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Identification of Actin and HSP 70 as Cyclosporin A Binding Proteins by Photoaffinity Labeling and Fluorescence Displacement Assays*

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A novel family of cyclosporin A (CsA) binding proteins was identified by using the biologically active, radioiodinated photoaffinity probe [D-Lys-N^{ϵ}-(4-azido-3-[^{125}I]iodophenyl)propionyl)]^{8}-CsA. In addition to cyclophilin, proteins with molecular masses of 43 kDa and approximately 50–55 kDa were labeled in Jurkat extracts and bovine calf thymus. Sequence analysis of the 43-kDa protein purified from calf thymus and subsequent Western analysis of CsA affinity-purified material from Jurkat extracts identified the 43-kDa component as actin. [D-Lys-N^{ϵ}-(5-dimethylamino-1-naphthalenesulfonyl)]^{8}-CsA, a fluorescent analogue of CsA, was prepared and used to measure the binding constants of cyclosporin derivatives to actin by means of a new fluorescence displacement assay. [D-Lys-N^{ϵ}-(5-dimethylamino-1-naphthalenesulfonyl)]^{8}-CsA and [N^{5}-*t*-butoxycarbonyl diaminobutyryl)]^{8}-CsA bind to bovine actin at physiologically relevant concentrations, with dissociation constants of 60 ± 33 and 570 ± 380 nM, respectively. Because the ATPase fragment of heat shock cognate 70 (HSC 70) is structurally related to actin, the yeast homologue SSA1 was tested and found to be radiolabeled by the cyclosporin A photoaffinity reagent. The binding constant for [D-Lys-N^{ϵ}-(5-dimethylamino-1-naphthalenesulfonyl)]^{8}-CsA to SSA1 was determined and is 53 ± 48 nM. These results indicate that actin and the 70-kDa heat shock protein family contain a structurally related domain for binding of cyclosporin A-related peptides.

Cyclosporin A (CsA **1**; Fig. 1)[1] is the immunosuppressive drug of choice for preventing organ-graft rejection (Borel, 1983). Recent studies on the mechanism of action of this cyclic undecapeptide have greatly clarified the events leading to immunosuppression. Cyclosporin A prevents activation of lymphokine genes essential for T cell proliferation such as IL-2, IL-4, and interferon-γ by disrupting calcium-dependent signalling transduction pathways in T cell activation (Crabtree *et al.*, 1989; Lin *et al.*, 1991; Flanagan *et al.*, 1991). This process was thought originally to result from the binding of the drug to cyclophilin (Handschumacher *et al.*, 1984). Subsequently, cyclophilin was discovered to be a peptidyl prolyl *cis-trans* isomerase (Fischer *et al.*, 1984), an enzyme that is believed to facilitate protein folding *in vivo* by catalyzing proline isomerization in newly synthesized proteins (Brandts *et al.*, 1975). Although inhibition of the peptidyl prolyl *cis-trans* isomerase activity of cyclophilin by CsA is competitive with small synthetic substrates (Kofron *et al.*, 1991, 1992), and the inhibition constant (K_i 6 nM) is in overall agreement with doses of the drug that cause immunosuppression, structure-activity data have demonstrated that inhibition of cyclophilin's peptidyl prolyl *cis-trans* isomerase activity does not always correlate with immunosuppression (Sigal *et al.*, 1991). Most notably, the weakly immunosuppressive analogue [MeAla]^{6}-CsA (0.4% relative to CsA activity), strongly inhibits cyclophilin (K_i 2 nM) whereas the moderately (30%) immunosuppressive analogue [MeBm$_2$t]^{1}-CsA (Aebi *et al.*, 1990; Rich *et al.*, 1990) poorly inhibits cyclophilin (K_i 690 nM). These exceptions may be explained by the recent discovery (Liu *et al.*, 1991) that the complex formed between CsA and cyclophilin inhibits the calmodulin-dependent phosphatase, calcineurin (Klee *et al.*, 1988).

In spite of this remarkable progress toward understanding the molecular basis for cyclosporin's mode of action, it is not yet clear whether inhibition of the phosphatase activity of calcineurin by the drug-cyclophilin complex is sufficient for causing immunosuppression, or if any of these biochemical events are important for the other biological activities of cyclosporin (Scolari *et al.*, 1987; Borel *et al.*, 1989). Two particularly serious side effects of this drug are nephrotoxicity and chronic rejection response, and the possibility exists that other CsA binding proteins are involved in these poorly understood processes.

