Rapamycin Sensitivity in *Saccharomyces cerevisiae* Is Mediated by a Peptidyl-Prolyl *cis-trans* Isomerase Related to Human FK506-Binding Protein

YIGAL KOLTIN,† LEO FAUCETTE, DERK J. BERGMSA, MARK A. LEVY, ROBERT CAFFERKEY, PAUL L. KOSER, RANDALL K. JOHNSON, and GEORGE P. LIVI*

Departments of Biomolecular Discovery, Molecular Genetics, Medicinal Chemistry, and Gene Expression Sciences, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

Received 24 September 1990/Accepted 7 December 1990

Rapamycin is a macrolide antifungal agent with structural similarity to FK506. It exhibits potent immunosuppressive properties analogous to those of both FK506 and cyclosporin A (CsA). Unlike FK506 and CsA, however, rapamycin does not inhibit the transcription of early T-cell activation genes, including interleukin-2, but instead appears to block downstream events leading to T-cell activation. FK506 and CsA receptor proteins (FKBP) and cyclophilin, respectively, have been identified and shown to be distinct members of a class of enzymes that possess peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. Despite the apparent differences in their mode of action, rapamycin and FK506 act as reciprocal antagonists in vivo and compete for binding to FKBP. As a means of rapidly identifying a target protein for rapamycin in vivo, we selected and genetically characterized rapamycin-resistant mutants of *Saccharomyces cerevisiae* and isolated a yeast genomic fragment that confers drug sensitivity. We demonstrate that the response to rapamycin in yeast cells is mediated by a gene encoding a 114-amino-acid, ~13-kDa protein which has a high degree of sequence homology with human FKBP; we designated this gene *RBPI* (for rapamycin-binding protein). The *RBPI* protein (RBP) was expressed in *Escherichia coli*, purified to homogeneity, and shown to catalyze peptidyl-prolyl isomerization of a synthetic peptide substrate. PPIase activity was completely inhibited by rapamycin and FK506 but not by CsA, indicating that both macrolides bind to the recombinant protein. Expression of human FKBP in rapamycin-resistant mutants restored rapamycin sensitivity, indicating a functional equivalence between the yeast and human enzymes.

Agents that inhibit T-cell activation include cyclosporin A (CsA) (19) and the recently discovered macrolide FK506 (31, 36). CsA was originally discovered as an antifungal agent, and FK506 was identified as an inhibitor of interleukin-2 (IL-2) production (20). Despite the structural dissimilarity between these two immunosuppressive drugs, recent reports suggest that the targets for both agents, cyclophilin and FK506-binding protein (FKBP), respectively, are peptidyl-prolyl *cis-trans* isomerases (PPIases), enzymes that promote protein folding in vitro (12, 15, 34, 35, 37, 40, 41). Although the endogenous function of PPIases is not known, the fact that the immunosuppressive action of CsA and FK506 is linked to inhibition of PPIase activity suggests that they may be required in the regulation of intracellular signaling events leading to T-cell activation (8, 12, 37).

Rapamycin, a macrolide antifungal agent with structural similarity to FK506 (32, 42), also exhibits immunosuppressive (3, 26, 38) as well as antineoplastic (9, 18) properties. Rapamycin and FK506 act as reciprocal antagonists in vivo (murine T cell activation [6]) and compete for binding to FKBP (15). Given these similarities, the mechanism of action of rapamycin remains enigmatic because, whereas FK506 (like CsA) acts to inhibit IL-2 transcription, rapamycin has no effect on the production of IL-2 but instead blocks later events required for T-cell activation (7).

Isolation and genetic characterization of rapamycin-resistant yeast mutants. *Saccharomyces cerevisiae* contains at least two genes which encode cyclophilin-related proteins (13, 21), one of which has been shown to possess PPIase activity (13) and to be the target for CsA (40). Cells of *S. cerevisiae* are exquisitely sensitive to rapamycin (10). In order to identify the target for rapamycin in yeast cells, we began by selecting rapamycin-resistant mutants in two sexuall compatible yeast strains with complementary nutritional markers (strains RS188 and DC6; Fig. 1). Spontaneous drug-resistant mutants arose in each strain at a frequency of ~10⁻⁷. The drug-resistant phenotype was specific for rapamycin and did not alter the cellular response to either CsA or FK506 (Fig. 1A), suggesting the presence of a unique drug target for rapamycin. Mutants were individually mated with the compatible drug-sensitive wild-type strain, and the resulting diploids were tested for sensitivity to rapamycin (Fig. 1B). Of 32 diploids formed with mutants of strain RS188 and 15 diploids formed with mutants of strain DC6, all were sensitive to rapamycin, suggesting that the mutations are recessive. This also suggests that rapamycin resistance results from the loss of function of a specific gene product(s) which normally acts as a drug target.

