Structure of Human Cyclophilin A in Complex with the Novel Immunosuppressant Sanglifehrin A at 1.6 Å Resolution*

Received for publication, February 11, 2005, and in revised form, March 9, 2005
Published, JBC Papers in Press, March 16, 2005, DOI 10.1074/jbc.M501623200

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Sanglifehrin A (SFA) is a novel immunosuppressant isolated from Streptomyces sp. that binds strongly to the human immunophilin cyclophilin A (CypA). SFA exerts its immunosuppressive activity through a mode of action different from that of all other known immunophilin-binding substances, namely cyclosporine A (CsA), FK506, and rapamycin. We have determined the crystal structure of human CypA in complex with SFA at 1.6 Å resolution. The high resolution of the structure revealed the absolute configuration at all 17 chiral centers of SFA as well as the details of the CypA/SFA interactions. In particular, it was shown that the 22-membered macrocycle of SFA is deeply embedded in the same binding site as CsA and forms six direct hydrogen bonds with CypA. The effector domain of SFA, on the other hand, has a chemical and three-dimensional structure very different from CsA, already strongly suggesting different immunosuppressive mechanisms. Furthermore, two CypA-SFA complexes form a dimer in the crystal as well as in solution as shown by light scattering and size exclusion chromatography experiments. This observation raises the possibility that the dimer of CypA/SFA complexes is the molecular species mediating the immunosuppressive effect.

More than 2 decades ago, the discovery of cyclosporine A (CsA) allowed spectacular progress in the field of organ transplantation (1). Since then the search for novel immunosuppressants that interfere with T cell activation/proliferation has resulted in the isolation of several new molecules from natural sources (2). The most prominent examples are FK506 and rapamycin. Besides their important therapeutic use, these drugs have also proven to be powerful tools for dissecting signal transduction pathways at the molecular level (3). It has been shown that the biological activity of CsA, FK506, and rapamycin is mediated by intracellular binding proteins called immunophilins. CsA binds to cyclophilins (e.g. cyclophilin A (CypA)), whereas FK506 and rapamycin bind to FK506-binding proteins (e.g. FK506-binding protein-12). Immunophilin binding is required but not sufficient for the immunosuppressive activity of these drugs. Each complex interacts with a third partner, the effector protein, to achieve the full biological response. Thus, the CypA-CsA and FK506-binding protein-12-FK506 complexes inhibit the serine/threonine phosphatase activity of calcineurin, thereby blocking the production of cytokines, including interleukin-2 (4). The FK506-binding protein-12-rapamycin complex inhibits the kinase FK506-binding protein-12-rapamycin-associated protein kinase (also known as RAPT or mTOR (5)) that is involved in interleukin-2 receptor-mediated T cell proliferation (6).

We have screened microbial broth extracts to identify CypA ligands with an immunosuppressive mechanism different from CsA (i.e. searching for the “rapamycin counterpart,” which binds to CypA). This search resulted in the isolation from Streptomyces sp. A92–308110 of a new class of compounds named sanglifehrins (7, 8). Among the 20 different sanglifehrins isolated so far, sanglifehrin A (SFA) is the most abundant component (Fig. 1A). The affinity of SFA for CypA in a cell-free assay is remarkably high (IC50 = 6.9 nM) (i.e. approximately 60-fold higher than that of CsA) (IC50 = 420 nM) (9). Furthermore, SFA displays potent immunosuppressive activity in the murine mixed lymphocyte reaction (IC50 = 170 nM), an in vitro immune response assay (9). The details of the mechanism by which SFA exerts its immunosuppressive activity at the molecular level are still under intense investigation. The first results describing the effects of SFA on T cells (10–12) and dendritic cells (13, 14) were published in the last few years. These data clearly indicate that SFA acts via a mode of action that is different from that of all other known immunophilin-binding compounds, namely CsA, FK506, and rapamycin.

