TCR–self antigen interaction to complete differentiation may well apply generally to TCRγδ-bearing T cells, independent of their individual receptor specificities.

However, the normal maturation of γδ T cells in nontransgenic $\beta_2M^{-/-}$ mice indicates that many γδ T cells do not require class I MHC in order to differentiate (*3, 4*). Thus, if all γδ cells are subject to positive selection, non-MHC antigens might function as selecting self restriction molecules for many TCRγδ. When the cell surface molecules [for example, heat shock proteins (*19*)] that restrict the responses of non-MHC–specific γδ T cells are identified, this possibility can be tested directly. However, the fact that the self antigens that select the $V_\delta5$ BID receptor map outside the MHC is consistent with this notion (*17*). If true, then the requirement for TCR engagement might be a universal property of all T cell differentiation.

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11. The mean percentage (±SEM) Tg^+ ($V_\gamma2^+$) cells in the thymus, spleen, and lymph nodes for the different groups of mice were determined. The mean percentage of Tg^+ thymocytes was 24 ± 4% (n = 15) for $\beta_2M^{-/-}$ mice, 28 ± 8% (n = 6) for $\beta_2M^{+/-}$ mice and 49 ± 6% (n = 15) for $\beta_2M^{+/+}$ mice. The corresponding mean absolute numbers of $V_\gamma2^+$ thymocytes were $1.5 \times 10^7 \pm 4 \times 10^6$ for $\beta_2M^{-/-}$, $2.8 \times 10^6 \pm 10^6$ for $\beta_2M^{+/-}$, and $6.2 \times 10^6 \pm 1.2 \times 10^6$ for $\beta_2M^{+/+}$. The mean percentage of $CD4^-CD8^-$ Tg^+ cells in spleen were 1 ± 0.2% (n = 5) for $\beta_2M^{-/-}$, 12 ± 3% (n = 4) for $\beta_2M^{+/-}$, and 17 ± 2.5% (n = 4) for $\beta_2M^{+/+}$. The average total number of cells per spleen (5×10^7 to 10^8) did not differ significantly among $\beta_2M^{-/-}$, $\beta_2M^{+/-}$, and $\beta_2M^{+/+}$ mice. Thus, the percentage values are directly proportional to the absolute numbers of Tg^+ splenocytes. The mean percentage of lymph node $CD4^-CD8^-$ Tg^+ cells were 1 ± 0.1% (n = 4) for $\beta_2M^{-/-}$, 32 ± 4% (n = 4) for $\beta_2M^{+/-}$ and 52 ± 6% (n = 7) for $\beta_2M^{+/+}$. Lymph nodes were quite comparable in size among different animals, indicating that the percentage values reflect the absolute numbers of Tg^+ cells present. However, the absolute numbers of Tg^+ lymph node cells were not compared because variable numbers of lymph nodes were recovered from individual animals.
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Targets for Cell Cycle Arrest by the Immunosuppressant Rapamycin in Yeast

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FK506 and rapamycin are related immunosuppressive compounds that block helper T cell activation by interfering with signal transduction. In vitro, both drugs bind and inhibit the FK506-binding protein (FKBP) proline rotamase. *Saccharomyces cerevisiae* cells treated with rapamycin irreversibly arrested in the G1 phase of the cell cycle. An FKBP-rapamycin complex is concluded to be the toxic agent because (i) strains that lack FKBP proline rotamase, encoded by *FPR1*, were viable and fully resistant to rapamycin and (ii) FK506 antagonized rapamycin toxicity in vivo. Mutations that conferred rapamycin resistance altered conserved residues in FKBP that are critical for drug binding. Two genes other than *FPR1*, named *TOR1* and *TOR2*, that participate in rapamycin toxicity were identified. Nonallelic noncomplementation between *FPR1*, *TOR1*, and *TOR2* alleles suggests that the products of these genes may interact as subunits of a protein complex. Such a complex may mediate nuclear entry of signals required for progression through the cell cycle.

IN HIGHER EUKARYOTES, THE IMMUnosuppressive compounds cyclosporin A (CsA), FK506, and rapamycin inhibit the intermediate steps in signal transduction between the cytoplasm and the nucleus. These three compounds are immunosuppressive because they block signal transduction pathways required for the activation of helper T cells (*1, 2*).

FK506 and rapamycin are related macrolides and share no chemical similarity with CsA, a cyclic undecapeptide (*2*). Although structurally unrelated, CsA and FK506 inhibit the same step in T cell activation, an antigen-stimulated, Ca^{2+}-dependent signal transduction step that acts through the nuclear factor of activated T cells (transcription factor NF-AT) (*3*). In contrast, rapamycin does not interfere with the response to an antigen but rather impairs a later step in T cell activation, the proliferative response to interleukin-2 (*4, 5*).

