Calcineurin is required for virulence of Cryptococcus neoformans

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Cyclosporin A (CsA) and FK506 are antimicrobial, immunosuppressive natural products that inhibit signal transduction. In T cells and Saccharomyces cerevisiae, CsA and FK506 bind to the immunophilins cyclophilin A and FKBP12 and the resulting complexes inhibit the Ca\textsuperscript{2+}-regulated protein phosphatase calcineurin. We find that growth of the opportunistic fungal pathogen Cryptococcus neoformans is sensitive to CsA and FK506 at 37°C but not at 24°C, suggesting that CsA and FK506 inhibit a protein required for C. neoformans growth at elevated temperature. Genetic evidence supports a model in which immunophilin–drug complexes inhibit calcineurin to prevent growth at 37°C. The gene encoding the C. neoformans calcineurin A catalytic subunit was cloned and disrupted by homologous recombination. Calcineurin mutant strains are viable but do not survive in vitro conditions that mimic the host environment (elevated temperature, 5% CO\textsubscript{2} or alkaline pH) and are no longer pathogenic in an animal model of cryptococcal meningitis. Introduction of the wild-type calcineurin A gene complemented these growth defects and restored virulence. Our findings demonstrate that calcineurin is required for C. neoformans virulence and may define signal transduction elements required for fungal pathogenesis that could be targets for therapeutic intervention.

Keywords: calcineurin/Cryptococcus neoformans/cyclosporin A/FK506/virulence

Introduction

Cryptococcus neoformans is an opportunistic pathogen that primarily afflicts immunocompromised patients. Infection initiates in the lung following inhalation, spreads hematogenously to the brain, and results in meningoencephalitis (Kwon-Chung \textit{et al.}, 1992; Cox and Perfect, 1993; Mitchell and Perfect, 1995). Of serious concern is an increased incidence of C. neoformans infections in AIDS patients who, following treatment, require lifelong suppressive therapy to prevent relapse. Existing treatments (amphotericin B and fluconazole) have toxic side effects, and drug-resistant strains are arising, necessitating identification of novel targets for therapeutic intervention. Several virulence factors have been identified (including capsule production, mating type, prototrophy, phenoloxidase activity and mannoside production), but none has yet led to therapeutic interventions (Kwon-Chung \textit{et al.}, 1982, 1992; Kwon-Chung and Rhodes, 1986; Perfect \textit{et al.}, 1993; Chang and Kwon-Chung, 1994; Chaturvedi \textit{et al.}, 1996).

Cryptococcus neoformans is a heterothallic basidiomyceteous fungus with several features that make it ideally suited for studies of fungal pathogenesis. First, it has a defined sexual life cycle (Kwon-Chung, 1976), and stable isogenic haploid strains of opposite mating type are available (Moore and Edman, 1993). Second, it is amenable to genetic analysis, transformation and gene disruption by homologous recombination (Edman, 1992; Perfect \textit{et al.}, 1992, 1993; Toffaletti \textit{et al.}, 1993; Chang and Kwon-Chung, 1994; Lodge \textit{et al.}, 1994). Third, virulence of strains can be readily assessed in animal models (Perfect \textit{et al.}, 1980; Kwon-Chung \textit{et al.}, 1982; Mody \textit{et al.}, 1988). Finally, \textit{C}. neoformans poses little threat to laboratory workers because it is ubiquitous and much of the population has been exposed without disease, eliminating a need for special containment facilities.

In previous studies to develop a murine model of \textit{C}. neoformans infections in the lung, animals were treated with the immunosuppressant cyclosporin A (CsA) and inoculated with \textit{C}. neoformans (Mody \textit{et al.}, 1988). Contrary to the simple expectation that immunosuppression would exacerbate infection, as it does in human patients, CsA instead protected mice from \textit{C}. neoformans infection. Although some evidence was presented suggesting that CsA inhibits \textit{C}. neoformans growth \textit{in vitro} (Mody \textit{et al.}, 1988), these findings proved difficult to reproduce in another laboratory with the same \textit{C}. neoformans strain (Perfect and Durack, 1985). Thus, whether or not CsA inhibits \textit{C}. neoformans growth has remained uncertain.

CsA, FK506 and rapamycin are immunosuppressants that inhibit signal transduction processes required for T-cell activation (reviewed in Heitman \textit{et al.}, 1992; Schreiber and Crabtree, 1992). CsA, FK506 and rapamycin are all natural products that also have potent antimicrobial activities. Previous studies revealed that the mechanisms of antifungal action of CsA, FK506 and rapamycin in the yeast \textit{Saccharomyces cerevisiae} are essentially identical to the immunosuppressive effects in T-cell proliferation (reviewed in Cardenas \textit{et al.}, 1994a). CsA and FK506/rapamycin diffuse into the cell and form complexes with the cyclophilin A and FKBP12 proteins, respectively. The resulting cyclophilin A–CsA and FKBP12–FK506 complexes inhibit calcineurin (Liu \textit{et al.}, 1991), a Ca\textsuperscript{2+}/calmodulin-activated serine/threonine-specific phosphatase required for stress responses or viability in yeast (Cuyt \textit{et al.}, 1991; Cuyt and Thorner, 1992; Nakamura \textit{et al.}, 1993; Breuder \textit{et al.}, 1994; Cunningham and Fink, 1994; Garrett-Engele \textit{et al.}, 1995; Hemenway \textit{et al.}, 1995;
Tanida et al., 1995), and for gene activation by the transcription factor NF-AT in T cells (Clipstone and Crabtree, 1992; O’Keeffe et al., 1992; Jain et al., 1993; Northrop et al., 1994). The targets of the FKBP12-rapamycin complex are the yeast TOR1 and TOR2 proteins and their mammalian homolog (RAFT1/FRAP/mTOR), novel kinase homologs required for cell cycle progression (Heitman et al., 1991a; Kunz et al., 1993; Brown et al., 1994; Helliwell et al., 1994; Sabatini et al., 1994; Sabers et al., 1995; Alarcon et al., 1996). Because the mechanisms of CsA, FK506 and rapamycin action are conserved from yeast to man, similar target proteins may mediate their effects in other organisms.