In addition to its interesting biological profile, SFA has a unique chemical structure. The compound consists of a 22-membered macrocyclic, bearing in position 23 a nine-carbon tether terminated by a highly substituted spirobicyclic moiety. The macrolide contains an E,E-diene, a short polypropionate fragment, and a tripeptide unit composed of valine and two unusual amino acids, piperazic acid and meta-tyrosine. It is the β-nitrogen of piperazic acid that is involved in the amide bond formation, which stands in contrast to all other piperazic acid containing natural products isolated so far. The unique chemical structure of sanglifehrin combined with its immunosuppressive activity generated broad interest. Chemical efforts resulted in the synthesis of several fragments and finally culminated with the total syntheses by Nicolaou et al. (15) and Paquette et al. (16). Finally, degradation and synthetic work on the SFA macrocycle, together with x-ray analysis of the 22-membered macrocycle alone bound to CypA, has been published recently (17).
human CypA and SFA. The high resolution of the x-ray data allowed the determination of the absolute configuration of all chiral centers of SFA and revealed the details of the CypA/SFA interactions. Furthermore, the structure of the CypA-SFA complex was compared with the structure of the CypA-CsA complex, since both immunosuppressants bind to the active site of CypA. Also, the finding that the CypA-SFA complex is present as a dimer in the crystal as well as in solution raises the possibility that the immunosuppressive effect of SFA might be mediated through a dimer of CypA-SFA complexes.

MATERIALS AND METHODS

Protein Preparation, Crystallization, and Data Collection—Human CypA was purified to homogeneity and SFA was obtained as described (7). The complex CypA-SFA was prepared by adding the ligand (100 mM solution in ethanol) in a 3-fold molar excess to the protein solution (12 mg/ml cyclophilin A, 20 mM NaCl, 20 mM Heps, 0.02% NaN₃, pH 7.0). Crystallization was performed using a standard vapor diffusion hanging drop set-up, with VDX crystallization plates and microtiter crystallization droplets. The crystals were obtained at 4°C in a reservoir solution containing 1.0 M magnesium acetate, 50 mM sodium cacodylate, pH 6.5, and 30% (w/v) polyethylene glycol 4000. The crystals were grown in a glass capillary for data collection at 20°C. X-ray diffraction data (347 frames of 1° rotation) were collected with a Rigaku rotating-anode generator (1.8 Å r.m.s. deviation bond angles (Å) 0.007
r.m.s. deviation bond angles (degrees) 1.183

| Parameters | Values |
|------------|--------|
| Reflections | |
| Measured (n) | 179,130 |
| Unique (n) | 41,593 |
| R pour | 0.075 (0.456) |
| Completeness (%) | 99.2 (100.0) |
| (I/σ(I)) a | 16.4 (1.9) |
| Refinement | |
| Resolution range (Å) a | 15.0 to 1.60 (1.64 to 1.60) |

* Numbers in parentheses are calculated using data from the highest resolution bin.
* Noncrystallographic symmetry.

The resulting 2F o - F c and F o - F c maps were of excellent quality for the missing ligands and allowed the clear determination of the absolute configurations at all 17 chiral centers of SFA. Further refinement with REFMAC version 5.0 (23), water insertion with ARP/wARP (24), and rebuilding steps yielded the final model, consisting of two complexes CypA-SFA (including residues 2–165 of CypA) and a total of 361 water molecules, which has an R-factor of 16.3% and R free of 18.5% (no σ cut-off, 15 to 1.60 Å, working set of 39,452 unique reflections, test set of 2096 reflections).

The quality of the model was assessed with PROCHECK version 3.3 (25) and REFMAC. 86.5% of the amino acids are in the most favored region, and 13.5% are in the additionally allowed region of the Ramachandran plot (no amino acids are in disallowed regions). The overall G-factor is 0.16, with r.m.s. bond lengths of 0.007 Å and r.m.s. bond angles of 1.18°. Buried solvent-accessible areas were calculated with the program AREAIMOL as implemented in the CCP4 suite (19), using a probe radius of 1.4 Å.

