Unexpected Side Chain Effects at Residue 8 of Cyclosporin A Derivatives Allow Photoswitching of Immunosuppression

Yixin Zhang‡, Frank Erdmann‡, Ria Baumgrass§§, Mike Schutkowski‡‡, and Gunter Fischer§§

From the §Max Planck Research Unit for Enzymology of Protein Folding, Weinbergweg 22, D-06120, Halle/Saale, Germany and the ¶Deutsches Rheuma-Forschungszentrum Berlin, Schumannstrasse 21/22, D-10117, Berlin, Germany

To dissect the enzyme inhibitory properties of the immunosuppressive cyclic undecapeptide cyclosporin A (CsA) and gain access to monospecific, non-calcineurin-inhibiting CsA derivatives, [d-Ser8]CsA was subjected to modifications at the d-Ser side chain. Thus, we modified a CsA residue flanking the calcineurin (CaN) and cyclophilin 18 (Cyp18) binding domains of CsA instead of the residues of the CaN binding domain in order to develop a new specificity-determining site within the cyclic peptide. The [O-(NH2(CH2)5NHC(O)CH2)-d-Ser 8]CsA (derivative 9), with an amino group on a tether, exhibits CsA-like inhibition of the peptidyl prolyl cis/trans isomerase activity of Cyp18 with an IC50 value of 3.2 ns, whereas the CaN inhibition by the Cyp18-derivative 9 complex is completely abolished. Consequently, this compound is not able to inhibit the proliferation and cytokine production of activated T cells. Structure-activity relationship studies with a series of [d-Ser8]CsA derivatives indicate that the positively charged side chain is an essential requirement for Cyp18-derivative 9 to be ineffective on CaN. Upon protecting the amino group in derivative 9 with the photolabile moiety 2-nitroveratryloxycarbonyl (NVOC), the Cyp18-[O-(NVOC)-NH(CH2)5NHC(O)CH2)-d-Ser 8]CsA (derivative 11) complex exhibits strong CaN inhibition and shows potent immunosuppressive activity. In stimulated T cells pretreated with derivative 11, a remarkable recovery of transcriptional activation of the nuclear factor of activated T cells (NFAT) has been achieved through light irradiation, as assessed with a NFAT reporter gene assay.

Cyclosporin A (CsA) is a standard immunosuppressive drug used to prevent allograft rejection after the transplantation of organs or bone marrow (1). CsA and another, structurally un-

related, immunosuppressive drug called FK506 bind to and inhibit the peptidyl-prolyl cis/trans isomerases (PPIases) cyclophilin (Cyp) and the FK506-binding protein (FKBP), respectively (2). Among the many known human PPIases, the most abundant cytosolic members, Cyp18 and FKBP12, have been found to be major receptors for CsA and FK506. However, such protein/ligand interactions are necessary but not sufficient for immunosuppression (3). The binary complexes of Cyp18-CsA and FKBP12-FK506 act as inhibitors for their common cellular target, Ser/Thr phosphatase calcineurin (CaN) (4, 5). In this case, the PPIases function as presenter molecules for the immunosuppressive drugs. CaN has been demonstrated to play a pivotal role in signaling in activated T cells. Thus, the CaN-mediated dephosphorylation of the nuclear factor of activated T cells (NFAT), NFAT nuclear translocation, and NFAT transcriptional activation (6, 7) provide an instance of a pathway that is essentially targeted by CsA and FK506 in immunosuppression (8).

Although earlier studies of the immunosuppressive mechanism of CsA and FK506 revealed the CaN-NFAT pathway in activated T cells, some recent evidence has suggested that Cyp18 plays a role in immune response on its own (9, 10). For instance, it has been reported that the immunosuppres- sive compound sanglifehrin A, which is also a potent inhibitor of Cyp18, exerts inhibitory effects on T cell proliferation in a CaN-independent manner (11–13). There is no indication that Cyp18 acts as a presenter protein for sanglifehrin A. Apart from its presence in the immune cells, Cyp18 appears to be abundantly expressed in all tissues (2) and is involved in many physiological processes (14). For example, it has been demonstrated that human immunodeficiency virus 1 incorporates host Cyp18 into its virion and that Cyp18 is required for the viral life cycle (15). CsA inhibits the association of Cyp18 with HIV-1 virions in a dose-dependent manner (16). Therefore, monospecific Cyp18 inhibitors that have no CaN inhibitory potency in their complex forms with Cyp18 and thus avoid the CaN inhibition associated with CsA treatment could facilitate the studies of various cellular functions of Cyp18 (16).

Previous work from our laboratory has shown that, for [dimethylaminomethyllethiosarcosine]CsA, the Cyp18-CsA derivative interaction did not lead to CaN inhibition, but the drug derivative potently inhibited CaN on its own (17). In contrast, several CsA derivatives have been shown to exhibit diminished immunosuppression, whereas the Cyp18 inhibition by these compounds is similar to that of CsA. Together with the crystal structures of the Cyp18-CsA-CaN complex (18, 19), structure-activity relationship studies have demonstrated that the protein binding surface of CsA involves two distinct parts of the molecule (Fig. 1). Modification of residues at positions 9 to 2 (termed the Cyp18 binding domain of CsA) reduces the affinity to Cyp18, whereas CaN inhibition by the Cyp18-CsA derivative complex could be impaired by changing the residues at positions 9–7 (termed the
with light of 366 nm resulting from chemical impurities. For irradiation of CsA derivative 11 the preparation (purity of chain on residue 8, CsA derivative 9 is more water-soluble than CsA. We analyzed the samples with analytic reverse phase-HPLC, and the常数 of Cyp18-CsA derivative-CaN several orders of magnitude.

