Inhibition of T Cell Signaling by Immunophilin–Ligand Complexes Correlates with Loss of Calcineurin Phosphatase Activity†

J. Liu,‡ M. W. Albers,‡ T. J. Wandless,‡ S. Luan,‡ D. G. Alberg,‡ P. J. Belshaw,‡ P. Cohen,§ C. MacKintosh,§ C. B. Klee,∥ and S. L. Schreiber*,‡

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, and MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Tayside, Scotland DD1 4HN

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ABSTRACT: Calcineurin, a Ca^{2+}, calmodulin-dependent protein phosphatase, was recently found to bind with high affinity to two different immunosuppressant binding proteins (immunophilins) with absolute dependence on the presence of the immunosuppressants FK506 or cyclosporin A (CsA) [Liu et al. (1991) *Cell 66*, 807–815]. The binding affinities of the immunophilin–drug complexes toward calcineurin and the stoichiometry of the resultant multimeric complexes have now been determined, and structural elements of FK506, CsA, and calcineurin that are critical for mediating their interactions have been identified. Analogues of FK506 (FK520, FK523, 15-*O*-demethyl-FK520) and CsA ($MeBm_2t^1$-CsA and $MeAla^6$-CsA) whose affinities for their cognate immunophilins do not correlate with their immunosuppressive activities have been prepared and evaluated in biochemical and cellular assays. We demonstrate a strong correlation between the ability of these analogues, when bound to their immunophilins, to inhibit the phosphatase activity of calcineurin and their ability to inhibit transcriptional activation by NF-AT, a T cell specific transcription factor that regulates IL-2 gene synthesis in human T cells. In addition, FKBP–FK506 and CyP–CsA do not inhibit members of the PP1, PP2A, and PP2C classes of serine/threonine phosphatases. These data suggest that calcineurin is the relevant cellular target of these immunosuppressive agents and is involved in Ca^{2+}-dependent signal transduction pathways in, among others, T cells and mast cells.

In addition to their applications in medicine (Showstack et al., 1989; Starzl et al., 1989), the immunosuppressants cyclosporin A (CsA) and FK506 have proven to be useful tools in the study of a family of signal transduction pathways (Schreiber, 1991; Hultsch et al., 1991). Both drugs inhibit the transcription of cytokine genes, such as the gene encoding interleukin-2 (IL-2), and the exocytosis of secretory granules by interfering with an intracellular step in signaling pathways emanating from the T cell receptor in T cells (Lin et al., 1991) and the IgE receptor in mast cells (Hultsch et al., 1991), respectively. The isolation and characterization of a cytosolic receptor for CsA, cyclophilin (CyP), offered the first insight to the mechanism of action of these drugs (Handschumacher et al., 1984). The findings that cyclophilin possesses rotamase (peptidyl-prolyl cis–trans isomerase) activity and that CsA is a potent inhibitor of that activity led to the suggestion that proline isomerization is involved in signal transduction and that CsA exerts its effects by inhibiting cyclophilin's rotamase activity (Fischer et al., 1989; Takahashi et al., 1989; Emmel et al., 1989). The connection between rotamase inhibition and immunosuppression appeared to be reinforced by the discovery that the cytosolic receptor for FK506 and rapamycin, FKBP, is also a rotamase that is potently inhibited by FK506 and rapamycin (Harding et al., 1989; Siekierka et al., 1989).

Subsequent studies, however, yielded evidence inconsistent with a causative relation between rotamase inhibition and immunosuppression. Genetic studies in yeast showed that neither CyP (Tropschug et al., 1989) nor FKBP (Koltin et al., 1991; Heitman et al., 1991) is essential for yeast survival. Thus, inhibition of the rotamase activities of FKBP and CyP could not explain the toxic effects of these drugs in yeast. In mammalian cells, two FKBP rotamase inhibitors, rapamycin and 506BD, were found to have cellular effects distinct from FK506 (Bierer et al., 1990a,b; Dumont et al., 1990a,b). Rapamycin has no effect on the CsA- and FK506-sensitive, Ca^{2+}-dependent signaling pathways in T cells and mast cells. Instead, rapamycin inhibits a later signaling pathway in the T cell activation cascade—a Ca^{2+}-independent pathway that originates at the IL-2 receptor. 506BD, on the other hand, does not inhibit Ca^{2+}-dependent or -independent signaling pathways but is a potent inhibitor of the action of both FK506 and rapamycin (Bierer et al., 1990b). Also, several CsA analogues were reported whose CyP binding affinities and immunosuppressive activities do not correlate (Sigal et al., 1991a,b). Among these analogues are $MeBm_2t^1$-CsA (Aebi et al., 1990), which is a weak inhibitor of the rotamase activity of CyP but retains significant immunosuppressive activities, and $MeAla^6$-CsA, which is a potent inhibitor of the rotamase activity of CyP but is a weak immunosuppressant (Sigal et al., 1991).