The effects of CsA, FK506, and rapamycin are thought to be mediated by the

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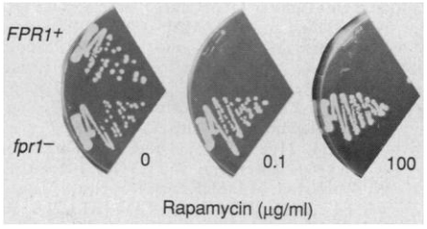

Fig. 1. Yeast strains that lack FKBP are resistant to rapamycin toxicity. Isogenic haploid strains that express (*FPR1*$^+$) or lack (*fpr1*$^-$) the FKBP proline isomerase were grown for 65 hours at 30°C on YEPD medium (*32*) containing rapamycin at 0, 0.1, or 100 μg/ml, prepared as described for FK506 medium (*16*). The *fpr1-1*, *fpr1-2*, and *fpr1-3* alleles (*16*) all conferred resistance to rapamycin. The strains are JK9-3dα (*FPR1*$^+$) and JH3-3b (*fpr1*$^-$) (*17*).

proline rotamases cyclophilin and FKBP (*6, 7*), which may be important for protein folding (*7–11*). In vitro, CsA inhibits cyclophilin, whereas FK506 and rapamycin inhibit FKBP (*5, 7, 8, 12*). However, the mechanism by which these compounds act as immunosuppressive drugs is unclear. Models that explain immunosuppressive drug action include the following: (i) inhibition of a proline rotamase activity that is responsible for folding components of signal transduction pathways (*7, 8*); (ii) inhibition of proteins other than cyclophilin or FKBP (minor immunophilins) (*13*); and (iii) formation of a rotamase-drug complex that poisons signal transduction (*2, 14, 15*).

Several observations suggest that immunosuppression is not attributable to rotamase inhibition. First, the drug concentration necessary to inhibit T cell activation is not sufficient to saturate the abundant rotamases (*5*); second, FK506 and rapamycin both inhibit FKBP rotamase activity, but each drug blocks a different signaling pathway (*3, 4, 5*); third, FK506 and rapamycin antagonize each other's effect on T cells (*4, 5*); and fourth, the FK506-rapamycin analog 506BD is a potent inhibitor of FKBP proline rotamase activity but is not immunosuppressive (*15*). Our studies investigate the action of rapamycin and FK506 in yeast.

Growth of isogenic haploid (Fig. 1) and diploid derivatives of *S. cerevisiae* strain JK9-3d (*16, 17*) was sensitive to the immunosuppressant rapamycin (*18*) with a minimum inhibitory concentration (MIC) of 0.05 to 0.1 μg/ml. Sensitivity to rapamycin was not dependent on strain, ploidy, respiratory competence, or amino acid auxotrophies, properties that affect sensitivity of yeast to CsA (*14*) and FK506 (*16, 19*). Cultures of strain JK9-3d treated with rapamycin yielded few viable cells, indicating that rapamycin is toxic to yeast. Moreover, the irreversible arrest of cell growth was predominantly (60 to 70%) in the G1 phase of the cell cycle, as determined from a terminal arrest phenotype of large unbudded cells (Fig. 2). CsA, FK506, and rapamycin, which block T cell activation and proliferation, produce an equivalent cell cycle arrest in cells of the immune system (*20*).

Because rapamycin binds and inhibits FKBP in vitro, isogenic haploid and diploid derivatives of strain JK9-3d that contain either the wild-type *FPR1* gene, which encodes FKBP, or disruption alleles *fpr1-1*, *fpr1-2*, or *fpr1-3* (*16*) were tested for rapamycin sensitivity. Strains that lack FKBP were fully resistant to concentrations of rapamycin 1000- to 2000-fold greater than the MIC (Fig. 1); haploid and homozygous diploid *fpr1* strains treated with rapamycin proceeded normally through the cell cycle. The cloned *FPR1* gene, in single or high copy number (*17*), complemented the *fpr1-1*, *fpr1-2*, and *fpr1-3* alleles and restored rapamycin sensitivity to the level observed with a single chromosomal copy of the gene. Overexpression of FKBP did not confer resistance or hypersensitivity to rapamycin (*21*). The observations that strains lacking the FKBP proline rotamase are viable (*16, 22*) and resistant to rapamycin, whereas strains that express FKBP are sensitive to rapamycin, support the model that rapamycin toxicity is mediated by a poisonous FKBP-rapamycin complex.

