

Rapamycin Antifungal Action Is Mediated via Conserved Complexes with FKBP12 and TOR Kinase Homologs in *Cryptococcus neoformans*

M. CRISTINA CRUZ,¹ LORA M. CAVALLO,^{1,2} JENIFER M. GÖRLACH,¹ GARY COX,³
JOHN R. PERFECT,^{3,4} MARIA E. CARDENAS,¹ AND JOSEPH HEITMAN^{1,2,3,4,5*}

Departments of Genetics,¹ Pharmacology and Cancer Biology,⁵ Microbiology,⁴ and Medicine,³ and Howard Hughes Medical Institute,² Duke University Medical Center, Durham, North Carolina 27710

Received 6 November 1998/Returned for modification 9 December 1998/Accepted 1 March 1999

Cryptococcus neoformans is a fungal pathogen that causes meningitis in patients immunocompromised by AIDS, chemotherapy, organ transplantation, or high-dose steroids. Current antifungal drug therapies are limited and suffer from toxic side effects and drug resistance. Here, we defined the targets and mechanisms of antifungal action of the immunosuppressant rapamycin in *C. neoformans*. In the yeast *Saccharomyces cerevisiae* and in T cells, rapamycin forms complexes with the FKBP12 prolyl isomerase that block cell cycle progression by inhibiting the TOR kinases. We identified the gene encoding a *C. neoformans* TOR1 homolog. Using a novel two-hybrid screen for rapamycin-dependent TOR-binding proteins, we identified the *C. neoformans* FKBP12 homolog, encoded by the *FRR1* gene. Disruption of the FKBP12 gene conferred rapamycin and FK506 resistance but had no effect on growth, differentiation, or virulence of *C. neoformans*. Two spontaneous mutations that confer rapamycin resistance alter conserved residues on TOR1 or FKBP12 that are required for FKBP12-rapamycin-TOR1 interactions or FKBP12 stability. Two other spontaneous mutations result from insertion of novel DNA sequences into the FKBP12 gene. Our observations reveal that the antifungal activities of rapamycin and FK506 are mediated via FKBP12 and TOR homologs and that a high proportion of spontaneous mutants in *C. neoformans* result from insertion of novel DNA sequences, and they suggest that nonimmunosuppressive rapamycin analogs have potential as antifungal agents.

Cryptococcus neoformans is an opportunistic fungal pathogen that causes systemic mycosis in immunocompromised patients (20, 45). Cryptococcosis in patients with AIDS is characterized by a poor response to therapy and a risk of recurrent disease requiring lifelong suppressive antifungal regimens. Current treatments include amphotericin B, flucytosine, and fluconazole. However, treatment failures and toxicity are common, and new antifungal agents are needed.

C. neoformans is sensitive to the immunosuppressants rapamycin, FK506, and cyclosporine (CsA) (48, 49). These compounds are natural products with antifungal activity and are also potent immunosuppressants used to treat graft rejection in transplant recipients (7, 38). Rapamycin is a macrolide originally discovered in a screen for antimicrobial activity against *Candida albicans* and later found to have potent immunosuppressive activity (62).

Rapamycin, FK506, and CsA suppress the immune system by inhibiting signal transduction steps required for T-cell activation (for reviews, see references 19 and 59). The mechanisms of action of these compounds have been studied in lymphocytes and *Saccharomyces cerevisiae* (for reviews, see references 11, 18, 32, and 59). These hydrophobic compounds diffuse into the cell and bind intracellular receptors known as immunophilins. The immunophilins are ubiquitous and conserved from yeast to humans. FK506 and rapamycin bind the immunophilin FKBP12, whereas CsA binds cyclophilin A. Although the cyclophilin and FKBP proteins have no sequence homology, both catalyze *cis-trans* peptidyl-prolyl isomerization, a rate-limiting

protein-folding step (for reviews, see references 24, 32, and 56).

Rapamycin and FK506 bind to the FKBP12 active site and inhibit prolyl isomerase activity, but this is not the mechanism of toxic action. Instead, FKBP12-rapamycin and FKBP12-FK506 complexes target proteins required for signal transduction and cell growth. The target of FKBP12-FK506 and cyclophilin A-CsA is calcineurin, a Ca²⁺-regulated protein phosphatase (8, 26, 41). In humans, calcineurin regulates nuclear localization of the transcription factor NFAT during the response to antigen presentation (for a review, see reference 53). In *S. cerevisiae*, calcineurin regulates cation homeostasis and cell integrity via the transcription factor Crz1/Tcn1, which regulates transcription of genes encoding ion pumps or cell wall biosynthetic enzymes (*FKS2*, *PMR2*, *PMCI*, and *PMR1*) (44, 65). In *C. neoformans*, calcineurin is also the target of FK506 and CsA and is essential for growth at 37°C, in 5% CO₂, or at alkaline pH, conditions found in the host (48). As a consequence, calcineurin is required for virulence of *C. neoformans* (48).

Rapamycin prevents proliferation of *S. cerevisiae* cells and T lymphocytes by inhibiting cell cycle progression from G₁ to S phase. An FKBP12-rapamycin complex is the active intracellular agent, but this complex does not inhibit calcineurin. Instead, FKBP12-rapamycin inhibits the TOR kinases (15, 33, 35, 40). The yeast *S. cerevisiae* expresses two TOR proteins, TOR1 and TOR2. A mammalian TOR homolog has also been identified (9, 21, 54, 55). The TOR proteins have homology to lipid and protein kinases and belong to a family of phosphatidylinositol kinase-related kinases that regulate the cell cycle. The TOR kinase domain is conserved and is essential for cell cycle progression (1, 17, 74). The TOR signaling pathway also regulates translation in yeast and mammalian cells (4, 5, 23). In

* Corresponding author. Mailing address: 322 Carl Building, Research Drive, Duke University Medical Center, Durham, NC 27710. Phone: (919) 684-2824. Fax: (919) 684-5458. E-mail: heitm001@mc.duke.edu.

mammalian cells, mTOR regulates translation initiation via the p70^{S6k} kinase and 4E-BP1/PHAS-I proteins (reviewed in reference 11). The *S. cerevisiae* TOR2 protein has an additional unique function involving actin cytoskeletal polarization (57, 58).

Recent models of mitogen-activated TOR signaling in mammalian cells invoke an initial activation of phosphatidylinositol 3-kinase, which signals to p70^{S6k} and 4E-BP1/PHAS-I via mTOR (28). The 4E-BP1/PHAS-I protein inhibits the eIF4E translation initiation factor, which recognizes the 5' N⁷-methylguanine cap to initiate translation. 4E-BP1/PHAS-I binds to and inhibits translational initiation by eIF4E, and the TOR kinase phosphorylates PHAS-I to release eIF4E and promote translation (12, 13, 30, 60). Rapamycin blocks translation by preventing PHAS-I phosphorylation and release of eIF4E. TOR also regulates phosphorylation of p70^{S6k} (14). Rapamycin blocks translation in yeast by destabilizing the eIF4E-associated translation factor eIF4G (4, 6, 23). Direct substrates of yeast TOR remain to be identified (for reviews, see references 29 and 66).

Biochemical, genetic, and structural studies have defined the molecular details of FKBP12-rapamycin inhibition of TOR. An X-ray crystal structure of an FKBP12-rapamycin-mTOR complex revealed that FKBP12-rapamycin binds to a small region on TOR, the FKBP12-rapamycin-binding domain (FRB domain) located adjacent to the carboxy-terminal kinase domain (22). Mutations in the FRB domain confer rapamycin resistance by blocking FKBP12-rapamycin binding to TOR (15, 17, 35, 43, 63, 74).

Here we describe the identification of the FKBP12 and TOR1 homologs from *C. neoformans*. We demonstrate that rapamycin antifungal action is mediated via FKBP12-rapamycin inhibition of TOR1. First, spontaneous FKBP12 and TOR1 mutations that confer rapamycin resistance destabilize FKBP12 or prevent FKBP12-rapamycin binding to *C. neoformans* TOR1. Second, disruption of the FKBP12-encoding gene *FRR1* confers rapamycin and FK506 resistance. Mutant cells lacking FKBP12 exhibited wild-type growth under a variety of different conditions and had no defects in mating, sporulation, or virulence. In summary, our studies reveal that a conserved TOR homolog is the target of FKBP12-rapamycin in *C. neoformans* and that the mechanism of rapamycin action is conserved from pathogenic fungi to yeasts and humans, and they suggest that nonimmunosuppressive rapamycin analogs have potential as novel antifungal agents. Finally, a high proportion of spontaneous mutations resulted from the insertion of novel sequences into the FKBP12 locus, which has important implications for the mutation, evolution, and virulence of this human pathogen.

MATERIALS AND METHODS

Strains, media, antisera, and compounds. The *S. cerevisiae* two-hybrid strains used here were PJ69-4A (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (37) and SMY4 (Y190 *TOR1-3 fpr1::ADE2*) (16). SMY87-4 is an isogenic *fpr1::hisG* derivative of strain PJ69-4A. The pathogenic *C. neoformans* serotype A strain H99 and its isogenic *Δade2* derivative M049 have been described previously (67). Strains JEC20 and JEC21 are isogenic *MATa* and *MATα* serotype D strains (46). Mutants C20F1, C20F2, C21F2, and C21F3 were as reported previously (48). *C. neoformans* strains were grown on rich (yeast extract-peptone-dextrose [YPD]) and synthetic media used for *S. cerevisiae* (61), and immunosuppressant and SLAD media were as described previously (27, 31). Regeneration medium for biolistic transformations was prepared as described previously (67). Capsule production was assessed in low-iron medium plus ethylenediaminedi(*o*-hydroxy)phenylacetic acid as described previously (71). Melanin production was assessed on Niger seed medium as described previously (3). The *S. cerevisiae* FKBP12 polyclonal antiserum was generated and characterized as described previously (17). The *C. neoformans* cyclophilin A polyclonal antiserum was produced by injecting a rabbit with *C. neoformans* cyclophilin A recombinant protein. FK506 was from Fujisawa, and

rapamycin was from the National Cancer Institute. Drug stock solutions were prepared in 90% ethanol–10% Tween 20.

