Cell-Specific Calcineurin Inhibition by a Modified Cyclosporin

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The immunosuppressive drug cyclosporin A (CsA) is widely used to suppress graft rejection following transplantation in humans. Its cellular effects are mediated by a complex of CsA and its intracellular, soluble receptor cyclophilin (Cyp).[1] The Cyp−CsA complex is a specific inhibitor of the protein phosphatase calcineurin (Cn).[2] Consequently, CsA has also been used to illuminate the cellular functions of Cn. For example, its use revealed that, in T cells, Cn mediates the nuclear translocation and activation of NFAT,[3] a transcription factor essential for T cell activation. As a therapeutic agent, CsA has several undesirable side effects including hypertension, CNS toxicity, and nephrotoxicity. These side effects result from CsA-mediated inhibition of Cn in cells outside the immune system.[4]

We now report the synthesis of α-cyclopentylsarcosine11−CsA (CsA*), a rationally modified CsA that does not bind to Cyp and consequently does not inhibit Cn in cells. We also describe a modified Cyp with compensatory mutations in its CsA-binding pocket (F113G, C115M, S99T; Cyp*) that promote high affinity complexation with CsA*. The resulting Cyp*−CsA* complex presents a composite surface that binds Cn with high affinity. As a result, Cn inhibition by CsA* is restored in cells expressing Cyp*. These results provide a method to control the inhibition of Cn spatially and temporally in animals by targeting the expression of Cyp* to specific cells and tissues.

Modifying receptors or ligands by adding even a few atoms can abolish their binding, often changing the function of the molecules involved. When these loss-of-interaction substituents are introduced into proteins by mutation[5] and small molecules by synthesis,[6] information about the cellular function of interacting molecules can be obtained. To achieve our goal of cell-specific Cn inhibition, it was essential that the modified receptor−ligand complex have a composite surface suited for Cn binding, since altered Cyp−CsA complexes can be envisioned that will no longer bind Cn (lower right schematic in Figure 1).

We designed a variant of CsA having two additional methylenes (CH_2) attached to the two methyl groups of the MeVal11 side chain, resulting in the cyclopentyl side chain of CsA*. The crystal structure of the CypA−CsA complex[7] shows residue 11 of CsA directly contacting Cyp, binding in a deep hydrophobic pocket in the active site of Cyp (Figure 2A). The additional atoms were expected to reduce the binding of CsA* to Cyp significantly, presumably through steric interactions between the cyclopentyl side chain and the receptor. To select possible receptors capable of binding CsA*, we generated computer models of complexes between CsA* and several Cyp mutants. On the basis of these models, we selected three mutations in residues lining the valine11 binding pocket of Cyp, one to remove the offending steric interaction (F113G) and two others (S99T, C115M) to improve the fit between the new receptor and ligand (Figure 2B). The synthesis of CsA* first required the asymmetric synthesis of α-cyclopentylsarcosine using a combination of the methods of Evans[8] and Dorrow[9] and the subsequent incorporation of this non-natural amino acid

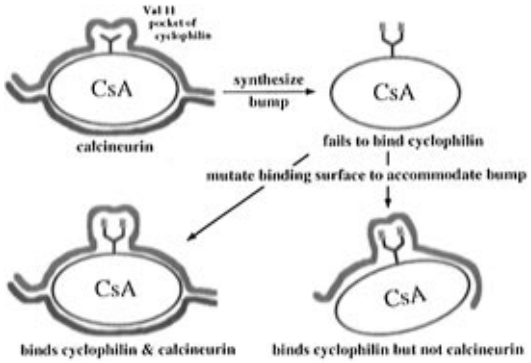

Figure 1. Strategy for producing selective inhibitors of calcineurin. A modified cyclosporin with additional atoms added to its cyclophilin binding surface abolishes its ability to bind to both cyclophilin and calcineurin. The cyclosporin binding surface of cyclophilin is re-engineered to have a complementary shape for the modified cyclosporin, allowing binding to the modified cyclophilin. The resulting complex may retain the ability to bind and inhibit calcineurin only if its composite surface is similar to that of the native Cyp−CsA complex (lower left vs lower right schematic).

