

Rapamycin and Less Immunosuppressive Analogs Are Toxic to *Candida albicans* and *Cryptococcus neoformans* via FKBP12-Dependent Inhibition of TOR

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***Candida albicans* and *Cryptococcus neoformans* cause both superficial and disseminated infections in humans. Current antifungal therapies for deep-seated infections are limited to amphotericin B, flucytosine, and azoles. A limitation is that commonly used azoles are fungistatic in vitro and in vivo. Our studies address the mechanisms of antifungal activity of the immunosuppressive drug rapamycin (sirolimus) and its analogs with decreased immunosuppressive activity. *C. albicans* *rbp1/rbp1* mutant strains lacking a homolog of the FK506-rapamycin target protein FKBP12 were found to be viable and resistant to rapamycin and its analogs. Rapamycin and analogs promoted FKBP12 binding to the wild-type Tor1 kinase but not to a rapamycin-resistant Tor1 mutant kinase (S1972R). FKBP12 and TOR mutations conferred resistance to rapamycin and its analogs in *C. albicans*, *C. neoformans*, and *Saccharomyces cerevisiae*. Our findings demonstrate the antifungal activity of rapamycin and rapamycin analogs is mediated via conserved complexes with FKBP12 and Tor kinase homologs in divergent yeasts. Taken together with our observations that rapamycin and its analogs are fungicidal and that spontaneous drug resistance occurs at a low rate, these mechanistic findings support continued investigation of rapamycin analogs as novel antifungal agents.**

Cryptococcus neoformans and *Candida albicans* are two common opportunistic fungal pathogens. Current antifungal agents in clinical use include amphotericin B, fluconazole, and flucytosine (29), which have side effects, lack fungicidal activity, or lack activity against emerging resistant mutants (66). Thus, additional antifungal agents are needed.

Several new antifungal agents are in development. The candins are 1,3- β -glucan synthase inhibitors and are potently active against *Candida* species and *Aspergillus fumigatus* (23, 40, 51, 52). The pneumocandin caspofungin acetate–MK-0991 is in phase III clinical trials for candida infections and has been approved for refractory aspergillosis. Broad-spectrum triazoles, including voriconazole, posaconazole, and ravuconazole, are being studied in human trials (50). Combination therapy with different antifungal agents may also improve therapy (20, 28, 62).

Rapamycin (sirolimus) is a natural product of the bacterium *Streptomyces hygroscopicus* originally discovered in a screen for antimicrobial activity against *C. albicans* and later found to have potent immunosuppressive activity (4, 63). Rapamycin diffuses into the cell and associates with the peptidyl-prolyl isomerase FKBP12. Rapamycin inhibits FKBP12 enzymatic activity; however, this inhibition is not related to

immunosuppressive or antifungal activity. The targets of the FKBP12-rapamycin complex are the TOR kinases. Two TOR proteins, Tor1 and Tor2, have been characterized in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, and a single TOR homolog has been identified in *C. albicans*, *C. neoformans*, *Drosophila melanogaster*, and humans (10, 18, 33, 39, 49, 56, 64). The Tor kinase has been functionally conserved from yeast to humans (2). Both immunosuppression in mammalian cells and the toxicity of rapamycin in *S. cerevisiae* and *C. neoformans* are mediated via FKBP12-dependent inhibition of Tor kinases (12, 18, 33, 34, 41). The FKBP12-rapamycin complex binds to a small region on Tor (the FKBP12-rapamycin binding domain [FRB domain]) adjacent to the carboxy-terminal kinase domain (17). Mutations in the FRB domain prevent FKBP12-rapamycin binding to TOR and confer rapamycin resistance (12, 15, 18, 34, 41, 59, 69). In addition, TOR proteins have a toxic effector domain that, when overexpressed, arrests cell growth (3).

Recent studies in the model yeasts *S. cerevisiae* and *S. pombe* reveal that the Tor kinases function in a nutrient-sensing pathway (reviewed in reference 54). Inhibition of Tor signaling by FKBP12-rapamycin induces autophagy (1, 38, 46), represses ribosomal protein gene expression (14, 53, 68), induces nitrogen catabolite-repressed genes (6, 8, 14, 31), and inhibits translation (5, 7). In *S. pombe*, rapamycin blocks mating in response to nutrient limitation (65). The Tor2 kinase is essential in *S. pombe* and *S. cerevisiae*, whereas in *S. pombe* the Tor1 kinase is required for mating and stationary-phase entry (64). Thus,

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TABLE 1. Strains used in this study

Strain	Genotype ^a	Source or reference
<i>C. albicans</i>		
SC5314	WT	27
BWP17	<i>ura3Δ::imm34/ura3Δ::imm34 his1::hisG/his1::hisG arg4::hisG/larg4::hisG</i>	66a
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	27
YJM126	<i>ade2/ade2 ura3Δ::ADE2/ura3Δ::ADE2</i>	This study
YAG116	<i>ura3Δ::imm434/ura3Δ::imm434 rbp1::CaURA3MX3F/RBP1</i>	This study
YAG134	<i>ura3Δ::imm434/ura3Δ::imm434 rbp1::MX3/RBP1</i>	This study
YAG171	<i>ura3Δ::imm434/ura3Δ::imm434 rbp1::MX3/rbp1::CaURA3MX3R</i>	This study
JRB12	SC5314 <i>TOR1-1/TOR1</i>	This study
JRB21	BWP17 <i>TOR1-2/TOR1</i>	This study
<i>C. neoformans</i>		
H99	Serotype A <i>MATα</i>	61
M049	H99 <i>ade2</i>	61
MCC1	M049 <i>frr1::ADE2 ade2</i>	18
JEC21	Serotype D <i>MATα</i>	43
CN111	JEC21 <i>TOR1-1</i>	18
<i>S. cerevisiae</i>		
JK9-3dα	<i>MATα trp1 his4 leu2-3,112 ura3-52 rme1 GAL⁺HMLa</i>	33
JHY3-3B	JK9-3dα <i>fpr1::URA3</i>	33
R17	JK9-3dα <i>TOR2-1 HIS⁺</i>	33
Y190	<i>MATa trp1-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80 URA3::GAL-lacZ LYS2::GAL-HIS3</i>	32
SMY4-1	Y190 <i>TOR1-3 fpr1::ADE2</i>	16

^a WT, wild type.

the TOR pathway likely serves as a global nutrient-sensing pathway that will be conserved in pathogenic fungi.

