subcloned into TBP derivatives into which we had inserted the relevant restriction sites. For oligo N4, the Kpn I site used for cloning was introduced by a second round of PCR with the following primer that overlaps the 5′ constant region of the mutated oligonucleotide (N4Kpn): TCT.ACT.*GGT.ACC*.-CCA.TCA.CAT.TTT.TCT.AAA.TTC.ACT.TAG.CAC.-AGG. None of the introduced sites changed the amino acid sequence of TBP. For oligo N1, the PCR fragment was digested with Bgl II; because the Bgl II site would have altered the protein sequence, we introduced a compatible Bcl I site into the TBP gene. The ligation product does not regenerate either site, and the amino acid sequence is not changed. For oligo N6, the PCR fragment was digested with Bam HI and cloned into the naturally occurring Bgl II site in TBP. All six libraries were constructed in a previously described *TRP1* centromeric vector that carried TBP and its own promoter on a 2.4-kb genomic fragment (*31*).

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25. Each library was transformed into yeast strain BYΔ2 (*31*), which contains a deleted TBP locus and a *URA3* centromeric plasmid that carried wild-type TBP; the plasmid encoding wild-type TBP can be removed from the resulting transformants by growth on 5-fluoro-orotic acid, which is selectively toxic to *URA3*$^+$ cells. To screen for temperature-sensitive mutants, we replica-plated transformants on medium that contained 5-fluoro-orotic acid at 23°C, 30°C, and 37°C.
26. The transcriptional patterns of A226T, V122S, and three double mutants were indistinguishable from that of F237R, which is shown in Fig. 3. Similar but less pronounced transcriptional patterns were observed for A226V, I230S, V232H, V232G, and V232K (*28*).
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29. The patch is defined by residues 226, 230, 232, and 237. Residue 122 (defined by V122S) (*28*) maps in the DNA binding region of TBP. The Pol II–specific phenotype of V122S may reflect a DNA binding defect similar to that observed in other TBP derivatives (*3*).
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Structure-Based Design of a Cyclophilin-Calcineurin Bridging Ligand

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The affinity of a flexible ligand that adopts a specific conformation when bound to its receptor should be increased with the appropriate use of conformational restraints. By determining the structure of protein-ligand complexes, such restraints can in principle be designed into the bound ligand in a rational way. A tricyclic variant (TCsA) of the immunosuppressant cyclosporin A (CsA), which inhibits the proliferation of T lymphocytes by forming a cyclophilin-CsA-calcineurin complex, was designed with the known three-dimensional structure of a cyclophilin-CsA complex. The conformational restraints in TCsA appear to be responsible for its greater affinity for cyclophilin and calcineurin relative to CsA.

Realizing the full impact of structural biology on basic research and medicine will require the ability to design high-affinity ligands to biological receptors with the use of structural information (*1*). One approach is to constrain the bound conformation of inherently flexible ligands by introducing structure-guided restraints into the ligand. In this way, greater thermodynamic stability of the receptor-ligand complex can be anticipated because the conformational entropy is reduced in the formation of the complex. A particularly challenging problem is posed by the immunosuppressive agents cyclosporin A (CsA), FK506, and rapamycin. These compounds are comprised of two distinct protein-binding surfaces that allow them to bind two proteins simultaneously. By enabling these multimeric complexes to form, the immunosuppressants inhibit specific signaling pathways that control the cell cycle (*2*). In the case of CsA, this property is the basis for the drug's revolutionary impact on clinical organ transplantation. Therefore, efforts to design novel "bridging" ligands not only serve to advance our understanding of the principles of structure-based ligand design, but also may lead to safer and more effective immunosuppressive agents.

It has been noted that the conformations of CsA and FK506, when bound to cyclophilin A and FK506-binding protein 12 (FKBP12), respectively, differ markedly from their unbound conformations in the solid state and in solution (*3*, *4*). Although the conformations adopted by these ligands while they are simultaneously bound to their immunophilin receptors and the protein phosphatase calcineurin have not yet been determined, residues that are important for calcineurin binding have been identified (*5*–*7*). We have used this information to prepare nonnatural immunophilin ligands that mediate immunophilin-ligand-calcineurin complex formation (*8*). We now report on the structure-based de-