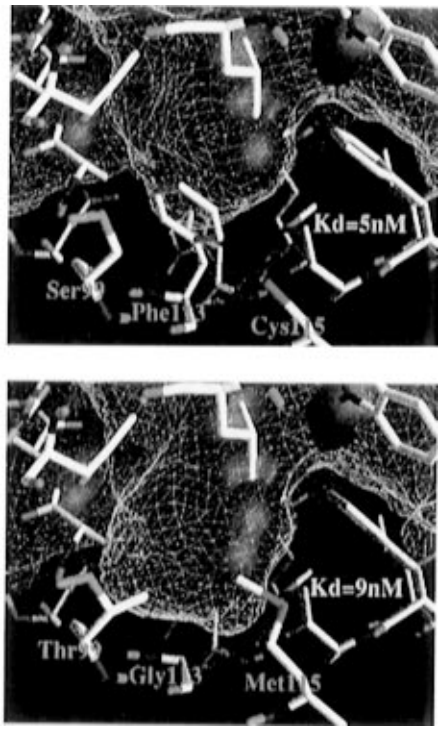

Figure 2. Graphical representation of the binding interfaces between Cyp, CsA, and variants. The solid blue surface and white mesh represent the solvent accessible surfaces of CsA and Cyp, respectively, (nonpolar hydrogens omitted). The contact side chains of Cyp are represented as tubular bonds and viewed from the inside of the protein, the lowest protrusion of solid blue corresponds to the side chain of residue 11 of CsA. (A) Cyp−CsA crystal structure. (B) Hypothetical model of Cyp-(S99T, F113G, C115M)−CpSar11−CsA (Cyp*−CsA*) complex.[10]

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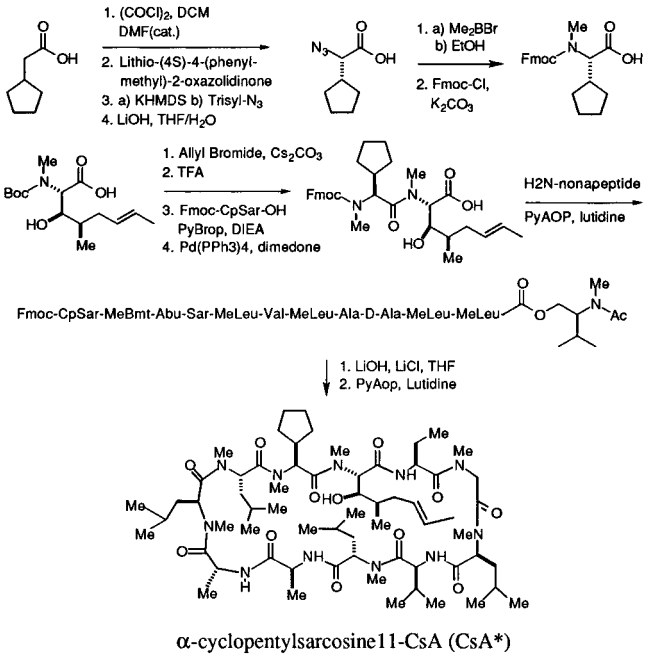

Figure 3. Synthesis of CpSar11−CsA. H_2N-nonapeptide (H_2N-Abu-Sar-MeLeu-Val-MeLeu-Ala-DAla-MeLeu-MeLeu-Ac-N-MeValinol ester) was synthesized from CsA as described previously.[10] Detailed experimental protocols and spectra are available from the authors upon request.

into the CsA macrocycle[10] (Figure 3). To determine the binding characteristics of CsA*, we purified recombinant CypA and Cyp(S99T, F113G, C115M) (Cyp*) expressed in *Escherichia coli* and measured the binding constants for CsA* with a fluorescence-binding assay.[10] As anticipated, CsA* has little affinity for wild-type Cyp ($K_d > 15\ \mu M$) and has a high affinity for the mutant Cyp* ($K_d = 9$ nM).