To search for additional CsA binding proteins in cells, we have developed a radiolabeled and photoreactive immunosuppressive CsA analogue, [D-Lys(A^{125}IPP)]^{8}-CsA **2** (Tung *et al.*, 1989). The 8 position was selected for functionalization because preliminary structure-activity studies indicated that several 8-position-modified CsA analogues retained immunosuppressive activity *in vitro*.[2] Derivative **2** was formed from [D-Lys]^{8}-CsA **3** (Colucci *et al.*, 1990) by acylating the ϵ-nitrogen of D-lysine^{8} with *N*-{3-(4-azido-3-[^{125}I]iodophenyl)propionyl}succinimide (A^{125}IPPS) **6** (Lowndes *et al.*, 1987). The photoaffinity reagent **2** has a specific activity of

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[1] The abbreviations used are: CsA, cyclosporin A; DTT, dithiothreitol; [D-Lys(AIPP)]^{8}-CsA, [D-Lys-N^{ϵ}-(4-azido-3-I-iodophenyl) propionyl)]^{8}-CsA; [D-Lys(Dns)]^{8}-CsA, [D-Lys-N^{ϵ}-(5-dimethylamino-1-naphthalenesulfonyl)]^{8}-CsA; [D-Dab(Boc)]^{8}-CsA, [N^{5}-*t*-butoxycarbonyldiaminobutyryl)]^{8}-CsA; PMSF, phenylmethanesulfonyl fluoride; EA, ethanolamine; A^{125}IPPS, *N*-{3-(4-azido-3-[^{125}I]iodophenyl)propionyl}succinimide; IL, interleukin; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran; Me, methyl; Bm$_2$t, *N*-methyl-4,4-dimethyl-4-butenyltheonine.

[2] M. L. Moss, R. E. Palmer, P. Kuzmic, B. E. Dunlap, W. Henzel, J. L. Kofron, W. S. Mellon, C. A. Royer, and D. H. Rich, unpublished results.

2010 Ci/mmol and retains partial biological activity of CsA (12%). We have utilized this radioiodinated probe to identify novel cyclosporin A binding proteins in the immune competent human cell line Jurkat and in bovine calf thymus. Herein, we determined the dissociation constants for protein-CsA analogues by using a new fluorescence displacement assay.

EXPERIMENTAL PROCEDURES

Materials

Bovine calf thymus was purchased from Pel Freeze. All column resins were obtained from Pharmacia LKB Biotechnolog Inc. Buffer components were purchased from commercially available sources. Cyclosporin A was obtained from Sandoz. SSA1 was a generous gift from Jeff Shilling and Dr. Elizabeth Craig, University of Wisconsin, Madison. The actin monoclonal antibody was obtained from Chemicon.

Methods

Purification of the 43-kDa Protein—All steps were performed at 4 °C unless otherwise specified. Bovine calf thymus (250 g) was homogenized in a Waring Blender in 250 ml of buffer that contained 20 mM Tris-Cl, pH 7.6, 100 mM NaCl, 5 mM dithiothreitol (DTT), and 20 mg of phenylmethanesulfonyl fluoride (PMSF). After saturation with ammonium sulfate to 40%, the supernatant was cleared by ultracentrifugation at 45,000 rpm using a Ti-50 rotor. The resuspended pellet was dialyzed overnight against 20 mM HEPES, pH 7.6, containing 1 mM PMSF. This material was applied to a Q-Sepharose Fast Flow column (2.6 × 60 cm) which was pre-equilibrated with 20 mM HEPES, pH 7.6. The column was washed with one column volume of the equilibrating buffer before running a 600-ml linear gradient from 0 to 0.6 M NaCl. Fractions containing the highest specific activity (See "Photolysis Experiments") were combined, and the protein was precipitated by addition of ammonium sulfate to 75% saturation. After centrifugation and dialysis against 20 mM HEPES, pH 7.6, 100 mM NaCl, the protein was applied to a Sephacryl S-200 column (2.6 × 100 cm) which had been pre-equilibrated with the same buffer. The fractions with the best specific activity were combined, dialyzed against 20 mM HEPES, applied to a Mono Q column, and eluted with a 0–500 mM NaCl gradient. Sequencing was performed on fractions containing the highest specific activity protein from the Sephacryl S-200 and Mono Q column chromatographies.

