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# Active site mutants of human cyclophilin A separate peptidyl-prolyl isomerase activity from cyclosporin A binding and calcineurin inhibition

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**Abstract**

Based on recent X-ray structural information, six site-directed mutants of human cyclophilin A (hCyPA) involving residues in the putative active site—H54, R55, F60, Q111, F113, and H126—have been constructed, overexpressed, and purified from *Escherichia coli* to homogeneity. The proteins W121A (Liu, J., Chen, C.-M., & Walsh, C.T., 1991a, *Biochemistry 30*, 2306–2310), H54Q, R55A, F60A, Q111A, F113A, and H126Q were assayed for cis–trans peptidyl-prolyl isomerase (PPIase) activity, their ability to bind the immunosuppressive drug cyclosporin A (CsA), and protein phosphatase 2B (calcineurin) inhibition in the presence of CsA. Results indicate that H54Q, Q111A, F113A, and W121A retain 3–15% of the catalytic efficiency ($k_{cat}/K_m$) of wild-type recombinant hCyPA. The remaining three mutants (R55A, F60A, and H126Q) each retain less than 1% of the wild-type catalytic efficiency, indicating participation by these residues in PPIase catalysis. Each of the mutants bound to a CsA affinity matrix. The mutants R55A, F60A, F113A, and H126Q inhibited calcineurin in the presence of CsA, whereas W121A did not. Although CsA is a competitive inhibitor of PPIase activity, it can complex with enzymatically inactive cyclophilins and inhibit the phosphatase activity of calcineurin.

**Keywords:** calcineurin; cyclophilin; cyclosporin A; immunosuppression; peptidyl-prolyl isomerase

The immunosuppressive drug cyclosporin A, a cyclic undecapeptide, blocks the activation of quiescent T-cells at a stage in signal transduction after T-cell receptors become involved. An 18-kDa protein, termed cyclophilin (CyP) due to its high affinity for cyclosporin A (CsA), has been proposed as the intracellular target (Handschumacher et al., 1984). CyP possesses enzymatic activity for cis–trans isomerization of peptidyl-prolyl bonds and may catalyze protein folding (Fischer et al., 1989; Takahashi et al., 1989; Schonbrunner et al., 1991). CsA is a potent inhibitor ($K_i = 10^{-9}$ to $10^{-8}$ M) of the PPIase activity of CyP (Kofron et al., 1991). Although attention focused initially on PPIase inhibition as the cause of in vivo immunosuppression, several lines of evidence now point to the CyP–CsA complex as the active immunosuppressive species (Tropschug et al., 1989; Friedman & Weissman, 1991; Liu et al., 1991b).

The structurally distinct immunosuppressant FK506 blocks T-cell activation at the same stage as CsA (Bierer et al., 1991). Similar to CyP, the FK506 binding protein (FKBP) is an active PPIase (Harding et al., 1989; Siekierka et al., 1989). Both the FKBP–FK506 and the CyP–CsA complexes have been shown to inhibit the in vitro activity of protein phosphatase 2B, calcineurin (Friedman & Weissman, 1991; Liu et al., 1991b). It has been speculated that calcineurin alters the phosphorylation state and consequently the localization of a cytosolic subunit of the transcription factor NFAT, which is essential for turning on genes encoding such cytokines as interleukin 2 ($IL_2$), $IL_3$, $IF_\gamma$, and granulocyte–macrophage colony-stimulating factor (Emmel et al., 1989; Flanagan et al., 1991).

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*Abbreviations:* hCyPA, human cyclophilin A; CyP, cyclophilin; CsA, cyclosporin A; PPIase, cis–trans peptidyl-prolyl isomerase; FKBP, FK506 binding protein; NFAT, nuclear factor from activated T-cells; IL, interleukin.

To understand the nature of the specific drug-immunophilin interactions and the mechanism by which the complexes inhibit calcineurin, structure/function studies of the ligands and proteins are required. The structure of CsA bound to CyP has recently been determined by NMR analysis (Fesik et al., 1991; Weber et al., 1991) and differs dramatically from CsA in solution (Kessler et al., 1990). Specifically, the 9,10-amide bond is trans when bound and cis in solution. Bound FK506 is likewise distinct from its unbound conformation (Tanaka et al., 1987; Van Duyne et al., 1991). Recently, two reports of the X-ray structure of recombinant human T-cell CyPA have appeared, one unliganded (Ke et al., 1991), the other with a bound tetrapeptide substrate (Kallen et al., 1991).

