Calcineurin is required for virulence of *Cryptococcus neoformans*

Audrey Odom^{1,2}, Scott Muir², Eric Lim²,
Dena L. Toffaletti³, John Perfect³ and
Joseph Heitman^{1,2,4,5}

Departments of ¹Genetics, ⁴Pharmacology and ³Medicine and
²Howard Hughes Medical Institute, Duke University Medical Center,
Durham, NC 27710, USA

⁵Corresponding author

A.Odom, S.Muir and E.Lim contributed equally to this work

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 Ca²⁺-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% CO₂ 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 *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 mannitol production), but none has yet led to therapeutic interventions (Kwon-Chung *et al.*, 1982, 1992; Kwon-Chung and Rhodes, 1986; Perfect *et al.*, 1993; Chang and Kwon-Chung, 1994; Chaturvedi *et al.*, 1996).

Cryptococcus neoformans is a heterothallic basidiomycetous 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 *et al.*, 1992, 1993; Toffaletti *et al.*, 1993; Chang and Kwon-Chung, 1994; Lodge *et al.*, 1994). Third, virulence of strains can be readily assessed in animal models (Perfect *et al.*, 1980; Kwon-Chung *et al.*, 1982; Mody *et al.*, 1988). Finally, *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 *C.neoformans* infections in the lung, animals were treated with the immunosuppressant cyclosporin A (CsA) and inoculated with *C.neoformans* (Mody *et al.*, 1988). Contrary to the simple expectation that immunosuppression would exacerbate infection, as it does in human patients, CsA instead protected mice from *C.neoformans* infection. Although some evidence was presented suggesting that CsA inhibits *C.neoformans* growth *in vitro* (Mody *et al.*, 1988), these findings proved difficult to reproduce in another laboratory with the same *C.neoformans* strain (Perfect and Durack, 1985). Thus, whether or not CsA inhibits *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 *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 *Saccharomyces cerevisiae* are essentially identical to the immunosuppressive effects in T-cell proliferation (reviewed in Cardenas *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 *et al.*, 1991), a Ca²⁺/calmodulin-activated serine/threonine-specific phosphatase required for stress responses or viability in yeast (Cyert *et al.*, 1991; Cyert and Thorner, 1992; Nakamura *et al.*, 1993; Breuder *et al.*, 1994; Cunningham and Fink, 1994; Garrett-Engle *et al.*, 1995; Hemenway *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'Keefe *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).

Fig. 1. *Cryptococcus neoformans* is sensitive to CsA and FK506 at elevated temperature. *C. neoformans* wild-type strains JEC20 and JEC21, the isogenic FK506/rapamycin-resistant mutant strains C20F1, C20F2 and C21F3, and the FK506-resistant mutant strain C21F2 were grown at 22, 30 or 37°C on YPD medium, and on YPD medium containing 1 µg/ml rapamycin, 0.1 µg/ml FK506 or 100 µg/ml CsA.

Table I. Drug minimum inhibitory concentrations in *C. neoformans*

Strain	Drug	22°C	30°C	37°C
H99 (MAT α)	CsA	>100 µg/ml	>100 µg/ml	1–5 µg/ml
	FK506	>100 µg/ml	>100 µg/ml	5 ng/ml
	rapa	1 µg/ml	1 µg/ml	1 µg/ml
JEC20 (MAT α)	CsA	>100 µg/ml	0.5 µg/ml	0.1 µg/ml
	FK506	>1 µg/ml	<0.1 µg/ml	<0.1 ng/ml
	rapa	1 µg/ml	1 µg/ml	1 µg/ml
JEC21 (MAT α)	CsA	>100 µg/ml	5 µg/ml	0.5 µg/ml
	FK506	>1 µg/ml	1 µg/ml	<0.1 ng/ml
	rapa	1 µg/ml	1 µg/ml	1 µg/ml

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 MAT α and MAT α *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

Table II. FK506-resistant *C.neoformans* mutants

Strain	CsA	FK506	Rapamycin
JEC20	S	S	S
JEC21	S	S	S
C20F1	S	R	R
C20F2	S	R	R
C21F2	S	R	S
C21F3	S	R	R

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 pCnTell (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 pharmacological 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 *CMP1* 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

