Cyclophilin Residues That Affect Noncompetitive Inhibition of the Protein Serine Phosphatase Activity of Calcineurin by the Cyclophilin·Cyclosporin A Complex†

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ABSTRACT: Mutation of three cationic surface residues of human cyclophilin A (hCyPA), R69, K125, and R148, to both anionic and neutral residues left its intrinsic peptidyl-prolyl isomerase (PPIase) activity and cyclosporin A (CsA) binding unaffected, but altered its ability to inhibit the serine phosphatase activity of calcineurin (CN). R69E was 13-fold less effective (K_i = 3400 nM) than wild-type hCyPA (K_i = 270 nM) in presenting CsA for calcineurin phosphatase inhibition, while R148E was 17-fold more effective (K_i ≤ 16 nM), and human CyPB was 13-fold better (K_i ≤ 21 nM), establishing that a composite drug/protein surface is being recognized. The phosphoserine phosphatase reaction catalyzed by CN using unlabeled phosphoserine RII19 peptide was coupled to a continuous spectrophotometric assay to measure inorganic phosphate production using the enzyme purine ribonucleoside phosphorylase and the substrate N^7-methyl-2-thioguanosine [Webb, M. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 4884–4887]. With this assay, we have determined that human cyclophilin A complexed with the immunosuppressive drug cyclosporin A is a noncompetitive inhibitor of calcineurin phosphatase activity. This mutational analysis identified hCyPA residues that interact with CN, and comparison to similar data on FKBP allowed us to begin to map out the CN recognition surface. The *p*-nitrophenylphosphatase activity of CN was stimulated ca. 3-fold by CyP·CsA, presumably reflecting altered active site geometry and selective access of this small substrate.

Cyclosporin A (CsA)[1] is the immunosuppressive drug which has allowed a dramatic increase in the number of survivors of organ transplants (Shevach, 1985; Wenger & Fliri, 1990). CsA and a more recently discovered, structurally unrelated macrolide, FK506 (Tanaka et al., 1987), each act to block activation and cell cycle progression of quiescent T-cells in response to foreign antigens. These hydrophobic immunosuppressive drugs penetrate T-cells and block Ca^{2+}-mediated signal transduction that would normally lead to transcriptional activation of genes for T- and B-cell growth factors such as IL2, IL4, and interferon (Emmel et al., 1989; Flanagan et al., 1991). Since the discovery of cyclophilin (CyP) (Handschumacher et al., 1984) and FKBP (FK506 binding protein) (Harding et al., 1989; Siekierka et al., 1989), which have specific high affinities for CsA and FK506, rapid advances have been made in our understanding of the mechanism of immunosuppression by these drugs. The 18-kDa hCyPA binds CsA with an affinity of ca. 10 nM (Liu et al., 1990; Kofron et al., 1991) and presents a distinct conformer of bound drug (Kofron et al., 1992) to a second protein, the heterodimeric protein serine/threonine phosphatase PP2B, or calcineurin (CN) (Liu et al., 1991b). The CyP·CsA complex inhibits the phosphatase activity of calcineurin assayed with phosphoserine protein and peptide substrates (Liu et al., 1991b; Swanson et al., 1992). One of the substrates for CN in T-cells is the cytoplasmic subunit of the transcription factor NFAT (McCaffrey et al., 1993). Dephosphorylation of NFAT permits translocation to the nucleus (Flanagan et al., 1991; Northrop et al., 1993), combination with jun/fos (Jain et al., 1992), and activation of transcription of the IL2 gene that initiates cell cycle progression, leading ultimately to the immune response (Shaw et al., 1988; Emmel et al., 1989). Thus, CsA bound to cyclophilin blocks T-cell activation and graft rejection by inhibiting the phosphatase activity of CN. The molecular actions of the immunosuppressant drugs CsA and FK506 have been reviewed (Etzkorn et al., 1993; Liu, 1993).

Among the issues in this T-cell signal transduction cascade are the specificity of drug and protein action and the molecular recognition of CN. The X-ray structures of human cyclophilin A (Kallen et al., 1991; Ke et al., 1991) and of the CyPA·CsA complex (Pflügl et al., 1993) have revealed key details of molecular architecture. We have reported on mutations in the hCyPA active site that abrogate its PPIase activity without affecting CsA binding or calcineurin inhibition, thus separating the protein-folding function of cyclophilin from its immunophilin function (Zydowsky et al., 1992). A similar mutant, F36Y, has been reported for FKBP12 (Wiederrecht et al., 1992). Neither CsA nor CyP alone is the physiologically relevant species for immunosuppression: only the complex is functional (Liu et al., 1991b). What was not clear was whether only the bound CsA contacts CN in immunosuppression or whether a composite surface of both CyP and CsA comes into contact with CN. We addressed this issue by mutating specific basic residues in loops residing on the protein surface near the CsA binding site as determined from the X-ray structure of hCyPA (Ke et al., 1991) and a model of the hCyPA·CsA complex (Fesik et al., 1992), Figure 1. In this work we have adapted a sensitive, continuous assay for inorganic phosphate production (Webb, 1992) to determine that the human T-cell hCyPA·CsA complex is a noncompetitive inhibitor of phosphoserine phosphatase activity of calcineurin. We then utilized this assay to evaluate the effects of mutation of charged surface

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[1] Abbreviations: hCyPA, human cyclophilin A; CsA, cyclosporin A; phosphatase, peptide serine phosphatase; PPIase, peptidyl-prolyl isomerase; CN, calcineurin; CaM, calmodulin; NFAT, nuclear factor for activation of T cells; ER, endoplasmic reticulum; pNPP, *p*-nitrophenyl phosphate; pNPPase, *p*-nitrophenylphosphatase; P_i, PO_4^{2-}; MESG, N^7-methyl-2-thioguanosine; PRPase, purine ribonucleoside phosphorylase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; cAMP, 3′,5′-cyclic adenosine monophosphate; PKA, protein kinase A; DTT, dithiothreitol; BSA, bovine serum albumen.