To determine the number of genes defined by our mutants, genetic complementation tests were performed by selecting diploids between all recessive mutants generated in both strains. All 480 diploids were found to be rapamycin resistant, suggesting that the mutations define a single gene. Sporulation and tetrad analysis (13 four-spore tetrads) of one of the diploids formed between two drug-resistant mutants yielded only drug-resistant progeny, indicating the presence of single gene mutations in those strains. We tentatively designated this gene *RBPI* (rapamycin-binding protein).

* Corresponding author.
† Present address: Department of Microbiology, Tel Aviv University, Ramat Aviv 69978, Tel Aviv, Israel.
Cloning and nucleotide sequence analysis of RBP1. To isolate the RBP1 gene, one drug-resistant mutant derived from RS188 was transformed with a yeast genomic library contained in the centromere-based vector YCP50, which carries the URA3 selectable marker (29). A total of 365 Ura+ transformants were assayed for growth in the presence of 40 ng of rapamycin per ml. One drug-sensitive transformant was isolated, and the activity of the transforming plasmid was confirmed by demonstrating cosegregation of rapamycin sensitivity and the Ura+ phenotype in cells grown in non-selective medium. The transforming plasmid contained an 8.1-kb insert (pRBP1; Fig. 2) and was found to confer drug sensitivity when reintroduced into a rapamycin-resistant mutant. Fragments of the insert were subcloned into YEp352 (17), and the rapamycin-responsive sequence was localized to a 2.6-kb region between the SacI and EcoRV sites (Fig. 2). (Chromosome blots indicated that this segment maps to chromosome XIV [data not shown].)

Confirmation that this region contains the active sequence was obtained by reverse genetics (30). Briefly, a 2.8-kb

![Figure 1](https://mcb.asm.org/)

**FIG. 1.** (A) Sensitivity of wild-type (WT) strain RS188 (MATa leu2-3,2-112 trp1-1 ura3-1 ade2-1 his3-11,15 can1-100; isogenic to strain W303-1a [1]) and a rapamycin-resistant mutant (M) derived from RS188 to FK506 (top row), CsA (middle row), and rapamycin (RAP, bottom row). The drug concentrations applied in each well (containing 100 μl) were (left to right): FK506, 100, 10, and 0.1 μg/ml; CsA, 1000, 100, 10, and 1 μg/ml; rapamycin, 80, 40, 20, and 10 ng/ml for the wild type and 8000, 800, 80, and 8 ng/ml for the mutant. (B) Sensitivity of wild-type (WT) strain DC6 (MATa leu2-3,2-112 his4 can1-100 gal2) (24), a resistant mutant (M) of RS188 selected on medium containing 40 ng of rapamycin per ml, and the diploid formed between the rapamycin-sensitive and -resistant strains (WT × M). The concentrations of rapamycin used to test the sensitivity of the wild-type and diploid strains were 160, 16, and 1.6 ng/ml, whereas 1600, 160, and 16 ng/ml were used for the mutant (lower left, top, and lower right, respectively).

![Figure 2](https://mcb.asm.org/)

**FIG. 2.** Restriction map of pRBP1 and representative genomic subclones used to identify the rapamycin-responsive region. The arrows indicate a region which was deleted and replaced with *S. cerevisiae* URA3. B, BamHI; P, PstI; Nc, NcoI; H, HindIII; Sc, SacI; K, KpnI; X, XhoI; N, NdeI; R, EcoRV; (B), BamHI/Sau3A.
FIG. 3. (A) Nucleotide sequence of RBP1, with the predicted amino acid sequence of the open reading frame. Coordinates on the right indicate nucleotide and amino acid positions. Asterisks show the termination codon. The underlined sequence indicates a putative TATAA element. Nucleotides in capital letters represent cDNA sequences, whereas lowercase letters denote genomic DNA sequences. Genomic and cDNA sequences were determined from both strands. (B) Alignment of the deduced amino acid sequence of the RBP1 gene product (RPB) with the sequence of human FKBP (hFKBP) (25, 35) and N. crassa FKB (nFKBP) (41). Dashes indicate identical amino acid sequences; periods indicate sequence gaps.

In order to rapidly identify the coding sequence, the 2.8-kb HindIII fragment (Fig. 2) was used to probe an S. cerevisiae DNA library made in AZAP (Stratagene). One cDNA clone was isolated containing a ~700-bp insert. DNA sequence analysis of this clone revealed a 342-bp open reading frame, which predicts a protein of 114 amino acids with a molecular mass of ~13,000 Da (Fig. 3A). The open reading frame is flanked by 5' and 3' untranslated sequences (UTRs) of 12 and 321 bp, respectively, with a putative poly(A) tract at the end of the 3' UTR. Analysis of the 5' UTR genomic sequence indicated the presence of a putative TATAA element at position -32 (Fig. 3A). The sequences adjacent to the AUG initiation codon match favorably with a consensus sequence (AAAAAAUGUCU) derived from the analysis of many yeast genes (5, 14).