Rapamycin inhibits the growth of several fungi, including *C. neoformans*, *C. albicans*, *Candida stelloidea*, *A. fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*, and *Penicillium* sp. (18, 24, 47, 67). We are interested in FKBP12 and TOR as targets for novel antifungal agents. The *C. albicans* *RBP1* gene encoding a homolog of the target protein FKBP12 was previously identified (25). We isolated *C. albicans* *rbp1/rbp1* and also *TOR1-1/TOR1* mutant strains that were viable and rapamycin resistant. These findings, and results using the yeast two-hybrid assay, demonstrate that rapamycin antifungal activity is exerted via FKBP12 and Tor1 homologs in *C. albicans*. In addition, we analyzed the antifungal activity of rapamycin analogs with reduced immunosuppressive activity against *C. albicans*, *C. neoformans*, and *S. cerevisiae* (22). Two analogs retain antifungal activity in vitro and promote FKBP12-Tor complexes. Further examination of rapamycin analogs as potential antifungal agents is warranted.

MATERIALS AND METHODS

Media, drugs, and strains. Media were prepared as previously described (55). 5-Fluoroorotic acid (5-FOA) medium was prepared as described previously (9) by using 625 μg of 5-FOA/ml and replacing uracil with 100 μg of uridine/ml. Rapamycin and its analogs (Abbott Labs) were added to media or disks from stock solutions in 90% ethanol–10% Tween 20. Strains used are listed in Table 1.

***RBP1* insertion-deletion constructs.** The *RBP1* disruption plasmids pAG70 and pAG71 contain the *CaURA3MX3* (30) disruption cassette replacing nucleotides (nt) 40 to 189 of the *RBP1* open reading frame (ORF). These plasmids are identical except that the *CaURA3MX3* cassettes are in an opposite orientation.

The *RBP1* disruption plasmids were constructed in three steps. First, the genomic *RBP1* locus and ~900 bp of flanking sequence was PCR amplified from the uracil auxotrophic strain YJM126 and subcloned into pGEM-T Easy (Promega), resulting in plasmid pAG54. Next, the *NotI* insert was subcloned into the *NotI* site of the yeast-*E. coli* shuttle vector pRS314 (*CEN ARS TRP1* [58]), resulting in plasmid pAG67. Finally, the *CaURA3MX3* disruption cassette was subcloned into plasmid pAG67 by in vivo PCR-directed recombination.

Primers. The primers used were as follows: JM37, CCTCGACATCATCTG CCC; PR80, CAAGGAGTCAACCACCCTAAG; PR83, TCTGAGTCTGGG TGTGGGTC; PR92, CAGCACTGGAAGGTATGAGTG; PR103, ATGTCTG AAGAAGTCCACAAATTGAAATTGTTCAAGAAGCAGCTGAAGCTTC GTACGC; PR104, GCACCACCTTTACCATAATTGTTAGTTAAAGAAA TATGCATAGGCCACTAGTGGATCTG; PR130, ATGTCTGAAGAAGCTT CCACAAATTGAAATTGTTCAAGGCATAGGCCACTAGTGGATCTG; PR131, TTAGCACCCCTTTACCATAATTGTTAGTTAAAGAAAATCA GCTGAAGCTTCGTACGC; JOHE2920, GTACGAGAATTCATGTCTGAA GAAGTCCACAA; JOHE2921, GCAACGGGATCCTTATTGACCATTAAAC ACCAAG; JOHE6244, TTTATGGCAGCAACAATGGCAGCATGCTTTGG AAGATGCTAGCAGGTTTTCTTTGGTGAACACAACACAGAAAAGAT GTTT; JOHE6245, TTTATGGCAGCAACAATGGCAGCATGCTTTGGAA GATGCTCGCAGGTTTTCTTTGGTGAACACAACACAGAAAAGATGT TT; JOHE6246, TTTATGGCAGCAACAATGGCAGCATGCTTTGGAAAGA TGCTATCAGGTTTTCTTTGGTGAACACAACACAGAAAAGATGTTT; JOHE6247, GGCAAGGTGTTCTTGAAGC; and JOHE6248, TACTTCTT ATTCCGATAGC.

PCR conditions. The *RBP1* ORF and ~900 nt of flanking genomic DNA was PCR amplified by using primers PR80 and PR83. The 50-μl reaction consisted of 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, 0.1% Triton X-100, 100 μg of genomic DNA, 0.5 μM PR80, 0.5 μM PR83, 0.2 mM concentrations of each deoxynucleoside triphosphate (dNTP), and 0.5 U of Vent polymerase (New England Biolabs). DNA amplification was initiated with a 4-min denaturation at 94°C followed by 30 amplification cycles (94°C for 1 min, 55°C for 15 s, and 72°C for 3 min) and was terminated with a 7-min 72°C extension.

The *CaURA3MX3* cassettes were PCR amplified for in vivo PCR-directed recombination by using the primer pairs PR103-PR104 (for the forward orientation of the disruption cassette) and PR130-PR131 (for the reverse orientation). These primers amplify the *CaURA3MX3* cassette (from plasmid pAG61 [30]) by adding 5'-terminal extensions homologous to nt 1 to 39 (PR103 and PR131) and 190 to 226 (PR102 and PR130) of the *RBP1* ORF. Each 100-μl reaction consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.1 mM MgCl₂, 0.1% gelatin, 100 ng of pAG61, 0.5 μM concentrations of each primer, 0.2 μM concentrations of each dNTP, and 2.5 U of REDTAQ DNA Polymerase (Sigma). DNA amplification was initiated with a 3-min 94°C denaturation followed by 30 cycles (94°C for 30 s, 55°C for 10 s, and 72°C for 3 min) and was terminated with a 7-min 72°C extension.

***RBP1* disruption.** The gene disruption cassettes from plasmids pAG70 and pAG71 were PCR amplified as described for the *RBP1* locus except that the denaturation time was reduced to 1 min. To disrupt the first *RBP1* ORF, the ~3.5-kb PCR product was transformed into *C. albicans* CAI4 (27) by electroporation (60). Prototrophic transformants were selected on minimal medium (synthetic dextrose [SD]). Homologous disruptants were verified by colony PCR (45) using primers PR92 and JM37 and Southern blotting. A resulting *rbp1::CaURA3MX3F/RBP1* strain, YAG116, was chosen.