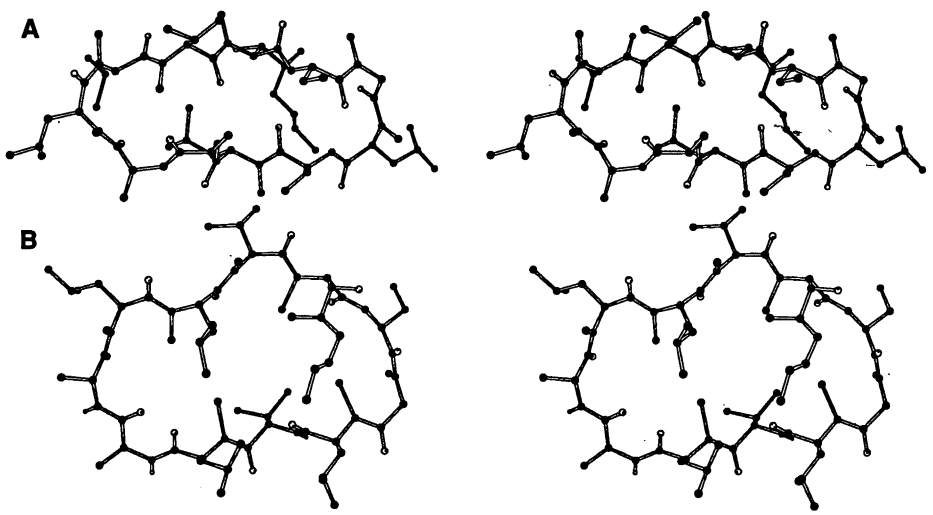

Fig. 1. (**A**) Stereodiagram of the unbound conformation of CsA in the solid state. (**B**) Stereodiagram of the cyclophilin A–bound conformation of CsA.

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sign of a tricyclic variant of CsA that exhibits enhanced bridging properties.

The structure of unbound CsA has been determined both in the solid state by x-ray crystallography (9) and in solution ($CHCl_3$) by nuclear magnetic resonance (NMR) methods (9–11). In both environments, the peptide forms a twisted β sheet that is comprised of four intramolecular hydrogen bonds and is capped by a type II′ β turn at Sar^3 and $MeLeu^4$. When CsA is bound to cyclophilin A (Fig. 1) (12, 13), the backbone of CsA is turned inside-out; the intramolecular hydrogen bonds and all elements of secondary structure observed in the unbound structure are absent. In light of these findings, it is evident why early efforts to prepare cyclophilin A ligands on the basis of the unbound conformation of CsA met with little success (14–16).

To avoid structural alterations of CsA that would interfere with binding, we chose to alter a segment that is not in contact with cyclophilin (17–19) and that can be modified without significant loss of immunosuppressive activity (20, 21) [that is, it can still bind to calcineurin (22)]. The L-Ala^7-D-Ala^8 dipeptide segment fulfills these criteria. Figure 2 shows the local structure of CsA residues 6 to 9 in their free and cyclophilin A–bound conformations. The relative orientations of the Cα–N and Cα–C bonds of residues L-Ala^7 and D-Ala^8, respectively, in the free and bound conformations are different. We desired a rigid template that would fix an amine substituent and a carboxyl moiety in the same relative orientations as found for these bonds in the bound CsA conformation. To facilitate the design process, we used the structural database searching program CAVEAT (23) to identify a rigid core structure (from the Cambridge structural database) to support these functional groups. The molecules identified by CAVEAT suggested that the bicyclic heterocycle **1** would provide an excellent framework for the amino and carboxyl groups (24). Superimposition of the amino and carboxyl moieties of **1** with the Cα–N bond of L-Ala^7 and the Cα–C bond of D-Ala^8, respectively, gives a root-mean-square deviation of 0.13 Å [within the errors inherent in the bound CsA structure (12, 13) (Fig. 2)].

