Catalytic and ligand binding properties of the FK506 binding protein FKBP12: effects of the single amino acid substitution of Tyr⁸² to Leu

Mary J. BOSSARD,* Derk J. BERGSMA,† Martin BRANDT,* George P. LIVI,‡ Wai-Kwong ENG,§, Randall K. JOHNSON and Mark A. LEVY*||

Departments of *Medical Chemistry, †Molecular Genetics, ‡Gene Expression Sciences and §Biomolecular Discovery, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406, U.S.A.

The binding of FK506 and rapamycin to their cytosolic receptor FKBP12 is an intermediate step in the paths leading to their potent immunosuppressive properties. One of the amino acids defining the hydrophobic binding cleft for the macrocycles is Tyr⁸², which is thought to form a hydrogen bond with the amide oxygens of the common piperidyl structural element within the two macrolides. To understand better the influence of this amino acid residue in catalytic activity (*cis-trans* peptidyl prolyl isomerization) and ligand binding properties, a Tyr⁸² to Leu site-specific modification of FKBP12 was prepared, purified and charac-

terized. Kinetic experiments have demonstrated that the Tyr⁸² to Leu modification has a greater effect on catalytic properties than on ligand binding affinities, a result which indicates that these inhibitors may not be binding as true transition-state analogues. In an additional test for cellular function, expression of both wild-type and mutant human FKBP12 in a strain of *Saccharomyces cerevisiae* rendered resistant to rapamycin by deletion of the gene encoding a cytosolic rapamycin binding protein (RBP1), the yeast homologue of FKBP12, restored wild-type drug sensitivity.

INTRODUCTION

The mechanisms by which cyclosporin A (CsA), FK506 and rapamycin achieve their potent immunosuppressive responses are thought to involve their binding to intracellular receptor proteins, referred to as immunophilins (for reviews of this research area, see [1–3]). CsA shows high-affinity association for members of the cyclophilin (CYP) class of immunophilin, whereas the structurally similar macrocycles FK506 and rapamycin both bind to members of a family of sequence-related proteins, respectively the FKBP (FK506 binding proteins) and RBP (rapamycin binding proteins). Despite the lack of sequence identity between the two classes of immunophilins, both the CYPs and the FKBP catalyse the *cis-trans* isomerization of peptidyl-prolyl bonds in peptides and proteins [1–3]. Neither the normal cellular function(s) of the immunophilins nor the biological significance of the catalytic activity as a peptide isomerase currently is understood.

The three natural products, FK506 ($K_{i,app} \sim 0.8$ – 2 nM), rapamycin ($K_{i,app} \sim 0.2$ – 0.5 nM) and CsA ($K_{i,app} \sim 2$ – 5 nM), are potent inhibitors of their respective binding protein's catalytic activities. However, a cellular response does not seem to result from a loss of these catalytic activities. The current model for the mechanisms of these drugs, which has evolved during the past 2 years, suggests that the binary complexes formed upon association of CsA with cyclophilin and FK506 or rapamycin with FKBP12 are the active cellular agents (see [1–3] for reviews of experiments leading to these conclusions). Each of these three compounds incorporates two important structural elements: a binding domain responsible for the molecule's high affinity for its cognate immunophilin, and an effector domain which physically contributes to the interaction of the immunophilin–ligand binary complex with a second, 'downstream' intracellular target [1].

The secondary target for the FK506–FKBP and CsA–CYP complexes has been proposed to be the Ca²⁺/calmodulin-dependent serine/threonine phosphatase known as calcineurin or PP2B [4,5]. No analogous direct intracellular receptor for the FKBP–rapamycin complex has been yet identified. However, the pathway influenced by the rapamycin–FKBP complex, which appears to be distinct from that involving CsA and FK506, might involve the phosphorylation and activation of pp70^{src} kinase [6,7].

The dual-domain structural hypothesis involving these potent peptidyl-prolyl *cis-trans* isomerase (PPIase) inhibitors and immunomodulators has prompted considerable interest in the mechanism of enzymic catalysis and the structures of immunophilin binary complexes with tight binding ligands. In principle, one approach to new immunoregulatory molecules could involve identification of novel, totally synthetic immunophilin binding domains to which a putative effector element ultimately could be tethered. The design of such binding domains should be facilitated by a more complete understanding of the catalytic mechanisms of FKBP and CYP (for one approach towards binding elements for FKBP, see [8]).

To achieve these objectives, there has been considerable effort in pursuing a better understanding of the interactions between the immunophilins, their respective immunosuppressive ligands, PPIase substrates and potential targets of the drug–protein complexes. N.m.r. and X-ray structural analyses of FKBP12, the predominant cytosolic member of this protein family, have revealed that its ligand binding site is composed of the aromatic side chains from several highly conserved amino acids that form a semi-symmetrical hydrophobic binding pocket [9–12]. Using site-directed mutagenesis, several charged surface residues of FKBP12 have been shown to influence the interaction between calcineurin and the FKBP12–FK506 complex [13]. Recent ran-

Abbreviations used: CYP, human cyclosporin A binding protein (cyclophilin); CsA, cyclosporin A; PPIase, peptidyl-prolyl *cis-trans* isomerase; FKBP, FK506 binding protein; RBP1, rapamycin binding protein from *Saccharomyces cerevisiae*; DTT, dithiothreitol; TFE, trifluoroethanol; DMSO, dimethyl sulfoxide; 4NA, 4-nitroaniline.

|| To whom correspondence should be addressed: Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, Mail Code UW-2103, King of Prussia, PA 19406, U.S.A.

dom mutagenesis studies have revealed that a single amino acid change (Tyr⁸⁹ to Asp) in yeast RBP1 conferred cellular resistance to rapamycin which correlated with the *in vitro* observation that the mutant RBP1 did not bind the drug [14]. Most interesting is that this residue is one of the aromatic amino acids comprising the binding cleft for FK506 and rapamycin [11,12]. Tyr⁸⁹ in RBP1, which corresponds to position 82 in human FKBP12, represents a tyrosine residue that is conserved throughout the FKBP family.