We have utilized the hCyPA X-ray structure and homologies to other CyPs to focus on putative active site residues and to test predictions about their effects on PPIase activity. Mutations are reported herein that inactivate catalysis but maintain CsA binding and calcineurin inhibition. These results lead to the conclusion that PPIase activity is not necessary for drug binding or phosphatase inhibition, and therefore immunosuppression may be independent of the PPIase activity of CyPs.

## Results

### *Selection of CyP residues for mutation*

The recently determined X-ray structure of hCyPA indicates that this 165-amino acid protein is comprised of eight strands of antiparallel $\beta$-sheet in a flattened $\beta$-barrel with two helices capping the top and bottom (Ke et al., 1991), as shown in Figure 1A. The single tryptophan at position 121 has been implicated as a key side chain in binding to CsA. For example mutagenesis of W121 to F121 reduced CsA affinity by 75-fold, whereas mutagenesis of F112 in *Escherichia coli* CyP to W112 enhanced drug sensitivity 23-fold (Liu et al., 1991a). Also, isotope-edited NMR studies on $^{13}$C-labeled CsA reveal nuclear Overhauser effects to this tryptophan (Kallen et al., 1991; Neri et al., 1991). Within the binding cleft identified by X-ray crystallography are the side chains of H54, R55, F60, Q111, F113, W121, and H126, which, we have noted (Ke et al., 1991), are highly conserved amongst several CyPs. The structure of hCyPA bound to tetrapeptide (Kallen et al., 1991) shows that the guanidinium side chain of R55 and the imidazole of H126 are in close proximity to the prolyl ring of the bound substrate. CsA competitively inhibits tetrapeptide Xaa-Pro isomerization, indicating that it binds in the PPIase active site (Kofron et al., 1991). These results have focused our attention on the region of hCyPA in Figure 1B in which the side chains of the mutated residues are shown. The side chains that were changed to produce inactive mutants and W121 are indicated with their van der Waals surfaces.

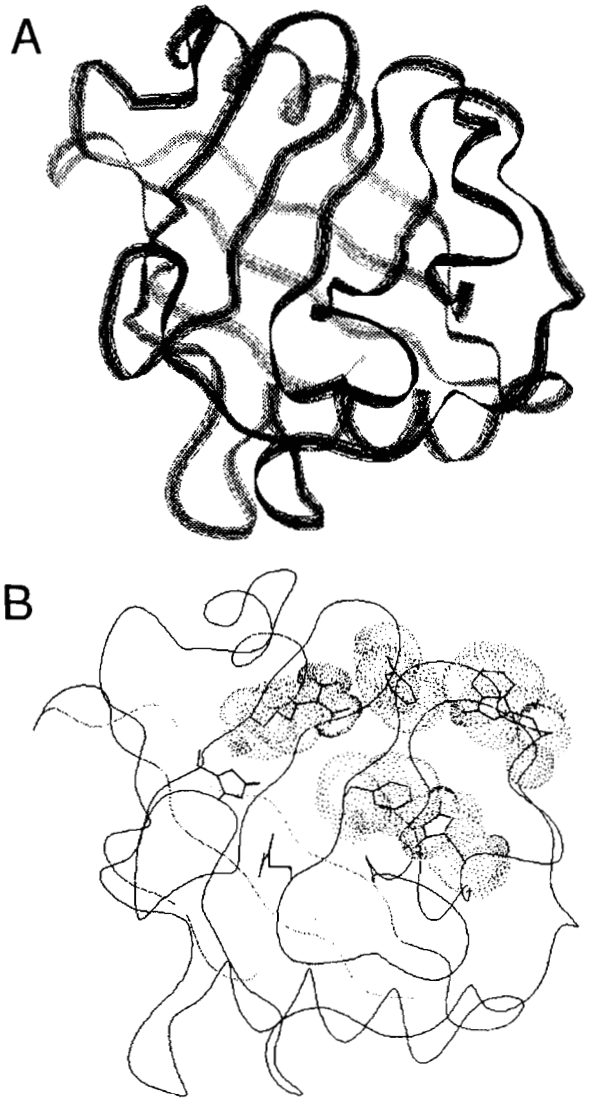


**Fig. 1. A:** Ribbon diagram of recombinant hCyPA. **B:** Schematic diagram of the hCyPA active site with side chains that were modified by mutagenesis. Mutated residues with less than 10% of wild-type activity are surfaced.

