Rapamycin Blocks Sexual Development in Fission Yeast through Inhibition of the Cellular Function of an FKBP12 Homolog*

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FKBP12 is a ubiquitous and a highly conserved prolyl isomerase that binds the immunosuppressive drugs FK506 and rapamycin. Members of the FKBP12 family have been implicated in many processes that include intracellular protein folding, transport, and assembly. In the budding yeast Saccharomyces cerevisiae and in human T cells, rapamycin forms a complex with FKBP12 that inhibits cell cycle progression by inhibition of the TOR kinases. We reported previously that rapamycin does not inhibit the vegetative growth of the fission yeast Schizosaccharomyces pombe; however, it specifically inhibits its sexual development. Here we show that disruption of the S. pombe FKBP12 homolog, fkh1⁺, at its chromosomal locus results in a mating-deficient phenotype that is highly similar to that obtained by treatment of wild type cells with rapamycin. A screen for fkh1 mutants that can confer rapamycin resistance identified five amino acids in Fkh1 that are critical for the effect of rapamycin in S. pombe. All five amino acids are located in the putative rapamycin binding pocket. Together, our findings indicate that Fkh1 has an important role in sexual development and serves as the target for rapamycin action in S. pombe.

Cyclosporin A (CsA), FK506, and rapamycin are microbial products that exhibit immunosuppressive activity (1). These three compounds bind with high affinity to cytoplasmic proteins termed immunophilins (2, 3). CsA binds an immunophilin called cyclophilin-18, whereas FK506 and rapamycin, which are structurally related, bind a different immunophilin called FKBP12. The immunosuppressive drugs form a drug-immunophilin complex, which binds and inhibits a third component. The complexes CsA-cyclophilin-18 and FK506-FKBP12 bind and inhibit the activity of the Ca²⁺-dependent protein phosphatase, calcineurin (4–6). The rapamycin-FKBP12 complex binds and inhibits the activity of the phosphatidylinositol-like kinase, TOR (7–10, 36, 37).

In addition to their immunosuppressive activity, CsA, FK506, and rapamycin have side effects that may stem, at least in part, from inhibition of the physiological function of the immunophilins. For example, in mammals, FKBP12 functions as a subunit of ryanodine calcium release channels and is thought to modulate intracellular Ca²⁺ levels in the heart (11–13). Mice deficient in FKBP12 show severe heart defects associated with loss of function of cardiac ryanodine receptors (14). Similarly, treatment with high doses of FK506 can lead to severe heart failure (15).

Although FKBP12 and cyclophilin-18 are unrelated in primary sequence, both classes of immunophilins exhibit a peptidyl prolyl-cis/trans-isomerization (PPIase) activity that accelerates a rate-limiting step in the folding of peptide and protein substrates in vitro (3, 16–18). The PPIase activity of the immunophilins is inhibited upon binding to their specific immunosuppressive drugs, suggesting an overlap between the PPIase-active site and the drug-binding site. According to atomic structure analyses of human cyclophilin-18 and FKBP12, both proteins contain a deep hydrophobic binding pocket (19–21). These pocket structures accommodate the specific immunosuppressive-acting ligands and model tetrapeptides used as pseudosubstrates.

The cellular functions of the immunophilins, as well as the relevance of the PPIase activity within the cellular environment, is not well understood. However, some of the important natural substrates of the immunophilins are now known (reviewed in Ref. 22). For example, the human cyclophilin-18, CyPA, binds the Gag polyprotein of the human immunodeficiency virus, type 1, virion (23–25). The human FKBP12 protein is physically associated with calcium release channels (11–13, 26), the type I tumor growth factor, transforming growth factor-β, receptor (27–30), and the transcription factor YY1 (31).

Genetic studies in the budding yeast Saccharomyces cerevisiae have played a critical role in elucidating the mode of action of the immunosuppressive drugs in higher eukaryotes (reviewed in Refs. 32 and 33). Similar to the effect of rapamycin in T cells and certain non-lymphoid cells, rapamycin treatment of S. cerevisiae cells results in a G₁ cell cycle arrest (34). S. cerevisiae cells contain one FKBP12 homolog, named FPR1 (34), also known as RBP1 (35). Disruption of FPR1 results in slightly slowly growing but viable cells that are completely resistant to rapamycin. This phenotype indicated that FPR1 is a nonessential gene and is the main mediator of the effect of rapamycin (34, 35). Later it was shown that Fpr1p forms a complex with rapamycin that binds and inhibits the functions of the TOR1 and TOR2 gene products in cell cycle progression (34, 36–39). Several proteins that interact physically with Fpr1p in the absence of rapamycin have been identified, and it has been suggested that their activity may be regulated by the interaction with Fpr1p. These include calcineurin (40), the biosynthetic enzyme aspartokinase (41), the high mobility group HMG 1/2 proteins (42), and the transcription factor homolog FAP1 (43).