Dynamic Light Scattering and Size Exclusion Chromatography—Dynamic light scattering experiments were performed with a DynaPro-801 molecular sizing instrument in conjunction with the software package “Dynamics (version 3.30),” both from Protein Solutions Ltd. The complexes were prepared as 1:1 mixtures of CypA and ligand (both at 56 μM) in 100 mM NaCl, 20 mM HEPES, pH 7.4, and measurements were performed at 20°C.

Size exclusion chromatography to detect monomer/dimer formation was done by injecting samples of 40-μl total volume containing 29 μM CypA and 126 μM ligand (or no ligand, as a reference) at pH 7.0. The collected fractions with CypA (detected at 210, 254, and 280 nm) were then subjected to liquid chromatography/mass spectrometry analysis for verification of complex formation and molecular weight of ligand.

RESULTS

The SFA-binding Site—We have determined the three-dimensional structure of CypA bound to SFA by x-ray crystallography. The 1.6 Å resolution data were phased by molecular replacement using CypA (21) as a search model, yielding a dimer of CypA-SFA complexes as solution. The atomic structure includes residues 2–165 of two CypA molecules, two SFA...
ligands and a total of 361 water molecules. The results of the crystallographic refinement are summarized in Table I. The high resolution of the x-ray data allowed the determination of the absolute configuration of all 17 asymmetric centers of the macrocyclic and spirobicyclic fragments of SFA (Fig. 1, A and B).

CypA is an enzyme that catalyzes the cis-trans isomerization of prolyl peptide bonds (1). Like CsA, SFA binds to the active site of CypA (Fig. 2). In particular, the piperazic acid (Pip) moiety of SFA occupies the same hydrophobic pocket of CypA that is used by the proline ring of the model substrate Ala-Ala-Pro-Ala (26), by the proline ring of dipeptides (27) and by the side chain of Me-Val11 for CsA (1, 28) (Fig. 3, A and B). This hydrophobic pocket is formed by the amino acids Phe60, Met61, Phe113, and Leu122 of CypA. The tripeptide moiety Pip-meta-Tyr-Val of SFA forms six direct hydrogen bond interactions (cut-off 3.2 Å) with four amino acids of the protein (Fig. 3). Three of these amino acids (Arg55, Gln63, and His126) make intermolecular hydrogen bonds through their side chains, whereas one amino acid (Asn102) forms an antiparallel β-sheet interaction with the ligand through its main chain. In detail, these hydrogen bonds are located between NE2-His126 and the meta-tyrosine hydroxy group (2.71 Å/2.73 Å), between the main-chain nitrogen of Asn102 and the main-chain carboxyl oxygen of meta-tyrosine (2.99 Å/2.96 Å), between the main-chain carboxyl oxygen of Asn102 and the main-chain nitrogen of meta-tyrosine (2.80 Å/2.85 Å), between NE2-Gln63 and the main-chain carboxyl oxygen of valine (3.03 Å/3.03 Å), between OE1-Gln63 and the α-nitrogen of the piperazic acid (3.06 Å/3.10 Å), and between NH2-Arg55 and the main-chain carboxyl oxygen of valine (3.21 Å/3.19 Å). In the latter list, distances are given between heteroatoms in the two independently refined complexes of the asymmetric unit (the averaged values are displayed in Fig. 3 A). In addition, SFA makes two water-mediated hydrogen bonds to the protein, namely between the main-chain carboxyl oxygen of valine and NH1-Arg55, NE2-His54, and CO-Gly72 and between the C31 hydroxyl and N-Trp121. The latter interaction and/or the side-chain reorientation of Trp121 enforced by the binding of SFA (see below) could account for the change in Trp121 fluorescence described previously (29). The 3-oxo-butyl side chain attached at C14 extends on the surface of the complex and does not form important interactions with the protein. The E,E-diene region C18–C22 of the macrocycle is not involved in direct contacts with the protein but forms vdW contacts with the meta-tyrosine of SFA in the dyad-related monomer. The linker region C24-C32 between the macrocycle and the spirobicyclic moiety makes vdW contacts with side-chain atoms from amino acids Ile57, Phe60, Thr119, and Trp121. The presence of the linker region of SFA imposes a side-chain reorientation on Trp121, compared with unliganded CypA.
from Ile57 and Phe60. Upon complex formation of the monomer, a total of 1232/1225 Å² of the solvent-accessible surface from CypA and SFA is buried (547/547 Å² from the protein molecules and 685/678 Å² from the SFA ligands). In the latter list, values are given for the two independently refined complexes of the asymmetric unit.