-189 gtgatttcactattatcctccatcaaccttctctggatataccacagctctcgtgacgttttctctctctccctccactaattgact
 -100 gggctgcctcttctctcccactctcacctgacagttgactcattttactcctggcctctgcgtcttcgcgcactcctaccaccaaacagtcaattccgctc
 1 ATGGCTTCCCCAGCCACTCAGACCGCTAATGCCATTGCGGCCATCAACACCGCTCGAATCTCGTTATCCCCGAGATCGACTTTACCCAGCACCCAGCTTG
 M A S P A T Q T A N A I A I N N R S N L V I P E I D F T Q H Q L E
 101 AGAACGGTGAAATCGTTAGCACCACTGAGAGGGTCATCAAAGATgtaagttaagtgagacgataaagcgggtgaaggcgggtggcggtgtgcgacccgtggagg
 N G E I V S T T E R V I K D intron 1
 201 ttaatcattcaaaatcggtgggggaatcgctatactgtctgcgaaagcgcgagtgaaaccagctaggcacaagaacagctctatgggtctatataacaact
 301 tcttgaagtttgtgcatggtgacaaacatacttctgtgatcatagCTTCAAGCCCCAGCGATGACGTGCCGACCCAGCAACCAATCTGGTCCAAGGTGGAT
 V Q A P A M Y V P T D D Q F W S K V D
 401 AAAACCAAGCCCGATATTGCATTTTTTAAAGAACCAATTTCTATCGAGAAGGGAGATTGACTGAAGAGCAAGCTTTGTATATCCTTGAAAAgttggctctatg
 K T K P D I A F L K N H F Y R E G R L T E E Q A L Y I L E K
 501 tgcgactttcagccggtcctggcgattggcggtgcgcatgatggggcagaaaactgacatgcgacacagAGGTGGAGAATCTTGAGATCAGAACCAAACT
 intron 2 G G E L L R S E P N L
 601 TGCTAGAAGTTGACGCGCCATTACTGgttaagttacgagtagattcgtgcatatcactgagtggggtatagTGTGCGGTGACATTACCGTCAATATatgt
 L E V D A P I T V intron 3 C G D I H G Q Y
 701 gtttatgcttaatcttttagtgatcaattgctgagtctgggaatagTACGATTGATGAAGCTCTTCGAGGTGCGGTGGGAATCCGGCAGATACTCGTTAT
 Y D L M K L F E V G G N P A D T R Y
 801 CTCTTTTGGGAGACTACGTTGATCGAGGATACCTTTTCTATTGAGgtatgcctatctgaattttattgaaatttaggttaacaagtttttgtagTGTGTG
 L F L G D Y V D R G Y F S I E intron 5 C V
 901 CTTTATTGTGGTCGCTCAAGATGTGGTATCCCGATACTCTTTTCTACTGAGAGGTAATCACGAGTGGCGACACTTGACCGACTATTTACCTTCAAGC
 L Y L W S L K M W Y P D T L F L L R G N H E C R H L T D Y F T F K L
 1001 TTGAGTGCAagctctgaattgctgccttaaacggtcataactaacgatatttttaggcaagCACAAGTACTCAGAGACTGTTTACAATGCCTTCGATGGAAAG
 E C K intron 6 H K Y S E T V Y N A C M E S
 1101 TTTCTGCAACTTGCCGCTGGCGGCGAGTTATGAACAAGCAATTTCTTGTGCATCCATGGTGGCTTGTGCCAGAGCTTCATACCCTCGATGATCTGCGATCT
 F C N L P L A A V M N K Q F L C I H G G L S P E L H T L D D L R S
 1201 gtacgttttccgttttcaatcgaagagtgacggtctcataaaaatgtcacATCAATCGGTTCCGAGAGCCTCCCCTCAAGGTCTCATGTGCGATATCCTTTG
 intron 7 I N R F R E P P T Q G L M C D I L W
 1301 GGCTGATCCGTTTAGAAGACTTTGGCTCAGAAAAAGACAAACGAGAACTTTTTCACAAATCACGTTCCGAGGTTGCAGTTATTCTTTACTTATAATGCTGCA
 A D P L E D F G S E K T N E N F L H N H V R G C S Y F F T Y N A A
 1401 TGTCAGTTCTTGGAGAGGAAACACCTGCTCTATTATTCGAGCTCACGAGGCTCAAGATGCCGGgtgagtgttcaacgtcttgaatgatcggaacat
 C Q F L E R N N L L S I I R A H E A Q D A G intron 8
 1501 gctgagaaatgaacaagttATCGAGATGTATCGAAAACTAAGACTACTGGCTTCCCTCCGTCATGACCATCTTTTCAGACCTTAACCTACCTTGATGTTT
 Y R M Y R K T K T T G F P S V M T I F S A P N Y L D V Y
 1601 ACTCTAACAGGCGCTGTGTTGAAGTACGAGTCAAACGTTATGAagtgagttttattatatacatgaaagaattgtcatttatggagttttagCATCC
 S N K A A V L K Y E S N V M N intron 9 I R
 1701 GACAATTCAATTGTACGCCCCCTACCTTATGGCTACCTAATTTATGGATGTGTTACCTGGAGTTTGCCTTTTCGTTGGCGAGAAGagtgagatattatg
 Q F N C T P H P Y W L P N F M D V F T W S L P F V G E K I
 1801 tgtaggggtgcgactcgaaactgacaatgagtagTCACGGATATGCTTATTGCAATTCTTAACTGTTGCACCAAGGAAGAGCTCGAGGAAGAGGACGAAGA
 intron 10 T D M L I A I L N C C T K E E L E E E D E E
 1901 GTTCCCTCTTAATGCTCCCCGACTACCGGATCCGGAACCTGCGAGCGGAAAGACGACAAATCATCAAGAACAATAATCTGGCGTTGGTCGCGATGCTCGA
 F P L N A P E P T D A E S A A E R R Q I I K N K I L A V G R M S R
 2001 GTATTCTCTCTGTTGCGTGAAGAATCTGAAAGGGTGTCTGAGCTTAAGAGTATTCTTGGCTCAAATGCTTTACCCGCTGGCATGCTTGGAGTGGCGCGG
 V F S L L R E E S E R V S E L K S I S G S N A L P A G M L A S G A E
 2101 AAGGTATTAAAGAAGCGATCCAGGGCTTTGAGGACGCGAGGAAAAGTGATATCGAGAATGAGAGACTTCCCCCTGACATCATCGACatcatgttctgtga
 G I K E A I Q G F E D A R K S D I E N E R L P P D I I D intron 11
 2201 tttttatcattaagcattgctaatgtgtctatagCCGTGATGAGGACAAGCCCGCATCACCTTCTGCCTCTCCCATTATGCCCCCACCCTCGAAGAT
 P D E D K P A S P S A S P I M P A T P E E I
 2301 CCCCAGCGAAATCCCTTACGACTCTCAATCACCGGTACTCTCGAACTCTTATCTCCAGCGCTATTGCTTCCGCGCTCTCCCGCTCTCCTGGCACCCCC
 P S E I P Y D S P I T G T P R T P I S S A I A S G S P G S P G T P
 2401 ACCAGCCCTTCCATTGGAGGTCCGCCACTCACTGCTTGGCAGCCCGGCCACTGGTCGTCGATCATCTTTGGCGACTACCAAGACTAGCCCCAGTACGCGAA
 T S P S I G G P P L T A W R P G H G R R T S L G T T K T S P S T R R
 2501 GGAGGAGTTTGGAGAACACGATGCACTTGATCCGAGATGTGGTGGGCGGTAAAGGATGCCCAGGGTATGGGCAGTTGGAGAGGCTCGCAGAGGTTATTTTC
 R S L E N T M H L I R D V V G G K D A Q G D G Q L E R L A E V I S
 2601 GAGTCCGACGAAGGGCGGTCAAGGCGAGTGAgagtagatgctggtttcgaacgatggaattgtttgaacgactgtttaacgaccctaagcccttctctga
 S P T K G G Q G E
 2701 tttctgaacacacgaacaattgtcaatcaatacgcattataaacacactgtgtagcctcaacaatccttttcttctcggagtttgtctgtgttctatga
 2801 atctatcctctactgtgacccttgagatgaaaagacactagaagaagtctgggaatttgggttctctcactttgtacaatacgcatactgtaattgc
 2901 attttgatatacaaatgtcaagtccttcgaagaaggtcataacgtcacactccatttggtcgtaactccagtcggttttacgacaaacttttttctgtt
 3001 tttcgcagtcactcaagactcatgttttggccaagacgcgggtcttaacctagcaagaatacagcataaacaagcatagcctctcaaatggg

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'-RACE product, as described in Materials and methods. The 5'- and 3'-untranslated regions and intronic sequences are in lower case and introns are numbered in order of occurrence from 5' to 3'. 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' 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.

disrupted by replacing a large fragment of the gene with the *ADE2* selectable marker to result in the calcineurin null mutation *cnal1Δ::ADE2* (Figure 4A). We took two measures to decrease non-homologous recombination.