As described in this paper, we had independently selected Tyr⁸² of human FKBP12 as an intriguing target for site-directed mutagenesis. Reinforcing our initial interest in this residue was the observation from X-ray structural analyses that the hydroxyl group of Tyr⁸² forms a hydrogen bond with the common piperidyl amide-oxygen structural element of both FK506 and rapamycin [11,12]. Together, these observations suggested that Tyr⁸² is a key residue involved in the binding of rapamycin and FK506 to FKBP12, leading to their pharmacological effects. To ascertain the functional role of this residue in the binding of these immunosuppressive molecules and in the PPIase activity of the protein, Tyr⁸² of human FKBP12 has been changed to leucine by site-specific mutagenesis. The kinetic and ligand binding characteristics of this modified enzyme are compared with those of wild-type FKBP12. In addition, expression of the human [Leu⁸²]FKBP12 in a yeast strain deficient in RBP1 has enabled us to test the effect of the Tyr⁸² to Leu mutation in a functional cellular assay.

EXPERIMENTAL

Materials

Restriction enzymes and a cDNA cloning kit were obtained from Boehringer Mannheim Biochemicals. α -Chymotrypsin (Type II; EC 3.4.21.1) was procured from Sigma Chemical Co. Peptide substrates were obtained from Bachem-Bioscience Inc. (Philadelphia, PA, U.S.A.) or American Peptide Co. (Santa Clara, CA, U.S.A.). Protein products were analysed by SDS/PAGE (15% gels) and visualized with Coomassie Blue. N-terminal sequence analysis was determined on an ABI gas phase sequencer (model 470a). All protein concentrations were estimated from total amino acid analyses. FK506 and rapamycin were prepared by fermentation and isolated at SmithKline Beecham Pharmaceuticals in Brockham Park, U.K.

Construction of *Escherichia coli* expression vectors

Oligonucleotide primers corresponding to the N- and C-termini of the FKBP12 cDNA protein encoded open reading frame were mixed with FKBP template cDNA, and the coding sequence was amplified by the PCR [15]. The 5'-specific primer (5'-ATACATATGGGAGTTCAAGTAGAACTATTTCTCTCTGGAGATGGAAGAACTTTTCCAAAGAGAGGCCAGACCTGCGTGGTGCCTACACCG-3'), which included an *Nde*I restriction site (CATATG) and contained the translation initiation codon (ATG) required for recombinant protein expression, was used for PCR amplification. To produce increased recombinant protein expression levels in *E. coli*, as previously described for the expression of human cyclophilin isoform 3 [16], the primer sequence included A-T-enriched codons which were substituted for many of the authentic G-C-containing codons. The 3'-specific primer (5'-AAGGGAGCTCTTATCATTCTAGTTTGTAGAAGTTCAACATCAAATACAAGAGTTGCA-TGTGGTGGG-3') contained a *Sac*I restriction site and a TGA

translation termination signal at its 5'-end, and several of the authentic G-C-containing codons were replaced by A-T-rich codons for the same amino acids. The DNA fragment that resulted from the PCR amplification was subcloned into the *Nde*I-*Sac*I sites of the *E. coli* expression vector pMG1, producing pMGFKBP_y, which encoded the wild-type FKBP12 protein. Construction of the human [Leu⁸²]FKBP12 mutant also was accomplished by PCR technology using the same primer as above. The 3'-specific primer (5'-AGGGAGCTCTCATTCTAGTTTGTAGAAGTTCAACATCAAATACAAGAGTTGCA-TGTGGTGGGATGATGCCTGGGTGCCAGTGGCACC-ATAGGCAT-3') included a Leu codon that replaced a Tyr codon at amino acid position 82. This primer also contained a *Sac*I site, TGA signal, and A-T-enriched codons as above. The PCR product was subcloned into plasmid pMG1, producing a pMGFKBP_L. The inserted genes of both clones were sequenced [17] to confirm that only the desired mutation or alterations were present.

Expression in *E. coli*

Cultures of *E. coli* AR-58 cells were transformed with pMGFKBP_y or pMGFKBP_L and grown at 30 °C in Luria broth (800 ml volume, including 100 µg/ml ampicillin) to an A_{550} of 0.7; 200 ml of Luria broth (preheated to 80 °C) was then added and the culture was transferred to 42 °C for an additional 3 h to induce expression [18]. The cells were collected by centrifugation and frozen at -80 °C until analysed or used as a source for protein purification. The expressed 12 kDa proteins FKBP12 and [Leu⁸²]FKBP12 were detected in the supernatant of cell lysates (see below) by SDS/PAGE.