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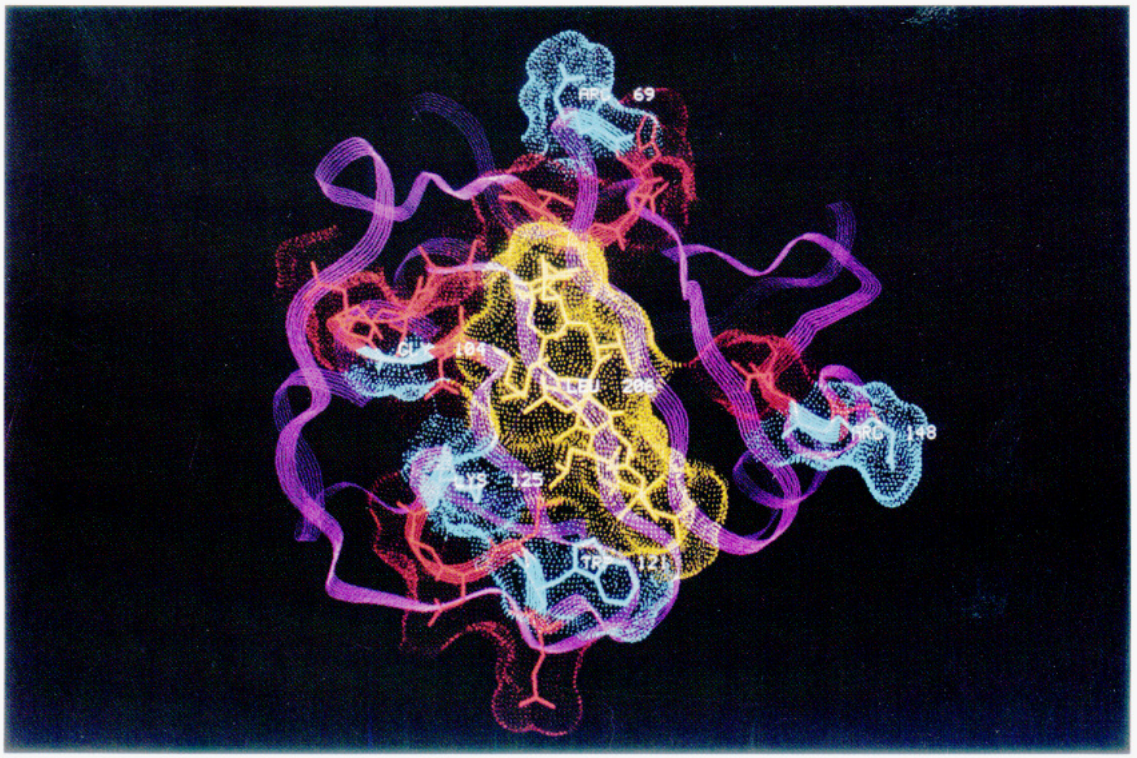


FIGURE 1: NMR structure of the CsA (red)·hCyPA (purple) complex (Thériault et al., 1993) highlighting the loops and helix (yellow) surrounding the CsA binding site. The mutated residues R69, W121, K125, and R148 that affect CN binding are shown in blue.

residues of cyclophilin on the noncompetitive inhibition of calcineurin.

EXPERIMENTAL PROCEDURES

General. All water was purified with a MilliQ system from Millipore. All commercial reagents were used without further purification unless otherwise noted. NMR spectra, obtained on a Varian VXR 500 FT NMR spectrometer at 500 MHz for ^{1}H, are reported as follows: chemical shift (multiplicity, number of protons, coupling constants, assignment). Phosphatase and pNPPase assays were performed in a 0.5-mL quartz cuvette (Helma) on a Perkin-Elmer Lambda 6 UV spectrometer equipped with a circulating-water-heated cuvette holder. Protein concentrations were determined either by UV using ϵ = 7920 M^{-1} for hCyPA and mutants or by Bradford assay (Bio-Rad) for hCyPB, W121A, and W121F.

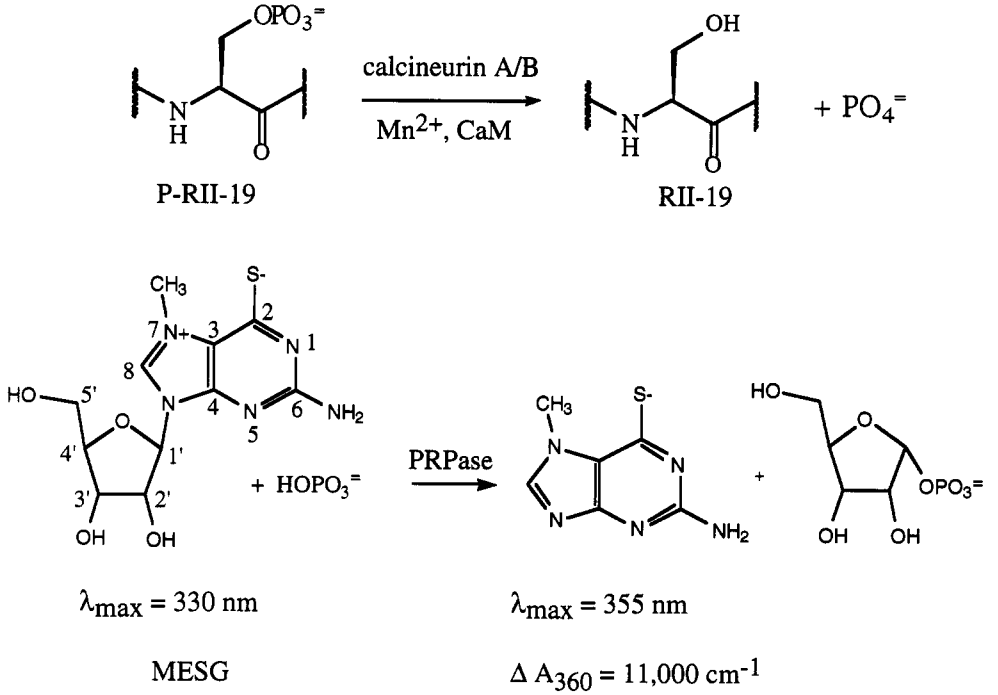
Mutagenesis of hCyPA. Site-directed mutagenesis of hCyPA cDNA (Liu et al., 1990) was accomplished by the Kunkel method (Kunkel, 1985) using a Mutagene kit from Bio-Rad. Primers used for mutagenesis and sequencing were synthesized by Alex Nusbaum (Harvard Medical School). The following mutagenic primers were used: K125Q, 5′-d(TGG TTG GAT GGC CAG CAT GTG GTG)-3′; K125E, 5′-d(TGG TTG GAT GGC GAG CAT GTG GTG)-3′; R69L, 5′-d(GGT GAC TTC ACA CTC CAT TTA GGC)-3′; R69E, 5′-d(GGT GGT GAC TTC ACA GAA CAT AAT GGC ACT GGT)-3′; R148L, 5′-d(CGC TTT GGG TCC CTG AAT GGC AAG ACC)-3′; R148E, 5′-d(CGC TTT GGG TCC GAG AAT GGC AAG ACC)-3′. Bases encoding mutated amino acids are underlined. The mutant constructs in pKen were purified using a plasmid Midi-prep kit from Qiagen, and the hCyPA genes were sequenced in full using Sanger's dideoxy method with a Sequenase v. 2.0 kit (U.S. Biochemicals) with the following primers: M13 universal, 5′-d(GTA AAA CGA CGG CCA GT)-3′; 5′-d(AG GGT TCC TGC TTT CAC)-3′; 5′-d(GCC AAG ACT GAG GCG TTG GAT GGC)-3′; internal reverse, 5′-d(GGA CTT GCC ACC AGT)-3′.