First, we used the *CNA1* genomic locus from the same strain used for transformation to decrease inhibition of homologous recombination by mismatch repair. Second, we used the *ADE2* gene from a divergent serotype D

C. neo.	MASPATQTANAIAAINNRSNLVIPEIDFTQHQL	33
human	msepik	5
yeast CMP1	mskdlnssrik-ikp-dsyik-drkk-l-kye-	33
C. neo.	ENGEIVSTTERVIKDVQAPAMYVPTDDQFWSKVDKTKPDIAFLKNHFYREGRLTEEQALY	93
A.nidulans	m-d-tq--l--v-e-----lnk-s-----dpeep--nlq--q-----d--w	61
N. crassa	m-d-sq--m--c-----fk-s-e--fedekv-----q--q-----w	61
human	aidpkl--d--v-a-pf-pshrl-akevfdndg-prv--..-a-lmk-----e-sv--r	63
yeast CMP1	--kvi--kd-p-as-p-itgki-s-eevfdsktglpnhs.--re--fh----sk----ik	92
C. neo.	ILEKGGELLRSEPNLLEVDAPITVCGDIHQYYDLMKLFEVGGNPADTRYLFLGDYVDRG	153
A.nidulans	-iga-tqi-k-----m-----v-----d-e-----	121
N. crassa	-ire-tk--a-----m-----v-----d-e-----	121
human	-ite-asi--q-k--di--v-----ff-----s-n-----	123
yeast CMP1	--nmstva-sk----klk---i-----l-----d-eid-----	152
C. neo.	YFSIECVLYLWSLKMWYPDTLFLLRGNHECRHLTDYFTFKLECKHKYSETVYNACMESFC	213
A.nidulans	-----a-i--n--w-----ri-e-i---	181
N. crassa	-----a-ih--k--w-----ai-e---	181
human	-----a-il--k-----e--q--i--r--d--da-d	183
yeast CMP1	a--f--li--y--lnnlgrfwm-----k--s--n--ml--dmev-d--cr--n	212
C. neo.	NLPLAAVMNKQFLCIHGGLSPELHTLDDLRSINRFREPPTQGLMCDILWADP.....L	266
A.nidulans	a-----e-ik--d-----h-----	234
N. crassa	c-----i-n-d-----	234
human	c----l-q--v-----in--i-kld--k--ay-p-----s-----	236
yeast CMP1	v----l-g-yf-v--i--ksve-vnk-----i-sr----l----venyddar	272
C. neo.	EDFGSEKTNENFLHNHVRGCSYFFTYNAACQFLERNLLSIIRAHEAQDAGYRMYRKTKT	326
A.nidulans	---q---gdy-i--s-----s-p--a--k--v-----r-	294
N. crassa	---q---tdf-v-----s-s--h--k-----r-	294
human	---n---q-h-t--t-----ys-p-v-e--qh-----l-----sq-	296
yeast CMP1	dgsefdqsede-vpnsl---fa--fk-s-k--ka-g-----kyn-v	332
C. neo.	TGFPSVMTIFSAPNYLDVYSNKA AVLKYESNMVNIROFNCTPHPYWLPNFMVFTWSLPE	386
A.nidulans	-----n-----n-----	354
N. crassa	-----n-----n-----	354
human	----li-----n-----n-----s-----	356
yeast CMP1	----li-m-----t-h-----e-----hms-----d-----	392
C. neo.	<u>VGEKITDMLIAILNCCTKEELEEEDEEFPLNAPEPT.....DAESAA</u>	428
A.nidulans	-----iv-----t-s-----d-tpstispa.--sppmpmdtv.....t--te	403
N. crassa	-----st-se--r-dsattspsgsas-alpsaanq.....pd-ie	403
human	----v-e--vny--i-sdd--gs-edg-dgataa.....	390
yeast CMP1	----v-s--vs--i-seq--dp-s-pkaaeetvkaranatketgtpsdekassailede	452
C. neo.	<u>ERROIKNKILAVGRMSRVFSLFREESERVSELKSISGSNALPAGMLASGAEGIKEAIOG</u>	488
A.nidulans	fk-ra-----i--l---qv-----t---taa-gr.---t-ml-----q--tn	462
N. crassa	fk-ra-----i--l---qv-----t---tv-gr.---t-ml-----q--ss	462
human	a-kev-r--r-i-k-a---v-----s-lt--gltpgtm--s-v-sg-kqtlqsX-ts	469
yeast CMP1	t--kalr-----iakv--m-sv-----k-ey--tmnagv.--r-a--r-t--lnetlst	511
C. neo.	<u>FEDARKSDIENERLPPDIIDPDEDKPASPSASPIMPATPEEIPSEIPYDSPITGTPTPTPI</u>	548
A.nidulans	-----v-lq-----shdevvrseeerrialdragheadndtglatvarrismvrr-	522
N. crassa	-----v-lq-----shdevvknqdeeraqaleratreadndkklqtl-rrls-s	518
human	--e-kg1-ri--m--rrdamps-anlnsinkaltse-ngtdsngsnssniq	521
yeast CMP1	--k--e-li--k--slseveqe-ikyeykilkaekkpql	553
C. neo.	SSAIASGSPGSPGTPTSPSIGGPPLTAWRPGHGRRTSLGTTKTSPTSTRRSLENTMHLIR	608
A.nidulans	rkipsttrr	531
C. neo.	DVVGKDAQGDGQLERLAEVISSPTKGGQGE	639
X=atveaieadeaikgfsqghk		

Fig. 3. *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 transformants were first selected at 24°C and then screened for the phenotype we predicted would result from calcineurin

Fig. 4. Disruption of the *C. neoformans* calcineurin A gene. (A) Diagram of calcineurin A gene disruptions. The restriction map of the genomic *CNA1* calcineurin A gene is depicted and the extent and 5'–3' orientation of the ORF, promoter and 3' region are indicated by the open arrow. The *cnal* Δ gene disruption is depicted in which an *Eco*RV fragment is deleted and replaced with the *ADE2* gene. The site at which the original *CNA1* clone was chimeric is indicated by two wavy lines. Restriction sites for *Eco*RI (RI), *Hind*III (HIII), *Bam*HI (B), *Xho*I (X) and *Eco*RV (RV) are indicated, and approximate sizes of restriction fragments in bp are given. (B) Confirmation of calcineurin *CNA1* disruption by PCR analyses. Genomic DNA from *CNA1* wild-type strain M049 (H99 $\Delta ade2$) (lane 2) and of four temperature-sensitive *ADE2*⁺ transformants with the *cnal* Δ ::*ADE2* construct (lanes 3–6) was prepared and PCR amplified with primers flanking the site of *ADE2* insertion in the *CNA1* gene. PCR products were analyzed on a 1% agarose gel and stained with ethidium bromide. The products from the wild-type *CNA1* gene and from the *cnal* Δ ::*ADE2* disruption allele are indicated by the arrowhead and arrow, respectively, to the right. Transformants in lanes 3, 4 and 5 result from double cross-overs (Δ allele only), and the transformant in lane 6 from a single cross-over event (resulting in the Δ allele plus a truncated inactive wild-type allele). The sizes of molecular weight standards (lane 1) are indicated in kb. (C) [¹²⁵I]Calmodulin binding to proteins from wild-type and calcineurin mutant *S. cerevisiae* and *C. neoformans*. Total proteins extracted from wild-type (CMP1, CMP2, lane 1), *cmp1* (Δ , CMP2, lane 2), *cmp2* (CMP1, Δ , lane 3), and *cmp1 cmp2* (Δ , Δ , lane 4) calcineurin A mutant strains of *S. cerevisiae* grown at 30°C, and from calcineurin A wild-type (*CNA1*, lanes 5 and 6) and calcineurin A mutant (*cnal* Δ , lanes 7 and 8) *C. neoformans* strains grown at 24°C (24°) or grown at 24°C and shifted to 37°C for 5 h (37°) were separated by 12.5% SDS–PAGE. A sample of purified bovine calcineurin A and B proteins (Bovine CN, Sigma) was analyzed in lane 9 as a control. Proteins were transferred to nitrocellulose and calmodulin-binding proteins identified by [¹²⁵I]calmodulin overlay and autoradiography. The positions of migration of the yeast CMP1 (solid arrowhead), yeast CMP2 (open arrowhead) and *C. neoformans* *CNA1* proteins (solid arrow) are indicated on the left. The ~50 kDa protein in lanes 1–4 and the ~40 kDa protein in lanes 5–8 are other calmodulin-binding proteins. Migration positions of pre-stained molecular weight markers are indicated to the right by arrows labeled with mass in kDa.

