently disrupted the *RBP1* gene and found that *rbp1/rbp1* mutants are viable and resistant to rapamycin, a drug related to FK506 that also forms a toxic complex with FKBP12 (Cruz *et al.*, 2001b).

Here the isogenic wild-type, *rbp1/RBP1* heterozygous, and *rbp1/rbp1* homozygous mutant cells were tested. Growth of the wild-type strain was potently inhibited by FK506 + fluconazole, whereas the *rbp1/rbp1* mutant strain lacking FKBP12 was completely resistant to the synergistic FK506–fluconazole combination (Figure 1, Table I; FIC = 2). The *rbp1/rbp1* mutant was as sensitive to CsA + fluconazole as wild-type cells (FIC = 0.16), indicating that the effects of the *rbp1* mutation are specific for the FKBP12 ligand FK506 (Figure 1, Table I). We conclude that FKBP12 mediates FK506 synergism with fluconazole.

These findings suggest that inhibition of calcineurin by the FKBP12–FK506 complex might be the molecular basis of the synergistic drug interaction. However, in previous studies FKBP12 has been implicated in the

function of mdr pumps heterologously expressed in *S.cerevisiae* (Hemenway and Heitman, 1996). *rbp1/rbp1* mutants lacking FKBP12 were not hypersensitive to fluconazole alone and thus do not have a general mdr pump defect. Our findings demonstrate that the effects of FK506 are not mediated by simple inhibition of FKBP12, and implicate the FKBP12–FK506 complex as the active agent.

Calcineurin is the target of FKBP12–FK506 in combination with fluconazole

To test if calcineurin is involved in action of the toxic FK506–fluconazole combination, a mutation was introduced into calcineurin that confers dominant resistance to FKBP12–FK506, but not to cyclophilin A–CsA. In previous studies in *C.neoformans*, we identified a mutation (*CNBI-1*) that results from a two amino insertion into the hinge region of the calcineurin B subunit (Fox *et al.*, 2001). The *CNBI-1* mutation has no effect on calcineurin activity and confers dominant resistance to the FKBP12–FK506 complex. If calcineurin is required for the observed synergistic interaction between FK506 and fluconazole in *C.albicans*, then the *CNBI-1* mutation should confer resistance to the drug combination.

The dominant FK506-resistant *CNBI-1* mutation was introduced into one allele of the calcineurin B gene in *C.albicans*. This was accomplished by transformation of *C.albicans* with a mutagenic 80-mer oligonucleotide that contains the *CNBI-1* mutation and also creates a *BsgI* restriction site. Transformants were selected on medium containing FK506 + fluconazole, and several were identified that remained sensitive to CsA + fluconazole. Three were shown to contain the *CNBI-1* mutation by PCR and restriction analysis with *BsgI*. In addition, when the *CNBI* locus was PCR amplified, cloned and sequenced from one such isolate, four of six clones contained the wild-type *CNBI* sequence and two of six contained the *CNBI-1* mutation. The resulting *CNBI-1/CNBI* strain is thus heterozygous for the introduced mutation, and this strain is resistant to the synergistic action of FK506 and fluconazole (Figure 1, Table I). The finding that the *CNBI-1* mutation renders *C.albicans* resistant to FK506 + fluconazole implicates calcineurin as the target of FKBP12–FK506 synergistic action with fluconazole.

Membrane perturbation increases cell entry by CsA and FK506

Two models were considered to explain how calcineurin inhibition is lethal to cells exposed to fluconazole. In the first, calcineurin is essential for cell viability but cell entry of CsA and FK506 is limited such that full inhibition of calcineurin is not achieved. In this model, exposure to fluconazole inhibits ergosterol biosynthesis, impairs the membrane, and promotes drug entry by compromising mdr pump function or membrane integrity. In the second model, calcineurin is not essential for cell viability unless the membrane is perturbed. In this second model, calcineurin is required to survive membrane stress.

We first tested if membrane perturbation affects cell entry of CsA or FK506. *Candida albicans* cells were cultured in the absence or presence of 50 µg/ml fluconazole for 20 h. Under these conditions cell growth was only partially inhibited by fluconazole and a similar

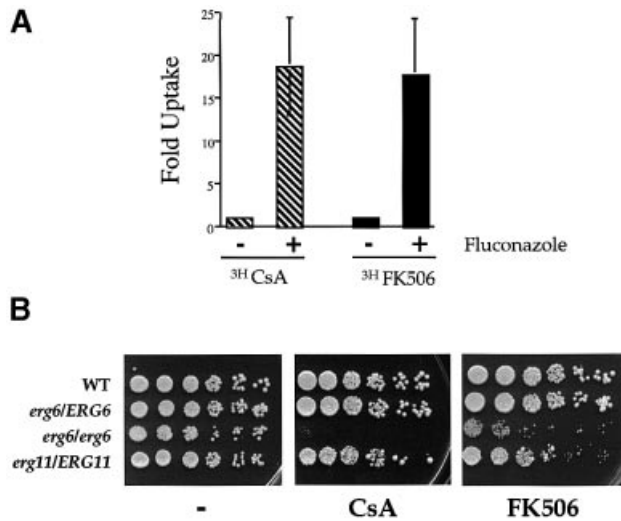


Fig. 2. Membrane perturbations enhance uptake and toxicity of CsA and FK506 in *C. albicans*. (A) Fluconazole treatment increases cell uptake of [³H]CsA and [³H]FK506. *Candida albicans* strain SC5314 was grown overnight in YPD medium with or without fluconazole (50 μ g/ml) at 30°C. [³H]CsA or [³H]FK506 uptake was measured as described in Materials and methods. The means of two independent experiments (three assays in total) were plotted as the fold of the base line concentration of [³H]CsA or [³H]FK506 that was taken up by *C. albicans* cells growing without fluconazole. (B) Five-fold serial dilutions of *C. albicans* wild-type (CAI4), *erg6/erg6* homozygous mutant (KPC8), *erg6/ERG6* heterozygous mutant (KPC1) and *erg11/ERG11* heterozygous mutant (4A) strains were spotted onto YPD solid medium with or without CsA (100 μ g/ml), FK506 (1 μ g/ml) or fluconazole (50 μ g/ml) and incubated at 37°C for 48 h.

number of cells could be obtained from either condition. Cells were washed, resuspended in fresh medium with or without fluconazole and incubated with tritiated CsA or FK506. Cells were pelleted, washed and cell-associated radioactivity measured by scintillation counting. Remarkably, culturing cells in fluconazole increased the entry of CsA or FK506 by ~20-fold (Figure 2A). These findings suggest that fluconazole might enhance the activity of both drugs by increasing their uptake or by limiting their export.

As a second measure of the effect of membrane perturbation on CsA/FK506 action, we tested *erg6/erg6* mutant cells lacking one of the late biosynthetic enzymes required for ergosterol biosynthesis (Jensen-Pergakes *et al.*, 1998). *ERG6* encodes the enzyme S-adenosylmethionine Δ -24-sterol-C-methyltransferase, which methylates side chains to convert zymosterol into ergosterol. Notably, *erg6* mutants of *S. cerevisiae* are hypersensitive to many toxins but not to CsA or FK506 (Gaber *et al.*, 1989). Here we found that an *erg6/erg6* *C. albicans* mutant is markedly hypersensitive to either CsA or FK506 whereas an *erg6/ERG6* heterozygous strain and also an *erg11/ERG11* strain were not (Figure 2B; Table I). In summary, the uptake or retention of CsA and FK506 is enhanced by fluconazole, and cells in which the membrane has been perturbed genetically or pharmacologically cannot tolerate loss of calcineurin function.