We reported previously (44) that rapamycin does not affect...
vegetative growth in the fission yeast, Schizosaccharomyces pombe, but severely inhibits its sexual development pathway. S. pombe cells are induced to enter the sexual development pathway under starvation conditions (45). If the sexual development pathway is chosen, cells of opposite mating type conjugate to form diploid zygotes that immediately undergo meiosis and sporulation (45). Rapamycin strongly inhibited sexual development at an early stage, before mating had occurred, but did not affect entry into stationary phase (44). More recently, we reported that S. pombe contains two TOR homologs, tor1 and tor2 (46). tor2 is an essential gene of as yet unknown function. tor1 is required under starvation and a variety of other stress conditions that include osmotic and oxidative stresses. Interestingly, one of the studied functions of the S. pombe TOR homologs appears to be inhibited by rapamycin (46).

To understand further the response of S. pombe to rapamycin, we isolated and characterized the S. pombe FKBP12 homolog. We found one FKBP12 homolog and named it fkh1 (46). We found that there are two TOR homologs, tor1 and tor2. tor2 is an essential gene of as yet unknown function. tor1 is required under starvation and a variety of other stress conditions that include osmotic and oxidative stresses. Interestingly, one of the studied functions of the S. pombe TOR homologs appears to be inhibited by rapamycin (46).

Rapamycin Inhibits the Function of FKBP12 in S. pombe

| Strain | Genotype | Source |
|--------|----------|--------|
| TA07   | leu1–32/leu1–32 ura4-D18/ura4-D18 ade6-M216/ade6-M210 h+ h- | Lab stock |
| TA06   | leu1–32 h- | Lab stock |
| TA16   | leu1–32 ura4-D18 ade6-M216 h+ h- | Lab stock |
| TA58   | leu1–32 ura4-D18 h+ h- | Lab stock |
| TA59   | leu1–32 ura4-D18 ade6-M216 fkh1::ura4' h+ h- | This study |
| TA77   | leu1–32 ura4-D18 fkh1::ura4' h+ h- | This study |
| TA94   | fkh1::ura4' fkh1::ura4' leu1–32/leu1–32 ura4-D18 ade6-M210/ade6-M216 h+ h- | This study |
| TA96   | fkh1::ura4' leu1–32 h- | This study |

Table I: S. pombe strains used in this study

Yeast Strains, Media, and Yeast Techniques—Yeast strains used in this paper are described in Table I. Media are used as described on the indicated medium (47). EMN-M contains no glucose. Transformation of S. pombe cells was performed by electroporation (48). Rapamycin was added to a final concentration of 0.2 mmol/L when spores were inoculated into liquid or agar-containing media, unless otherwise indicated. In one experiment, cells were cultured in liquid or agar-containing media, unless otherwise indicated. An equal volume of the drug vehicle solution (1% Me2SO/methanol) was used as a control in all experiments. Assays for mating or sporulation efficiency were carried out as follows. Cells were grown at 30 °C in EMN medium to a density of 5 × 10^6~1 × 10^7 cell/ml. The cultures were then washed three times with 2% glucose. After washing, 5 ml containing 5 × 10^6 cells were spotted on EMN, EMN-lowG, or ME medium (see Ref. 44 for detailed description of medium composition). After 3 days of incubation at 30 °C, a toothpick was used to pick some of the cells from the center of each patch, and the cells were briefly sonicated and examined microscopically. The percentage of mating was calculated by dividing the number of zygotes, asci, and spore pairs by the number of total cells. The percentage of sporulation was calculated by dividing the number of ascus and spore pairs by the number of total cells. The efficiency of pairing was determined by following the method described (47). A aliquots of whole cell extracts containing 40 μg of protein were fractionated by SDS-polyacrylamide gels and transferred to membrane filters. The immobilized proteins were detected using the PerkinElmer Life Sciences ECL system. The Fkh1 proteins were detected with polyclonal antibodies raised against S. cerevisiae FKBP12, the kind gift of J. Heitman, Duke University Medical Center.