Here we report that CsA, FK506 and rapamycin have potent antifungal activity against the opportunistic fungal pathogen C. neoformans. Remarkably, CsA and FK506 are not toxic to C. neoformans at 24°C in vitro, but are markedly toxic at 37°C. Analysis of drug-resistant C. neoformans mutants supports a model in which CsA and FK506 bound to cryptococcal homologs of cyclophilin A and FKBP12 inhibit a common target required for temperature-resistant growth. To test if this target is calcineurin, the C. neoformans gene encoding the calcineurin A catalytic subunit was cloned, sequenced and disrupted. Calcineurin mutant strains were viable but exhibited temperature-, CO₂- and pH-sensitive growth in vitro and were no longer pathogenic in a rabbit model of cryptococcal meningitis. These mutant phenotypes were complemented by re-introduction of the wild-type calcineurin A gene. Our studies identify calcineurin as the target of CsA and FK506 antifungal activity and demonstrate that calcineurin is required for three distinct virulence traits of C. neoformans, possibly as a component of a signal transduction cascade required for fungal pathogenesis.

**Results**

**Cyclosporin A and FK506 are toxic to C. neoformans at elevated temperature by inhibition of a common target protein**

We first tested whether CsA affects growth of several strains of C. neoformans in different liquid or solid media at different temperatures. Remarkably, we discovered that CsA does not inhibit C. neoformans growth at lower temperatures but markedly inhibits growth at 37°C for all strains tested (Figure 1). Different liquid or solid media had little effect. These observations resolve earlier discrepant studies on CsA action, which were performed at different growth temperatures (Perfect and Durack, 1985; Mody et al., 1988). Our observations suggest that CsA inhibits a protein required for C. neoformans growth at elevated temperature.

Because CsA could inhibit either calcineurin or a cyclophilin required for growth at 37°C, we tested the effects of FK506, which also inhibits calcineurin but not cyclophilins. As shown in Figure 1, FK506 was also toxic to C. neoformans cells grown at 37°C but was not toxic to cells grown at 22°C. The effects of CsA and FK506 were quantified by determining the minimum inhibitory concentrations (MIC) for each drug at different temperatures, revealing that CsA and FK506 are 100- to 10 000-fold more toxic at 37°C compared with 22°C (Table I).

Taken together, these pharmacological findings suggest that calcineurin, the only known common target of CsA and FK506, might be required for C. neoformans growth at elevated temperature in vitro.

As a next step to determine the mechanism of action and targets of CsA and FK506, we took a genetic approach. Spontaneous CsA- or FK506-resistant mutants were isolated in an isogenic pair of MATα and MATα C. neoformans strains suitable for genetic analyses (strains JEC20 and JEC21, see Materials and methods). The majority of these mutants (596/600) were found to be resistant to both CsA and to FK506, but not to unrelated compounds [5-fluoro-orotic acid (5-FOA), cycloheximide]. These findings are consistent with a common target protein, such as calcineurin, that is now resistant to both drugs or no longer essential at 37°C in the drug-resistant mutants.

In this genetic screen, we also identified four unusual mutants that were resistant to FK506 but not to CsA (Figure 1, Table II). Three of these FK506-resistant mutants (C20F1, C20F2 and C21F3) were also resistant to rapamycin (Figure 1), a drug structurally related to FK506 that is also toxic when bound to FKBP12. This
observation suggests that FKBP12 might be altered in these mutant strains. Additional findings support this interpretation. First, these mutations segregate as single nuclear mutations following genetic crosses to the isogenic wild-type strain and are recessive in heterokaryons derived from mutants crossed to wild-type. More importantly, all three mutations were partially complemented by heterologous expression of yeast FKBP12 from the *C. neoformans* actin promoter borne by the telomeric plasmid pCnTe1 (data not shown; for details, see Materials and methods). In *S. cerevisiae*, FKBP12 mutations are known to confer a similar recessive FK506/rapamycin-resistant phenotype (Heitman et al., 1991a). Taken together, our observations suggest that a common FK506-binding protein, such as FKBP12, mediates FK506 and rapamycin toxicity in *C. neoformans*.

One mutant (C21F2) was resistant to FK506 but not to rapamycin. In this mutant, drug resistance was conferred by a mutation that was dominant by heterokaryon analysis and segregated independently of the recessive FK506/ rapamycin-resistant mutations in genetic crosses (data not shown). By analogy with calcineurin mutations in *S. cerevisiae* that confer dominant FK506 resistance (Cardenas et al., 1995), this mutation might alter *C. neoformans* calcineurin to prevent inhibition by FKBP12– FK506. In summary, these pharmaceutical and genetic studies support a model in which FKBP12–FK506 and cyclophilin A–CsA complexes block *C. neoformans* growth at 37°C by inhibiting a common target, which we propose is calcineurin.

**Cloning the *C. neoformans* gene encoding the calcineurin A catalytic subunit**

Our resulting model is that CsA and FK506 are toxic to *C. neoformans* at 37°C by inhibiting calcineurin, and that calcineurin is only required for growth at elevated temperature. We tested this model by cloning the *C. neoformans* gene encoding calcineurin, disrupting this gene by homologous recombination, and testing if this results in temperature-sensitive growth.

Genes encoding the calcineurin A catalytic subunit have been isolated from *S. cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Neurospora crassa*, *Drosophila melanogaster*, mouse and human, revealing significant overall identity, especially within the domain of calcineurin A that interacts with the calcineurin B regulatory subunit (see Figure 3). Four degenerate pools of oligonucleotides corresponding to highly conserved peptides in this region were synthesized and used as primers in low stringency PCR reactions with a *C. neoformans* cDNA library as template. All three possible primer pairs yielded fragments of the predicted size that co-migrated with PCR products using *S. cerevisiae* genomic DNA or the cloned *S. cerevisiae* calcineurin A gene CMPI as templates. Sequence analysis of several independent cloned fragments revealed a single unique sequence with striking identity to known calcineurin A genes. Based on this sequence, internal primers were synthesized, and a PCR product with perfect homology to the gene was obtained and used as a probe to clone the gene from a *C. neoformans* strain H99 genomic EMBL3 library.