Calcineurin is not essential in *C. albicans*

We next sought to directly test between the two models, one in which calcineurin is essential under all conditions

and the other in which calcineurin only becomes essential during membrane stress. To address these models, the genes encoding the calcineurin B regulatory subunit were disrupted. Calcineurin B is encoded by a single gene in *C. albicans* and is known to be essential for calcineurin activity and function in other organisms. The *CNB1* gene was identified from sequence traces at the Stanford *Candida albicans* genome project website. Recently a novel and facile gene disruption approach has been developed for *C. albicans* that also allows a direct test for essential genes (Enloe *et al.*, 2000). This approach utilizes the *ura3-ARG4-ura3* marker (*UAU1*). Following disruption of the first allele, spontaneous mitotic crossing over or gene conversion events occur. This then allows the selection of Arg⁺ Ura⁺ isolates in which one allele of the gene is disrupted with the *ura3-ARG4-ura3* marker and the other allele is disrupted with the recombined and now *URA3* wild-type marker (Figure 3). When this approach is applied to essential genes, the resulting Arg⁺ Ura⁺ isolates are trisomic and still contain a wild-type copy of the gene.

We applied this gene disruption approach to the *CNB1* gene. A PCR product spanning the *UAU1* marker and containing 60 bp of homology flanking the 5' and 3' regions of the *CNB1* gene was transformed into the *ura3/ura3 arg4/arg4 his1/his1* strain BWP17. Two independently derived *CNB1/cnb1 Δ ::ura3-ARG4-ura3* strains were obtained, allowed to grow on YPD medium for many generations, and Ura⁺ Arg⁺ colonies were isolated and analyzed. In both cases, *cnb1 Δ ::URA3/cnb1 Δ ::ARG4* mutant strains that lacked any copy of the wild-type *CNB1* gene were obtained. The *cnb1/CNB1* heterozygous and the *cnb1/cnb1* homozygous mutant strains were confirmed by PCR and Southern blot analysis (Figure 3). We conclude that calcineurin B is not essential in *C. albicans*.

Calcineurin mediates cell survival during membrane perturbation by fluconazole

Next the sensitivity of the *cnb1/cnb1* mutants to fluconazole, both alone and combined with CsA or FK506 was tested. Remarkably, the *cnb1/cnb1* mutants lacking calcineurin were hypersensitive to fluconazole alone (Figure 4, Table I). The minimum inhibitory concentration of the *cnb1/cnb1* mutants for fluconazole was equivalent to that of wild-type cells exposed to fluconazole and CsA or FK506. Two independent *cnb1/cnb1* mutants exhibited a similar level of azole hypersensitivity. There was no further effect on fluconazole action when the calcineurin mutants were also exposed to CsA or FK506 (Figure 4). These findings provide direct evidence that calcineurin is the target of CsA and FK506 action with fluconazole and indicate that inhibition of calcineurin is the predominant or exclusive mechanism by which CsA and FK506 enhance azole action.

We next sought to demonstrate unequivocally that the introduced *cnb1/cnb1* mutation is responsible for the azole hypersensitive phenotype. One concern is that because the approach used to generate these mutant strains can involve mitotic crossover events that homozygose the distal arm of the chromosome, other polymorphic genetic loci in the parental strain can be a confounding influence. To address this, the wild-type *CNB1* locus with 5' and 3' untranslated regions was cloned into an integrating *HIS1* vector. This

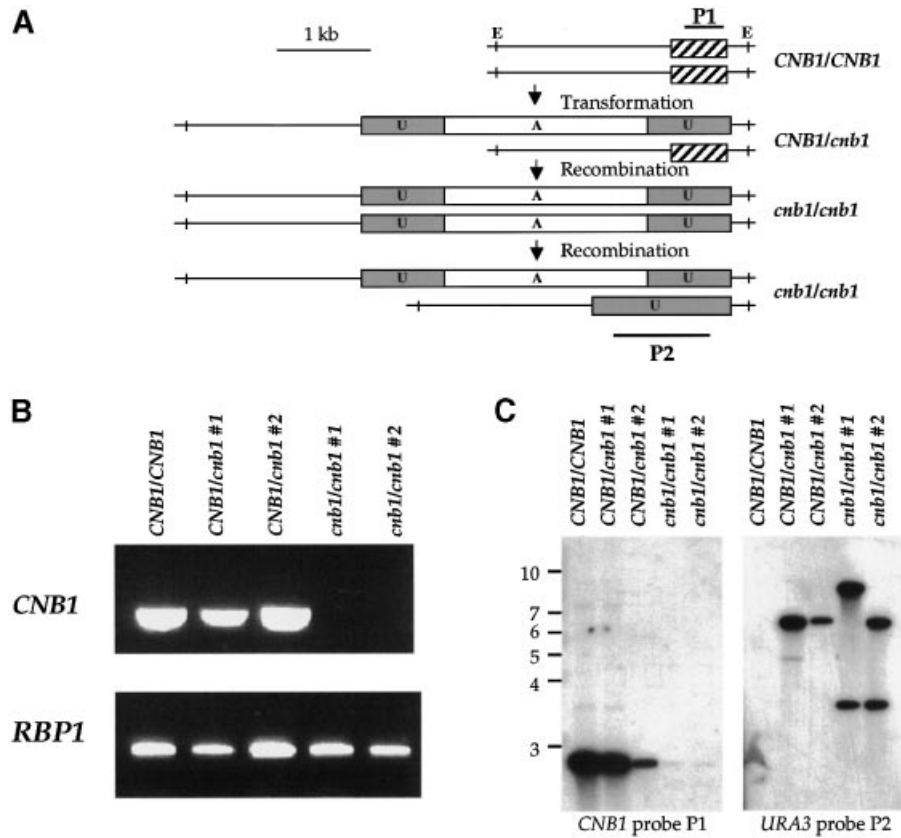


Fig. 3. Disruption of the *C. albicans* calcineurin B genes. (A) Disruption alleles of the *CNB1* gene. One *CNB1* wild-type allele was replaced by transformation and homologous integration with the *UAU1* cassette in strains DAY343 (*CNB1/cnb1* #1) and DAY345 (*CNB1/cnb1* #2). ‘U’ indicates the *ura3* portions and ‘A’ the *ARG4* portions of the *UAU1* cassette. Arg⁺ Ura⁺ isolates containing the homozygous *cnb1/cnb1* mutation [strains DAY364 (*cnb1/cnb1* #1) and DAY365 (*cnb1/cnb1* #2)] were identified from each heterozygous strain. (B) PCR verification of the *CNB1* gene deletions. Genomic DNA from the wild-type *CNB1/CNB1* strain BWP17, the *cnb1/CNB1* heterozygous strains DAY343 and DAY345, and the *cnb1/cnb1* strains DAY364 and DAY365 was PCR amplified with primers PR146/147 to the *CNB1* gene (515 bp product) or primers to the *RBP1* gene (375 bp product) as a control. (C) Southern blot analysis of *CNB1* alleles. Genomic DNA from the wild-type *CNB1/CNB1* strain BWP17, the *cnb1/CNB1* heterozygous strains DAY343 and DAY345 and the *cnb1/cnb1* homozygous strains DAY364 and DAY365 was cleaved with *EcoRV* (E) and analyzed by Southern blotting with probes to the *URA3* or *CNB1* gene. The *URA3* probe hybridized to the *CNB1* disruption allele in strains DAY343 and DAY345 (~6.2 kb) and to *cnb1* disruption alleles in strain DAY364 and DAY365 (~9.0, 6.2 and 3.6 kb). An interchromosomal recombination event between the proximal portion of the *URA3* gene in one allele and the distal portion of the *URA3* gene in the other yielded one *cnb1Δ::URA3* allele and one larger ~9 kb tandem *cnb1Δ::ura3-ARG4-ura3-ARG4-ura3* duplication in strain DAY364. Positions of DNA markers are shown on the left in kb. P1 and P2 indicate probes used to detect the *CNB1* and *URA3* genes.