To select for loss of the *CaURA3F* ORF in strain YAG116, these cells were grown overnight in rich medium plus uridine, ~10⁵ cells were plated on 5-FOA medium, and 5-FOA-resistant cells were assayed by Southern blotting and colony PCR with primers PR80 and PR92 to verify the loss of the *CaURA3* ORF. A resulting *rbp1::MX3/RBP1* strain, YAG134, was used for a second round of gene disruption using the *RBP1* disruption construct from plasmid pAG71. As above, homologous disruptants were verified by colony PCR with primers PR80 and JM37 and Southern blotting. Strain YAG171 was shown to have the genotype *rbp1::MX3/rbp1::CaURA3MX3R* (Fig. 1B and C).

Mutagenesis of *C. albicans* TOR1. *C. albicans* *TOR1-1* and also *TOR1-2* mutant strains (JRB12 and JRB21) were created by transformation of strain SC5314 or BWP17 with 81-mer oligonucleotides designed to carry the wild-type *TOR1* sequence or a single base pair change (A→C or G→T) in the Tor1 FRB domain which result in single amino acid substitutions (S→R or S→I) and also disrupt an *NheI* restriction site. The wild-type partial sequence of *C. albicans* Tor1 was found in the Genome Project at Stanford University. Transformants were selected on yeast extract-peptone-dextrose (YPD) plates containing 0.1 μg of rapamycin/ml. Multiple resistant isolates were obtained with the mutagenic oligonucleotides, whereas none were obtained with the wild-type oligonucleotide.

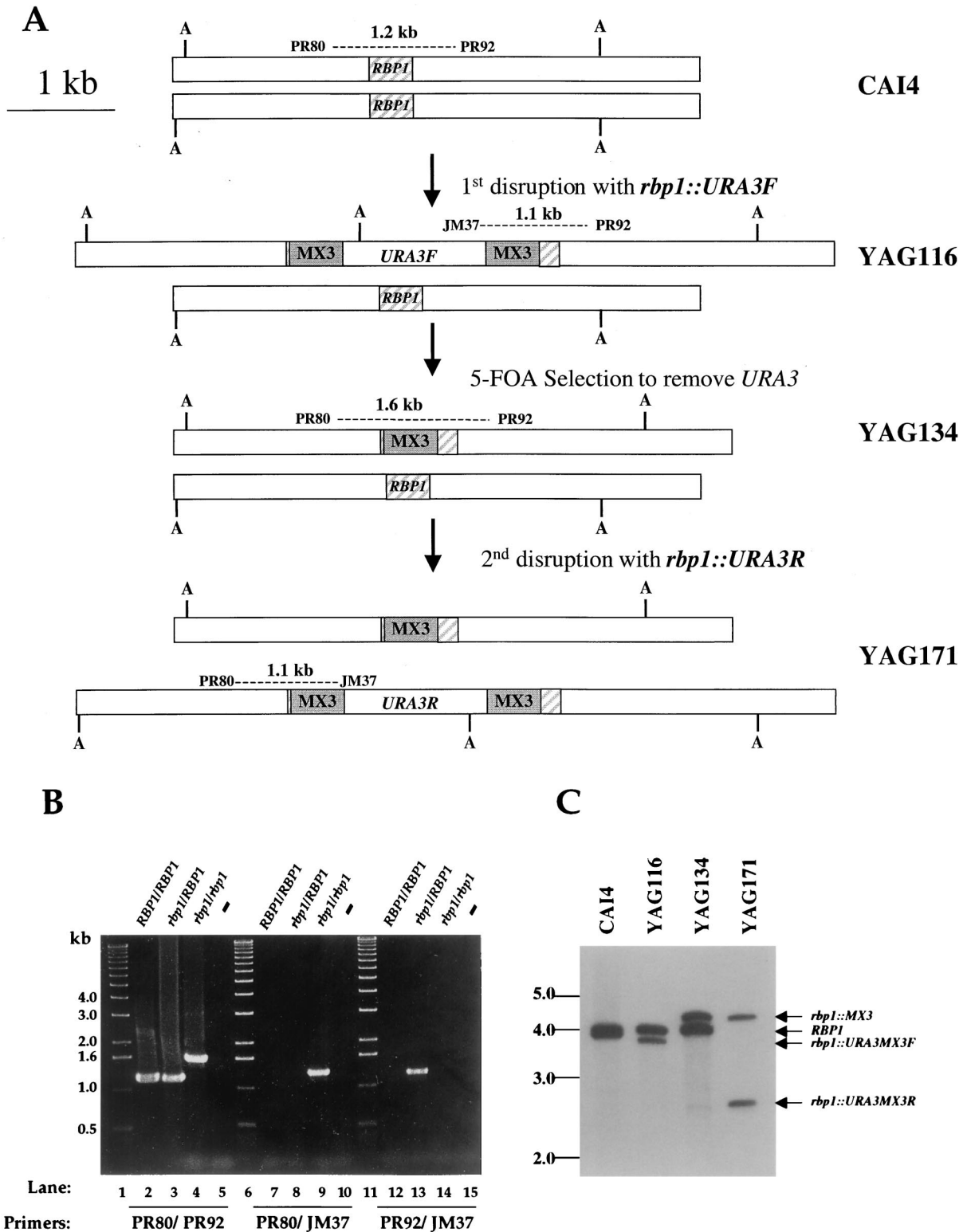


FIG. 1. Disruption of the *C. albicans* *RBP1* genes encoding FKBP12. (A) Disruption alleles of the *RBP1* gene. The *RBP1* gene is depicted on a 5-kb genomic fragment including the 375-bp *RBP1* ORF. One *RBP1* wild-type allele was replaced with the *CaURA3MX3* forward cassette, followed by treatment with 5-FOA to counterselect *URA3*. A second transformation replaced the second *RBP1* allele with the *CaURA3MX3* reverse cassette. Dashed lines represent PCR products used to verify homologous replacement of *RBP1*. Gray boxes indicate *MX3* direct repeats. (B) PCR verification of homologous integration of insertion. Primers PR80 and PR92 amplified an ~1.2-kb product containing the *RBP1* ORF. Primers PR80 and JM37 amplified the *rbp1::CaURA3MX3R* allele. Primers PR92 and JM37 amplified the *rbp1::CaURA3MX3F* allele. Lanes: 1, 6, and 11, 1-kb markers; 2, 7, and 12, *RBP1/RBP1* strain CAI4; 3, 8, and 13, *rbp1/RBP1* strain YAG116; 4, 9, and 14, *rbp1/rbp1* strain YAG171; 5, 10, and 15, no-DNA controls. (C) Southern analysis of *RBP1* and *rbp1* alleles. Genomic DNA from the wild-type *RBP1/RBP1* strain CAI4,

