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Human and *Escherichia coli* Cyclophilins: Sensitivity to Inhibition by the Immunosuppressant Cyclosporin A Correlates with a Specific Tryptophan Residue†

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ABSTRACT: The human T-cell protein cyclophilin shows high affinity for and is the proposed target of the major immunosuppressant drug cyclosporin A (CsA). Cyclophilin also has peptidyl prolyl cis-trans isomerase activity that is inhibited by CsA with an IC_{50} of 6 nM, while by contrast a homologous PPIase from *Escherichia coli* has been found to be much less sensitive to CsA, shown here to be 500-fold less potent at an IC_{50} of 3000 nM. This *E. coli* rotamase lacks the single highly conserved tryptophan residue of eukaryotic cyclophilins, and we show here that mutation of the natural F112 to W112 enhances *E. coli* rotamase susceptibility to CsA inhibition by 23-fold. Correspondingly, the human W121 mutations to F121 or A121 yield cyclophilins with 75- and 200-fold decreased sensitivity to CsA, while k_{cat}/K_m values of rotamase activity in a tetrapeptide assay drop only 2- and 13-fold, respectively. This complementary gain and loss of CsA sensitivity to mutation to or from tryptophan validate the indole side chain as a major determinant in immunosuppressant drug recognition and the separation of PPIase catalytic efficiency from CsA affinity.

The immunosuppressant drug cyclosporin A (CsA),[1] a central therapeutic agent in organ transplant medicine (Borel et al., 1989; Showstack et al., 1989; Starzl et al., 1989), binds with high affinity [IC_{50} value of 5-200 nM (Handschumacher et al., 1984; Harding et al., 1986)] to a cytosolic 18-kDa protein, cyclophilin (CyP), in human T cells (Handschumacher et al., 1984) and blocks T-cell activation by selective blockade of transcriptional activation of T-cell genes for synthesis of cytokines IL-2, IL-4, and GMCSF (Elliott et al., 1984; Kronke et al., 1984) by specifically inhibiting the function of such transcriptional activators as the nuclear factor of activated T cells (Emmel et al., 1989). In 1988 cyclophilin was further identified as a peptidyl prolyl cis-trans isomerase (PPIase) or rotamase with catalytic activity at accelerating rates of interconversion of cis and trans rotamers of Xaa-Pro amide bonds in small peptides and proteins (Fischer et al., 1989; Takahashi et al., 1989). Much effort is underway to determine possible physiologic substrates for this potential "foldase" activity and to determine if the potent inhibition of such enzymatic activity by CsA is the relevant biological readout in immunosuppression.

In addition to cloning, expressing, and purifying wild-type and mutant human T-cell cyclophilins (Liu et al., 1990), we have recently expressed and purified an *Escherichia coli*

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[1] Abbreviations: wtHCyP, wild-type human cyclophilin; wtECyP, wild-type *Escherichia coli* cyclophilin; HCWF, human cyclophilin W121 to F121 mutant; HCWA, human cyclophilin W121 to A121 mutant; ECFW, *Escherichia coli* cyclophilin F112 to W112 mutant; CsA, cyclosporin A.

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protein that also has high rotamase activity in a synthetic tetrapeptide rotamase assay but was much less sensitive, at least by 200-fold, to inhibition by CsA (Liu & Walsh, 1990). This *E. coli* rotamase, which could be involved in folding of proteins in periplasm (Liu & Walsh, 1990), has ca. 35% homology to the eukaryotic cyclophilin family (Kawamukai et al., 1989) and lacks both the four cysteine residues of human cyclophilin and the single conserved tryptophan residue whose fluorescence is enhanced on CsA binding (Handschumacher et al., 1984).

To address the determinants for tight binding of cyclosporin A to human cyclophilin and much weaker binding to *E. coli* rotamase, we have initiated structure/function studies by mutagenesis. Herein, we report on the striking effects of introduction of tryptophan into the *E. coli* rotamase at position 112 and its corresponding removal at residue 121 in the human T-cell protein, thus identifying the single tryptophan residue as a major determinant in the binding of the immunosuppressant drug CsA by cyclophilin.