Two models were proposed to rationalize these observations. The first suggests that minor immunophilins, yet to be identified, mediate the actions of FK506 and CsA (Sigal et al., 1991). A second model assumes an obligate role for immunophilin–immunosuppressant complexes in interfering with the actions of a key component of signal transduction (Tropschug et al., 1989; Bierer et al., 1990a,b; Schreiber, 1991; Koltin et al., 1991; Heitman et al., 1991). The similar biological properties of FK506 and CsA (their main distinctions are their potencies and sensitivities to 506BD and rapamycin) suggested that the FKBP–FK506 and CyP–CsA complexes may interact

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‡ Department of Chemistry, Harvard University.
§ Department of Biochemistry, University of Dundee.
∥ Laboratory of Biochemistry, National Cancer Institute.

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with a common cellular target, which might be a key component of the drug-sensitive, Ca^{2+}-dependent signaling pathways. Using fusion proteins between glutathione *S*-transferase and the immunophilins and tissue extracts, a common target protein, the Ca^{2+}, calmodulin-dependent protein phosphatase calcineurin (Klee et al., 1987), was found to bind specifically to FKBP–FK506 and CyP–CsA complexes, resulting in modulation of calcineurin's protein phosphatase activity (Liu et al., 1991). These studies suggested that calcineurin is the physiologic, secondary target of both FK506 and CsA, which act through their primary immunophilin targets. Such a hypothesis requires linking the biochemical data with studies of the cellular properties of FK506 and CsA. We now report investigations of several minor structural variants of FK506 and CsA that bind to their cognate immunophilins (and inhibit their rotamase activity) in a manner that does not correlate with their immunosuppressive properties. An excellent correlation has been found between the ability of immunophilin–ligand complexes to inhibit the phosphatase activity of calcineurin and their ability to inhibit gene transcription in human T cells mediated by NF-AT, a T cell specific transcription factor that regulates IL-2 gene synthesis. In addition to these correlative studies, a more detailed understanding of the structural basis for, and specificity of, immunophilin–ligand complexes to calcineurin has been achieved.

Materials and Methods

Materials

FK520, FK523, and 15-demethyl-FK520 (Figure 2) were generous gifts from H. Fliri (Sandoz, Basel, Switzerland). 506BD was synthesized as previously described (Somers et al., 1991). $MeBm_2t^1$-CsA (Figure 2) was synthesized essentially as described in Aebi et al. (1990), with the substitution of Castro's BOP reagent (Castro et al., 1975) in the place of propyl phosphonic anhydride as the coupling reagent in the macrocyclization step. $MeAla^6$-CsA (Figure 2) was synthsized by an analogous protocol (Gooley et al., 1991). The unusual amino acid MeBmt, which is a component of $MeAla^6$-CsA and CsA, was synthesized according to Lubell et at. (1990). Recombinant human FKBP12 was provided by R. F. Standaert and M. K. Rosen (Standaert et al., 1990). Recombinant human cyclophilin was prepared as previously described (Liu et al., 1990). Jurkat T lymphocyte line stably transfected with a plasmid containing the *Escherichia coli lacZ* gene attached to a minimal promoter of the IL-2 gene (−72 to +47) linked to a trimer of the NF-AT binding site (−280 to −257 of the IL-2 gene) was kindly provided by G. R. Crabtree (Fiering et al., 1990). Calmodulin was purchased from Sigma. Bovine brain calcineurin and a ^{32}P-labeled substrate [DLDVPIPGRFDRRV(^{32}P)SVAAE] were prepared as described (Hubbard & Klee, 1991). The catalytic subunits of cAMP-dependent protein kinase (PKA) from bovine cardiac muscle (Reimann & Beham, 1983), protein phosphatase 1 (PP1), PP2A, and PP2C (from rabbit skeletal muscle) were purified to homogeneity as previously described (Cohen et al., 1988; MacGowan & Cohen, 1988). ^{32}P-Labeled casein used in the PP2C assay was prepared by incubation of 2 mg of casein in 1 mL of buffer A [50 mM Tris-HCl, pH 7.0 (25 °C), 0.1 mM EGTA, 10% glycerol, 0.1% β-mercaptoethanol] with 10 mM $Mg(OAc)_2$, 0.1 mM [γ-^{32}P]ATP (with specific radioactivity of 10^6 cpm/mol), and 2 units of PKA for 16 h at 30 °C. A 110-μL solution containing 100 mM EDTA, 500 mM NaF, and 10 mM sodium pyrophosphate, pH 7.0, was added to stop the reaction. Following a 10-min incubation at 30 °C, the mixture was centrifuged at 12000*g* for 2 min. The supernatant was loaded onto a 15 cm × 1 cm Sephadex G50 column equilibrated with buffer A, and subsequent fractions of ^{32}P-labeled casein were counted and used in phosphatase assays for PP2C.