Purification of FKBP12 and [Leu⁸²]FKBP12

Both proteins were purified to apparent homogeneity in a procedure involving three chromatographic steps. All manipulations were carried out at 2–6 °C. *E. coli* cells from a 1 litre culture expressing either authentic FKBP12 or [Leu⁸²]FKBP12 were suspended in buffer A (2 mM CaCl₂, 10 mM MgCl₂, in 50 mM Tris/HCl, pH 8.0) containing 5% glycerol and 2 mM dithiothreitol (DTT), and were broken by sonication. The suspension was centrifuged for 30 min at 10000 *g* and the resulting 50 ml supernatant was applied to a 5 cm × 10 cm column of DEAE-Sephacel which had been equilibrated with buffer A. The column was washed with approximately 120 ml of buffer A, followed by a linear gradient in buffer A of 0–500 mM NaCl over a total of 600 ml. Fractions were analysed for human FKBP12 by SDS/PAGE and/or for rapamycin-sensitive PPIase activity. The pH of the combined fractions (50–70 ml) which contained the desired protein(s) was adjusted to 6.0 with acetic acid, and the resulting solution was applied to a 5 cm × 10 cm column of CM-Sephadex equilibrated with 20 mM sodium acetate, pH 6.0. The column was washed with 200 ml of the equilibration buffer, followed by a gradient from 0 to 500 mM NaCl over 700 ml in the same buffer. Fractions containing rapamycin-sensitive PPIase activity were concentrated by ultrafiltration (Amicon; YM-3 membrane) to a volume of less than 5 ml. The sample was chromatographed on a 1.5 cm × 90 cm column of Sephadex G50 (fine) equilibrated with 20 mM sodium phosphate, pH 7.1, also using the equilibration buffer for column elution, yielding homogeneous samples of FKBP12 (wild-type FKBP12 or [Leu⁸²]FKBP12) by SDS/PAGE analysis (results not shown). N-terminal sequence analysis for the first 18 amino acids of each FKBP12 was identical to that originally reported: (M)GVQV-ETISPGDGRTPFKR.

PPIase activity and inhibition studies

PPIase activity ($k_{\text{cat.}}/K_m$) of the FKBP12 proteins was determined using synthetic peptides (see Table 1) as substrates in the chymotrypsin coupled reaction as described for the cyclophilins [16,19]. Except as noted, assays were performed in 1 ml at 10 °C in 35 mM Hepes, pH 7.8, with 25 μ M substrate and monitored at 390 nm with Perkin–Elmer Lambda 4C or Lambda 6 spectrophotometers; assays were initiated by addition of α -chymotrypsin ($\geq 250 \mu$ g) and monitored for 5–10 min. Absorbance as a function of time from individual PPIase assays was analysed by a first-order exponential function corresponding to the *cis* to *trans* isomerization [19] of peptide substrate. The net-rate constants were normalized to protein concentration determined by total amino acid content. Determination of the pH dependence of $k_{\text{cat.}}/K_m$ was conducted with the substrate succinyl-Ala-Phe-Pro-Phe-4NA (where 4NA is 4-nitroanilide) in constant-ionic-strength buffer (50 mM Mes, 25 mM Tris, 25 mM ethanolamine with 0.1 M NaCl) covering a pH range of 4.5–10.5 [20,21]. Kinetic data on $k_{\text{cat.}}/K_m$ over the pH range were analysed by the WAVL and HBBELL programs described by Cleland [22]. Solvent isotope effects on $k_{\text{cat.}}/K_m$ were determined by diluting protein stock solutions into deuterated buffers; all reactions were determined in 35 mM Hepes, pH 7.8, containing 90% ²H₂O at 10 °C using the substrate succinyl-Ala-Phe-Pro-Phe-4-NA.

The substrate succinyl-Phe-Leu-Pro-Phe-4NA was used for determination of apparent inhibition constants with FK506 and rapamycin because of its relatively high catalytic efficiency with [Leu⁸²]FKBP12 (see Table 1), enabling assays to be conducted at significantly lower concentrations of the enzymes. In experiments to determine inhibitory potencies, an aliquot of FK506 or rapamycin in ethanol was added to the incubation mixture prior to initiation of the reaction. The percentage of ethanol in the reactions was held constant at 0.5% of the final volume, an amount shown to have minimal effect on FKBP12 PPIase activities. Inhibition of enzymic activity was determined using eqn. (1), describing a reversible tight-binding inhibitor [23]:

$$v_i = (v_o/2E_t)\{[(K_{i,\text{app.}} + I_t - E_t)^2 + 4 K_{i,\text{app.}} E_t]^{0.5} - (K_{i,\text{app.}} + I_t - E_t)\} \quad (1)$$

where v_i and v_o are the enzymic rates in the presence and absence respectively of inhibitor, I_t and E_t are the total concentrations of inhibitor and enzyme respectively, and $K_{i,\text{app.}}$ is the apparent equilibrium inhibition constant. Data were analysed using eqn. (1) because the estimated $K_{i,\text{app.}}$ values were consistently less than the PPIase concentrations. Fitting of data to eqn. (1) was accomplished with a non-linear regression procedure using the Marquardt algorithm [24] in the SAS statistical analysis software package (SAS Institute, Inc., Cary, NC, U.S.A.).