Materials and Methods

Mutagenesis. The site-directed mutagenesis was accomplished by using a method similar to the "megaprimer" polymerase chain reaction (PCR) (Sarkar & Sommer, 1990). In the first round of PCR reaction, the mutagenic primer was used together with the 5′ primer to generate a fragment. This fragment with mutation incorporated then served as a megaprimer in a second round of PCR together with the 3′ primer of the corresponding cyclophilin genes. The primers used were as as follows: 5′ primer for HCyP, 5′-GCCTACGAATTCAGAAGGAGATATACATATGGTTAACCCCACCGTGTTCTTC-3′; mutagenic primer for HCWF, 5′-GCCATCCAAGAACTCAGTCTT-3′; mutagenic primer for HCWA, 5′-TGCCATCCAAAGCCTCAGTCTTG-3′; mutagenic primer for ECFW, 5′-CATGGTCAAGCCAGGCGTTATC-3′. Mutated regions are underlined. The 3′ primer for HCyP (Liu et al., 1990) and the 5′ and 3′ primers for ECyP (Liu & Walsh, 1990) have been previously described. The purification of the mutant proteins is similar to that for the wild-type human and *E. coli* rotamases (Liu & Walsh, 1990). From SDS–PAGE stained with Commassie brilliant blue, each mutant was over 95% in homogeneity. The protein concentrations were determined by UV absorbance at 280 nm (Gill & Hippel, 1989). On the basis of the protein sequence, the calculated extinction coefficients are 8730 M^{-1} cm^{-1} for wtHCyP, 3040 M^{-1} cm^{-1} for both HCWF and HCWA, 5120 M^{-1} cm^{-1} for wtECyP, and 10810 M^{-1} cm^{-1} for ECFW.

Fluorescence Spectroscopy. One milliliter of buffer (35 mM HEPES, pH 8.0) containing 300 nM protein in the presence or absence of CsA was subjected to fluorescence analysis with Shimadzu RF-5000 spectrofluorophotometer. The excitation wavelength was set at 290 nm, the emission range was from 300 to 400 nm, and the bandwidth was set at 10 nm. The experiments were carried out at room temperature. The emission maximum of each enzyme was noted. Fluorescence titration of tryptophan-related cyclophilin mutants with CsA was performed by addition of aliquots of cyclosporin A [100 μM stock in 40% (v/v) ethanol] to each sample, and fluorescence readings were taken at each emission maximum, i.e., 324 nm for human wild-type, HCWF, HCWA, and *E. coli* wild-type enzymes and 352 nm for ECFW. The fluorescence change upon the addition of CsA is plotted against CsA concentration. Since Handschumacher et al. (1984) reported fluorescence enhancement for bovine cyclophilin with excitation at 289 nm and emission at 340 nm, readings at 340 nm were also taken (not shown), and the fluorescence changes were parallel to the data reported here.

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                human 110-SQFFICTAKTE-WLDGKHV-127
               bovine     SQFFICTAKTE-WLDGKHV
             porcine      SQFFICTAKTE-WLDGKHV
               mouse      SQFFICTAKTE-WLDGKHV
             hamster      SQFFICTAKTE-WLDGKHV
                 rat      SQFFICTAKTE-WLDGKHV
Echinococcus granulosus   SQFFITTAVTS-WLDGKHV
    NinaA of Drosophila   CQFYVTTVGAK-WLDGKHT
              yeast       SQFFITTVPCP-WLDGKHV
            yeast-b       SQFFITTTEEASWLDGKHV
     Neurospora crassa    SQFFVTTVPTS-WLDGRHV
Salmonella typhimurium    SQFFINVADNA-FLDHGQR
      Escherichia coli 101-SQFFINVADNA-FLDHGQR-118
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FIGURE 1: Sequence alignment of the tryptophan-containing region of cyclophilin-like proteins from eukaryotes and prokaryotes. Sequences are taken from human (Haendler et al., 1987), bovine (Harding et al., 1986), porcine (Takahashi et al., 1989), mouse (Hasel & Sutcliffe, 1990), hamster (Bergsma & Sylvester, 1990), rat (Danielson et al., 1988), *Echinococcus granulosus* (Lightowlers et al., 1989), *Drosophila* NinaA (Schneuwly et al., 1989), yeast (Haendler et al., 1989), yeast b (Koser et al., 1990), *N. crassa* (Tropschug et al., 1988), *Salmonella typhimurium* (Tran et al., 1990), and *E. coli* (Kawamukai et al., 1989).