Methods

Phosphatase Assay for Calcineurin. The assay buffer (Manalan & Klee, 1983) consisted of 40 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mg/mL bovine serum albumin, and 0.5 mM dithiothreitol. A typical assay mixture contained 30–300 nM calcineurin, 1 molar equiv of calmodulin (when present), 10–50 molar equiv of FK506, CsA, or their analogues, 0.3–10 molar equiv of immunophilin (relative to calcineurin present), and 1 μM phosphopeptide substrate [DLDVPIPGRFDRRV(^{32}P)SVAAE]. For each assay, the immunophilin–ligand complex was preincubated with calcineurin with or without calmodulin for 20–30 min at 4 °C before the substrate was added to initiate the assay. The assay was carried out at 30 °C for 10–15 min.

Phosphatase Assays for PP1, PP2A, and PP2C. For this set of assays, stock solutions (300 μM) of FK506, rapamycin, and CsA were prepared in methanol. Three micromolar solutions of each immunophilin were prepared in the assay buffer. The immunophilin–ligand complexes were formed by adding 1% (v/v) of 300 μM stock solutions of FK506, rapamycin, or CsA to 3 μM of the appropriate immunophilin(s) in the assay buffer and incubating the mixture for at least 30 min at 4 °C.

PP1 and PP2A were assayed (30 μL) using [^{32}P]-phosphorylase *a* in the following assay buffer: 50 mM Tris-HCl, pH 7.5 (20 °C), 0.1 mM EGTA, 0.03% (v/v) Brij-35, 0.1% (v/v) β-mercaptoethanol and 1 mg/mL bovine serum albumin. The enzyme and the inhibitor were mixed together and incubated for 2 min at 4 °C; the reaction was started by the addition of [^{32}P]phosphorylase *a*, and the mixture was incubated for 10 min at 30 °C. Two hundred microliters of 20% trichloroacetic acid was then added to stop the reaction. The samples were vortexed and centrifuged at 12000*g* for 2 min, followed by counting of 200 μL of supernatant. PP2C was assayed as described above except that 6 μM ^{32}P-labeled casein was used as the substrate and the assay buffer contained 10 mM $MgCl_2$.

Kinetic Analysis To Derive the Intrinsic K_i's of Immunophilin–Immunosuppressant Complexes for Calcineurin Phosphatase Activity. Given the 1:1:1 binding stoichiometry in the immunophilin–ligand–calcineurin ternary complex and that the interactions between the free immunophilns (R) or immunosuppressants (L) and calcineurin are negligible under the assay conditions (see Results section), the association of the FKBP–FK506 or CyP–CsA complexes to calcineurin–calmodulin is represented by eq 1. (R represents immunophilin receptor; L, immunosuppressant ligand; and C, calcineurin-related phosphatase, including calcineurin alone, calmodulin-bound calcineurin, or the 43-kDa calcineurin fragment; K_1 is the dissociation constant of the ligand to immunophilin; K_2 is the dissociation constant of the immunophilin–ligand complex to calcineurin.)

$$\mathrm{R} + \mathrm{L} + \mathrm{C} \overset{K_1}{\rightleftharpoons} \mathrm{R\text{–}L} + \mathrm{C} \overset{K_2}{\rightleftharpoons} \mathrm{R\text{–}L\text{–}C} \qquad (1)$$

