sequence studies (*7*). Since all amino acids in the sequence were identified, this would appear to suggest noncovalent binding. Because of the homology of HC with RBP and BLG, we suggest that the chromophoric group in HC may be a retinoid, which would only be removed by extraction with organic solvents. Previous studies have suggested that HC might play a role in excretion, since large amounts are found in the urine of patients with tubular proteinuria, presumably as a result of a failure of the kidney to catabolize the protein, as well as in a high concentration in the plasma of patients maintained on hemodialysis (*5*). HC may be a vehicle for the controlled excretion of retinol metabolites.

Other workers have suggested that two intracellular retinoid-binding proteins, cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRAB), and two lipid-binding proteins, P2 myelin protein and Z protein, show sequence similarity (*16*). In the region where the intracellular proteins are most similar (near the NH_2-terminus) they have a small amount of similarity with RBP, BLG, and HC. Their similarity in other regions is not sufficient to justify the conclusion that they are all related, but it appears possible that the intracellular and extracellular retinol-binding proteins might also have a common, though distant, relationship.

Note added in proof: Feigelson (*18*) has noted a significant, but limited, sequence relationship of the three proteins discussed here with α_2-globulin, and the structure of BBLG at 2.8-Å resolution shows similarities to RBP (*19*).

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Cyclosporin A Binding to Calmodulin: A Possible Site of Action on T Lymphocytes

Abstract. *Cyclosporin A, a potent immunosuppressive agent, has been widely used to treat patients with solid organ transplants. Although its precise mechanism of action is unknown, it appears to inhibit subsets of T lymphocytes at an early stage in cell activation. Fluorescent, fully active derivatives of cyclosporin A and calmodulin, a protein that binds calcium and is therefore essential to normal cell function, were utilized to demonstrate that cyclosporin A binds to calmodulin. Flow cytometry showed that the calmodulin inhibitors R24571 and W-7 competitively inhibited binding of cyclosporin A to cloned T lymphocytes. Cyclosporin A inhibited the calmodulin-dependent activation of phosphodiesterase in a dose-dependent manner. Binding of cyclosporin A to calmodulin may prevent the latter's role in the activation of the second messengers and enzymes required for effective cell proliferation and function in the immune response.*

Cyclosporin A (CsA) has become a principal immunosuppressive agent in solid organ transplantation and offers the possibility of an effective treatment for graft-versus-host disease, autoimmune disorders, malaria, and schistosomiasis, disorders that may affect over 1 billion people worldwide (*1*). There has been

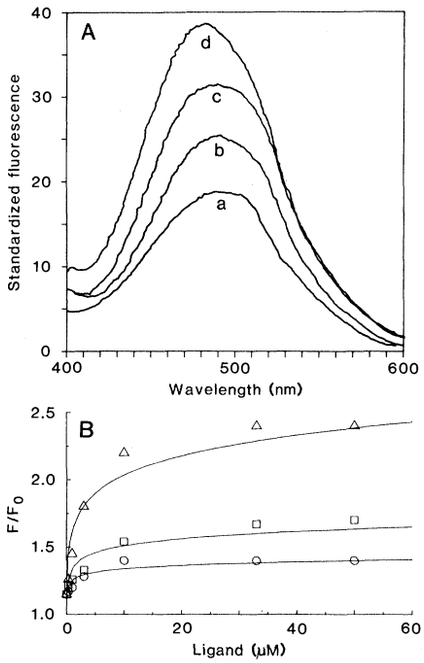

Fig. 1. (A) Fluorescence emission spectra of $1 \times 10^{-6}M$ dansylated calmodulin alone (a) and in the presence of $3 \times 10^{-5}M$ W-7 (b), $3 \times 10^{-5}M$ CsA (c), and $3 \times 10^{-5}M$ R24571 (d). Spectra obtained with insulin (0.1 mg/ml) and an ethanol control were identical to the spectrum of dansylated calmodulin alone. Solutions contained 0.2*N* KCl, 1.0 m*M* $CaCl_2$, and 50 m*M* 4-morpholinepropanesulfonic acid (MOPS) in glass-distilled water buffered to *p*H 7.3 at 25°C. Insulin was initially solubilized with HCl and buffered to *p*H 7.3. All studies were performed on a Mark I spectrophotometer (Farrand Optical). Maximum excitation wavelength was 345 nm for all ligands tested. Maximum increase in fluorescence occurred within minutes. Maximum emission wavelength was 495 nm, although a small blue shift in wavelength was noted with R24571. Control solutions of MOPS buffer, CsA, R24571, W-7, and ethanol without dansylated calmodulin yielded no intrinsic fluorescence. (B) Increase in fluorescence versus concentration of ligand. Ligands R24571 (△), W-7 (○), and CsA (□) were initially solubilized in 99 percent ethanol to a concentration of $10^{-2}M$. Serial dilutions of each ligand were made from 5×10^{-5} to $1 \times 10^{-8}M$ in MOPS buffer and incubated with 1 μ*M* dansylated calmodulin. The increase in fluorescence for dansylated calmodulin (F/F_0) was calculated at each concentration of ligand; where F is observed fluorescence of dansylated calmodulin bound to ligand minus fluorescence of ligand alone and F_0 is fluorescence of unbound dansylated calmodulin.