One strongly hybridizing isolate was obtained from a screen of ~80 000 plaques. Sequence analysis confirmed identity to other calcineurin A genes. However, this clone was chimeric, and sequence identity diverged at a Sau3A site near the N-terminal end of the predicted open reading frame (ORF). By Southern analysis, the gene was present on an ~7000 bp EcoRI fragment, and the complete gene was recovered from a size-selected EcoRI genomic library. Both strands of the ORF and 5′- and 3′-untranslated regions were sequenced. To confirm the sequence and exon–intron borders, several cDNA fragments were retrieved by PCR from a *C. neoformans* cDNA library and sequenced. Finally, the 5′ end of the gene was determined by RACE (see Materials and methods) using total RNA from strain H99 as template.

The sequence of the *C. neoformans* calcineurin A gene CNA1 and its predicted protein product are depicted in Figure 2. The CNA1 ORF is punctuated by multiple small introns with consensus donor, acceptor and branch splice sites (underlined in Figure 2). As shown in Figure 3, the predicted *C. neoformans* CNA1 gene product exhibits marked sequence identity with the calcineurin A proteins from other organisms, especially with calcineurin A from *A. nidulans* (70% identity) and *N. crassa* (73% identity). The overall order of the protein domains is conserved, including the catalytic region, the calcineurin B-binding domain, the calmodulin-binding domain and the auto-inhibitory domain (underlined in Figure 3).

The predicted product of the *C. neoformans* calcineurin A gene differs in that the protein is longer than other calcineurins at the C-terminus and contains a longer N-terminal end of similar length to and limited identity with the CMP1 calcineurin A subunit of *S. cerevisiae*. Several findings indicate that the *C. neoformans* calcineurin A protein is longer on the C-terminal end. First, the genomic and cDNA sequences from divergent serotype A and D strains differ by multiple silent third base polymorphisms in this region, consistent with an ORF (data not shown). Second, the last exon of the assigned ORF ends with two in-frame stop codons. Finally, the encoded protein migrates at the expected molecular mass (described below). The marked identity between the predicted *C. neoformans* CNA1 gene product and calcineurin A from other microorganisms and vertebrates indicates that this is the authentic calcineurin A gene and that calcineurin may be regulated by calcineurin B and calmodulin in *C. neoformans*. Finally, Southern analysis under both high and low stringency conditions revealed only a single calcineurin A gene in *C. neoformans* (data not shown), as is also the case in *A. nidulans* and *N. crassa*.

**Disruption of the *C. neoformans* calcineurin A gene by homologous recombination**

To determine the phenotype of *C. neoformans* mutants lacking calcineurin, the calcineurin A CNA1 gene was
Calcineurin is required for virulence in C. neoformans.

Fig. 2. Sequence of C. neoformans calcineurin A CNA1 gene and protein. The DNA sequence of the C. neoformans genomic locus encoding the calcineurin A catalytic subunit CNA1 is depicted and was determined from the genomic clone, multiple cDNA clones and a 5'H11032-RACE product, as described in Materials and methods. The 5'H11032- and 3'H11032-untranslated regions and intronic sequences are in lower case and introns are numbered in order of occurrence from 5'H11032 to 3'H11032. Exons of the CNA1 open reading frame are in upper case with the translated protein sequence below the DNA sequence in upper case single letter amino acid abbreviations. The approximate 5'H11032 mRNA start site determined by RACE, consensus splice donor (GTNNGY), branch (CTRAY) and acceptor (YAG) sites, and two in-frame stop codons at the end of the ORF are underlined.

First, we used the CNA1 genomic locus from the same strain used for transformation to decrease inhibition of the ADE2 selectable marker to result in the calcineurin null mutation cna1Δ::ADE2 (Figure 4A). We took two homologous recombination by mismatch repair.

Disrupted by replacing a large fragment of the gene with the ADE2 selectable marker to result in the calcineurin null mutation cna1Δ::ADE2 (Figure 4A). We took two measures to decrease non-homologous recombination.

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Cryptococcus neoformans calcineurin shares striking identity with other calcineurins. The predicted protein sequence of the CNA1 calcineurin A catalytic subunit from *C. neoformans* was aligned with the sequences of calcineurin A from *A. nidulans*, *N. crassa*, humans and *S. cerevisiae* (yeast CMP1 protein). Identical amino acids are indicated by dashes. Non-identical amino acids are in lower case. In some cases, gaps were introduced to maximize alignment and are indicated by periods. Amino acid numbers are to the right. The highly conserved calcineurin B-binding, calmodulin-binding and autoinhibitory domains are underlined. A small non-homologous insert in the human protein is indicated by an X, and its sequence is depicted at the bottom.

strain to reduce gene conversion at the ade2 locus. DNA was introduced by biolistic transformation into the *C. neoformans* serotype A strain M049, an isogenic non-reverting ade2 mutant of the pathogenic serotype A strain H99. Because homologous recombination occurs at low frequency in *C. neoformans*, adenine prototrophic trans-DNA was introduced by biolistic transformation into the *C. neoformans* serotype A strain M049, an isogenic non-reverting ade2 mutant of the pathogenic serotype A strain H99. Because homologous recombination occurs at low frequency in *C. neoformans*, adenine prototrophic transformants were first selected at 24°C and then screened for the phenotype we predicted would result from calcineurin reversion of the ade2 mutant of the pathogenic serotype A strain 2580.
mutation, namely temperature-sensitive growth. Remarkably, 16 of 200 ADE⁺ transformants with cna1Δ::ADE2 (8%) were viable at 24°C but failed to grow at 39°C in vitro. This finding provides further support that CNA1 is the only gene encoding calcineurin A in C. neoformans. PCR analyses of genomic DNA from the isogenic wild-type and candidate disruption strains (both with and without the temperature-sensitive phenotype) revealed that the CNA1 calcineurin A gene had been replaced with the cna1Δ::ADE2 deletion mutation in temperature-sensitive transformants but not in temperature-resistant transformants, and that all transformants with the cna1Δ allele exhibited a temperature-sensitive phenotype (Figure 4B and data not shown). Similar findings were obtained with a second cna1::ADE2 disruption allele in which the ADE2 gene was inserted between two XhoI sites in the CNA1 gene (data not shown). These findings support the conclusion that the cna1 mutation alone confers a temperature-sensitive phenotype.