These mutants were not cross-resistant to FK506 plus fluconazole and are therefore not *rbp1/rbp1* mutants. Homologous recombination was verified by colony PCR with primers JOHE 6247 and JOHE 6248, followed by digestion of the 1.5-kb PCR product with the restriction enzyme *NheI*. The presence of a 1.5-kb uncleaved fragment indicated the presence of the mutant *TOR1-1* allele. Genomic DNA flanking the *TOR1* and *TOR1-1* alleles was isolated by PCR from genomic DNA from strain JRB12 and cloned, and restriction digestion with *NheI* and sequence analysis of multiple clones revealed five wild-type and six mutant alleles of the eleven analyzed. A similar analysis of the wild-type *TOR1* strain SC5314 yielded six wild-type and no mutant *TOR1* sequences.

Two-hybrid assays. The yeast *S. cerevisiae* two-hybrid strain used was SMY4 (Y190 *TOR1-3 fpr1::ADE2*) (16). The two-hybrid fusion plasmids used were pGAD424 and pGBT9 (26). The *C. albicans* *RBPI* gene was fused to the GAL4 activation domain [GAL4(AD)] in plasmid pGAD424. First, the *RBPI* gene was amplified with primers JOHE2920 and JOHE2921 by using plasmid pAG54 as a template. The PCR product was cleaved with *EcoRI* and *BamHI* and cloned in the corresponding sites in plasmid pGAD424, yielding plasmid pMCC4. Plasmids pML80 (*TOR1*) and pML82 (*TOR1-1*) expressing the *S. cerevisiae* *TOR1* gene or the *TOR1-1* mutant allele, respectively, fused to the GAL4 DNA-binding domain [GAL4(BD)] in plasmid pGBT9 were as described previously (41). Plasmids expressing the GAL4(BD)-*C. neoformans* *TOR1* FRB domain, the GAL4(BD)-*C. neoformans* *TOR1-1* mutant FRB domain, and the GAL4(AD)-*C. neoformans* FKBP12 were as described previously (18). The two-hybrid strain SMY4 was cotransformed with the two-hybrid fusion plasmids, cells were grown on SD-Leu-Trp synthetic medium, and the β -galactosidase activity was assayed by using the chromogenic substrate chlorophenol- β -D-galactopyranoside (CPRG) (16). For two-hybrid growth assays, cells were resuspended in top agar (0.7% Bacto agar in water) on the surface of synthetic SD-Leu-Trp-His medium with 10 mM 3-aminotriazole. Disks without or with 1 μ g of rapamycin or an analog were placed on the surface, and cells were incubated for 2 to 3 days at 30°C. Colony formation indicated GAL4-dependent expression of the *GAL-HIS3* reporter gene fusion by FKBP12-ligand-TOR interactions.

Growth inhibition assays. *C. albicans*, *C. neoformans*, or *S. cerevisiae* cells were cultured in YPD liquid medium overnight, and $\sim 10^6$ cells were resuspended in 3 ml of top agar (0.7% Bacto agar in water), which was poured onto the surface of YPD medium (90-mm plates) and allowed to solidify. Disks without or with 1 μ g of rapamycin or an analog were placed on the surface. Cultures were incubated for 24 to 36 h at 30°C. Experiments to determine MICs and minimal fungicidal concentrations (MFCs) were performed by the broth microdilution method according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) (44). The optical density at 600 nm (OD₆₀₀) was measured with a Beckman spectrophotometer after incubation for 48 h (*C. albicans*) or 72 h (*C. neoformans*) at 30°C. The MIC was defined as the lowest drug concentration in which a visual turbidity of $\leq 80\%$ inhibition was observed compared to that produced by the growth control. The MFC was determined as previously described (21). Briefly, 100- μ l aliquots from wells with growth inhibition were plated onto Sabouraud agar plates. The lowest concentration that yielded three or fewer colonies was recorded as the MFC.

RESULTS

Disruption of the *C. albicans* *RBPI* genes by homologous recombination. *S. cerevisiae* cells treated with rapamycin irreversibly arrest growth, and the effect of rapamycin is mediated by the prolyl-isomerase FKBP12 (33). The FKBP12-rapamycin complex binds to and inhibits the TOR kinases, and strains that lack FKBP12 are viable and rapamycin resistant (33). The human pathogenic yeast *C. albicans* is markedly sensitive to rapamycin (63). We found that rapamycin has fungicidal activity (Table 2), and in a standard Luria-Delbrück fluctuation test the spontaneous rate of rapamycin resistance was found to be low (~ 1 in 10^8 cells). The presumed target of rapamycin in this

fungus is the FKBP12 homolog encoded by the *RBPI* gene (25). To test this hypothesis, a homozygous *rbp1/rbp1* mutant strain was constructed.

C. albicans is diploid, and both *RBPI* alleles were mutated by replacing 150 nt within the *RBPI* ORF (i.e., nt 40 to 189) with the *URA3*-containing disruption cassette *CaURA3MX3* (30). To ensure that gene disruption was the result of two independent events, two different *RBPI* disruption plasmids were constructed in which nt 40 through 189 of the *RBPI* ORF were replaced with the *CaURA3MX3* cassette in opposite orientations (Fig. 1A). Ca. 900 bp of genomic sequence flanking the *RBPI* ORF were present to facilitate homologous recombination. The *rbp1 Δ ::CaURA3MX3* allele in the forward orientation (Fig. 1A) was PCR amplified and electroporated into the *ura3/ura3* strain CAI4 to generate the *rbp1::CaURA3MXF/RBPI* heterozygous mutant strain, YAG116. To reuse the *CaURA3MX3* cassette for the second round of gene deletion, the *rbp1/RBPI* YAG116 cells were treated with 5-FOA to select for loss of the *URA3* ORF by recombination of the flanking direct repeats (designated as *MX3*). A 5-FOA resistant, uracil auxotrophic *rbp1::MX3/RBPI* strain, YAG134, was transformed with the *rbp1::CaURA3MX3* allele in the reverse orientation (Fig. 1A), resulting in the homozygous *rbp1/rbp1* mutant YAG171. The genotypes of wild-type (CAI4), *rbp1/RBPI* heterozygous (YAG116 and YAG134), and *rbp1/rbp1* homozygous (YAG171) strains were verified by both colony PCR (Fig. 1B) and Southern blotting (Fig. 1C).