Ala6]CsA complex. Similarly, Me-Leu4 fits tightly into a hydrophobic pocket of CaN (19) and stabilizes the Cyp18-CsA-CaN complex. Consequently, [N-methyl-Ile4]CsA (SDZ NIM811) and [N-methyl-Val4]CsA, which lack the stabilizing interaction, have been proved to be monospecific Cyp18 inhibitors with only minor effects on CaN activity. However, an inspection of the nature of CsA substitutions resulting in a monospecific Cyp18 inhibitor reveals considerable synthetic effort. Moreover, to design CsA derivatives whose specificity can be regulated in situ (as discussed later), the C–C bond-based side chain branching in positions 4 and 6 of CsA indicates the difficulty of employing simple chemical interventions to achieve switching between the inhibitory types based on the structures of these known monospecific CsA analogues.

In cells treated with inhibitors, modulation of the target protein activity would be of considerable value when controlled in timing, location, and amplitude (22, 23). Although the precondition can be achieved by photomodulation, caged CsA derivatives are still lacking. Furthermore, caged CsA derivatives, when properly designed, could principally be used to recover the inhibited cellular CaN phosphatase activity upon photolysis and to differentiate Cyp18 inhibition from the combined Cyp18-CaN effects of CsA, avoiding time-dependent drug partitioning into cells. Such a compound would be expected to allow the release of the T cell proliferation after photolysis. Given that most residues within CsA are important for contacting Cyp18 and CaN, modifications at these positions in CsA and their chemical features (as discussed above) are not suitable for making derivatives selective for Cyp18-CaN complexes. This approach is based on the hypothesis that some functional groups on the residue 8 of CsA, which flanks the Cyp18 and CaN binding domains of CsA, could interfere with the CaN contacting surface in the Cyp18-CsA derivative complex (18, 19). The introduction of photolabile 2-nitroveratryl oxy carbonyl (NVOC) protecting group changes the dissociation constant of Cyp18-CsA derivative-CaN several orders of magnitude, whereas the effect can be reversed upon photolysis.

EXPERIMENTAL PROCEDURES

Preparation of CsA Derivatives from [d-Ser6]CsA

The CsA derivatives with modified residue 8 were synthesized from [d-Ser6]CsA without protecting the hydroxyl of MeBmt as reported by Eberle et al. (24). Reverse phase-HPLC was applied for monitoring the reactions and for preparative separations. The identities of all derivatives were confirmed with electrospray ionization mass spectrometry. We analyzed the samples with analytic reverse phase-HPLC, and the purities of all CsA derivatives are >95%. Possessing a hydrophilic side chain on residue 8, CsA derivative 9 is more water-soluble than CsA and can be easily separated from its CaN inhibitory precursors during the preparation (purity of >99%), thus preventing immunosuppression resulting from chemical impurities. For irradiation of CsA derivative 11 with light of 366 nm in situ, a handheld UV lamp (type 2-5062, Dr. Grobel UV-Elektronik, Ettlingen, Germany) was used. The emission spectrum of the handheld UV lamp shows a narrow band of ~366 nm without a detectable emission in the UV-C and UV-B ranges (supplemental Fig. S1, available in the on-line version of this article). For irradiation of CsA derivative 11 with light of 366 nm in vitro, a HGXE lamp (Hamamatsu E7536) equipped with a monochromator (model 101, Photon Technology International) has been used. Because derivative 11 is more hydrophobic than derivative 9, derivative 11 and its photolysis product, derivative 9, possess different retention times on HPLC. The photolysis of derivative 11 has been monitored by using analytic HPLC. The cleavage of the photolabile NVOC group of derivative 11 was monitored by using analytic HPLC.

PP1ase Activity Assay

The PP1ase activity of recombinant human Cyp18 was measured with a protease-coupled assay on a Hewlett-Packard 8452a diode array spectrophotometer according to Fischer et al. (25), with succinyl-Ala-Phe-Pro-Phe-4-nitroanilide as the substrate and α-chymotrypsin (Merck) as the auxiliary protease in 35 mM HEPES buffer, pH 7.8, at 7 °C. After a 10-min incubation of Cyp18 (2.5 μM) with an inhibitor of the desired concentration, protease and substrate were added. The final substrate concentration was 40 μM. The decrease in fluorescence was monitored at 390 nm. The rate was calculated according to first order reaction. The residual enzymatic activities were plotted versus the inhibitor concentrations and used for calculating the IC50.

CaN Phosphatase Activity Assay

Materials—ScintiPlates coated with streptavidin were purchased from PerkinElmer Life Sciences. The biotinylated RI1 peptide (biotin-DLDPVPIGRDFRDRSVSAAE-OH), a partial sequence of the RI1 subunit of the bovine cAMP-dependent protein kinase A, was synthesized with Fmoc (N-(9-fluorenylmethoxycarbonyl)-based solid phase peptide synthesis. The catalytic subunit of protein kinase A was purchased from New England Biolabs (Frankfurt, Germany). Calmodulin, buffer, and salts were purchased from Sigma. Expression and purification of recombinant human CaN was performed as described (26).

CaN Activity Assay Using RI1 Phosphopeptide Substrate—The biotinylated RI1 peptide was phosphorylated by protein kinase A. The reaction mixture containing 700 μM peptide, 100 μM of [γ-32P]ATP, and 10 kilounits of protein kinase A in a final volume of 100 μl (in the buffer containing 20 mM MIES, pH 6.5, 0.4 mM EDTA, 0.2 mM EGTA, 50 μM CaCl2, and 2 mM MgCl2) was incubated for 1 h at 30 °C. The phosphorylated peptide was separated from [γ-32P]ATP by 1 ml of an RP-C2 cleanup extraction column (Amchro, Sulzbach, Germany). The eluted peptide was lyophilized and reconstituted with deionized water.