Isolation of Rapamycin-resistant fkh1 Mutants—fkh1 mutants were obtained by PCR-based mutagenesis. Conditions for PCR-based random mutagenesis of fkh1 were essentially as described (54). Briefly, 5 ng of

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plasmid pR22 were taken for PCR amplification of fkh1′ cDNA with 0.2 μg of primers 92 (5'-GGAATTCATATGGGGTTGCGAAA GCAAGT-TATTTC, NdeI site is underlined) and 51 (5'-TGACCATGGGGAAGA GATCC). The PCR buffer contained 10 mM Tris-HCl (pH 8.7), 50 mM KCl, 5 μM MgSO4, 2.5 mM MgCl2, 5 units of Taq polymerase, 250 μM each of dNTP, and an excess of 1.5 μM of one dNTP nucleotide concentration over the others. Four separate PCRs were performed, and in each reaction a different dNTP was present in excess. 25 cycles of PCR were performed with the following temperature profile: 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s. The four PCRs were pooled and fractionated in a 1.5% agarose gel and eluted. The resultant 550-bp DNA fragments were digested with NdeI and ligated with a NdeI-SmaI digested pREP1 S. pombe vector. The ligation product was used for PCR amplification with primers 189 (5'-GAAATGC- CATCAGCGGTGTTTCCG) and 190 (5'-TCATCCATGGCGCAATCTT-GTGC). These DNA fragments containing the mutated fkh1′ cDNA flanked by pREP1 sequences were co-transformed with pREP1 into the S. pombe strain TA77 (leu1-32 ura4-D18 fkh1::ura4+ his2). Transformants were plated on minimal medium and after 4 days of incubation at 30 °C replica-plated to minimal medium with or without 0.2 μg/ml rapamycin. After an additional 5 days of incubation at 30 °C, the plates were exposed to iodine vapor. Iodine vapor is routinely used to detect sporulating colonies. Spores are darkly stained by iodine vapor, whereas vegetative cells remain unstained. In the presence of rapamycin (44) or in Δfkh1 colonies (this study), no dark staining is observed since the sexual development pathway is blocked prior to conjugation. Plasmid DNA was isolated from Δfkh1 transformants that stained dark in the presence of rapamycin and used for re- transformation of TA77 and transformation of bacterial cells for plasmid amplification. Plasmids that conferred rapamycin resistance phenotype upon re-transformation were further subjected to DNA sequence analysis.

RESULTS

Identification of the FKBP12 Homolog in the S. pombe Genome—Most of the S. pombe genome has been sequenced through the coordination of the Sanger Center, UK. Based on sequence comparisons, we identified one S. pombe FKBP12 homolog on chromosome II and named it fkh1′ (for FKBP12 homolog). The open reading frame of fkh1′ is interrupted by 4 introns of 182, 128, 105, and 48 base pairs. fkh1′ encodes a putative 112-amino acid protein with a predicted mass of 12 kDa. We cloned the fkh1′ cDNA by PCR amplification using a fission yeast cDNA library as a template (see “Experimental Procedures”). Sequence analysis confirmed that the 4 introns predicted in the genomic sequence are spliced out in the cDNA clone.

Analysis of the predicted amino acid sequence encoded by fkh1′ reveals that this gene is very similar to its S. cerevisiae homolog, FPR1 (72% overall identity). The similarity between fkh1′ and the human FKBP12 homolog is comparable to the similarity between FPR1 and the human FKBP12 (55% overall identity). fkh1′ encodes all the amino acids required for rapamycin binding as predicted by the high resolution structure of the human FKBP12-rapamycin complex (Ref. 21 and see Fig. 5).