Isolation and characterization of the *C. neoformans* gene encoding TOR1. Amplification by PCR of *C. neoformans* cDNA was the initial approach used to characterize the *C. neoformans* TOR homolog. Degenerate oligonucleotide primers were designed based on conserved regions in mammalian mTOR and yeast TOR1 and TOR2. The forward primer was 5'-CA(G/A)GCITGGGA(T/C)ITITA(T/C)TA (775), and the reverse primer was 5'-TC(G/A)AA(G/A)C A(G/A)TCICCC(A/G)TCIATGATG (781) (I is inosine). PCR amplification was performed under conditions of moderate annealing stringency with a pooled *C. neoformans* cDNA library from the serotype D strain B3501 (Stratagene). PCR conditions were as follows: 3 min at 95°C; 35 cycles of 30 s at 95°C, 1 min at 45°C, and 2 min at 72°C; and a final 10-min 72°C step. A 795-bp product was cloned and sequenced. The remaining portions of the *C. neoformans* TOR1 gene were isolated by screening three size-selected subgenomic libraries of the serotype A H99 strain and by analysis of a 5' RACE (rapid amplification of cDNA ends) product. Total RNA for the cDNA RACE was prepared from *C. neoformans* H99 grown in YPD at 30°C. RACE was performed with the Gibco BRL 5' Race system (version 2.0) and the TOR1-specific primers 5'-CTAGAGTCACGAG GGGG (2121), 5'-CTTTTCCAAGACCTTGGAGATG (2122), and 5'-CTGA TGGAATCTCCTGGG (2123). Sequence analysis of the TOR1 gene and the *FRR1* gene described below was performed with a Perkin-Elmer Applied Biosystems DNA sequencer model 377 (Amplitaq dye terminator chemistry).

Two-hybrid screen to clone the *C. neoformans* FKBP12 homolog. Plasmids expressing the GAL4 DNA binding domain [GAL4(DB)]-*C. neoformans* TOR1 FRB domain protein fusion were used to screen a two-hybrid cDNA library from *C. neoformans* H99. The two-hybrid library construction was as follows. H99 was grown overnight at 30°C in YPD medium to saturation. Aliquots were transferred to fresh medium (1:50 dilution) and grown for another 6 h under the following conditions: YPD medium at 30°C, YPD medium at 37°C, and nitrogen starvation SLAD medium at 30°C. Total RNA and poly(A)⁺ RNAs were isolated and pooled such that the total population consisted of 50% mRNA from YPD at 30°C, 37% mRNA from YPD at 37°C, and 13% mRNA from SLAD medium at 30°C. The two-hybrid cDNA library was constructed by using the Clontech MATCHMAKER library kit; the cDNA population was size fractionated on a column to be larger than 300 bp, and H99 cDNAs were then fused to the GAL4 activation domain [GAL4(AD)] in plasmid pGAD10.

To fuse the TOR1 FRB domain to the GAL4(DB), the FRB domain was amplified with primers 5'-GACGTTTCAGGAGCTTGAGCTG (849) and 5'-GC GAGAGCACTCTCCCAAGATCGTGG (889) and cloned in the two-hybrid vector pGBT9, yielding plasmid pGBT9-CnTOR FRB. SMY87-4, an isogenic *fpr1::hisG* derivative of strain PJ69-4A, was used as the two-hybrid strain to screen the *C. neoformans* cDNA library. SMY87-4 was cotransformed with the GAL4(DB)-*C. neoformans* TOR1 FRB domain and the two-hybrid cDNA library described above. In addition, a URA3⁺ plasmid (pTR17) was introduced to express a dominant, rapamycin-resistant allele of the TOR2 gene (*TOR2-1*). Cells were grown on synthetic medium lacking leucine, tryptophan, uracil, and adenine (SD-Leu-Trp-Ura-Ade) and supplemented with 1 μg of rapamycin per ml. The cells were replica plated to synthetic medium lacking histidine (SD-Leu-Trp-Ura-His) and supplemented with 3 mM 3-aminotriazole and 1 μg of rapamycin per ml. β-Galactosidase activity was assayed by using chlorophenol-β-D-galactopyranoside (CPRG) as described previously (16).

Site-directed mutagenesis of *C. neoformans* TOR1. The *C. neoformans* GAL4(DB)-TOR1 (Ser1846Leu) mutant was created by PCR overlap mutagenesis (36) with plasmid GAL4(DB)-TOR1 wild-type FRB domain as a template by using primers 5'-GGAAGAAGCTTTAAAGCACTAC (1113) and 5'-GTAGT GCTTTAAAGCTTCTTCC (1114) (mutations are in boldface) and flanking primers 5'-CTTACCGGAATTCATCCAACGCCACGCCAGTCTATAC (1115) and 5'-CTCGCACTGCAGCTATGGAACAGCAATATCCAAGTC TCG (1116). First-round PCR overlap products were gel purified as templates for second-round PCR with flanking primers. The PCR protocol was 3 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final 5-min 72°C step. The resulting PCR product was cleaved with *EcoRI* and *PstI*, cloned into these sites in plasmid pGBT9, and confirmed by sequencing.

Disruption of the *C. neoformans* FRR1 gene. The *FRR1* gene was disrupted by inserting a 3,000-bp *KpnI/SmaI* fragment spanning the *C. neoformans* *ADE2* gene (blunted with T4 DNA polymerase and deoxynucleoside triphosphates) into an *RsvII* site within the *FRR1* gene in a 5-kb *EcoRI* genomic fragment cloned in pBluescript (Stratagene), yielding plasmid pMCC1 bearing the *frr1::ADE2* disruption allele. Approximately 4 μg of plasmid DNA was purified on a Qiagen column, adsorbed onto the surface of gold microprojectiles, and introduced by biolistic transformation into strain M049, an isogenic *Δade2* derivative of strain H99. Adenine-prototrophic transformants were selected on synthetic medium (1 M sorbitol) lacking adenine at 30°C and colony purified. ADE⁺ transformants were tested for the ability to grow on YPD medium lacking or containing 1 μg of rapamycin per ml or 1 μg of FK506 per ml (at 37°C).

Southern blot analysis. *C. neoformans* genomic DNA was prepared as described previously (52). DNA (~10 μg) was digested to completion with restriction enzymes (New England Biolabs), fractionated by electrophoresis in a 0.8% agarose gel, transferred to a nylon membrane, and fixed by UV cross-linking (Stratalinker; Stratagene). The membrane was hybridized overnight with gel-

purified DNA probes labelled with ^{32}P by random priming (Pharmacia Biotech), and the membranes were then washed, dried, and exposed to film.

Western blot analysis. *C. neoformans* cultures were grown in YPD medium at 30°C for 24 h, and cells were then pelleted and lysed by glass bead agitation in a bead beater in 20 mM Tris-Cl (pH 7.4)–100 mM KCl. Protein concentrations were determined by the Bradford assay (Bio-Rad) with bovine serum albumin as a standard. Equivalent amounts of protein (~100 µg/ml) were fractionated by sodium dodecyl sulfate–17% polyacrylamide gel electrophoresis (SDS–17% PAGE), transferred to nitrocellulose membranes, and probed with rabbit polyclonal antiserum directed against yeast FKBP12 or the *C. neoformans* cyclophilin A protein as a loading control. Detection was performed with the ECL system (Amersham Corp.).

Analysis of *frr1* mutants. Mutant strains were assayed for capsule and melanin production. *C. neoformans* cells were incubated on liquid low-iron medium plus ethylenediaminedi(*o*-hydroxy)phenylacetic acid for 48 h, and the polysaccharide capsule was stained with a standard India ink preparation. To assess melanin production, *C. neoformans frr1* strains were grown at 30°C for 72 h on Niger seed agar, on which strains that produce melanin are brown, while strains that do not produce melanin are white. Sequence analysis of the mutant strains C20F1, C20F2, and C21F3 was done as follows. First, genomic DNA was isolated according to a protocol described previously (51) and used as a template for duplicate PCR amplifications with oligonucleotides 5'-GCATGGGATCCCATGGGTGTTACTGTTGAG (1769) and 5'-GTACGAGAATTCCTAGTTGACC TTGAGGAG (1770) for strain C21F3 and oligonucleotides 5'-CGTTGCAAC AGAATTAACCTG (2785) and 1770 for strains C20F1 and C20F2. The PCR conditions were 35 cycles of 94°C for 1 min, 47°C for 1 min, and 72°C for 3 min. The resulting DNA fragments spanning the *frr1* gene were cloned into the pCR2.1 TA cloning vector (Invitrogen) and subsequently sequenced in duplicate with a Perkin-Elmer Applied Biosystems DNA sequencer model 377 (Amplitaq dye terminator chemistry).

Rabbit model of cryptococcal meningitis. New Zealand White rabbits weighing 2 to 3 kg were housed in separate cages and provided with water ad libitum and Purina rabbit chow. Isogenic *FRR1* wild-type (M049 *ADE2* reconstituted) and *frr1* mutant *C. neoformans* serotype A strains were prepared by growth for 72 h on YPD medium and resuspended in 0.015 M phosphate-buffered saline. Eight rabbits were administered cortisone acetate at 2.5 mg/kg intramuscularly 1 day prior to inoculation with *C. neoformans* and then daily for 14 days. Twenty-four hours following initiation of steroid treatment, rabbits were anesthetized with intramuscular xylazine and ketamine and then inoculated intracisternally with 0.3 ml of yeast suspension through a 25-gauge needle on a 3-ml syringe. Three rabbits received wild-type strain H99, and five rabbits received the isogenic *frr1* mutant strain. The rabbits were sedated with ketamine and xylazine on days 4, 7, 10, and 14 after inoculation, and cerebrospinal fluid (CSF) was withdrawn. Quantitative yeast cultures were performed by plating serial dilutions of CSF in phosphate-buffered saline on YPD medium and incubating for 3 days at 30°C.

