TOR Mutations Confer Rapamycin Resistance by Preventing Interaction with FKBP12-Rapamycin*

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The antifungal, immunosuppressive compound rapamycin arrests the cell cycle in G1 in both yeast cells and T-lymphocytes. Previous genetic studies in yeast identified mutations in three genes, FPR1 (FKBP12), TOR1, and TOR2, which confer rapamycin resistance, and genetic findings implicated the TOR proteins as direct targets of FKBP12-rapamycin. Consistent with this model, we find that modulating TOR1 and TOR2 expression alters rapamycin sensitivity. We describe several TOR2 mutations that confer rapamycin resistance. These mutations prevent FKBP12-rapamycin binding to TOR2, as assayed by the two-hybrid system. We find that TOR1 and the mammalian TOR homologue (mTOR) also bind FKBP12-rapamycin, and mutations corresponding to those in TOR2 similarly block FKBP12-rapamycin binding to TOR and that a composite protein-drug surface contacts the TOR proteins. These studies confirm that the TOR proteins are direct targets of FKBP12-rapamycin, reveal that drug-resistant mutations prevent this association, and define structural features of these complexes.

RAPAMYCIN is a natural product with both antifungal and immunosuppressive activities (reviewed in Refs. 1–3). In mammalian T-lymphocytes, rapamycin blocks an unknown step in the signal transduction pathway initiated by interleukin-2 (IL-2), leading to G1 cell cycle arrest via inhibition of cyclin D- and cyclin E-dependent p33cdc2 and p34cdc2 kinase activities (4–7). This arrest may result from a block in IL-2-stimulated Cdk-inhibitor p27kip1 degradation (7). Rapamycin also prevents the phosphorylation and activation of the p70 S6 kinase (8, 9).

Several studies have shown that rapamycin and the structurally related macrolide FK506 are mutually antagonistic inhibitors of T-cell activation (10, 11). The intracellular receptor for FK506 is the cytoplasmic 12-kDa cis-trans peptidyl-prolyl isomerase FKBP12 (for FK506 Binding Protein; Refs. 12 and 13). Prolyl isomerization is a rate-limiting step in protein folding, and two classes of enzymes catalyze this reaction, the FKBP51s and cyclophilins. Despite their similar activity, cyclophilins and FKBP51s share no homology; remarkably, though, cyclophilin A is the intracellular receptor for the immunosuppressant cyclosporin (14–16). FKBP12 also binds rapamycin (17), but FK506 and rapamycin inhibit distinct T-cell signaling pathways; FK506 prevents IL-2 expression in response to antigen presentation to the T cell receptor while rapamycin prevents the subsequent autocrine response to IL-2 (10, 11, 17).

Early models suggested that immunosuppression resulted from inhibition of FKBP12 enzymatic activity; however, several lines of evidence argue against this. First, several FK506 analogs inhibit isomerase activity but are not immunosuppressive (18, 19). Additionally, rapamycin is toxic to the yeast Saccharomyces cerevisiae, but mutants lacking yeast FKBP12 (fpr1) are viable and drug resistant (20). The relevant target of both FK506 and cyclosporin, the Ca2+/calmodulin-regulated protein phosphatase calcineurin, was identified using FKBP12-FK506 and cyclophilin-calcineurin affinity chromatography (16). Inhibition of calcineurin by these complexes prevents T-cell activation by blocking the nuclear import of the cytoplasmic subunit of NFAT, a transcription factor that regulates transcription of genes involved in T-cell activation (21–23). Genetic screens in yeast identified mutations in three genes, FPR1 (encodes FKBP12), TOR1, and TOR2, which confer rapamycin resistance (20). TOR1 and TOR2 (for Target Of Rapamycin) encode large proteins related to phosphatidylinositols and protein kinases (24–26), and TOR2 has been shown to have an associated phosphatidylinositol 4-kinase activity (27). TOR2 is essential whereas TOR1 is not. Depletion of both TOR genes leads to a G1 cell cycle arrest resembling that imposed by rapamycin (20, 25). The genetic finding of nonallelic non-complementation between tor1, tor2, and fpr1 mutations led to a model in which the FKBP12-rapamycin complex physically interacts with the TOR proteins (20), analogous to the interaction between FKBP12-FK506 and calcineurin. This model has recently been confirmed by the discoveries that TOR2 and the mammalian TOR homologue (mTOR, also known as FRAP, RAFT1, and RAPT1) bind FKBP12-rapamycin in vitro (27–31) and that FKBP12-rapamycin interacts with a small piece of TOR2 or mTOR in the two-hybrid system (32, 33). It is not yet clear how binding of FKBP12-rapamycin to TOR arrests the cell cycle.