The observation that rapamycin and FK506 inhibit each other's effects on T cells suggests that the two drugs compete for binding to the FKBP proline rotamase (*4, 5*). If FK506 and rapamycin do indeed compete for FKBP in vivo, then FK506 might prevent formation of an FKBP-rapamycin complex and protect yeast cells from rapamycin toxicity. To test this hypothesis, we used yeast strain JH6-1b, which is sensitive to rapamycin and resistant to FK506. Strain JH6-1b, a derivative of strain JK9-3dα, (i) expresses FKBP, which is essential for rapamycin sensitivity, (ii) is haploid, thus its colony-forming efficiency is not decreased by FK506 (*16*), and (iii) is a *TRP1* prototroph; FK506 inhibits import of amino acids in yeast and prototrophic strains are FK506-resistant (*19*). As predicted, an excess of FK506 antagonized the toxic effect of rapamycin and partially restored growth of JH6-1b (*23*) (Fig. 3). The simplest interpretation is that the FKBP proline rotamase is a target for both FK506 and rapamycin in yeast cells and that FK506 competitively inhibits formation of

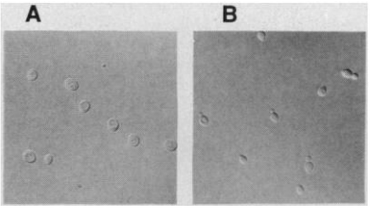

Fig. 2. Arrest of yeast cell cycle by rapamycin. A culture of diploid strain JK9-3da/α (*17*) was grown to early logarithmic phase in synthetic complete medium (*32*), divided, and treated (**A**) with a rapamycin solution (0.1 μg/ml final concentration) or (**B**) with an equal volume of a blank solution (ethanol with 10% Tween 20). After 5 hours of incubation at 30°C, cells were adhered to polylysine-treated glass slides. Samples were protected with glass cover slips affixed with a drop of Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, Alabama), allowed to harden for 12 hours, and analyzed by photomicroscopy with Nomarski optics. The G1 arrest was most pronounced with a diploid grown in synthetic complete medium.

Table 1. Rapamycin-resistant mutants. Growth was assessed after 52 hours at 30°C on YEPD medium containing rapamycin at the indicated concentrations. Resistance is denoted as follows: "+" > "+/−" > "−/+" > "−." For the *fpr1-7* allele, plasmid YCp50::FPR1 (*17*) was mutagenized in vitro with hydroxylamine (*32*) and introduced into *fpr1-2* strain JH2-1b (*17*), with selection for uracil prototrophy, and a mutant resistant to rapamycin (0.1 μg/ml) was identified by replica plating on synthetic uracil-deficient medium (*32*) containing rapamycin. In this case, denatured plasmid DNA served as the template for sequencing. All other alleles are spontaneous, chromosomal mutations. Sequencing templates of chromosomal *fpr1* mutations were obtained by asymmetric PCR amplification of genomic DNA with primers flanking the *FPR1* locus (*24*). Mutations are designated by the wild-type amino acid followed by the position in the sequence and then the amino acid substitution. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe, G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q. Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Data that were not determined are indicated by n.d.

Allele	Growth on rapamycin (μg/ml)				FKBP sequence
	0	0.1	1	10	
fpr1-4	+	+	+	−	M1I
fpr1-5	+	+	+	−	P23R
fpr1-6	+	+	+/−	−	H32P
fpr1-7	+	+	n.d.	n.d.	G35D
fpr1-8	+	+	+	+	G65D
fpr1-9	+	+	−/+	−	G65C
fpr1-10	+	+	+	−/+	G65V
fpr1-11	+	+	+/−	−	L81S
fpr1-12	+	+	+	+	E109ochre (UAA)
tor1-1	+	+	+	+	WT
TOR1-2	+	+/−	+/−	−/+	WT
tor2-1	+	+	+	+	n.d.
fpr1-3	+	+	+	+	*fpr1::URA3-3*
FPR1	+	−	−	−	WT