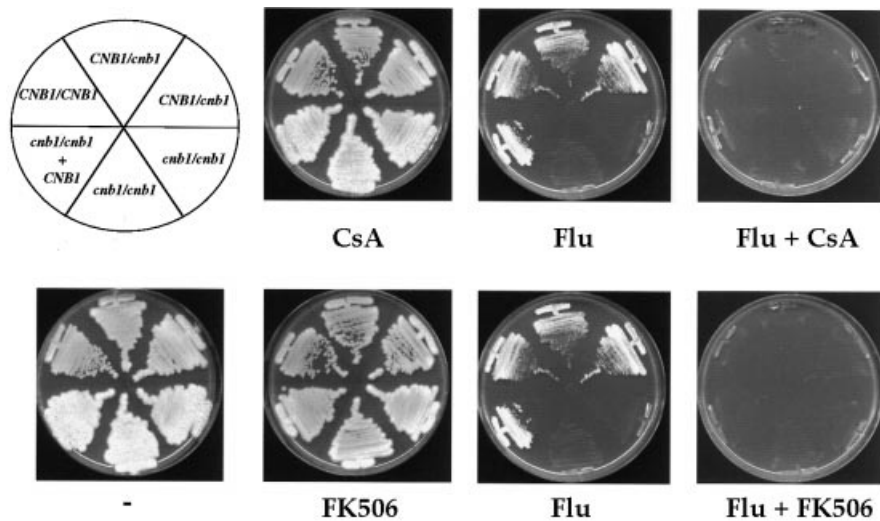


Fig. 4. *Candida albicans* calcineurin mutant strains are viable and fluconazole hypersensitive. The wild-type (BWP17), *cnb1/CNB1* heterozygous (DAY343, DAY345) and *cnb1/cnb1* homozygous (DAY364, DAY365) mutants, and the *cnb1/cnb1* + *CNB1* complemented strain MCC85 were grown on YPD solid medium with or without CsA (100 µg/ml), FK506 (10 µg/ml) and fluconazole (50 µg/ml), and incubated at 37°C for 72 h.

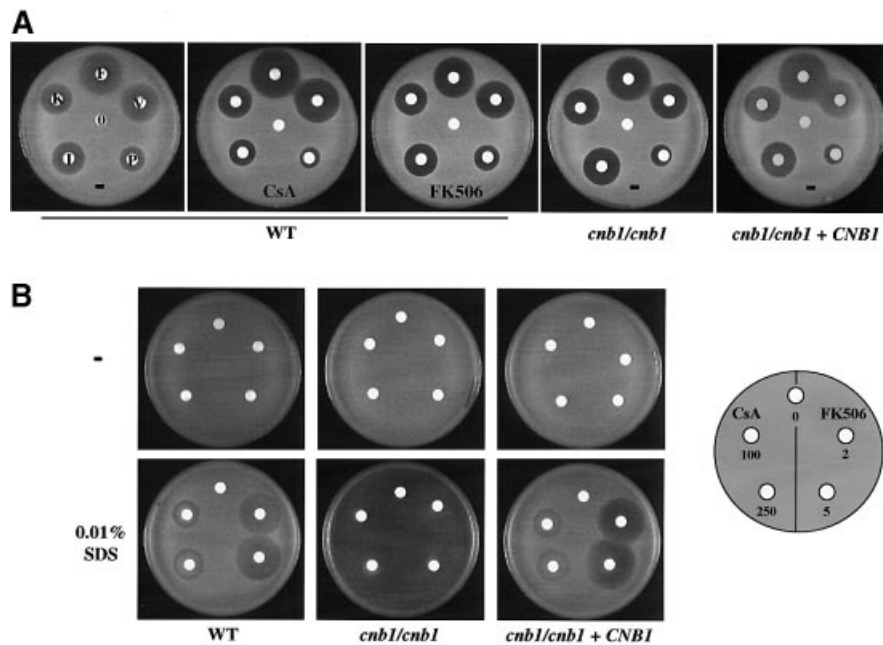


Fig. 5. Calcineurin protects *C. albicans* from membrane stress exerted by other azoles or SDS. (A) Disks were spotted with H₂O (0), 5 μ g fluconazole (F), 160 μ g ketoconazole (K), 16 μ g itraconazole (I), 80 ng posaconazole (P), or 60 ng voriconazole (V) and placed on the surface of YPD solid medium containing $\sim 1 \times 10^6$ cells of *C. albicans* in top agar with no drug, 100 μ g/ml cyclosporin A, or 1 μ g/ml FK506. Strains were wild-type (SC5314), the *cnb1/cnb1* mutant strain (DAY364), or the *cnb1/cnb1 + CNB1* complemented strain (MCC85). Cells were incubated at 37°C for 3 days and photographed. (B) Disks containing water (0), 2 or 5 μ g of FK506 or 100 or 250 μ g of CsA were placed on YPD medium without (–) or with 0.01% SDS and containing $\sim 1 \times 10^6$ cells of *C. albicans* in top agar. Strains were wild-type (SC5314), the *cnb1/cnb1* mutant strain (DAY364), or the *cnb1/cnb1 + CNB1* complemented strain (MCC85). Cells were incubated at 37°C for 3 days and photographed.

plasmid was targeted to the *his1* genomic locus by cleavage with *Nru*I; the *cnb1/cnb1 his1/his1* mutant cells were transformed and His⁺ transformants were selected. In multiple independent isolates, the azole hypersensitive phenotype was complemented and the minimum inhibitory concentration of fluconazole was restored to the wild-type level (Figure 4). These findings demonstrate that mutation of calcineurin B confers the observed azole hypersensitive phenotype.

A variety of azoles are synergistic with FK506

Fluconazole is one of several structurally distinct azoles that all target the enzyme lanosterol 14 α demethylase required for ergosterol biosynthesis. Several azoles, including itraconazole, ketoconazole and fluconazole are clinically approved for systemic fungal infections and have different antifungal spectrums of action. For example, itraconazole has activity against *Aspergillus fumigatus* whereas fluconazole is not active clinically for use against this ubiquitous pathogen. A number of newer azoles are in late stage clinical trials nearing clinical approval, including voriconazole, posaconazole and ravuconazole. Here we tested if calcineurin inhibitors are also synergistic with other azoles.

In disc diffusion assays on solid medium, both CsA and FK506 were clearly synergistic with all of the azoles tested and led to clear rather than turbid halos (Figure 5A), in part indicative of fungicidal activity. The diameters of the growth inhibition halos were not markedly increased, suggesting that the predominant effect of calcineurin inhibition is to kill cells exposed to azoles that would otherwise have exhibited residual growth. These findings

suggest that calcineurin becomes essential when cells are exposed to any of these azole compounds. Consistent with this interpretation, all of the azoles tested produced clear rather than turbid halos on a lawn of *cnb1/cnb1* homozygous mutant cells and this effect was complemented by reintroduction of the wild-type *CNB1* gene (Figure 5A). These observations further support the model that calcineurin is the target of CsA and FK506 action in *C. albicans*. The fact that newer azoles such as voriconazole and posaconazole may have increased activity and broader spectrums of action suggests their combination with calcineurin inhibitors might be even more efficacious.