Rotamase Activity Assay and Cyclosporin A Inhibition. The modified rotamase assay (Liu & Walsh, 1990) of Fischer et al. (1984) was used. In a typical assay, 970 μL of a stock solution containing 35 mM HEPES (pH 8.0), 0.01% Triton X-100, 100 μM *N*-suc-Ala-Ala-Pro-Phe-*p*-NA, and 6–45 nM wild-type or mutant cyclophilin proteins was equilibrated in a 1-mL cuvette at 10 °C for over 15 min (the specific protein concentrations were so chosen that they give a 8–10-fold rate acceleration over background). The concentrations used were 6 nM wtHCyP, 12 nM HCWF, 45 nM HCWA, 12 nM ECFW, and 9 nM wtECyP. The reaction was initiated by addition of 30 μL of 10 mg/mL α-chymotrypsin (Sigma) in 1 mM HCl. The progress of the reaction was followed at 390 nm in a Hewlett-Packard Diode Array UV spectrophotometer (HP 89500). The data were collected every 0.5 s for a total of 90–1200 s and were iteratively fitted with a first-order rate equation to give a first-order rate constant by using the HP kinetics program. To obtain the inhibition data with CsA, 1–10 μL of 0.1 μM to 10 mM CsA stock solutions in 40% (v/v) aqueous ethanol was added to the assay mixture prior to the addition of chymotrypsin.

A set of nine (*N*-succinyl-*C*-nitroanilide) tetrapeptide substrates, AXPF, with various X residues were used to explore the substrate specificity of the cyclophilin mutants. The assay is the same as outlined above except that a fixed concentration of the proteins was used (either 12 or 24 nM depending on the activity the protein toward a specific substrate).

Results and Discussion

Sequence analysis around the conserved trytophan residue reveals high homology in eukaryotic cyclophilins but divergence in the two prokaryotic sequences (Figure 1). Because the *E. coli* rotamase had a phenylalanine (Kawamukai et al., 1989) rather than the single Trp at the position corresponding to residue 121 in the human enzyme (Haendler et al., 1987; Harding et al., 1986), the *E. coli* F112W mutant was constructed and the protein was purified to homogeneity for comparison with the *E. coli* wild-type rotamase. As seen in Figure 2a, the F112W mutant protein has a much higher fluorescence emission than *E. coli* rotamase, even substantially increased over the human wild-type cyclophilin. On addition of CsA there is no change in *E. coli* wild-type rotamase fluorescence (contributed presumably by tyrosine residues) but a substantial enhancement in the F112W mutant, permitting