### *PPIase activity and CsA affinity of mutants*

The histidines at positions 54 and 126 were converted to glutamine. The phenylalanines at 60 and 113, the arginine at 55, and glutamine at 111 were all mutated to alanine. The encoded CyPs were expressed in *E. coli* and purified to homogeneity (Liu et al., 1990).

Table 1 summarizes the catalytic efficiency, $k_{cat}/K_m$, of the mutant CyPs, assayed with *N*-succinyl-AAPF-*p*-nitroanilide as the substrate (Fischer et al., 1989). These values were then normalized to the $k_{cat}/K_m$ value of wild-type enzyme (16 $\mu M^{-1}$ $s^{-1}$). Circular dichroism (CD) spectra provided evidence for the native-like conformation of these mutant CyPs (data not shown). Except for Q111A, the spectra were virtually superimposable with the spectrum of wild-type human CyP, indicating

**Table 1.** *PPIase activity, inhibition by CsA, and calcineurin inhibition in the presence of CsA by mutant hCyPA proteins in order of decreasing PPIase activity*

| hCyPA | $k_{cat}/K_m$ ($\mu M^{-1} s^{-1}$) | PPIase, % of wild type | $K_i$ CsA (nM) | $K_d$ by fluorescence | Calcineurin inhibition |
|---|---|---|---|---|---|
| WT | 16.0 | 100.0 | 17 ± 2 | <10 nM | + |
| H54Q | 2.4 | 15.0 | 40 ± 10 | NF[c] | ND |
| Q111A | 2.4 | 15.0 | 130 ± 20 | ND | ND |
| W121A | 1.4 | 8.7 | 290 ± 20[a] | ND | – |
| F113A | 0.48 | 3.0 | 190 ± 30 | <10 $\mu$M | + |
| H126Q | 0.084 | 0.53 | ND[b] | WF[d] | + |
| F60A | 0.051 | 0.32 | ND | <10 $\mu$M | + |
| R55A | 0.016 | 0.10 | ND | <10 nM | + |

[a] This $K_i$ value was obtained after 18–20 h preincubation of W121A with CsA. No calcineurin inhibition was observed under these conditions.
[b] Not determined.
[c] No detectable fluorescence change.
[d] Weak fluorescence change.

proper folding of the mutant proteins. Two of the targeted mutant CyPs, H54Q and Q111A, had 15% of wild-type activity. CsA binding, as measured by inhibition of PPIase activity, was also not substantially affected. However, the activity of Q111A may only represent the proportion of properly folded enzyme, because the CD spectrum of the Q111A mutant showed a weaker minimum at 223 nm than wild type. Thus, H54 and Q111 do not appear to have crucial roles in PPIase activity.

Intermediate effects were observed for the F113A and W121A mutants. F113A has approximately 3% of the wild-type $k_{cat}/K_m$. The 33-fold drop in catalytic efficiency still permitted assay of enzyme inhibition. Increasing the enzyme concentration from 7 nM with wild-type CyPA to 310 nM with F113A allowed determination of the $K_i$ for CsA of 190 nM. The W121A mutant (Liu et al., 1991a) exhibited similar PPIase activity (9% of wild type), whereas CsA affinity was about 80-fold lower than wild type ($K_i$ = 1,350 nM). When W121A was incubated overnight with CsA, much tighter binding was measured in the PPIase assay ($K_i$ = 290 nM). This enzyme therefore shows slow-binding behavior with CsA.

The remaining three mutant hCyPA proteins R55A, F60A, and H126Q displayed dramatically lower PPIase catalytic efficiency, all below 1% of wild type. The activities of these three mutants were down from wild-type enzyme by 190–1,000-fold, suggesting that each of these residues may have an important role in catalysis. However, these values for $k_{cat}/K_m$ in Table 1 are above nonenzymatic background levels. It should also be noted that the residual activity may be due to contamination by endogenous *E. coli* CyPs (Liu & Walsh, 1990; Hayano et al., 1991); therefore, these three mutants may be even less active than the data in Table 1 indicate.