To characterize molecular interactions between FKBP12-rapamycin and TOR proteins, we identified additional rapamycin-resistant yeast mutants. We describe here several novel TOR2 mutations and employ the two-hybrid system to examine interactions between wild-type and mutant TORs and the FKBP12-rapamycin complex. Our studies confirm that the TORs physically interact with FKBP12-rapamycin, that TOR mutations confer rapamycin resistance by preventing this interaction, and that a composite FKBP12-rapamycin surface contacts TOR.
FKBP12-Rapamycin-TOR Complexes

**TABLE I**

| Genotype\(^a\) | Plasmid | MIC\(^b\) ng/ml |
|--------------|--------|---------------|
| TOR1 TOR2   | Vector | 20            |
| TOR1 TOR2   | TOR2-2CEN | 50            |
| \(\Delta\)tor1 TOR2 | TOR2-2\(\mu\) | 250           |
| \(\Delta\)tor1 TOR2 | Vector | 5             |
| \(\Delta\)tor1 TOR2 | TOR2-CEN | 50            |
| \(\Delta\)tor1 TOR2 | TOR2-2\(\mu\) | 100           |

\(^a\) Strains were isogenic except at the TOR1 locus.

**RESULTS**

TOR Gene Expression Levels Alter Rapamycin Sensitivity—As a genetic test of the model that TOR is the target of FKBP12-rapamycin, we examined the effects of altering TOR gene expression on rapamycin toxicity. Rapamycin sensitivity increased 4-fold in a strain lacking the nonessential TOR1 gene (Table I). Overexpression of the essential TOR2 gene from low-copy or multi-copy plasmids increased rapamycin resistance by 2.5–20-fold (Table I), while increasing FKBP12 had no effect (data not shown). These findings suggest the TOR proteins are limiting for FKBP12-rapamycin action and that TOR1 may compete with TOR2 for FKBP12-rapamycin.

Isolation of Rapamycin-resistant Mutants—To further explore rapamycin action, we isolated rapamycin-resistant yeast mutants. We biased our screen to avoid rescaling mutations...
FKBP12-Rapamycin-TOR Complexes

FKBP12-Rapamycin-TOR Complexes Do Not Require FKBP12 Prolyl Isomerase Activity—We determined if FKBP12 prolyl isomerase activity was required for FKBP12-rapamycin binding to TOR proteins. A mutation of human FKBP12, F36Y, is known to reduce prolyl isomerase activity 1000-fold but has no effect on FK506 binding or calcineurin inhibition (46). Yeast and human FKBP12 share 54% identity (47) and have superimposable tertiary structures (48, 49). We therefore tested the effects of the corresponding mutation, F43Y, on yeast FKBP12-rapamycin-TOR interactions in the two-hybrid system. Western blot confirmed that the GAL4(AD)-FKBP12F43Y fusion protein was expressed, albeit at a somewhat lower level than wild-type GAL4-FKBP12 (Fig. 3C). Nonetheless, the GAL4(AD)-FKBP12F43Y mutant fusion protein interacted in a rapamycin-dependent fashion at nearly wild-type levels with all three TOR proteins (Fig. 5). Thus, FKBP12 prolyl isomerase activity is not required for rapamycin binding or association of the FKBP12-rapamycin complex with TOR1, TOR2, or mTOR.