Determination of kinetic constants of substrates

Kinetic constants ($k_{\text{cat.}}$ and K_m) were determined using the modified chymotrypsin-coupled assay described by Kofron et al. [25]. Concentrated peptide substrate solutions were prepared in anhydrous trifluoroethanol (TFE) containing 400–500 mM LiCl. Assays were initiated by addition of substrate in TFE to a solution containing all other assay components pre-equilibrated to 0 °C, the temperature at which the assays were conducted. The final concentration of TFE in all assays was constant at 2.5%. Initial reaction velocities were determined by fitting the enzymic progress curves to the integrated rate equation as described [25] using the KineTic program obtained from BioKin Ltd. (Madison, WI, U.S.A.). The complete initial velocity data sets were analysed by the HYPER program described by Cleland [22].

[³H]Rapamycin binding assay

[³H]Rapamycin (8.6 mCi/mg) was prepared by a fermentation process at SmithKline Beecham Pharmaceuticals, Brockham Park, UK. Samples of the radiolabelled compound were purified by silica gel t.l.c. prior to use in binding assays; purified compound was stored in benzene at –80 °C. For use in binding assays, the benzene was removed under a stream of argon and the [³H]rapamycin was dissolved in a small volume of ethanol. A 5 μ l aliquot of the ethanolic solution containing 40000 d.p.m. of the [³H]rapamycin was added to a solution of buffer B (100 mM NaCl, 20 mM sodium phosphate, 1 mM EDTA, 0.015% Triton X-100, pH 7.3) containing 5 nM FKBP in a final volume of 200 μ l; the final concentration of [³H]rapamycin in the incubation was 12 nM. After incubating the sample for 5 min at room temperature, an aliquot (90 μ l) of the mixture was applied to a 2 ml minicolumn of Sephadex LH-20 resin which had been equilibrated with buffer B. The columns were eluted with buffer B to separate protein-bound from non-bound radioligand. Fractions containing the protein-associated [³H]rapamycin were counted for radioactivity in Ecoscint cocktail using a Beckman LS-3801 scintillation counter.

In competition experiments with unlabelled inhibitors, FK506 or rapamycin in ethanol was added to the buffered enzyme solution 5 min prior to introduction of the [³H]rapamycin; the final ethanol content was held at a constant 5% of the final incubation volume. Final evaluation for protein-associated radiolabel was carried out as detailed above.

Fluorescence studies

Protein fluorescence was monitored using approx. 0.6 μ M FKBP in 10 mM sodium phosphate buffer, pH 7.0, at 20 °C with a Perkin–Elmer MPF-66 spectrophotometer interfaced to a Perkin–Elmer 7500 computer. Uncorrected emission spectra were collected in the ratio mode using a 290 nm filter with both excitation and emission slits set at 5 nm.

Functional evaluation of yeast sensitivity to rapamycin

Plasmids were constructed to express FKBP12, [Leu⁸²]FKBP12 and RBP1 in *Saccharomyces cerevisiae* as previously described [26,27]. Briefly, a cassette was generated by PCR amplification from pMGFKBP_L using two primers: 5'-AACACTCGAGAA-TATGGGAGTTCAAGTAGAACT-3' and 5'-TTTTCCATGGAGCTCTTATCATTCTAGTTTCTAG-3'. The first 5'-specific primer contained an *Xho*I restriction site plus an ATG codon (underlined). The second 3'-specific primer contained an *Nco*I site plus two consecutive translation termination signals (underlined). The PCR-generated fragment was cut with *Xho*I and *Nco*I, gel-purified and subcloned into the *Xho*I and *Nco*I polylinker sites of the yeast expression plasmid p138NB [27]. p138NB contains the *TRP1* selectable marker and partial sequence for maintenance at high copy number, with expression driven by the copper-inducible *CUP1* gene promoter. The polylinker is present downstream of the promoter, followed by the yeast *CYC1* transcriptional terminator. Sensitivity to rapamycin was determined by measuring the zones of cellular growth inhibition in an agar plate assay as previously described [26]. Briefly, yeast cells were spread on SC-Trp medium containing 150 μ M CuSO₄ [28] and aliquots (100 μ l) of rapamycin solution in 1:1 dimethyl sulfoxide (DMSO)/methanol were added to 7 mm wells embedded in the agar. After incubation of the plates for 2 days at 30 °C, zones of growth inhibition were measured and the results were correlated against the concentration of compound originally added to the individual wells.

Figure 1 Proposed mechanism for the PPIase activity of FKBP12: induced fit, distortion and/or desolvation catalysis

RESULTS AND DISCUSSION

Catalysis by PPIase

The isomerization of peptidyl prolyl bonds catalysed by the immunophilins can be visualized as depicted in Figure 1. Protein mediation is not required for this process, as the isomerization occurs spontaneously in solution. Based in part on the sensitivity of CYP PPIase activity to thiol reagents [29,30], it was originally proposed that immunophilin-mediated catalysis involved an attack upon the amide carbonyl of the substrate by a nucleophile, presumably a thiol, forming a protein-associated tetrahedral intermediate [31]. Alternatively, PPIase catalysis could occur without proceeding through a covalent intermediate between enzyme and substrate. Desolvation of the amide bond upon binding within a hydrophobic site, such as the ligand binding cleft of FKBP12, and polarization of the amide bond through electrostatic interactions with protein amino acids could decrease the bond order between the carbonyl carbon and the prolyl nitrogen. This combination of physical interactions would be expected similarly to lower the activation barrier to bond rotation.