Thus

$$K_1K_2 = \frac{[\mathrm{R}][\mathrm{L}]}{[\mathrm{R\text{–}L}]}\frac{[\mathrm{R\text{–}L}][\mathrm{C}]}{[\mathrm{R\text{–}L\text{–}C}]} = \frac{[\mathrm{R}][\mathrm{L}][\mathrm{C}]}{[\mathrm{R\text{–}L\text{–}C}]} \qquad (2)$$

We have

$$[\mathrm{C}]_o = [\mathrm{C}] + [\mathrm{R\text{–}L\text{–}C}] \qquad (3)$$

$$[R]_o = [R] + [R\text{–}L] + [R\text{–}L\text{–}C] \quad (4)$$

$$[L]_o = [L] + [R\text{–}L] + [R\text{–}L\text{–}C] \quad (5)$$

$$I = \frac{[R\text{–}L\text{–}C]}{[C]_o} \quad (6)$$

where I is the fractional inhibition of total calcineurin activity present in the assay mixture and $[X]_o$ is the total concentration of species X (X = C, R, or L).

From eqs 2–6, K_2 can be solved in terms of $[L]_o$, $[R]_o$, $[C]_o$, K_1, and I

$$K_2 = \frac{[R][L][C]}{K_1[R\text{–}L\text{–}C]} = \frac{1 - I}{K_1 I}[R][L] \quad (7)$$

where

$$[L] = [[L]_o - [R]_o - K_1 + \{([R]_o - [L]_o + K_1)^2 + 4K_1[L]_o - 4[C]_o K_1 I\}^{1/2}]/2$$

$$[R] = [[R]_o - [L]_o - K_1 + \{([L]_o - [R]_o + K_1)^2 + 4K_1[R]_o - 4[C]_o K_1 I\}^{1/2}]/2$$

For the data analysis, K_1 is taken as K_i of the ligand for the rotamase activity of its immunophilin receptor, and K_2 is taken as the K_i of the immunophilin–ligand complex for the phosphatase activity of calcineurin.

Determination of K_i for FK506 and CsA Analogues. The rotamase assay and calculations of inhibition constants of various analogues of FK506 and CsA were carried out as previously described (Bierer et al., 1990a).

Cellular Assay for Signaling Inhibition by Immunophilin Ligands. The FGL cell line (Fiering et al., 1990), a T lymphoblastoid cell line stably transfected with an NF-AT-βGal construct, was used in these studies. Cells were activated for 5 h with 20 ng/mL phorbol 12-myristate 13-acetate and 1.5 μg/mL ionomycin in the presence of drug analogues at various concentrations in a 96-well culture dish (10^5 cells/well). After activation, cells were harvested by centrifugation at 12000 rpm in a microcentrifuge and assayed for β-galactosidase activity. The enzyme assay was as described (Bierer et al., 1990a) except for a few modifications. Briefly, 360 μL of the enzyme reaction buffer containing 100 mM Na_2HPO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 0.1% Triton X-100, and 0.5 mM 4-methylumbelliferyl β-D-galactoside was added to the cells and incubated for 1 h at 25 °C. The reaction was stopped by adding 150 μL of stop solution (300 mM glycine and 15 mM EDTA, pH 11.3), and the β-galactosidase activity was determined by fluorescence measurements (355-nm excitation/460-nm emission).

Results

Inhibition of Calcineurin Phosphatase Activity by FKBP–FK506 and CyP–CsA Complexes: Binding Stoichiometry and Inhibition Constants. Previous work has established that both FKBP–FK506 and CyP–CsA bind competitively to a common target protein, calcineurin, both in the presence and in the absence of calmodulin; calmodulin appeared to enhance the binding affinity of calcineurin to both FKBP–FK506 and CyP–CsA complexes. As a first step to unravel the molecular interactions between calcineurin and the two immunophilin–ligand complexes, we determined the binding stoichiometry and the binding affinities of FKBP–FK506 and CyP–CsA to calcineurin. To determine the binding stoichiometry, three concentrations of calcineurin were tested. The titration curve using 300 nM calcineurin is shown in Figure 1. Upon addition