When Expressed in S. cerevisiae, fkh1′ Functions Similarly, but Not Identically, to the S. cerevisiae FKBP12 Homolog—The S. cerevisiae FKBP12 protein, Fpr1p, binds to rapamycin. FKBP12-rapamycin complexes bind the TOR proteins and thus inhibit some of their functions (see Introduction). Since the S. pombe fkh1′ gene shows a significant level of homology with FPR1, we examined whether fkh1′ can replace FPR1 in mediating the effect of rapamycin in S. cerevisiae. To this goal, we expressed fkh1′ cDNA in S. cerevisiae using ADH1 promoter-driven vector, pCM189 (51). Wild type and Δfpr1 S. cerevisiae cells were transformed with pCM189-fkh1′, and the transformants were streaked onto plates containing 0.08 μg/ml rapamycin (Fig. 1). As described previously, the wild type S. cerevisiae cells did not form colonies in the presence of rapamycin, whereas Δfpr1 cells were completely resistant to the lethal effect of the drug (34) (Fig. 1). Expression of fkh1′ in Δfpr1 cells restored rapamycin sensitivity (Fig. 1, plate 2), indicating that fkh1′, like FPR1, is capable of mediating the effect of rapamycin in S. cerevisiae cells. It is therefore most likely that the gene product of fkh1′ forms a toxic complex with rapamycin that binds and inhibits the S. cerevisiae TOR proteins.

Unexpectedly, following a prolonged incubation, cells expressing pCM189-fkh1′ exhibited slow growth in the presence of rapamycin, either in the genetic background of wild type or Δfpr1 cells (Fig. 1, plates 3 and 4). Thus, the expression of fkh1′ under the strong ADH1 promoter can slightly increase rapamycin resistance in S. cerevisiae cells. Overexpression of FPR1 from the same expression vector did not exhibit such an effect (see Fig. 2A), consistent with previous findings (55).

We also screened an S. pombe cDNA library for genes that can confer rapamycin resistance in S. cerevisiae cells (see “Experimental Procedures”). Wild type S. cerevisiae was transformed with the S. pombe cDNA library, and the transformants were plated on rapamycin-containing plates. Sequence analysis of one of the isolated cDNA clones revealed that it encoded fkh1′. The weak rapamycin resistance phenotype conferred by overexpression of fkh1′ was observed in several S. cerevisiae strains, including RS188N and JK9–3d (see “Experimental Procedures” for full genotypes), indicating that this suppression activity is not strain-specific.

We examined the ability of fkh1′ to suppress rapamycin sensitivity at different drug concentrations ranging from 10 to 150 ng/ml. S. cerevisiae wild type cells transformed with either pCM189-fkh1′ or pCM189-FPR1 were streaked on rapamycin-containing plates, and their growth was monitored. Cells transformed with pCM189-fkh1′ grew faster on 10 ng/ml rapamycin than on 100 ng/ml rapamycin (Fig. 2A) and did not form colonies on 150 ng/ml rapamycin (data not shown). pCM189-FPR1 had no significant suppression activity at any drug concentration.

One possibility to explain the dosage-dependent suppression of fkh1′ is that the gene product, Fkh1, forms a complex with rapamycin that does not inhibit the S. cerevisiae TOR proteins as efficiently as Fpr1p-rapamycin complexes. According to such a model, Fpr1p-rapamycin complexes would compete with Fkh1-rapamycin complexes. Thus overproduction of Fpr1p could counteract the weak rapamycin resistance associated with fkh1′. To investigate this, wild type cells were co-transformed with pCM189-FPR1 and pCM189-fkh1′, and the resulting transformants were streaked on rapamycin-containing plates. The results demonstrate that increased levels of FPR1 abolished the fkh1′-dependent rapamycin resistance (Fig. 2B).

These findings support our suggestion that the slight decrease in rapamycin sensitivity in cells overexpressing Fkh1 results from reduced ability in inhibiting the TOR proteins.

Cells Disrupted for fkh1′ Exhibit a Maturing Deficient Pheno-
Thus, although vector also suppressed the mating defect of S. cerevisiae associated with an inability to arrest in G1 in response to nitrogen starvation (46, 59). We analyzed the DNA content of cin-treated cells are defective in their ability to arrest in G1 in response to starvation conditions. Newly, in some sterile mutants, the defect in mating is not required for meiosis/sporulation. When S. pombe cells enter stationary phase they become smaller and round and can maintain their viability over long periods (56). Some of the S. pombe mutants that are impaired in sexual development are also impaired in their ability to acquire normal stationary phase morphology and physiology. These include mutants of the cAMP-dependent pathway (57, 58) and mutants of the Spc1-Wis1 stress-activated mitogen-activated protein kinase pathway (59–61). In such mutants, the sterile phenotype may stem from an inability to sense or respond properly to starvation conditions. Recently, we reported (46) that null mutants of the S. pombe TOR homolog, tor1, are defective both in sexual development and entrance into stationary phase. In contrast, rapamycin-treated cells can enter stationary phase properly (44). Here we found that Δfkh1 cells arrested growth as relatively small cells and remained viable over a long period (see “Experimental Procedures” and data not shown). We therefore conclude that fkh1+ is not required for entrance into stationary phase.