Murine model of systemic cryptococcosis. *FRR1* wild-type and *frr1* mutant *C. neoformans* strains were used to infect 4- to 6-week-old female BALB/c mice (Charles River Laboratories, Raleigh, N.C.) by lateral tail vein injections. Ten mice were infected with 10^7 yeast cells of each strain in a volume of 100 µl, and the mice were monitored by twice-daily inspections. Mice that appeared to be moribund or in pain were sacrificed by CO₂ inhalation.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *C. neoformans TOR1* genomic and cDNA sequences and for the *C. neoformans FRR1* genomic and cDNA sequences are AF098972, AF098973, AF097888, and AF097889, respectively.

RESULTS

Cloning of the *C. neoformans TOR1* homolog. In *S. cerevisiae*, rapamycin action is mediated by the FKBP12 prolyl isomerase, which binds to and inhibits the TOR kinase homologs to block cell cycle progression (15, 33, 39, 40). We have tested whether homologous FKBP12 and TOR proteins mediate rapamycin action in *C. neoformans*.

The *C. neoformans TOR1* homolog was identified by PCR amplification under reduced-stringency conditions with primers to conserved TOR sequences and DNA from a *C. neoformans* serotype D strain B3501 cDNA library as a template. A 795-bp product was obtained, purified, and cloned. Sequence analysis revealed a unique open reading frame encoding 265 amino acids with marked identity to other TOR proteins. Southern blot analysis confirmed that this sequence was derived from *C. neoformans* and hybridized to a single genomic locus, and this fragment was used as a probe to clone the *TOR1* gene. Three size-selected subgenomic libraries were constructed from genomic DNA of the serotype A strain H99, and several screens were performed to complete the cloning of *C.*

neoformans TOR1 (see Materials and Methods). In addition, the 5' end of the gene and the approximate transcription start site were established by 5' RACE (see Materials and Methods). The complete sequence of the *C. neoformans TOR1* homolog was determined from genomic and 5' RACE cDNA clones.

The open reading frame of the *C. neoformans TOR1* gene spans 8,439 bp and encodes a 2,360-amino-acid protein. Twenty-six introns were identified by comparing *TOR1* genomic and cDNA sequences and by alignment with the homologous TOR proteins from *S. cerevisiae*, *Schizosaccharomyces pombe*, and humans. The introns have an average size of 52 bp and have consensus splice donor (GTNNGY) and acceptor (YAG) sites, with the exception of intron 25 (with GCAAGT as splice donor) and intron 20 (with AAG as splice acceptor). CTRAY intronic branch sites were present in 25 of the 26 introns.

The overall size and order of established protein domains are similar in the *C. neoformans TOR1* protein and the TOR proteins of *S. cerevisiae* and humans. The *C. neoformans TOR1* homolog is ~200 amino acids shorter than the mammalian and *S. cerevisiae* counterparts because it lacks an N-terminal region and one short internal segment. The C-terminal region of the *C. neoformans TOR1* homolog contains the conserved FRB and kinase domains. The *C. neoformans TOR1* kinase domain has 78, 77, and 73% identity to the TOR1, TOR2, and mTOR proteins, respectively (Fig. 1). Within the *C. neoformans TOR1* FRB domain, Ser1862 is homologous to the serine residue implicated in rapamycin binding to the *S. cerevisiae TOR1* and TOR2 proteins and mammalian mTOR (Ser1972 in TOR1, Ser1975 in TOR2, and Ser2035 in mTOR) (10, 15, 17, 22, 35, 43, 63, 74). In addition, TOR residues that bind rapamycin in the crystal structure of the mTOR-rapamycin-FKBP12 complex (Trp2101, Asp2102, Tyr2105, Phe2108, Leu2038, Tyr2038, and Gly2039) are conserved in the *C. neoformans TOR1* FRB domain, with the sole exception that Thr2098 of mTOR is replaced by Gln1925 in *C. neoformans TOR1* (Fig. 1). All presumptive TOR active-site residues are conserved (Arg2276 and Asp2294 in TOR1, Asp2279 in TOR2, and Asp2338 and Asp2357 in mTOR) (10, 17, 58, 74). These findings indicate that a bona fide TOR homolog has been identified in *C. neoformans*, with conserved structural features important for FKBP12-rapamycin binding and enzymatic activity.

Cloning of the *C. neoformans FKBP12* homolog. Because FKBP12 is the intracellular receptor for rapamycin and FK506 in other organisms, we sought to identify the FKBP12 homolog from *C. neoformans*. Previous attempts to clone the gene by several approaches were unsuccessful. We therefore developed a novel two-hybrid screen to isolate the *FRR1* (for FK506 and rapamycin resistance) gene, encoding the *C. neoformans FKBP12* homolog.

For this two-hybrid cloning approach, the FRB domain of the *C. neoformans TOR1* protein was fused to the GAL4(DB). The resulting GAL4(DB)-TOR1 fusion protein was used as bait to identify proteins that interact with cryptococcal TOR1 in the presence of rapamycin. Second, a *C. neoformans* cDNA two-hybrid library was constructed by fusing cDNAs isolated from the *C. neoformans* serotype A strain H99 to the GAL4(AD). Third, we constructed a customized two-hybrid *S. cerevisiae* strain, isogenic with strain PJ69-4A (37), which had three important features: first, the FKBP12 gene was deleted to reduce competition for rapamycin; second, the strain has three GAL4-dependent reporter genes, *ADE2*, *HIS3*, and *lacZ*; and third, because our screen requires rapamycin, which is normally toxic to *S. cerevisiae*, a plasmid expressing a dominant, rapamycin-resistant TOR2 mutant was introduced.

The two-hybrid screen was performed by cotransforming the

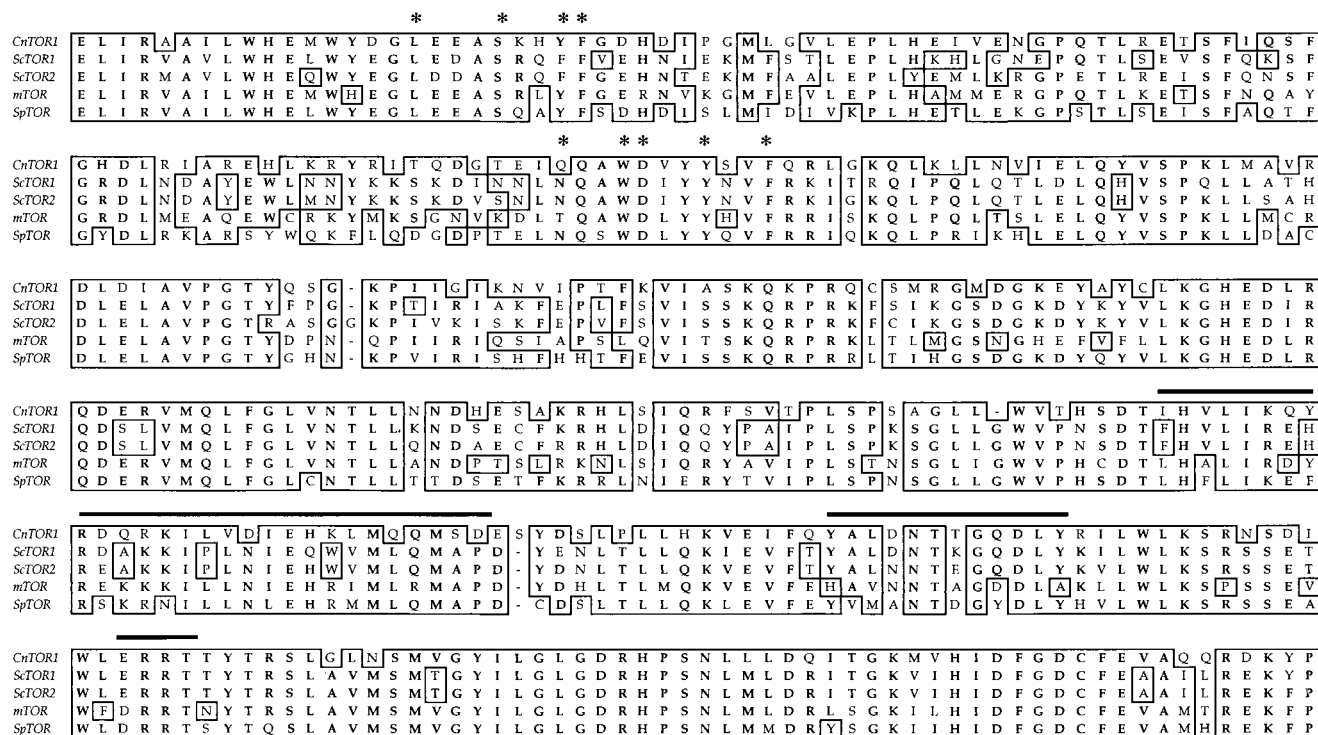


FIG. 1. *C. neoformans* TOR1 kinase and FRB domains are highly conserved. The predicted *C. neoformans* TOR1 (CnTOR1) protein sequence from amino acid residues 1842 to 2200 is aligned with analogous regions from *S. cerevisiae* TOR1 (ScTOR1) (amino acids 1952 to 2309) (15), *S. cerevisiae* TOR2 (ScTOR2) (amino acids 1955 to 2313) (40), mammalian mTOR (amino acids 2015 to 2372) (54), and *S. pombe* TOR (SpTOR) (amino acids 1814 to 2171). The positions of kinase conserved motifs are underlined. Asterisks indicate conserved amino acid residues of the FRB domain of TOR.