To confirm that the calcineurin A gene mutation abrogates calcineurin expression, proteins from isogenic C. neoformans CNA1 wild-type and cna1Δ deletion strains were fractionated by SDS–PAGE, transferred to nitrocellulose and analyzed by [125I]calmodulin overlay (Figure 4C). Extracts from S. cerevisiae strains expressing or lacking the yeast calcineurin A CMP1 and CMP2 subunits, respectively (Figure 4C). Extracts from the C. neoformans CNA1 wild-type strain H99 contained an abundant calmodulin-binding protein of ~80 kDa that was absent in extracts from the isogenic cna1Δ mutant strain (Figure 4C). These findings confirm that the C. neoformans calcineurin A gene has indeed been disrupted and that the size of the CNA1 protein is in good agreement with that predicted from the CNA1 gene sequence. In addition, we conclude that the CNA1 protein: (i) is the only calcineurin A catalytic
calcineurin is temperature sensitive in vitro indicates that these strains contain only the original FK506 and CsA are not toxic in cells lacking calcineurin. Inhibited further by either FK506 or CsA, indicating that calcineurin encoded levels of CO2 and the physiological alkaline pH present in 2582 temperatures, further indicating that calcineurin encoded levels of CO2 and the physiological alkaline pH present in elevated temperatures. (37°C), growth of the calcineurin wild-type strain H99 (wild-type H99, A and C), the isogenic calcineurin A deletion mutant strain cna1Δ ADE2 (cna1Δ, B and D) and the calcineurin cna1Δ mutant strain transformed with the wild-type CNA1 gene (cna1Δ + CNA1, data not shown) were grown in triplicate in liquid RPMI medium at 30°C for 0–72 h in an atmosphere of air at pH 7.0 (●), air at pH 7.3 (▲) or 5% CO2 at pH 7.0 (○) and the number of surviving colony-forming units (CFU) measured by dilution and plating on YPD medium and averaged for the triplicate cultures.

subunit in C.neoformans, (ii) represents a major calmodulin-binding protein in C.neoformans and (iii) is expressed at both 24 and 37°C (Figure 4C).

**Growth of C.neoformans mutants lacking calcineurin is temperature sensitive in vitro**

Given the temperature sensitivity of CsA and FK506 effects, we have investigated the consequences of loss of calcineurin function on growth at low and high temperatures. As shown in Figure 5, growth of the isogenic CNA1 wild-type and the cna1Δ calcineurin mutant strains was indistinguishable at 24°C. In contrast, the calcineurin mutant strain was severely growth impaired at 37°C and inviable at 39°C (Figure 5A). At intermediate conditions (37°C), growth of the cna1Δ mutant strain was not inhibited further by either FK506 or CsA, indicating that FK506 and CsA are not toxic in cells lacking calcineurin. In addition, the extent of growth inhibition conferred by calcineurin deletion was equivalent to that imposed by CsA or FK506 in the calcineurin wild-type strain at all temperatures, further indicating that calcineurin encoded by the CNA1 gene is the sole target of FK506 and CsA in C.neoformans.

When the CNA1 gene encoding wild-type calcineurin A was re-introduced into the cna1Δ mutant strain, growth at both 37 and 39°C was restored and this growth was sensitive to FK506 (Figure 5C). PCR analyses of genomic DNA from these transformants confirmed that both the original cna1Δ mutation and an ectopic copy of the CNA1 calcineurin A gene were now present (Figure 5B). Thus, the growth defect conferred by the cna1Δ mutation is recessive and is complemented by the wild-type CNA1 gene. In contrast, we have isolated a number of suppressor mutations that can restore growth of the cna1Δ mutant strain at 37°C but not at 39°C (Figure 5C). PCR analysis indicates that these strains contain only the original cna1Δ mutation (Figure 5B). In these cases, growth at 37°C is resistant to FK506, indicating that these suppressor mutations partially bypass the requirement for calcineurin for growth at elevated temperature. Further analysis of these suppressor mutations should permit the identification of downstream elements of the calcineurin signaling cascade in C.neoformans. Taken together, our findings indicate that calcineurin plays a central role in growth at elevated temperature in vitro.

**C.neoformans calcineurin mutants are CO2 and pH sensitive and exhibit altered responses to cations**

To infect mammalian hosts, C.neoformans must survive not only at elevated temperature, but also in the increased levels of CO2 and the physiological alkaline pH present in host tissues. Remarkably, we find that 5% CO2 profoundly inhibits the growth of the cna1Δ calcineurin mutant strains in liquid RPMI medium in vitro (Figure 6D). In contrast, the isogenic calcineurin wild-type strain proliferates normally in the presence of CO2 and both strains proliferate normally in air (~0.04% CO2) (Figure 6C and D). Similarly, the cna1Δ calcineurin mutant strains were unable to...
proliferate in RPMI medium at pH 7.3 (Figure 6B), whereas both calcineurin wild-type and cnaΔ calcineurin mutant strains proliferated normally in RPMI at pH 7.0 in vitro (Figure 6A and B). Transformation of the calcineurin mutant strain with the wild-type CNA1 calcineurin A gene restored growth in either the presence of CO2 or at pH 7.3 (data not shown), confirming that both the CO2- and the pH-sensitive growth defects are attributable to loss of calcineurin function. These findings link calcineurin to the ability of C. neoformans to survive three distinct environmental conditions required for host infection and pathogenesis, suggesting that calcineurin may coordinate the regulation of virulence traits in this organism.

In S. cerevisiae, calcineurin is required for resistance to the toxic effects of either Li+ or Mn2+ cations. C. neoformans mutants lacking calcineurin, or wild-type cells exposed to CsA or FK506, are also sensitive to 50 mM Li+ cations (data not shown). In contrast, the C. neoformans calcineurin mutant was resistant to 10 mM Mn2+ ions whereas the isogenic calcineurin wild-type strain was Mn2+ sensitive (data not shown), precisely the opposite of the phenotype conferred by calcineurin mutations in S. cerevisiae. Both mutant phenotypes were complemented when the cnaΔ mutant strain was transformed with the wild-type calcineurin A gene (not shown). These observations indicate that calcineurin plays a general role in regulating cation homeostasis in microorganisms.