A further point emerges when one examines the ability of CsA to bind to R55A, F60A, or H126Q mutants. Because of the low residual activity (and the possibility of endogenous *E. coli* CyP contamination), $K_i$ measurement was not possible with the assay used. Instead, the mutants were bound to a CsA matrix to demonstrate affinity for the drug. All of the mutants tested bound to the matrix, whereas none of the molecular weight marker proteins bound under the same conditions. The proteins were specifically eluted with free CsA and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). This assay did not distinguish between high- and low-affinity binding; rather it showed that these mutants were folded, bound drug, and were suitable for calcineurin inhibition assays.

Fluorescence enhancement of the single Trp in CyP has previously been used to measure CsA binding constants (Handschumacher et al., 1984; Liu et al., 1991a). Optimal conditions for obtaining accurate $K_d$ values use protein concentrations near the dissociation constant. If saturation of the fluorescence signal is reached, the binding constant may be calculated from: $K_d = [\mathrm{CsA}]_{50\%} - \frac{1}{2}[\mathrm{CyP}]_0$, where $[\mathrm{CsA}]_{50\%}$ is the concentration at 50% of fluorescence saturation.[1] In practice, data collection is limited by the sensitivity of the fluorescence signal and the low solubility of CsA. Fluorescence changes observed for wild type and the mutants were all less than 50 fluorescence units when 500 nM of protein was used (Fig. 3). Quantitation of the binding constants was not possible in this concentration range. R55A showed saturation of fluorescence enhancement and appeared to bind drug almost as well as wild type. F60A, F113A, and H126Q required much higher concentrations of CsA, ca. 4 $\mu$M, to approach saturation. At these levels, precipitation of CsA

[1] This corrects an error in the calculation of $K_d$ given in Figure 2 of Liu et al. (1991a).

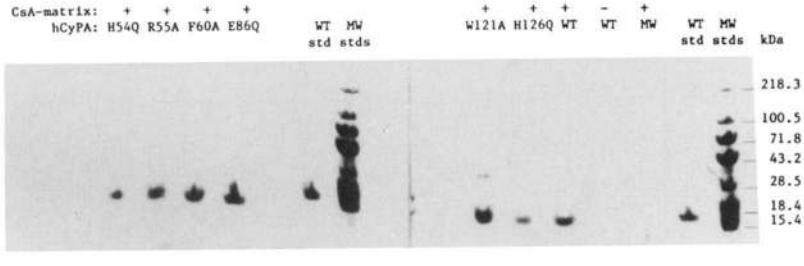


**Fig. 2.** SDS-PAGE gels of mutant hCyPA proteins eluted with CsA from CsA affinity matrix beads. Treatment of the protein with beads is indicated by a + sign. Left to right: H54Q+, R55A+, F60A+, E86Q+, MW stds, W121A+, H126Q+, WT–, MW stds+, WT std, MW stds. MW, molecular weight; std, standard; WT, wild type.