Calcineurin promotes survival during membrane but not cell wall stress

Calcineurin was found to be important for cells to survive perturbations of the cell membrane but not of the cell wall. The finding that calcineurin mutant cells were hypersensitive to azoles, which deplete ergosterol from the membrane, suggested that other perturbations to the cell wall might exert a toxic effect, but did not exclude that cell-wall stress might also be lethal to calcineurin mutant strains. To address this, we studied the sensitivity of the wild-type, *cnb1/cnb1* mutant and the *cnb1/cnb1 + CNB1* strains to 0.01% SDS, which perturbs membrane lipids, or to antifungal agents that inhibit enzymes involved in cell-wall biosynthesis. Remarkably, we observed that combination of 0.01% SDS with either CsA or FK506 was toxic to wild-type cells, the *cnb1/cnb1* mutant strain was hypersensitive to 0.01% SDS alone, and reintroduction of the wild-type *CNB1* gene complemented this mutant phenotype (Figure 5B). In contrast, there was no difference in

Table II. Non-immunosuppressive FK506 and CsA analogs are synergistic with fluconazole

<i>C. albicans</i> strain	MIC alone ($\mu\text{g/ml}$)				MIC combined			FIC index		
	Flu	FK506	CsA	L-685,818	Flu + FK506	Flu + CsA	Flu + L-685,818	Flu + FK506	Flu + CsA	Flu + L-685,818
Wild-type (SC5314)	>16	>3.12	>12.5	>12.5	1/ \leq 0.19	1/1.56	2/0.39	0.045	0.093	0.078
<i>CNB1-1/CNB1</i> (YAG237)	>16	>12.5	>12.5	>12.5	>16/>12.5	2/3.12	>16/>12.5	2	0.19	2
<i>rbp1Δ/rbp1Δ</i> (YAG171)	>16	>3.12	>12.5	>12.5	>16/>3.12	4/0.78	>16/>12.5	2	0.16	2

<i>C. albicans</i> strain	MIC alone ($\mu\text{g/ml}$)					MIC combined				FIC index			
	Flu	FK506	CsA	209-825	211-810	Flu + FK506	Flu + CsA	Flu + 209-825	Flu + 211-810	Flu + FK506	Flu + CsA	Flu + 209-825	Flu + 211-810
Wild-type (BWP17)	>16	>12.5	>12.5	>3.12	>3.12	2/0.39	4/1.56	4/0.78	8/1.56	0.078	0.19	0.25	0.50

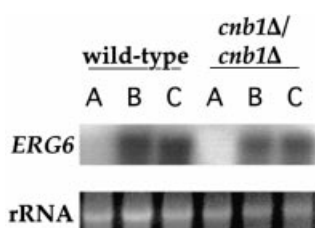


Fig. 6. *ERG6* gene induction in response to azoles is calcineurin independent. Transcription of the *ERG6* gene was assayed in wild-type (SC5314) and *cnb1/cnb1* mutant cells (DAY364) by northern blotting. Liquid cultures of cells were harvested after 8 h of either (A) no treatment, (B) 10 $\mu\text{g/ml}$ fluconazole, or (C) 10 $\mu\text{g/ml}$ fluconazole + 1 $\mu\text{g/ml}$ FK506.

sensitivity to the chitin synthase inhibitor nikkomycin Z, or to the β -1,3 glucan synthase inhibitor caspofungin, of the wild type compared with the calcineurin B mutant strain (data not shown). These additional findings support the conclusion that calcineurin is essential for cell survival during membrane but not cell-wall stress.

Calcineurin is not necessary for induction of the *ERG6* gene by azoles

Previous studies have revealed that azoles stimulate transcription of the genes encoding enzymes in the ergosterol biosynthetic pathway, likely in response to depletion of ergosterol in the cell membrane (Henry *et al.*, 2000; Kennedy and Bard, 2001; Leber *et al.*, 2001). The hypothesis that calcineurin might be necessary for this transcriptional induction was tested by assaying expression of the *ERG6* gene by northern blotting in wild-type and calcineurin mutant cells exposed to azoles in the absence or presence of the calcineurin inhibitor FK506 (Figure 6). As previously reported, the *ERG6* gene was clearly induced in response to fluconazole exposure (Figure 6). *ERG6* gene induction still occurred in wild-type cells exposed to fluconazole in the presence of FK506 or in calcineurin B mutant cells (Figure 6), indicating that calcineurin is not essential for induction of the *ERG6* gene in response to fluconazole.

In addition, ergosterol levels were quantified by a novel method involving heptane extraction of non-saponifiable lipids and the unique spectrophotometric absorption profile of ergosterol (Arthington-Skaggs *et al.*, 1999). This analysis revealed that fluconazole abolishes ergosterol production whereas calcineurin was not required (data not shown).

Non-immunosuppressive FK506 and CsA analogs are synergistic with azoles

A disadvantage of FK506 and CsA for antimicrobial therapy is that immunosuppression by these drugs causes a predisposition to, and exacerbates fungal infections (Perfect and Durack, 1985). In previous studies, we identified non-immunosuppressive analogs of CsA (209-825, 211-810) and FK506 (L-685,818) that do not inhibit mammalian calcineurin, yet retain antifungal activity mediated via fungal cyclophilin A, FKBP12 and calcineurin (Odom *et al.*, 1997b; Cruz *et al.*, 2000a). Previous studies implicate the fungal FKBP12 protein in similar species-specific effects, namely that the FK506 analog L-685,818 can inhibit bovine calcineurin when bound to yeast FKBP12 but not when bound to human FKBP12 (Rotonda *et al.*, 1993). Thus, in the case of the FK506 analog L-685,818, additional contacts from yeast FKBP12 to calcineurin mitigate the deleterious effect of the introduced C-18 hydroxyl group on the analog that protrudes into a hydrophobic calcineurin surface. Thus, these analogs take advantage of structural differences between host and fungal targets to spare immune function yet impair fungal growth.

We tested if these non-immunosuppressive FK506 and CsA analogs also exhibit synergistic action with fluconazole against *C. albicans*. As shown in Table II, the FK506 analog L-685,818 exhibited potent synergistic activity with fluconazole. *rbp1/rbp1* and *CNB1-1/CNB1* mutants that are resistant to FK506–fluconazole synergism were also resistant to L-685,818 in combination with azole (Table II), indicating that this analog exerts its effects via FKBP12-dependent inhibition of fungal calcineurin. The CsA analogs 209-825 and 211-810 exhibited similar synergistic activity with fluconazole (Table II).

Some azole resistant mutant strains are sensitive to FK506 + fluconazole

A significant issue with clinical use of azole antifungal agents is the emergence of resistant mutants. Defined molecular mechanisms include alterations in ergosterol biosynthetic enzymes or increased *mdr* expression (White *et al.*, 1998). Here we tested if calcineurin inhibitors render azole-resistant strains sensitive.