The following amino acids have a nonhydrogen atom closer than 4 Å to the ligand SFA in both independently refined monomers: Arg55, Ile57, Gly59, Phe60, Met61, Gln63, Gly72, Thr73, Ala101, Asn102, Ala103, Gln111, Phe113, Thr119, and His126. The average $B$-factor for the ligand (11.1 Å²) is lower than the average $B$-factor for the protein (15.4 Å²), consistent with the fact that excellent electron density for all nonhydrogen atoms of SFA is visible. The most flexible regions of SFA, with $B$-factors $> 15$ Å², are the 3-oxo-butyl side chain and the ethyl group attached to the spirobicycle at C40.

Overall, the x-ray crystal structure of SFA bound to CypA confirms that the affinity of the ligand for CypA is primarily mediated by the 22-membered macrocycle, whereas the spiropcyclic unit remains essential for the immunosuppressive activity.

**Comparison of the CypA-SFA and CypA-CsA Complexes: A Case of Induced Fit and Binding with Opposite Peptide Backbone Directions**—A least squares superposition of CypA in complex with SFA (monomer A in the present structure) and CsA (accession code 1CWA), using residues 2–165, is 0.30 Å. The largest difference in the position of backbone atoms occurs for the 68–73 loop, with maximal deviations of 1.7 and 1.4 Å for the Cos of Asn71 and Gly72, respectively. In addition, there are two important side-chain differences in the ligand binding pockets that occur for Arg55 (Fig. 3, A and B) and Trp121. These latter differences are essential for binding the two respective ligands. The side chain of Arg55 mediates a hydrogen bond with the carbonyl oxygen of valine (SFA), rotates into a new position in the CypA-CsA complex in order to form a hydrogen bond with the carbonyl oxygen of MeLeu12 (CsA) and to avoid a steric clash with Val15 (CsA). In return, the latter position of Arg55 from the Cyp-CsA complex would lead to a steric clash with the ester and diene groups of SFA. The side chain of...
Val for SFA superimpose with the carbonyl oxygens of MeVal\(^{11}\) and MeBmt\(^{1}\) for CsA, respectively (gen bond with NE2-Gln\(^{63}\), superimpose. The situation is very O-Asn\(^{102}\) (Fig. 3). The piperazine ring and the valine side chain of SFA superimpose with the CsA side chains of MeVal\(^{11}\) and Abu\(^{1}\), respectively.

Interestingly, the parts of the backbone moieties of the tripeptides Pip-meta-Tyr-Val (SFA) and MeVal\(^{11}\)-MeBmt\(^{1}\)-Abu\(^{2}\) (CsA) that undergo key interactions with CypA superimpose closely in space, although the peptide directionality is from left to right for CsA and from right to left for SFA. The main-chain carbonyl oxygens of meta-Tyr and Val for SFA superimpose with the carbonyl oxygens of MeVal\(^{11}\) and MeBmt\(^{1}\) for CsA, respectively (cf. black circles). The main-chain nitrogens of meta-Tyr (SFA) and Abu\(^{2}\) (CsA), although not coincident (cf. black arrows), are both located such that they can accept a hydrogen bond from O-Asn\(^{102}\) (Fig. 3). The piperazine ring and the valine side chain of SFA superimpose with the CsA side chains of MeVal\(^{11}\) and Abu\(^{1}\), respectively.