C. neoformans two-hybrid cDNA library and the plasmid expressing the GAL4(DB)-TOR1 FRB domain fusion protein into the two-hybrid yeast strain lacking FKBP12 and expressing TOR2-1. Transformed cells were grown on synthetic medium lacking leucine, tryptophan, uracil, and adenine (SD-Leu-Trp-Ura-Ade) and supplemented with 1 μg of rapamycin per ml. Twelve ADE⁺ colonies were isolated. Of the 12 cDNA clones recovered, 9 activated reporter gene expression when reintroduced with the GAL4(DB)-TOR1 bait plasmid into the two-hybrid strain. Sequence analysis revealed that all nine contain overlapping or identical portions of the same gene, which we named *FRR1*. The sequence of the *C. neoformans* *FRR1* gene predicts a 108-amino-acid protein, which has sequence identity with FKBP12 proteins of yeast, fungi, and mammals (Fig. 2). The predicted amino acid sequence of the *C. neoformans* FKBP12 protein is aligned in Fig. 3 with sequences of conserved FKBP12 proteins from other organisms. This FKBP12 protein has 65, 57, and 59% identity with FKBP12 homologs from *S. cerevisiae*, *C. albicans*, and humans, respectively. Importantly, residues of the rapamycin- and FK506-binding pocket are highly conserved or invariant between FKBP12 proteins from *C. neoformans*, *S. cerevisiae*, and humans (Fig. 3).

The genomic *FRR1* locus was cloned by screening a sub-genomic library from strain H99 with the *FRR1* cDNA as a probe. A 5.1-kb genomic clone spanning the *FRR1* open reading frame was obtained. Sequence analysis revealed that the *FRR1* coding sequence is punctuated by five introns. All five introns were small (96, 89, 50, 53, and 52 bp) and contained consensus splice donor, acceptor, and branch sites.

FKBP12-rapamycin binds to wild-type TOR1 but not to a rapamycin-resistant TOR1 mutant. Previous studies have

characterized *TOR* mutations that confer rapamycin resistance by preventing FKBP12-rapamycin binding (15, 17, 35, 43, 63, 74). We isolated several spontaneous *C. neoformans* mutations that confer rapamycin but not FK506 resistance. Because mutations in the TOR FRB domain confer rapamycin resistance in other organisms, these mutants might harbor alterations in the *C. neoformans* TOR1 FRB domain. In one mutant, duplicate PCR amplification and sequence analysis revealed a Ser1846Leu substitution in TOR1 at the conserved serine residue implicated in FKBP12-rapamycin binding to yeast TOR1 and TOR2.

We used the yeast two-hybrid system to test if the Ser1862Leu mutation confers rapamycin resistance by blocking FKBP12-rapamycin binding to *C. neoformans* TOR1. The GAL4(DB) was fused to wild-type and to mutant FRB (Ser1862Leu) domains of *C. neoformans* TOR1. Plasmids expressing the GAL4(DB)-TOR1 FRB domain fusion protein, the GAL4(DB)-TOR1 (Ser1862Leu) mutant FRB domain, and the GAL4(AD)-yeast or *C. neoformans* FKBP12 fusion proteins were coexpressed in a two-hybrid host (SMY4 [Y190 *TOR1-3 fpr1::ADE2*]) in the presence or absence of rapamycin. Protein-drug-protein interactions were monitored by measuring expression of the *GAL4-lacZ* reporter gene by assaying β-galactosidase activity (Fig. 4). Rapamycin-dependent interactions between wild-type *C. neoformans* TOR1 and FKBP12 were detected. In contrast, the Ser1862Leu mutant TOR1 protein failed to interact with FKBP12-rapamycin (Fig. 4). Thus, the Ser1862Leu mutation confers rapamycin resistance in *C. neoformans* by preventing FKBP12-rapamycin binding to TOR1.

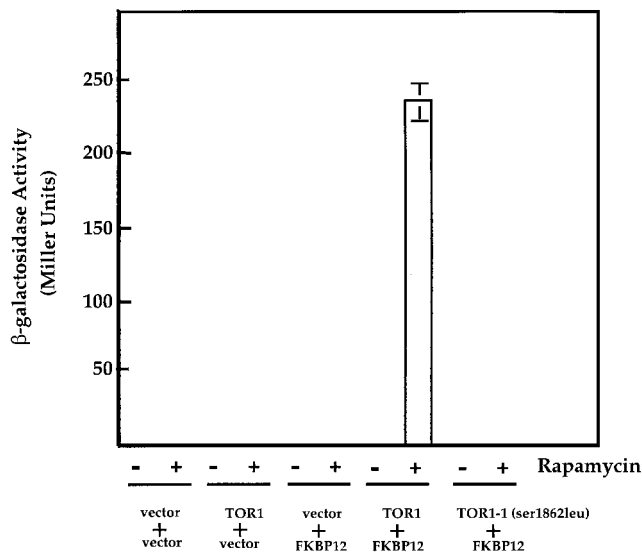


FIG. 4. A rapamycin-resistant TOR1 mutation prevents FKBP12-rapamycin binding. The GAL4(DB)-TOR1 FRB domain wild-type and Ser1862Leu mutant fusion proteins were coexpressed with the GAL4(AD)-FKBP12 fusion protein in the two-hybrid host strain SMY4 (*fpr1 TOR1-3*), with or without rapamycin (1 μ g/ml). β -Galactosidase activity was measured by CPRG assay, and the values were determined in triplicate.

firming that the wild-type *FRR1* locus had been replaced by the *frr1::ADE2* disruption allele through homologous recombination without ectopic integration in all four transformants that were analyzed (Fig. 5B). Thus, FKBP12 is not essential for viability in *C. neoformans*.

To confirm that disruption of the *FRR1* gene abrogates FKBP12 expression, total proteins were isolated from isogenic *FRR1* wild-type and *frr1* mutant strains, fractionated by SDS-15% PAGE, and transferred to a nitrocellulose membrane. Polyclonal antibodies raised against the yeast FKBP12 protein (17) were used to detect the *C. neoformans* FKBP12 protein. By Western analysis, this polyclonal antiserum recognizes a 12-kDa protein that is present in extracts from wild-type *FRR1* strains and missing in *frr1* mutant strains (Fig. 6D). These findings confirm that the *FRR1* gene has been disrupted by homologous recombination, that the size of the FKBP12 protein is in agreement with that predicted from the *FRR1* gene sequence, that the FKBP12-encoding gene is unique in *C. neoformans*, and that a rabbit polyclonal antiserum against *S. cerevisiae* FKBP12 cross-reacts with the *C. neoformans* FKBP12 homolog.

frr1::ADE2 mutants lacking FKBP12 were resistant to the toxic effects of FK506 and to rapamycin (Fig. 5C). In plate assays, the MIC of either FK506 or rapamycin was 0.1 to 1.0 μ g/ml in *FRR1* wild-type strains and >100 μ g/ml in the *frr1::ADE2* mutant strains lacking FKBP12. Quantitative MIC and minimum fungicidal concentration determinations in liquid culture further reveal that *frr1* mutants are completely resistant to the toxic effects of both drugs (data not shown; see also references 48 and 49). Thus, FKBP12 mediates FK506 and rapamycin action in *C. neoformans*.

Spontaneous FKBP12 mutations confer rapamycin resistance in *C. neoformans*. In previous studies, we identified recessive mutations that render *C. neoformans* rapamycin and FK506 resistant (48) (Fig. 6A). Because both drugs are toxic when bound to FKBP12, we hypothesized that the FK506-rapamycin resistance phenotype might be attributable to

FKBP12 mutations. To address this, the *FRR1* gene was PCR amplified from genomic DNA isolated from the rapamycin-FK506-resistant *C. neoformans* mutant strains C20F1, C20F2, and C21F3 that were isolated from the congenic serotype D *MATa* strain JEC20 and *MAT α* strain JEC21 (48). A PCR product of the expected size was obtained from the C21F3 mutant, whereas larger PCR products were produced from mutant strains C20F1 and C20F2 (Fig. 6C). The *FRR1* gene from the C21F3 mutant strain was cloned and sequenced, revealing a single amino acid substitution, Trp60Arg (*frr1-3*), in the conserved tryptophan residue at the base of the FKBP12 hydrophobic drug-binding pocket (33, 69, 70) (Fig. 3). Two independent PCR amplifications of the *FRR1* gene from the C21F3 mutant were carried out, confirming that the mutation is present in genomic DNA and is not a PCR artifact.

PCR, Southern blot analysis (Fig. 6B and C), and DNA sequence analysis (data not shown) revealed that the *FRR1* loci in the C20F1 (*frr1-1*) and C20F2 (*frr1-2*) rapamycin-FK506-resistant mutant strains have undergone insertions of two different DNA sequences. An ~780-bp sequence has inserted in the C20F2 mutant, and ~2,200 bp has been inserted in the C20F1 mutant. Because these were spontaneous mutations, this finding suggests that an unusual genomic instability is occurring in this human fungal pathogen, which may involve mobilization of novel transposable elements (22a).

Western blot analysis with antiserum to *S. cerevisiae* FKBP12 showed that these rapamycin-resistant mutant strains, including the C21F3 (*frr1-3*) mutant containing the Trp60Arg FKBP12 point mutation, all fail to express FKBP12 protein (Fig. 6D). On the other hand, the C21F2 mutant strain, which is resistant to FK506 but not to rapamycin, contained a wild-type FKBP12 gene and expressed FKBP12; in this *FKR1-1* mutant strain a calcineurin mutation may be responsible for the dominant FK506-resistant phenotype.

Phenotypic analysis of *C. neoformans* mutants lacking FKBP12. In addition to rapamycin and FK506 resistance (Fig. 5C), we addressed several other possible phenotypes conferred by FKBP12 mutations in *C. neoformans*. No differences in growth rates in liquid or solid or in rich or minimal media were observed between wild-type and *frr1* mutant strains; in contrast, *S. cerevisiae* FKBP12 mutant strains exhibit a modest growth defect in rich media (34). *C. neoformans* FKBP12 mutant strains also had no growth defect at low (24°C) or high (37°C) temperature, exhibited no novel auxotrophic requirements, grew normally on different carbon sources (glucose, galactose, and glycerol), were not resistant or hypersensitive to cycloheximide, and had no defects in the melanin or capsule virulence factors (data not shown).