The G-actin used in the fluorescence displacement assay was purified by a slightly modified procedure. To increase the stability of the protein during purification and improve the overall yield of actin, the homogenization buffer was changed to 20 mM Tris-Cl, pH 7.6, 0.5 mM DTT, 0.2 mM ATP, and 0.1 mM $CaCl_2$. All purification steps were the same as previously described through the Q-Sepharose chromatography. Fractions containing actin from the Q-Sepharose column were combined and dialyzed against the homogenization buffer. The actin was polymerized after dialysis by the addition of 50 mM KCl and 2 mM $MgCl_2$ to the buffer. The F-actin was pelleted by centrifugation at 100,000 × *g* for 4 h. The pellet was resuspended in the homogenization buffer and then poured into a Dounce homogenizer and subjected to several gentle strokes with an A-size pestle. This suspension was frozen in liquid nitrogen and stored at −72 °C.

Sequencing of the 43-kDa Protein—Automated Edman Degradation was performed with Applied Biosystems Models 470A and 473A protein sequencers equipped with on-line phenylthiohydantoin analyzers. Sequence interpretation was performed on a VAX 8750 as described by Henzel *et al.* (1987). Cyanogen bromide (CNBr) was used to generate peptide fragments. The protein was solubilized in 0.1 ml of 7% formic acid, and the reaction was initiated by addition of a small crystal of CNBr. The vessel was capped and kept at room temperature for 17 h. Peptides obtained from the cleavage reaction were separated on a Synchrom column (2 × 100 mm, 4000 Å) from Synchrompac by using a 50-min linear gradient of 0.1% trifluoroacetic acid to 70% 1-propanol at a flow rate of 0.2 ml per min.

Photolysis Experiments—Typically, 5–50 μg of protein was brought to a final volume of 50 μl with 20 mM HEPES, pH 7.6, and 100 mM NaCl and was treated with 3 μl (150,000 cpm of [D-Lys(A^{125}IPPS)]^8-CsA in EtOH) for 2 h at room temperature in a borosilicate conical centrifuge tube. The tubes were placed in an ice bath for 5 min, and then a solution (10% by volume) that contained 3% activated charcoal and 0.3% dextran was added. After low speed centrifugation to pellet the charcoal and dextran, the material was photolyzed for 5 min at 4 °C with a Rayonet photochemical reactor from Southern N.E. Ultraviolet Co using a wavelength of 350 nM. A 40-μl aliquot was removed, added to 10 μl of loading dye, and analyzed directly on a discontinuous 12% SDS-polyacrylamide gel electrophoresis Laemmli system. The gels were stained with Coomassie Brillant Blue and subjected to autoradiography for 1–2 days with an intensifying screen. Specific activities were determined by cutting out the 43-kDa band from the gel and determining the number of cpm bound to the 43-kDa protein with a Cobra γ counter from Packard. The specific activity was defined as: cpm in the 43-kDa protein/total cpm/mg of protein.

Purification of CsA Binding Proteins from Jurkat Extracts—Jurkat cells (1×10^9) grown in RPMI 1640 medium with 5% fetal calf serum and antibiotics were washed with phosphate-buffered saline and then resuspended in 10 ml of ice-cold lysis buffer (35 mM HEPES, pH 7.3, 50 mM NaF, 0.1 mM DTT, 0.015% Triton X-100, and 1 mM PMSF). After breakage of the cells with a polytron, the cell debri was pelleted by centrifugation at 2000 rpm for 10 min followed by ultracentrifugation for 30 min at 37 K in a Ti-50 rotor. One half of the extract was incubated with Affi-Gel-CsA resin prepared by coupling [D-Lys]^8-CsA to cyanogen bromide-activated Affi-Gel 10, while the remaining extract was mixed with a control resin that had been capped with ethanolamine (Affi-Gel-EA). The resin was washed, and the proteins were eluted and run on a 10 or 12% SDS polyacrylamide gel. Proteins were detected by either Coomassie Blue staining or silver staining as indicated.