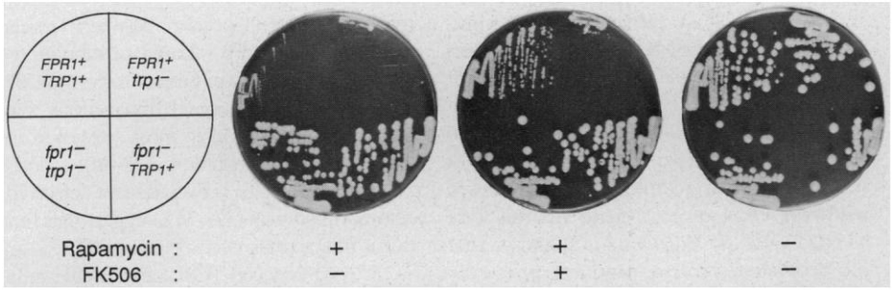

Fig. 3. Antagonism of rapamycin toxicity by FK506. Isogenic haploid derivatives of strain JK9-3d (*17*) were grown for 96 hours at 30°C on YEPD medium (*32*) that contained either rapamycin (0.1 μg/ml), rapamycin (0.1 μg/ml) plus FK506 (50 μg/ml), or neither drug (presence or absence indicated by + or −). Antagonism is seen with the *FPR1*$^+$ *TRP1*$^+$ strain JH6-1b (upper left quadrant); this is the only strain that is both sensitive to rapamycin (*FPR1*$^+$) and resistant to the toxic effects of FK506 (*TRP1*$^+$) (*16, 19*). All other strains are controls. The strains are JH6-1b (*FPR1*$^+$ *TRP1*$^+$), JK9-3dα (*FPR1*$^+$ *trp1*$^-$), JH10 (*fpr1*$^-$ *TRP1*$^+$), and JH3-3b (*fpr1*$^-$ *trp1*$^-$) (*17*).

the toxic FKBP-rapamycin complex. The reversal of rapamycin toxicity by FK506 and the observation that FKBP overexpression confers FK506 resistance (*16*) suggest that an FKBP-FK506 complex is only weakly toxic, if at all.

To study the interaction of FKBP with rapamycin and identify other proteins that contribute to rapamycin toxicity, we isolated rapamycin-resistant yeast mutants. Spontaneous independent mutants resistant to rapamycin (0.1 μg/ml) were isolated from a and α haploid derivatives of strain JK9-3d (*17*). Genetic crosses between rapamycin-resistant mutants and the rapamycin-sensitive parental strain demonstrated that the majority of the mutations (15 of 18) were fully recessive (diploids were rapamycin-sensitive). Two mutations, labeled *tor1-1* and *tor2-1* for target of rapamycin, were recessive at 24°C and partially dominant at 30°C or 37°C. The one fully dominant mutant, *TOR1-2*, was isolated from a strain (JK9-3d) that carried *FPR1* on a multicopy plasmid (*17*). Sporulation and dissection of these diploid strains revealed a pattern of segregation, indicating that rapamycin resistance was conferred by a single nuclear mutation; the segregation pattern among the four meiotic products was two rapamycin-resistant (R) and two rapamycin-sensitive (S) segregants, that is, 2R:2S. The resistant haploids were resistant to rapamycin at 24°, 30°, and 37°C.

The rapamycin-resistant mutants, including the disruption alleles *fpr1-2* and *fpr1-3* (*16*), were crossed to determine which mutants contain alleles of *FRP1* and the number of complementation groups. The 15 fully recessive mutations were allelic with *FPR1* as indicated by (i) failure to complement the *fpr1-2* or *fpr1-3* allele (diploids resistant to rapamycin); (ii) linkage to *FPR1* based on meiotic segregation opposite the *fpr1-2* or *fpr1-3* allele (4R:0S); (iii) complementation by cloned *FPR1* (restoration of rapamycin sensitivity); (iv) exhibition of the *fpr1* phenotype of slow growth on YEPD medium (*16*); and (v) DNA sequence analysis of the *FPR1* gene (Table 1). An additional *fpr1* allele that conferred rapamycin resistance was isolated after mutagenesis of a plasmid-borne copy of *FPR1* (Table 1).

The DNA sequence of *FPR1* was determined for each of the rapamycin-resistant mutants (Table 1) (*24*). As expected from the genetic complementation and segregation analyses, the majority of the mutants contained mutations within *FPR1*. Eight unique missense mutations and one unusual nonsense mutation in *FPR1* were identified (Table 1 and Fig. 4). One missense mutation (P23R) altered a residue conserved in yeast and human FKBP but not in *Neurospora crassa* or bacterial FKBP homologs (*25*). The remaining missense mutations were of invariant (G35D; G65D, C or, V) or highly conserved (H32P, L81S) residues in the FKBP proteins from humans, *N. crassa*, and yeast, and in the two bacterial proteins with marked similarity to FKBP (*9, 10, 16, 25*). In contrast to *fpr1* null alleles (*fpr1-1, fpr1-2*, and *fpr1-3*), most *fpr1* missense alleles did not confer resistance to rapamycin at 10 μg/ml, indicating that the encoded proteins (P23R, H32P, G65C, G65V, and L81S) were expressed and retained some rapamycin-binding activity (Table 1). The nonsense mutation *fpr1-12* resulted in a truncated FKBP that lacked six COOH-terminal amino acids.