***C. albicans* FKBP12 homolog Rbp1 is required for rapamycin antifungal action.** The FKBP12 homolog Rbp1 was found to mediate rapamycin action in *C. albicans*. The wild-type *RBPI/RBPI* parental strain and the *rbp1/RBPI* heterozygous mutant were sensitive to inhibition by rapamycin, whereas the *rbp1/rbp1* homozygous mutant was rapamycin resistant (Fig. 2A). The *rbp1/RBPI* heterozygous strain readily gave rise to rapamycin-resistant colonies. Consistent with the interpretation that these isolates arise from spontaneous homozygosis that produces *rbp1/rbp1* mutants by mitotic recombination, these isolates were prototrophic, cross-resistant to another drug that targets Rbp1 (FK506 plus fluconazole), and the majority lacked the wild-type *RBPI* gene by PCR analysis. In summary, the FKBP12 homolog Rbp1 mediates rapamycin antifungal activity against *C. albicans*.

The *rbp1/rbp1* mutant strain grew more slowly than the isogenic wild-type strain on rich medium. However, this growth defect was corrected when the medium was supplemented with uridine (400 μ M) and cytosine (3 μ g/ml), suggesting that this is the result of incomplete *Ura3* expression and not the *rbp1* mutation. *rbp1/rbp1* mutant strains had no defect in filamentous or invasive growth or growth at different temperatures or on minimal medium (not shown).

***C. albicans* *TOR1-1* mutation confers rapamycin resistance.** To test if the Tor1 kinase is the target of the FKBP12-rapamycin complex, two different point mutations were introduced

heterozygous *rbp1/RBPI* strains YAG116 and YAG134, and homozygous *rbp1/rbp1* strain YAG171 was cleaved with *AflIII* (A), electrophoresed in a 0.8% gel, transferred to a nylon membrane, and hybridized to a random-primed ³²P-labeled 375-bp gel-purified fragment spanning the *RBPI* gene. The probe hybridizes to the *RBPI* wild-type alleles in strain CAI4 (4.0 and 3.9 kb), to *rbp1::URA3MX3F* and *RBPI* alleles in strain YAG116 (3.6 and 4.0 kb), to *rbp1::MX3* and *RBPI* alleles in strain YAG134 (4.2 and 4.0 kb), and to *rbp1::MX3* and *rbp1::URA3MX3R* alleles in strain YAG171 (4.2 and 2.7 kb). The positions of DNA markers are shown on the left in kilobases.

TABLE 2. FKBP12 and TOR mutations confer resistance to rapamycin and analogs^a

Strain ^a	MICs and MFCs ($\mu\text{g/ml}$) ^b of:									
	Rapamycin		Analog							
	MIC	MFC	2		18		19		23	
			MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
<i>C. neoformans</i> WT	0.19	0.39	3.12	3.12	>100	>100	>100	>100	1.56	3.12
<i>C. neoformans frr1</i>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>C. neoformans TOR1-1</i>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>C. albicans</i> WT	<0.09	0.39	6.25	12.5	>100	>100	>100	>100	0.78	0.78
<i>C. albicans rbp1/rbp1</i>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>C. albicans TOR1-1/TOR1</i>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

^a WT, wild type.^b Drug dilutions were from 100 to 0.09 $\mu\text{g/ml}$.

into the FRB domain of *C. albicans* Tor1 to result in an amino acid substitution (serine to isoleucine or arginine) at a serine residue implicated in rapamycin-FKBP12 binding to fungal and mammalian Tor proteins (11, 12, 15, 17, 18, 34, 41, 59, 69).

These amino acid substitutions were introduced into the *C. albicans TOR1* gene by transformation with a single-stranded mutagenic 80-mer oligonucleotide and selection on rapamycin-containing medium. PCR amplification with primers