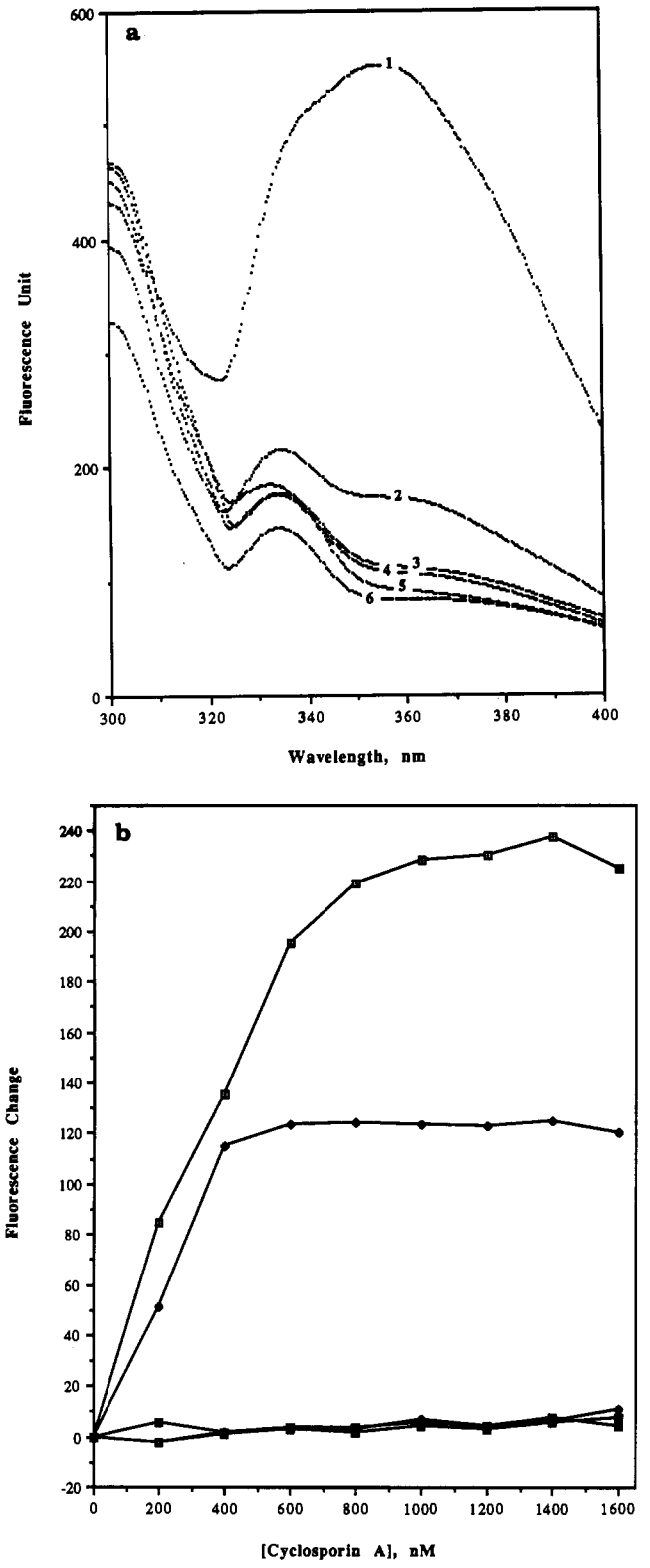

FIGURE 2: (a) Fluorescence spectra of tryptophan-related cyclophilin mutants. The spectra were recorded in the absence of CsA. (1) ECFW; (2) human wild type; (3) HCWA; (4) HCWF; (5) *E. coli* wild type; (6) buffer. Each sample has an emission maximum of 324 nm except ECFW, which is 352 nm, indicating a different microenvironment around the tryptophan residue in the ECFW mutant protein as compared to human cyclophilin. (b) Fluorescence titration of tryptophan-related cyclophilin mutants with CsA: (⊡) ECFW; (◆) human wild type; (□) HCWF; (◇) HCWA; (■) *E. coli* wild type.

the titration as shown in Figure 2b and suggesting that the F112W protein may have gained sensitivity to CsA. The effect of CsA on inhibition of rotamase activity using *N*-succinyl-AAPF-*p*-nitroanilide as substrate (Fischer et al., 1984) was then examined as depicted in Figure 3. The wild-type *E. coli* enzyme showed an IC_{50} for CsA of ca. 3000 nM while the F112W mutant displayed an IC_{50} of 130 nM, a reasonable correlation with fluorescence enhancement data, and a 23-fold gain in sensitivity to CsA by the Phe to Trp mutation. The catalytic efficiencies, k_{cat}/K_m ratios, of the wild type and the F112W mutant acting as rotamases, on the other hand, are essentially equal (Figure 4), further corroborating the dissociation of rotamase catalytic activity from sensitivity to cyclosporin A inhibition.