Fluorescence Binding Experiments—The fluorescence binding experiments were carried out on a Greg PC fluorometer (ISS, Inc., Champaign, IL). Excitation was at 350 nm, and the emission was monitored at 460 nm using a Y-42 cut-off filter (Hoya Optics, Inc., Freemont, CA) to eliminate any scattering of light. Data for the heat shock protein fluorescence binding experiments was collected on a Perkin-Elmer MPF-4 fluorimeter with a Perkin-Elmer 150 xenon power supply. The excitation and emission wavelengths were set to 350 and 500 nM, respectively.

G-actin was prepared from frozen stocks of F-actin by dialysis overnight against 10 mM Tris-Cl, pH 7.6, 0.5 mM dithiothreitol, 0.2 mM $CaCl_2$, and 0.1 mM ATP. Actin (3.2–7 mg/ml) was diluted 10- and 100-fold into the dialysis buffer. Aliquots from these stock solutions along with dialysis buffer (50 μl total volume) was added to 1.93 ml of 10 mM Tris-Cl, pH 7.6, immediately before addition of [D-Lys(Dns)]^8-CsA **4** (40 μl, 10 μM stock solution dissolved in 0.47 M dry THF/LiCl).

The yeast cytoplasmic heat shock protein 70 (Hsp 70), a mixture of SSA1 and SSA2, was provided as a 1.2 mg/ml solution dissolved in 20 mM HEPES, pH 7.6, 50 mM KCl, and 10% glycerol. The stock solution was diluted 10- and 100-fold into this same buffer. The protein and buffer (118 μl total volume) were added to 1.86 ml of 10 mM Tris-Cl, pH 7.6, immediately before addition of the fluorophore **4** (40 μl, 10 μM stock solution dissolved in 0.47 M dry THF/LiCl). Fluorescence intensity values were measured 50 s after addition of [D-Lys(Dns)]^8-CsA **4** to actin or Hsp 70.

[D-Lys(Dns)]^8-CsA **4** was prepared by dissolving [D-Lys]^8-CsA (Colucci *et al.*, 1990) and 1.3 equivalents of 5-dimethylamino-1-naphthalenesulfonyl chloride in dry DMF. After one equivalent of triethylamine was added, the reaction was allowed to proceed at room temperature for 3 h. The [D-Lys(Dns)]^8-CsA **4** was purified by preparative silica gel thin-layer chromatography (chloroform/methanol 20:1). A 1 mM stock solution of [D-Lys(Dns)]^8-CsA **4** was made up in dry THF and stored for no longer than 3 days. Dilutions were made into dry THF/LiCl, 0.47 M, immediately before use.

RESULTS

Photoaffinity Labeling of Crude Extracts—Extracts from calf thymus and the human leukemic cell line Jurkat were treated separately with the photolabel [D-Lys(A^{125}IPP)]^8-CsA **2** (Fig. 1) to screen for novel cyclosporin A binding proteins. Three distinct bands were previously identified in mouse thymocyte cytosols with molecular masses of 55, 43, and 37 kDa, respectively (Tung *et al.*, 1989). In addition to cyclophilin, primarily proteins with M_r of 50–55,000 and 43,000 were detected in Jurkat extracts (Fig. 2, *lane 1*). Minor components at both 37 and 90 kDa were also identified (Fig. 2, *lane 1*). Bovine calf thymus contained almost exclusively the 43-kDa protein and cyclophilin (*lane 3*). In addition, all bands could be displaced by nonradioactive cyclosporin A (*lane 2*).

Diethylamide-A^{125}IPP reagent **7**, prepared as a control com-

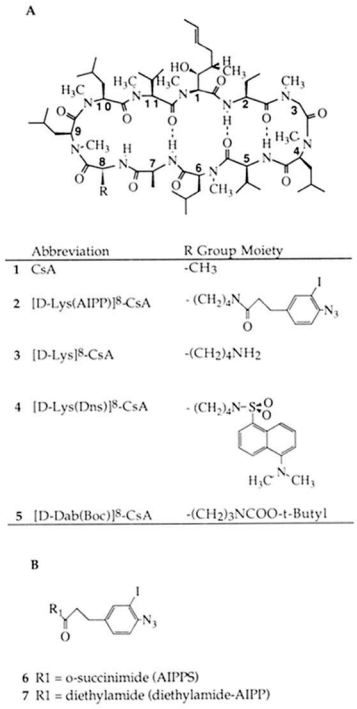

FIG. 1. **Structures of photoaffinity reagents and cyclosporin A analogues.** *A*, structure of CsA and 8-position analogues of CsA. *B*, structure of AIPPS and the photoaffinity reagent diethylamide-AIPP.