Trp\(^{121}\) rotates into a new position in the CypA-CsA complex in order to form a hydrogen bond with the carbonyl oxygen of MeLeu\(^{9}\) (CsA). In return, the latter position of Trp\(^{121}\) from the CypA-CsA complex would lead to a steric clash with the linker that extends between the macrocycle and spirobicycle of SFA. These two fundamental side-chain adaptations can be regarded as examples of induced fit in order to bind two chemically very different ligands.

Interestingly, the parts of the backbone moieties of the tripeptides Pip-meta-Tyr-Val (SFA) and MeVal\(^{11}\)-MeBmt\(^{1}\)-Abu\(^{2}\) (CsA) that undergo key interactions with CypA superimpose closely in space, although the peptide directionality is opposite (Fig. 4). In particular, the carbonyl groups of valine (SFA) and MeBmt\(^{1}\) (CsA), both mediating the hydrogen bond with NE2-Gln\(^{63}\), superimpose. The situation is very similar for the carbonyl groups of meta-Tyr (SFA) and MeVal\(^{11}\) (CsA), both mediating the hydrogen bond (weaker in the case of CsA) with the backbone nitrogen N-Asn\(^{102}\). Also, the backbone nitrogens of meta-Tyr (SFA) and Abu\(^{2}\) (CsA) are positioned in such a way that they both can mediate a hydrogen bond with CO-Asn\(^{102}\).

Whereas both immunosuppressants SFA and CsA, despite their completely different chemical structures, fit nicely into the active site of CypA, their effector domains are clearly very different, already strongly suggesting that the respective immunosuppressive mechanisms are different. Indeed, a superposition with the ternary complex CypA-SFA complexes, respectively (cf. black circles). The main-chain nitrogens of meta-Tyr (SFA) and Abu\(^{2}\) (CsA), although not coincident (cf. black arrows), are both located such that they can accept a hydrogen bond from O-Asn\(^{102}\) (Fig. 3). The piperazine ring and the valine side chain of SFA superimpose with the CsA side chains of MeVal\(^{11}\) and Abu\(^{1}\), respectively.

Structure of the Local Dimer of CypA-SFA Complexes: The Dimer Hypothesis—The two CypA-SFA complexes are related by an approximate local dyad axis (rotation angle = 176°, calculated from the least squares superposition of protomers A and B using residues 2–165), which is located between the two SFA ligands and oriented almost parallel to the longest dimension of SFA (Fig. 5, A and B). The slight disturbance of the C2 symmetry can be explained by crystal packing (see below). With the exception of the spirobicyclic and \(\alpha\)-ketobutyrate moieties (which extend toward the outside of the dimeric complex), all of the remaining parts of the SFA ligands are deeply buried in the dimer. Upon dimer formation, a total of 1504 Å\(^{2}\) of the solvent-accessible surface from the two monomers is buried (721 Å\(^{2}\) from the protein molecules and 783 Å\(^{2}\) from the SFA ligands). Interestingly, these values are even larger than the ones for monomer complex formation itself (see above). The two SFA ligands make extensive vdW contacts with each other along the local dyad. In particular, the meta-tyrosine side chain is in close proximity to the \(E,E\)-diene fragment of the neighboring SFA molecule, and the C26–C28 fragments form vdW contacts with each other. In addition, a number of water-mediated hydrogen bonds are formed between the two SFA ligands (e.g. between the C41 carbonyl oxygen and the C31 hydroxy group), between a SFA ligand and the neighboring protein molecule of the dimer (e.g. between the C17 hydroxy group and the Asn\(^{102}\) side chain), and between the two protein molecules. Finally, a direct hydrogen bond links NE1-Trp\(^{A121}\) and CO-ArgB\(^{148}\) (distance 3.1 Å).
The two CypA-SFA complexes in the asymmetric unit were refined without noncrystallographic symmetry restraints. They show an r.m.s. deviation of 0.26 Å for the Ca positions of residues 2–165 after least squares superposition. The main side-chain differences in proximity of the SFA binding site occur for Trp<sub>121</sub>, Lys<sub>125</sub>, and Arg<sub>148</sub>. These differences can be explained by differences in crystal contacts: for protomer A, Arg<sub>148</sub> is involved in a salt bridge with Glu<sub>B</sub><sup>148</sup> of a neighboring protomer B in the lattice and thus adopts a different side-chain conformation than Arg<sub>B</sub><sup>148</sup>. As a consequence, Trp<sub>B</sub><sup>121</sup>, which stacks on Arg<sub>B</sub><sup>148</sup>, adopts a different side-chain conformation than Trp<sub>A</sub><sup>121</sup>. Finally, Lys<sub>A</sub><sup>125</sup>, which stacks on Trp<sub>A</sub><sup>121</sup>, adopts a different side-chain conformation than Lys<sub>B</sub><sup>125</sup>. Since the side-chain conformations of Trp<sub>A</sub><sup>121</sup> and Lys<sub>B</sub><sup>125</sup> are not influenced by crystal packing, they probably represent the state for the dimer in solution.