Finally, in some sterile mutants, the defect in mating is associated with an inability to arrest in G1 in response to nitrogen starvation (46, 59). We analyzed the DNA content of starved Δfkh1 cells and rapamycin-treated cells. The results shown in Fig. 3C demonstrate that neither Δfkh1 nor rapamycin-treated cells are defective in their ability to arrest in G1 under nitrogen starvation conditions.

Our findings indicate that fkh1+ is required specifically for an early stage of the sexual development pathway. Like treatment with rapamycin (44), Δfkh1 cells are specifically defective in their ability to undergo meiosis but can undergo meiosis/sporulation. As in rapamycin-treated cells, Δfkh1 cells do not show other defects associated with abnormal responses to starvation, such as entrance into stationary phase or arrest in G1 in response to nitrogen deprivation. Taken together, the phenotype of Δfkh1 cells is extremely similar to that of rapamycin-treated cells.

Isolation of Rapamycin-resistant fkh1 Mutants—The close similarity between the phenotypes of Δfkh1 cells and rapamycin-treated cells suggests that the direct target of rapamycin in
S. pombe cells is Fkh1. We hypothesized that if Fkh1 is the target for rapamycin action, then we might isolate fkh1 mutants that can confer rapamycin resistance. Such mutants are expected to be impaired in rapamycin binding but to retain activity necessary for the sexual development pathway.

We randomly mutated fkh1+ cDNA using error-prone PCR (see “Experimental Procedures”). Strain TA77 (leu1 ura4 Δfkh1 h86) was transformed with a mixture of plasmids bearing mutated fkh1 DNA, plated on minimal medium in the absence of rapamycin. After colonies had developed they were replicated to 0.2 μg/ml rapamycin-containing plates. Colonies that underwent sexual development despite the presence of rapamycin were identified by exposure to iodine vapor (see “Experimental Procedures”). Of 25,000 transformants, 28 clones showed plasmid-dependent sporulation on rapamycin-containing plates. Sequence analysis revealed that 16 of these 28 clones carried single missense mutations at one of the following positions: Phe-47, Cys-49, Leu-56, Ile-92, or Phe-100. The remaining 12 mutants carried 2–4 missense mutations. In each case at least one mutation occurred at one of the critical positions Phe-47, Cys-49, Leu-56, Ile-92, or Phe-100 (see Table II). Since iodine vapor analysis suggested that all mutations conferred similar rapamycin resistance phenotype, representatives of mutants of each of the five critical mutations were chosen for further analysis as follows: F47S, C49R, L56F, I92F, and F100L. Quantitative assessment of the mating efficiencies of these mutants demonstrated that all fully complemented the mating deficiency phenotype and conferred similar rapamycin resistance phenotype, representing most deeply into the protein (21). Cys-49 in Fkh1, corresponding to the HuFKBP12 amino acid residue that surrounds the portion of rapamycin that penetrates most deeply into the protein (21). Cys-49 in Fkh1, corresponding to the HuFKBP12 amino acid residue that is not mapped to the very core of the rapamycin binding pocket but resides in a close vicinity. The identification of rapamycin-resistant fkh1 mutants carrying mutations near or at the predicted rapamycin-binding pocket argues that the rapamycin resistance of these mutants is due to impaired rapamycin binding.

We were curious if the fkh1 mutants were also impaired in rapamycin binding in S. cerevisiae cells. If so, the fkh1 mutants could not restore rapamycin sensitivity in S. cerevisiae Δfpr1 mutants. We cloned each of the fkh1 mutants into an S. cerevisiae expression vector and transformed it into Δfpr1 strain (Fig. 6). Although the wild-type fkh1+ gene could efficiently restore rapamycin sensitivity in Δfpr1 mutants, the F47S mutant completely failed to restore rapamycin sensitivity, suggesting that this mutation is strongly impaired in rapamycin binding. Δfpr1 cells transformed with F100L and C49R grew well on plates containing 25 ng/ml rapamycin but poorly on plates containing 100 ng/ml rapamycin, suggesting that the F100L and C49R mutants are partially impaired in rapamycin binding in S. cerevisiae cells. Somewhat surprisingly, the mutant L56F efficiently restored rapamycin sensitivity, suggesting that it can efficiently form a toxic complex with rapamycin in S. cerevisiae.