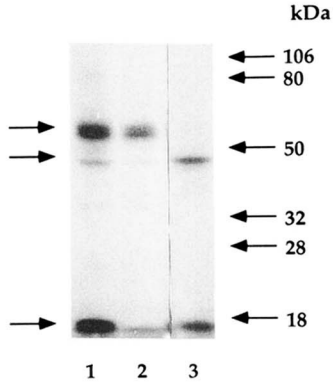

FIG. 2. **Identification of cyclosporin A binding proteins in Jurkat extracts.** Jurkat extracts were prepared by breakage of cells with a polytron followed by high speed centrifugation at 100,000 × *g* to remove nuclei and particulate matter. Extracts were prepared in 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.5 mM dithiothreitol. The *arrows* on the left side of the panel indicate the positions of cyclophilin (18 kDa), the 43-kDa protein, and the 55-kDa protein. *Lane 1*, Jurkat extract photolyzed with [D-Lys(A^{125}IPPS)]^8-CsA; *lane 2*, same as *lane 1* except for the addition of the nonradioactive competitor, CsA (100 μM); *lane 3*, bovine calf thymus extract photolyzed with [D-Lys(A^{125}IPPS)]^8-CsA.

pound by coupling diethyl amine to A^{125}IPPS, did not radiolabel the 43-kDa band in thymus nor the 55- and 43-kDa bands in Jurkat (data not shown). These results establish that labeling of the proteins requires the presence of both the cyclosporin A and the AIPPS moieties in the photoaffinity label **2**.

Purification and Identification of the 43-kDa Protein—The 43-kDa protein was purified from bovine calf thymus by fractionation with ammonium sulfate followed by chromatography over Q-Sepharose. A typical elution profile from the Q-Sepharose chromatography is shown in Fig. 3*A*. Cyclophilin emerged from the column in the first protein peak, whereas the 43-kDa CsA-binding protein was eluted near the end of

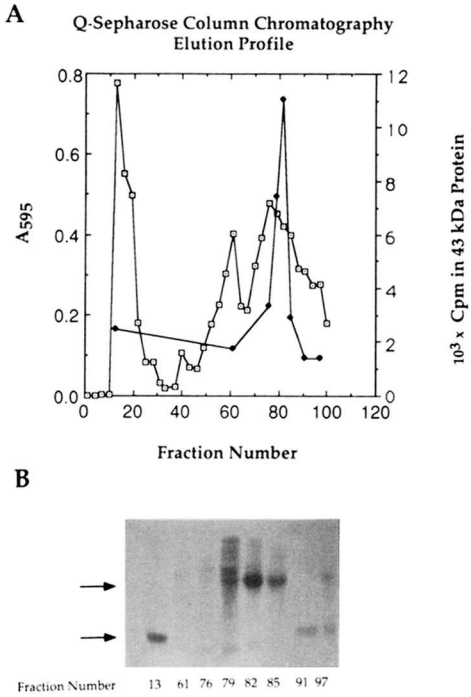

FIG. 3. **Elution profile from Q-Sepharose chromatography.** Aliquots from specific fractions from the Q-Sepharose column were photolyzed with [D-Lys(A^{125}IPP)]^8-CsA as described under "Methods." To determine the specific 43-kDa binding activity, the photolyzed samples were subjected to SDS-12% poyacrylamide gel electrophoresis. The 43-kDa band was detected by autoradiography of the dried gel. *A*, elution profile from the Q-Sepharose column. Protein concentration (□), indicated as absorbance at 595 nm, was quantified by the BPA assay. Cpm (◆) indicate the amount of radioactivity as determined by γ counting incorporated into the 43-kDa band after gel electrophoresis. *B*, autoradiogram of the gel with *arrows* indicating the positions of cyclophilin (18 kDa) and the 43-kDa protein.

the salt gradient (Fig. 3*B*). After further purification of the 43-kDa protein by Sephacryl S200 chromatography, fractions with the highest specific activity were chosen for sequence analysis. When the protein was found to be N-terminally blocked, CNBr was used to generate peptide fragments. These were purified, sequenced, and analyzed as described under "Methods." Two proteins were identified. The sequenced peptide fragments *X*KAGRAGDDAPRAVFPSIVGRPR and GQKDSYVGDEAQSKRGILTL (where *X* equals cysteine not identified during sequencing) established that the major component was γ-cytosolic actin, 42 kDa (actin migrates with an apparent M_r of 43,000 after photolysis). The identity of the second protein was determined when sequence analysis revealed that peptides MADGT*X*QDAAIVGYKDSPSVW-AAVPG and DLRTKSTGGAP were identical to portions of profilin, 15-kDa. Homogeneous fractions from the final purification step (Mono Q chromatography) were sequenced after cyanogen bromide cleavage and found to contain only γ-cytosolic actin.