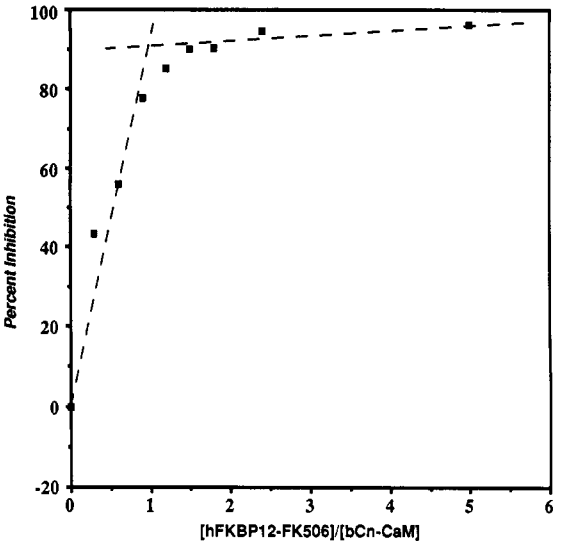


FIGURE 1: Titration of calmodulin-bound bovine brain calcineurin with hFKBP12–FK506. The titration was carried out with 300 nM calmodulin–calcineurin, 30 μM FK506, and various concentrations of the FKBP.

Table I: Inhibition Constants (K_i) of Human FKBP–FK506 and Human CyP–CsA for Three Forms of Bovine Calcineurin[a]

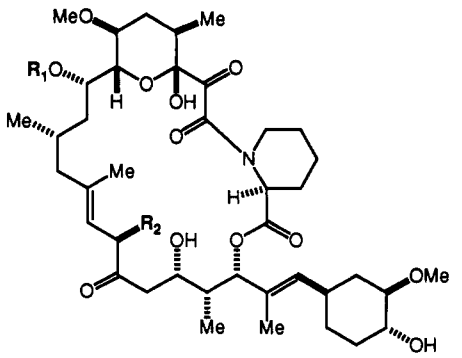
immunophilin–ligand complex	bCn	bCn–CaM	CnA′
hFKBP12–FK506	40	32	40
hCyPA–CsA	191	33	32

[a] Abbreviations: bCn, bovine brain calcineurin; CaM, calmodulin; CnA′, 43-kDa bovine CnA fragment complexed with the B subunit of calcineurin.

of 1 molar equiv of the FKBP–FK506 complex, over 80% of calcineurin's protein phosphatase activity is inhibited, indicating a 1:1 binding stoichiometry between FKBP–FK506 and calcineurin–calmodulin (calcineurin is a heterodimeric protein comprised of a 1:1 ratio of A and B subunits). A similar 1:1 molar ratio is also observed with FKBP–FK506 in the absence of calmodulin. Similar results were obtained with the CyP–CsA complex (data not shown). These results suggest that, in the pentameric complex of immunophilin–ligand–calcineurin A–calcineurin B–calmodulin, the ratio between each component is 1:1:1:1:1 [assuming that immunophilin–ligand complexation does not alter the 1:1 stoichiometry of calcineurin and calmodulin (Klee et al., 1988)].

Inhibition constants for the two human immunophilin–ligand complexes against bovine calcineurin were measured in the presence or absence of calmodulin. As shown in Table I, the K_i of FKBP12–FK506 for calcineurin–calmodulin is 32 nM. The K_i value increases to 40 nM for calcineurin alone. For the CyP A–CsA complex, the K_i value is 33 nM for calcineurin–calmodulin. For calcineurin alone, the K_i is much higher (191 nM). The calmodulin-enhanced affinity of calcineurin to the FKBP–FK506 complex is in agreement with previous observations (Liu et al., 1991).

Inhibition of Calcineurin by FK506 Analogues. FK520, FK523, and 15-*O*-demethyl-FK520 differ as a result of minor structural changes in the "effector domain" of FK506. FK520 and FK523 differ from FK506 in that the allyl group in FK506 at position 21 is an ethyl group in FK520 and a methyl group in FK523 (Figure 2). 15-*O*-Demethyl-FK520 has two structural alterations: an allyl to ethyl change as in FK520 and replacement of the 15-methoxy group in FK506 with a hydroxyl group (Figure 2). For each analogue, we determined the binding constant for FKBP, the IC_{50} of NF-AT-driven