The nuclear magnetic resonance (NMR) solution structure of human FKBP and the x-ray crystal structure of the FKBP-FK506 complex have been deduced (*26*). Yeast and human FKBP share 54% identity and many conservative amino acid substitutions. Residues that compose the drug-binding site of human FKBP (Y26, W59, and Y82) are conserved in yeast FKBP (Y33, W66, and Y89) (*16, 26*) (Fig. 4). We used the structures of the human FKBP as a guide to understanding the mutations that rendered yeast FKBP insensitive to rapamycin. In Fig. 5, these mutations are indicated at the corresponding positions in the NMR structure of human FKBP. Three mutations altered G65; this residue is adjacent to W66, which in human FKBP (W59) lies directly above the pipecolinic (homoproline) ring of FK506 in the FKBP drug-binding site (*26*).

On the basis of molecular modeling, we conclude that steric interference with other side chains should occur when any amino acid other than glycine is substituted at position 58 of human FKBP (corresponding to G65 of yeast FKBP). However, because yeast FKBP mutants in which residue G65 has been substituted by valine or cysteine retain partial rapamycin sensitivity in vivo, small uncharged side chains may be accommodated by local structural alterations. Two other mutations, H32P and G35D, corresponding to H25 and G28 in human FKBP, are predicted to disrupt the positioning of other residues that lie in the drug-binding site (Y26 and Y82 of human FKBP). Thus, the missense mutations support the assignment of the drug-binding site as determined by NMR and x-ray crystal

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                    10        20        30        40        50
Yeast:     MSEVIEGNVKIDRISPGDGATFPKTGDLVTIHYTGTLENGQKFDSSVDRG
Human:     -------GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRN
N. crassa: MTIPQLDGLQIEVQQEGQGTRETRRGDNVDVHYKGVLTSGKKFDASYDRG
N. meningitidis: ------MGSLIIEDLQAEFGKEAVKGKEITVHYTGWLEDGTKFDSSLDRR
L. pneumophila: GVVVLPSGLQYKVINAGNGVKPGK-SDTVTVEYTGRLIDGTVFDSTEKTG
Consensus: -------G--i--i--G-G----k-G---tVHYTG-LedG-KFDSS-DRg
Mutations: ----------------------r--------p--d---------------