The scintillation proximity concept was applied for measuring CaN activity using ScintiStrip surfaces coated with streptavidin. Preincubation of calmodulin (50 μM), CaN (1.22 μM), and inhibitors of desired concentrations in the assay buffer (40 mM Tris/HCl, pH 7.5, 100 mM NaCl, 6 mM MgCl2, 0.5 mM dithiothreitol, 1 mM CaCl2, and 0.1 mg/ml bovine serum albumin) was carried out for 30 min at 30 °C in a 96-well microtiter plate (Costar, Bademuenster, Germany). Biotinylated RI1 phosphtpeptide was added to a final concentration of 100 nM, and the total assay volume was 100 μl. After incubation at 30 °C for 30 min, 90 μl of the reaction mixture was transferred to the ScintiPlates coated with streptavidin. The streptavidin immobilized the biotinylated peptide in the wells. After 20 min at 22 °C, the well was washed once with water, and the biotinylated RI1 phosphtpeptide-associated [32P] was measured in a MicroBeta top counter (Wallac, Turku, Finland).
**Regulation of Cyp18/CaN Interaction by CsA Residue 8**

**Immunosuppression**

*T Cell Proliferation—* Peripheral blood mononuclear cells (PBMCs) were labeled with 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester before stimulation with plate-bound anti-CD3 plus anti-CD28 antibodies (2 x 10^6 cells/ml) in the presence of inhibitors or MeSO as control in 24-well plates. Inhibitors were dissolved in MeSO and added to a desired final concentration with 0.5% MeSO in each cell sample. The cells were cultured for 5 days and analyzed directly on proliferating cells by flow cytometry.

*Intracellular Cytokine Production—* PBMCs (2 x 10^6 cells/ml) were preincubated with inhibitors at various concentrations at 37 °C for 10 min in 24-well plates. Inhibitors were dissolved in MeSO and added to a desired final concentration with 0.5% MeSO in each cell sample. PBMCs were stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin (both Sigma) for 5 h with the addition of brefeldin A (5 μg/ml) for the last 3 h of stimulation. Cells were then fixed in 2% paraformaldehyde for 20 min, permeabilized by washing in PBS supplemented with 0.5% saponin and 1% fetal calf serum, incubated with anti-cytokine phospho-Dyltyophospholamino-conjugated antibody, and measured by flow cytometry.

**Luciferase Reporter Gene Assays**

Jurkat T cells transiently transfected with the NFAT-luciferase reporter plasmid (Stratagene) by electroporation (Anaxa, Cologne, Germany) were cultured in RPMI 1640 with 10% fetal calf serum and 2 mM L-glutamine for 16 h at 37 °C in 5% CO2. The cells were preincubated with inhibitors for 20 min, additionally incubated for 30 min with or without light irradiation at 366 nm by the hand-held UV lamp, and then stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin for 5 h. After cell lysis, the level of the extracted luciferase from these cells was determined by bioluminescence measurement using the luciferase assay system (Promega). In addition, a β-galactosidase plasmid was cotransfected as an internal standard. Under our conditions, irradiation in the UV-A range does not exhibit significant influence on the transcriptional activity of NFAT. Moreover, the transcriptional activity of AP-1 is also not affected (supplemental Fig. S2, available in the on-line version of this article).

**RESULTS**

The synthesis of CsA derivatives 2–12 was carried out by regioselective modification of the hydroxyl group of d-Ser\(^8\) of [d-Ser\(^8\)]CsA (derivative 1) without protecting the hydroxyl group of MeBmt\(^1\) (24). The compounds investigated in this study are shown in Fig. 1. The starting material [d-Ser\(^8\)]CsA can be obtained from fermentation (28).

**Acidic Modifications—** First we investigated the influences of acidic functional groups attached to the d-Ser\(^8\) oxygen of [d-Ser\(^8\)]CsA on both the direct Cyp18 inhibition and the CaN inhibition by the Cyp18-CsA derivative complex.

The inhibitory efficacies of the CsA derivatives toward Cyp18 PPIase activity were measured using a protease-coupled assay (29). As shown in Table I, CsA and the four derivatives [O-phosphoryl-d-Ser\(^8\)]CsA (derivative 3), [O-H-phosphoryl-d-Ser\(^8\)]CsA (derivative 4), [O-carboxymethyl-d-Ser\(^8\)]CsA (derivative 6), and [O-(HOOC/CH\(_2\)_NHC(O)/CH\(_2\)_N-d-Ser\(^8\)]CsA (derivative 7) inhibit Cyp18 with very similar IC\(_50\) values. Obviously, negative charges at CsA residue 8 do not have a significant influence on the inhibition of Cyp18 PPIase activity.

The CaN phosphatase activities toward 32P-labeled biotinylated 19-residue phosphopeptide (RII phosphopeptide) were measured using a scintillation assay (27). The CaN inhibitions by Cyp18-CsA derivative complexes were measured by addition of 10 μM inhibitor and Cyp18 of desired concentrations. Neither CsA and its position 8-substituted derivatives nor Cyp18 alone produces any detectable inhibition of CaN. Because of the high concentrations of CsA and its derivatives (10 μM) and their high binding affinities to Cyp18 (0.9–100 nM; Table I), the concentrations of the Cyp18-CsA derivative complexes are assumed to be equal to that of Cyp18. CsA and the derivatives 4, 6, and 7 in their complexes with Cyp18 show potent CaN inhibition (Table I). However, the Cyp18-derivative 3 complex inhibits CaN phosphatase activity 50-fold less effectively than the Cyp18-CsA complex. We synthesized an unsupervised analogue of [O-phosphoryl-d-Ser\(^8\)]CsA (derivative 3), namely [O-(dimethylphosphoryl)-d-Ser\(^8\)]CsA (derivative 5), and measured its inhibitory efficiency to demonstrate that this remarkable effect is contributed mainly by the dianionic phosphate moiety. As shown in Table I, derivative 5 possesses potent Cyp18 inhibition like derivative 3, whereas the complex of Cyp18-derivative 5 exhibits 10-fold higher potency in CaN inhibition than the Cyp18-derivative 3 complex.