considerable investigation of CsA's primary effect, the inhibition of subsets of T lymphocytes. The precise subcellular site of its action, however, is unknown, although cyclophilin, an unidentified 16-kD protein that binds to CsA, has been isolated from a murine thymoma cell line, calf thymus, and a wide variety of other tissue types (*2*).

Functional analyses of lymphocyte subsets make it clear that CsA exerts its primary effects on T lymphocytes at an early stage of activation to prevent cell proliferation, secretion of interleukin 2, and expression of receptors for interleukin 2 on cytotoxic T lymphocytes. Suppressor T cells appear to be relatively resistant to the effects of CsA (*3*).

In vitro models of T-cell activation have demonstrated that proliferation in response to concanavalin A (Con A), phytohemagglutinin (PHA), the OKT3 monoclonal antibody, and calcium ionophore A23187 is CsA-sensitive, while proliferation in response to phorbol-12-myristate-13-acetate (PMA) is relatively CsA-resistant. Proposed mechanisms of action for CsA have thus included receptor blockade or competition with Con A, PHA, and the OKT3 monoclonal antibody or blockade of calcium influx by calcium ionophore A23187 (*4*).

Recently it was found that PHA and calcium ionophore both increased intracellular calcium and that the proliferative response of T lymphocytes to these agents was inhibited by trifluoroperazine (TFP), a calmodulin inhibitor. Proliferation of B lymphocytes, however, did not require an increase in intracellular calcium and was not inhibited by TFP. In another study it was observed that CsA did not affect the increase in intracellular calcium secondary to mitogen stimulation of T lymphocytes (*5*).

Since calmodulin, a 16- to 18-kD protein, is intimately involved in early events of cell activation and requires an increase in intracellular calcium for its activity, we investigated the possibility that CsA binds to calmodulin by using dansylated derivatives of calmodulin and CsA and fluorometric and flow cytometric techniques. Dansylated calmodulin undergoes a calcium-dependent increase in baseline fluorescence secondary to ligand binding (*6*); dansylated CsA, a fully immunosuppressive derivative of native CsA with similar binding kinetics, undergoes an increase in fluorescence upon binding to cells which can be detected by ultraviolet fluorescence flow cytometry (*7*).

We initially analyzed the binding of native CsA to dansylated calmodulin and compared its binding to the known hydrophobic calmodulin inhibitors W-7 [*N*-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide] and calmidazolium (R24571) and to a known peptide that does not bind to calmodulin (insulin). Representative fluorescence emission spectra for dansylated calmodulin (Fig. 1A) demonstrate that it undergoes a large increase in fluorescence when incubated with native CsA, W-7, or R24571, while insulin has no effect. Curves showing the binding of R24571, W-7, and native CsA to dansylated calmodulin were then generated; Fig. 1B depicts representative results of one of three experiments. The fluorescence emission spectra and the observed increase in fluorescence of dansylated calmodulin when incubated with R24571 and W-7 were similar to those reported by other investigators (*6*). Similar binding curves were generated for native CsA incubated with decreasing concentrations of dansylated calmodulin. Apparent dissociation constants (K_d's) were determined at each concentration of dansylated calmodulin; the apparent K_d was the concentration of CsA producing a half-maximal increase in the fluorescence of dansylated calmodulin. In Fig. 2 the apparent K_d's are plotted against the calmodulin concentration, yielding a straight line with the *y*-intercept indicating an approximate true K_d of $2 \times 10^{-7} M$. This K_d is remarkably similar to the K_d's for binding of CsA to T lymphocytes (2×10^{-7} to $3 \times 10^{-7} M$) and to cyclophilin ($1 \times 10^{-7} M$) (*7, 8*).

The fluorescence emission spectrum and the observed increase in the fluorescence of dansylated CsA when incubated with approximately equal concentrations of bovine calmodulin and calf thymus cyclophilin were then examined. The fluorescence emission spectrum of CsA was, as expected, similar to that of calmodulin with respect to maximum excitation wavelength and maximum emission wavelength (345 and 495 nm, respectively). When 0.1 μM CsA was incubated with 25 μM calmodulin the observed fluorescence, F/F_0, increased from 1.00 to 2.28 (128 percent). Incubation with 25 μM cyclophilin yielded an increase in observed fluorescence from 1.00 to 2.22 (122 percent).