mutation, namely temperature-sensitive growth. Remarkably, 16 of 200 *ADE2*⁺ transformants with *cnal* Δ ::*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 *cnal* Δ ::*ADE2* deletion mutation in temperature-sensitive transformants but not in temperature-resistant transformants, and that all transformants with the *cnal* Δ allele exhibited a temperature-sensitive phenotype (Figure 4B and data not shown). Similar findings were obtained with a second *cnal*::*ADE2* disruption allele in which the *ADE2* gene was inserted between two *Xho*I sites in the *CNA1* gene (data not shown). These findings support the conclusion that the *cnal* 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 *cnal* Δ 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 two yeast calcineurin A catalytic subunits were analyzed in parallel, revealing a doublet of 64 and 66 kDa calmodulin-binding proteins corresponding to the known 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 *cnal* Δ 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

Fig. 5. Disruption of the *C. neoformans* calcineurin A gene confers temperature-sensitive growth. (A) The wild-type *C. neoformans* strain H99 (WT) and the isogenic calcineurin A deletion mutant strain *cna1Δ::ADE2* (Δ) were grown on YPD medium containing no drug (-), 1 μ g/ml FK506 (FK506) or 100 μ g/ml CsA (CsA) for 96 h at 24, 37 and 39°C. (B) Confirmation of calcineurin *CNA1* re-introduction by PCR. Genomic DNA was prepared from *CNA1* wild-type strain H99 (lane 1), and from the *cna1Δ* strain alone (lane 2) or containing suppressor mutations (Sup8, Sup24) (lanes 3 and 4) or the transformed wild-type *CNA1* gene (lane 5), amplified by PCR with flanking primers and analyzed on a 1% agarose gel. The products from wild-type *CNA1* gene and from the *cna1Δ::ADE2* disruption are indicated by the arrowhead and arrow, respectively, to the left. Molecular weight size standards are indicated in kb (lane 6). (C) Isogenic calcineurin A wild-type (*CNA1*), calcineurin A mutant (*cna1Δ*) and the calcineurin A mutant strains containing the *sup8* suppressor mutation (*cna1Δ* + SUP8) or the wild-type calcineurin A gene (*cna1Δ* + *CNA1*) were grown for 4 days on YPD medium at 30, 37 and 39°C without or with 1 μ g/ml FK506.

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

Fig. 6. Disruption of the *C. neoformans* calcineurin A gene confers CO₂- and pH-sensitive growth. The wild-type *C. neoformans* 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.

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 *cna1* 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 *cna1Δ* 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 *cna1Δ* 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 wild-type or mutant *C. neoformans* strains. Survival of *C. neoformans* in the CNS was determined by removal of cerebral spinal fluid (CSF) and quantification of colony-forming units by serial dilution and plating. Previous studies have established the value of this model system in studies of *C. neoformans* virulence and the action of antifungal agents (Perfect *et al.*, 1980, 1983; Perfect and Durack, 1985; Lodge *et al.*, 1994).

As shown in Figure 7, whereas wild-type *C. neoformans* strain H99 persists for up to 12 days in the CSF of immunosuppressed animals, the isogenic *cna1Δ* calcineurin mutant strain was cleared rapidly from the immunosuppressed hosts. By days 2 and 5 following inoculation, survival of the calcineurin mutant strain was reduced ~400- and ~10 000-fold respectively compared with the isogenic wild-type strain, as measured by quantitative colony counts (Figure 7). In comparison, virulence was largely restored in the *cna1Δ* strain by re-introduction of the wild-type calcineurin A gene. Virulence was not restored to the wild-type level at the latest time point, which we attribute to the fact that while the introduced ectopic calcineurin A wild-type gene fully restored growth to the wild-type level on solid YPD medium at 39 and at 37°C in liquid medium, it did not fully restore growth at 39°C in liquid RPMI medium. This temperature is similar

Fig. 7. Calcineurin is required for virulence of *C. neoformans*. Rabbits immunosuppressed with steroids were inoculated intrathecally with the wild-type calcineurin A *CNA1* *C. neoformans* serotype A strain H99 (●, wild-type H99), the isogenic calcineurin A *cna1Δ* mutant strain (■, *cna1Δ*) or the isogenic *cna1Δ* mutant strain into which the wild-type *CNA1* gene had been re-introduced (▲, *cna1Δ* + 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 *cna1Δ* strain compared with wild-type H99 at all time points ($P < 0.01$ by T-test for unpaired means) and the *cna1Δ* + CNA1 counts were only lower than wild-type on day 12 ($P < 0.03$).

to normal rabbit body temperature and emphasizes the importance of yeast growth rate for virulence. In summary, our findings reveal that calcineurin is required for growth *in vivo* in a mammalian host at a clinically relevant site of infection.

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 in other organisms, the Ca²⁺/calmodulin-dependent protein phosphatase calcineurin, might be required for growth of *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

Fig. 8. Proposed signal transduction pathways regulating *C. neoformans* virulence. This figure outlines our hypotheses concerning the mechanisms of action of CsA, FK506 and rapamycin (Rapa) and the roles of the target proteins, cyclophilin A, calcineurin A and B, FKBP12 and TOR in immunosuppressive drug action, virulence and growth. CAM and B indicate calmodulin and the calcineurin B regulatory subunit, respectively. We propose that cyclophilin A and FKBP12 homologs mediate the inhibitory actions of CsA and FK506/rapamycin on calcineurin and TOR, respectively. In the model, unknown targets required for virulence are dephosphorylated by calcineurin in response to $\text{Ca}^{2+}/\text{CAM}$ activation stimulated by an unknown extracellular signal. By analogy with yeast and T cells, we propose the FKBP12–rapamycin target is a TOR homolog required for growth in response to an extracellular cue, such as nutrients.

grow at elevated temperature, in the presence of CO_2 and at physiological pH *in vitro* and for survival and virulence in an infected mammalian host.