Given that F112W replacement in the *E. coli* enzyme markedly enhances the susceptibility to CsA inhibition, we turned to the complementary studies with the human cyclophilin where one would predict that mutagenesis of the single, conserved Trp residue should go the other way and decrease sensitivity to CsA. To this end the human W121F and W121A mutant cyclophilins were constructed, expressed, and purified to homogeneity in 10-mg quantities for evaluation by fluorescence and activity assays. As shown in Figure 2a, both human mutant proteins have the expected diminished fluorescence and show no change in residual fluorescence enhancement on titration with CsA (Figure 2b). The replacement of Trp by Phe or Ala does have dramatic effects on the potency of CsA in inhibition of rotamase activity. As shown in Figure 3, the IC_{50} of CsA for wild-type (recombinant) human cyclophilin was 6 nM while the W121F mutant had an IC_{50} of 430 nM, a drop of ca. 75-fold. The W121A cyclophilin was even more affected with an IC_{50} of 1220 nM, reflecting a decrease of about 200-fold in sensitivity to CsA. Examination of rotamase catalytic efficiency (Figure 4) indicates the human W121F mutant is impaired only about 2-fold in k_{cat}/K_m with *N*-succinyl-AAPF-*p*-NA as substrate while the W121A enzyme is about 13-fold less efficient a catalyst.

The substrate specificity of each of the human and *E. coli* wild-type and mutant enzymes was assessed with eight other tetrapeptide substrates (Figure 4) in which the A-P dipeptide, the site of cis–trans isomerization, was replaced by V-P, L-P, E-P, F-P, G-P, K-P, H-P, and W-P sequences, respectively. It was reasoned that any changes in rank order of the nine substrates might have been anticipated if the mutations were at the rotamase active site. For example, the W121A change in the human cyclophilin might have created space to more readily accommodate an A to W change in the "P1 site" of the tetrapeptide substrate. In the event, however, there was no change in rank order of catalytic efficiency profiles among the three human enzymes and the two *E. coli* proteins, although there is indeed a difference in substrate specificity between the human and *E. coli* rotamases (Figure 4).

The experiments reported here establish that the tryptophan at residue 121 in the human T-cell cyclophilin is a major determinant for high-affinity interaction with the immunosuppressant drug cyclosporin A. The reciprocal behavior of human and *E. coli* enzymes with predictable loss or complementary gain of drug sensitivity by removal or introduction of the side chain of tryptophan at that locus in the protein increases confidence that the bicyclic indole side chain is in close contact with some portions of the hydrophobic cyclic undecapeptide when bound to cyclophilin. Further studies of these mutants with modified CsA analogues should allow identification of the portion in the drug that is in contact with wild-type and mutant cyclophilins. The wild-type *E. coli* rotamase is some 500-fold less sensitive (3000 vs 6 nM) than human T-cell cyclophilin to CsA, but the F112W *E. coli*