**Basic Modifications—** The consequences of incorporating free amino groups on flexible tethers of variable length in position 8 were assessed with derivatives 8 and 9. As compared with CsA, the Cyp18 inhibition by derivatives 8 and 9 demonstrates only a small change in IC\(_50\) values (Table I and Fig. 2A), whereas CaN inhibition shows an abrupt deterioration associated with the long side chain in derivative 9. Whereas the Cyp18-derivative 8 complex inhibits CaN phosphatase activity as potently as the Cyp18-CsA complex (Table I), the Cyp18-derivative 9 complex is inactive, showing <5% CaN inhibition at a Cyp18 concentration as high as 10 μM (Table I and Fig. 2A).

Coprecipitation experiments of CaN with GST-Cyp18 in the presence of derivative 9, using CsA as a positive control, have been performed. CaN could only be coprecipitated together with GST-Cyp18 by glutathione-Sepharose in the presence of CsA, but not in the presence of derivative 9 or the CsA derivative-free control (Fig. 2B). These results demonstrated that not only the functional but also the physical interactions of Cyp18-derivative 9 with CaN are much weaker than that of Cyp18-CsA with CaN.
The PPIase activity of Cyp18 was measured in a protease-coupled assay at 7 °C with succinyl-Ala-Phe-Pro-Phe-4-nitroanilide as the substrate. The CaN phosphatase activities were measured using a scintillation assay and a 33P-labeled biotinylated RII phospopeptide as the substrate at 30 °C in the presence of 1.22 nM CaN, 50 nM calmodulin, 10 μM CaA derivative, and Cyp18 at different concentrations.}

### Table I

| Abbreviations | Inhibitors | Inhibition of Cyp18 by CaA derivatives (IC50) | Inhibition of CaN by Cyp18-CaA derivative complexes (IC50) |
|---------------|------------|--------------------------------------------|--------------------------------------------------|
| CsA           | Cyclosporin A | 3.7                                       | 100                                             |
| 1             | (n-Ser⁸)CsA | 2.3                                       | 40                                              |
| 2             | (O-Methyl-n-Ser⁸)CsA | 3.9                                   | 120                                             |
| 3             | (O-Phosphoryl-n-Ser⁸)CsA | 5.0                                   | 5000                                            |
| 4             | (O-H-Phosphoryl-n-Ser⁸)CsA | 7.3                                   | 130                                             |
| 5             | (O-(Dimethylphosphoryl)-n-Ser⁸)CsA | 2.6                                   | 450                                             |
| 6             | O-Carboxymethyl n-Ser⁸CsA | 0.9                                   | 220                                             |
| 7             | (O-(HOOC(CH₂)₅NHC(O)CH₂)-n-Ser⁸)CsA | 5.0                                   | 200                                             |
| 8             | (O-2-Aminoethoxyethyl)-n-Ser⁸CsA | 12.7                                    | 100                                             |
| 9             | (O-(Boc-NH(CH₂)₅NHC(O)CH₂)-n-Ser⁸)CsA | 3.2                                   | No inhibition*                                  |
| 10            | (O-(Boc-NH(CH₂)₅NHC(O)CH₂)-n-Ser⁸)CsA | 100                                   | 2000                                            |
| 11            | (O-(NVOCNH(CH₂)₅NHC(O)CH₂)-n-Ser⁸)CsA | 10                                    | 330                                             |
| 12            | (O-(Ac-NH(CH₂)₅NHC(O)CH₂)-n-Ser⁸)CsA | 8.1                                    | 2000                                            |

* S.D. <20 %.
** S.D. <10 %.
*** <5 % inhibition of CaN at 10 μM of Cyp18-derivative 9 complex.

**Fig. 2.** There is no physical and functional interaction between CaN and Cyp18-derivative 9. A, CaN inhibition (left) by Cyp18-CsA (square) and Cyp18-derivative 9 complexes (cycle) and Cyp18 inhibition (right) by CsA (gray) and derivative 9 (black). The CaN phosphatase activities were measured using a scintillation assay and a 32P-labeled, biotinylated RII phospopeptide as substrate at 30 °C in the presence of 1.22 nM CaN, 50 nM calmodulin, and Cyp18 at the desired concentrations. The Cyp18 PPIase activities were measured using a protease-coupled assay with succinyl-Ala-Phe-Pro-Phe-4-nitroanilide as the substrate.

---

To demonstrate that the dramatic impairment observed in CaN inhibition assay is caused mainly by the electrostatic effect involving the protonated amino group on the residue 8 of derivative 9 (Fig. 3), we tested the Cyp18 inhibitory efficiencies of derivatives 7 and 12 and the CaN inhibition by the Cyp18-derivative 7 and Cyp18-derivative 12 complexes (Fig. 1 and Table I). Instead of the amino group in derivative 9, derivative 7 has a carboxyl group at the end of the side chain, and derivative 12 possesses an acetyl-protected amino group. Like CsA, both derivative 7 and derivative 12 are potent inhibitors of Cyp. Furthermore, both the Cyp18-derivative 7 complex and the Cyp18-derivative 12 complex display potent CaN inhibition, as compared with the Cyp18-derivative 9 complex. Thus, neither a neutral nor an acidic functional group affects the Cyp18-CaA-CaN tertiary complex formation significantly. Similarly, with a photoremovable protecting group, NVOC, on the amino group of derivative 9 at residue 8, derivative 11 exhibits high Cyp18 inhibitory efficiency with an IC50 value of 10 nM, as well as potent CaN inhibition by the Cyp18-derivative 11 complex with an IC50 value of 330 nM.