A number of different experiments have discounted the associative mechanism involving a covalent intermediate in favour of the second possibility outlined above, referred to as catalysis by distortion and/or desolvation [32–34]. The most important of

these results come from the interpretation of kinetic isotope effects with both classes of PPIases [33,34] and from the substitution of potential amino acid nucleophiles by mutagenesis [32,35]. One depiction of PPIase catalysis by distortion is presented in Figure 1, where a general acid (designated H-Enz), which co-ordinates to the amide nitrogen in the transition state, facilitates isomerization by lowering the barrier to rotation. Our observation of an inverse solvent ^2H isotope effect with both FKBP12 (0.84 ± 0.09 ; see Table 2) and [Leu 82]FKBP12 (0.86 ± 0.07) indicates that desolvation of substrate upon binding to both proteins contributes to a lowering of the amide bond rotational barrier in the transition state, consistent with the proposed mechanism shown in Figure 1.

One of our first mechanistic studies with FKBP12 involved the pH-dependence of its PPIase activity on $k_{\text{cat.}}/K_m$ using the standard chymotrypsin-coupled assay. Upon increasing the pH to 10.5, a decrease in catalytic efficiency was observed above pH 9.5 as shown in Figure 2. These data were best evaluated using the WAVL function [22] describing a catalytic mechanism that decreases from a maximal level at low pH ($1700 \pm 200 \text{ s}^{-1} \cdot \text{mM}^{-1}$) to a lower, yet constant, value ($900 \pm 100 \text{ s}^{-1} \cdot \text{mM}^{-1}$) upon a deprotonation event at higher pH. Greater loss of activity was observed as the pH was further increased to 11.2. However, independent fluorescence studies of wild-type FKBP12 showed a shift in the protein's emission spectrum from that characteristic of the native state (324–328 nm; see discussion below) to one more similar to that of free tryptophan (348–352 nm; results not shown) at pH 11, suggesting that the lower catalytic efficiency near pH 11 may result from a loss of protein structural integrity.

Analysis of our data ranging to pH 10.5 indicated that protonation of a functionality with a pK estimated to be 9.8 ± 0.2 is required for maximal catalytic activity. The side chain of a tyrosine residue ($pK \sim 9.6$) in FKBP12 was the most likely functionality to give rise to this observation. Furthermore, it was thought that a tyrosine hydroxy group might be acting as a hydrogen-bond donor to the prolyl nitrogen, in the form of a general acid, contributing to the energetics of binding and/or catalysis. While others have reported no effect on catalysis with changes in pH, it appears that those studies did not extend the experimental pH range above 10 [35,36].

With intent to understand better the structural and physical interactions contributing to the energetics of ligand binding and catalysis, we decided to give further attention to the tyrosines in FKBP. Originally, the desired approach was to change independently each of the protein's three tyrosine residues (at positions 26, 80 and 82) by site-directed mutagenesis; these modified proteins could then be evaluated in functional assays to provide information relevant to our objectives. Shortly after we had initiated these experiments, n.m.r. [9,10] and X-ray [11] structural data on the FKBP–FK506 complex became available. These results indicated both Tyr 26 and Tyr 82 to be integral components of FKBP's hydrophobic binding domain for FK506.

Figure 2 Dependence of FKBP PPIase activity on pH

The PPIase activities of FKBP12 (29 nM) and [Leu 82]FKBP12 (250 nM) were determined at various pH values using succinyl-Ala-Phe-Pro-Phe-4 NA as substrate in the standard chymotrypsin-coupled assay in constant ionic strength buffer. The log of the net enzyme activity (in units of $\text{s}^{-1} \cdot \text{mM}^{-1}$) for FKBP12 (○) was evaluated as a function of pH using the mathematical model (WAVL) describing a decrease in catalytic efficiency to a lower non-zero level upon increasing pH [22]. With [Leu 82]FKBP12 (□), a minimal change in catalytic activity was observed over the same pH range.

Table 1 Specificity of FKBP12 and [Leu⁸²]FKBP12 with peptide substrates

The percentage of peptide present as the *cis* isomer was estimated from the absorbance changes in the fast and the slow phases of the chymotrypsin cleavage reaction in absence of any FKBP. Background rates represent spontaneous first-order rates for isomerization of *cis* to *trans* isomers in the absence of FKBP. PPIase activity was determined with 27 nM FKBP12 and 250 nM [Leu⁸²]FKBP12 at 10 °C using the standard chymotrypsin-coupled assay with synthetic peptides of sequence succinyl-Yaa-Xaa-Phe-Pro-4-NA. Data represent the mean of three or four assays per substrate.

Substrate	<i>cis</i> rotamer (%)	10 ⁻³ × background rate (s ⁻¹)	k_{cat}/K_m catalytic rate (s ⁻¹ · μM ⁻¹)		Ratio of rates (wild type/mutant)
			FKBP12	[Leu ⁸²]FKBP12	
Suc-AFPF-4 NA	21–23	4.7	2.2	0.17	13
Suc-ALPF-4 NA	9–10	6.9	3.6	0.30	12
Suc-LLPF-4 NA	9–11	7.8	3.5	0.30	13
Suc-VLPF-4 NA	10–12	5.5	2.6	0.32	8.2
Suc-FLPF-4 NA	10–11	6.7	4.9	0.80	6.4
Suc-RLPF-4 NA	14–16	5.2	1.5	0.80	1.9
Suc-SLPF-4 NA	12–13	6.1	1.7	0.94	1.9

In these structures, Tyr⁸² seemed to be particularly intriguing since its hydroxy group was found to form a hydrogen bond with the C₈ carbonyl of FK506, an amide oxygen whose bound orientation was seen to be perpendicular to that of the adjacent ketone. Since the relative orthogonal orientation of the keto-amide carbonyls in FK506 bound to FKBP was found to be similar to that proposed for the amide carbonyl of substrate in the transition state during catalysis (see Figure 1), we chose to investigate the functional involvement of Tyr⁸² in ligand binding and catalysis.