For these experiments, we employed a set of 17 isolates obtained from a single patient over a 2-year period during

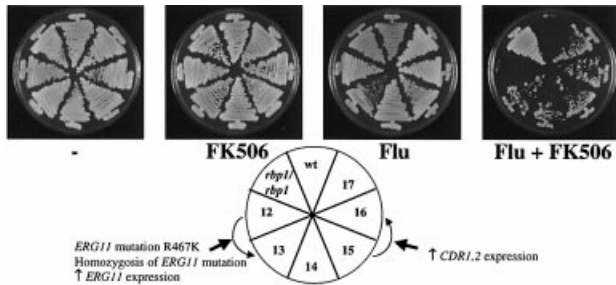


Fig. 7. Some azole resistant strains are sensitive to FK506 + fluconazole. Wild-type strain SC5314, *rbp1/rbp1* mutant strain YAG171 and six representative *C. albicans* isolates, 12–17, from a series of increasingly fluconazole-resistant isolates from an HIV patient were plated on YPD medium alone or containing 10 $\mu\text{g/ml}$ FK506, 50 $\mu\text{g/ml}$ fluconazole, or both 10 $\mu\text{g/ml}$ FK506 and 50 $\mu\text{g/ml}$ fluconazole. Previously described changes in the expression pattern of *ERG11* mRNA and *CDR1/2* mRNAs are labeled.

which increased doses of fluconazole were required to control multiple episodes of oral candidiasis (White, 1997; White *et al.*, 1997). Isolates 1–12 were sensitive to FK506 + fluconazole and isolates 13–15 were initially sensitive, but resistant mutants rapidly arose during growth on solid medium (Figure 7). By comparison, isolates 16 and 17 were only slightly more resistant to the azole–FK506 combination (Figure 7, not shown).

These alterations in sensitivity to FK506–fluconazole are correlated with known molecular events giving rise to changes in azole sensitivity (White, 1997; White *et al.*, 1997). First, increases in *MDR1* expression in isolates 2 and 3 that are correlated with a modest increase in the MIC for fluconazole from 1 to 10 $\mu\text{g/ml}$ had no effect. Secondly, alterations in the *ERG11* gene that occurred in isolates 13–15 and that increase the MIC to 30 $\mu\text{g/ml}$ confer partial resistance to fluconazole–FK506. Finally, the increase in *CDR1* and *CDR2* expression that occurred in isolates 16 and 17 and is correlated with an increase in the MIC to 100 $\mu\text{g/ml}$ only conferred a modest further protection from the synergistic action of FK506 with fluconazole. These observations suggest that combination of calcineurin inhibitors with azoles can partially but not completely overcome drug resistance.

FK506 and fluconazole exhibit synergistic toxicity against other fungal species

FK506 and fluconazole exhibit synergistic action against *C. albicans*, but not against *S. cerevisiae*, which diverged from a common ancestor ~200 million years ago. We

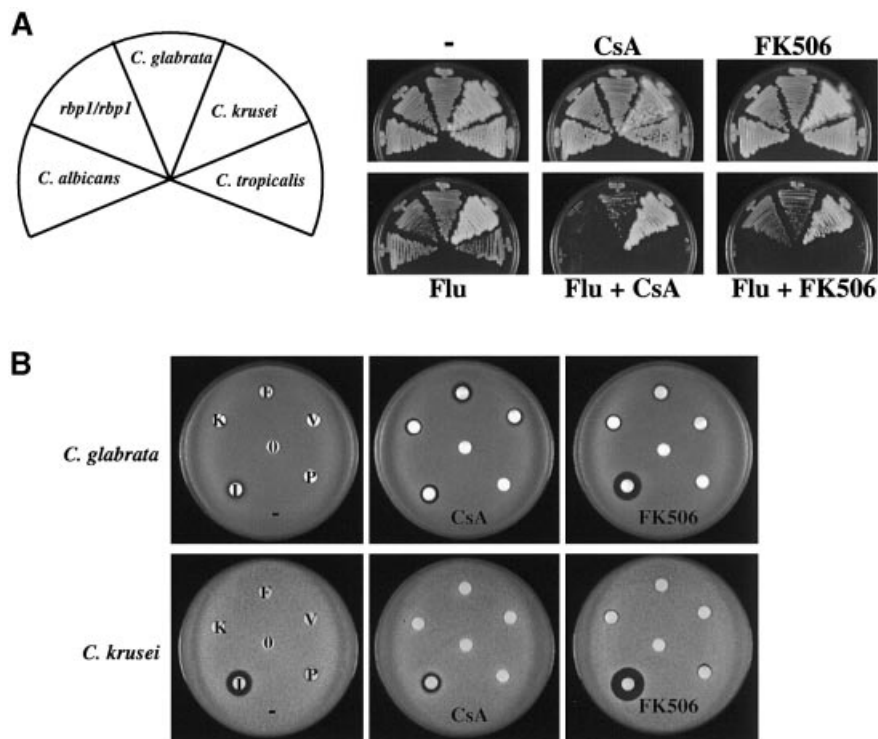


Fig. 8. FK506–fluconazole synergism in other fungi. (A) Wild-type and an *rbp1/rbp1* mutant *C. albicans* and isolates of *C. glabrata*, *C. krusei* and *C. tropicalis* strains were grown on YPD medium alone (–), or with 100 $\mu\text{g/ml}$ CsA, 10 $\mu\text{g/ml}$ FK506, 10 $\mu\text{g/ml}$ fluconazole (Flu), 100 $\mu\text{g/ml}$ CsA + 10 $\mu\text{g/ml}$ fluconazole, or 10 $\mu\text{g/ml}$ FK506 + 10 $\mu\text{g/ml}$ fluconazole for 48 h at 37°C. (B) Cells ($\sim 1 \times 10^9$) of the *C. glabrata* and *C. krusei* strains used in (A) were plated in top agar on YPD rich medium alone (–), or containing 100 $\mu\text{g/ml}$ CsA or 10 $\mu\text{g/ml}$ FK506. Discs containing 5 μg fluconazole, 160 ng ketoconazole, 16 μg itraconazole, 80 ng posaconazole, 60 ng voriconazole or water were placed on the top agar and the cells were incubated for 48 h at 37°C and photographed.

therefore tested a variety of different fungal species to establish which are hypersensitive to the combination of FK506 or CSA with fluconazole (Figure 8). First, in contrast to *S.cerevisiae*, the related sibling species *S.bayanus* is sensitive to the drug combination (not shown). Similarly, several pathogenic species of *Candida* related to *C.albicans*, including *C.tropicalis* and *C.parapsilosis* were also sensitive to fluconazole–FK506 synergism (Figure 8A, not shown). More distantly related *Candida* species, including *C.lusitaniae* and *C.guilliermondii* were hypersensitive to azoles alone (not shown). In contrast, *C.krusei* was completely resistant to FK506–fluconazole synergism and *C.glabrata* was partially resistant, which is of particular interest because fluconazole is poorly active, and resistant mutants frequently arise with these species (Figure 8A). Both *C.krusei* and *C.glabrata* are moderately sensitive to itraconazole, and in a disc diffusion assay FK506 and itraconazole were synergistic against both of these species (Figure 8B). In summary, these observations suggest that agents that target calcineurin might have broad applicability in conjunction with azoles against many, but not all, pathogenic fungi related to *C.albicans* and *S.cerevisiae*.

Discussion

CsA and FK506 exert synergistic toxic effects in combination with fluconazole in *C.albicans*. Our studies establish that FK506 and CsA actions are exerted via the *C.albicans* homologs of the conserved target proteins, the phosphatase calcineurin and the prolyl isomerase FKBP12. *Candida albicans* mutants lacking the calcineurin B regulatory subunit are viable and hypersensitive to fluconazole. Thus, either pharmacological or mutational inactivation of calcineurin sensitizes *C.albicans* cells to fluconazole. Although CsA and FK506 can inhibit mdr pump action in some settings, CsA and FK506 action in *C.albicans* is attributable to inhibition of calcineurin and not of mdr pumps. Pharmacological or genetic manipulations that reduced the membrane sterol ergosterol increased import (or prevented export) and sensitized *C.albicans* cells to CsA and FK506. However, this finding does not account for the observed synergistic drug actions since calcineurin is not essential. Taken together, our observations establish calcineurin as the conserved target of CsA and FK506 antifungal actions in *C.albicans*, *S.cerevisiae* and *C.neoformans*. We note that Sanglard and colleagues have recently independently reached similar conclusions (D.Sanglard, personal communication).