FKBP12 Surface Residues Bind TOR1 and TOR2—We assayed the contribution of FKBP12 surface residues to the formation of FKBP12-rapamycin-TOR complexes, focusing on residues adjacent to but distinct from the active site/ligand binding pocket (Asp-48, Arg-49, Phe-94). These studies were guided by the structure of the human FKBP12-rapamycin complex (50) and previous studies on the FKBP12-FK506-calcinurein complex (39, 51–53). Western blot confirmed that the GAL4(AD)-FKBP12 mutant fusion proteins were expressed at levels comparable to the wild-type FKBP12 fusion protein.
FIG. 2. Schematic of TOR mutations and two-hybrid constructs with their ability to bind to FKBP12. Panel A, Full-length TOR2 depicting the region used in the two-hybrid system, including the specific mutations analyzed in this study (left). The interaction of these fusions with GAL4(AD)-FKBP12 in medium with or without 1.0 μg/ml rapamycin as measured by the activity of the β-galactosidase reporter is shown in the table on the right. Panels B and C, similar to panel A, diagramming the regions and mutations of TOR1 (B, left) and mTOR (C, left) and their interaction with GAL4(AD)-FKBP12 (right). Panel D, a schematic of full-length TOR2, indicating other segments of TOR2 that were tested in the two-hybrid system (left) and their interaction (+) or lack of interaction (−) with GAL4(AD)-FKBP12 in the presence or absence of 1.0 μg/ml rapamycin (right). PI, phosphatidylinositol.
A hydrophobic substitution of an FKBP12 acidic surface residue, D48V, had only a minor 2–4-fold effect on FKBP12-rapamycin binding to TOR1 or TOR2 (Fig. 5, A and B). Substitution of two other surface residues, R49I and F94V, alone and in combination, had a more severe impact on binding of the mutant FKBP12-rapamycin complex to TOR1 and TOR2. There are, however, subtle differences between the TOR1 and TOR2 complexes with FKBP12-rapamycin (Fig. 5, A and B). For example, the F94V mutation impaired formation of the FKBP12-rapamycin-TOR1 complex, but this effect was more pronounced with the R49I,F94V double mutant. In contrast, the FKBP12-rapamycin-TOR1 complex was more sensitive to the R49I mutation, and the F94V mutation on its own had little or no effect on association with TOR1. Rapamycin-stimulated binding of GAL4(AD):FKBP12 to GAL4(BD)-mTOR was less affected by these mutations (Fig. 5C), possibly as a result of the relatively stronger interaction (−7-fold; Fig. 2C) with FKBP12-rapamycin compared with TOR1 or TOR2.

The GAL4(AD):FKBP12 mutant fusion proteins were also expressed in an FKBP12 mutant strain and tested for functional complementation. The degree to which these fusion proteins complemented to restore rapamycin sensitivity in vivo (Fig. 6) correlated with ability to bind to GAL4(BD)-TOR2 fusion proteins (Fig. 5), indicating that binding to TOR2 is directly correlated with toxicity. Taken together, these findings reveal that a composite FKBP12-drug surface contacts the TOR proteins.

**DISCUSSION**

Our finding that rapamycin stimulates the formation of complexes between FKBP12 and both the TOR1 and TOR2 proteins, taken together with other recent reports of FKBP12-rapamycin binding to mTOR (28–30, 33), to a TOR2 fragment (32), or to intact TOR2 (27) confirms the original model, based on genetic evidence, that the TOR proteins are the direct targets of FKBP12-rapamycin (2, 20). Moreover, the finding that drug-resistant TOR mutants no longer bind to FKBP12-rapamycin further implicates FKBP12-rapamycin binding to the TOR proteins (especially to TOR2, which is essential) as the critical event in rapamycin toxicity.

Our findings reveal that while FKBP12 prolyl isomerase activity is dispensable, FKBP12 surface residues are important for FKBP12-rapamycin binding to TOR1 and TOR2. FKBP12-rapamycin binding to TOR2 was more sensitive to perturbation by FKBP12 mutations compared with binding to TOR1, suggesting that although TOR1 and TOR2 are highly related, there are structural differences in the FKBP12-rapamycin-TOR interfaces. Notably, residues Arg-49 and Phe-94 of yeast FKBP12, and the corresponding residues Arg-42 and His-87 of human FKBP12, have been previously implicated in FKBP12-FK506 binding to calcineurin (39, 51–53). Taken together, these findings suggest that some of the same FKBP12 surface residues are important for the two distinct FKBP12-drug inhibitor complexes to interact with different targets, calcineurin, and the TOR proteins.