Flow cytometric techniques were used to directly assess competition for T-lymphocyte receptors between dansylated CsA and the calmodulin inhibitors R24571 and W-7. Cloned T-lymphocyte lines derived from one-way, mixed-lymphocyte reactions were used to provide homogeneous cell populations for analysis. Figure 3 depicts a double-reciprocal plot of dansylated CsA binding to cloned T lymphocytes when incubated alone or with native CsA, R24571, or W-7. Both R24571 and W-7 competitively inhibited

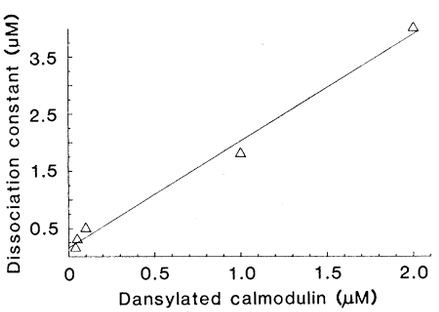

Fig. 2. Apparent K_d's of CsA binding in the presence of decreasing concentrations of dansylated calmodulin. Native CsA in MOPS buffer solution (final concentrations, 3×10^{-5} to $1 \times 10^{-9} M$) were incubated with 0.1 ml of dansylated calmodulin (final concentrations, 2, 1, 0.1, 0.05, and 0.04 μM). Binding curves were generated and the native CsA concentration producing a half-maximal increase in the fluorescence of dansylated calmodulin was the apparent K_d. Plotting the apparent K_d against the calmodulin concentration yields a straight line relation with the *y*-intercept equal to the probable true K_d as the calmodulin concentration approaches zero. The true K_d by this method was approximately $2 \times 10^{-7} M$ [0.158 ± 0.2 μM by linear regression analysis (mean ± standard error; IBM SYSTAT program)].

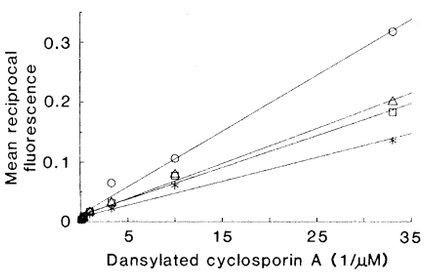

Fig. 3. Double-reciprocal plot of dansylated CsA fluorescence versus concentration. Cloned T lymphocytes (2.5×10^4 per milliliter) were incubated for 1 hour at 37°C with decreasing concentrations of dansylated CsA (final concentration, 1×10^{-5} to $1 \times 10^{-9} M$) and $1 \times 10^{-5} M$ native CsA, $1 \times 10^{-5} M$ R24571, $1 \times 10^{-5} M$ W-7, or an ethanol control. All reagents were initially solubilized in 99 percent ethanol to a concentration of $10^{-2} M$ and diluted to the appropriate test concentrations with the MOPS buffer solution. Cells were then analyzed on a fluorescence-activated cell sorter (FACS II, Bectin Dickinson) for the increase in mean cell fluorescence secondary to binding of dansylated CsA (excitation wavelength, 350 nm; emission wavelength, 480 to 540 nm). Cells incubated in MOPS buffer, CsA, R24571, W-7, or ethanol alone served as controls. Double-reciprocal plots were generated for dansylated CsA with ethanol (*), with native CsA (○); with R24571 (△), and with W-7 (□). Straight-line relations were obtained for dansylated CsA alone and with the three inhibitors. All four lines approached a similar *y*-intercept (maximum fluorescence), indicating competitive inhibition of dansylated CsA binding by native CsA, R24571, and W-7 (*7*).

binding of dansylated CsA to cell receptors in a dose-dependent manner similar to that of native CsA. These findings are in contrast to previous results that showed no change in binding of dansylated CsA to T lymphocytes with excess concentrations of PHA, Con A, indomethacin, or amphotericin B (*7*). Figure 4 shows that CsA inhibited the calmodulin-dependent activation of phosphodiesterase.

Using a calmodulin radioimmunoassay system involving a high-affinity polyclonal antiserum, we observed that purified cyclophilin has immunologic cross-reactivity with calmodulin. Cyclophilin was as competitive as known quantities of calmodulin for the calmodulin antibody.