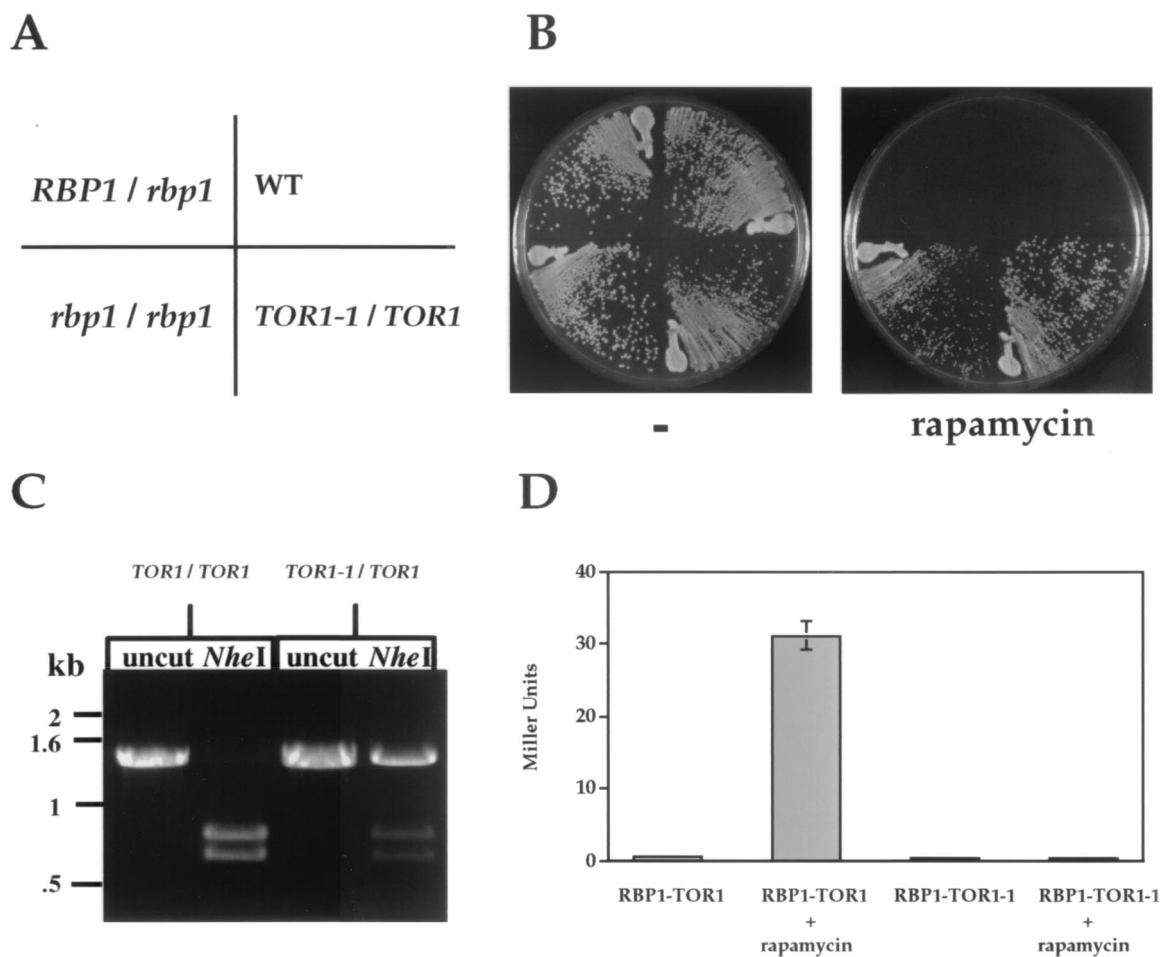


FIG. 2. *C. albicans rbp1/rbp1* and *TOR1-1/TOR1* mutants are rapamycin resistant. (A and B) The wild-type (SC5314), *rbp1/RBP1* heterozygous (YAG116), *rbp1/rbp1* homozygous (YAG171), and *TOR1-1/TOR1* heterozygous (JRB12) mutants were grown on YPD medium without or with 1 μg of rapamycin/ml for 24 h at 30°C. (C) Genomic DNA from the wild type (SC5314) and the *TOR1-1/TOR1* mutant strain (JRB12) was PCR amplified, cleaved with *NheI*, and electrophoresed, demonstrating that strain JRB12 is heterozygous for the *TOR1-1* and *TOR1* alleles. (D) *C. albicans* FKBP12 homolog binds rapamycin and forms a complex with Tor1. The *S. cerevisiae* GAL4(BD)-TOR1 wild-type and S197R mutant fusions were coexpressed with the *C. albicans* GAL4(AD)-FKBP12 fusion protein (Rbp1) in the two-hybrid host strain SMY4 (*fpr1 TOR1-3*), with or without rapamycin (1 $\mu\text{g/ml}$). β -Galactosidase activity was measured by CPRG assay in duplicate.

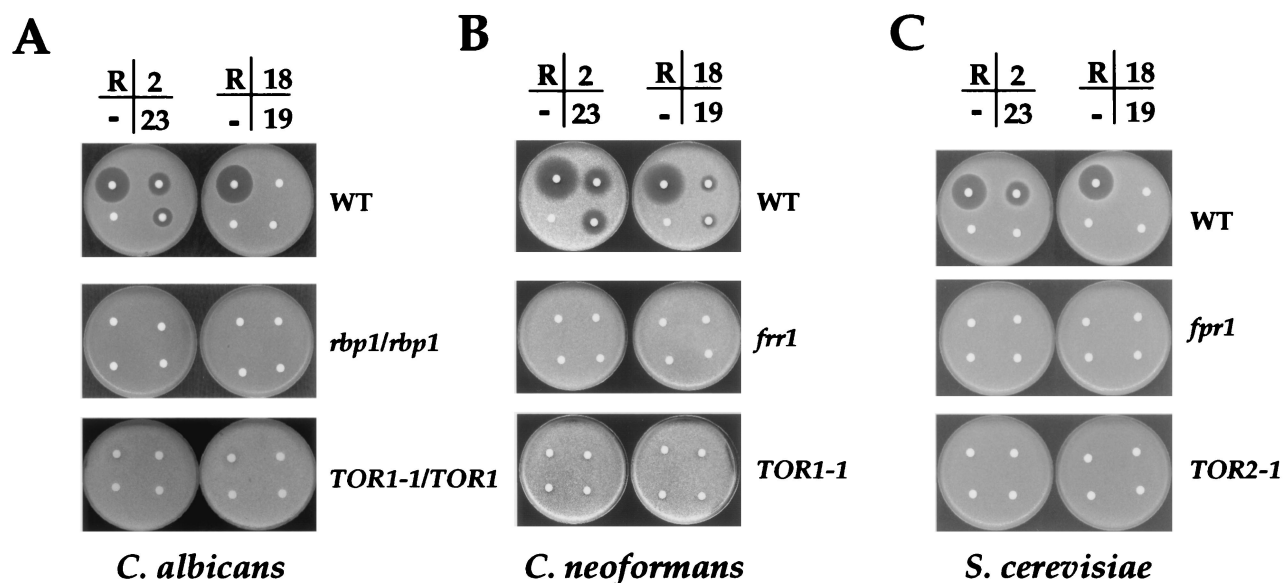


FIG. 3. The antifungal activity of rapamycin and its analogs is mediated via FKBP12 and TOR proteins. *C. albicans* (A), *C. neoformans* (B), and *S. cerevisiae* (C) wild-type and FKBP12 (*rbp1/rbp1*, *frr1*, or *fpr1*) and TOR (*TOR1-1/TOR1*, *TOR1-1*, and *TOR2-1*) mutant strains were grown in YPD liquid medium overnight, and $\sim 10^6$ cells were resuspended in the top agar on the surface of YPD medium. Disks without (-) or with 1 μ g of rapamycin (R) or analogs 2, 18, 19, and 23 were placed on the surface of the medium and incubated for 24 to 36 h at 30°C.

flanking the site of mutation and digestion with *NheI* (a site present in wild-type *TOR1* is destroyed by the introduced mutation) revealed that both wild-type *TOR1* and the *TOR1-1* or *TOR1-2* mutant allele were present (Fig. 2C and data not shown). The PCR products for strain JRB12 (*TOR1-1/TOR1*) were cloned; of 11 clones, 5 were digested by *NheI* and contained the wild-type *TOR1* sequence, whereas 6 were *NheI* resistant and contained the mutant sequence. The resulting heterozygous *TOR1-1/TOR1* (JRB12) and *TOR1-2/TOR1* (JRB21) cells were viable and rapamycin resistant, indicating that the Tor1 kinase is the target of the Rbp1-rapamycin complex and that the *TOR1-1* and *TOR1-2* mutations confer dominant drug resistance (Fig. 2A).