Our findings indicate that whereas all the fkh1 mutants confer complete rapamycin resistance in S. pombe, they are not identical in their ability to restore rapamycin sensitivity in Δfpr1 S. cerevisiae cells. Of all the mutations only the F47S mutation completely abolished the ability of fkh1 to restore rapamycin resistance in Δfpr1. The differences in the behavior of the fkh1 mutants in the two yeast systems may not be surprising since in S. pombe rapamycin seems to inhibit directly the FKBP12 function, whereas in S. cerevisiae rapamycin exerts its effect by forming a complex with FKBP12 that inhibits the TOR proteins.

**DISCUSSION**

The immunophilins, FKBP5s, and cyclophilins, are highly conserved from bacteria to human and have been found to be both widely distributed and abundantly expressed. In *in vitro*, these proteins exhibit PPInase activity that accelerates the refolding of denatured proteins (16–18). Given these properties, it has been suggested that immunophilins may play a general role in protein folding (reviewed in Ref. 3). However, more recent studies strongly suggest that immunophilins play specialized roles, dependent on their ability to selectively bind to other proteins. For example, the mammalian FKBP12 specifically interacts with the ryanodine calcium release channel, altering its sensitivity to Ca2+ and stabilizing its closed state (11–13). Studies in S. cerevisiae are consistent with the suggestion that the immunophilins do not carry out a general, housekeeping role, since mutants lacking all the immunophilins are viable (62).

Little is known about the functions of the immunophilins in S. pombe, a yeast that is distantly related to S. cerevisiae. Only two members of the family have been subjected to detailed analysis as follows: wsc2+, a heat shock-inducible 40-kDa cyclophilin that is involved in cell cycle regulation (63), and fkb39+, a 39-kDa FKBP homolog that is localized to the nucleus (64). The isolation and expression of a cyclophilin-18
FIG. 5. A comparison of FKBP12 sequences and positions of rapamycin-resistant fkh1 mutants. The predicted amino acid sequence of fkh1' (SpFKBP) is aligned with the S. cerevisiae Fpr1p (ScFKBP) and the human FKBP12 homolog (HuFKBP). Asterisks denote the positions in SpFKBP12 in which single point mutations confer rapamycin resistance phenotype. Boxes indicate the positions that are predicted to interact most closely with rapamycin according to structural studies of the HuFKBP12-ramapycin complex (21).

FIG. 6. Expression of fkh1 mutants in Δfpr1 S. cerevisiae cells. S. cerevisiae Δfpr1 cells transformed with vector only (−), fkh1', or fkh1 bearing the indicated mutations were streaked on plates containing rapamycin as indicated. Plates were photographed after 3 days of incubation at 30 °C.

homolog has been reported (63, 65). In the present study we isolated the S. pombe FKBP12 homolog fkh1' and demonstrated that it is specifically required for an early step of the sexual development pathway.

In S. cerevisiae, the FKBP12 homolog, FPR1, has a critical role in mediating the effect of rapamycin to the TOR proteins (see Refs. 38 and 39 and reviewed in Ref. 66). The amino acid sequence encoded by fkh1' is highly similar to that of FPR1. Consistently, fkh1' can replace FPR1 in mediating the effect of rapamycin in S. cerevisiae (Fig. 1). Slight differences do appear between fkh1' and FPR1, since overexpression of fkh1' but not of FPR1 can slightly reduce the sensitivity to rapamycin in S. cerevisiae cells (Fig. 2A). We suggest that fkh1' reduces sensitivity in S. cerevisiae by forming Fkh1-ramapycin complexes that are not as efficient in inhibiting the S. cerevisiae TOR proteins as Fpr1p-ramapycin complexes (Fig. 2B).

One of the key observations that led to the currently accepted model for rapamycin mode of action in S. cerevisiae was that cells disrupted for FPR1 are viable and rapamycin-resistant (34, 35). Unlike this finding, disruption of fkh1' does not result in rapamycin resistance in S. pombe. Unexpectedly, the phenotype of Δfkh1 mutants highly resembled that of cells treated with rapamycin; Δfkh1 cells are defective in an early step of the sexual development pathway, before mating occurs, but show no defects in later steps such as meiosis or sporulation. In S. pombe, sexual development is a process induced only under starvation conditions (see Introduction). However, neither the sterility of rapamycin-treated cells (44) nor the sterility of Δfkh1 mutants is associated with defects in response to starvation. The strong similarity between the phenotype of Δfkh1 mutants and that of rapamycin-treated wild type cells suggests that rapamycin inhibits sexual development directly by inhibiting the cellular function of fkh1'.