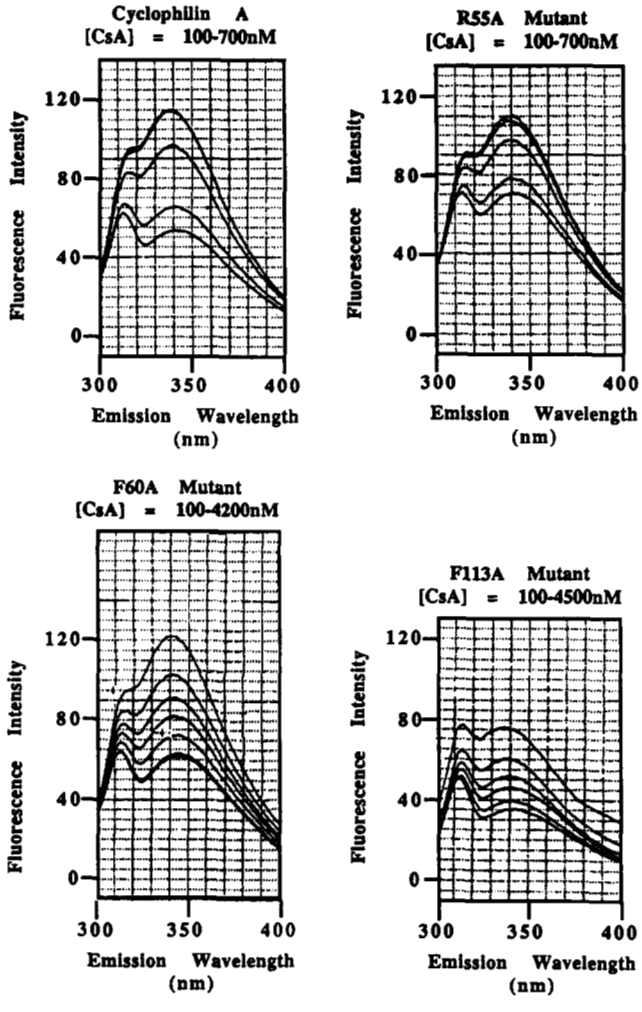


**Fig. 3.** Fluorescence enhancement of hCyPA and three mutants—R55A, F60A, and F113A—by titration with CsA.

began to interfere with the signal, but sufficient data were obtained to verify binding of these four mutants to CsA. By fitting the data to a theoretical binding curve, estimates for the upper limits of the dissociation constants were obtained (Table 1).

### *Calcineurin inhibition studies*

The CsA affinity matrix and PPIase assays identified five mutants (R55A, F60A, F113A, W121A, and H126Q) that were of prime interest for evaluation of inhibition of calcineurin phosphatase activity. Dephosphorylation of the phosphoserine form of the 19-residue peptide from the regulatory subunit ($R_{II}$) of the cAMP-dependent protein kinase A (Blumenthal et al., 1986) was monitored in the presence of the appropriate CyP, CsA, calmodulin, and $Ca^{2+}$ (Swanson et al., 1992). The mutants, R55A, F60A, F113A, and H126Q were compared to wild-type hCyPA and to the W121A mutant, which binds CsA with lower affinity (Fig. 4). Even after overnight incubation of W121A with CsA (see PPIase inhibition results above), the complex did not inhibit calcineurin's phosphatase activity. None of the CyPs nor CsA alone inhibited phosphatase activity of calcineurin. Possible contaminating *E. coli* CyPs could not inhibit calcineurin, since these proteins lack sufficient CsA binding activity (Liu & Walsh, 1990). The data of Figure 4 show that four of the active site mutants exhibited calcineurin inhibition in the presence of CsA. These mutants, in addition to defining residues that contribute to catalytic efficiency, show a separation of high-affinity CsA binding and phosphatase inhibition from PPIase catalytic activity.

## Discussion

The X-ray structural analysis (Ke et al., 1991) of recombinant human T-cell CyP shows a region with a shallow crevice containing conserved basic residues and hydrophobic aromatic side chains (Fig. 1B). This pocket has been implicated in PPIase catalysis by the location of bound *N*-acetyl-AAPA-amidomethyl-coumarin (Kallen et al., 1991) and by the recent evidence that a CsA analogue is a competitive inhibitor of tetrapeptide substrates (Kofron et al., 1991). To evaluate structural predictions, we determined PPIase activity and CsA recognition and ultimately determined the activity of the mutants for calcineurin inhibition. These studies should contribute to understanding the immunosuppressive function of CsA. The retention of substantial catalytic efficiency and CsA sensitivity in the H54Q and Q111A mutants argues

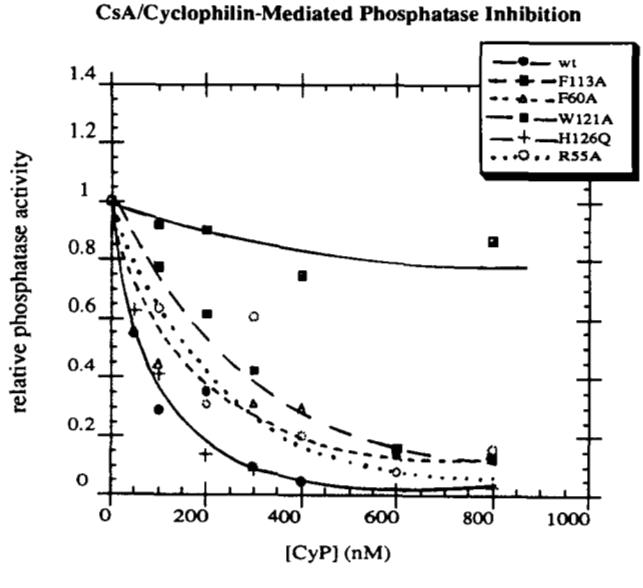


**Fig. 4.** Percent inhibition of [$^{32}$P]orthophosphate release from [$^{32}$P]$R_{II}$ peptide by calcineurin as a function of mutant hCyPA concentrations in the presence of 5 $\mu$M CsA.

against significant roles for these residues. Likewise the F113A and W121A mutations, while down 33- and 11-fold in $k_{cat}/K_m$, respectively, are still sufficiently active to point to their minor roles in cis–trans PPIase activity.