Candida albicans *erg6/erg6* mutants were found to be hypersensitive to CsA and FK506. This mutant strain accumulates the sterol precursor zymosterol, which supports viability but alters the membrane and impairs activity of integral membrane proteins. These include the tryptophan transporter and the mdr pump Pdr5 that can export FK506 (Gaber *et al.*, 1989; Kralli and Yamamoto, 1996; Egner *et al.*, 1998; Kaur and Bachhawat, 1999; Kontoyiannis, 2000). Thus, reducing ergosterol in the membrane is likely to increase intracellular CsA and FK506 levels by impairing mdr pump action. Thus azoles might reduce the doses of CsA and FK506 necessary for synergistic action, which could be important in clinical settings in which drug levels are limiting.

Clinical implications of FK506–azole synergism

There are several clinical implications that stem from the finding that calcineurin inhibitors enhance azole activity against *C.albicans* and other pathogenic fungi. First, a common issue with azoles is that they exhibit largely fungistatic rather than fungicidal activity. This results in an inability to efficiently eradicate fungal infections, particularly in hosts with compromised immunity. This fungistasis may contribute to emergence of azole-resistant isolates in immunocompromised hosts with large yeast burdens. Combination antifungal drug treatments that render azoles fungicidal might dramatically increase success, with shorter therapy courses and a lesser risk of drug resistance.

A second clinical implication is that a cohort of patients with fungal infections are already treated with both calcineurin inhibitors and azoles. For instance, organ and bone marrow transplant recipients receive calcineurin inhibitors as part of an immunosuppressive regime to prevent graft rejection and azoles to treat concomitant fungal infections. In addition, the fact that both azoles and CsA are metabolized by the hepatic P450 system leads to interesting drug interactions. In fact, ketoconazole treatment has been advocated as a method to reduce the daily dose of CsA necessary to achieve therapeutic levels and thereby to reduce drug costs (Sobh *et al.*, 2001). Furthermore, recent clinical studies reveal that the spectrum of *C.neoformans* infections is shifting from CNS to skin involvement in transplant recipients treated with FK506 (tacrolimus) (Husain *et al.*, 2001), possibly because at lower skin temperatures, calcineurin is no longer required for the stress survival and virulence of this pathogenic fungus. Further epidemiological studies are warranted to examine whether calcineurin inhibitors, alone or in conjunction with azoles, are already conferring an antifungal benefit in transplant recipients.

FK506 is synergistic with azoles via distinct mechanisms in different fungi

We find that FK506 is synergistic with azoles via different molecular mechanisms in *C.albicans* compared with *C.neoformans*. Here we show that in *C.albicans*, FK506 exerts its actions via its known target proteins FKBP12 and calcineurin. In contrast, in previous studies we found that FK506 and azoles exhibited a less dramatic synergistic action in *C.neoformans* that is independent of FKBP12 and calcineurin and which may involve the known action of FK506 on mdr pumps (Del Poeta *et al.*, 2000). Our studies demonstrate that one mechanism predominates in *C.neoformans* (possibly mdr-related) and the other in *C.albicans* (calcineurin-dependent), but we cannot exclude that the two may coexist in other fungi not yet studied. Our findings argue against both being operative in *C.neoformans* or *C.albicans*. First, calcineurin and FKBP12 mediate the effects of FK506 with azoles in *C.albicans* and there is no need to invoke a second action on mdr pumps. Secondly, in *C.neoformans* the effects of FK506 with azoles are independent of FKBP12 and calcineurin, suggesting that an action on another target, such as the known ability of FK506 to inhibit the ability of mdr pumps to extrude azoles, could be involved in this organism.

Calcineurin plays a conserved role in fungal survival during stress

Our findings, and previous reports in other fungi, reveal that calcineurin plays a global role in stress responses necessary for fungal cell survival. Importantly, calcineurin is required for cell survival during different stresses in different organisms. For example, in *C.neoformans* calcineurin is required for growth at 37°C and in conditions that mimic the host and, as a consequence, calcineurin is required for virulence of this ubiquitous human fungal pathogen (Odom *et al.*, 1997a; Cruz *et al.*, 2000b; Cruz *et al.*, 2001a; Fox *et al.*, 2001). Calcineurin is also necessary for hyphal elongation during mating and haploid fruiting in *C.neoformans* (Cruz *et al.*, 2001a). Calcineurin plays a role in cold stress responses in the fission yeast *Schizosaccharomyces pombe* (Yoshida *et al.*, 1994). In the budding yeast *S.cerevisiae*, calcineurin is essential during cation stress and recovery from pheromone cell cycle arrest (Nakamura *et al.*, 1993; Breuder *et al.*, 1994; Hemenway *et al.*, 1995; Moser *et al.*, 1996; Withee *et al.*, 1997). Our findings indicate that calcineurin functions to promote cell survival during membrane stress in *C.albicans*, possibly in conjunction with a cell membrane stress response pathway analogous to the cell wall integrity pathway in *S.cerevisiae* (Smits *et al.*, 2001).

A conserved cell membrane stress sensing pathway

In fungi, the fungal ergosterol biosynthetic pathway is governed by a global homeostatic mechanism that responds to changes in ergosterol levels and the presence of pathway inhibitors. Studies with reporter gene assays revealed that the *ERG1*, *ERG9* and *ERG11* genes are transcriptionally induced in response to azoles (Henry *et al.*, 2000; Kennedy and Bard, 2001; Leber *et al.*, 2001). In addition, in genome array analysis in *S.cerevisiae*, inhibition of ergosterol biosynthesis resulted in the global induction of the ergosterol biosynthetic pathway (Bammert and Fostel, 2000). Similarly, treatment of *C.albicans* cells with itraconazole globally induces the ergosterol biosynthetic pathway (De Backer *et al.*, 2001). These studies suggest that a homeostatic mechanism responds to changes in the levels of products or intermediates in the ergosterol pathway and induces gene expression when products decrease or intermediates accumulate. Antifungal drugs that target different enzymes in the biosynthetic pathway, and which lead to the accumulation of different biosynthetic intermediates, all have a similar action, arguing that ergosterol itself is sensed (Henry *et al.*, 2000).

How ergosterol in the cell membrane is sensed is not known, but could involve oxysterol binding proteins known to control the ergosterol biosynthetic pathway in *S.cerevisiae* (Beh *et al.*, 2001). The pathway is likely to converge on two transcription factors, Upc2 and Ecm22, which bind to a conserved sterol response element and induce multiple *ERG* genes in response to sterol limitation (Vik and Rine, 2001). Our studies reveal that calcineurin is required for cells to survive cell membrane stress in response to azole exposure, but calcineurin is not required for the transcriptional response of the *ERG6* gene to azoles (Figure 6). Further studies will be required to establish if calcineurin is activated by Ca²⁺ fluxes during membrane

stress or functions in a basal signaling capacity that is necessary for cell survival under membrane stress conditions.

Related cholesterol sensing homeostatic mechanisms in humans

Similar mechanisms enable human cells to sense cholesterol and transcriptionally and post-transcriptionally regulate sterol biosynthesis (Goldstein and Brown, 1990; Brown and Goldstein, 1997, 1999). Several mammalian proteins, including the key cholesterol biosynthetic enzyme HMG-CoA reductase and the SREBP transcription factor cleavage activating protein (SCAP), contain sterol binding domains that sense and respond to intracellular cholesterol levels (Hua *et al.*, 1996). Similar sterol sensing domains have been identified in other mammalian proteins (reviewed in Lange and Steck, 1998). These studies illustrate that sterol sensing is conserved from yeasts to mammals, and that molecular mechanisms employed to sense and regulate cholesterol in mammalian cells may have originated in fungi to sense ergosterol.