Protein Expression and Purification. The mutant hCyPA cDNAs were cloned into the expression vector pKen, overexpressed in XA90F′lac^{Q1} (both gifts from Gregory Verdine, Harvard University), and purified as described (Zydowsky et al., 1992) with minor modification as follows. The dialyzed $(NH_4)_2SO_4$ supernatant was loaded onto a DEAE-Sepharose column equilibrated with 20 mM Tris·HCl, pH 8.0, and eluted with a gradient from 0 to 250 mM KCl in 20 mM Tris·HCl, pH 7.8, over 300 mL at 1mL/min. Protein-containing fractions were collected and concentrated to ca. 10 mL, loaded onto a Sephadex G-50 column (80 × 2.5 cm), and eluted with 25 mM Hepes, 250 mM KCl, and 0.02% NaN_3, pH 7.8. Protein-containing fractions were combined and concentrated to 3–10 mg/mL, diluted to 25% glycerol, and stored at –80 °C.

Enzyme Assays. The peptidyl-prolyl isomerase, CsA inhibition, and ^{32}P-Ser-RII peptide/CN phosphatase assays were performed as described previously (Zydowsky et al., 1992).

Continuous Spectrophotometric CN Phosphatase Assay. (A) Synthesis of MESG. MESG was synthesized from 2-amino-6-chloropurine nucleoside (Sigma) by the method of Webb (Broom & Robins, 1964; Webb, 1992) with a modified purification procedure. The crude product (30–40 mg/4.0 mL of water per injection) was purified by high-pressure liquid chromatagraphy (HPLC) on a Bio-Rad Hi-Pore 318 (C-18) 21.5 mm × 25 cm column using water as eluant at 8.0 mL/min. Each peak was analyzed for phosphorylation activity with inorganic phosphate catalyzed by purine ribonucleoside phosphorylase. The peaks at 22.6 min from several injections were combined, lyophilized, and stored as a 16.6 mM solution in pure water at –80 °C. The structure of MESG was confirmed by ^{1}H-NMR (Broom & Milne, 1975) (500 MHz, D_2O): δ 3.85 (dd, J = 4.1, 12.9 Hz, 1, 5′H), 3.96

Scheme 1



(dd, J = 2.8, 12.9 Hz, 1, 5'H), 4.26 (m, 1, 4'H), 4.28 (s, 3, $7CH_3$), 4.37 (t, J = 5.3, 5.7 Hz, 1, 3'H), 4.66 (dd, J = 3.7, 4.9 Hz, 1, 2'H), 6.07 (d, J = 3.7, 1, 1'H), 9.22 (s, 1, 8H).

(*B*) *Purification of Purine Ribonucleoside Phosphorylase* (*PRPase*). PRPase (E.C. 2.4.2.1) (Sigma) was purified by fast protein liquid chromatagraphy (FPLC) on a HiLoad Q-Sepharose 16 mm x 10 cm column (Pharmacia) with a gradient from 50 mM Tris·HCl, pH 7.6, to 50 mM Tris·HCl and 0.5 M KCl, pH 5.8, at 2.0 mL/min for a total of 400 mL. The eluted fractions were analyzed by 10% SDS–PAGE. Fractions that contained protein bands with the same mobility (≈29 kDa) were combined and checked for phosphorylase activity using MESG and inorganic phosphate as substrates. The active fractions were concentrated by use of Amicon (Pharmacia) and stored in 50% glycerol at −80 °C.

(*C*) *Preparation of Phosphoserine-RII Peptide.* The RII19 peptide (DLDVPIPGRFDRRVSVAAG) was synthesized by Charles Dahl (Harvard Medical School). This peptide was phosphorylated on the serine residue by 3',5'-cAMP-dependent protein kinase A (PKA, Sigma). The kinase reaction was performed in 50 mM Mops and 10 mM $MgCl_2$, pH 7.1. PKA (2.5 mg, 5.0 nM units/mg) was dissolved in 1.0 mL of 1 M DTT, 0.125 mL of 50 mg/mL BSA, 50 μL of 1.0 mM 3',5'-cAMP, and 2.5 mL water for 10 min at room temperature. Then 25.0 mL of 100 mM Mops/20 mM $MgCl_2$, 15.5 mL of water, 1.0 mL of 0.1 M ATP (0.02 mmol), and 5.0 mL of 6.6 μM (33 μmol) RII peptide were added in order. The reaction mixture was incubated at 30 °C for 8 h while the reaction was monitored by reverse-phase HPLC at 214 nm on a Vydac C18 peptide and protein column (4.6 mm × 25 cm) using a gradient from 100% A to a 60:40 mixture of A and B at 1.0 mL/min over 30 min, where A is a 9:1 mixture of 20 mM aq $NH_4^+OAc^-$ and CH_3CN and B is a 1:9 mixture of 20 mM aq $NH_4^+OAc^-$ and CH_3CN. The phosphopeptide eluted at a retention time of 13.7 min, and the starting RII peptide eluted at 16.2 min. The kinase reaction was driven to completion by adding another 0.5 mg of PKA preincubated for 10 min in 3.7 mL of 0.27 M DTT, 0.013 mM 3',5'-cAMP, and 0.085 mg/mL BSA. Then another 1.0 mL of 0.1 M ATP was added, and the reaction mixture was incubated for another 14 h to 90% completion. The reaction mixture was filtered using a Centriprep30 (Pharmacia) to remove protein before purification by preparative HPLC on a Bio-Rad Hi-Pore 318 C18 column (21.5 mm × 25 cm) using the same gradient system at 9.9 mL/min. The phosphopeptide peaks from several runs were collected and lyophilized (twice again from water to remove residual $NH_4^+OAc^-$ salt) to give 21 mg (29% yield).

(*D*) *Continuous CN Phosphatase Assay.* This assay was carried out in the buffer 50 mM Mops, 1 mM $MnCl_2$, 1 mM DTT, 0.5 mg/mL BSA, and 42 units/mL calmodulin (Sigma) in a total volume of 300 μL. To 237 μL of buffer incubated at 30 °C were added 20 μL of 2.93 mM MESG (final concentration, 0.2 mM), 30 μL of 0.1 mg/mL PRPase (final concentration, 10 μg/mL) and 3 μL of 2.5 μM CN (final concentration 25 nM), mixed and incubated at 30 °C for 4 min. The reaction was started by adding 10 μL of P-RII19 peptide from 0 to 115 μM final concentrations. The reaction was monitored every 0.5 s at 360 nm for 3 min, and the initial rate was obtained from the linear part of the curve. For the CyP·CsA inhibition assay, excess CsA (gift from Sandoz) was used at 5 μM (15 μL of 2 mM CsA/EtOH per final 6 mL of assay mixture). Varying concentrations of CyP in 25 mM Hepes, 250 mM KCl, and 0.02% NaN_3, pH 7.8 (10 μL), were added with PRPase, MESG, and CN, mixed and preincubated at 30 °C for 4 min. The reaction was started by adding 10 μL of P-RII19 peptide (typical concentration, 47.7 μM). The inhibition constants were determined by subtracting a background of 0.0001 s^{-1} from each rate and solving for K_i using eq 1 (and eq 2 if necessary), given in Results, with the curve-fitting program Kaleidagraph on a Macintosh IICX.