The binding of CsA to dansylated calmodulin, the binding of calmodulin to dansylated CsA, and the competitive inhibition of binding of dansylated CsA to T lymphocytes by R24571 and W-7 provide direct evidence that CsA binds to T lymphocyte–associated calmodulin. The observed inhibition of calmodulin-dependent phosphodiesterase activity by CsA may be the first in vitro demonstration of the inhibition of an enzyme system by this immunosuppressive agent. The results of the radioimmunoassay and the binding of cyclophilin to dansylated CsA suggest that cyclophilin, the CsA-binding protein, may be calmodulin or a very closely related calmodulin-like protein molecule. Although cyclophilin shows no significant sequence homology to calmodulin in the first five to ten amino acid residues, cyclophilin may be a structural analog to calmodulin or may copurify with it, which could account for the cross-reactivity in the calmodulin radioimmunoassay (*9*). A CsA-calmodulin interaction could explain the findings that first messengers such as alloantigen, T-cell mitogens, and calcium ionophore, despite creating a calcium flux into the cell, are unable to activate T cells in the presence of CsA but that PMA, which acts directly on protein kinases at a step in activation beyond calmodulin activation, is relatively CsA-resistant (*10*).

The ability of CsA to bind to calmodulin may effectively remove calmodulin-dependent cellular activities (kinases, phosphorylases, cyclic nucleotides, and prostaglandins) from the activation sequence of susceptible T lymphocytes, thus attenuating the normal immune response at an early stage of lymphocyte triggering. This would be consistent with the narrow time frame of CsA's effectiveness (only in early cell activation) and the relative resistance of "activated" T cells to the agent (*11*). It would also be consistent with our data that demonstrates a paradoxical increase in the binding of cyclosporin to activated, CsA-resistant cloned T cells, since actively dividing cells have increased cytoplasmic calmodulin (*7, 12, 13*). In contrast, activation of suppressor T cells may follow a calmodulin-independent pathway (similar to B lymphocytes) or involve a higher "resting" concentration of cytoplasmic calmodulin, thus demonstrating the relative resistance of these cells to CsA in most in vitro models (*3*).

In summary, we have demonstrated binding of CsA to calmodulin by fluorometric techniques with an apparent K_d similar to that of CsA binding to T lymphocytes and cyclophilin, competitive inhibition of CsA binding to T lymphocytes by calmodulin inhibitors, inhibition of calmodulin-dependent phosphodiesterase activity by CsA, and cross-reactivity of cyclophilin in a calmodulin radioimmunoassay. This may be the first observation of CsA binding to a known cytoplasmic protein and of inhibition by CsA of an enzyme system dependent on that protein for optimal activity. Interruption of the T-lymphocyte activation pathway by CsA may occur by inactivation of calmodulin despite a calcium flux into the cell, thus preventing the signals to activate cyclic nucleotides, protein kinases, and phospholipase A_2, the second messengers that are necessary to initiate synthesis of protein, messenger RNA, DNA, and prostaglandins.

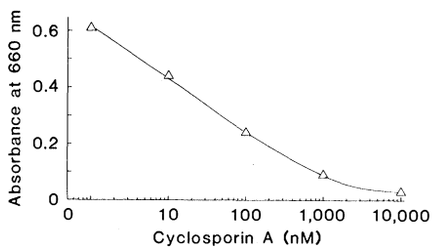

Fig. 4. Calmodulin-dependent phosphodiesterase activity versus CsA concentration. A modification of a three-step phosphodiesterase assay was used to determine calmodulin-dependent activation of phosphodiesterase and its inhibition by CsA. Activator-deficient bovine brain 3′,5′-cyclic-nucleotide phosphodiesterase (PDE) and 2 m*M* cyclic AMP in 0.5 ml of tris buffer solution (40 m*M* tris-chloride, 0.1 m*M* $MnCl_2$, and 0.01 m*M* $CaCl_2$ in glass-distilled water; pH 7.5) were incubated alone, with bovine brain calmodulin (0.5 U), with calmodulin (0.5 U) plus increasing concentrations of native CsA (1×10^{-9} to 1×10^{-5}*M*), or with CsA alone for 10 minutes at 30°C. The reaction was stopped by placing test tubes in a boiling water bath for 2 minutes and then cooling them. The 5′-AMP in the reaction product was then cleaved to adenosine and inorganic phosphate by incubation with 5′-nucleotidase (0.05 ml) for 10 minutes. This reaction was stopped by adding 0.05 ml of trichloroacetic acid (55 percent, weight to volume) and 0.15 ml molybdic acid solution and centrifuged until clear. Clear supernatants were decanted into test tubes with Fiske-SubbaRow reagent (0.1 ml) and the blue color reaction with the inorganic phosphorus present was allowed to develop for 10 minutes. Absorbance at 660 nm was then measured spectrophotometrically. Absorbance is plotted against CsA concentration for both PDE activity alone and with calmodulin. Baseline activator-deficient PDE activity was unchanged by addition of CsA. PDE activity with calmodulin, however, was inhibited by the addition of CsA and half-maximal inhibition occurred at approximately 1×10^{-7}*M* CsA (*13*).

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