Distinct pathways sense cell wall and cell membrane integrity

In *S.cerevisiae* and other fungi, the cell wall integrity pathway senses cell wall perturbations and promotes survival by transcriptional and post-transcriptional mechanisms (Smits *et al.*, 2001). Several integral membrane proteins (Hcs77, Wsc2/3/4, Mid2) have been implicated as sensors (Rajavel *et al.*, 1999; Philip and Levin, 2001). The small GTPase Rho1 is activated in response to cell wall perturbations (Bickle *et al.*, 1998; Delley and Hall, 1999) and in turn activates the Pkc1-Bck1-Mkk1/2-Mpk1 MAP kinase cascade (Irie *et al.*, 1993; Lee *et al.*, 1993; Kamada *et al.*, 1996; de Nobel *et al.*, 2000). One target of this pathway is the transcription factor Rlm1, which plays a central role in regulating gene expression in response to cell wall perturbations (Jung and Levin, 1999).

Several findings indicate that the cell wall integrity signaling pathway is distinct from an ergosterol sensing cell membrane integrity pathway. First, by genome array analysis, increased Mpk1 activity was found to enhance expression of genes in the cell wall integrity pathway but not ergosterol biosynthetic genes (Jung and Levin, 1999). Secondly, studies in *C.albicans* and *S.cerevisiae* reveal that the ergosterol biosynthetic genes are globally induced by azoles whereas the cell wall integrity signaling pathway is not (Bammert and Fostel, 2000; De Backer *et al.*, 2001). Thirdly, our findings reveal that calcineurin is essential for *C.albicans* to survive membrane perturbation by azoles, SDS, or the *erg6* mutation but does not promote cell survival during cell wall perturbation by inhibitors of chitin or β -1,3 glucan synthesis. Thus, fungal cells have distinct mechanisms to sense perturbations in the cell wall and the cell membrane. Agents that target either pathway might find clinical utility in conjunction with drugs that target ergosterol or its biosynthesis compared with drugs that target cell wall biosynthetic enzymes. Further studies to elucidate the cell membrane integrity pathway, and the role of calcineurin in this pathway, will be of considerable interest.

Materials and methods

Strains and media

Candida albicans strains used in this study were SC5314, the *ura3/ura3* strain CA14, and the *ura3/ura3 arg4/arg4 his1/his1* auxotrophic strain BWP17 (Wilson *et al.*, 1999). Strains were grown on YPD rich medium or synthetic defined dextrose medium, which were prepared as previously described (Sherman, 1991). The *rbp1/RBP1* heterozygous and *rbp1/rbp1* homozygous *C. albicans* mutant strains were prepared as recently described (Cruz *et al.*, 2001b). The *erg6/ERG6* (KPC1) and *erg6/erg6* (KPC8) strains were generously provided by Martin Bard and the *erg11/ERG11* (4A) mutant by Ted White. The fluconazole resistant strain series (isolates 1–17) were generously provided by Ted White and Spencer Redding. Finally, a variety of different *Saccharomyces* and *Candida* species isolates were provided by John McCusker, Wiley Schell or Laura Young (Duke University).

Construction of the CNB1-1/CNB1 and *cnb1/cnb1* mutant strains

The *CNB1-1/CNB1* mutant strain YAG237 was constructed by direct transformation of *C. albicans* strain SC5314 by electroporation with 1 µg of the synthetic oligonucleotide PR148 TATTGTGATGAAAA-TGATGGTTGGTAAAAATTTGAAGTGCAGGACGAGGAACTAC-AACAAATAGTGGATAAGACTTTAA (Moerschell *et al.*, 1988, 1991; Yamamoto *et al.*, 1992; Thompson *et al.*, 1998). PR148 is identical to nucleotides 345 to 418 of the sense strand of the *CNB1* ORF except the underlined nucleotides, which introduce a 6 bp insertion to result in a two amino acid insertion between residues K127 and D128 in the hinge region of calcineurin B that confers FK506 resistance in *C. neoformans* (Fox *et al.*, 2001). A *BsgI* restriction site is also introduced by this mutation. Transformants were selected on YPD plates containing 50 µg/ml fluconazole and 1 µg/ml FK506. Three isolates were obtained that were resistant to FK506 + azole, but sensitive to CsA + azole or rapamycin. Homologous transformants were identified by PCR of the *CNB1* locus with primers PR146 (5'-CTAACGCAAGTATTCTTGATG) and PR147 (5'-TCAGAACATATTTAATGTCAAAG) and subsequent digestion of the PCR products with *BsgI*. The digestion products were resolved on a 2% Metaphor (BME, Rockland, ME) gel. The PR146/147 product was 521 bp long and was cleaved by *BsgI* to yield 375 and 146 bp products. The *CNB1* locus was PCR amplified with primers PR163/164 (see sequences below) and TA cloned. Four of six isolates were digested by *BsgI* and contained the desired mutation, while the other two out of six were resistant to *BsgI* and lacked the mutation, indicating that a *CNB1-1/CNB1* heterozygous strain had been obtained.

The *cnb1/cnb1* mutants (DAY364 and DAY365) were created as follows. First, the *UAU1* cassette was amplified from pBME101 (Enloe *et al.*, 2000) using primers PR161 (5'-GAAGTTGATTTTAT-AATATTACTTAAATGCTGTCTACATGAACTAAACCAAACTGTTG-TTTgtggaattgtgagcgata) and PR162 (5'-CCAAATCGTATACTTTA-CTACAACCTACCAGTACATTCGACTTCAACAACACTACTGACAAAAG-Tttccagtcacgacgtt). These primers amplify the *UAU1* cassette with 61 and 62 nt flanking sequences with homology to *CNB1*. Secondly, this PCR product was used to transform strain BWP17 (Wilson *et al.*, 1999) by selection for Arg⁺ transformants. The Arg⁺ heterozygous *CNB1/cnb1* strains (DAY343 and DAY345) were identified via colony PCR using primers PR163 (5'-ATTATAGGTGTACAGCAGGTATTG), PR164 (5'-TGCTAAATATTTATAGTGCCTG) and Arg4det (Enloe *et al.*, 2000). All three primers were included in a single PCR to allow detection of the wild-type *CNB1* and mutant *cnb1Δ::UAU1* alleles within the same reaction.

PCR mixtures contained 1 µl of a 10 µM stock of PR164 and Arg4det, 2 µl of a 10 µM stock of PR163, 5 µl of 10× PCR buffer (Sigma), 2 µl of a mixture of 10 mM dNTP (Sigma), 3 µl of 25 mM MgCl₂ and 35.5 µl of water. A small portion of cells from a colony (~1 × 10⁴) was added to the reaction and the mixture was incubated at 94°C for 10 min. 0.5 µl of *Taq* DNA polymerase (Sigma) was then added and the samples were overlaid with mineral oil. PCR consisted of 31 cycles of 94°C for 45 s, 50°C for 60 s and 72°C for 5 min. A final extension at 72°C for 10 min followed by a 4°C incubation completed the PCR.

To obtain homozygous *cnb1/cnb1* derivatives, strains DAY343 and DAY345 were patched in large quadrants onto YPD plates and grown for 2 days at 30°C. The patches were then replica plated onto SC-arg-*uri* and SC-*uri* medium and grown for 5 days at 30°C. Approximately 100-fold more colonies grew on the SC-*uri* medium than the SC-arg-*uri* medium, similar to results previously described (Enloe *et al.*, 2000). Twenty-four Arg⁺ Uri⁺ colonies were picked and screened via colony PCR as described

above. A homozygous mutant was identified from each heterozygous strain, demonstrating that the *CNB1* gene is not essential. The resulting strains were analyzed by Southern blotting with ³²P random primer-labelled *URA3* (1700 bp, primers PR183/JM41, template pAG61) and *CNB1* (515 bp, primers PR146/147, template BWP17) gene probes.