We investigated the immunosuppressive effect of derivative 9 on the proliferation of human T cells. Isolated PBMCs were labeled with 5-(and 6)-carboxyfluorescein-diacetate-succinimidyl ester and stimulated by plate-bound anti-CD3-CD28, allowing the cytometric discrimination and determination of cell numbers in the individual generation of proliferating cells. As
Production of cytokines in T cells was investigated using the CaN inhibition assay, as shown in Fig. 4B. To investigate whether derivative 9 is a monospecific Cyp18 inhibitor, we tried to determine the drug concentration at which derivative 9 fully retains the inhibition of the PPIase activity of Cyp18, which is also indicative of the formation of a tight binary Cyp18-derivative 9 complex. Importantly, the introduction of a variety of amino protecting groups restores the CsA-like behavior in enzyme inhibition (Table I) and immunosuppression.

DISCUSSION

Our results show that CsA derivatives reveal that minor differences in chemical modifications in the D-Ala8 side chain of CsA can lead to abrupt changes in the inhibitory specificity of the respective analogues. Interestingly, when a free amino group on a tether (D-CH2CONH(CH2)5NH2) in the side chain of residue 8 (derivative 9) is presented, functional and physical interactions of CaN with the Cyp18-derivative 9 complex as well as immunosuppression, which is normally observed in other position 8-substituted CsA derivatives, is completely abolished. In contrast, compound 9 fully retains the inhibition of the PPIase activity of Cyp18, which is also indicative of the formation of a tight binary Cyp18-derivative 9 complex. Importantly, the introduction of a variety of amino protecting groups restores the CsA-like behavior in enzyme inhibition (Table I) and immunosuppression.

The formation of the Cyp18-CsA-CaN complex is mediated through multiple contacts between Cyp18 and the Cyp18 binding domain of CsA, between CaN and the CaN binding domain of CsA, and an interface between Cyp18 and CaN. Although plenty of chemical genetic studies have illustrated the interactions of CsA derivatives with both proteins, the existing data on enzyme inhibition by residue 8 derivatives are rather limited (21, 32, 33). However, this position was considered to have the highest tolerance for various modifications (18). Consequently, the side chain of position 8 was thought to be well suited for the modifications by attaching groups for cross-linking, fluorescence labeling, and membrane permeability perturbation to investigate the CsA derivatives that could mediate immunosuppression in a CsA-like manner (33–35). Our design is based on the fact that residue 8 has little effect on either Cyp18 inhibitory potency or CaN inhibition by the Cyp18-CsA derivative complex, whereas large functional groups exhibit weak to moderate influence. As the amino group of derivative 9 is protected by acetyl, the Cyp18 inhibition by the resulting derivative 12 is similar to that of CsA, whereas a 20-fold decrease of CaN inhibition by the Cyp18-derivative 12 complex is observed as compared with that of the Cyp18-CsA complex. In the CsA derivative 10, the amino group in derivative 9 is blocked by the large and hydrophobic tert-butoxycarbonyl (Boc) group. Derivative 10 exhibits an ~30-fold decrease of Cyp18 inhibition, as compared with CsA and derivative 9. A 20-fold reduction of CaN inhibition by the Cyp18-derivative 10 complex is also observed, as compared with Cyp18-CsA. The photolabile functional group NVOC (derivative 11) exhibits only weak effects on both Cyp18 inhibition and CaN inhibition.

Photoswitching of T Cell Activation in CsA Derivative 11-Pretreated Jurkat T Cells—Despite their structural similarities, derivatives 11 and 9 exhibit dramatically different CaN inhibition in their complex forms with Cyp18. We examined the photoconversion reaction of derivative 11 to derivative 9 with light irradiation at 366 nm (23). As monitored with analytic reverse phase-HPLC, the cleavage of the photolabile NVOC group of derivative 11 was complete (>95%) after 30 min of irradiation at 366 nm, either in ethanol solution or in Jurkat T cell lysate (data not shown). As expected, derivative 11 exhibits potent immunosuppressive activity, inhibiting the proliferation and cytokine production of activated T cells (Fig. 4) and supplemental Fig. S3, available in the on-line version of this article), in contrast to its photolysis product, derivative 9. We investigated the influences of light irradiation on derivative 11-pretreated Jurkat T cells. As shown in Fig. 6, light irradiation of cells for 30 min results in significant enhancements of the NFAT reporter gene activity. Especially at a relatively low drug concentration (20 nM), irradiation fully recovers the inhibited production of the NFAT reporter gene.

Photolabile modifications by attaching groups for cross-linking, fluorescence labeling, and membrane permeability perturbation to investigate the CsA derivatives that could mediate immunosuppression in a CsA-like manner (33–35). Our design is based on the fact that residue 8 has little effect on either Cyp18 inhibitory potency or CaN inhibition by the Cyp18-CsA derivative complex, whereas large functional groups exhibit weak to moderate influence. As the amino group of derivative 9 is protected by acetyl, the Cyp18 inhibition by the resulting derivative 12 is similar to that of CsA, whereas a 20-fold decrease of CaN inhibition by the Cyp18-derivative 12 complex is observed as compared with that of the Cyp18-CsA complex. In the CsA derivative 10, the amino group in derivative 9 is blocked by the large and hydrophobic tert-butoxycarbonyl (Boc) group. Derivative 10 exhibits an ~30-fold decrease of Cyp18 inhibition, as compared with CsA and derivative 9. A 20-fold reduction of CaN inhibition by the Cyp18-derivative 10 complex is also observed, as compared with Cyp18-CsA. The photolabile functional group NVOC (derivative 11) exhibits only weak effects on both Cyp18 inhibition and CaN inhibition.