          60        70        80        90        100       110
SPFQCNIGVGQVIKGWDVGIPKLSVGEKARLTIPGPYAYGPRGFPGLIPPNSTLVFDVELLKVN
KPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE
EPLNFTVGQGQVIKGWDEGLLGMKIGEKRKLTIAPHLAYGNRAVGGIIPANSTLIFETELVGIKGVQKGE
QPLTITLGVGQVIKGWDEGFGGMKEGGKRKLTIPSEMGYGATRRGGVIPPHATLIFEVELLKVYE
KPATFQVS--QVIPGWTEALQLMPAGSTWEIYVPSGLAYGPRSVGGPIGPNETLIFKIHLISVKKSS
-P--f--G-gQVIkGWdEG---M--G-k-kLTIp---AYG-r--gG-IPPn-TLiF-vELlkv-
--------------d---------------s----------------------------stop
                c
                v
```

Fig. 4. Rapamycin resistance is conferred by mutations in highly conserved FKBP residues. FKBP-homologous sequences from yeast (*16, 22*), human (*10*), *N. crassa* (*9*), and two bacterial proteins (*25*) are aligned. In some cases, short gaps were introduced to maximize overlap. The *L. pneumophila* sequence is residues 120 to 233 of the macrophage infection potentiator (mip) protein. Amino acid number designations are from the yeast FKBP, and every tenth amino acid is underlined. The consensus is shown beneath the five aligned FKBP sequences. In the consensus, invariant residues (present in all five proteins) are capitalized and underlined; residues that occur in four out of five sequences are capitalized; less-conserved residues (three out of five matches) are indicated by lowercase letters. FKBP mutations that confer rapamycin resistance in yeast are indicated below the consensus sequence.

structures and further indicate that FK506 and rapamycin have the same binding site. The *fpr1-12* nonsense mutation removes six COOH-terminal residues, a region that is unlikely to participate in drug binding, because in human FKBP these residues lie outside the drug-binding site (Fig. 5).

The three remaining rapamycin-resistant mutations (*tor1-1, TOR1-2*, and *tor2-1*) are not alleles of *FPR1*. Under conditions in which they are recessive (24°C), *tor1-1* and *tor1-2* complemented *fpr1-3* and were not complemented by the cloned *FPR1* gene. Furthermore, the *tor1-1* and *tor2-1* mutations segregated independently from *fpr1* (*27*), and *tor* mutant strains were found to contain a wild-type *FPR1* gene on the basis of sequence analysis of PCR-amplified genomic DNA (Table 1) (*24*) and, in contrast to *fpr1* mutants, did not grow slowly. Because the *tor1-1* and *tor2-1* mutations are recessive at 24°C and semidominant at 30°C or 37°C, these mutations are unlikely to cause simple loss of function.

Examination of crosses between the *tor1-1* and *tor2-1* mutants initially suggested that the two mutations were allelic because they failed to complement; a *tor1-1*×*tor2-1* diploid was resistant to rapamycin (10 μg/ml) under conditions where the two mutations are recessive (24°C). However, the two mutations segregated independently in meiosis (*27*) and therefore defined two independent genes (*TOR1* and *TOR2*). Nonallelic noncomplementation (*28–31*), in which the mutant phenotype of two recessive mutations is still expressed in a diploid that contains a wild-type copy of each gene (in this case *tor1-1/TOR1 TOR2/tor2-1*), suggests that the respective protein products physically interact (*28–30*). The single fully dominant rapamycin-resistant mutant was shown to be a second allele of *TOR1* (*TOR1-2*) on the basis of segregation opposite *tor1-1*; of 18 tetrads dissected from a *tor1-1*×*TOR1-2* diploid, all were 4R:0S.

Nonallelic noncomplementation was also observed between *tor2-1* and *fpr1-12*. Under conditions where the *tor2-1* mutation is recessive (24°C), the *tor2-1*×*fpr1-12* diploid was resistant to rapamycin (10 μg/ml). This finding suggests that the fpr1-12 protein is expressed and that the remaining portion of FKBP (residues 1 through 108) is sufficient for some functional interaction with at least the tor2-1 mutant protein. Crosses between *tor1-1* or *tor2-1* and other *fpr1* alleles did not reveal any other clear examples of nonallelic noncomplementation.