pNPPase Assay. This assay was performed in the same buffer as in the continuous CN phosphatase assay in a total volume of 300 μL. To 270 μL of buffer, as above, was added 3 μL of CN (final concentration, 25 nM), and the mixture was preincubated at 30 °C for 4 min. The reaction was started by adding 10 μL of *p*-nitrophenyl phosphate at final concentrations from 3.15 to 47.5 mM. For CyP·CsA stimulation, excess CsA (Sandoz) at 5 μM was included in the preincubated

mix at 30 °C. Cyclophilins hCyPA and R148E (10 μL) at varying concentrations were added with CN and preincubated at 30 °C for 4 min. The reaction was initiated by adding 10 μL of pNPP (final concentration, 5 mM).

RESULTS

Noncompetitive Inhibition of the Peptide Serine Phosphatase Activity of Calcineurin. In the initial report that the phosphatase activity of calcineurin was a target for both of the immunophilin·drug complexes, hCyPA·CsA and FKBP12·FK506, Liu et al. (Liu et al., 1991b) observed inhibition of CN-catalyzed dephosphorylation of a ^{32}P-labeled phosphoserine 19-residue peptide, but a 3–4-fold activation of *p*-nitrophenyl phosphate hydrolysis. This observation was confirmed when we examined both human cyclophilin A and B (Swanson et al., 1992). These contrasting results suggested noncompetitive inhibition by drug·immunophilin complexes which would be a key constraint on the mechanism. Non-active-site binding of the complexes would explain the selectivity of inhibition of calcineurin (PP2B) versus other phosphatases (Liu et al., 1992).

To determine the mode of inhibition by the CyP·CsA complex, competitive or noncompetitive, the discontinuous assay using [^{32}P]Ser-RII19 residue peptide from the regulatory subunit of protein kinase A, as previously described (Swanson et al., 1992; Zydowsky et al., 1992), was not well-suited to accurate kinetic evaluation. While qualitative correlations hold, there was too much scatter in the data to be confident of distinguishing one pattern of inhibition from another.

We then turned to a nonradioactive assay that has recently been developed by Webb for continuous assay of inorganic phosphate in which the P_i produced by the enzyme of interest is captured by the enzyme purine ribonucleoside phosphorylase (PRPase) in the presence of a purine riboside substrate (Webb, 1992). The specific ribonucleoside used is N^7-methyl-2-thioguanosine (MESG) (Broom & Robins, 1964; Broom & Milne, 1975), which on phosphorolysis yields ribose 1-phosphate and the free base, N^7-methyl-2-thioguanine. The quaternized N^7-riboside substrate yields a neutral base product with a blue shift in λ_{max} and a ΔA_{360} of 11 000 M^{-1} cm^{-1}, producing sensitivity to P_i at 1 μM concentrations (Webb, 1992). As shown in Scheme 1, the P-RII19 is acted on by the calcineurin A/B dimer, in the presence of calmodulin and Mn^{2+}; PRPase serves as the coupling enzyme. In addition to purification of the commercially available PRPase and synthesis of MESG, P-RII19 peptide substrate for calcineurin had to be produced on a several-milligram scale. As noted in Experimental Procedures, 21 mg of P-RII19 could be prepared by enzymatic phosphorylation with 3′,5′-cAMP-dependent protein kinase A followed by HPLC purification. We found that bovine brain CN reconstituted from lyophilized powder and stored at −20 °C or lower required incubation at 4 °C for at least 6 h to reach full activity. A similar activation time was required for reconstitution of A and B CN from different species (Ueki & Kincaid, 1993). With this assay, the K_m for P-RII19 was 110 μM, compared to 23 μM determined originally by the ^{32}P-release assay (Chan et al., 1986). A modest background rate (0.000 10 s^{-1}) of N^7-methyl-2-thioguanine production in the absence of either P-RII19 peptide or CN was subtracted from each initial velocity.

This continuous coupled assay proved satisfactory for determination of the inhibition pattern of CN by the CyP·CsA complex. Data for wild-type recombinant human T-cell cyclophilin A in the presence of saturating CsA are shown in Figure 2. We have previously determined the K_i value of CsA to be 17 nM for hCyPA by inhibition of peptidyl-prolyl isomerase activity under the conditions used in the assay (Zydowsky et al., 1992). For the CN inhibition studies, CsA was held constant at 5 μM and the cyclophilin concentration was varied. For each cyclophilin concentration, the concentration of P-RII19 was varied from 1.0 to 10 μM. The double-reciprocal plots from 0 to 1.5 μM hCyPA·CsA complex yield a family of lines that converge on the *x*-axis, reflecting pure noncompetitive inhibition (Figure 2). A replot of $1/v$ vs $1/[P\text{-RII19}]$ without background correction yields a K_i value of 336 nM for the CsA·hCyPA complex. CN inhibition under the same conditions used for analysis of the CyP mutants gave K_i = 270 ± 40 nM (Figure 3a) with a background of 0.000 13 s^{-1} subtracted (344 nM without). Neither CsA alone nor hCyPA alone produces inhibition. Appropriate controls were performed to show that the CN phosphatase activity was rate-limiting, not PRPase activity. Given the establishment of noncompetitive inhibition, all subsequent inhibition constants for hCyPA mutants and for wild-type hCyPB were determined by fitting the observed rates of P-RII19 peptide dephosphorylation vs CyP·CsA concentration to the rate equation for noncompetitive inhibition (Fersht, 1985):

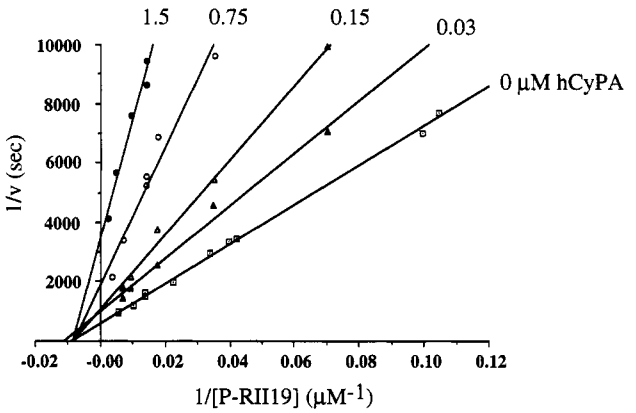


FIGURE 2: Lineweaver–Burke plots ($1/v$ vs $1/[S]$) at five inhibitor (hCyPA·CsA) concentrations used to determine the mode of inhibition of the phosphatase activity of calcineurin. The lines intersect on the *x*-axis, showing noncompetitive inhibition.