FIG. 3. Crystal structure of the Cyp18-CsA complex (1CWA) and the side chains on the residues 8 of CsA derivatives 8 and 9. The Arg148 (R148) of Cyp18 is presented as a stick-and-ball. The electrostatic surface potential is indicated by colors (blue, acidic; red, basic). The CsA structure is presented as a stick. The D-Ala8 of CsA is highlighted in yellow. The distance between the β-carbon of D-Ala8 of CsA and the carbon of the Arg148 guanidino group is 10.07 Å.
on the hypothesis that the residue 8 could influence the Cyp18-CaN interface within the Cyp18-CsA-CaN complex for the following reasons. (a) It is close to an open, presumably water-filled cavity around Ala7-D-Ala8 visible in the crystal structures of the Cyp18-CsA-CaN complex (18, 19). (b) It flanks the Cyp18 binding domain and CaN binding domain of the CsA molecule with no participation of the D-Ala8 methyl group in essential protein-drug contacts. (c) The residue 8 is involved in a kink (19) of the cyclic peptide near the interface between Cyp18 and CaN in the tertiary complex structure.

Fig. 4. CsA derivative 9 did not inhibit the proliferation and intracellular cytokine production of stimulated PBMCs. A, the 5-(and 6)-carboxyfluorescein-diacetate-succinimidyl ester (CFSE)-labeled human PBMCs were stimulated with immobilized anti-CD3 plus anti-CD28 antibodies and then cultured for 5 days and measured by flow cytometry. The histograms show the particular generations after cell division of PBMCs pretreated with CsA (100 nM), derivative 9 (1 μM), light-irradiated derivative 11 (1 μM), and non-irradiated derivative 11 (1 μM). B, PBMCs were treated with brefeldin A 2 h after stimulation with PMA/ionomycin, cultured further for 3 h, and then fixed. Intracellular IL-2 and IFN-γ production was determined by staining with anti-IL-2-phosphatidylethanolamine and anti-IFN-γ-Cy5 antibodies and measuring with flow cytometry. The histograms show the cytokine producers and non-producers of stimulated PBMCs pretreated with CsA derivatives or Me2SO. The percentages of IL-2 or IFN-γ producing cells relative to the total cell count are given above the markers.

FIG. 4.

Regulation of Cyp18/CaN Interaction by CsA Residue 8
Given that the amino group in derivative 9 interferes with Cyp18-CsA-CaN complex formation, cationic surface residues on Cyp18 and CaN are the most likely candidates to account for the dramatic effect. It was reported that mutations of several cationic surface residues of Cyp18 did not change its PPIase activity but altered the CaN inhibitory efficiency of the Cyp18-CsA complex (36). Among these mutations, R148E is 17-fold more effective than wild type Cyp18, whereas R69E and K125E impair the binding affinity. There might be a cationic region on the surface of CaN, which contacts with the Arg^{148} of Cyp18 and prevents optimal Cyp18-CaN interaction through static electric expulsion. The R148E mutation of Cyp18 diminishes the energy expense and enhances the binding affinity. CaS is a hydrophilic cyclic peptide. A hydrophilic tether at the solvent-exposed residue 8 side chain of derivative 9 would preferentially extend to the aqueous environment. Analyzing the crystal structure of the Cyp18-CsA complex (37) (Fig. 3), we found that the distance between the β-carbon of CsA d-Ala^8 and the Arg^{148} guanidino of Cyp18 is 10.1Å. The fully extended lengths of the side chains of derivatives 9 and 8 are 12.1 and 8.4 Å, respectively. Apparently, the amino group on the side chain of derivative 9 possesses a larger effective radius than that of derivative 8. Therefore, we assume that the amino group at the end of the long side chain of derivative 9 could access the cationic surface on Cyp18 around Arg^{148} efficiently, increasing the electrostatic expulsion between Cyp18 and CaN. Whereas the amino group of derivative 9 is replaced by an acidic or a neutral group (as in derivative 7 or derivative 12, respectively), the affinity between CaN and the Cyp18-CsA derivative is recovered. These results demonstrate that the amino group, but not the steric hindrances or other functional groups within the residue 8 side chain of derivative 9, have contributed markedly to this effect. Moreover, on the one hand the similar Cyp18 inhibitions by CaS, derivative 8, and derivative 9 demonstrate that the amino side chain does not cause a significant conformational reorganization in CsA on its own. On the other hand, the potent CaN inhibition by the Cyp18-derivative 8 complex, as well as the immunosuppressive activity of [D-Lys^8]CsA (33), also indicates that a positively charged group on a short tether at position 8 does not have significant influence on the formation of a Cyp18-CsA derivative-CaN complex. Eventually, the hypothesis regarding a repulsive surface on CaN against Cyp18 Arg^{148} is further proved by the Cyp18-CsA-CaN crystal structure (18). A direct contact between the Arg^{148} of Cyp18 and the Arg^{122} of CaN represents an unfavorable contact between the two cationic surfaces of both proteins.

Structure-function relationships in the Cyp18 and CaN inhibition of residue 8 CsA derivatives indicate that a tethered basic group could play a unique role in mediating the interaction of a Cyp18-drug complex with CaN (Table 1). As for acidic groups, phosphate in the side chain of residue 8 has shown a significant influence on CaN inhibition by a Cyp18-CsA derivative complex. However, minor CaN inhibition is still present. Alterations in the ionization state by esterification (derivative 5) or the phosphate group mimic (derivative 4) restore the potent CaN inhibition of the CsA derivative upon Cyp18 binding. Obviously, the dianionic state of the phosphate group may corrupt the formation of the Cyp18-CsA derivative-CaN tertiary complex.

As for neutral groups, hydrophilic substitutions have little effect on the binding affinity of a CsA derivative to Cyp18, whereas their influences on CaN inhibition by a Cyp18-CsA derivative complex could differ from each other remarkably (Table 1). In agreement with previously published results, upon introducing Boc-protected amino group at position 8, the resulting [Nβ-Boc-d-1,3-diaminopropionic acid^8]CsA ([(d-
Regulation of Cyp18/CaN Interaction by CsA Residue 8

nM for both the Cyp18-CsA complex and the Cyp18-derivative Kd with \[\text{Dab8}]CsA (21) as well as derivative 10 in their complexes with \[\text{Dab8}]CsA have been shown to inhibit Cyp18 like CsA, the CsA derivative 10 possesses a significantly reduced Cyp18 inhibitory effect.