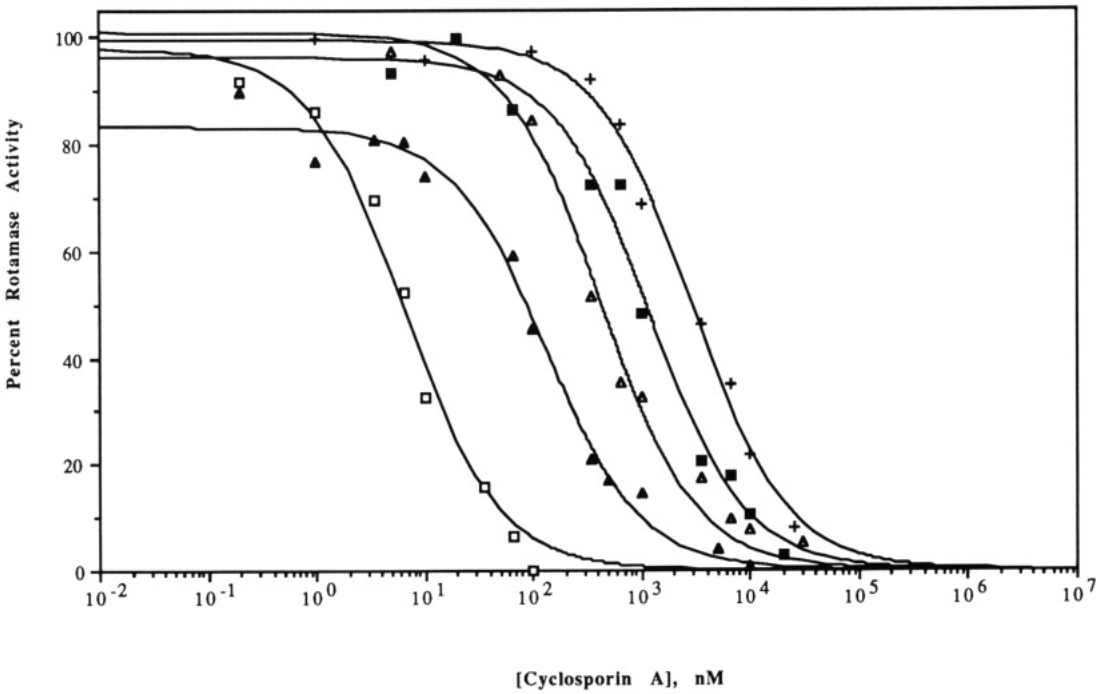

FIGURE 3: Inhibition of tryptophan-related cyclophilin mutants with cyclosporin A. The inhibition curves and the corresponding IC_{50} values were derived by fitting the data according to Cleland (1979) $\{V = V_{max}/(1 + [CsA]/IC_{50})\}$. The IC_{50}'s were 6.6 ± 1.0 nM for wtHCyP (□), 430 ± 20 nM for HCWF (△), 1220 ± 170 nM for HCWA (■), 3000 ± 250 nM for wtECyP (+), and 130 ± 20 nM for ECFW (▲).