We summarize our findings in the model in 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 CO_2 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 CO_2 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 CO_2 - and pH-resistant growth is intriguing given the known role of calcineurin in ion, pH and Ca^{2+} 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 CO_2 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 carbonic anhydrase homolog or, following spontaneous CO_2 hydration, be required to regulate pumps that extrude the resulting H^+ and HCO_3^- ions from the cytoplasm to allow continued growth in the presence of CO_2 . 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-Engle *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 Mn^{2+} sensitivity in *S. cerevisiae* but Mn^{2+} tolerance in *C. neoformans*, suggesting that calcineurin may differentially regulate homologs of the yeast Mn^{2+} and Ca^{2+} 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 the 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 FKBP12 (Rotonda *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

inhibits fungal growth. Further studies of this and other analogs are warranted.

Our studies are the first to implicate calcineurin in the virulence of a human pathogen. Calcineurin previously has been identified in a number of other microorganisms. For example, in *S.cerevisiae* calcineurin is required for survival during cation stress (Nakamura *et al.*, 1993; Breuder *et al.*, 1994), recovery from pheromone arrest (Cyert *et al.*, 1991; Cyert and Thorner, 1992) and for viability of some mutant strains (Parent *et al.*, 1993; Breuder *et al.*, 1994; Garrett-Engele *et al.*, 1995; Hemenway *et al.*, 1995; Tanida *et al.*, 1995). Calcineurin is also inappropriately activated and toxic in mutant yeast strains with altered Ca^{2+} homeostasis (Cunningham and Fink, 1994, 1996). In *S.pombe*, calcineurin is induced by nitrogen starvation and is required for growth at low temperature, mating and proper cytokinesis (Yoshida *et al.*, 1994; Plochocka-Zulinska *et al.*, 1995). In *A.nidulans*, calcineurin is essential and regulates cell cycle progression (Rasmussen *et al.*, 1994). Thus, calcineurin plays diverse roles in different microorganisms. Our studies reveal that the mechanisms of action of CsA and FK506 are highly conserved from yeast to pathogenic fungi to man. Other yeasts and pathogenic fungi whose growth is known to be inhibited by CsA or FK506, including *Coccidioides immitis* (Kirkland and Fierer, 1983), *Aspergillus fumigatus* (Hatanaka *et al.*, 1988), *N.crassa* (Tropschug *et al.*, 1989) and *Aspergillus niger* (Kirkland and Fierer, 1983), are likely to be sensitive to both drugs via inhibition of calcineurin. If so, the spectrum of calcineurin inhibitors might include many pathogenic yeast and fungi.

An important technical advance of our studies was the high rate of homologous recombination. In previous studies, the *CAP59* gene was disrupted at a frequency of 1 in 10 000 (Chang and Kwon-Chung, 1994). By comparison, the *N-myristoyl transferase* (NMT) gene was disrupted at a frequency of ~3% (Lodge *et al.*, 1994). As in that study, we employed a genomic clone isolated from the strain used for transformation to avoid mismatches during homologous recombination. To limit gene conversion at *ade2*, we used an *ADE2* gene from a serotype D strain that is polymorphic with the recipient serotype A *ade2* gene. Our findings indicate that it is now feasible to disrupt any non-essential gene in this pathogenic organism.

Previous observations have linked fungal pathogenicity to CO_2 - and temperature-regulated physiology. In *C.neoformans*, polysaccharide capsule is induced by CO_2 , and mutants that lack a capsule or do not induce capsule in response to CO_2 are avirulent (Granger *et al.*, 1985; Kwon-Chung and Rhodes, 1986). A *C.neoformans* strain with a temperature-sensitive mutation in an unidentified gene was avirulent in mice (Kwon-Chung *et al.*, 1982). In the pathogenic yeast *Candida*, virulent *C.albicans* strains grow at 42°C whereas closely related but avirulent *C.stellatoidea* strains do not (Kamiyama *et al.*, 1989). Similarly, some *S.cerevisiae* strains isolated from immunocompromised human patients can grow at 42°C *in vitro* whereas many laboratory strains do not (McCusker *et al.*, 1994a). The ability of *S.cerevisiae* to grow at elevated temperature has also been correlated with enhanced survival following intravenous inoculation in mice (McCusker *et al.*, 1994b). Finally, in the fungal pathogen *Histoplasma capsulatum*, a morphological transition from the avirulent mycelial to

the virulent yeast is induced by shift from 25 to 37°C and is associated with a heat shock response (Lambowitz *et al.*, 1983; Maresca *et al.*, 1994). Moreover, the relative pathogenicity of *H.capsulatum* isolates is correlated with ability to grow at elevated temperatures (Medoff *et al.*, 1986). Thus, temperature-resistant growth is linked to pathogenesis.

The association of temperature-resistant growth and virulence is not limited to pathogenic fungi and yeasts. The heat shock response is thought to be involved in pathogenesis and differentiation of parasites, such as *Trypanosoma brucei* and *Leishmania major*, the causative agents of African sleeping sickness and zoonotic cutaneous leishmaniasis, respectively (Van Der Ploeg *et al.*, 1985). Following transmission from the insect vectors at 25°C, these parasites adapt to and survive in mammalian hosts at 37°C or higher. Similarly, virulence of pathogenic bacteria has been associated with temperature (reviewed in Maurelli, 1989). Invasion of mammalian cells by the Gram-negative bacterium *Shigella* is blocked by growth at 30°C and is restored by growth at 37°C (Maurelli *et al.*, 1984). A number of *Shigella* virulence genes have been identified that are transcriptionally induced at 37°C compared with 30°C. These genes are controlled by the *virR* repressor which, when mutated, results in loss of invasion (Maurelli and Sansonetti, 1988). Virulence of enteroinvasive *Escherichia coli*, which causes dysentery, and *Yersinia* species are similarly temperature regulated (Bolin and Wolf-Watz, 1988; Isberg *et al.*, 1988; Small and Falkow, 1988).

Our studies define a novel function for calcineurin in pathogenesis of the opportunistic fungal pathogen *C.neoformans*. Genetic, biochemical and structural information available for calcineurin, and clinical experience in the use of calcineurin inhibitors and analogs can now be brought to bear on signal transduction cascades regulating fungal pathogenesis as targets for novel antifungal agents.