The antagonistic activity of derivative 9 toward CsA-inhibited T cell proliferation has been investigated. Both 506BD, a compound designed to preserve the common FKBP binding domain of FK506 but lacking the immunosuppressive activity, and rapamycin act as potent antagonists of FK506 immunosuppression (38, 39). In contrast, although derivative 9 and \[\text{N-methyl-Ala6}]CsA (3) inhibit Cyp18 as potently as CsA, they are not able to antagonize CsA. We found that the Cyp18 concentration had a significant influence on the antagonizing potency of derivative 9 in vitro (Fig. 5). At a low concentration of Cyp18, derivative 9 competes with CsA for Cyp18 binding. It reduces the inhibitory efficiency of Cyp18-CsA and recovers the CaN phosphatase activity.

However, at high Cyp18 concentrations derivative 9 does not affect CaN inhibition by Cyp18-CsA, an observation similar to that from the antagonism experiments with T cells (3, 31). Thus, the extraordinarily high concentration of Cyp18 in cells (2) as well as the thermodynamically favorable formation of the Cyp18-CsA-CaN complex could prevent Cyp18 monospecific inhibitors such as derivative 9 and \[\text{N-methyl Ala6}]CsA from antagonizing CsA. With \(K_d\) values of 100 nM for the Cyp18-CsA-CaN complex and 9 nM for both the Cyp18-CsA complex and the Cyp18-derivative 9 complex (40), a computational simulation has also demonstrated that high Cyp18 concentrations of T cells (2) can abolish the antagonizing effect of derivative 9 (data not shown).

Because the inhibition and immunosuppression profile of the photolabile derivative 11 resembles that of CsA, we tested the possibility of a photoconversion of derivative 11 to the non-immunosuppressive derivative 9. Light-based deprotection of masked functional groups of biomolecules (caged compounds) has been realized as a simple, non-invasive technique to generate concentration jumps of substrates, ligands, and proteins (22). Different from the classical caging method in which irradiation of a biologically inactive compounds removes the photoprotective group and with the subsequent appearance of active species, uncaging of the immunosuppressive derivative 11 could result in reduced immunosuppressive activity in a T cell proliferation assay. This is particularly interesting for a photoregulation of inhibitory molecules, because it would allow a recovery of target protein activity in the inhibitor-treated cells in parallel with the protein activation approaches in which uncaging leads to the release of a second messenger such as calcium (22) or inositol 1,4,5-trisphosphate (41).

As analyzed by analytical HPLC, irradiation of derivative 11 with light of 366 nm for 30 min results in removal of the NVOC group (> 95%) and converts derivative 11 to derivative 9 in a quantitative reaction when dissolved either in ethanol or at Jurkat T cell lysate (23). The photoswitching of the activation of Jurkat T cells pretreated with derivative 11 was investigated with an NFAT reporter gene assay, because NFAT is a major transcription factor in antigen-receptor signaling regulated by the CaN protein phosphatase activity (6). Because the CaN-inhibitor derivative 11 is already present in the cell prior to irradiation and stimulation, the recovery of CaN activity by irradiation is a sequential reaction with a lag phase at the beginning. Thus, a quantitative comparison between experimental data using incubation of PBMCs by derivative 9 and experiments with derivative 11 photoconverted within cells is not possible. As shown in Fig. 6, a 3–4-fold enhancement of the NFAT reporter gene luciferase activity has been observed in the irradiated cells as compared with that in the non-irradiated cells. When assessed by a reporter gene assay, irradiation alone does not stimulate the essential transcription factor AP-1 under our conditions (supplemental Fig. S2). The results depicted in Fig. 6 are aimed toward demonstrating proof of the principle of photomodulating compound 11-mediated CaN inhibition in situ.

Similarly, upon treating PBMCs with an in vitro irradiated sample of derivative 11, the activity of derivative 11 to suppress cell proliferation is remarkably reduced (Fig. 4A). Because the cells have not been exposed to the light of 366 nm, a direct effect from the UV-A light on the cellular effects could be excluded. Whereas a photoregulation of T cell transcriptional activation has been realized in our study, the interesting mode of action of derivative 9 and its caged derivatives suggests many further applications, for instance, to control the timing of immune response (42) and to reactivate cellular CaN with three-dimensional spatial resolution by using two-photon photomodulation (43).

In summary, our work represents a novel strategy for designing Cyp18 monospecific CsA derivatives. Instead of changing the CsA residues responsible for direct contacts with CaN, we examined the capability of the residue 8 side chain to modulate the interactions between Cyp18 and CaN. CsA derivative 9 is a potent and monospecific Cyp18 inhibitor. It is not immunosuppressive, because the Cyp18-derivative 9 complex does not inhibit CaN phosphatase activity. Furthermore, a photoresponsive CsA derivative (derivative 11) has been designed, and photoswitching of drug activity has been achieved in situ in activated T cells.

Acknowledgments—We thank A. Schierhorn, T. Pfeiffer, and M. Kipping for mass spectroscopic measurements. We gratefully acknowledge M. Heidler and I. Kunze for excellent technical assistance.