Purification of the 43- and 55-kDa Proteins from Jurkat Extracts—A cyclosporin A affinity resin was used to purify CsA binding proteins in a one-step purification procedure from Jurkat cells. Whole cell extracts were prepared and incubated with either the Affi-Gel-CsA affinity resin or a control Affi-Gel resin that was capped with ethanolamine. The eluted proteins from the column were further separated by SDS-polyacrylamide gel electrophoresis. In addition to cyclophilin, proteins with M_r of 43,000, 50,000–55,000, and approximately 90,000 were detected (Fig. 4). Western analysis revealed that the 43-kDa protein is actin (data not shown).

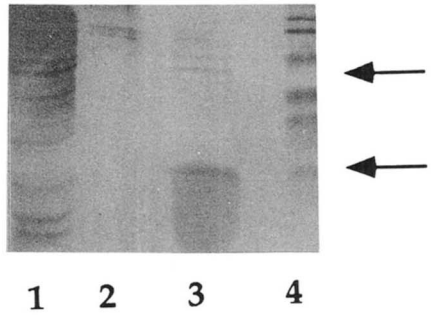

FIG. 4. **Identification of the 43-kDa protein from Jurkat extract.** CsA binding proteins were purified from whole cell Jurkat extracts using a CsA-affinity matrix. *A*, proteins bound to the matrix were eluted and run on a 12% SDS-polyacrylamide gel. The protein bands were detected by silver staining. *Lane 1*, whole Jurkat extract (50 μg); *lane 2*, EA affinity-purified material; *lane 3*, CsA affinity-purified material; *lane 4*, molecular weight markers of 106,000, 80,000, 49,500, 32,500, 27,500, and 18,500.

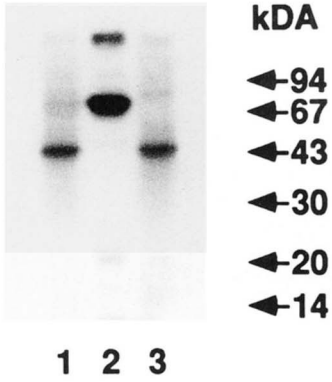

FIG. 5. **Photoaffinity labeling of SSA1 with [D-Lys(A^{125}IPP)]^8-CsA in the presence or absence of purified G-actin.** Samples were preincubated with [D-Lys(A^{125}IPP)]^8-CsA in 10 mM HEPES, pH 7.6, 0.5 mM dithiothreitol, 0.2 mM $CaCl_2$ for 2 h at 25 °C before photolysis. Carrier protein, soybean trypsin inhibitor (20 μM) was added to all samples. *Lane 1*, G-actin at a concentration of 1.4 μM; *lane 2*, SSA1, 2 μM; *lane 3*, actin (1.4 μM) and SSA1 (2 μM).

Binding of Cyclosporin A to Actin-related Proteins—SSA1,[3] the cytosolic Hsp 70 protein from yeast that is structurally related to actin (Kabsch *et al.*, 1990), was photolyzed in the presence of either [D-Lys(A^{125}IPP)]^8-CsA **2** or the diethylamide control photoreagent **7**. SSA1 was labeled by [D-Lys(A^{125}IPP)]^8-CsA **2** in the presence of a 3-fold excess of soybean trypsin inhibitor or bovine serum albumin, which were added to serve as control proteins that do not specifically bind CsA (data not shown). These results establish that SSA1 is labeled by CsA photoaffinity reagent **2** in a specific manner. However, when an excess of Hsp 70 was photolyzed in the presence of G-actin, only actin was radiolabeled (Fig. 5). In both experiments, diethylamide-AIPP **7** was not incorporated into the proteins, which established that the CsA moiety was needed for efficient labeling of actin and Hsp 70. Whereas addition of nonradioactive CsA (10 μM) to to the photoaffinity labeling reaction mixture prevented incorporation of the radiolabel **2** into 1 μM cyclophilin, a 50 μM or 25-fold excess of cyclosporin was required for protection of SSA1 or actin. In contrast, a 2-fold excess (2 μM) of cold photoreagent **2** could protect actin completely (1 μM) from being labeled by [D-Lys(A^{125}IPP)]^8-CsA **2** (data not shown). When the binding assay was performed in the presence of a 2-fold excess of bovine serum albumin, actin was the only detectably labeled protein (data not shown).