To test whether the new receptor−ligand combination is able to inhibit the cellular function of Cn, we used a cellular assay for NFAT-signaling[6] where human T cells (Jurkat) were transiently transfected with a reporter gene responsive to the transcription factor NFAT either alone or in combination with Cyp or Cyp*. Stimulation of these cells with phorbol ester (PMA) and ionomycin mimics signaling through the T cell receptor and activates NFAT by a Cn-dependent pathway. As anticipated, CsA* has little or no effect on reporter gene expression at concentrations up to 400 nM in cells expressing either endogenous cyclophilins alone or coexpressing recombinant, wild-type cyclophilin, and yet in cells expressing Cyp*, CsA* potently inhibits NFAT signaling with $IC_{50} = 25$ nM (Figure 4). Thus, we have made cells conditionally sensitive to a small molecule ligand, dependent on the expression of a dominant allele of its receptor protein.

In a previous study we engineered a similar Cyp−CsA-based receptor−ligand pair: Cyp(S99T, F113A)−MeIle11−CsA.[10] In this NFAT-signaling assay, we found that although MeIle11−CsA potently inhibits NFAT-signaling in cells expressing CypA-(S99T, F113A), MeIle11−CsA still inhibited NFAT-signaling in cells transfected with wild-type CypA ($IC_{50} \approx 300$ nM, data not shown). Presumably, this is due to the high concentration CypA ([CypA] > K_d), which promotes formation of the Cyp−

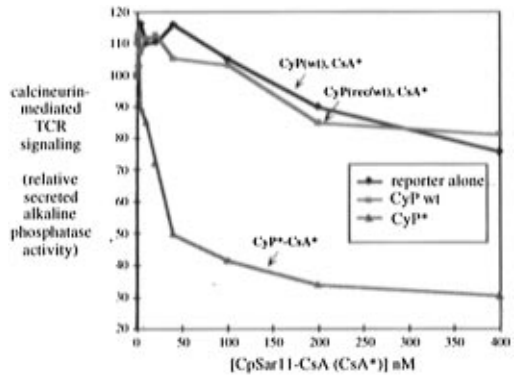

Figure 4. Cellular assay for Cn-mediated NFAT-signaling.[13] CpSar11−CsA has little effect on NFAT-signaling even at the highest concentrations tested yet potently inhibits signaling in human Jurkat-TAg T cells expressing Cyp(S99T, F113G, C115M) (Cyp*). In this assay, CsA has an IC_{50} of 15 nM in cells transfected with NFAT−SEAP alone (data not shown).

MeIle11−CsA complexes and therefore inhibition of Cn. The bulkier CpSar11−CsA used here provides greater selectivity for inhibiting Cn only in cells expressing Cyp*.

Cyclophilins are expressed ubiquitously and have been found in virtually all organisms and tissues. Cn is expressed in many tissues with the highest levels of expression occurring in the brain.[11] The roles of Cn in cells outside the immune system are not known with certainty. The ability to inhibit Cn in a tissue-specific fashion, for example, through the expression of Cyp* under the control of a tissue-specific promoter in transgenic animals, may provide a method to dissect the many roles of Cn in different cells types. Indeed, the use of a T cell specific promoter in a transgenic mouse was recently used to direct the expression of a designed protein that is activated by a synthetic ligand.[12] Since cyclosporin affects many cell subsets within the immune system, the question of whether inhibition of Cn in T cells or other calcineurin-dependent immune cells alone (e.g., B cells, mast cells, neutrophils) is sufficient to prevent rejection of transplanted organs or ameliorate autoimmune disorders has particular relevance to the development of new immunosuppressants. This technique may also find applications in human gene therapy, for example, in the treatment of graft-vs-host disease following bone marrow transplantation. In this scenario, Cyp* would be expressed in the transplanted bone marrow by gene transfer ex vivo so that CsA* could be used tissue specifically in vivo. Since many signaling proteins are expressed ubiquitously yet have context-dependent roles in signaling, this approach to cell-specific targeting may provide a widely useful experimental window to study cells and tissue subsets within an organism.

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(13) CypA and mutants were subcloned from pGEX-based expression vectors into pBJ5, a eukaryotic expression vector containing an N-terminal FLAG epitope tag. The signaling assay was performed essentially as previously described.[6] The data for each transfection are presented as percent SEAP activity relative to the signal for [CpSar11-CsA] = 0 (triplicate readings).