REFERENCES

1. Boriel, J. F. (1989) Pharmacol. Rev. 41, 259–371
2. Fischer, G. (1994) Angew. Chem. Int. Ed. Engl. 33, 1415–1436
3. Sigal, N. G., Dumont, F., Durette, P., Siekierka, J. J., Peterson, L., Rich, D. H., Dunlap, B. E., Staruch, M. J., Melino, M. R., Koprak, S. L., Williams, D., Witzel, B., and Pisano, J. M. (1991) J. Exp. Med. 173, 619–628
4. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) Cell 66, 807–815
5. Schreiber, S. L., and Crabbe, G. R. (1992) Nature 360, 411–420
6. Zhang, L. H., and Liu, J. O. (2001) Cell Biochem. Biophys. 30, 115–151
7. Colgan, J., Asmal, M., Neagu, M., Yu, B., Schneidkrantz, J., Lee, Y., Sokolskaja, E., Andreotti, A., and Luban, J. (2004) Immunity 21, 189–201
8. Brainin, K. N., Mallis, R. J., Fulton, D. B., and Andreotti, A. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1899–1904
9. Zhang, L. H., and Liu, J. O. (2001) J. Immunol. 166, 5611–5618
10. Zhang, L. H., Youn, H. D., and Liu, J. O. (2001) J. Biol. Chem. 276, 43534–43540
11. Clarke, S. J., McStay, G. P., and Halestrap, A. P. (2002) J. Biol. Chem. 277, 34793–47979
12. Fischer, G., and Aumuller, T. (2003) Rev. Physiol. Biochem. Pharmacol. 148, 153–180
13. Luban, J. (1996) Cell 87, 1157–1159
14. Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C. T., Sodroski, J., and Gottleber, H. G. (1994) Nature 372, 363–365
15. Baumgrras, R., Zhang, Y., Erdmann, F., Thiel, A., Radbruch, A., and Fischer, G. (2004) J. Biol. Chem. 279, 2479–2479
16. Haizl, Q., Kim, H. Y., Liu, Y., Zhao, Y., Mordrano, A., Liu, J. O., and Ke, H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12037–12042
17. Jin, L., and Harrison, S. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13522–13526
18. Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Alberg, D. G., Belshaw, P. J., Cohen, P., MacKintosh, C., Klee, C. B., and Schreiber, S. L. (1992) Biochemistry 31, 3898–3901
19. Nelson, P. A., Akselband, Y., Kawamura, A., Su, M., Tung, R. D., Rich, D. H., Ichikura, V., Rosborough, S. L., DeCenzo, M. T., Livingston, D. J., and Harding, M. W. (1993) J. Immunol. 150, 2139–2147
20. Adams, S. R., and Tien, Y. R. (1993) Annu. Rev. Biochem. 55, 755–784
21. Marriot, G. (1994) Biochemistry 33, 9092–9097
22. Kieber, M. K., Hiestand, P., Jutzi-Enne, A. M., Niningen, F., and Zillman, H. R. (1995) J. Med. Chem. 38, 1853–1864
23. Fischer, G., Bang, H., and Meech, C. (1984) Biomed. Biochim. Acta 43.
26. Mondragon, A., Griffith, E. C., Sun, L., Xiong, F., Armstrong, C., and Liu, J. O. (1997) *Biochemistry* **36**, 4934–4942
27. Baumgrass, R., Weiward, M., Erdmann, F., Liu, J. O., Wunderlich, D., Grabley, S., and Fischer, G. (2001) *J. Biol. Chem.* **276**, 47914–47921
28. Traber, R., Hofmann, H., and Kiefl, H. (1989) *J. Antibiot. (Tokyo)* **42**, 591–597
29. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefer, T., and Schmid, F. X. (1989) *J. Mol. Biol.* **237**, 476–478
30. Thiel, A., and Radbruch, A. (1999) *Arthritis Res.* **1**, 25–29
31. Zenke, G., Strittmatter, U., Fuchs, S., Quesniaux, V. F., Brinkmann, V., Schulze, W., Zurini, M., Enz, A., Billich, A., Sangier, J. J., and Fehr, T. (2001) *J. Immunol.* **166**, 7165–7171
32. Wenger, R. M. (1988) *Annu. Rev. Pharmacol. Toxicol.* **28**, 31–54
33. Wenger, R. M. (1988) *Transplant Proc.* **20**, 313–318
34. Moss, M. L., Palmer, R. E., Kuzmic, P., Dunlap, B. E., Henzel, W., Kofron, J. L., Mellon, W. S., Royer, C. A., and Rich, D. H. (1992) *J. Biol. Chem.* **267**, 22054–22059
35. Schote, U., Ganz, P., Fahr, A., and Seelig, J. (2002) *J. Pharm. Sci.* **91**, 856–867
36. Etzkorn, F. A., Chang, Z. Y., Stolz, L. A., and Walsh, C. T. (1994) *Biochemistry* **33**, 2380–2388
37. Mikol, V., Kallen, J., Pfahl, G., and Walkinshaw, M. D. (1993) *J. Mol. Biol.* **234**, 1119–1130
38. Dumont, F. J., Staruch, M. J., Kopra, S. L., Melino, M. R., and Sigal, N. H. (1999) *J. Immunol.* **162**, 251–258
39. Hultsch, T., Albers, M. W., Schreiber, S. L., and Hohman, R. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 6229–6233
40. Fanghanel, J., and Fischer, G. (2003) *Biophys. Chem.* **100**, 351–366
41. Li, W., Llopis, J., Whitney, M., Zlokarnik, G., and Tsien, R. Y. (1998) *Nature* **392**, 936–941
42. Kiani, A., Rao, A., and Aramburu, J. (2000) *Immunity* **12**, 359–372
43. Furuta, T., Wang, S. S., Dantzker, J. L., Dore, T. M., Bybee, W. J., Callaway, E. M., Denk, W., and Tsien, R. Y. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 1195–1200
Unexpected Side Chain Effects at Residue 8 of Cyclosporin A Derivatives Allow Photoswitching of Immunosuppression
Yixin Zhang, Frank Erdmann, Ria Baumgrass, Mike Schutkowski and Gunter Fischer

J. Biol. Chem. 2005, 280:4842-4850.
doi: 10.1074/jbc.M409178200 originally published online November 30, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409178200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/12/16/M409178200.DC1

This article cites 43 references, 15 of which can be accessed free at
http://www.jbc.org/content/280/6/4842.full.html#ref-list-1