The fact that the crystallographically observed CypA-SFA dimer is stabilized by numerous interactions raised the possibility that this dimer also exists in solution. Toward this end, monomer/dimer formation was followed by size exclusion chromatography as well as dynamic light scattering. Using the latter method, CypA alone has an apparent molecular mass of 14 kDa, the actual mass being 18 kDa. In contrast, the apparent molecular mass of the CypA-SFA complex was 31 kDa, clearly confirming the existence of a dimer in solution. In a further experiment, a SFA derivative was tested that retains a high affinity for CypA but for which dimer formation is predicted to be impaired. The apparent molecular mass of (283,27S)-dihydroxysanglifehrin A (33) in complex with CypA is 17 kDa, confirming the prediction, based on the crystal structure, that the two additional hydroxyl groups would lead to steric clashes with themselves and thus block dimerization (in the intact dimer, the atoms C26 and C27 are at distances of 3.8 and 4.0 Å with their corresponding partners) (Fig. 5B). Also, size exclusion chromatography (followed by liquid chromatography/mass spectroscopy for verification of complex formation and molecular mass of ligand) showed that CypA complexed with the latter derivative displayed a retention time (6.5–7 min), which is very similar to unliganded CypA and CypA-CsA and thus corresponds to a monomer. CypA bound to the 22-membered SFA macrocycle (17) alone showed the same result, again indicating a monomer. The complex CypA-SFA, on the other hand, had a clearly shorter retention time of 5 min, which indicates probable dimer formation.

The spirobicycle linker, which is missing in the case of the 22-membered SFA macrocycle alone, mediates important vdW contacts within the dimer and also induces a side-chain conformation of Trp<sub>121</sub> (Fig. 2), which enables interactions with the other monomer. The latter influence on Trp<sub>121</sub> could be denoted as the “Trp switch,” which yields the intersubunit hydrogen bond between NE1-Trp<sub>121</sub> and O-Arg<sub>B</sub><sup>148</sup>. Importantly, both derivatives of SFA (dihydroxy-SFA and the macrocycle) lack immunosuppressive activity in the murine mixed lymphocyte reaction, although their affinity for CypA is unaffected (Table II). 42-N-methyl-SFA, on the other hand, is an example of a derivative retaining immunosuppressive activity (Table II) and still forming a dimer in solution. The apparent molecular mass of the CypA-42-N-methyl SFA complex using the dynamic light scattering method was 26 kDa. Taken together, these results suggest that the ability of SFA derivatives to form a dimer similar to the one observed by crystallography might be related to their immunosuppressive activity as measured in the murine mixed lymphocyte reaction assay.