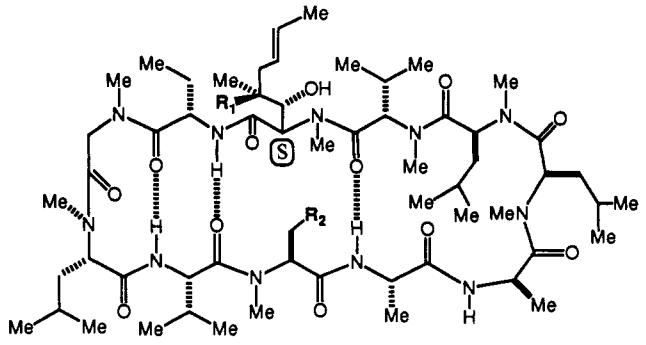


1. FK506: R_1 = Me, R_2 = allyl;

2. FK520: R_1 = Me, R_2 = Et;

3. FK523: R_1 = Me, R_2 = Me;

4. 15-O-DeMe-FK520, R_1 = H, R_2 = Et.



5. CsA: R_1 = H, R_2 = i-Pr;

6. MeBm$_2$t^1-CsA: R_1 = Me, R_2 = i-Pr;

7. MeAla^6-CsA: R_1 = H, R_2 = H.

FIGURE 2: Structures of FK506, FK520, FK523, and 15-*O*-demethyl-FK520 (**1–4**) and structures of CsA, MeBm$_2$t^1-CsA, and MeAla^6-CsA (**5–7**).

Table II: Immunophilin Binding, Calcineurin Inhibition, and Signal Transduction Inhibition by CsA and FK506 Analogues

compd no.	compound[a]	K_i (nM) for immunophilins	K_i (nM) for calcineurin	IC_{50} (nM) for NF-AT activity[b]
1	FK506	1.0	34	0.5
2	FK520	5.0	89	0.8
3	FK523	0.80	230	1.2
4	15-*O*-DeMe-FK250	15	1.6×10^3	$>8.0 \times 10^3$
5	CsA	6.0	40	3.5
6	MeBm$_2$t^1-CsA	500	13	29
7	MeAla^6-CsA	9.0	$>1.0 \times 10^3$	3.2×10^3

[a] For structures of each analogue, see Figure 2. [b] As measured by NF-AT-driven β-galactosidase activity.

expression of β-galactosidase activity in Jurkat cells, and the inhibition constant (K_i) for calcineurin phosphatase activity. As shown in Table II (compounds **1–4**), whereas FK520 and FK523 bind to FKBP with affinities similar to that of FK506, they displayed decreased ability to inhibit T cell signaling relative to FK506. Although 15-*O*-demethyl-FK520 has a 15-fold lower affinity for FKBP, its relative cellular activity is considerably lower than can be accounted for by its affinity for FKBP. When the inhibition of calcineurin was examined for each analogue, an excellent correlation between affinity for calcineurin and immunosuppressive activity was observed (Table II).

Table III: Effects of Immunophilin–Ligand Complexes on Four Classes of Phosphatases

immunophilin–ligand complexes (1 μM)	% of control activity			
	PP1	PP2A	PP2C	PP2B (Cn)
FKBP12–FK506	103	117	81	5.7
CyP A–CsA	122	125	81	6.1

Inhibition of Calcineurin by CsA Analogues Whose Binding Affinities to CyP Do Not Correlate with Their Immunosuppressive Activities. MeBm$_2$t^1-CsA and MeAla^6-CsA are minor structural variants of CsA, yet exhibit strikingly aberrant and distinct behavior when their abilities to bind CyP and to elicit an immunosuppressive response were compared (Sigal et al., 1991). In the current studies, we found that the addition of a methyl group to the MeBmt side chain results in significant reduction of its binding affinity to cyclophilin but with a markedly smaller effect on its T cell signaling inhibitory properties (Table II and Figure 2). In MeAla^6-CsA, deletion of an isopropyl group (Leu to Ala change) has a relatively small effect on its binding to CyP but causes a dramatic decrease in its cellular activity. Thus, these two analogues provide an important test for the relevance of inhibition of calcineurin to the immunosuppressive activity of CsA. Indeed, the K_i for the MeBm$_2$t^1-CsA–CyP A complex for calcineurin–calmodulin is 13 nM [lower than K_i's associated with FKBP–FK506 and CyP–CsA (Table I); we suggest this high affinity for calcineurin can compensate for the lower affinity of MeBm$_2$t^1-CsA to CyP], and the K_i for the MeAla^6-CsA–CyP A complex is greater than 1 μM (Table II), yielding an excellent correlation between their calcineurin inhibition activities and cellular activities.