Materials and methods

Strains, media and compounds

The *S.cerevisiae* strains employed here were as described (Cardenas *et al.*, 1994b). Most *C.neoformans* strains were isogenic derivatives of the serotype A strain H99 or of the isogenic pair of MATa and MAT α serotype D strains JEC20 and JEC21 (Moore and Edman, 1993). In addition, drug sensitivity was assessed for the serotype A strain 145A (Mody *et al.*, 1988). The *C.neoformans* strains were routinely grown using rich (YPD) and synthetic medium as for *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/l KH_2PO_4 , and 4% agar and was adjusted to pH 7.2 with KOH prior to autoclaving. Regeneration medium for biolistic transformations was as described (Toffaletti *et al.*, 1993). 5-FOA medium to select *ura5* auxotrophic *C.neoformans* mutants and plasmid loss experiments was as described (Kwon-Chung *et al.*, 1992). Where indicated, 10 $\mu\text{g}/\text{ml}$ cycloheximide was added for 5-FOA plus cycloheximide media. Canavanine- and cycloheximide-resistant *C.neoformans* mutants were selected as described for *S.cerevisiae* (Sikorski and Hieter, 1989). FK506 was from Fujisawa, L-685,818 from Merck and rapamycin from the NCI.

Isolation and genetic and biochemical analyses of drug-resistant mutants

Spontaneous independent mutants resistant to 100 $\mu\text{g}/\text{ml}$ CsA or 1 $\mu\text{g}/\text{ml}$ FK506 in YPD at 37°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 $\mu\text{g}/\text{ml}$).

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 canavanine. In other organisms, recessive mutations confer resistance to these agents. *ura5* mutations were 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 canavanine or canavanine 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 *URA*⁺ *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. *ura5* derivatives of FK506-resistant mutant strains were crossed to the isogenic *ade2* *lys2* strains JEC170 (MAT α) or JEC171 (MAT α) 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, a second independent feature of MAT α /MAT α 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 (Heitman 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'-ACTCGCCCA-ACATGTCTATGTCTGAAGTAATTGAAGGTAA) and 48 (5'-CGCG-GGATCCGAATTCAAAATAATCACTGCTCATATATAG) to provide 5' overlap with the actin promoter sequence, and the actin promoter gene sequence was amplified with primers 387 (5'-GAATTCCTGGAAGCG-AGTTG) and 418 (5'-GTTACCTTGAATTACTTCAGACATAGACAT-GTTGGGCGAGTT). The ~600 bp and ~900 bp first round PCR products were gel purified, combined and PCR amplified with the flanking primers 387 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 pCnTel1 (Edman, 1992), to yield plasmid pAOT1 with the yeast FKBP12 gene expressed by the *C.neoformans* actin promoter.

The control plasmid pCnTel1 and the yFKBP12 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 not sufficient to inhibit all available calcineurin.

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. Forward primers were 292 (HEAQDAGYRMY peptide; 5'-CAYGARGCICARGAYGCGIGITAYA/CGIATGTA) and 293 (NVMNIRQF peptide; 5'-AAYGTIATGAAYATIC/AGICARTT) and reverse primers were 294 (NVMNIRQF peptide; 5'-AAYTGICG/TIATRTTCATACRTT) and 295 (FMDVFTW peptide; 5'-CCAIG-

TRAAIACRTCCATRAA). 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 JK9-3da. PCR reaction conditions were 5 min at 94°C, 70 cycles of 30 s at 94°C, 30 s at 30°C and 90 s at 72°C, and a final 5 min 72°C step. 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'-CCATCGATAGATCTCGGAAAACCTAAGACCACCT-GGC) and 330 (5'-CCGGGTACCTCGAGGTTAGGCAGCCAATAA-GGATGGGG), 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 *Bam*HI-*Sau*3A 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 *Bam*HI fragment that bore the desired target sequence (based on PCR amplification with primers 329–330) was subcloned into the *Bam*HI site in pUC18. The sequence of the insert was determined using appropriate subcloned fragments and synthetic primers. The original clone was incomplete and chimeric at a *Sau*3A site, and the intact gene was obtained by colony hybridization from a size-selected *Eco*RI 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'-AAGATGGTCATGACGGAGGGG). Following second strand synthesis and ligation to the Marathon cDNA adapter, a nested PCR was performed using an internal *CNA1*-specific primer (392: 5'-GTATCTGCCGGATTCCACCG) 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 inserting a 2400 bp *Xho*I *ADE2* fragment (blunted with Klenow and dNTPs) between two *Eco*RV sites in the *CNA1* gene in the 3.5 kb *Bam*HI genomic fragment cloned in pUC18, yielding plasmid pcna1 Δ ::*ADE2*. Approximately 5 µg of supercoiled disruption plasmid DNA was purified on a Qiagen column, absorbed onto the surface of gold microprojectiles and introduced by biolistic transformation into strain M049, an *Ade2* 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 a 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 once with chloroform. DNA was ethanol precipitated, dried and resuspended in 50 µl of TE.

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

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 *ADE2* insertion served as primers, including 377 (5'-AAGCTTATG-ATGTTTGATCCG) and 566 (5'-CGAAGAGTGCACGGCTCATA), 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 result 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 ~100 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 pUC18 was introduced into the *cna1Δ::ADE2* mutant strain by biolistic 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 10⁴/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 time points (0, 6, 12, 24, 48 and 72 h), samples were taken from the culture and diluted onto YEPD agar 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 *CNA1* wild-type (H99) and *cna1Δ* mutant *C. neoformans* serotype A strains were prepared by growth for 96 h at 24°C on Sabouraud'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 intracranially 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 Breuder 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 plasmids. We thank Robin Wharton, Joe Nevins, 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.

References

- Alarcon, C.M., Cardenas, M.E. and Heitman, J. (1996) Mammalian RAFT1 kinase domain provides rapamycin-sensitive TOR function in yeast. *Genes Dev.*, **10**, 279–288.
- Bolin, I. and Wolf-Watz, H. (1988) The virulence plasmid-encoded Yop2b protein of *Yersinia pseudotuberculosis* is a virulence determinant regulated by calcium and temperature at transcriptional level. *Mol. Microbiol.*, **2**, 237–245.
- Breuder, T., Hemenway, C.S., Movva, N.R., Cardenas, M.E. and Heitman, J. (1994) Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. *Proc. Natl Acad. Sci. USA*, **91**, 5372–5376.
- Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S. and Schreiber, S.L. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*, **369**, 756–759.
- Cardenas, M.E., Lorenz, M., Hemenway, C. and Heitman, J. (1994a) Yeast as model-T cells. *Perspect. Drug Discovery Design*, **2**, 103–126.
- Cardenas, M., Hemenway, C., Muir, R.S., Ye, R., Fiorentino, D. and Heitman, J. (1994b) Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. *EMBO J.*, **13**, 5944–5957.
- Cardenas, M., Muir, R.S., Breuder, T. and Heitman, J. (1995) Targets of immunophilin-immunosuppressant complexes are distinct highly conserved regions of calcineurin A. *EMBO J.*, **14**, 2772–2783.
- Chang, Y.C. and Kwon-Chung, K.J. (1994) Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol. Cell. Biol.*, **14**, 4912–4919.
- Chaturvedi, V.P., Lynn, T., Niehaus, N.G. and Wong, B. (1996) Stress tolerance and pathogenic potential of a mannitol-mutant of *Cryptococcus neoformans*. *Microbiology*, **142**, 937–947.
- Clipstone, N.A. and Crabtree, G.R. (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature*, **357**, 695–697.
- Cox, G.M. and Perfect, J.R. (1993) Fungal infections. *Curr. Opin. Infect. Dis.*, **6**, 422–426.
- Cox, G.M., Rude, T.H., Dykstra, C.C. and Perfect, J.R. (1995) Actin gene from *Cryptococcus neoformans*: structure and phylogenetic analysis. *J. Med. Vet. Mycol.*, **33**, 261–266.
- Cunningham, K.W. and Fink, G.R. (1994) Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca²⁺ ATPases. *J. Cell Biol.*, **124**, 351–363.
- Cunningham, K.W. and Fink, G.R. (1996) Calcineurin inhibits VCX1-dependent H⁺/Ca²⁺ exchange and induces Ca²⁺ ATPases in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **16**, 2226–2237.
- Cyert, M.S. and Thorner, J. (1992) Regulatory subunit (*CNB1* gene product) of yeast Ca²⁺/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. *Mol. Cell. Biol.*, **12**, 3460–3469.