**C. neoformans mutants lacking calcineurin A are not pathogenic**

Our findings suggested that calcineurin might be required for virulence of C. neoformans, which requires survival in mammalian hosts, whose body temperatures are equal to or exceed 37°C and in which alkaline pH and CO2 at high levels are present. To test this hypothesis, we employed an animal model of cryptococcal meningitis in which rabbits are immunosuppressed with steroids and inoculated intrathecally with the C. neoformans wild-type strain H99. Where the isogenic calcineurin A wild-type strain (■, wild-type H99), the isogenic calcineurin A cnaΔ mutant strain (■, cnaΔ) or the isogenic cnaΔ mutant strain into which the wild-type CNA1 gene had been re-introduced (▲, cnaΔ + CNA1). CSF was removed on days 2, 5, 7 and 12 following inoculation, and the number of surviving organisms (expressed as the mean of log10 CFU/mL CSF from three rabbits at each time point, CFU colony-forming units) determined by serial dilution and plating on YPD medium with growth at 24°C for 4 days. Counts were significantly lower in the cnaΔ strain compared with wild-type H99 at all time points (P < 0.01 by T-test for unpaired means) and the cnaΔ + CNA1 counts were only lower than wild-type on day 12 (P < 0.05).

**Discussion**

Our findings reveal that the immunosuppressive antifungal natural products CsA, FK506 and rapamycin are toxic to the opportunistic fungal pathogen C. neoformans. Interestingly, CsA and FK506 toxicity was only manifested under conditions of elevated growth temperature. This observation, and additional genetic evidence, suggested that the only known common target of CsA and FK506 is calcineurin, which might be required for growth in C. neoformans at 37°C but not at 24°C in vitro. Because pathogenesis requires survival at the body temperature of the infected mammalian host, this further suggested that calcineurin might be required for pathogenesis of C. neoformans in vivo.

Here we describe the isolation of the CNA1 gene encoding the calcineurin A catalytic subunit of C. neoformans. Disruption of the calcineurin A CNA1 gene abrogates calcineurin expression, confers temperature-, CO2- and pH-sensitive conditional growth in vitro, and prevents pathogenicity in vivo in an animal model of cryptococcal meningitis. Re-introduction of the wild-type calcineurin A gene complemented these growth defects and largely restored virulence. Thus, calcineurin is required for the opportunistic fungal pathogen C. neoformans to
Our pharmacological and genetic findings support the tolerance in grow at elevated temperature, in the presence of CO2 and cations in both yeast and growth in response to an extracellular cue, such as nutrients. membrane or vacuolar H+ pumps PMR1, VCX1/HUM1 and PMC1 in C.neoformans (Cunningham and Fink, 1996, Pozos et al., 1996). Our findings also suggest that inhibitors of calcineurin, or of other elements of this cascade, might be therapeutically useful. However, CsA exacerbates cryptococcal meningitis in the rabbit model system (Perfect and Durack, 1985), most likely as a consequence of immunosuppression and because CsA does not cross the blood–brain barrier. Although FK506 does penetrate the CNS, FK506 also exacerbates C.neoformans meningitis in rabbits, most likely as a result of immunosuppression (Odom et al., 1997). Thus, potent immunosuppression by the presently available agents outweighs any beneficial antifungal effect. Non-immunosuppressive CsA and FK506 analogs have been described. One FK506 analog, L-685,818, is known potently to inhibit bovine calcineurin when bound to S.cerevisiae FKBP12 but not when bound to human calcineurin (Dumont et al., 1993). As a result, L-685,818 is not immunosuppressive and is a potent antagonist of FK506 actions in vitro and in vivo (Dumont et al., 1992). We find that L-685,818 is toxic to C.neoformans. L-685,818 is, like FK506, toxic at 37°C but not at 24°C, and FK506-resistant mutants are cross-resistant to L-685,818, indicating a conserved mechanism of action (Odom et al., 1997). Our findings establish that this non-immunosuppressive analog takes advantage of structural differences between host and fungal FKBP12 and calcineurin and, as a result, spares host immune function yet grow at elevated temperature, in the presence of CO2 and at physiological pH in vitro and for survival and virulence in an infected mammalian host.

We summarize our findings in the model of Figure 8. Our pharmacological and genetic findings support the hypothesis that in C.neoformans, as in other systems, CsA, FK506 and rapamycin diffuse into the cell, bind cyclophilin A and FKBP12, and form protein–drug complexes that inhibit components of signal transduction cascades. Our findings indicate that calcineurin is the common and universal target of cyclophilin A–CsA and FKBP12–FK506. Based on the known roles of calcineurin in mediating calcium-activated signal transduction in T cells and in yeast, an attractive hypothesis is that calcineurin is activated by an increase in intracellular calcium and calmodulin in response to an unknown extracellular signal present in the infected host. Activated calcineurin would then in turn dephosphorylate specific proteins required for survival at elevated temperature, growth in the presence of CO2 and at alkaline pH, and pathogenesis. In this model, other elements that might be required for virulence include calmodulin, increased intracellular calcium and the machinery that provides it, and one or more specific calcineurin substrates. The finding that calcineurin is required under three distinct conditions present in the infected host suggests that calcineurin may be a central regulator of virulence in C.neoformans.

A less likely alternative hypothesis is that calcineurin is not part of a signal transduction pathway required for virulence, but is instead required to maintain activity of components required for growth at high temperature and CO2 and pH resistance. Such a model would require that some proteins differ in phosphorylation state at 37 and 24°C, or that activity at 37°C requires dephosphorylation whereas activity at 24°C does not. One means to test these and other models, and to identify downstream effectors of calcineurin, would be to characterize suppressor mutations that restore growth to calcineurin mutants at 37°C. In summary, our studies reveal that calcineurin is required for virulence and may delineate the first elements of a signal transduction cascade required for fungal pathogenesis.