In both *Drosophila* and yeast, nonallelic noncomplementation occurs between mutant forms of the α and β subunits of tubulin, two proteins that form a heterodimer (*28, 29*). Although protein-protein interactions have been proposed to explain most cases of nonallelic noncomplementation (*28–30*), another possible explanation is two additive defects in a common pathway (*31*). We suggest that functional interactions occur between TOR1 and TOR2 and between TOR2 and FKBP, either as members of a metabolic or enzymatic pathway or as a protein-protein complex. One model to explain α and β tubulin mutant interactions is that a mutant α-β complex poisons tubulin polymerization (*28*). By analogy, the *tor1-1*×*tor2-1* or *tor2-1*×*fpr1-12* mutant combinations could produce a hyperactive species that overcomes the cytotoxicity of the FKBP-rapamycin complex.

The finding that *S. cerevisiae* lacking the FKBP proline rotamase is viable and fully resistant to rapamycin toxicity supports a model in which an FKBP-rapamycin complex mediates rapamycin toxicity in yeast. Like the toxicity of rapamycin, toxicity of CsA in both *N. crassa* and *S. cerevisiae* appears to be mediated through a rotamase-drug complex; in this case the rotamase is cyclophilin (*14*). Similar rotamase-drug complexes may poison signal transduction pathway components in T cells to produce immunosuppression. The finding that FK506 protects yeast from rapamycin suggests that the FKBP-FK506 and the FKBP-rapamycin complexes have different physiological effects, providing an explanation for the observation that these two structurally related immunosuppressive drugs have different effects on T cells despite binding to the same protein.

That cyclophilin and FKBP are abundant proteins expressed in cells other than T cells raises the paradox as to how CsA, FK506, and rapamycin can interact with such nonspecific targets to mediate highly specific immunosuppressive effects. Because both cyclophilin and FKBP are dispensable for growth in yeast (*16*), widespread inhibition of rotamases might have only minor effects in most tissues in animals. The finding that the TOR1 and TOR2 gene products are required for rapamycin toxicity in yeast suggests that T cell–specific factors, possibly homologs of TOR1 and TOR2, may be necessary to render FKBP an effective target for rapamycin and that cell types resistant to rapamycin may not express these factors.

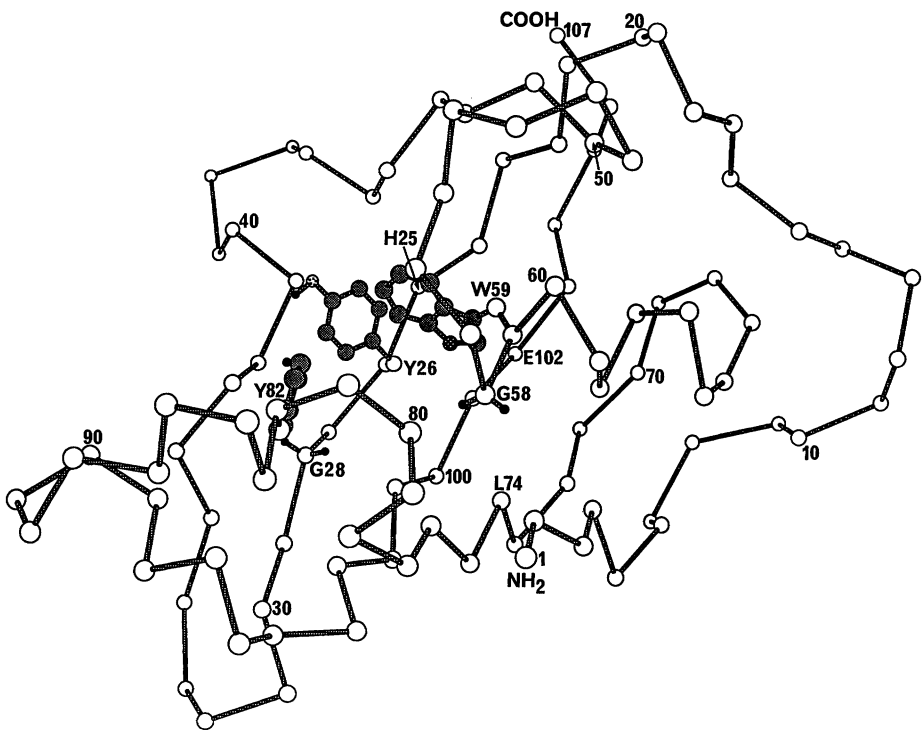

Fig. 5. Rapamycin resistance by alteration of the FKBP drug-binding site. Shown is an α-carbon tracing of human FKBP; the drug-binding site is to the upper left (*26*). The FK506-binding site is composed of amino acids Y26, W59, and Y82; the side chains (shaded atoms) of these residues have been included in the α-carbon tracing. The nearby residues H25, G28, G58, and L74 correspond to the amino acids of yeast FKBP (H32, G35, G65, and L81) that, once mutated, confer rapamycin resistance (Table 1 and Fig. 4). By molecular modeling of human FKBP, we expect substitutions at these sites to disrupt the structure of the drug-binding site. The α-carbon tracing of human FKBP was provided by T. Wandless and S. Schreiber (*26*).

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17. The *S. cerevisiae* strains used in all experiments were JK9-3d, a/α, a, or α *leu2-3,112 ura3-52 rme1 trp1 his4 HML*a (the diploid JK9-3da/α is homozygous for all markers except mating type) (*16*), or isogenic derivative of JK9-3d. Isogenic derivatives with only the changes indicated were as follows: JH1-2a, α *fpr1::URA3-1*; JH2-1b, α *ade2 fpr1::ADE2-2*; JH3-3b, α *fpr1::URA3-3* (*16*); JH6-1b, α *TRP1*; JH10, α *fpr1::URA3-3 TRP1*. The alleles *fpr1::URA3-1, fpr1::ADE2-2*, and *fpr1::URA3-3* are the same as *fpr1-1, fpr1-2*, and *fpr1-3* (*16*), respectively. Single and high copy number plasmids bearing *FPR1*, in both cases contained in a 2-kb Eco RI fragment, were YCp50::FPR1 and pYJH23, respectively. The plasmid YCp50::FPR1 is *amp cen ars URA3 FPR1*. The plasmid pYJH23 is *amp 2μ URA3 FPR1* (*16*).
18. Rapamycin was originally isolated as an antifungal compound and described to have potent activity against *Candida albicans* [H. Baker, A. Sidorowicz, S. N. Sehgal, C. Vezina, *J. Antibiot.* **31**, 539 (1978)].
19. FK506 inhibits transport of some amino acids in *S. cerevisiae*. Thus, auxotrophic strain JK9-3d (*trp1 his4*) is sensitive to FK506, and prototrophic derivatives (*TRP1* or *HIS4*) are resistant to FK506 (J. Heitman and M. N. Hall, unpublished data). For unknown reasons, a derivative that is prototrophic for only one amino acid (*TRP1*) becomes resistant although it still depends on import of another amino acid (*his4*). A possible explanation is that multiple auxotrophies are necessary to reach a threshold beyond which amino acids become limiting. Sensitivity of auxotrophic JK9-3d to FK506 appears not to be mediated through an FKBP-FK506 complex because FK506 still inhibit amino acid transport in an *fpr1* mutant.