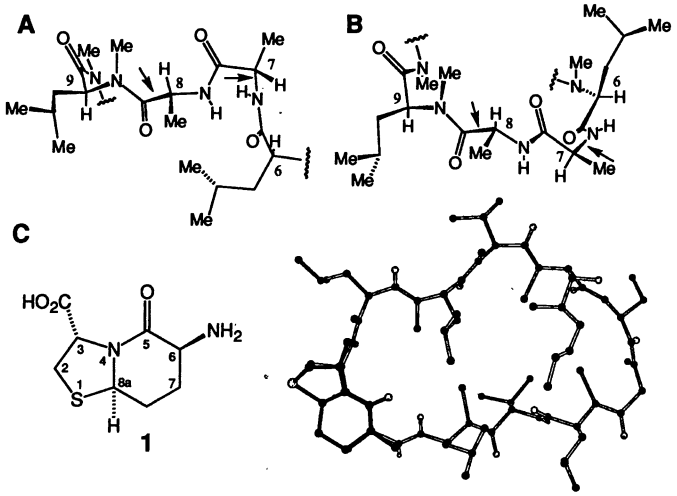

Fig. 2. (**A**) Local structure of CsA residues 6 to 9 in the unbound conformation. (**B**) Local structure of CsA residues 6 to 9 in the cyclophilin A–bound conformation. The arrows indicate the relative orientations of the Cα–N and Cα–C bonds of residues L-Ala^7 and D-Ala^8, respectively. (**C**) Heterocycle **1** superimposed on CsA (cyclophilin A–bound) residues L-Ala^7-D-Ala^8. Me, methyl group.

Heterocycle **7**, a protected derivative of **1**, was prepared in a manner analogous to a route developed by Bartlett and co-workers for the C6 epimer of **7** (Fig. 3) (25). Thus, the oxazolidinone **2** (26), which is derived from L-glutamic acid, was converted to aldehyde **4** (27). Condensation of this aldehyde with D-cysteine methyl ester provided the desired heterocycle **7** in 70% yield for the two steps, as well as a small amount of epimer **6**. The stereochemistry of **7** was assigned through its two-dimensional nuclear Overhauser enhancement spectroscopy (2D-NOESY) NMR spectrum (28). Cleavage of the carbobenzyloxy (Cbz) group in **7** and successive peptide coupling steps yielded **9**, which was condensed with hexapeptide **10** to give the linear peptide **11** in 95% yield. The COOH- and NH_2-termini of **11** were deprotected with trifluoroacetic acid (TFA) and the product was made cyclic. The conformationally biased target that we term tricyclic CsA (TCsA), in 41% yield, was the product of this reaction.

The TCsA molecule was designed to destabilize the ground-state conformation of CsA that is observed in organic solvents. Therefore, it is noteworthy that the NMR spectrum of TCsA ($CHCl_3$) is quite complicated, which indicates that the molecule exists in multiple, slowly interconverting conformations in solution. This observation is in contrast to the relatively simple spectrum observed for CsA ($CHCl_3$), where essentially only one conformation is populated (9), and it suggests that TCsA and CsA have different conformational properties.

We compared the cyclophilin A binding affinity of TCsA to that of CsA by determining each compound's ability to inhibit the rotamase activity of cyclophilin A (29). The affinity of TCsA [inhibition constant (K_i) = 2 ± 0.5 nM] for cyclophilin A is three times that of CsA for cyclophilin A (K_i = 6 ± 0.5