Does Fkh1 form a toxic complex with rapamycin that inhibits the functions of the S. pombe TOR proteins? We have recently determined that the S. pombe tor1' gene is required under various stress conditions, including starvation, whereas tor2' is an essential gene (46). Since rapamycin does not affect entry into stationary phase or response to stress conditions, it appears that most, if not all, of the S. pombe TOR functions are not inhibited by the drug. Sterility is the sole phenotype common to rapamycin treatment and loss of function of TOR activ-

ity. However, the sterile phenotypes of tor1 mutants and rapamycin-treated cells seem to be unrelated. In tor1 mutants the sterile phenotype is likely to be associated with the inability to respond to starvation conditions, whereas rapamycin-treated cells are specifically defective in the sexual development pathway (46). Are the phenotypes of tor1 and fkh1 mutants related? Thus far we have failed to show any genetic link between tor1 and fkh1 mutants; overexpression of tor1' does not alleviate the sterility of Δfkh1 cells, and overexpression of fkh1' does not alleviate the sterility of Δtor1 cells.

The rapamycin-binding site of FKBP12, revealed through structure analyses of HuFKBP12 (21), is composed of aromatic side chains that form a hydrophobic pocket. In this work we have exploited the yeast genetic system to identify residues in Fkh1 involved in the response to rapamycin. We identified 5 amino acid residues in Fkh1 that are critical for the effect of rapamycin in S. pombe. Four of the five amino acids identified, Phe-47, Leu-56, Ile-92, and Phe-100, correspond to conserved amino acid residues of HuFKBP12 that most closely interact with rapamycin. The fifth amino acid, Cys-49, corresponding to Cys-48 in HuFKBP12, is not mapped to the very core of the ligand-binding pocket but in a close vicinity. The C49R mutation, however, conferred complete rapamycin resistance in S. pombe, and our studies in S. cerevisiae suggested that it diminished rapamycin binding (Fig. 6). Since all the rapamycin-resistant fkh1 mutants are mutated at amino acid residues potentially important for rapamycin binding, it is likely that the rapamycin resistance phenotype stems from an inability of the mutant proteins to bind the drug. This suggestion awaits further support from binding experiments of rapamycin to the wild type and mutant Fkh1 proteins.

Notably, all the rapamycin-resistant fkh1 mutants completely restored mating in Δfkh1 mutants (Fig. 4). This finding suggests that the Fkh1 rapamycin-binding site and the putative active site do not completely overlap. In particular, mutation at the Phe-100 amino acid residue did not impair the cellular activity of Fkh1 in sexual development, despite its being one of the most conserved amino acid residues in FKBP12 sequences (21). The effects of mutations at positions corresponding to Phe-100 in human FKBP12 (F99Y) and in S. cerevisiae FPR1 (F106Y) have been studied previously (67, 68). Like the F100L mutation in Fkh1, neither the F99Y mutation nor the F106Y mutation affected the protein function in vivo. The F99Y mutant supported the ryanodine channel function (67), and the F106Y mutant complemented the slow growth phenotype observed in Δfpr1 (68). It is also interesting to note that the HuFKBP12 F99Y mutant and the S. cerevisiae F106Y mutant show reduced PPIase activity in the in vitro peptide cleavage assay (67, 68). More recently, however, it was demonstrated that the S. cerevisiae Fhe-106 mutant retained PPIase activity in a different assay that uses ribonuclease T1 as a substrate (68). It has thus been suggested by Dolinski et al. (68)

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that the Phe-106 mutant may retain PPIase activity toward large substrates, leaving it an open question whether the PPIase activity observed in vitro is relevant for the cellular activity in vivo.

The finding that fkh1" is required for sexual development provides a novel, genetically amenable system to study the future we intend to exploit this system to identify the substrate(s) for Fkh1 cellular function. In the future, a similar approach will be utilized to isolate fkh1 mutants that are rapamycin-resistant. In the future, a similar approach will be used to support sexual development, thus determining the amino acid residues critical for Fkh1 activity in this pathway.

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