For this, we engineered recombinant FKBP12 containing Leu for Tyr at position 82. Both the mutant and the wild-type FKBP cDNAs were subcloned into an *E. coli* expression vector, producing pMGFKBP_L and pMGFKBP_Y respectively. These plasmids, when introduced into *E. coli*, expressed significant levels of both the mutant and the wild-type proteins as soluble cellular components. Milligram quantities of both recombinant FKBP12 and [Leu⁸²]FKBP12 were purified to apparent homogeneity by a modified protocol [37] as determined by SDS/PAGE (results not shown) and amino acid sequence analysis.

Comparisons between the catalytic efficiencies of wild-type FKBP12 and [Leu⁸²]FKBP12 with a selection of peptide substrates are presented in Table 1. In general, the PPIase activity of the mutant FKBP12 with all of the peptide substrates evaluated in the chymotrypsin-coupled assay was lower than that of the wild-type enzyme. Within a series of peptides, represented by the sequences succinyl-Yaa-Xaa-Phe-Pro-4 NA in Table 1, the loss of activity with [Leu⁸²]FKBP12 was greatest for those substrates having hydrophobic amino acids at the Yaa position. Differences in the relative rates of PPIase activity with the substrates presented in Table 1 may be related to the energetic contributions of desolvation as applied towards catalysis by the wild-type and mutant enzymes. These results indicate that Tyr⁸² is important for maximal PPIase activity, but is not absolutely required for the catalysis by FKBP12.

Evaluation of the PPIase activity (k_{cat}/K_m) of [Leu⁸²]FKBP12 as a function of pH produced a profile that was much flatter than that observed with FKBP12 (Figure 2). While there was a small decline in the PPIase activity of the mutant moving from low to high pH, no clear pK break was detected. Comparing results from the pH profiles for FKBP12 and [Leu⁸²]FKBP12 suggests that the loss of activity at high pH observed in wild-type FKBP12 most likely results from the deprotonation of Tyr⁸². While a Tyr at amino acid position 82 is not required for FKBP12 to catalyse the PPIase reaction, the contributing side

Table 2 Kinetic and inhibition constants for FKBP-12 and [Leu⁸²]FKBP12

Initial velocity data (K_m and k_{cat}) were determined with 6.2 nM FKBP12 and 250 nM [Leu⁸²]FKBP12 at 0 °C using the modified protocol with chymotrypsin as described by Kofron et al. [25]. $(V/K)^H$ and $(V/K)^H$ are the ratios in the absence and presence respectively of per-deuterated solvent. Apparent inhibition constants were determined with 13.5 nM FKBP12 and 62.5 nM [Leu⁸²]FKBP12 according to the protocol in the Experimental section using succinyl-Phe-Leu-Pro-Phe-4 NA as substrate. For comparison, $K_{i,\text{app}}$ values for FK506 and rapamycin with wild-type FKBP12 were determined to be 0.8–2.0 nM and 0.3–0.6 nM respectively, using the alternative substrate succinyl-Ala-Phe-Pro-Phe-4 NA.

Kinetic data	FKBP12	[Leu ⁸²]FKBP12
K_m for Suc-ALPF-4 NA (mM)	0.9–1.3	0.7–1.0
k_{cat} for Suc-ALPF-4 NA (s ⁻¹)	1000–1300	20–24
k_{cat}/K_m at 0 °C (s ⁻¹ · μM ⁻¹)	1.2	0.21
k_{cat}/K_m at 10 °C (s ⁻¹ · μM ⁻¹)	3.6	0.30
$(V/K)^H/(V/K)^H$	0.84 ± 0.09	0.86 ± 0.07
$K_{i,\text{app}}$ for FK506 (nM)	0.6 ± 0.3	2.1 ± 1.1
$K_{i,\text{app}}$ for rapamycin (nM)	0.4 ± 0.2	0.9 ± 0.3

chain hydroxy group appears to facilitate the enzyme-catalysed reaction when protonated, possibly by the mechanism depicted in Figure 1.

Using the modified chymotrypsin-coupled assay and protocols of data analysis developed by Kofron et al. [25], kinetic data with the substrate succinyl-Ala-Leu-Pro-Phe-4 NA were fitted to equations describing the PPIase kinetic progress curves; the results from these experiments are summarized in Table 2. Most interesting from these experiments is the observation that the difference in k_{cat}/K_m between the wild-type and mutant enzymes almost entirely results from a change in k_{cat} (i.e. V_{max}), with little if any discernible modulation of the Michaelis constant K_m . The decrease in V_{max} with no change in K_m determined with [Leu⁸²]FKBP12 is consistent with a chemical mechanism by distortion. As suggested in Figure 1, a decrease in the barrier to rotation about the peptidyl-prolyl amide bond in the transition state could result from Tyr⁸² acting as a general acid.