The severe losses (about three orders of magnitude) in catalytic efficiencies of the mutants, R55A, F60A, and H126Q, focus attention on this area of the protein surface. Residues F60 and W121 are aromatic side chains that exhibit nuclear Overhauser effects to bound CsA (Neri et al., 1991). A precise role for the phenyl side chain of F60 in catalysis or in drug binding is not yet clear. The R55 and H126 side chains may be involved in hydrogen bonding with either the inhibitor, CsA, or peptide substrates. Figure 1B indicates the van der Waals surfaces of R55, F60, F113, H126, and W121, which define at least part of the active site.

In a recent NMR and X-ray structure, both R55 and H126 were identified as candidates for coordination to the Ala-Pro amide of bound *N*-Ac-AAPA-amidomethylcoumarin (Kallen et al., 1991). In particular, because Nδ of His 126 resides near the Ala-Pro amide carbonyl, Kallen et al. originally proposed that His 126 may assist in nucleophilic attack by a water molecule. However, this route would suggest generation of a tetrahedral intermediate during catalysis of cis–trans isomerization. Evidence against a tetrahedral intermediate includes (1) the lack of a solvent isotope effect, (2) the pH independence of $k_{cat}/K_m$, and (3) the normal secondary deuterium isotope effect for the isomerization (Harrison et al., 1990; Harrison & Stein, 1990). These arguments against a tetrahedral intermediate were summarized recently (Stein, 1992), and the proposed water molecule was ruled out after refinement of the X-ray structure (Kallen et al., 1992). The basic residues, R55 and H126, are clearly involved in catalytic acceleration, but their roles are yet to be understood.

On the other hand, a direct twist or distortion mechanism (Harrison & Stein, 1990; Liu et al., 1990) has also been suggested for PPIase's calculated $10^6$ catalytic enhancement over the nonenzymatic rate (Kofron et al., 1991). Mutagenesis of FKBP, in which sterically conservative changes were made, revealed less drastic activity losses (Park et al., 1992) than in this work on CyP. Both mutagenesis results are consistent with a twistase mechanism.

The separation of PPIase catalysis from CsA recognition and binding in these mutants led us to investigate the action of the immunophilin–drug complexes on calcineurin. The R55A, F60A, and H126Q mutants in complex with CsA still inhibit the phosphatase activity of calcineurin. Because these mutants have drastically impaired PPIase activity, cis–trans isomerization activity may not be required to present a specific conformer of CsA. Determination of the conformation of CsA bound to these mutants may answer this question. This is of interest given that CsA is a slow-binding inhibitor and undergoes two-step binding to a tightened complex with wild-type hCyP, indicating that the drug may be isomerized upon binding (Kofron et al., 1991; K.S. Anderson, L.D. Zydowsky, J. Liu, C.H. Baker, R.E. Handschumacher, & C.T. Walsh, unpubl.). It is likely that the mutant CyPs selectively bind the *trans* conformation of CsA, which exists in solution (Kessler et al., 1990; Altschuh et al., 1992) and has recently been shown to be the active species for PPIase inhibition (Kofron et al., 1992). In this view, the CyP active site serves solely as a scaffolding apparatus to present bound drug in an active conformation. Alternately, the mutant enzymes may catalyze the isomerization of the drug sufficiently to populate the conformer needed for presentation and phosphatase inhibition.

Because these mutant CyP–CsA complexes inhibit calcineurin, they are candidates for structural determination of the bound CsA conformation. The W121A mutant is of particular interest because it is an active PPIase ($10^5$ rate enhancement over background), but does not inhibit calcineurin's phosphatase activity even in the presence of 5 μM CsA, which is well above the $K_i$ of 290 nM. The lack of phosphatase inhibition implies that the W121 indole side chain may interact directly with calcineurin. Additional structural and mutagenesis information will yield further understanding of the immunosuppressive architecture of the CyP–CsA complex that is recognized by calcineurin.