Re-introduction of the wild-type CNB1 gene

The *cnb1/cnb1* mutant strain DAY364 was complemented as follows. The *C. albicans* wild-type *CNB1* gene on a 1 kb fragment was amplified from genomic DNA of strain BWP17 by standard PCR with primers JOHE7326 (5'-GATTCCATATGATTCATTGACTATTGCTTGT) and JOHE7327 (5'-GCAACGGAGCTCTCAGAACATATTTAATGTCAA). The resulting PCR product was digested with *NdeI* and *SacI*, and ligated into the *NdeI* and *SacI* sites in plasmid pGEM-HIS1 (Wilson *et al.*, 1999), generating pMCC15. Plasmid pMCC15 was digested with *NruI* and transformed into the *cnb1/cnb1* homozygous strain DAY364 to generate the *cnb1/cnb1* + *CNB1* complemented strain MCC85. Integration was confirmed by PCR with primers PR146/PR147.

Immunosuppressive drug import assays

Candida albicans strain SC5314 was grown overnight in YPD medium with or without fluconazole (50 µg/ml) at 30°C. Cells were centrifuged and the pellet was washed with YPD medium. Cells (2 × 10⁷) were placed in fresh YPD medium with or without fluconazole and with or without [³H]CsA (0.5 µCi) or [³H]FK506 (2 µCi) and incubated for 2 h at 37°C with constant shaking. Cells were then centrifuged and the pellet was washed twice with 1× PBS. Cells were resuspended in liquid scintillation fluid and counted in a Beckman counter.

Quantitative dilution assays

Saturated cultures of *C. albicans* strains were diluted into YPD liquid medium. Five-fold serial dilutions of cell suspensions containing ~31250, 6250, 1250, 250, 50 and 10 cells were spotted onto YPD solid medium with or without CsA (100 µg/ml), FK506 (1 µg/ml) and/or fluconazole (50 µg/ml) and incubated at 37°C for 48 h.

Synergism of novel azoles or SDS with cyclosporin A and FK506

Candida albicans wild-type strain SC5314 and mutant strains BWP17, DAY364 (*cnb1/cnb1*), DAY343 (*CNB1/cnb1*), MCC85 (*cnb1/cnb1* + *CNB1*), a clinical isolate of *C. glabrata*, MMRL361, and a clinical isolate of *C. krusei*, MMRL70, were grown overnight in liquid YPD medium. Cells (~1 × 10⁶) were suspended in top agar (0.7% Bacto Agar) and spread onto YPD medium containing no drug, 1 µg/ml FK506, or 100 µg/ml CsA. BBL™ concentration disks (0.6 mm; Becton Dickinson and Company) were placed on the plates after solidification of the top agar and 5 µg fluconazole, 160 ng ketoconazole, 16 µg itraconazole, 80 ng posaconazole, 60 ng voriconazole, or water were spotted on the disks. Sensitivity to membrane stress was tested on YPD medium containing 0.01% SDS.

Northern blotting

Overnight cultures of strains SC5314 (*CNB1/CNB1*) and DAY364 (*cnb1Δ/cnb1Δ*) were diluted into 150 ml YPD and grown to OD₆₀₀ = 0.5. Either no drug, 10 µg/ml fluconazole, or 10 µg/ml fluconazole + 1 µg/ml FK506 were added to 50 ml aliquots of each strain. At time 0, 2, 4, 8 and 16 h, 2 × 10⁸ cells were removed from each flask and stored at -70°C. After 16 h, equal numbers of cells from each flask (determined by hemocytometer count) were plated onto YPD plates to estimate survival of the cells under each treatment. Total RNA was purified from the samples by hot phenol extraction (Collart and Oliviero, 1998) and 10 µg of each sample was fractionated on a 1% agarose-MOPS gel containing formaldehyde. Northern blots were probed with a [³²P]dCTP-labeled *ERG6* probe.

Testing synergistic effects on a panel of azole-resistant strains

A series of 17 previously reported *C. albicans* isolates with increasing resistance to fluconazole (White, 1997; White *et al.*, 1997) were plated onto YPD medium containing no drug, 100 µg/ml cyclosporin A, 10 µg/ml FK506, 50 µg/ml fluconazole, 50 µg/ml fluconazole and 100 µg/ml CsA, or 50 µg/ml fluconazole and 10 µg/ml FK506, and incubated at 37°C for 3 days.

Testing synergistic effects on related Candida species

Candida albicans strain SC5314, YAG171 (*rbp1/rbp1*), *C. tropicalis* strain MMRL2017, *C. parapsilosis* strain MMRL1599, *C. krusei* strain

MMRL70, *C. guilliermondii* strain MMRL2021, *C. glabrata* strain MMRL361, and *C. lusitanae* strain DUMC117.96 were plated onto YPD medium containing no drug, 100 µg/ml CsA, 10 µg/ml FK506, 50 µg/ml fluconazole, 50 µg/ml fluconazole and 100 µg/ml CsA, or 50 µg/ml fluconazole and 10 µg/ml FK506.

Antifungal drug activity testing by NCCLS criteria

Drug interactions were assessed with a checkerboard titration, according to the National Committee for Clinical Laboratory Standards (Del Poeta *et al.*, 2000). *In vitro* testing was in RPMI 1640 medium with L-glutamine and without sodium bicarbonate, buffered at pH 7.0 with 0.165 M MOPS. Uracil was added at 40 mg/l. Aliquots of 50 µl of each drug at a concentration of 4× the target final concentration were dispensed in wells of a microtiter plate (96-well Cell Culture Cluster, flat-bottom, Constar, Cambridge, MA) to provide 77 drug combinations. Additional rows were used to determine the MIC of each agent alone and for the growth control well (drug-free).

The final drug concentrations tested were: fluconazole from 16 to 0.01 µg/ml (11 dilutions); CsA from 12.5 to 0.04 µg/ml (7 dilutions); FK506 from 12.5 to 0.19 µg/ml or from 3.12 to 0.04 µg/ml, as indicated (7 dilutions); L-685,818 from 12.5 to 0.19 µg/ml (7 dilutions); 209-825 from 3.12 to 0.04 µg/ml (7 dilutions); and 211-810 from 3.12 to 0.04 µg/ml (7 dilutions).

The yeast inocula (100 µl) were prepared according to the proposed standard, added to each well, and the microtiter plates incubated at 30°C without shaking. Readings were performed following a 48 h incubation. Before the readings, each plate was shaken for 5 min using an Easy-Shaker EAS 2/4 (SLT, Lab-instruments) and the OD₆₀₀ of each well was read on a microtiter plate reader (Molecular Devices Thermomax, Menlo Park, CA).

The MIC of both drugs, alone or in combination, was defined as the lowest drug concentration in a well producing a decrease in absorbance ≤100% compared with the control well. Drug interactions were classified as synergistic (FIC <1.0), additive (FIC = 1), autonomous (FIC between 1 and 2) or antagonistic (FIC >2), based on the fractional inhibitory concentration (FIC) index. The FIC index is the sum of the FICs for each of the drugs, which in turn is defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone. For calculation purposes, an MIC >16, >12.5, >3.12, ≤0.19, ≤0.04 and ≤0.01 were assumed to be 32, 25, 6.25, 0.09, 0.02 and 0.005, respectively.

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