Relative affinities of FK506 and rapamycin for FKBP12 and [Leu⁸²]FKBP12

The PPIase catalytic sites of both the FKBP12 and the CYPs are thought to physically coincide with the binding regions for their complementary immunosuppressive compounds. This concept

Table 3 Binding of [^3H]rapamycin to FKBP12 and [Leu^{82}]FKBP12

Samples of purified FKBP12 and [Leu^{82}]FKBP12 (5 nM) were incubated in the presence of 40 000 d.p.m. of [^3H]rapamycin for 5 min. Protein-associated and non-associated radiolabel were separated on columns of Sephadex LH-20 as described in the Experimental section. In the competition experiments, the proteins were incubated in the presence of 500 nM non-labelled FK506 or rapamycin for 5 min prior to the addition of the [^3H]rapamycin.

Protein	Competitive ligand	Protein-associated radiolabel (d.p.m.)
FKBP12	None	5133
FKBP12	500 nM FK506	57
FKBP12	500 nM Rapamycin	35
[Leu^{82}]FKBP12	None	4904
[Leu^{82}]FKBP12	500 nM FK506	955
[Leu^{82}]FKBP12	500 nM Rapamycin	52

Figure 3 Fluorescence of wild-type FKBP12 and [Leu^{82}]FKBP12 in the absence and presence of rapamycin

Emission spectra for wild-type FKBP12 (a) and [Leu^{82}]FKBP12 (b) were determined upon excitation at 278 nm. All curves represent constant protein concentrations of $0.6 \mu\text{M}$. FKBP12 emission in the absence of additional ligand (—) is compared with the signal in the presence of increasing concentrations of rapamycin (---), at 0.14, 0.28, and $0.56 \mu\text{M}$ for FKBP12 and at $0.56 \mu\text{M}$ for [Leu^{82}]FKBP12. The fluorescence intensity between 318 and 328 nm associated with FKBP12 decreased progressively with increasing concentrations of rapamycin; no decrease in emission was observed with [Leu^{82}]FKBP12 in the presence of a stoichiometric concentration of rapamycin.

has been supported by competitive inhibition of CYP and FKBP PPIase activities, as demonstrated respectively using an analogue of CsA [25] and a synthetic molecule incorporating the structural 'binding domain' common to FK506 and rapamycin (M. A. Levy and M. J. Bossard, unpublished work). The observation of competitive kinetics supports the use of PPIase inhibition to estimate relative ligand binding affinities, despite the uncertain cellular function and significance of this catalytic activity.

The inhibition constants for FK506 and rapamycin with FKBP12 and [Leu^{82}]FKBP12 are included in Table 2. Removal of the aromatic hydroxy group from position 82 in wild-type FKBP by site-directed mutagenesis decreases the affinities of both FK506 and rapamycin by only 2–3-fold, consistent with loss of the hydrogen bond suggested from the X-ray structural analyses of the complexes [11,12]. The side chain of Leu is anticipated to provide approximately the same hydrophobic character as the aryl substituent of Tyr^{82} in wild-type FKBP12. As expected from the inhibition constants, both FK506 and rapamycin have been shown to still bind to [Leu^{82}]FKBP12 using our [^3H]rapamycin/Sephadex LH-20 binding assay (Table 3). However, FK506 was less effective at preventing radioligand

from binding to the mutant compared with wild-type FKBP12, presenting the possibility that the tightest protein–ligand complex may involve this unique hydrogen bond.

Effects of ligand binding upon protein fluorescence

Wild-type FKBP12 has a fluorescence spectrum dominated by the protein's single Trp. Unlike the case of CYP, where CsA induces an increase in protein spectral emission due to the increased hydrophobic environment upon ligand association [29,32,38], quenching of fluorescence occurs upon binding of FK506 or rapamycin to FKBP12. The fluorescence data presented in Figure 3(a) showing the decrease in emission of FKBP12 upon increasing substoichiometric concentrations of rapamycin is representative of this response. Maximal fluorescence suppression occurs at a 1:1 stoichiometry of ligand and protein. Initially, the observation of fluorescence quenching was a surprising result, since introduction of either compound into the binding cleft was expected to increase hydrophobicity around the Trp, which in turn could be expected to enhance the protein fluorescence. FK506 also has been shown to quench the fluorescence of FKBP12 (results not shown; [35]).

A possible explanation for these fluorescence data has come from comparative studies with the Leu^{82} site-specific mutant. [Leu^{82}]FKBP12 has a fluorescence spectrum which is indistinguishable from that of the wild-type immunophilin (Figure 3b). However, no quenching of maximal fluorescence emission was observed in the presence of stoichiometric levels of either rapamycin or FK506; the data with rapamycin are presented in Figure 3(b). This leads us to propose that the mechanism of fluorescence quenching in the presence of FK506 or rapamycin occurs by the transfer of energy from an excited electronic state of Trp to a high-energy state of Tyr^{82} which has been polarized as a consequence of its hydrogen bonding to amide carbonyls in both ligands. Ligand-induced suppression of tryptophan fluorescence by a mechanism of energy transfer to a tyrosine which becomes ionized or polarized upon binding the quenching agent has been proposed previously for other systems such as liver alcohol dehydrogenase [39].