In addition, FKBP12 was not required for mating, either unilaterally (*MATa frr1* or *MAT α frr1* mutant strains crossed to *MAT α* or *MATa FRR1* wild-type strains) or bilaterally (*frr1* mutant crossed to an *frr1* mutant), since abundant filaments, basidia, and viable basidiospores were produced. Thus, FKBP12 is not required for response to pheromone, cell fusion, filamentation, nuclear migration, basidium formation, nuclear fusion, meiosis, or sporulation (data not shown). FKBP12 was not required for production, secretion, or response to the mating pheromone MF α 1, because conjugation tubes were observed to the same extent when a plasmid expressing the MF α 1 pheromone was introduced into a *MATa frr1* mutant strain and into the isogenic *MAT α FRR1* wild-type strain (data not shown). Finally, FKBP12 was not required for haploid fruiting, because filaments were observed to similar extents when a *pGAL7-STE12* expression plasmid was introduced into a *MAT α frr1* mutant strain and into the isogenic *MAT α FRR1* wild-type strain (data not shown). In summary, FKBP12 is

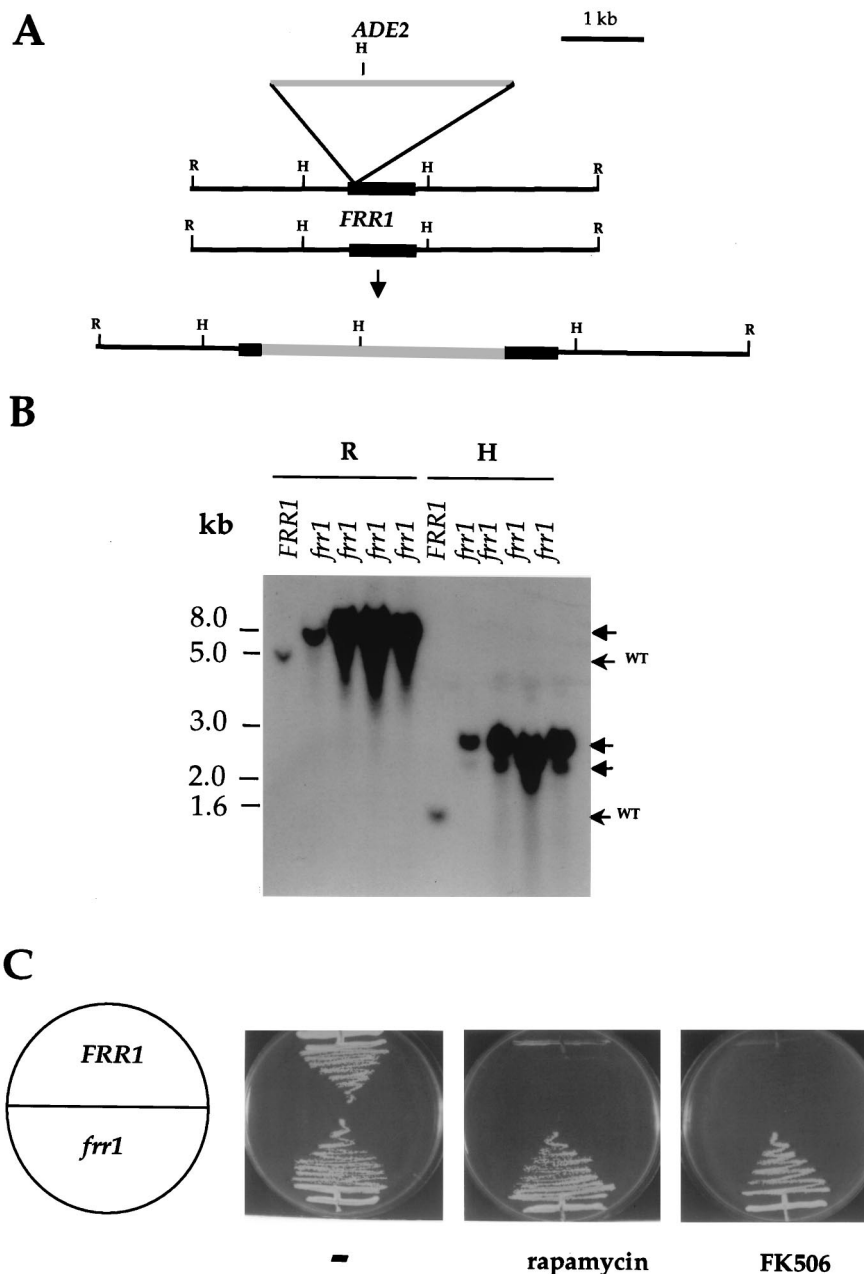


FIG. 5. Disruption of the *C. neoformans* *FRR1* gene encoding FKBP12. (A) Diagram of the *frr1::ADE2* gene disruption. The restriction map of the genomic *FRR1* gene is shown. The *FRR1* gene was disrupted by inserting a blunt-ended *ADE2* gene into an *RsrII* restriction site. R, *EcoRI*; H, *HindIII*. (B) Confirmation of the *frr1::ADE2* disruption by Southern analysis. Genomic DNA from the isogenic *FRR1* wild-type strain M049 (*H99 Δade2*) and four *frr1::ADE2* disruption mutant strains was cleaved with *EcoRI* (R) and *HindIII* (H), electrophoresed in a 0.8% agarose gel, and transferred to nitrocellulose. The membrane was hybridized to a random-primed ³²P-labelled 700-bp gel-purified fragment spanning the *FRR1* gene. Positions of DNA size markers are shown on the left. Note that there is a *HindIII* site in the *ADE2* marker; hence, two fragments arise from the *frr1::ADE2* allele. In addition, integration of tandem copies of the *frr1::ADE2* disruption allele results in more intense hybridization with the *frr1::ADE2* allele than with the wild-type (WT) locus. (C) An *frr1::ADE2* mutant strain is rapamycin and FK506 resistant. Wild-type *FRR1* strain M049 and an isogenic *frr1* disruption mutant lacking FKBP12 were grown for 72 h on YPD medium containing 1 μg of rapamycin or FK506 per ml at 37°C.

dispensable for normal vegetative growth and growth during stress and for physiological and differentiation events during haploid filamentation and mating.

FKBP12 is not required for *C. neoformans* virulence. Given that FKBP12 is the target of two potent antifungal drugs that bind and inhibit enzymatic activity, we tested if FKBP12 is involved in *C. neoformans* virulence. For this purpose, we em-

ployed a rabbit model of cryptococcal meningitis (50). Rabbits were immunosuppressed with corticosteroids and inoculated intrathecally with isogenic *FRR1* wild-type and *frr1* mutant strains. Survival of *C. neoformans* cells was determined by removal of CSF and quantification of CFU by serial dilution and plating. As shown in Fig. 7A, both the wild-type and FKBP12 mutant strains persisted in the CSF of the immuno-