**Analysis of the deduced RBP1 protein sequence.** Alignment of the predicted RBP1 protein sequence with the sequences of cloned PPlases from several other sources revealed that it contains striking homology to human FKB (24, 35) (Fig. 3B) as well as to an FKB from Neurospora crassa (41). In fact, greater than 53% of the deduced yeast protein sequence is identical to that of human FKB. Furthermore, the sequences of both proteins align precisely at their carboxyl-terminal ends, although the RBP1 protein contains six additional N-terminal amino acids (Fig. 3B). The RBP1 protein exhibits no significant homology to either yeast or human cyclophilin (13, 21, 37).
4-nitroanilide as rates are computer by 0.50 FK506 200 0.50 FK506 50 0.0055 2.3 0.50 Rapamycin 0.50 FK506 200 0.0055 2.3 0.50 FK506 400 0.0042

FIG. 4. Purification of yeast RBP expressed in E. coli. The RBP cDNA was enzymatically amplified by the polymerase chain reaction (PCR) (27) with oligonucleotide primers specific for the 5' and 3' sequences of the encoded protein. The PCR primers were equipped with NdEl or SacI restriction sites at their 5' ends to allow subcloning of the PCR-generated DNA fragment into the E. coli expression vector pMG1. Expression is driven by the phage p17 promoter, which is heat-inducible in the E. coli lysogenic strain AR58 (33). Cells carrying the RBP expression plasmid were grown in LB-ampicillin at 32°C, shifted to 42°C for 3 h, and harvested for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein products were subjected to 20% SDS-PAGE and visualized by staining with Coomassie blue. Lanes 1 and 6, Molecular weight standards (Bio-Rad); lane 2, complete E. coli extract, uninduced; lane 3, complete E. coli extract, induced; lane 4, supernatant from 20,000 × g centrifugation of ruptured cells, induced; lane 5, RBP following sequential chromatography on DEAE-Sepharose, CM-Sephadex treatment, and gel filtration on Sephadex G-50. All purification procedures were conducted at 2 to 6°C. E. coli cells containing the expressed RBP were suspended in 40 ml of buffer A (20 mM Tris, 5 mM β-mercaptoethanol, 5% glycerol [pH 8.5]) and ruptured by sonication on ice. The soluble fraction from a 20,000 × g centrifugation (lane 4) was applied to a column (2.5 by 18 cm) of DEAE-Sepharose equilibrated with buffer A. The column was washed with 100 ml of buffer A, and the RBP was eluted in a 0 to 400 mM NaCl gradient in buffer A over a total of 400 ml. At this stage, a rapamycin-sensitive PPlase activity (data not shown) was detected in fractions containing the expressed ~13-kDa protein by SDS-PAGE analysis. Combined fractions containing RBP were adjusted to pH 6.0 with acetic acid and applied to a column (2.5 by 15 cm) of CM-Sephadex Fast-Flow which had been equilibrated with 20 mM sodium phosphate (pH 6.0) buffer. The ~13-kDa protein eluted in the void volume upon washing with equilibration buffer. The RBP solution was concentrated by ultrafiltration (YM-5 membrane, Amicon Corp.) to 2.0 ml and fractionated on a column (1.5 by 85 cm) of Sephadex G-50-fine (20 mM sodium phosphate, 100 mM NaCl [pH 7.2]), yielding a sample of homogeneous RBP (lane 5).

TABLE 1. PPlase activity of purified yeast RBP

<table>
<thead>
<tr>
<th>Purified RBP (μg/ml)</th>
<th>Inhibitor</th>
<th>Inhibitor concn (nM)</th>
<th>Isomerization rate, k (s⁻¹)</th>
<th>% RBP PPlase activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>None</td>
<td>0.0042</td>
<td>NA²</td>
</tr>
<tr>
<td>0.25</td>
<td>None</td>
<td>0.0333</td>
<td>100</td>
<td>3.2</td>
</tr>
<tr>
<td>0.50</td>
<td>Rapamycin</td>
<td>0.0055</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>0.50</td>
<td>FK506</td>
<td>0.0055</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>0.50</td>
<td>FK506</td>
<td>0.0050</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>0.50</td>
<td>CsA</td>
<td>0.0604</td>
<td>101</td>
<td></td>
</tr>
</tbody>
</table>

— protein concentrations were estimated by the method of Bradford (2) with bovine serum albumin as the calibration standard.
— PPlase activities were determined at 10°C in 35 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.8) with succinyl-Ala-Ala-Pro-Phe-4-nitroanilide as the substrate by the previously described chromotryptic-coupled assay (11). Isomerization rates of cis to trans rotomer conversion were obtained by computer fitting the change in absorbance (A) to a first-order exponential function of incubation time (t): A = A₀ - A₀ e⁻ᵏᵗ, where A₀ is the final absorbance at infinite time, C is the range of the slow phase in the cis to trans isomerization, and k is the first-order rate constant for isomerization.
— percent activity remaining was calculated as 100 times the ratio between the net isomerization rates catalyzed by RBP in the presence and absence of inhibitor; net rates are calculated as the difference in experimentally derived values for k (presented in the table) in the presence and absence of purified RBP.