Specificity of FK506-FKBP and CsA–CyP for the Calcineurin Class of Protein Phosphatase. It was found that the phosphatase activity of calcineurin toward a phosphopeptide substrate is inhibited only by FKBP–FK506 and CyP–CsA complexes; free ligands or unbound immunophilins did not inhibit calcineurin phosphatase activity at concentrations used in the assays. However, it remained unknown whether only the calcineurin (PP2B) class of serine/threonine phosphatase is sensitive to FKBP–FK506 and CyP–CsA, since there is significant homology between the "core" regions of the catalytic subunit of calcineurin (calcineurin A) and those of PP1 and PP2A (Guerini & Klee, 1989; Kincaid et al., 1988; Ito et al., 1989). Therefore, the various immunophilins, ligands, and their complexes were tested in assays of three other classes of serine/threonine phosphatases, PP1, PP2A, and PP2C. It was found that the unligated immunophilins or free ligands have no effect on these phosphatase activities (data not shown). Furthermore, neither FKBP–FK506 nor CyP–CsA has a significant effect on the other three classes of phosphatases (Table III).

Inhibition of the 43-kDa Calcineurin A Fragment Complexed with Calcineurin B. Limited proteolytic digestion of calcineurin with clostripain or chymotrypsin yields a 43-kDa calcineurin A fragment that is missing the calmodulin binding and the autoinhibitory domains (Hubbard & Klee, 1989). Both immunophilin–ligand complexes were found to inhibit the phosphatase activity of the 43-kDa fragment with K_i values of 40 and 32 nM for FKBP–FK506 and CyP–CsA, respectively. This eliminates the possibility that the calmodulin binding domain and C-terminal autoinhibitory domain are primary recognition elements for the binding of FKBP–FK506

and CyP–CsA complexes. In the absence of calcineurin A and calmodulin, neither immunophilin–ligand complex was found to bind to the isolated calcineurin B subunit (data not shown), indicating that the A subunit of calcineurin plays an important role in the binding of the two immunophilin–ligand complexes.

Discussion

Previously, the protein phosphatase calcineurin was identified as a common target for both FK506 and CsA by demonstrating a specific, high-affinity association of calcineurin with the two corresponding immunophilin–ligand complexes (Liu et al., 1991). In this study, chemical and biological aspects of the interactions between the two immunophilin–ligand complexes and calcineurin isolated from bovine brain have been characterized. We find that the FKBP-FK506 and CyP–CsA complexes bind to calcineurin in a 1:1 ratio. In the presence of calmodulin, both FKBP–FK506 and CyP–CsA have similar affinities for calcineurin (inhibitory constants K_i of 32 and 33 nM, respectively). In the absence of calmodulin the K_i's of the two complexes increase to 40 nM for FKBP–FK506 and 191 nM for CyP–CsA, in agreement with previous observations using affinity matrix adsorption (Liu et al., 1991). The K_i values for both FKBP–FK506 and CyP–CsA are higher than the respective IC_{50} values of FK506 and CsA in immunosuppression assays (Kino et al., 1987) and the signal transduction assay described herein. The K_i values determined in this study reflect the intrinsic binding constants between the immunophilin–ligand complexes and calcineurin, independent of the concentration of the immunophilins or calcineurin. In a cellular context, however, the IC_{50} is determined not only by these intrinsic affinities but also by given concentrations of the immunophilins and calcineurin. Indeed, the effects of FK506 and CsA on the cellular phosphatase activity of calcineurin in human T lymphocytes have recently been reported to correlate quantitatively with their respective antiproliferative properties (Fruman et al., 1992). Finally, since calcineurin from human lymphocytes is not readily available in preparative quantities, we used bovine brain calcineurin, which might be less sensitive to the human immunophilin–ligand complexes used in these studies.

In this work, the interaction between FKBP–FK506 or CyP–CsA and calcineurin is studied by following the inhibition of calcineurin's serine/threonine phosphatase activity. It has been found that the same complexes also stimulate, rather than inhibit, the tyrosine phosphatase-like activity of calcineurin when *p*-nitrophenyl phosphate is used as the substrate (Liu et al., 1991). In fact, there have been several reports of calcineurin's tyrosine phosphatase activity, although the requirement for high concentrations of transition metal ions suggests this activity may have no physiological relevance. Although unlikely, it remains to be determined if calcineurin gains tyrosine phosphatase activity without high concentrations of transition metal ions when it is bound in FKBP–FK506 or CyP–CsA.