This interesting observation of localized biophysical and electronic contributions influencing the spectral characteristics of FKBP12 should find utility in probing the immediate environments about the single Trp and Tyr^{82} . In working towards the objective of preparing novel FKBP12 binding domains to which potential immunosuppressive effectors can be tethered, the effects of alternative ligands on the fluorescence properties of FKBP12 can be studied. Molecules that interact similarly to FK506 or rapamycin would be expected to induce fluorescence quenching in wild-type FKBP12, while those which show dissimilar effects on protein fluorescence must be interacting in an alternative structural manner. The availability of information on the molecular details of ligand–protein interactions from such studies should be useful in establishing new strategies toward the identification of novel immunosuppressive molecules.

Functional evaluation of the Tyr^{82} to Leu mutation in yeast

RBP1 is the predominant cytoplasmic FKBP found in *S. cerevisiae*; deletion of the *RBP1* gene in haploid cells results in cellular resistance to rapamycin [26,40]. Rapamycin sensitivity can be restored upon expression of RBP1 or wild-type human FKBP12, indicating a functional equivalence between the fungal and human proteins [26]. To study the functional effect of the Tyr^{82} to Leu mutation in a cellular assay, [Leu^{82}]FKBP12 was expressed in a yeast strain which had been rendered rapamycin-

Figure 4 Sensitivity of yeast strains to rapamycin

RBPI-deficient cells of *S. cerevisiae* (strain MM3) were transfected with plasmids expressing no FKBP protein (□), human FKBP12 (○), yeast RBP1 (■) or [Leu⁸²]FKBP12 (●). The cells were tested for viability upon exposure to rapamycin using an agar plate diffusion assay described in the Experimental section. Yeast devoid of RBP1 (□) were viable at all concentrations of rapamycin, as demonstrated by no inhibitory zone of cell proliferation. Expression of RBP1, wild-type FKBP12 or [Leu⁸²]FKBP12 restores rapamycin sensitivity, indicating a functional equivalence among the three proteins.

resistant by deletion of *RBPI*. The rapamycin response of RBP1-deficient yeast cells transfected with expression plasmids for FKBP12, [Leu⁸²]FKBP12 and RBP1 are presented in Figure 4. The results from these experiments demonstrate that sensitivity to rapamycin is restored upon expression of each of these FKBP forms. In these experiments, the sensitivity of the cells containing [Leu⁸²]FKBP12 was indistinguishable from that of yeast expressing FKBP12, a result consistent with the only 2–3-fold lower affinity for rapamycin of the Tyr⁸² to Leu⁸² mutant compared with wild-type FKBP12 (see Table 2). For further comparison, rapamycin inhibition of authentic RBP1 PPIase activity has been estimated to have a $K_{i,app}$ value of 0.8–2 nM.

Interestingly a Tyr⁸⁹ to Asp change in RBP1 has recently been shown to result in rapamycin resistance [14]. This amino acid in RBP1 corresponds to Tyr⁸² in human FKBP12. In this case, fungal resistance results from an inability of the mutant RBP1 to bind the macrocyclic antifungal effectively, as demonstrated using the [³H]rapamycin binding assay. Presumably, introduction of an acidic functionality into the predominantly hydrophobic ligand binding cleft of RBP1 upon replacement of Tyr⁸⁹ with Asp disrupts the molecular interactions necessary for favourable protein–drug association.

Conclusions

The mechanistic, kinetic and cellular experimental results obtained with the [Leu⁸²]FKBP12 mutant protein are consistent with the concept that the cellular response of rapamycin does not result from inhibition of the FKBP12 PPIase activity. Rather, like CsA and FK506, rapamycin must be composed of two structural elements: one which confers high affinity for its congener immunophilin, the ‘binding domain’, and a second which contributes to interaction of the immunophilin–drug binary complex with a secondary cellular target. These results

confirm that Tyr⁸² is involved in the direct interactions between FKBP12 and both substrates and inhibitors, having a greater influence on PPIase catalytic efficiency than on ligand binding affinity.

Our comparative results with the native and Leu⁸²-containing proteins indicate that Tyr⁸² has a significant effect on V/K for FKBP12 PPIase activity, most of which is manifest in the catalytic process as described by k_{cat} , not the Michaelis constant (K_m). A smaller effect than that seen on V/K was observed on the binding affinities of rapamycin and FK506 upon changing Tyr⁸² to Leu. These observations raise the question of whether the bound orientations of FK506 and rapamycin can be considered to be true mimics of the transition state (which is represented by V/K) encountered during PPIase catalysis, as has been proposed solely from the relative orientations of the two ligands deduced in the FKBP12 structural studies. Thus the functional results we have observed with [Leu⁸²]FKBP12 are consistent with some, but not all, of the implications that have been drawn from structural analyses alone.

It is hoped that the approaches summarized in this paper can be further utilized to extend our understanding of the key protein and ligand structural elements involved in the cellular pathways, ultimately leading to a better understanding of the natural intracellular functions of the CyPs and FKBP, as well as of cellular response and immunosuppression. As an example, FKBP12/FKBP13 chimeras were used recently to identify several amino acid residues of the binding proteins which contribute to the FK506-induced affinity for calcineurin [41,42]. Evaluation of cellular sensitivity to rapamycin upon expression of these chimeric proteins in the RBP1-deficient strain of yeast could indicate whether the same structural features of FKBP12 are involved in the interaction with the rapamycin–FKBP putative downstream target. Our abilities to evaluate selected mutant immunophilins both *in vitro* and *in vivo* present unique opportunities to learn more about these complicated interactive properties.

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