$$v = \{([CN]_0[\text{P-RII19}]_0 k_{cat})/(1 + [\text{CyP·CsA}]_0/K_i)\}/([\text{P-RII19}]_0 + K_m) \quad (1)$$

Since a high concentration of CsA (5 μM) was used to ensure saturation of the complex, the concentration of $[CyP·CsA]_0$ was assumed to be equal to $[CyP]_0$ unless the independently determined value of K_i for CsA was greater than 100 nM. In the case of W121F (K_i(CsA) = 490 nM), the initial concentration of the CyP·CsA complex was calculated for each concentration of CyP as follows:

$$[CyP] = [CyP]_0 - [CyP·CsA] \quad \text{and} \quad [CsA] = [CsA]_0 - [CyP·CsA]$$

substituting these expressions into the equation for K_i,

$$K_i = [CyP][CsA]/[CyP·CsA]$$

and rearranging to solve for [CyP·CsA] gives a quadratic equation,

$$0 = [CyP·CsA]^2 - \{K_i + [CsA]_0 + [CyP]_0\}[CyP·CsA] + [CyP]_0[CsA]_0$$

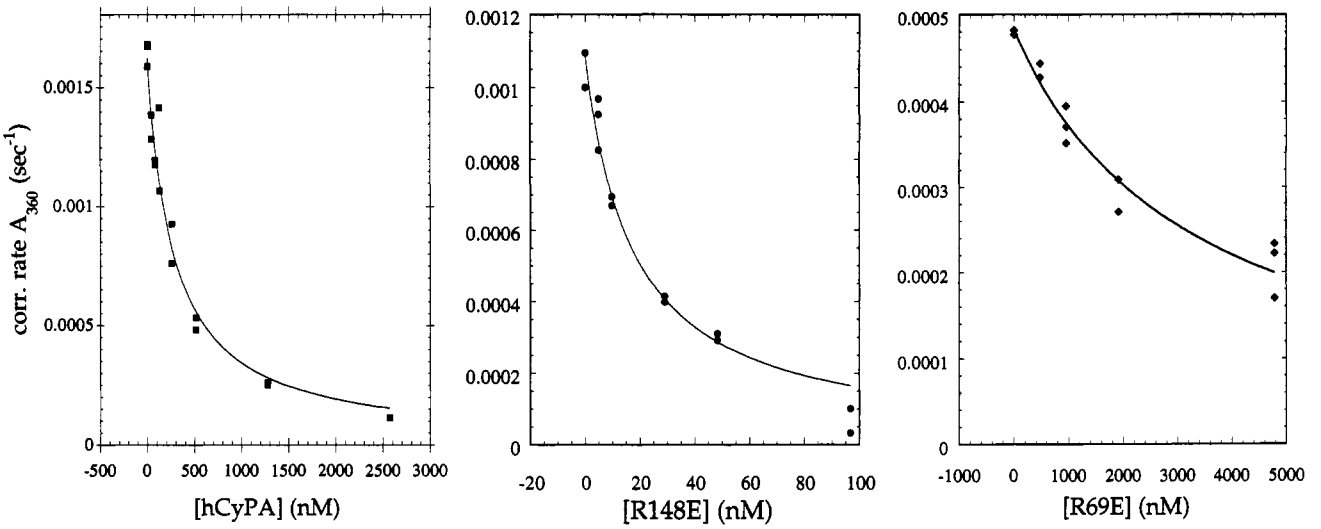


FIGURE 3: Inhibition of calcineurin phosphatase activity (v vs [hCyPA] at 5 μM CsA) for wild-type hCyPA (a, left) and two glutamate-containing mutants, R148E (b, center) and R69E (c, right). Inhibition constants (K_i, Table 1) were determined by fitting the data to the equation for noncompetitive inhibition: $v = \{([CN]_0[P\text{-}RII19]_0k_{cat})/(1 + [CyP\cdot CsA]_0/K_i)\}/([P\text{-}RII19]_0 + K_m)$.

for which the solution is.

$$[CyP\cdot CsA] = 1/2\{([CsA]_0 + K_i + [CyP]_0) - (([CsA]_0 + K_i + [CyP]_0)^2 - 4[CsA]_0[CyP]_0)^{1/2}\} \quad (2)$$

At the other extreme, K_i values for tight-binding inhibitors of CN cannot be accurately determined since the concentration of the inhibitor, [CyP·CsA], is depleted by binding to the enzyme (CN), which must be used at 25 nM for sensitivity. The values listed in Table 1 for R148E hCyPA and for wild-type hCyPB thus represent an upper limit on the affinity for CN.

The ER isoform of human cyclophilin, hCyPB, has been cloned (Price et al., 1991) and shown to be more potent than hCyPA in IC_{50} estimates (Swanson et al., 1992). We have now determined the K_i value for the CsA·hCyPB complex to be $\leq 21 \pm 3$ nM (Table 1), demonstrating that the hCyPB·CsA complex is an inhibitor of the phosphatase activity at 13-fold lower concentration than hCyPA.

Surface Loop Mutants of Human CyPA for Calcineurin Inhibition. Previously, we discovered that the W121A mutation impaired the ability of hCyPA to present bound CsA to CN (Zydowsky et al., 1992), independent of the 10-fold lower affinity for CsA (Liu et al., 1991a). We have now used the coupled assay to show that W121A hCyPA is at least 18-fold less potent and the mutant W121F is 7-fold weaker than wild type in phosphatase inhibition (Table 1). In this study, we broadened the search for residues on the hCyPA surface that may interact with CN. Figure 1 shows a ribbon diagram of hCyPA with four β-turn-containing loops—IPGF 57–60, RHNGT 69–73, NAGPNTNG 102–108, and SRNG 147–150—and one α-helix—EWLDGK 120–125 (highlighted in yellow)—which surround the drug-binding site. Within two of these loops and the helix are basic residues which were chosen for mutagenesis: R69, K125, and R148, shown with solvent-accessible surfaces in blue (also shown: W121). To assess whether the positive charge on any of these three hCyPA residues makes a significant contribution to the wild-type hCyP·CsA affinity for CN, these three residues were converted to residues containing either neutral side chains, R69L, K125Q, and R148L, or acidic side chains, R69E, K125E, and R148E, by oligonucleotide mutagenesis. Since we wanted to analyze only the effect of surface charge differences, the replacement residues were chosen so as not to disrupt protein structure; that is, no Pro, Gly, or β-branched residues were used. All of the mutated residues are within 20 Å of the Cα of MeLeu^6 of CsA (known to be an important immunosuppressive pharmacophore (Sigal et al., 1991)) bound to hCyPA. In the case of R148L, only the double mutant R69L/R148L was prepared, but it could be assessed by comparison with the single mutant R69L. Each of the six hCyPA mutants of Table 1 was overproduced in *Escherichia coli*, purified to homogeneity, and evaluated for function. Attempts to prepare mutations at G104, also in a surface loop, failed at the protein overproduction stage, possibly due to protein folding problems.