- Cyert, M.S., Kunisawa, R., Kaim, D. and Thorner, J. (1991) Yeast has homologs (*CNA1* and *CNA2* gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. *Proc. Natl Acad. Sci. USA*, **88**, 7376–7380.
- Dumont, F.J. et al. (1992) The immunosuppressive and toxic effects of FK-506 are mechanistically related: pharmacology of a novel antagonist of FK-506 and rapamycin. *J. Exp. Med.*, **176**, 751–760.
- Edman, J.C. (1992) Isolation of telomere-like sequences from *Cryptococcus neoformans* and their use in high-efficiency transformation. *Mol. Cell. Biol.*, **12**, 2777–2783.
- Garrett-Engle, P., Moilanen, B. and Cyert, M.S. (1995) Calcineurin, the Ca^{2+} /calmodulin dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H^{+} -ATPase. *Mol. Cell. Biol.*, **15**, 4103–4114.
- Granger, D.L., Perfect, J.R. and Durack, D.T. (1985) Virulence of *Cryptococcus neoformans*, regulation of capsule synthesis by carbon dioxide. *J. Clin. Invest.*, **76**, 508–516.
- Hatanaka, H., Kino, T., Miyata, S., Inamura, N., Kuroda, A., Goto, T., Tanaka, H. and Okuhara, M. (1988) FR-900520 and FR-900523, novel immunosuppressants isolated from a *Streptomyces* II. Fermentation, isolation and physico-chemical and biological characteristics. *J. Antibiot.*, **41**, 1592–1601.
- Heitman, J., Movva, N.R. and Hall, M.N. (1991a) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*, **253**, 905–909.
- Heitman, J., Movva, N.R., Hiestand, P.C. and Hall, M.N. (1991b) FK506-binding protein proline rotamase is a target for the immunosuppressive agent FK506 in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **88**, 1948–1952.
- Heitman, J., Movva, N.R. and Hall, M.N. (1992) Proline isomerases at the crossroads of protein folding, signal transduction and immunosuppression. *The New Biologist*, **4**, 448–460.
- Heitman, J., Koller, A., Cardenas, M.E. and Hall, M.N. (1993) Identification of immunosuppressive drug targets in yeast. *Methods*, **5**, 176–187.
- Helliwell, S.B., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R. and Hall, M.N. (1994) TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol. Biol. Cell*, **5**, 105–118.
- Hemenway, C.S., Dolinski, K., Cardenas, M.E., Hiller, M.A., Jones, E.W. and Heitman, J. (1995) *vph6* mutants of *Saccharomyces cerevisiae* require calcineurin for growth and are defective in vacuolar H^{+} -ATPase assembly. *Genetics*, **141**, 833–844.
- Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*, **77**, 61–68.
- Isberg, R.R., Swain, A. and Falkow, S. (1988) Analysis of expression and thermoregulation of the *Yersinia pseudotuberculosis* *inv* gene with hybrid proteins. *Infect. Immun.*, **56**, 2133–2138.
- Jain, J., Miner, Z. and Rao, A. (1993) Analysis of the preexisting and nuclear forms of nuclear factor of activated T cells. *J. Immunol.*, **151**, 837–848.
- Kamiyama, A., Niimi, M., Tokunaga, M. and Nakayama, H. (1989) Adansonian study of *Candida albicans*: intraspecific homogeneity excepting *C. stellatoidea* strains. *J. Med. Vet. Mycol.*, **27**, 229–241.
- Kirkland, T.N. and Fierer, J. (1983) Cyclosporin A inhibits *Coccidioides immitis* *in vitro* and *in vivo*. *Antimicrob. Agents Chemother.*, **24**, 921–924.
- Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N.R. and Hall, M.N. (1993) Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G_1 progression. *Cell*, **73**, 585–596.
- Kwon-Chung, K.J. (1976) Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. *Mycologia*, **68**, 942–946.
- Kwon-Chung, K.J. and Rhodes, J.C. (1986) Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. *Infect. Immun.*, **51**, 218–223.
- Kwon-Chung, K.J., Polachek, I. and Popkin, T.J. (1982) Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *J. Bacteriol.*, **150**, 1414–1421.
- Kwon-Chung, K.J., Varma, A., Edman, J.C. and Bennett, J.E. (1992) Selection of *ura5* and *ura3* mutants from the two varieties of *Cryptococcus neoformans* on 5-fluoroorotic acid medium. *J. Med. Vet. Mycol.*, **30**, 61–69.
- Lambowitz, A.M., Kobayashi, G.S., Painter, A. and Medoff, G. (1983) Possible relationship of morphogenesis in pathogenic fungus, *Histoplasma capsulatum*, to heat shock response. *Nature*, **303**, 806–808.
- Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*, **66**, 807–815.
- Lodge, J.K., Jackson-Machelski, E., Toffaletti, D.L., Perfect, J.R. and Gordon, J.I. (1994) Targeted gene replacement demonstrates that myristoyl-CoA:protein *N*-myristoyltransferase is essential for viability of *Cryptococcus neoformans*. *Proc. Natl Acad. Sci. USA*, **91**, 12008–12012.
- Maresca, B., Carratu, L. and Kobayashi, G.S. (1994) Morphological transition in the human fungal pathogen *Histoplasma capsulatum*. *Trends Microbiol.*, **2**, 110–114.
- Maurelli, A.T. (1989) Temperature regulation of virulence genes in pathogenic bacteria: a general strategy for human pathogens? *Microb. Pathogen.*, **7**, 1–10.
- Maurelli, A.T. and Sansonetti, P.J. (1988) Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. *Proc. Natl Acad. Sci. USA*, **85**, 2820–2824.
- Maurelli, A.T., Blackmon, B. and Curtiss, R.I. (1984) Temperature-dependent expression of virulence genes in *Shigella* species. *Infect. Immun.*, **43**, 195–201.
- McCusker, J.H., Clemons, K.V., Stevens, D.A. and Davis, R.W. (1994a) Genetic characterization of pathogenic *Saccharomyces cerevisiae* isolates. *Genetics*, **136**, 1261–1269.
- McCusker, J.H., Clemons, K.V., Stevens, D.A. and Davis, R.W. (1994b) *Saccharomyces cerevisiae* virulence phenotype as determined with CD-1 mice is associated with the ability to grow at 42°C and form pseudohyphae. *Infect. Immun.*, **62**, 5447–5455.
- Medoff, G., Maresca, B., Lambowitz, A.M., Kobayashi, G., Painter, A., Sacco, M. and Carratu, L. (1986) Correlation between pathogenicity and temperature sensitivity in different strains of *Histoplasma capsulatum*. *J. Clin. Invest.*, **78**, 1638–1647.
- Mendoza, I., Rubio, F., Rodriguez-Navarro, A. and Pardo, J.M. (1994) The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **269**, 8792–8796.
- Mitchell, T.G. and Perfect, J.R. (1995) Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. *Clin. Microbiol. Rev.*, **8**, 515–548.
- Mody, C.H., Toews, G.B. and Lipscomb, M.F. (1988) Cyclosporin A inhibits the growth of *Cryptococcus neoformans* in a murine model. *Infect. Immun.*, **56**, 7–12.
- Moore, T.D.E. and Edman, J.C. (1993) The α -mating type locus of *Cryptococcus neoformans* contains a peptide pheromone gene. *Mol. Cell. Biol.*, **13**, 1962–1970.
- Nakamura, T., Liu, Y., Hirata, D., Namba, H., Harada, S.-i., Hirokawa, T. and Miyakawa, T. (1993) Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. *EMBO J.*, **12**, 4063–4071.
- Northrop, J.P., Ho, S.N., Chen, L., Thomas, D.J., Timmerman, L.A., Nolan, G.P., Admon, A. and Crabtree, G.R. (1994) NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature*, **369**, 497–502.
- Odom, A., Del Poeta, M., Perfect, J. and Heitman, J. (1997) The immunosuppressant FK506 and its nonimmunosuppressive analog L-685,818 are toxic to *Cryptococcus neoformans* by inhibition of a common target protein. *Antimicrob. Agents Chemother.*, **41**, 156–161.
- O'Keefe, S.J., Tamura, J.-i., Kincaid, R.L., Tocci, M.J. and O'Neill, A.E. (1992) FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature*, **357**, 692–694.
- Parent, S.A., Nielsen, J.B., Morin, N., Chretien, G., Ramadan, N., Dahl, A.M., Hsu, M.-J., Bostian, K.A. and Foor, F. (1993) Calcineurin-dependent growth of an FK506- and CsA-hypersensitive mutant of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.*, **139**, 2973–2984.
- Perfect, J.R. and Durack, D.T. (1985) Effects of cyclosporin in experimental cryptococcal meningitis. *Infect. Immun.*, **50**, 22–26.
- Perfect, J.R., Lang, S.D.R. and Durack, D.T. (1980) Chronic cryptococcal meningitis: a new experimental model in rabbits. *Am. J. Pathol.*, **101**, 177–194.
- Perfect, J.R., Rude, T.H., Penning, L.M. and Johnson, S.A. (1992) Cloning the *Cryptococcus neoformans* *TRP1* gene by complementation in *Saccharomyces cerevisiae*. *Gene*, **122**, 213–217.
- Perfect, J.R., Toffaletti, D.L. and Rude, T.H. (1993) The gene encoding phosphoribosylaminoimidazole carboxylase (*ADE2*) is essential for growth of *Cryptococcus neoformans* in cerebrospinal fluid. *Infect. Immun.*, **61**, 4446–4451.