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21. Data are not shown. FKBP was overexpressed by providing *FPR1* on multicopy plasmid pYJH23 in strain JK9-3dα (*17*).
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23. Antagonism between FK506 and rapamycin in T cells also requires an excess (100- to 500-fold of the limiting amount) of the competing compound (*4, 5*).
24. Oligonucleotides for polymerase chain reaction (PCR) were: 5′ oligo (P130), 5′-AAATTGCCGGAAGAGAGACG-3′; 3′ oligo (P132), 5′-ACGTATGCTTCTCCTTGTC-3′. Asymmetric PCR amplification reactions contained 50 pM P130 (330 ng), 1 pM P132 (6.6 ng), 50 μM dNTP (N = A, G, T, and C), and 10 μl of a 1:100 dilution of yeast genomic DNA (approximately 200 to 400 ng) in a final reaction volume of 100 μl. The reaction mixtures were overlaid with 10 μl of mineral oil and PCR-amplified with a Perkin-Elmers thermocycler for 30 cycles consisting of 95°C for 1 min, 45°C for 1 min, and 70°C for 2 min. Yeast genomic DNA was purified as described [C. S. Hoffman and F. Winston, *Gene* **57**, 267 (1987)]. Oligonucleotides for sequencing were P132 (above), P142 (5′-TAAGGCTCAGATACTTACC-3′), and P133 (5′-TTCACCAACAGACAACTT-3′).
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27. An *fpr1*×*tor1-1* diploid yielded 4 4R:0S, 1 2R:2S, and 14 3R:1S tetrads: *fpr1*×*tor2-1* diploid, 3 4R:0S, 6 2R:2S, and 22 3R:1S; *tor1-1*×*tor2-1* diploid, 6 4R:0S, 4 2R:2S, and 18 3R:1S.
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Factor XI Activation in a Revised Model of Blood Coagulation

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Coagulation factor XI is activated in vitro by factor XIIa in the presence of high molecular weight kininogen (HMWK) and a negatively charged surface. Factor XII deficiency is not associated with bleeding, which suggests that another mechanism for factor XI activation exists in vivo. A revised model of coagulation is proposed in which factor XI is activated by thrombin. In the absence of cofactors, thrombin is more effective (k_{cat}/K_m = 1.6 × 10^5) than factor XIIa (1.7 × 10^4) in activating factor XI. Dextran sulfate enhances activation of factor XI by thrombin 2000-fold; part of this effect is due to autoactivation of factor XI by activated factor XI.

IN THE CASCADE (*1*) OR WATERFALL (*2*) hypothesis of hemostasis, blood coagulation proceeds through a series of reactions in which plasma zymogens of serine proteases are sequentially activated by limited proteolysis, culminating in the generation of thrombin, which then cleaves fibrinogen to produce fibrin. The putative "intrinsic" pathway of coagulation is initiated when blood is exposed to a negatively charged surface, which activates factor XII in a reaction involving high molecular weight kininogen (HMWK) and prekallikrein (*3*). Activated factor XII (factor XIIa) then activates factor XI (*4*) and factor XIa, in turn, is responsible for the activation of factor IX (*5*). It is apparent that factor XI is important in normal hemostasis because individuals deficient in factor XI suffer a variable bleeding diathesis that may be particularly severe after surgery (*6*). In contrast, deficiency of factor XII, HMWK, or prekallikrein is not associated with bleeding, suggesting that an alternative mechanism for the activation of factor XI exists in vivo. In a recent, revised model of coagulation, factor XI is not required for the initiation of coagulation, but instead, functions to sustain hemostasis after coagulation has been induced by the exposure of plasma to tissue factor at a site of injury (*7*). We therefore investigated the ability of thrombin, an enzyme generated late in the coagulation cascade, to activate factor XI.

Factor XI is a 160-kD homodimer composed of two identical 83-kD polypeptides connected by a disulfide bond. Each factor XI monomer is cleaved by factor XIIa at a single site, resulting in a 47-kD heavy chain and a 35-kD light chain, the latter of which contains the catalytic serine-protease site (*4*). Initial experiments demonstrated that thrombin cleaves factor XI to yield a pattern

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