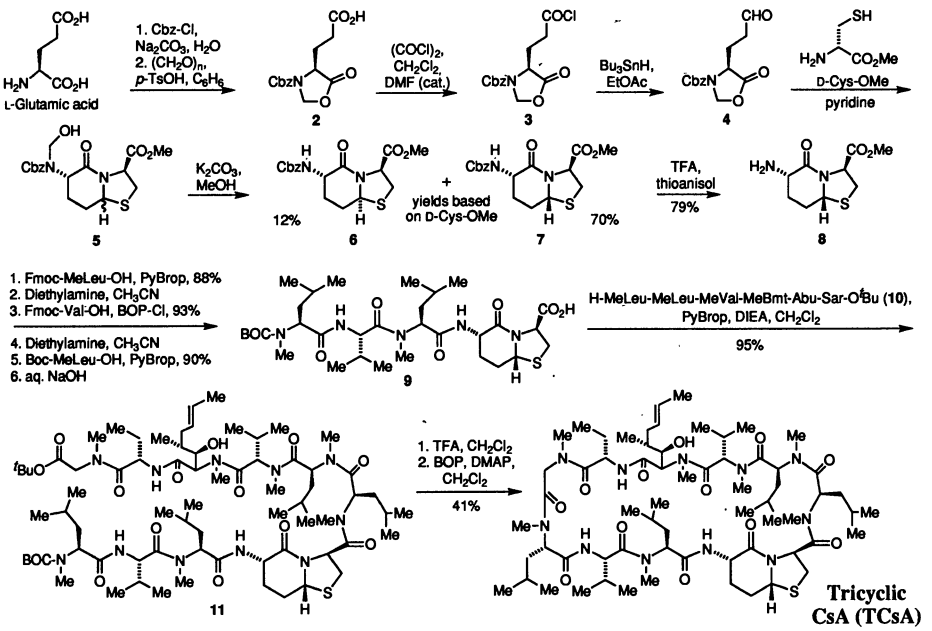

Fig. 3. Synthesis of TCsA. Cbz, carbobenzyloxy; *p*-TsOH, *p*-toluene sulfonic acid; DMF, dimethylformamide; EtOAc, ethyl acetate; TFA, trifluoroacetic acid; Fmoc, fluorenylmethyloxycarbonyl; Boc, *tert*-butoxycarbonyl; DIEA, diisopropylethylamine; DMAP, dimethylaminopyridine; PyBrop, bromotris-pyrrolidine-phosphonium hexafluorophosphate; Bmt, butenyl-methyl-threonine; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; BOPCl, *N,N*-bis(2-oxo-3-oxazolidinyl)phosphonic chloride; Bu, butyl group; and Me, methyl group.

nM) (*30*, *31*). Because the L-Ala^7-D-Ala^8 fragment of CsA makes no direct contact with cyclophilin A, it is reasonable to expect that the bicyclic fragment of TCsA also does not contact the protein. This expectation suggests that the greater binding affinity of TCsA is due to the conformational properties of TCsA induced by the bicyclic fragment, rather than the result of favorable direct contacts between the protein and the bicyclic fragment.

Although we were less certain that the L-Ala^7-D-Ala^8 region (of CsA) of the cyclophilin A–CsA complex does not contact calcineurin, the binding data suggest that this speculation has merit. The affinity of cyclophilin A–TCsA for calcineurin [K_i = 78 ± 14 nM; inhibition of phosphatase activity (*32*)] is approximately two to three times that of cyclophilin A–CsA for calcineurin (K_i = 198 ± 16 nM) (*33*). In addition, TCsA is twice as potent an inhibitor of the T cell receptor signaling pathway, as judged by its ability to inhibit the induction in a cellular assay of β-galactosidase activity regulated by the nuclear factor of activated T cells (NFAT) [50% inhibition concentration (IC_{50}) for TCsA = 2 nM; IC_{50} for CsA = 4 nM] (*34*, *35*). [NFAT activity appears to be dependent on the calcineurin-mediated dephosphorylation of the cytoplasmic component of NFAT (*36*–*38*).]

The collective inhibitory data from biochemical and cellular assays reveal a systematic enhancement in the cyclophilin A– and calcineurin-binding properties of TCsA relative to those of CsA. The enhancement is small (two- to threefold) but statistically meaningful. The basis for the enhancement is most likely the reduced entropic cost of binding resulting from the designed conformational restraints (conformation entropy). Thus, the results demonstrate the feasibility of using structural information to improve the already high affinity interactions provided by a natural system. The degree of enhancement, however, is a reminder of the enormous complexity of receptor-ligand interactions in the solution phase. For example, it is a significant challenge to correctly anticipate the differential effect of the molecular restraint in TCsA on the entropy of solvation or desolvation of the ligand versus that of the receptor-ligand complex (solvation entropy).

The structure-based studies described herein resulted in the preparation of a single target molecule. The TCsA compound is a structural variant of CsA with signaling inhibitory properties greater than those of CsA. These studies suggest that the analysis of a ligand bound to its receptor can provide valuable information for the rational design of new ligands. This capability augurs well for the structure-based design of new cellular probes and therapeutic agents.

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An Antiviral Soluble Form of the LDL Receptor Induced by Interferon

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Interferons, which induce several intracellular antiviral proteins, also induce an extracellular soluble protein that inhibits vesicular stomatitis virus (VSV) infection. This 28-kilodalton soluble protein was purified to homogeneity and identified by protein sequencing as the ligand-binding domain of the human 160-kilodalton low density lipoprotein receptor (LDLR). The existence of an antiviral soluble LDLR was confirmed by immunoaffinity chromatography with monoclonal antibody to LDLR. This soluble receptor mediates most of the interferon-triggered antiviral activity against VSV, apparently by interfering with virus assembly or budding, and not by inhibiting virus attachment to cells.

Interferons (IFNs) are virus- and mitogen-induced cytokines that function by several mechanisms to induce resistance against many types of viral infections. IFNs induce several proteins, some of which have a known role in mediating the antiviral state. Among the antiviral proteins induced are the Mx proteins, 2′,5′-oligoadenylate synthetase, protein kinase (P68), and the RNA binding protein 9-27 (*1*–*4*). Many other IFN-induced proteins have been detected (*5*), but their role in establishing the antiviral state is either indirect or unclear. The majority of IFN-induced proteins are