[3] The SSA1 obtained from Dr. Elizabeth Craig's laboratory is a mixture of SSA1 and SSA2.

These displacement studies, which were carried out with nonradioactive CsA, indicate that actin binds more tightly to the AIPP analogue **2** than to CsA **1**. To determine if other analogues would compete for binding to actin more effectively than CsA, displacement studies were performed with a cyclosporin A derivative that contains another hydrophobic side chain in the 8 position, [D-Dab(Boc)]^8-CsA **5**. This analogue, which binds tightly to cyclophilin (K_i 2 nM) and has 14% of the immunosuppressive activity of CsA in an Il-2 secretion assay, prevented labeling of actin at concentrations as low as 10 μM. On the other hand, 50 μM CsA was required to protect actin from being labeled by the photoreagent **2** (Fig. 6). It is apparent from these experiments that the presence of a hydrophobic or aromatic residue at the 8 position of CsA increases the affinity for actin in comparison with CsA itself.

Fluorescence Binding Assays—In order to determine the dissociation constants for the complexes formed of various CsA analogues to G-actin and other CsA binding proteins, we devised a competition binding assay. [D-Lys(Dns)]^8-CsA **4**, a fluorescent analog that has 10–50% the immunosuppressive activity of CsA (Hess *et al.*, 1985) was synthesized and used as the reporting ligand. The dissociation constant for the CsA **4**-actin complex first was determined essentially by the method of Inglese *et al.* (1989) by titrating increasing concentrations of actin against a fixed concentration (200 nM) of the fluorescent derivative (Fig. 7, *open circles*). Critical to the success of the competition binding assay is the method for

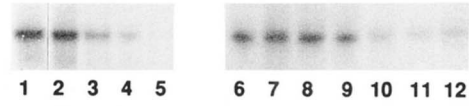

FIG. 6. **Labeling of actin in the presence of cyclosporin A or [D-Dab(Boc)]^8-CsA.** Samples of actin purified through the Q-Sepharose column chromatography (4.3 μg) were preincubated for 2 h with nonradioactive competitors cyclosporin A or [D-Dab(Boc)]^8-CsA and then treated with 200,000 cpm of [D-Lys(A^{125}IPPS)]^8-CsA for 5 min before cooling the samples to 4 °C and adding charcoal. Samples were then photolyzed and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography as described under "Experimental Procedures." *Lanes 1–5*, actin photolyzed with [D-Lys(A^{125}IPPS)]^8-CsA in the presence of 0, 10, 20, 50, and 100 μM CsA; *lanes 6–12*, actin photolyzed with [D-Lys(A^{125}IPPS)]^8-CsA and 0, 1, 2, 5, 10, 20, and 50 μM [D-Dab(Boc)]^8-CsA.

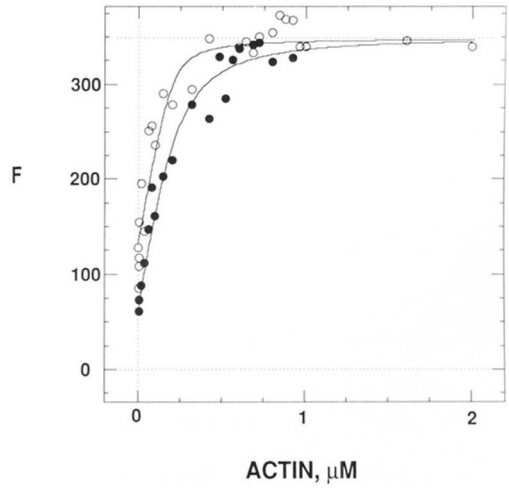

FIG. 7. **Fluorescence intensity measurements observed in binding of [D-Lys(Dns)]^8-CsA and [D-Dab(Boc)]^8-CsA to actin.** The binding curves represent the best least-squares fit to a recursive equilibrium equation as described in the text (K_d for [D-Dab(Boc)]^8-CsA = 570 ± 380 nM). *Open circles*, [D-Lys(Dns)]^8-CsA alone (200 nM); *filled circles*, mixture of [D-Lys(Dns)]^8-CsA (200 nM) and [D-Dab(Boc)]^8-CsA (1 μM).