Vol. 11, 1991
NOTES 1721

Although this initial evaluation cannot distinguish the relative binding affinities of rapamycin and FK506 to RBP, both compounds can be classified as tight-binding inhibitors (4) since the concentration of either macrolide required to abolish catalytic activity is comparable to the concentration of the purified ~13-kDa protein (0.05 μg/ml, ~38 mM) used in the assays (Table 1). These observations are similar to the tight-binding interactions demonstrated by both rapamycin (Kᵣ, 0.7 ± 0.2 mM) and FK506 (Kᵣ, <5 nM) with human recombinant FKBP (22a) as well as FK506 with FKBP derived from the Jurkat T-cell line (16). In contrast, the PPlase activity of purified yeast RBP is not inhibited by relatively high concentrations of CsA, a tight-binding inhibitor of the cyclophilin PPlases (16).

FUNCTIONAL EXPRESSION OF HUMAN FKBP IN YEAST CELLS. Given the similarity between yeast RBP and human FKBP, we next investigated the effect of FKBP expression on drug sensitivity. As shown in Fig. 5, expression of human FKBP in rapamycin-resistant yeast mutants restores sensitivity to rapamycin. At the same time, there was no effect on FK506 sensitivity, and FK506 had no impact on rapamycin sensitivity when both drugs were used in combination (data not shown). Restoration of rapamycin resistance suggests a functional equivalence between the yeast and human enzymes. Functional expression of human FKBP in S. cerevisiae should provide a means to rapidly generate valuable structure-function information on this important drug target.

CONCLUSIONS. We conclude that yeast RBP is an FKBP-related PPlase, distinct from the reported yeast cyclophilins, which acts as a cellular target for rapamycin. Despite the inhibition of purified RBP (or "rapaphilin") by FK506, the absence of RBP in S. cerevisiae does not appear to affect sensitivity to FK506 in vivo (Fig. 1A). This suggests that the mechanism of FK506 cytotoxicity in yeast cells may be different than that of rapamycin. While FK506 may indeed act to inhibit RBP in vivo, the fact that rapamycin-resistant mutants remain sensitive to FK506 suggests that its antifungal effect may also involve other proteins in the cell.

As reported previously (40), cyclophilin-deficient yeast mutants are resistant to the cytotoxic effects of CsA. Like-
wise, rapamycin cytotoxicity requires the presence of RBP. An analogous observation has been made with topoisomerase I, which is not essential for viability in *Saccharomyces cerevisiae* (39). The topoisomerase I inhibitor camptothecin produces potentially lethal DNA lesions by interacting with its target enzyme, and yet topoisomerase deletion mutants are completely resistant to camptothecin (10, 28). Similarly, PPlase inhibitors apparently produce a lethal event by interacting with their target enzymes. The fact that mutations in *RBP1* are recessive and nonlethal suggests that the interaction of rapamycin with its target protein may also involve other essential components in the cell, including potential substrates for the yeast FKBP-related PPlase. Thus, the association of RBP and rapamycin may result in the formation of a toxic binary complex. Alternatively, RBP may be involved in an essential function, but other rapamycin-insensitive enzymes might compensate for the absence of this particular PPlase.

One possible role for PPlases, including the yeast RBP that we have described, is in the folding of nascent polypeptides as they exit the ribosome. Interestingly, the other yeast protein with which RBP shows the greatest degree of homology (25% identity within an 88-amino-acid sequence) is ribosomal protein 59, the product of the *CRY1* gene, which has been shown to be the target for cryptopleurine (22). Further analysis of the yeast RBP may facilitate our understanding of the biological processes in which these unique proteins are involved and, when compared with human FKBP, may reveal some of the molecular mechanisms of immunosuppression.

We thank Ganesh Sathe and Felicia Watson for synthetic oligonucleotides, Peter Kmetz for DNA sequencing, and Mitchell Gross, Edward Arcuri, Gordon Moore, Martin Rosenberg, and especially Russell Greig for advice and support throughout this study.

REFERENCES

the AUG startcodons in *Saccharomyces cerevisiae* mRNAs. Nucleic Acids Res. 15:3581–3593.