***C. albicans* FKBP12 homolog binds to wild-type Tor1 but not to a Tor1-1 mutant.** The yeast two-hybrid system was used to test whether the *C. albicans* FKBP12 homolog Rbp1 interacts with the Tor1 protein. Protein-drug-protein interactions were monitored by measuring β -galactosidase expression from a *GAL-lacZ* reporter gene (Fig. 2D). Rapamycin-dependent interactions were detected between wild-type *S. cerevisiae* Tor1 and the *C. albicans* FKBP12 homolog Rbp1. In contrast, the *S. cerevisiae* rapamycin-resistant Tor1-1 S1972R mutant protein failed to interact with FKBP12-rapamycin (Fig. 2D). The S1972R mutation does not affect the stability of the Tor1-1 mutant as previously reported (41). Thus, the S1972R mutation prevents the formation of the FKBP12-rapamycin-TOR complex. These findings indicate that the *C. albicans* FKBP12 homolog Rbp1 forms a complex with rapamycin that interacts with the TOR kinase at the FRB domain in vivo.

Nonimmunosuppressive rapamycin analogs are toxic to *C. albicans*, *C. neoformans*, and *S. cerevisiae* via FKBP12-dependent inhibition of TOR. Previously, several rapamycin derivatives were prepared, and their immunosuppressive and antifungal activities were determined (22). Analog 2 is 1,2,3,4-tetrahydro-rapamycin. Analogs 18 [(*S*)-2-methyl-thienyl], 19

[(*S*)-NOHCOOibu], and 23 [(*S*)-NOHCON-piperidyl] are 7-substituted rapamycin analogs. We assessed the fungal activities of these rapamycin analogs with diminished immunosuppressive activities by determining MICs and MFCs using the NCCLS criteria, as well as a drug diffusion assay. Analog 2 was fungicidal to wild-type *C. albicans*, *C. neoformans*, and *S. cerevisiae* but not to the isogenic FKBP12 or TOR mutants (Fig. 3, Table 2). Analog 23 was also toxic and fungicidal to *C. albicans* and *C. neoformans* via FKBP12-dependent inhibition of Tor but showed no activity against *S. cerevisiae* (Fig. 3). Analogs 18 and 19 were weakly toxic to *C. neoformans* in the drug diffusion assay (Fig. 3B); however, the MIC was >100 μ g/ml (Table 2). Analogs 18 and 19 had no activity against either *C. albicans* or *S. cerevisiae* (Fig. 3A and C; Table 2). In summary, analogs 2 and 23 are the most toxic to *C. neoformans* and *C. albicans*, and they share a common mechanism of antifungal action with rapamycin involving FKBP12-dependent inhibition of Tor kinases.

The yeast two-hybrid system was used to test whether rapamycin analogs mediate cryptococcal FKBP12-TOR interactions similar to the way rapamycin does. A two-hybrid host strain whose growth is rendered resistant to rapamycin by a *TOR1-3* mutation and which lacks endogenous yeast FKBP12 was employed for these studies (SMY4-1). This specialized two-hybrid reporter strain was transformed with plasmids expressing the *C. neoformans* FKBP12 fused to GAL4(BD) [GAL4(BD)-FKBP12] and the *C. neoformans* Tor1 wild-type protein or the Tor1-1 mutant protein fused to the protein GAL4(AD) [GAL4(AD)-TOR1]. A large halo of colonies was observed surrounding the disks containing rapamycin (Fig. 4A), which is indicative of GAL4-dependent expression of the *GAL-HIS3* reporter gene fusion as a consequence of FKBP12-rapamycin-Tor1 interactions. Rapamycin analogs 2 and 23, and to a lesser extent analogs 18 and 19, also promoted this interaction (Fig. 4A). These observations were quantitated by mon-