By analogy with other systems, the targets of calcineurin might include ion pumps or transcription factors, which could regulate expression of other proteins required for virulence. The role of calcineurin in CO2- and pH-resistant growth is intriguing given the known role of calcineurin in ion, pH and Ca2+ homeostatic mechanisms in other organisms such as S.cerevisiae (Nakamura et al., 1993; Cunningham and Fink, 1994, 1996; Hemenway et al., 1995; Tanida et al., 1995). Little is known about how CO2 affects C.neoformans growth beyond that it potently induces capsule formation in vivo (Granger et al., 1985). Calcineurin could regulate the activity of a fungal carbon anhydrase homolog or, following spontaneous hydrolysis, be required to regulate pumps that extrude the resulting H+ and HCO3– ions from the cytoplasm to allow continued growth in the presence of CO2. Calcineurin is required for growth at alkaline pH in both C.neoformans and S.cerevisiae, possibly via regulation of the plasma membrane or vacuolar H+-ATPase (Mendoza et al., 1994; Garrett-Engele et al., 1995; Hemenway et al., 1995; Tanida et al., 1995). Calcineurin is required for resistance to Li+ cations in both yeast and C.neoformans, possibly via regulation of conserved proteins, such as homologs of the yeast Na+/Li+ pump PMR2A. In contrast, calcineurin mutations confer Mn2+ sensitivity in S.cerevisiae but Mn2+ tolerance in C.neoformans, suggesting that calcineurin may differentially regulate homologs of the yeast Mn2+ and Ca2+ pumps PMR1, VCX1/HUM1 and PMC1 in C.neoformans (Cunningham and Fink, 1996, Pozos et al., 1996).
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**Materials and methods**

\textbf{Strains, media and compounds}

The \textit{S.cerevisiae} strains employed here were as described (Cardenas et al., 1994b). Most \textit{C.neoformans} strains were isogenic derivatives of the serotype A strain H99 or of the isogenic pair of MATa and MAT\(\alpha\) serotype D strains JEC20 and JEC21 (Moore and Edman, 1993). In addition, drug sensitivity was assessed for the serotype A strain H99 (Mody et al., 1988). The \textit{C.neoformans} strains were routinely grown using rich (YPD) and synthetic medium as for \textit{S.cerevisiae} as described (Sherman, 1991), and immunosuppressant-containing medium was prepared as described (Heitman et al., 1993). V8 mating-starvation medium contained 5% V8 vegetable juice (Campbells Soup Co.), 0.5 g/LKH\(_2\)PO\(_4\), and 4% agar and was adjusted to pH 7.2 with KOH prior to autoclaving.

\textbf{Isolation and genetic and biochemical analyses of drug-resistant mutants}

Spontaneous independent mutants resistant to 100 \(\mu\)g/ml CsA or 1 \(\mu\)g/ml FK506 in YPD at 37\(^\circ\)C were isolated in strain H99 and in the isogenic strain pair JEC20 and JEC21 and tested for resistance to CsA, FK506 and rapamycin (1 \(\mu\)g/ml).
We devised methods to select meiotic recombinants to analyze segregation of other unlinked markers of interest in the cross. We first isolated derivatives of strains JEC20, JEC21, C20F1, C20F2, C21F2 and C21F3 that were resistant to 5-FOA, 10 μg/ml cycloheximide or 60 μg/ml canavine. In other organisms, recessive mutations confer resistance to cycloheximide. ura5 mutant derivatives were then known to confer 5-FOA resistance in C.neoformans serotype D strains (Kwon-Chung et al., 1992). Strains of opposite mating type and resistant to different drugs were co-cultured on V8 starvation-mating medium for 7–14 days at room temperature. Hyphae were removed, germinated on YPD medium and replica-plated to 5-FOA plus cycloheximide, 5-FOA plus canavine or canavine plus cycloheximide media. The most suitable combination proved to be 5-FOA plus cycloheximide. By this means, random 5-FOA- and cycloheximide-resistant meiotic recombinants were obtained, and the segregation pattern of FK506 resistance mutations crossed to wild-type or to each other was ascertained. One other recombinant selection was employed in some cases in which 5-FOA-resistant Ura4* LYS* mutant strains were crossed to ura5 (5-FOA) lys1 or lys2 mutant strains (JEC157, JEC168 or JEC169) and ura5 LYS* meiotic recombinants selected on 5-FOA medium lacking lysine. In both cases, unselected auxotrophic markers, mating type and drug resistance mutations segregated in an approximate ratio of 1:1.

To determine if mutations conferred dominant or recessive drug resistance, we developed a method to isolate and analyze heterokaryons derived from a genetic cross. ura3 derivatives of FK506-resistant mutant strains were transformed with a yeast isogene ade2-1 or MATa on V8 mating medium and incubated for 7–14 days at room temperature. Hyphae were removed and cultured on synthetic dextrose medium lacking uracil, adenine and lysine. Colonies with the odd ruffled morphology characteristic of heterokaryons (Whelan and Kwon-Chung, 1986) were streak purified and confirmed to be self-filamenting on V8 medium, as a second independent feature of MATa/ MATa heterokaryons. In parallel, the isolated heterokaryons were tested for growth on YPD medium containing 1 μg/ml FK506.

Heterologous expression of yeast FKBP12

The S.cerevisiae gene encoding FKBP12 (Hestman et al., 1991b) was fused to the C.neoformans actin gene promoter (Cox et al., 1995) by PCR overlap (Horton et al., 1989) as follows. The yeast FKBP12 gene was PCR amplified with oligonucleotides 389 (5'-ACTGCGCCAACATGTCTATGCTGAAAAATTGAGAAGTAA and 48 (5'-CGCCGGGATCCAAAATAATCACTGCTCATATATAG) to provide 5' overlap with the actin promoter sequence, and the actin promoter gene sequence was amplified with primers 387 (5'-GGAATCCGGGATCCAAAATAATCACTGCTCATATATAG) and 418 (5'-GTACCTTGAATTCTGGAATGDTAGATACAT- GTTTGCGGAGAATGTT) and 48. The resulting second round ~1500 bp PCR product was cloned in the TA system (Invitrogen), released with EcoRI and cloned in the EcoRI site of the C.neoformans URA5 telomeric plasmid pCrnTe1 (Edman, 1992), to yield plasmid pAOT1 with the yeast FKBP12 gene fused to the C.neoformans actin promoter (Horton et al., 1992). The control plasmid pCrnTe1 and the yeast FKBP12 expression plasmid pAOT1 were linearized with the rare-cutting Meganuclease I-SceI (Omega-Nuclease, Boehringer) to reveal the linear telomeric ends of the vector. Linear DNA was introduced by electroporation-mediated transformation (Edman, 1992) into ura5 mutant derivatives of strains JEC20, C20F1, C20F2 and C21F3. Ura+ transformants were selected and tested for growth on synthetic medium lacking uracil and containing 1 or 10 μg/ml FK506 or 1 μg/ml rapamycin. Yeast FKBP12 complemented and restored rapamycin sensitivity in the C20F1, C21F2 and C21F3 mutants. Rapamycin resistance was restored in isolates that had lost the URA5 plasmid following passage on YPD medium and selection on 5-FOA. Yeast FKBP12 did not restore FK506 sensitivity, either because the yeast FKBP12–FK506 complex does not inhibit C.neoformans calcineurin or because the level of yeast FKBP12 expressed is too low.