## Materials and methods

### *Mutagenesis*

Site-directed mutagenesis of hCyPA cDNA was accomplished by the Kunkel method (Kunkel, 1985) using a Mutagene Kit from BioRad. Human CyPA cDNA was cloned into M13mp19. This construct was passed through CJ236, a *dut⁻ung⁻* strain of *E. coli* from BioRad. The hybrid DNA constructs were introduced into XL1B, a *dut⁺ung⁺ E. coli* strain. Primers used for mutagenesis and sequencing were synthesized by Alex Nusbaum (Harvard Medical School). The mutagenic primers used were: H54Q, 5′-C TGC TTT CAG AGA ATT ATT-3′; R55A, 5′-TCC TGC TTT CAC GCA ATT ATT CCA-3′; F60A, 5′-ATT ATT CCA GGG GCT ATG TGT CAG-3′; Q111A, 5′-ACA AAT GGT TCC GCG TTT TTC ATC-3′; F113A, 5′-TCC CAG TTT GCC ATC TGC ACT GCC-3′; and H126Q, 5′-GAT GGC AAG CAG GTG GTG TTT GGC-3′. Bases encoding mutated amino acids are underlined. The mutant constructs were sequenced in full with the following primers: M13 universal, 5′-ATC CTA AAG CAG ACG GGT CCT GGC-3′, 5′-GCC AAG ACT GAG GCG TTG GAT GGC-3′, 5′-ACT GGA GAG AAA GGA-3′, 5′-AAT GGC ACT GGT GGC-3′, and the reverse primer 5′-GGA CTT GCC ACC AGT-3′. The F60A and H126Q mutant cDNAs were sequenced by Lori Wirth (Dana

Farber Molecular Biology Core Facility) using Applied Biosystems Inc. Automated Tac Dyedeoxy Terminator Sequencing with the following primers: M13 universal (H126Q), 5′-TGG AAT TGT GAG CGG-3′ (pHN1 for F60A), and the reverse primer 5′-CCG CTT CTG CGT TCT G-3′ for both.

### *Protein expression and purification*

The mutant hCyPA cDNAs were cloned into the expression vector pKen or pHN1$^+$, overexpressed in XA90F′$lac^{Q1}$ (gifts of Gregory Verdine, Harvard University) and purified as described with minor modifications (Liu et al., 1990). Instead of collecting the pellet of a 40–60% (w/v) ammonium sulfate precipitation, the supernatant after the 0–40% ammonium sulfate precipitation was dialyzed twice against 2 L of 20 mM Tris-HCl, pH 8.0, overnight. Following DEAE-Sepharose CL-6B column chromatography, the eluant was concentrated to 10 mL, loaded onto a Sephadex G-50 column (80 × 2.5 cm), and eluted with 10 or 25 mM $KP_i$, pH 7.5, 250 mM KCl, 0.02% $NaN_3$. Protein-containing fractions were combined and concentrated. The proteins were diluted 1:1 with glycerol and stored at −80 °C.

### *Circular dichroism*

CD spectra of all hCyPA proteins at ca. 60 μM concentration in 10 mM $KP_i$, 250 mM KCl, pH 7.5, 0.02% $NaN_3$:glycerol (1:1) were obtained on an AVIV CD Spectrometer, model 62 DS. The data were obtained in the range of 210–260 nm with a step size of 0.5 nm at 15 °C. The intensity at 223–224 nm in the spectrum of each mutant protein was corrected for concentration and compared with the spectrum of the wild type.

### *Enzyme assay*

The PPIase assay was performed essentially as described (Liu et al., 1990). Amounts of mutant hCyPA protein (appropriate to give rates from 0.05 to 0.3 AU/s) in 10 μL of 20 mM Tris-HCl, pH 7.8, were added to a solution of *N*-succinyl-AAPF-*p*-nitroanilide (final concentration = 100 μM) in 0.68% DMSO, 35 mM HEPES (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid), pH 8.0, precooled to 8 °C. The reaction was initiated by addition of 25 μL of 10 mg/mL chymotrypsin in 10 mM HCl with rapid inversion for a total assay volume of 1.0 mL. The temperature was controlled at 8 °C during the assay. First-order rate constants ($k_{obs}$) were obtained by nonlinear least-squares fitting of the data to a simple exponential curve. The $k_{cat}/K_m$ values were obtained as the slope of the plot of $k_{obs}$ vs. enzyme concentration. PPIase inhibition was performed by addition of 5 μL of CsA in ethanol prior to addition of substrate as above to give final concentrations varying from 10 to 5,000 nM. Using a nonlinear least-squares program (KaleidaGraph on a Macintosh IIcx), the data were fit to the equation (Williams & Morrison, 1979)

$$k_{obs} = k_{uncat} + (k_{cat}/2K_m)\{[(E_0 - I_0 - K_i)^2 + 4E_0K_i]^{1/2} + (E_0 - I_0 - K_i)\}.$$