DISCUSSION

The present report describes the x-ray structure of the novel immunosuppressive natural product SFA bound to human CypA at 1.6 Å. The x-ray structure not only allowed the determination of the absolute configuration at all chiral centers of SFA but also revealed the details of the binding mode of SFA to CypA. In particular, it showed that the macrocycle of SFA forms six direct hydrogen bonds with CypA. The x-ray structure also revealed that the chemical structure of SFA is nicely suited to enable strong interactions with the active site of CypA (or possibly of CypA homologs possessing similar binding sites).

The hydrogen bond to NE2-His<sup>125</sup>, for example, can only be formed because the hydroxyl group on the tyrosine side chain is not in the usual para-position but rather in the meta-position of the aromatic ring. The removal of this hydroxyl group leads to a significant reduction in the affinity for CypA as measured in a cell-free assay, as well as to substantial loss of the immunosuppressive activity (34). Similarly, the hydrogen bond with the side-chain atom OE1-Glu<sup>25</sup> is only possible because the β-nitrogen of the piperazic acid, instead of the usual α-nitrogen, is involved in the amide bond formation. This feature is unique among all piperazic acid-containing natural products.

The observation that two CypA-SFA complexes form an intimate dimer in the crystal and in solution has led to the hypothesis that this dimer formation is related to the immunosuppressive effect. Again, many aspects of the chemical structure of SFA are nicely tailored toward promoting dimer formation. For instance, the meta-tyrosine ring forms vdW interactions with the E,E-diene of the other monomer, and the linker to the spirobicycle induces an important modification of the side chain conformation of Trp<sub>121</sub>. The dimer hypothesis states that SFA derivatives that induce a dimeric structure similar to the CypA-SFA complex should retain immunosuppressive activity, whereas SFA derivatives that block dimer formation (or form a different dimer) are not immunosuppressive. In summary, this x-ray structure assists in clarifying the mechanism of action of SFA and may help to identify the effector protein mediating the biological effects of SFA.

Acknowledgments—We thank J. Causevic and M. Zurini for protein purification and light scattering experiments as well as G. Hegy for size exclusion chromatography and liquid chromatography/mass spectroscopy experiments. We thank C. Maurer, C. Vedrine, and V. Quesniaux for CsA/CypA ELISA measurements. Experimental assistance from the staff of the Swiss-Norwegian Beam Lines at the European Synchrotron Radiation Facility, Grenoble, is acknowledged. We thank M. Walkinshaw for initial interest and discussions in the project.