The rapamycin-resistant TOR2 mutations and FKBP12 residues identified here are likely to be relevant for studies of...
FKBP12-rapamycin-TOR structures. The minimal FKBP12-rapamycin binding domain of TOR would be amenable for structural determination by either NMR or crystallography. Our studies define Trp-2042 and Phe-2049 as residues that, with Ser-1975, are likely to lie on the TOR interface with FKBP12-rapamycin. Similarly, FKBP12 residues Arg-49 and Phe-94 likely lie on the FKBP12-rapamycin interface with TOR2. These mutations should serve to guide analysis of FKBP12-rapamycin-TOR structures and models.

There are several biological correlates to our analysis of FKBP12-rapamycin-TOR interactions in the two-hybrid system. The relative binding affinities of mutant FKBP12-rapamycin complexes to TOR2 (Fig. 5) were well correlated with functional complementation and restoration of rapamycin sensitivity in vivo (Fig. 6). Importantly, we found that FKBP12-rapamycin binding to TOR1 occurs at 10-fold lower rapamycin concentrations compared to TOR2 (Fig. 4). We believe this comparison of relative binding affinity is valid based on the following findings. First, the same highly homologous domain from TOR1, TOR2, and mTOR was fused to GAL4. Second, by Western blot, the GAL4-TOR1 and GAL4-TOR2 fusion proteins were equivalently expressed (Fig. 3). Importantly, two biological observations also support the interpretation that TOR1 binds to FKBP12-rapamycin with higher affinity than TOR2. First, yeast strains lacking TOR1 are rapamycin hypersensitive, indicating that TOR1 normally effectively competes with TOR2 for FKBP12-rapamycin. Second, in yeast strains in which both TOR1 and TOR2 are required for viability, rapamycin sensitivity is increased 10-fold (27).

Recent studies from our laboratory reveal that while FKBP12-rapamycin binds TOR2, it does not inhibit the phosphatidylinositol 4-kinase activity associated with TOR2 (27). Importantly, the TOR1 and TOR2 domains that interact with FKBP12-rapamycin are distinct from and do not overlap the putative kinase domain. A data base search using the BLAST program of NCBI with the 1886–2081 region of TOR2 returns strong homology to only TOR1 and mTOR and very weak homology to another yeast protein, ESR1/MEC1 (54, 55). Significantly, this region shows no similarity to any other phosphatidylinositol 3- or phosphatidylinositol 4-kinases, indicating that it is outside the putative active site. FKBP12-rapamycin binding to TORs may inhibit interactions with other proteins that may, for example, be important for intracellular localization (27).

We also found that yeast FKBP12-rapamycin binds mTOR, the mammalian homologue of the yeast TOR proteins, indicating that the FKBP12-TOR interaction surface is highly conserved from yeast to man. Mutation of the conserved serine residue in mTOR abolished binding by FKBP12-rapamycin. Further studies will be required to establish mTOR functions in rapamycin-sensitive signaling cascades in vivo. One approach would be to test if introduction of the mTOR mutant into T-lymphocytes confers rapamycin-resistant IL-2 signaling. Alternatively, mTOR might provide TOR1 or TOR2 function in yeast or render yeast rapamycin resistant when mutated. Such studies are in progress.

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Fig. 5. FKBP12 mutations alter interactions with the TOR proteins. Panels A–C, GAL4(AD)-FKBP12 fusion proteins encoding the indicated mutation were tested for interaction with wild-type TOR2 (A), TOR1 (B), or mTOR (C) GAL4(BD) fusion proteins. , 0.0 μg/ml rapamycin; , 0.1 μg/ml; , 1.0 μg/ml.

Fig. 6. FKBP12 mutants that fail to bind the TOR proteins do not complement Δfpr1. The FKBP12 mutant fusion proteins were expressed in the FKBP12-deficient strain JHY-2-1c (fpr1::ADE2) and grown on synthetic medium lacking leucine (SD-Leu) and supplemented with the indicated concentration of rapamycin for 2 days at 30°C.
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