To assure that the mutant proteins were properly folded and functional, the peptidyl-prolyl isomerase (PPIase) activity of each mutant was measured and compared with that of wild-type enzyme. We expected that R69, K125, and R148 would not be crucial for the PPIase activity of hCyPA, and indeed, all the mutants have k_{cat}/K_m ratios in the range of 9.2–15 μM^{-1} s^{-1} (Table 1) compared with the range of 11–16 μM^{-1} s^{-1} observed for different preparations of wild type. The effect of CsA as an inhibitor of the PPIase activity was then measured, and as anticipated for mutations outside the active site and the drug-binding pocket, the high affinity for CsA is unimpaired, with K_i values ranging from 9 to 62 nM compared to 17 nM for wild-type hCyPA under the same conditions. Thus all six mutant proteins are fully active in PPIase and immunosuppressant drug binding properties.

The ability of each mutant hCyPA·CsA complex to inhibit the phosphatase activity of bovine brain CN was then measured with the continuous assay discussed above. Surprisingly, the data of Figure 3b show that R148E cyclophilin mutant has a 17-fold *improved* noncompetitive inhibition of phosphatase activity, $K_i \leq 16$ nM vs 270 nM for wild-type hCyPA, so glutamate at position 148 may be an important contact site for CN. The double mutant R69L/R148L is 4-fold less effective (K_i = 1000 nM) than wild type, and comparison with the single mutant R69L (K_i = 880 nM) suggests that the R148L mutation alone had little deleterious effect. On the other hand, the R69E cyclophilin mutant (Figure 3c) is some 13-fold impaired in its ability to inhibit the phosphatase activity (K_i = 3400 nM), while the neutral change R69L is only 3.3-fold less effective. The mutation at K125Q impairs the ability to inhibit CN by 6.3-fold, while the negatively charged K125E has no effect.

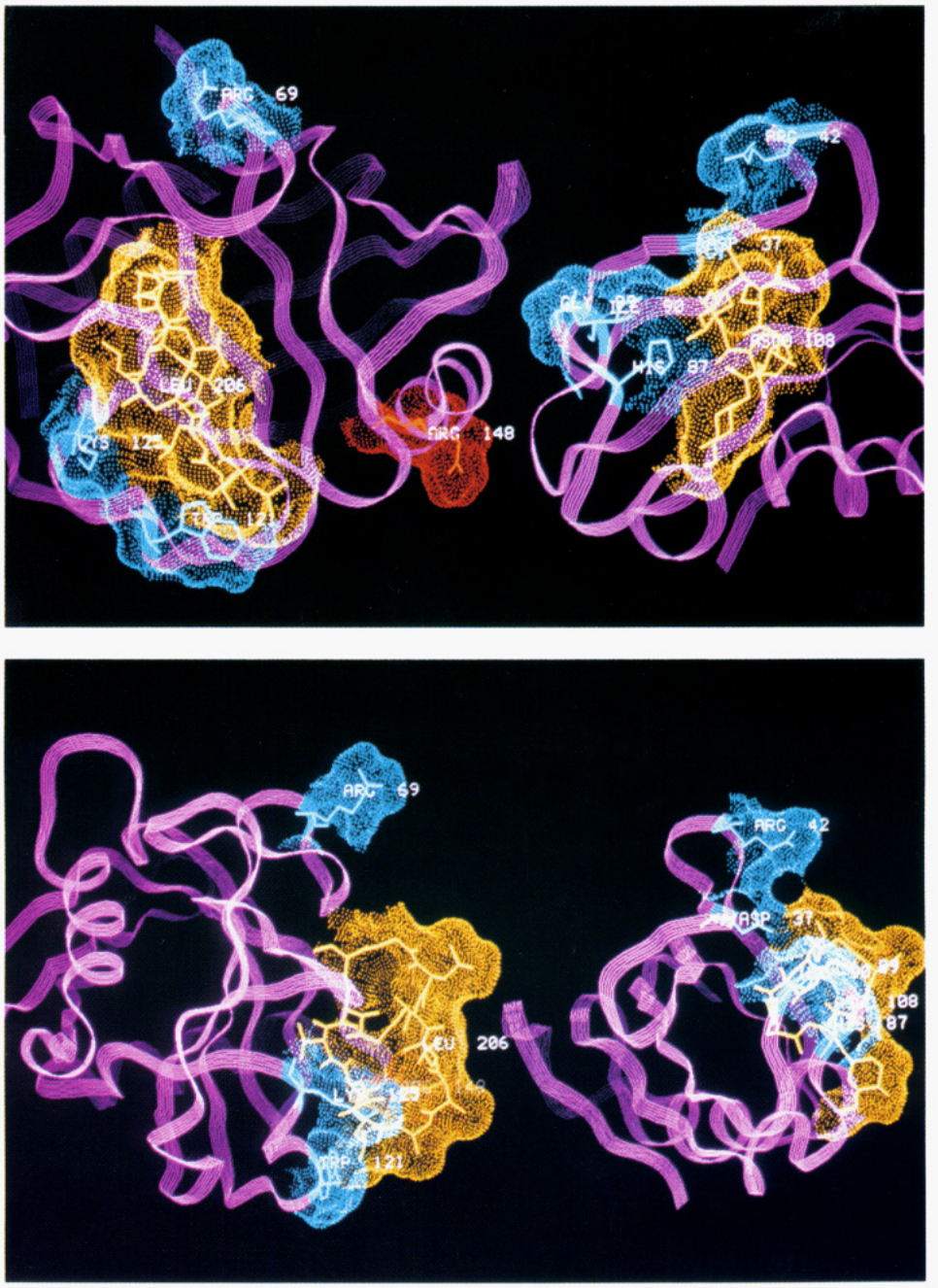


FIGURE 4: (a, top) (Left) NMR solution structure of the CsA (yellow)·hCyPA (purple) complex (Thériault et al., 1993) showing residues R69, W121, K125, and R148. Mutants that decrease CN affinity are surfaced in blue, and R148 is surfaced in red because the R148E mutant has increased CN affinity. (Right) NMR solution structure of the ascomycin (yellow, an FK506 analogue)·FKBP12 (purple) complex (Meadows et al., 1993) showing the residues D37, R42, H87, G89, and I90 in blue that have been mutated to decrease CN affinity (Aldape et al., 1992; Rosen et al., 1993; Yang et al., 1993). (b, bottom) (Left) hCyPA·CsA and (right) ascomycin·FKBP12 complexes rotated by 90° around the vertical axis.