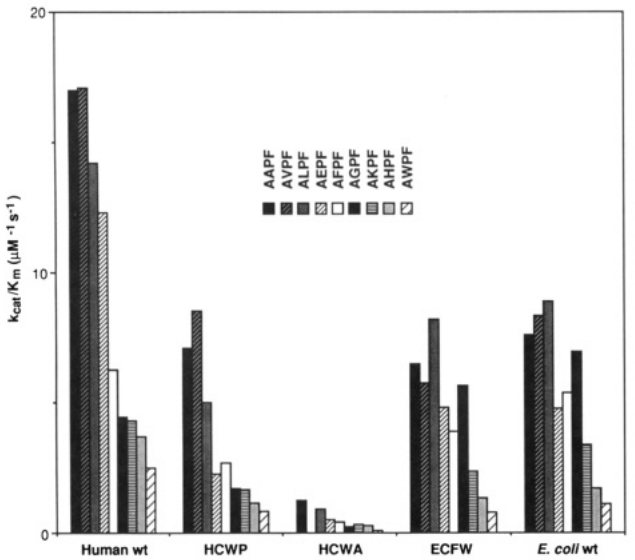

FIGURE 4: Rotamase activity and substrate specificity of wild-type and mutant cyclophilins. The vertical axis is the catalytic efficiency of the proteins (K_{cat}/K_m in $\mu M^{-1} s^{-1}$). Under the assay conditions as described under Materials and Methods, $[S] \ll K_m$; therefore, the overall observed rate $k_{obs} = k_{unc} + k_{cyp} = k_{unc} + (k_{cat}/K_m)[CyP]$, in which k_{unc} is the uncatalyzed rate constant of cis to trans isomerization and k_{cyp} is the rotamase-catalyzed rate constant. Thus, the catalytic efficiency can be derived from $k_{cat}/K_m = (k_{obs} - k_{unc})/[CyP]$. The k_{obs} and k_{unc} values are an average of three repeated assays. To assure accurate estimate of k_{cat}/K_m's, the same protein preparation was used with different substrates. The substrate specificity of calf thymus cyclophilin has been determined with eight of the nine tetrapeptide substrates used in this study (Harrison & Stein, 1990). The bovine cyclophilin exhibited a lower rotamase activity but the same substrate specificity (Harrison & Stein, 1990) compared to the wild-type recombinant human cyclophilin.

mutant ($IC_{50} \approx 130$ nM) actually becomes 10-fold more sensitive than the human W121A mutant cyclophilin. That W121A alteration confers about a 3.5 kcal/mol decrease in specific interaction energy for CsA. The structural consequences in the end will be revealed by NMR and X-ray analyses of cyclophilin alone and in complex with an immunosuppressant drug, and these sets of mutant proteins should be useful in that work.

CsA has been shown to exert its toxicity to *Neurospora crassa* and *Saccharomyces cerevisiae* through its binding to cyclophilin (Tropschug et al., 1989). The question then arises as to how the CsA-producing fungi *Cylindrocarpon lucidum* and *Tolypocladium inflatum* (Borel et al., 1989) avoid the toxicity of CsA given that cyclophilins seem to be a class of ubiquitous proteins present in all organisms (Liu & Walsh, 1990). The work described here points to a possible strategy the fungi might use to solve the dilemma, i.e., by replacement of the tryptophan with a phenylalanine in its cyclophilin. It remains to be seen whether the tryptophan at position 121 in human cyclophilin is indeed absent in cyclophilin(s) from these CsA-producing fungi.

The decoupling of cyclosporin A sensitivity and rotamase activity, quantified in these studies with wild-type and mutant human and *E. coli* cyclophilins, may suggest that the CsA binding site is separate from the active site for cis–trans Xaa–Pro amide bond isomerization. As yet, the poor K_m values of small peptide substrates have prevented the determination of whether CsA acts as a competitive or noncompetitive inhibitor. On the other hand, it is possible that CsA binds to a more extended region of the active site than does the tetrapeptide substrate and the Trp residue is in the extended portion. If CsA is binding and inhibiting at an allosteric site thus serving as a allosteric inhibitor, it may render doubtful the relevance of rotamase activity as the relevant biological readout blocked by CsA. It is imaginable that rotamase inhibition is a necessary but not a sufficient requirement for immunosuppression by CsA. Upon binding of CsA there may be a major conformational change in the receptor to allow the CyP–CsA complex to further interact with other cellular proteins which eventually lead to inhibition of the transcriptional activation of such lymphokine genes as IL-2. In this connection, in studies on the immunosuppressant macrolide FK506 and its high-affinity interaction with its receptor FKBP,

also possessing rotamase activity (Harding et al., 1990; Siekierka et al., 1990; Standaert et al., 1990), attention has recently focused on gain of function rather than loss of function (i.e., enzyme inhibition) as the immunosuppressive readout in an FK506–FKBP complex (Bierer et al., 1990). The mutant cyclophilins described here, for example, human W121A with a 200-fold decrease in CsA sensitivity and only 6% rotamase activity (using AAPF) and also W121F with a 75-fold decrease in CsA sensitivity but still 50% rotamase activity, may also be useful in transfection studies to assess how the altered catalytic rates affect biological readouts, from immunosuppression on the one hand to putative protein folding activity on the other.

Added in Proof

Recent NMR experiments using ^{13}C-labeled CsA have demonstrated that Trp121 in recombinant human cyclophilin resides in close proximity to the MeLeu side chain at position 9 of CsA in the CsA–CyP complex (Fesik et al., 1990), in full agreement with the conclusions made in this paper.

Acknowledgments

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