The significant homology between the catalytic subunits of calcineurin and the protein phosphatases PP1 and PP2A (Ito et al., 1988) raised the possibility that FKBP–FK506 and CyP–CsA may have inhibitory effects on other protein phosphatases as well. No inhibition of the catalytic subunits of the other three known classes of serine/threonine phosphatases was seen with either free immunosuppressants or immunophilins or their complexes (Table III). The results obtained with the 43-kDa truncated calcineurin eliminate the calmodulin binding domain and autoinhibitory domain is FKBP–FK506 or CyP–CsA recognition sites. In addition, affinity experiments showed that calcineurin B by itself does not bind to FKBP–FK506. A detailed understanding of the interactions between immunophilin–drug complexes and calcineurin–calmodulin awaits high-resolution crystallographic analyses.

An earlier report had noted the lack of a correlation between cyclophilin binding and immunosuppression with several CsA analogues, including the two synthesized in the current studies. This was interpreted as evidence that cyclophilin A may not be the relevant receptor for CsA. The hypothesis that CsA (and FK506 and rapamycin) causes a gain in the function of cyclophilin (and FKBP) following drug binding (Tropschug et al., 1989; Bierer et al., 1990a,b; Schreiber, 1991; Koltin et al., 1991; Heitman et al., 1991) and the identification of calcineurin as a common target of CyP–CsA and FKBP–FK506 suggest that a more meaningful correlation should take into account the calcineurin binding properties of immunophilin–drug complexes. In order to ensure that the cellular effects of drug analogues are specific to the T cell receptor signaling pathway, we analyzed the ability of drug analogues to inhibit transcriptional activation mediated by NF-AT. The IL-2 gene has been shown to be under the control of the T cell specific transcription factor NF-AT, which is activated following antigen binding to the T cell receptor. The actions of CsA and FK506 have been previously examined with the assay used in this study, which uses the human leukemia T cell line Jurkat stably transfected with a plasmid encoding β-galactosidase under the control of a promoter containing three consecutive NF–AT binding sites. Activation of this cell line results in the drug-sensitive synthesis of a β-galactosidase reporter gene, thus offering a mechanistic specificity not utilized in previous studies of drug analogues (Sigal et al., 1991).

The correlation of cellular effects and immunophilin–ligand inhibition of calcineurin's phosphatase activity with analogues of both FK506 and CsA strongly supports a physiologic role for calcineurin as a cellular target of the immunosuppressive drugs. CsA analogues that either bind strongly to cyclophilin but are not immunosuppressive or bind weakly to cyclophilin but are immunosuppressive are seen to behave as anticipated when examined in the context of their cyclophilin complex (Table II). The cyclophilin complex with the high-affinity (but nonimmunosuppressive) ligand MeAla^6-CsA does not inhibit calcineurin, but the cyclophilin complex with the low-affinity (but immunosuppressive) ligand MeBm$_2$t^1-CsA is the most potent inhibitor of calcineurin measured to date. In the former case, the high-affinity cyclophilin ligand is not able to mediate interactions between cyclophilin and calcineurin (faulty effector domain), whereas in the latter case, weak cyclophilin binding (faulty immunophilin binding domain) is compensated for by an unusually high affinity of this cyclophilin complex to calcineurin. Invoking a role for calcineurin as the cellular target of immunophilin–drug complexes also provides an explanation for an otherwise difficult-to-rationalize observation in the FK506 series. Simply removing the methyl group of the 15-methoxy substituent of FK520 renders the molecule inactive as an immunosuppressant and inhibitor of signal transduction (faulty effector domain), yet has only a minor effect of FKBP binding [the X-ray structure of FKBP–FK506 (Van Duyne et al., 1991) reveals the 15-methoxy substituent does not contact FKBP—instead, it is part of the composite, calcineurin binding surface]. As seen in Table II, the corresponding FKBP complex has little effect on the phosphatase activity of calcineurin. In addition to providing strong support for a physiologic role for calcineurin, the results of the current studies are in full agreement with the view of immunophilin

ligands as comprised of both immunophilin binding domains and "effector elements" (the structural elements that interact with calcineurin) (Schreiber, 1991) and help to further define these domains. Immunophilin ligands require effective interactions with both their cognate immunophilin and calcineurin in order to behave as "molecular glue"—a property now recognized as essential for biological function.

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