Isolation and characterization of the C.neoformans gene encoding calcineurin A

Calcineurin A sequences from several organisms (Figure 3) were aligned and pools of oligonucleotides corresponding to highly conserved peptides were synthesized (Odom et al., 1996). Primers were made from chlorophyll aFPV MRV peptide; 5'-CAYGARGGCICARGAYGCIGGITAYA/CGIATGTA) and 293 (NVNMRQF peptide; 5'-AAYGTIATGAAYATIC/AGICARTT) and reverse primers were 294 (NVNMRQF peptide; 5'-AAYTGICG- TIAATRTCATIACRTT) and 295 (FMDVFTWP peptide; 5'-CAAG-

TRAIAACRTCTCATRAA). PCR reactions were performed under conditions of low annealing stringency using primer pairs 292–294, 293–295 and 292–295 with a pooled C.neoformans cDNA library from strain B3501 (Stratagene) and S.cerevisiae genomic DNA from strain JYK3-3a. PCR reaction conditions were 5 min at 94°C, 70 cycles of 30 s at 94°C, 1 min 30 s at 50°C and an additional 5 min at 72°C. PCR products of the expected sizes (160 bp for 292–294, 80 bp for 293–295 and 210 bp for 292–295) that co-migrated were obtained from C.neoformans cDNA and S.cerevisiae genomic DNA and cloned with the TA system (Invitrogen) and sequenced, revealing a unique C.neoformans sequence with identity to other calcineurin A proteins. Two primers with perfect homology (underlined) to the internal sequence, 329 (5'-CACCAGTAGATCTGCAGAATTAGACCACT- GCC) and 330 (5'-CCGGTGATCCCTCAGGTTAGGACACAAATAAAGGATGGG), were used to amplify a portion of the C.neoformans calcineurin A gene. This PCR product was gel purified, random primer labeled and used as a probe to isolate the corresponding genomic locus from a C.neoformans strain H99 genomic library cloned as partial BamHI–Sau3A fragments in the λ vector EMBL3. A screen of ~80 000 plaques yielded one strongly hybridizing clone. λ DNA was prepared with a Qiagen λ DNA prep kit, and an internal 3.5 kb BamHI fragment that bore the desired target sequence (based on PCR amplification with primers 329–330) was subcloned into the BamHI site in pUC18. The sequence of the insert was determined using appropriate subcloned fragments and synthetic primers. The original clone was incomplete and chimeric, so the intact gene was obtained by cloning hybridization from a size-selected EcoRI library of H99 genomic DNA cloned in plasmid pUC18. The DNA sequence depicted in Figure 2 was completed from this intact 6.9 kb clone. In addition, a number of partial cDNA clones were retrieved from the C.neoformans cDNA library using primers designed against the genomic exonic sequences. DNA sequence analysis confirmed the predicted intron– exon borders and revealed a number of DNA polymorphisms between the genomic CNA1 locus from serotype A strain H99 and cDNA clones from serotype D strain B3501, as previously observed for other genes.

5'-RACE analysis

Total RNA was prepared from C.neoformans strain H99 grown in YPD medium at 30°C. RACE was performed using the Marathon cDNA amplification kit (Clontech). First strand synthesis was with the CNA1-specific primer 363 (5'-AAAGFFGTGTCATGACGGGAGGG). Second strand synthesis and ligation to the Marathon cDNA adapter, a nested PCR was performed using an internal CNA1-specific primer (392: 5'-GTATCTGCAGGATCCACACCGC) and the adapter primer 1 (AP1) and a PCR program of 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, 2 min at 72°C, and a final 5 min at 72°C step. The resulting PCR product was cloned in the TA cloning kit (Invitrogen) and sequenced.

Disruption of the calcineurin A CNA1 gene

A deletion mutation in the CNA1 calcineurin A gene was constructed by correcting a 2400 bp Jolol ADE2 fragment (blunted with Klow and dNTPs) between two EcoRV sites in the CNA1 gene in the 3.5 kb BamHI genomic fragment cloned in pUC18, yielding plasmid pCA1A:ADE2. Approximately 5 μg of supercoiled disruption plasmid DNA was purified on a Quagen column, absorbed onto the surface of gold microprojectiles and introduced by biolistic transformation into strain M049, an Δade2 isogenic derivative of strain H99. Adenine prototrophic transformants were selected on synthetic regeneration medium (1 M sorbitol) lacking adenine at 24°C, streak purified and tested for growth at 24, 37 and 39°C on YPD medium.

Genomic DNA for PCR analyses was prepared as follows. Strains were grown as dense patches on YPD medium, resuspended in 500 μl of buffer (50 mM Tris–HCl pH 7.5, 20 mM EDTA, pH 7.5, 1% SDS), broken with 0.5 g of glass beads agitated for 3–5 cycles (1 min vortex, 1 min on ice) with a bead beater, and heated at 70°C for 10 min. Then 200 μl of 3 M KAc and 150 μl of 5 M NaCl were added, incubated for 20 min on ice and microcentrifuged. Nucleic acids were recovered from the resulting supernatants by addition of one-third volume of 30% PEG 3000 in 1.5 M NaCl and microcentrifugation for 5 min at 4°C. Pellets were resuspended in 100 μl of TE, treated for 5 min at 37°C with 8 ng of RNase, twice extracted with phenol:chloroform (1:1) and then with chloroform, and ethanol precipitated, dried and resuspended in 50 μl of TE.