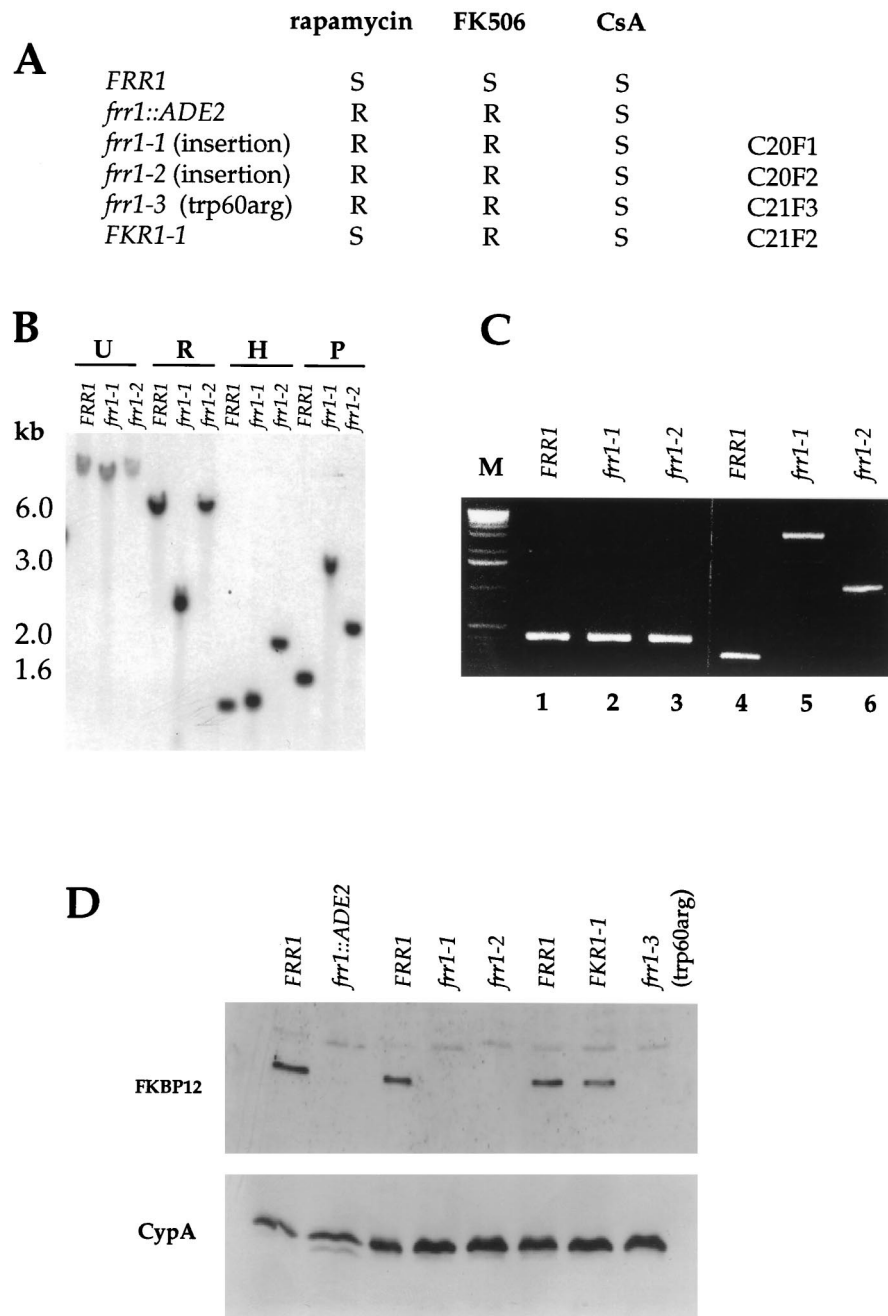


FIG. 6. Spontaneous *frr1* mutations confer rapamycin and FK506 resistance in *C. neoformans*. (A) *FRR1* wild-type (H99, JEC20, and JEC21) and isogenic *frr1::ADE2*, *frr1-1*, *frr1-2*, *frr1-3*, and *FKR1-1* mutant strains were grown on YPD medium containing rapamycin (1 μ g/ml), FK506 (1 μ g/ml), or CsA (100 μ g/ml). R and S, drug resistant and sensitive, respectively. (B) Southern analysis of genomic DNA from isogenic wild-type *FRR1* and *frr1* rapamycin-FK506-resistant mutant strains. Genomic DNA was cleaved with *EcoRI* (R), *HindIII* (H), or *PstI* (P), electrophoresed in a 0.8% agarose gel, transferred to nitrocellulose, and hybridized to a 700-bp *FRR1* gene probe. Positions of DNA size markers are shown on the left. (C) PCR amplification of the *frr1* mutant locus in strains C20F1, C20F2, and C21F3. Genomic DNA was PCR amplified with a pair of primers directed against the N-terminal region of the FKBP12 gene (primers 1 and 2) or against the C-terminal region of the FKBP12 gene (primers 3 and 4). The longer PCR products in lanes 5 and 6 resulting from strains C20F1 and C20F2 were cloned and sequenced, revealing novel DNA sequences of \sim 2,200 and \sim 780 bp that have inserted into the FKBP12 locus in strains C20F1 and C20F2 and are flanked by FKBP12 gene sequence in both cases. Lane M, markers. (D) Rapamycin-resistant mutants fail to express FKBP12. Protein extracts from wild-type and isogenic rapamycin-resistant mutants were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed with rabbit polyclonal antiserum against yeast FKBP12. Strains were *FRR1* wild-type serotype A strain H99 and the isogenic *frr1::ADE2* mutant strain, wild-type *FRR1* serotype D strain JEC20 and the isogenic *frr1-1* (C20F1) and *frr1-2* (C20F2) mutant strains, and wild-type *FRR1* serotype D strain JEC21 and the isogenic *FKR1-1* (C21F2) and *frr1-3* (C21F3) mutant strains. One hundred micrograms of protein from the same extracts was analyzed by Western blotting with antiserum against the *C. neoformans* cyclophilin A protein (CypA) as a loading control.

suppressed animal host for the duration of the experiment (14 days); although there were initial moderate reductions in the colony counts of the *frr1* mutant strain compared to the wild type in CSF obtained on days 4, 7, and 10 of infection, these

differences were not statistically significant, and CSF yeast counts were similar by 2 weeks of infection. By comparison, several other mutations (*gpa1*, *cna1*, and *nmt1*) have a profound impact on *C. neoformans* survival, with sustained reduc-

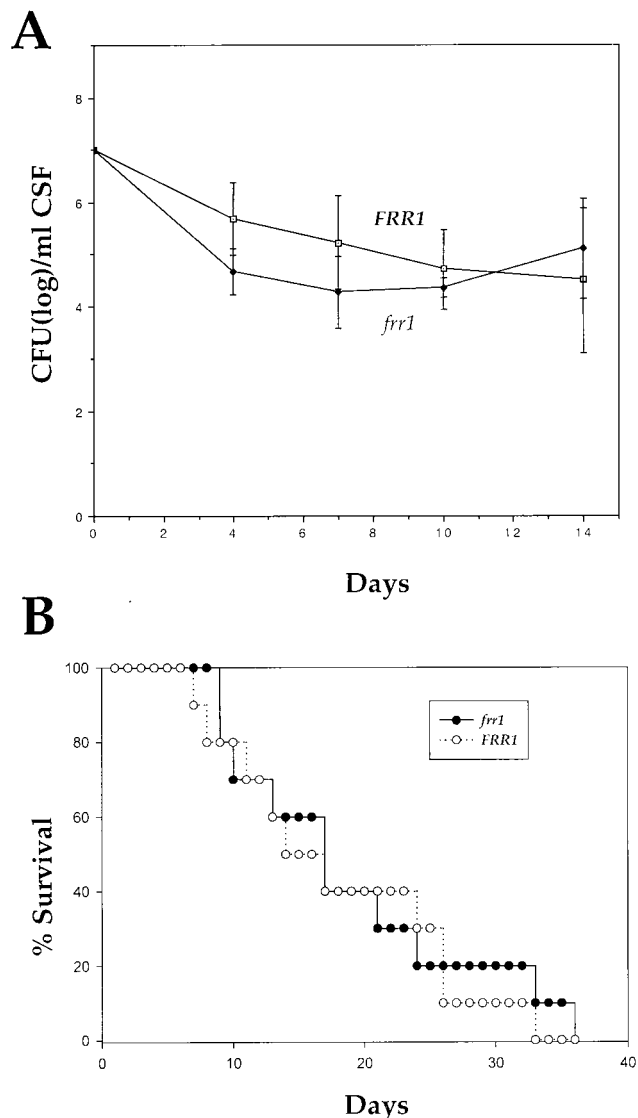


FIG. 7. FKBP12 is not required for virulence of *C. neoformans*. (A) Rabbits were immunosuppressed with steroids and inoculated intrathecally with wild-type *FRR1* *C. neoformans* M049 expressing FKBP12 (○) (wild-type M049 *ADE2* reconstituted) and the isogenic *frr1::ADE2* mutant strain lacking FKBP12 (●). CSF was removed on days 4, 7, 10, and 14 following inoculation, and the number of surviving *C. neoformans* cells (expressed as the mean log₁₀ CFU per milliliter of CSF from eight rabbits at each time point) was determined by serial dilution and plating on YPD medium with growth for 3 days at 30°C. Error bars indicate the standard error of the mean. (B) Mice (10 each) were injected in the lateral tail vein with 10⁷ cells of the *FRR1* wild-type strain H99 or the isogenic *frr1::ADE2* mutant strain lacking FKBP12. Survival was monitored and plotted with respect to time.

tions of yeast counts by >10,000-fold during similar experiments (3, 42, 48).

We also tested the possible effects of the *frr1* mutation on virulence in the murine model of cryptococcal infection. Groups of 10 mice were injected in the lateral tail vein with 10⁷ cells of the isogenic *FRR1* wild-type strain expressing FKBP12 or the *frr1* mutant strain lacking FKBP12. Survival was monitored and plotted. In this murine model system, the wild-type and *frr1* mutant strains were equally virulent, resulting in 50% mortality by day 13 to 17 and 100% mortality by day 33 to 36

(Fig. 7B). Thus, in two different animal model systems, FKBP12 mutant strains were as virulent as the isogenic wild-type parental strain. These observations are in accord with the finding that FKBP12 mutations conferred no defects in known virulence factors of *C. neoformans*, including prototrophy, growth at 37°C, capsule, melanin, or mating.

DISCUSSION

We have elucidated the mechanisms of action of the antifungal drug rapamycin in *C. neoformans*. Previous studies demonstrated that rapamycin is toxic to this organism, and genetic analysis of drug-resistant mutants provided evidence that an FKBP12 homolog might mediate rapamycin action (48), as is the case in *S. cerevisiae* and in T lymphocytes. To address this hypothesis, we isolated the *C. neoformans* genes encoding FKBP12 (*FRR1*) and the target of the rapamycin kinase homolog (*TOR1*).

The *C. neoformans* FKBP12 homolog was identified with a novel two-hybrid screen for rapamycin-dependent protein binding to the *C. neoformans* TOR1 homolog. The *C. neoformans* FKBP12 protein has marked identity with known FKBP12 proteins (Fig. 3). Disruption of the FKBP12-encoding *FRR1* gene did not result in a loss of viability and conferred both rapamycin and FK506 resistance (Fig. 5), confirming that FKBP12 is required for both rapamycin and FK506 antifungal action in *C. neoformans*. Three spontaneous rapamycin-FK506-resistant mutants were found to harbor mutations in the *FRR1* gene encoding FKBP12 (Fig. 6). One mutation results from a single amino acid substitution, Trp60Arg, which destabilizes FKBP12. This tryptophan residue is conserved in all known FKBP12 proteins, lies at the base of the hydrophobic drug-binding/active-site pocket, and mediates rapamycin, FK506, and substrate binding to FKBP12.

Previous studies on FKBP12 function in *S. cerevisiae* provide a framework to consider FKBP12 functions in other organisms. For example, yeast mutants lacking FKBP12 are viable and are rapamycin and FK506 resistant. On the other hand, yeast FKBP12 mutants exhibit only subtle additional phenotypes, including a modest growth defect, enhanced recovery from pheromone arrest, and alterations in the regulation of the metabolic enzyme aspartokinase (2, 16, 34). By comparison, *C. neoformans* mutants lacking FKBP12 are also viable and are resistant to rapamycin and to FK506. Hence, in both organisms, FKBP12 mediates drug action. On the other hand, FKBP12 mutations in *C. neoformans* conferred no growth defects during vegetative growth or under stress conditions and had no effect on mating, responses to pheromone, filamentation, meiosis, sporulation, or virulence.