Effect of CyP·CsA Complexes on p-Nitrophenylphosphatase Activity of CN. While CyP·CsA or FKBP·FK506 inhibit the phosphatase activity of calcineurin toward phosphocasein or the phosphoserine-RII peptide, they actually stimulate the pNPPase activity of CN. Liu et al. (Liu et al., 1991b) and Swanson et al. (Swanson et al., 1992) had reported 2–4-fold stimulation by wild type hCyPA·CsA complexes. We have repeated and confirmed these results for both hCyPA and hCyPB (data not shown), consistent with the noncompetitive inhibition results presented above. Curiously, the pNPPase activity of CN is not stimulated, but rather is inhibited, by the R148E hCyPA mutant (Figure 5), in contrast to its improved inhibition of the phosphatase activity.

DISCUSSION

In this study we have adapted a sensitive, continuous assay for inorganic phosphate detection (Webb, 1992) to measure the protein serine phosphatase activity of bovine calcineurin. We then demonstrated that the hCyPA·CsA complex is a noncompetitive inhibitor of CN and quantitated the K_i values of various isoforms and mutants of cyclophilins. The CyP·CsA complex, and most likely also the FKBP12·FK506 complex, acts as a noncompetitive inhibitor of the protein serine phosphatase activity of calcineurin, the target of drug action in T-cell signal transduction. Noncompetitive inhibition is consistent with the differential behavior of CyP·CsA complexes for inhibition of phosphoserine peptide

Table 1: Biochemical Characterization of the Human Cyclophilin A Proteins[a]

hCyPA	k_{cat}/K_m (μM^{-1} s^{-1})	K_i of CsA for CyP (nM)	K_i of CyP·CsA for CN (nM)	K_{rel} for CN
WT	16	17 ± 2	270 ± 40	1.0
W121A	1.4	290 ± 20	≥5000[b]	≥18
W121F	9.2	490 ± 20	2300 ± 200[c]	8.5
R69E	11	21 ± 4	3400 ± 400	13
R69L	11	34 ± 6	880 ± 50	3.3
K125Q	15	19 ± 2	1700 ± 100	6.3
K125E	11	18 ± 6	370 ± 70	1.4
R148L/R69L	9.2	40 ± 6	1000 ± 100	3.7
R148E	9.2	62 ± 8	≤16 ± 3[d]	0.06
hCyPB	5.3	60 ± 14[e]	≤21 ± 3[d]	0.08

[a] The values for K_i of CyP·CsA for CN were measured using the MESG-coupled phosphatase assay under saturating CsA conditions (5000 nM). [b] See also Zydowsky et al. (1992). [c] The concentration of [W121F CyP·CsA]$_0$ complex was calculated as given in eq 2 in Results. [d] These inhibitors fall into the tight-binding regime where $[I]_{free} \neq [I]_0$ because of depletion to [EI] and [EIS]. The K_i values given represent an upper estimate. [e] This K_i value was recalculated from the original IC_{50} data (Price et al., 1991) using the equation for competitive inhibition (Zydowsky et al., 1992).

hydrolysis versus stimulation of pNPPase activity. Most recently it has been shown that ^{125}I-labeled immunophilins hCyPA and FKBP12, in the presence of CsA or FK506, respectively, cross-link only with the B subunit of CN, but the CNA subunit is required for the interaction (Li & Handschumacher, 1993). This result is also consistent with noncompetitive inhibition and may indicate binding of CyP·CsA to CNB. This type of inhibition gives a rationale for selective inhibition of calcineurin by immunophilin·drug complexes without effect on other phosphatases (Liu et al., 1992) despite clear homology in their catalytic domains (Berndt et al., 1987).

It has been established previously that inhibition of the phosphatase activity of calcineurin is sensitive to the structure of cyclosporin A and congeners presented in the CyP·CsA complex (Sigal et al., 1991; Liu et al., 1992). NMR and X-ray analysis of the complex indicated a structure/function correlation with protruding surface of bound drug conformer (Fesik et al., 1991, 1992; Weber et al., 1991; Wüthrich et al., 1991; Spitzfaden et al., 1992; Pflügl et al., 1993; Thériault et al., 1993). A similar relationship holds for FK506 analogues presented by FKBP12 (Van Duyne et al., 1991; Meadows et al., 1993). A priori it seemed likely that the drug·immunophilin complexes may present a composite surface for recognition by calcineurin since neither drug nor immunophilin alone produces significant inhibition. As an initial test of this prediction and to identify which residues of CyP might contact CN, we used the X-ray structure of hCyPA (Ke et al., 1991) and a model of hCyPA·CsA (Fesik et al., 1992) to scan surface residues within 20 Å of the α-carbon of the MeLeu^6 residue of bound cyclosporin A, a proposed contact site for calcineurin. Three surface residues in loops surrounding the drug pocket with positively charged side chains, R69, K125, and R148, were noted as potential sites of electronic interaction with calcineurin. Each of these three residues was changed to a residue containing a neutral side chain, R69L, K125Q, and R148L, to assess the consequences of the loss of charge and to a negatively charged glutamate residue to assess the consequences of the opposite charge.

The mutants of human CyPA described in Table 1 and Figure 3 all retain PPIase activity and high affinity for CsA, but they split into two groups when assayed for interaction with CN. All three of the mutated positions affect noncompetitive inhibition of CN to some extent. R148E hCyPA is

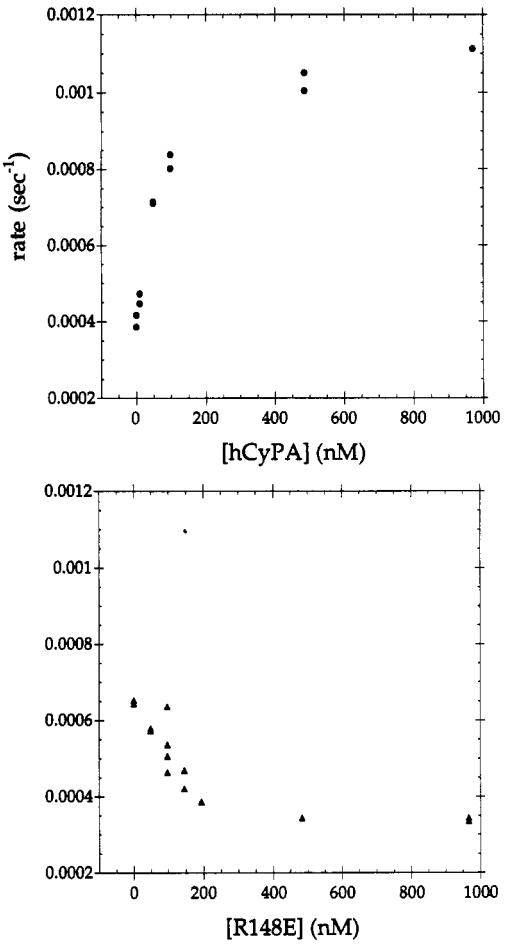


FIGURE 5: Stimulation by the hCyPA·CsA complex (top) and inhibition by the R148E hCyPA·CsA complex (bottom) of calcineurin pNPPase activity.

a dramatically improved inhibitor (Figure 3b), while the R69E mutation shows a large loss in the ability to inhibit CN (Figure 3c), and mutants at K125 and R69L show moderate losses. The neutral change R148L shows almost no effect on CN inhibition since the K_i value of the double mutant R148L/R69L is about the same as that of the single mutant R69L. The improved inhibition by the R148E hCyPA·CsA complex is intriguing in that such a mutant cyclophilin would presumably make CsA a more potent immunosuppressant. Primary sequence comparison of hCyPA and hCyPB (Price et al., 1991) shows two negatively charged residues, D179 and D182, flanking R181 of hCyPB at the position analogous to R148 of hCyPA:

hCyPA 146-GSRN

hCyPB 179-DSRD

These negatively charged residues may be the reason why hCyPB is a 13-fold better inhibitor of CN. Human CyPB is likely not the relevant immunosuppressant binding protein in T-cells since it resides in the ER (Price et al., 1991), but these results may be useful in the design of improved immunosuppressant drugs.