Genomic DNA prepared from wild-type, temperature-resistant (ts) and temperature-resistant C.neoformans strains was analyzed by PCR to determine whether the CNA1 gene had been disrupted. PCR reactions

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contained ~0.05 µg of genomic DNA (0.05 µl of standard genomic DNA prep) and were amplified with a PCR program consisting of 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C, and a final step of 5 min at 72°C. Synthetic oligonucleotides corresponding to sequences in the CNA1 gene flanking the site of the ΔCNA1::ADE2 mutation served as primers, including 3' (5'-AAGCTTAGTGGTATCCCG) and 5' (5'-CGAAGATTCGACCGGCTCATA), which yield PCR products of ~2000 and ~2500 bp for the CNA1 wild-type and cna1Δ mutant alleles, respectively. Two types of homologous integration events resulting in ts mutants were observed. In the first, the chromosomal CNA1 gene had been replaced by the deletion allele by a double cross-over event. These isolates were inviable at 39°C and did not give rise to temperature-resistant revertants at this temperature. In the other type, both the deletion allele and a truncated wild-type gene were present, resulting from integration of the entire disruption plasmid at the chromosomal CNA1 gene by a single cross-over event. In this case, revertants viable at 39°C were readily obtained that were calcineurin wild-type (Csa-FK506-sensitive) and ade− and presumably resulted from homologous recombination between the gene duplication. In subsequent analyses, we employed mutants of the first type in which the calcineurin A gene had been disrupted and replaced precisely.

[125I]Calmodulin overlay blot
Proteins from S. cerevisiae and C. neoformans strains were extracted by glass bead homogenization as described (Cardenas et al., 1994b), and ~0.2 µg of total protein extracts were separated by SDS-PAGE (10%) and transferred to a nitrocellulose membrane. The membrane was blocked overnight in block/wash buffer (10 mM imidazole pH 7.3, 100 mM KCl, 5 mM CaCl2, 5% bovine serum albumin, 0.05% Tween-20 and 0.02% NaN3), transferred to fresh buffer containing 1×106 c.p.m. of [125I]calmodulin, and incubated at room temperature for 2 h with gentle agitation. The membrane was washed twice in block/wash buffer, air dried and exposed to film overnight at –80°C.

Re-introduction of the wild-type CNA1 gene
The wild-type CNA1 gene on a 6.9 kb EcoRI fragment in plasmid pH C18 was introduced into the cna1Δ ADE2 mutant strain by homologous transformation and selection for growth on YPD regeneration agar at 39°C. Growth of these temperature-resistant colonies was sensitive to CsA and FK506, indicating that calcineurin activity had been restored by the introduced CNA1 wild-type gene. In control transformations performed in parallel with no transforming DNA, we found no colonies surviving at 39°C, indicating that re-introduction of the CNA1 gene is required to complement the cna1Δ mutation and restore growth at 39°C. Genomic DNA was prepared and analyzed by PCR as described above, confirming that both the original cna1Δ mutation and an ectopic copy of the wild-type CNA1 gene were now present (Figure 5).

In vitro growth kinetics of calcineurin mutant strains in air versus CO2
The in vitro growth rate at 30°C in 5% CO2 and air at pH 7 and pH 7.3 was determined for the isogenic wild-type CNA1 strain H99, the cna1Δ mutant strain and the cna1Δ mutant strain transformed with the wild-type CNA1 gene (cna1Δ + CNA1). The media was RPMI-1640 (Sigma) supplemented with 54 mM NaMOPS and adjusted to pH 7.0 or pH 7.3 for growth studies in air (RPMI-air). For growth studies in CO2, RPMI-1640 was prepared with either 10 mM NaHCO3 and 44 mM NaMOPS or 29 mM NaHCO3 and 25 mM NaMOPS and adjusted to pH 7.0 or pH 7.3, respectively (RPMI-CO2). An overnight YEPD broth culture of each strain of C. neoformans was resuspended in 0.8% NaCl and inoculated into RPMI-air and RPMI-CO2 that had been warmed to 30°C at a final cell density of ~106/ml. The cells were transferred to 25 cm2 tissue culture flasks (Corning) and allowed to incubate at 30°C in air or 5% CO2. At various points (0, 6, 12, 24, 48 and 72 h), samples were taken from the culture and diluted onto YEPD plates for assessment of quantitative yeast counts. All cultures were done in triplicate.

Animal model of cryptococcal meningitis
New Zealand White rabbits weighing 2–3 kg were housed in separate cages and provided with water ad libitum and Purina rabbit chow. Isogenic C. neoformans wild-type (H99) and cna1Δ mutant C. neoformans serotype A strains were prepared by growth for 96 h at 25°C on Saboraud’s agar medium containing 100 µg/ml chloramphenicol and resuspended in 0.015 M phosphate-buffered saline (PBS). In three different experiments, six or nine rabbits were administered cortisone acetate 2.5 mg/kg intramuscularly 1 day prior to inoculation with C. neoformans and then daily for 12–14 days. Twenty four hours following initiation of steroid treatment, rabbits were anesthetized with xylazine and ketamine intramuscularly and inoculated intracereurally with 0.3 ml of yeast suspension through a 25 gauge needle on a 3 ml syringe. Three rabbits received wild-type strain H99, three rabbits the cna1Δ deletion mutant strain and three rabbits the cna1Δ mutant strain transformed with the wild-type CNA1 gene (cna1Δ + CNA1). Rabbits were sedated with ketamine and xylazine on days 2, 5, 7 and 12 after inoculation, and CSF was withdrawn. Quantitative yeast cultures were performed by plating serial dilutions of CSF in PBS on YPD medium, incubating at 24°C for 72 h, and counting colony-forming units.

Acknowledgements
We thank Tamara Breeder and James Joseph for technical assistance, Maria Cardenas and Clara Alarcon for discussions and advice, Chris Mody and Mary Lipscomb for discussions about CsA toxicity and strains, June Kwon-Chung and Tracy Moore for suggestions, and Jeff Edman for strains and plasmid. We thank Robin Wharton, Joe Nevens, Tony Means, John McCusker, Steve Garrett, Dan Lew, Maria Cardenas, Mike Lorenz and Clara Alarcon for comments on the manuscript. Supported in part by Public Health Service Grant AI28388 and Veterans Administration Research Center on AIDS and HIV infection (J.P.). J.H. is an assistant investigator of the Howard Hughes Medical Institute.

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Received on December 5, 1996, revised on January 29, 1997