In both *S. cerevisiae* and T lymphocytes, rapamycin binds the FKBP12 protein, forming a protein-drug complex that inhibits the TOR kinases. To address whether such a mechanism also explains rapamycin antifungal action in a human pathogen, we isolated and characterized the *C. neoformans* TOR1 gene, which encodes an ~280-kDa protein that has marked identity with *S. cerevisiae* TOR1 and TOR2 and the mammalian mTOR homolog (Fig. 1). In the yeast two-hybrid system, the *C. neoformans* FKBP12 and TOR1 proteins physically interact only in the presence of rapamycin (Fig. 4). A spontaneous rapamycin-resistant mutant that results from a point mutation in the FRB domain of TOR1 (Ser1862Leu) prevents FKBP12-rapamycin binding to TOR1 (Fig. 4). Analogous mutations in the FRB domain of mTOR and the *S. cerevisiae* TOR1 and TOR2 confer rapamycin resistance by similar mechanisms (10, 15, 17, 35, 43, 63).

Our studies demonstrate that rapamycin antifungal action is

mediated by the formation of FKBP12-rapamycin complexes that inhibit a TOR kinase homolog. These studies extend our understanding of the rapamycin mechanism of action from the ascomycetous yeast *S. cerevisiae* to the evolutionarily divergent pathogenic basidiomycete *C. neoformans*. Hence, the mechanism of action and targets of rapamycin are conserved from nonpathogenic yeasts to pathogenic fungi and humans. Given that rapamycin has potent antifungal activity against other human-pathogenic fungi and yeasts, such as *C. albicans* and *Aspergillus fumigatus*, FKBP12 and TOR homologs are likely conserved targets of rapamycin in these pathogens (25).

Previous studies on rapamycin action and the TOR kinases in budding and fission yeasts provide a framework to consider the physiological roles of TOR in other organisms. The *S. cerevisiae* TOR1 and TOR2 proteins share a conserved role in regulation of translation (4, 23). Inhibition of this TOR function by FKBP12-rapamycin underlies the effects of rapamycin on inducing G₁ or G₀ cell cycle arrest (4, 33) and the stimulation of starvation-related events, including vacuole-mediated protein degradation (47) and meiosis (75). The role of TOR in regulating translation has been conserved from *S. cerevisiae* to humans (4, 5). Given that rapamycin is toxic to *C. neoformans* and mediates its effects via FKBP12-dependent inhibition of a TOR homolog, the TOR proteins may also play an essential translation function in *C. neoformans*. The *S. cerevisiae* TOR2 protein has evolved a second function to regulate actin cytoskeletal polarization (57, 58), which may be shared by the *C. neoformans* TOR1 homolog or a TOR2 homolog recently identified by expressed sequence tag database sequencing (53a).

The role of TOR in the fission yeast *Schizosaccharomyces pombe* is different (72). Rapamycin has no effect on *S. pombe* vegetative growth but inhibits mating in response to nutrient deprivation (72). Thus, the essential translation function of TOR may be mediated by other redundant functions in *S. pombe*, as is known to occur in several mammalian cell types. The unique signaling role of TOR during nutrient deprivation and mating in fission yeast is not known.

Our studies on spontaneous rapamycin-resistant *C. neoformans* mutants provide insight into mutagenic mechanisms operating in this human pathogen. Of the four spontaneous rapamycin-resistant mutants characterized here, two resulted from common single-nucleotide transition mutations. More importantly, two of the four mutants result from insertions of two distinct novel sequences into the *FRR1* locus. Sequence analysis and Southern blots suggest that these may represent novel transposable elements. We note that this is a significant difference between *S. cerevisiae* and *C. neoformans* in that all *S. cerevisiae* rapamycin-resistant mutants isolated resulted from single nucleotide changes (33) whereas 50% of the *C. neoformans* rapamycin-resistant mutants isolated resulted from novel insertions at the FKBP12 locus. Further studies on these novel insertion sequences are warranted to establish if these are transposable elements, which could have clear implications for the evolution of virulence, the emergence of drug resistance, and mechanisms to escape attack by the vertebrate immune system in this important human pathogen.

Our studies provide support for development of rapamycin and analogs as antifungal agents. Several features make rapamycin ideal for further development. First, rapamycin is potentially toxic to *C. neoformans*, and it exhibits fungicidal activity, which likely to be important in the development of new antifungal agents, especially for AIDS. Second, rapamycin is orally active, which is critical for outpatient antifungal therapy. Third, while rapamycin itself is unlikely to be of clinical benefit in fungal infection, because immunosuppression predisposes individuals to cryptococcal meningitis, a collection of nonimmu-

nosuppressive rapamycin analogs are available. Fourth, rapamycin has broad-spectrum antifungal activity against several human pathogens, including *C. albicans*, *C. neoformans*, and *A. fumigatus* (48, 73).

In summary, our studies define the molecular mechanism of rapamycin antifungal action in the fungal pathogen *C. neoformans*, reveal a novel mechanism of spontaneous mutagenesis in a pathogen, and suggest that nonimmunosuppressive antifungal rapamycin analogs have potential as novel antifungal agents.

ACKNOWLEDGMENTS

We thank Andrew Alspaugh, N. Shane Cutler, Arturo Verrotti, James Walker, and Ping Wang for comments on the manuscript, Phil James for yeast strain PJ69-4A, and Cristl Arndt for technical assistance.

This work was supported in part by RO1 grants AI41937 and AI39115 (to M.E.C., J.R.P., and J.H.), and supplement AI41937-S1 (to M.C.C. and J.H.) from the NIAID and by K01 award CA77075 from the NCI (to M.E.C.). Joseph Heitman is a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology and an associate investigator of the Howard Hughes Medical Institute.

REFERENCES

- Alarcon, C. M., M. E. Cardenas, and J. Heitman. 1996. Mammalian RAFT1 kinase domain provides rapamycin-sensitive TOR function in yeast. *Genes Dev.* **10**:279–288.
- Alarcon, C. M., and J. Heitman. 1997. FKBP12 physically and functionally interacts with aspartokinase in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **17**:5968–5975.
- Alspaugh, J. A., J. R. Perfect, and J. Heitman. 1997. *Cryptococcus neoformans* mating and virulence are regulated by the G-protein α subunit GPA1 and cAMP. *Genes Dev.* **11**:3206–3217.
- Barbet, N. C., U. Schneider, S. B. Helliwell, I. Stansfield, M. F. Tuite, and M. N. Hall. 1996. TOR controls translation initiation and early G1 progression in yeast. *Mol. Biol. Cell* **7**:25–42.
- Beretta, L., A.-C. Gingras, Y. V. Svitkin, M. N. Hall, and N. Sonenberg. 1996. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.* **15**:658–664.
- Berset, C., H. Trachsel, and M. Altmann. 1998. The TOR (target of rapamycin) signal transduction pathway regulates the stability of translation initiation factor eIF4G in the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **95**:4264–4269.
- Borel, J. F. 1976. Comparative study of *in vitro* and *in vivo* drug effects on cell-mediated cytotoxicity. *Immunology* **31**:631–641.
- Breuder, T., C. S. Hemenway, N. R. Movva, M. E. Cardenas, and J. Heitman. 1994. Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. *Proc. Natl. Acad. Sci. USA* **91**:5372–5376.
- Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber. 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* **369**:756–759.
- Brown, E. J., P. A. Beal, C. T. Keith, J. Chen, T. B. Shin, and S. L. Schreiber. 1995. Control of p70 S6 kinase by kinase activity of FRAP *in vivo*. *Nature* **377**:441–446.
- Brown, E. J., and S. L. Schreiber. 1996. A signaling pathway to translational control. *Cell* **86**:517–520.
- Brunn, G. J., P. Fadden, T. A. J. Haystead, and J. J. C. Lawrence. 1997. The mammalian target of rapamycin phosphorylates sites having a (Ser/Thr)-pro motif and is activated by antibodies to a region near its COOH terminus. *J. Biol. Chem.* **272**:32547–32550.
- Brunn, G. J., C. C. Hudson, A. Sekulic, J. M. Williams, J. Hosoi, P. J. Houghton, J. J. C. Lawrence, and R. T. Abraham. 1997. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* **277**:99–101.
- Burnett, P. E., R. K. Barrow, N. A. Cohen, S. H. Snyder, and D. M. Sabatini. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci. USA* **95**:1432–1437.
- Cafferkey, R., P. R. Young, M. M. McLaughlin, D. J. Bergsma, Y. Koltin, G. M. Sathe, L. Faucette, W.-K. Eng, R. K. Johnson, and G. P. Livi. 1993. Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. *Mol. Cell. Biol.* **13**:6012–6023.
- Cardenas, M., C. Hemenway, R. S. Muir, R. Ye, D. Fiorentino, and J. Heitman. 1994. Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. *EMBO J.* **13**:5944–5957.