These results establish that the protein serine/threonine phosphatase 2B, CN, is recognizing a composite surface of immunosuppressive drug and immunophilin protein in the CsA·CyP complex. Figure 4 (left) shows a view of the X-ray surface of human CyPA in purple complexed with bound CsA in yellow in which the R69, K125, and W121 solvent-accessible surfaces are in blue and the R148 surface, for which glutamate

is preferred, is in red. The approximately triangular distribution of the three affected residues surrounding the drug-binding site may indicate that CsA becomes completely buried when bound to CN, consistent with the drug acting as a "molecular glue" (Schreiber, 1992).

The gain or loss of the CN-binding activity of these mutant hCyPA proteins can now be correlated with parallel studies of the immunosuppressive complex FK506·FKBP12. In the discovery of CN as the physiologically relevant target for immunosuppression (Clipstone & Crabtree, 1992; Fruman et al., 1992; O'Keefe et al., 1992), both cyclophilin and FKBP were found to inhibit CN when they were complexed with their specific immunosuppressant drugs (Liu et al., 1991b). Since the drugs and their binding proteins are dissimilar, yet they compete with each other for binding CN, some three-dimensional aspect of the surfaces of the two complexes must be similar. The 3D structures of both the CyP·CsA and FK506·FKBP complexes determined by X-ray crystallography (Van Duyne et al., 1991; Pflügl et al., 1993) and NMR (Meadows et al., 1993; Thériault et al., 1993) in conjunction with mutagenesis studies now permit us to make this comparison.

Aldape et al. (Aldape et al., 1992) made 34 mutations in human FKBP12 and found that mutations at three sites, D37, R42, and H87, yield impaired CN recognition. While D37V was 195-fold impaired in CN inhibition, it was approximately 600-fold weaker in binding FK506. The D37 side chain is known to make contact with the C10 hemiketal hydroxy of bound FK506 (Van Duyne et al., 1991). The R42K and H87V mutants had no loss of PPIase activity or FK506 affinity but were 107- and 4.2-fold less potent CN inhibitors, respectively, establishing that a composite surface of the FKBP12·FK506 complex involving charged residues recognizes CN. Perhaps R69 of hCyPA and R42 of FKBP12 play similar roles in their convergent inhibition of calcineurin, as shown in Figure 4.

Yang et al. have observed that FKBP12 is several hundredfold more potent than isoform FKBP13 (Jin et al., 1991) in presenting FK506 for inhibition of calcineurin (Yang et al., 1993). A double mutant, P89G/K90I, in a surface loop did not affect the K_i for FK506, but could make FKBP13 competent to inhibit CN with a 115-fold gain in potency (Rosen et al., 1993). The converse switch in FKBP12, G89P/I90K, gave a corresponding loss of CN inhibition (Yang et al., 1993). A C18-hydroxy analogue of FK506 that could not be presented by human FKBP12 could be presented by yeast FKBP12 for calcineurin inhibition (Rotonda et al., 1993), perhaps due to tighter interactions of CN with the presenting immunophilin. These structure/function studies on the cyclophilin and FKBP classes of immunophilins begin to yield a picture of similar molecular surfaces of drug and protein that interact with calcineurin. To date essentially nothing is known about residues in calcineurin that are contacted, except that the regulatory subunit CNB is required for inhibition by CyP·CsA (Haddy et al., 1992) and directly contacts hCyPA (Li & Handschumacher, 1993).

The FKBP12·ascomycin solution NMR structure (Meadows et al., 1993) is displayed at the right of Figure 4 with D37, R42, H87, G89, and I90 solvent-accessible surfaces in blue. Ascomycin, or FK520, is an active immunosuppressive analogue of FK506 with an ethyl vs an allyl group on the surface. Since both CyP and FKBP are highly basic proteins ($pI \approx 9$) (Harding et al., 1989; Liu et al., 1990) and each has basic residues found to be important for interaction with CN, we may now hypothesize that the common recognition interface includes electronic interactions and/or hydrogen bonds. Ubiquitous hydrophobic interactions are also likely to be important at the interface with calcineurin. The view of the two complexes in Figure 4a aligns the exposed surfaces of the bound drugs and the R69 hCyPA and R42 FKBP12 residues found to be important for CN recognition. Figure 4b shows the same orientations of each complex rotated by 90° around the vertical axes. The recognition sites appear to be oblong, including the long axis of each of the exposed drug surfaces, with positive charges on one edge and a preferred negative charge on the opposite side of hCyPA. In this orientation, E107 FKBP12 (not shown) is in the same region as R148E hCyPA, which may help explain the higher affinity of FKBP12·FK506 for CN. The similarity of the highlighted regions begins to identify the shape and charge distributions in a preliminary map of the CN recognition surface by the two immunophilin·drug complexes. Mutations in hCyPA at the upper left of CsA and the lower left of FK506 in FKBP12 will be needed to verify this model.

The differential effect of drug·immunophilin complexes on phosphoserine peptide/protein hydrolysis (inhibition) compared to the *p*-nitrophenylphosphatase effect (stimulation) was initially surprising, showing an actual increase in pNPPase V_{max} by CyP·CsA (Liu et al., 1991b; Swanson et al., 1992). Given noncompetitive inhibition of phosphoserine hydrolysis, one may assume that the access of large substrates (phosphorylated proteins) to the phosphatase active site may be blocked. But small substrates such as pNPP may still be able to diffuse in and see an altered active site geometry of CN that allows for more efficient phosphoryl transfer to water (Figure 5). The pNPPase stimulation assay may be the most convenient screen for evaluating CsA and FK506 analogues as potential immunosuppressants. On the other hand, the R148E hCyPA complex, a more potent inhibitor of peptide phosphatase activity, inhibits the pNPPase activity, so the stimulation of pNPPase activity by tight-binding drug·immunophilin complexes may not be fully predictive of phosphoprotein recognition by CN. The MESG coupled phosphopeptide assay described here should serve as a sensitive and predictive assay for immunosuppressants that act on CN.

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