### *CsA affinity matrix binding*

The CsA affinity matrix beads were obtained as a gift from Mark Albers in Prof. Stuart Schreiber's group (Harvard University). Small portions (ca. 40 μL) of the beads were washed twice with 0.5 mL of buffer (25 mM $KP_i$, pH 7.5, 250 mM KCl, 0.02% $NaN_3$). The beads were spun at 14,000 rpm in an Eppendorf centrifuge and the solvent decanted via a 26-gauge needle at each step. The beads were incubated overnight at 4 °C with 20 μL of 1 mg/mL CyP in buffer. The proteins were eluted from the beads with 15 μL of 100 μM CsA in 10% ethanol/buffer. The eluted proteins were analyzed by 8–25% gradient SDS-PAGE on a Pharmacia PhastGel system.

### *Fluorescence titrations*

Tryptophan fluorescence enhancements of CyP upon binding to CsA have been reported (Handschumacher et al., 1984), and fluorescence titrations have been used to determine affinities (Liu et al., 1990). The modified titration procedure for estimating mutant CyP proteins affinities for CsA involved constant protein concentrations of 500 nM (35 mM HEPES, pH 8.0) and addition of CsA aliquots (100 μM and 1.74 mM stock solutions in ethanol). Final CsA concentrations were varied from 0 to 700 nM for wild-type and mutant R55A hCyPA and varied from 0 to 4200 nM for F60A and 0 to 4500 nM for F113A. The 1.0-mL buffered protein solutions were equilibrated at 15 °C for at least 5 min following addition of each CsA aliquot. Measurements were made on a Shimadzu RF-500 spectrofluorophotometer at 15 °C. Emission spectra were recorded over a range of 300–400 nm with the excitation wavelength set to 280 nm and slit widths of 5 nm. After subtraction of background fluorescence due to addition of CsA/ethanol aliquots, the fluorescence changes from unliganded CyP were calculated, and maximum $K_d$ values were estimated based on comparison of data points to the binding curve:

$$\Delta F_{obs} = [(K_d + E_0 + I_0)/(2I_0)] \times \{1 - [1 - (4E_0I_0/(K_d + E_0 + I_0)^2)]^{1/2}\}\Delta F_{sat},$$

where $E_0$ is the concentration of hCyPA and $I_0$ is the total concentration of CsA at that data point.

*Phosphatase inhibition*

Calmodulin and bovine calcineurin were purchased from Sigma. The $R_{II}$ peptide was synthesized by Charles Dahl (Harvard Medical School). $^{32}$P-labeled $R_{II}$ peptide (DLDVPIPGRFDRRVpSVAAE) was prepared via the published method (Blumenthal et al., 1986) and purified from ATP via filtration through a C-18 Waters Sep-Pak cartridge. Calcineurin-catalyzed [$^{32}$P]orthophosphate release from [$^{32}$P]$R_{II}$ peptide was assayed at 30 °C in a final volume of 100 μL of buffer (40 mM Tris-HCl, pH 7.8, 100 mM NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.05 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 80 nM calmodulin). The final concentrations of mutant CyPs were in the range of 0–800 nM and each concentration point was assayed in the absence and in the presence of 5 μM CsA. All components of the reaction were mixed and incubated at 30 °C for 10 min. The reaction was started upon addition of [$^{32}$P]$R_{II}$ peptide (final concentration: 8 μM). Aliquots of 20 μL were removed at 0 and 10 min and bound to Whatman P81 ion-exchange chromatography paper, which was immediately submerged in 75 mM $H_3PO_4$ solution to stop the reaction. The filter papers were washed four times with 75 mM $H_3PO_4$ to remove any [$^{32}$P]orthophosphate and mixed with 3 mL of scintillation cocktail for counting. The ratio of $^{32}$P· released with CsA vs. without CsA after 10 min was plotted against CyP concentration.

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