REFERENCES

1. Kallen, J., Mikol, V., Quesniaux, V. F. J., Walkinshaw, M. D., Schneider-Scherzer, E., Schoergendorfer, K., Weber, K., and Firl, H. G. (1997) Biotechnology, Vol. 7 (Rehm, H.-J., and Reed, G., eds) pp. 536–591, VCH, Weinheim, Germany
2. Anderson, P. S., Kenyon, G. L., and Marshall, G. R. (eds) Perspectives in Drug Discovery and Design, Vol. 2 (1994) pp. 3–248, ESCOM Science Publishers, Leiden, The Netherlands
3. Schreiber, S. L., and Crabtree, G. R. (1992) Immunol. Today 13, 136–142
4. Liu, J., Farmer Jr., J. D., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1993) Cell 66, 807–815
5. Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) Nature 369, 756–758
6. Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G., and Schreiber, S. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87,
7. Sanglier, J.-J., Quesniaux, V., Fehr, T., Hofmann, H., Mahnke, M., Memmert, K., Schuler, W., Zenke, G., Geschwind, L., Maurer, C., and Schilling, W. (1999) *J. Antibiot.* **52**, 466–473
8. Fehr, T., Kallen, J., Oberer, L., and Sanglier, J.-J. (1999) *J. Antibiot.* **52**, 474–479
9. Zenke, G., Strittmatter, U., Fuchs, S., Quesniaux, V. F. J., Brinkmann, V., Schuler, W., Zarini, M., Enz, A., Billich, A., Sanglier, J.-J., and Fehr, T. (2001) *J. Immunology* **166**, 7165–7171
10. Zhang, L.-H., and Liu, J. O. (2001) *J. Immunology* **166**, 5611–5618
11. Zhang, L.-H., Youn, H.-D., and Liu, J. O. (2001) *J. Biol. Chem.* **276**, 43534–43540
12. Allen, A. Zheng, Y., Gardner, L., Safford, M., Horton, M. R., and Powell, J. D. (2004) *J. Immunology* **172**, 4797–4803
13. Steinschulte, C., Taner, T., Thomson, A. W., Bein, G., and Hackstein, H. (2003) *J. Immunology* **171**, 542–546
14. Wolfman, A. M., Schlagwein, N., van der Kooij, S. W., and van Kooten, C. (2004) *J. Immunology* **172**, 6482–6489
15. Nicolau, K. C., Murphy, F., Barluenga, S., Ohshima, T., Wei, H., Xu, J., Gray, D. L. F., and Baudoin, O. (2000) *J. Am. Chem. Soc.* **122**, 3830–3838
16. Paquette, L. A., Duan, M., Konekti, I., and Kempmann, C. A. (2002) *J. Am. Chem. Soc.* **124**, 4257–4270
17. Sedrani, R., Kallen, J., Cabrejas, L. M., Papageorgiou, C. D., Senia, P., Rohrbach, S., Wagner, D., Thai, B., Erne, A. M., France, J., Berer, L., Rihs, G., Zenke, G., and Wagner, J. (2003) *J. Am. Chem. Soc.* **125**, 3849–3859
18. Otwinowski, Z., and Minor, W. (1997) *Methods Enzymol.* **276**, 307–326
19. Collaborative Computational Project 4 (1994) *Acta Crystallogr.* Sect. *D* **50**, 760–763
20. Bruenger, A. T. (1993) *X-PLOR Version 3.1: A System for X-ray Crystallography and NMR*, Yale University Press, New Haven, CT
21. Kallen, J., Spitzfaden, C., Zarini, M., Wider, G., Widmer, H., Wuethrich, K., and Walkinshaw, M. D. (1991) *Nature* **353**, 276–278
22. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr.* Sect. *A* **47**, 110–119
23. Murshudov, G., Vagin, A. A., and Dodson, E. J. (1997) *Acta Crystallogr.* Sect. *D* **53**, 240–255
24. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) *Nature Struct. Biol.* **6**, 458–463
25. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* **26**, 307–326
26. Collaborative Computational Project 4 (1994) *Acta Crystallogr.* Sect. *D* **50**, 760–763
27. Zhao, Y., and Ke, H. (1996) *Biochemistry* **35**, 7362–7368
28. Mikol, V., Kallen, J., Pfuegl, G., and Walkinshaw, M. (1993) *J. Mol. Biol.* **234**, 1119–1130
29. Pemberton, T. J., and Kay, J. E. (2003) *FEBS Lett.* **555**, 335–340
30. Ke, H. (1992) *J. Mol. Biol.* **228**, 539–550
31. Huai, Q., Kim, H. Y., Liu, Y., Zhao, M., Mandragon, A., Liu, J. O., and Ke, H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12037–12042
32. Jin, L., and Harrison, S. C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 13522–13526
33. Metternich, R., Denni, D., Thai, B., and Sedrani, R. (1999) *J. Org. Chem.* **64**, 9632–9639
34. Baenste, R., Wagner, J., and Zenke, G. (2001) *Bioorg. Med. Chem. Lett.* **11**, 1609–1612