17. Cardenas, M. E., and J. Heitman. 1995. FKBP12-rapamycin target TOR2 is a vacuolar protein with an associated phosphatidylinositol-4 kinase activity. *EMBO J.* **14**:5892–5907.
18. Cardenas, M. E., M. Lorenz, C. Hemenway, and J. Heitman. 1994. Yeast as model-T cells. *Perspect. Drug Discov. Design* **2**:103–126.
19. Cardenas, M. E., A. Sanfridson, N. S. Cutler, and J. Heitman. 1998. Signal-transduction cascades as targets for therapeutic intervention by natural products. *Trends Biotechnol.* **16**:427–433.
20. Casadevall, A., and J. R. Perfect. 1998. *Cryptococcus neoformans*. ASM Press, Washington, D.C.
21. Chiu, M. I., H. Katz, and V. Berlin. 1994. RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proc. Natl. Acad. Sci. USA* **91**:12574–12578.
22. Choi, J., J. Chen, S. L. Schreiber, and J. Clardy. 1996. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science* **273**:239–242.
- 22a. Cruz, M. C., and J. Heitman. Unpublished data.
23. Di Como, C. J., and K. T. Arndt. 1996. Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes Dev.* **10**:1904–1916.
24. Dolinski, K., and J. Heitman. 1997. Peptidyl-prolyl isomerases (PPIases), p. 359–369. *In* S. Toozé (ed.), *Guidebook to molecular chaperones and protein folding catalysts*. Oxford University Press, Oxford, United Kingdom.
25. Ferrara, A., R. Cafferkey, and G. P. Livi. 1992. Cloning and sequence analysis of a rapamycin-binding protein-encoding gene (*RBP1*) from *Candida albicans*. *Gene* **113**:125–127.
26. Foor, F., S. A. Parent, N. Morin, A. M. Dahl, N. Ramadan, G. Chretien, K. A. Bostian, and J. B. Nielsen. 1992. Calcineurin mediates inhibition by FK506 and cyclosporin of recovery from α -factor arrest in yeast. *Nature* **360**:682–684.
27. Gimeno, C. J., P. O. Ljungdahl, C. A. Styles, and G. R. Fink. 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68**:1077–1090.
28. Gingras, A.-C., S. G. Kennedy, M. A. O'Leary, N. Sonenberg, and N. Hay. 1998. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev.* **12**:502–513.
29. Hall, M. 1996. The TOR signalling pathway and growth control in yeast. *Biochem. Soc. Trans.* **24**:234–239.
30. Hara, K., Yonezawa, M. T. Kozlowski, T. Sugimoto, K. Andrabi, Q.-P. Weng, M. Kasuga, I. Nishimoto, and J. Avruch. 1997. Regulation of eIF-4E BP1 phosphorylation by mTOR. *J. Biol. Chem.* **272**:26457–26463.
31. Heitman, J., A. Koller, M. E. Cardenas, and M. N. Hall. 1993. Identification of immunosuppressive drug targets in yeast. *Methods Companion Methods Enzymol.* **5**:176–187.
32. Heitman, J., N. R. Movva, and M. N. Hall. 1992. Proline isomerases at the crossroads of protein folding, signal transduction, and immunosuppression. *New Biol.* **4**:448–460.
33. Heitman, J., N. R. Movva, and M. N. Hall. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253**:905–909.
34. Heitman, J., N. R. Movva, P. C. Hiestand, and M. N. Hall. 1991. FK506-binding protein proline rotamase is a target for the immunosuppressive agent FK506 in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **88**:1948–1952.
35. Helliwell, S. B., P. Wagner, J. Kunz, M. Deuter-Reinhard, R. Henriquez, and M. N. Hall. 1994. TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol. Biol. Cell* **5**:105–118.
36. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51–59.
37. James, P., J. Halladay, and E. A. Craig. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**:1425–1436.
38. Kino, T., H. Hatanaka, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, and H. Imanaka. 1987. FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, physicochemical and biological characteristics. *J. Antibiot.* **40**:1249–1255.
39. Koltin, Y., L. Faucette, D. J. Bergsma, M. A. Levy, R. Cafferkey, P. L. Koser, R. K. Johnson, and G. P. Livi. 1991. Rapamycin sensitivity in *Saccharomyces cerevisiae* is mediated by a peptidyl-prolyl *cis-trans* isomerase related to human FK506-binding protein. *Mol. Cell. Biol.* **11**:1718–1723.
40. Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N. R. Movva, and M. N. Hall. 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G₁ progression. *Cell* **73**:585–596.
41. Liu, J., J. D. Farmer, W. S. Lane, J. Friedman, I. Weissman, and S. L. Schreiber. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**:807–815.
42. Lodge, J. K., E. Jackson-Machelski, D. L. Toffaletti, J. R. Perfect, and J. I. Gordon. 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.
43. Lorenz, M. C., and J. Heitman. 1995. TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. *J. Biol. Chem.* **270**:27531–27537.
44. Matheos, D. P., T. J. Kingsbury, U. S. Ahsan, and K. W. Cunningham. 1997. Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae*. *Genes Dev.* **11**:3445–3458.
45. Mitchell, T. G., and J. R. Perfect. 1995. Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. *Clin. Microbiol. Rev.* **8**:515–548.
46. Moore, T. D. E., and J. C. Edman. 1993. The α -mating type locus of *Cryptococcus neoformans* contains a peptide pheromone gene. *Mol. Cell. Biol.* **13**:1962–1970.
47. Noda, T., and Y. Ohsumi. 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* **273**:3963–3966.
48. Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, and J. Heitman. 1997. Calcineurin is required for virulence of *Cryptococcus neoformans*. *EMBO J.* **16**:2576–2589.
49. Odom, A., M. D. Poeta, J. Perfect, and J. Heitman. 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.
50. Perfect, J. R., S. D. R. Lang, and D. T. Durack. 1980. Chronic cryptococcal meningitis: a new experimental model in rabbits. *Am. J. Pathol.* **101**:177–194.
51. Perfect, J. R., D. L. Toffaletti, and T. H. Rude. 1993. The gene encoding phosphoribosylaminoimidazole carboxylase (*ADE2*) is essential for growth of *Cryptococcus neoformans* in cerebrospinal fluid. *Infect. Immun.* **61**:4446–4451.
52. Pitkin, J. W., D. G. Panaccione, and J. D. Walton. 1996. A putative cyclic peptide efflux pump encoded by the TOXA gene of the plant-pathogenic fungus *Cochliobolus carbonum*. *Microbiology* **142**:1557–1565.
53. Rao, A., C. Luo, and P. G. Hogan. 1997. Transcription factors of the NF-AT family: regulation and function. *Annu. Rev. Immunol.* **15**:707–747.
- 53a. Roe, B., D. Kupfer, J. Lewis, K. Buchanan, D. Dyer, and J. Murphy. 29 September 1998, posting date. *Cryptococcus neoformans* cDNA sequencing. [Online.] <http://www.genome.ou.edu/cneo.html>. [19 April 1999, last date accessed.]
54. Sabatini, D. M., H. Erdjument-Bromage, M. Lui, P. Tempst, and S. H. Snyder. 1994. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78**:35–43.
55. Sabers, C. J., M. M. Martin, G. J. Brunn, J. M. Williams, F. J. Dumont, G. Wiederrecht, and R. T. Abraham. 1995. Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J. Biol. Chem.* **270**:815–822.
56. Schmid, F. X. 1993. Prolyl isomerase—enzymatic catalysis of slow protein-folding reactions. *Annu. Rev. Biophys. Biomol. Struct.* **22**:123–143.
57. Schmidt, A., M. Bickle, T. Beck, and M. N. Hall. 1997. The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell* **88**:531–542.
58. Schmidt, A., J. Kunz, and M. N. Hall. 1996. TOR2 is required for organization of the actin cytoskeleton in yeast. *Proc. Natl. Acad. Sci. USA* **93**:13780–13785.
59. Schreiber, S. L., and G. R. Crabtree. 1992. The mechanism of action of cyclosporin A and FK506. *Immunol. Today* **13**:136–142.
60. Scott, P. H., G. J. Brunn, A. D. Kohn, R. A. Roth, and J. C. Lawrence. 1998. Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc. Natl. Acad. Sci. USA* **95**:7772–7777.
61. Sherman, F. 1991. Getting started with yeast. *Methods Enzymol.* **194**:3–21.
62. Singh, K., S. Sun, and C. Vezina. 1979. Rapamycin (AY-22,989), a new antifungal antibiotic. IV. Mechanism of action. *J. Antibiot.* **32**:630–645.
63. Stan, R., M. M. McLaughlin, R. T. Cafferkey, R. K. Johnson, M. Rosenberg, and G. P. Livi. 1994. Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue. *J. Biol. Chem.* **269**:32027–32030.
64. Standaert, R. F., A. Galat, G. L. Verdine, and S. L. Schreiber. 1990. Molecular cloning and overexpression of the human FK506-binding protein FKBP. *Nature* **346**:671–674.
65. Stathopoulos, A. M., and M. S. Cyert. 1997. Calcineurin acts through the *CRZ1/TCN1*-encoded transcription factor to regulate gene expression in yeast. *Genes Dev.* **11**:3432–3444.
66. Thomas, G., and M. N. Hall. 1997. TOR signalling and control of cell growth. *Curr. Opin. Cell Biol.* **9**:782–787.
67. Toffaletti, D. L., T. H. Rude, S. A. Johnston, D. T. Durack, and J. R. Perfect. 1993. Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. *J. Bacteriol.* **175**:1405–1411.
68. Tropschug, M., E. Wachter, S. Mayer, E. R. Schonbrunner, and F. X. Schmid. 1990. Isolation and sequence of an FK506-binding protein from *N. crassa* which catalyses protein folding. *Nature* **346**:674–677.
69. Van Duyne, G. D., R. F. Standaert, P. A. Karplus, S. L. Schreiber, and J. Clardy. 1991. Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex. *Science* **252**:839–842.

70. **Van Duyne, G. D., R. F. Standaert, P. A. Karplus, S. L. Schreiber, and J. Clardy.** 1993. Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J. Mol. Biol.* **229**:105–124.
71. **Vartivarian, S. E., E. J. Anaissie, R. E. Cowart, H. A. Sprigg, M. J. Tingler, and E. S. Jacobson.** 1993. Regulation of cryptococcal capsular polysaccharide by iron. *J. Infect. Dis.* **167**:186–190.
72. **Weisman, R., M. Choder, and Y. Koltin.** 1997. Rapamycin specifically interferes with the developmental response of fission yeast to starvation. *J. Bacteriol.* **179**:6325–6334.
73. **Wong, G. K., S. Griffith, I. Kojima, and A. L. Demain.** 1998. Antifungal activities of rapamycin and its derivatives, prolylrapamycin, 32-desmethylrapamycin, and 32-desmethoxyrapamycin. *J. Antibiot.* **51**:487–491.
74. **Zheng, X.-F., D. Fiorentino, J. Chen, G. R. Crabtree, and S. L. Schreiber.** 1995. TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. *Cell* **82**:121–130.
75. **Zheng, X. F., and S. L. Schreiber.** 1997. Target of rapamycin proteins and their kinase activities are required for meiosis. *Proc. Natl. Acad. Sci. USA* **94**:3070–3075.