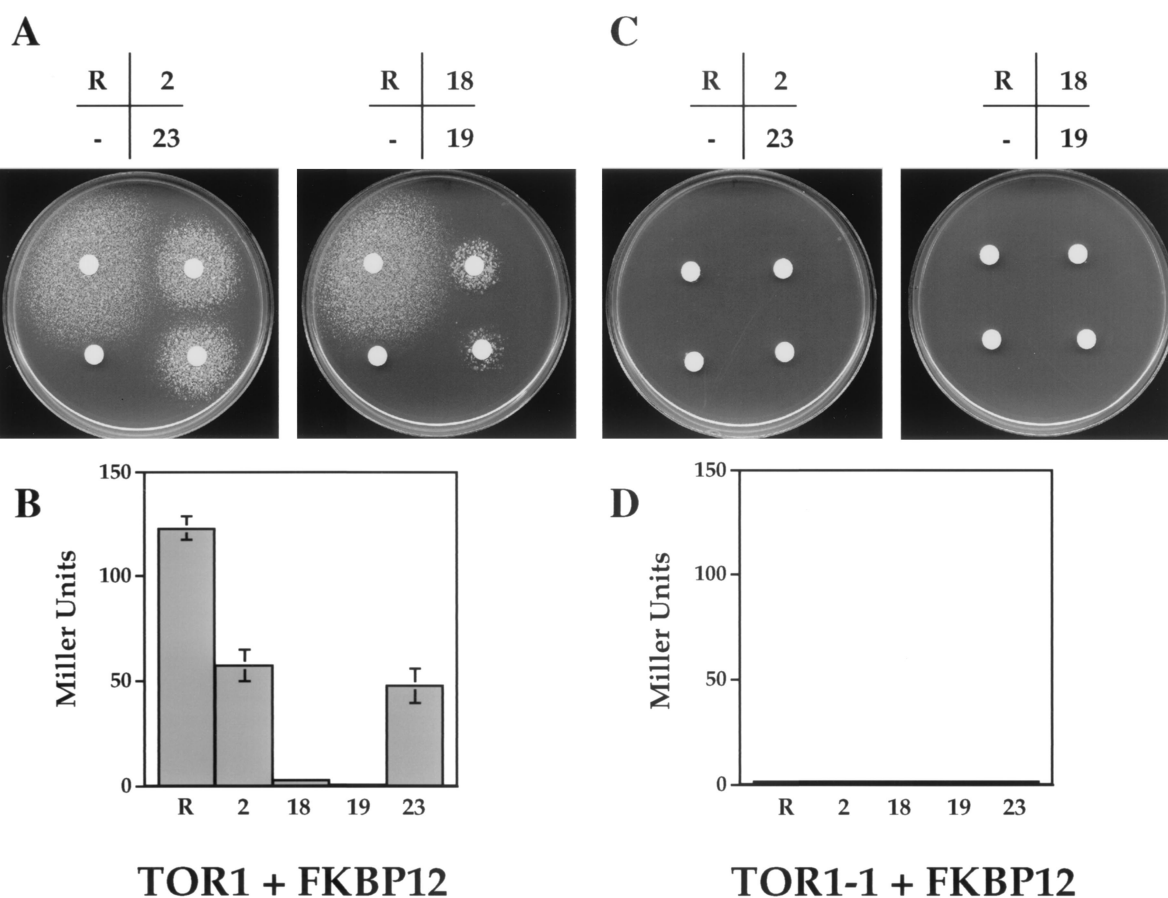


FIG. 4. Rapamycin and analogs promote FKBP12-TOR interactions. The wild-type GAL4(BD)-TOR1 FRB (A and B) and the mutant GAL4(BD)-TOR1-1 FRB (Ser1862Leu) domains (C and D) were coexpressed with the GAL4(AD)-FKBP12 fusion protein in the two-hybrid host SMY4 (*fpr1::hisG TOR1-3*). (A and C) Cells were resuspended in top agar on the surface of medium lacking histidine and disks bearing rapamycin, the analogs 2, 28, 19, or 23, or no drug were placed on the surface. Growth indicates GAL4-dependent expression of the *GAL-HIS3* reporter gene and results from the formation of FKBP12-drug-Tor complexes. (B and D) β -Galactosidase activity was measured by the CPRG assay and was determined in duplicate.

itoring expression of the *GAL-lacZ* reporter gene (Fig. 4B). A similar concentration of analogs 2 and 23 promoted ~50% the level of reporter gene expression compared to rapamycin itself, whereas analogs 18 and 19 had significantly less activity. No growth on medium lacking histidine or the *GAL-lacZ* reporter gene was observed when a mutation that prevents FKBP12-rapamycin binding to TOR was introduced into the GAL4-TOR1 fusion protein (Fig. 4C and D). In summary, rapamycin analogs 2 and 23 promote FKBP12 binding to the wild-type Tor1 kinase but not to a Tor1-1 mutant.

DISCUSSION

The identification of novel drug targets is of great importance in the field of medical mycology. We have previously demonstrated that rapamycin is toxic to *C. neoformans* via FKBP12-rapamycin inhibition of the Tor1 kinase (18). Here we assessed the antifungal activities and mechanisms of action of rapamycin and its analogs with diminished immunosuppressive activities against *C. neoformans* and *C. albicans*.

We established that rapamycin and two less immunosuppressive analogs are toxic to *C. neoformans* and *C. albicans* via FKBP12-dependent inhibition of Tor1. First, we constructed a

C. albicans rbp1/rbp1 mutant by a sequential two-step method by using two different *rbp1::URA3* disruption alleles in which the marker gene was in opposite orientations. Second, point mutations were introduced into the FRB domain of the *C. albicans* Tor1 homolog by transformation with mutagenic 80-mer oligonucleotides. Both classes of mutants are resistant to rapamycin and its analogs, indicating that antifungal activity is mediated via binding to the *C. albicans* FKBP12 homolog Rbp1 and Tor1. Third, rapamycin promoted interactions between the *C. albicans* FKBP12 homolog and wild-type *S. cerevisiae* Tor1 in the two-hybrid assay. Rapamycin analogs 2 and 23 also promoted strong interactions between the cryptococcal FKBP12 and Tor1 homologs. Our studies extend the understanding of the conserved antifungal mechanism of action of rapamycin and its analogs from the basidiomycete *C. neoformans* to the ascomycetous human pathogen *C. albicans*.

Although analogs 2 and 23, with the highest toxicity to *C. neoformans* and *C. albicans*, can only achieve <50% of the antifungal activity of rapamycin itself, they were previously found to be ~1,000-fold less immunosuppressive than rapamycin in a human T-cell proliferation assay (22). Additional work is warranted to develop or to test existing rapamycin ana-

logs (42) with a greater selective toxicity for lower eukaryotes. There is a clear advantage for the use of analogs of known immunosuppressive drugs, such as rapamycin, since there is a wealth of structural, molecular, and pharmacological information on the activity of these compounds (13, 37, 57). Rapamycin was approved by the Food and Drug Administration as an immunosuppressant for renal transplant recipients in August 1999. New studies are investigating the efficacy of using rapamycin alone or in combination with FK506 and cyclosporine A in other transplant conditions. Interestingly, we have found previously that cyclosporine A and FK506, and also analogs of these compounds with reduced or no immunosuppressive activity, are toxic to *C. neoformans* (19, 48). Additional advantages of rapamycin and its analogs are their oral activity, fungicidal activity, and the fact that spontaneous drug resistance is infrequent *in vitro*.

The use of rapamycin as an antifungal agent in an *in vivo* setting was reported by earlier studies wherein rapamycin was found to protect 50% of mice from an otherwise-lethal infection with *C. albicans* (4) and to improve the survival of mice with invasive aspergillosis (35, 36). These studies and our findings suggest that patients receiving rapamycin may benefit from both the immunosuppressive and the antifungal activities of rapamycin. It remains to be tested if rapamycin or its analogs with decreased immunosuppressive activity confer a beneficial effect in animal models of candidiasis and cryptococcal meningitis or in transplant recipients currently receiving rapamycin.

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