- Plochocka-Zulinska,D., Rasmussen,G. and Rasmussen,C. (1995) Regulation of calcineurin gene expression in *Schizosaccharomyces pombe*. *J. Biol. Chem.*, **270**, 24794–24799.
- Pozos,T.C., Sekler,I. and Cyert,M.S. (1996) The product of *HUM1*, a novel yeast gene, is required for vacuolar $\text{Ca}^{2+}/\text{H}^{+}$ exchange and is related to mammalian $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers. *Mol. Cell. Biol.*, **16**, 3730–3741.
- Rasmussen,C., Garen,C., Brining,S., Kincaid,R.L., Means,R.L. and Means,A.R. (1994) The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*. *EMBO J.*, **13**, 2545–2552.
- Rotonda,J., Burbaum,J.J., Chan,H.K., Marcy,A.I. and Becker,J.W. (1993) Improved calcineurin inhibition by yeast FKBP12–drug complexes. *J. Biol. Chem.*, **268**, 7607–7609.
- Sabatini,D.M., Erdjument-Bromage,H., Lui,M., Tempst,P. and Snyder,S.H. (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell*, **78**, 35–43.
- Sabers,C.J., Martin,M.M., Brunn,G.J., Williams,J.M., Dumont,F.J., Wiederrecht,G. and Abraham,R.T. (1995) Isolation of a protein target of the FKBP12–rapamycin complex in mammalian cells. *J. Biol. Chem.*, **270**, 815–822.
- Schreiber,S.L. and Crabtree,G.R. (1992) The mechanism of action of cyclosporin A and FK506. *Immunol. Today*, **13**, 136–142.
- Sherman,F. (1991) Getting started with yeast. *Methods Enzymol.*, **194**, 3–21.
- Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
- Small,P.L.C. and Falkow,S. (1988) Identification of regions on a 230-kilobase plasmid from enteroinvasive *Escherichia coli* that are required for entry into HEP-2 cells. *Infect. Immun.*, **56**, 225–229.
- Tanida,I., Hasegawa,A., Iida,H., Ohya,Y. and Anraku,Y. (1995) Cooperation of calcineurin and vacuolar H^{+} -ATPase in intracellular Ca^{2+} homeostasis of yeast cells. *J. Biol. Chem.*, **270**, 10113–10119.
- Toffaletti,D.L., Rude,T.H., Johnston,S.A., Durack,D.T. and Perfect,J.R. (1993) Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. *J. Bacteriol.*, **175**, 1405–1411.
- Tropschug,M., Barthelmess,I.B. and Neupert,W. (1989) Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*. *Nature*, **342**, 953–955.
- Van Der Ploeg,L.H.T., Giannini,S.H. and Cantor,C.R. (1985) Heat shock genes: regulatory role for differentiation in parasitic protozoa. *Science*, **228**, 1443–1446.
- Whelan,W.L. and Kwon-Chung,K.J. (1986) Genetic complementation in *Cryptococcus neoformans*. *J. Bacteriol.*, **166**, 924–929.
- Yoshida,T., Toda,T. and Yanagida,M. (1994) A calcineurin-like gene *ppb1⁺* in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. *J. Cell Sci.*, **107**, 1725–1735.

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