dissolving the CsA analogues in anhydrous 0.47 M LiCl/tetrahydrofuran, which is then added to the buffer. This method sets the conformation of the CsA analogue predominantly into one conformation that is close to the bioactive conformation (Kofron *et al.*, 1992). Erratic results were obtained when dansyl analogue **4** was dissolved in 95% ethanol, because cyclosporin A and its derivatives exist as slowly interconverting mixtures of multiple conformations in protic solvents. The binding constant of [D-Lys(Dns)]^8-CsA **4** to actin was calculated as 60 ± 33 nM (five determinations) by nonlinear least-squares fit of fluorescence intensities.

A new method for data analysis was utilized to determine the dissociation constants for the cyclosporine analogues relative to the fluorescent analogue **4** (Kuzmic *et al.*, 1992). The competition binding assay is illustrated for the complex of actin and [D-Dab(Boc)]^8-CsA **5**, an 8-position analogue with a hydrophobic tertiary butyl group on the N terminus of the aminobutyryl side chain. A mixture of [D-Dab(Boc)]^8-CsA **5**(1 μM) and [D-Lys(Dns)]^8-CsA **4** (200 nM) was added to actin (0–2 μM), and fluorescence intensities were measured, which were fitted to Equation 1 by using the Marquardt algorithm (Marquardt, 1963). In Equation 1, F_o is the fluorescence at zero protein concentration, ΔF the asymptotic fluorescence change, K_{d1} the dissociation constant, and $[R]$ the concentration of unbound (free) protein. For each set of concentrations, $[R]$ was obtained as a solution of the cubic Equation 2 by using a recurrent formula (Kuzmic *et al.*, 1992). The results indicate that the K_d for [D-Dab(Boc)]^8-CsA **5** is 570 ± 380 nM. Cyclosporin A, in contrast, could not displace the [D-Lys(Dns)]^8-CsA (200 nM) at concentrations as high as 2 μM which means that the K_d for CsA is greater than or equal to 1 μM.

$$F = F_o + \Delta F \frac{[R]}{[R] + K_{d_1}} \tag{1}$$

$$[R]^3 + [R]^2(K_{d_1} + K_{d_2} + [L_1]_o + [L_2]_o - [R]_o) + [R](K_{d_1}K_{d_2} + K_{d_1}[L_2]_o + K_{d_2}[L_1]_o - [R]_o(K_{d_1} + K_{d_2})) + K_{d_1}K_{d_2}[R]_o = 0 \tag{2}$$

The labeling experiments suggested that actin may bind better than SSA1 to photoreagent **2** and other 8-position analogues of CsA. To test the binding affinity of the yeast cytoplasmic Hsp 70s to [D-Lys(Dns)]^8-CsA **4**, a titration experiment was performed with SSA1. The amount of the protein was varied from 0–1 μM and the fluorophore **4** was kept at a constant concentration of 200 nM. Analysis of the data as previously described for actin gave a K_d of 53 ± 48 nM.

DISCUSSION

Our understanding of how cyclosporin A and FK 506 affect specific events during T cell activation has increased greatly in recent years. Liu and coworkers (1991) discovered that the CsA-cyclophilin complex and FK 506-FKBP complex inhibit the phosphatase activity of the calmodulin-dependent phosphatase, calcineurin, and have suggested that this process may be linked to the suppression of transcription of both lymphokine and nonlymphokine genes. Another immunosuppressant rapamycin, which is structurally similar to FK 506, forms a complex with FKBP that does not inhibit calcineurin's phosphatase activity, nor does rapamycin interfere with the transcription of genes such as IL-2, IL-4, and IL-2R (Liu *et al.*, 1991; Dumont *et al.*, 1990). This indicates that inhibition of these transcriptional events is not the only mechanism for causing immunosuppression and that these compounds may disrupt multiple processes required for T cell activation.

Moreover, immunosuppression is only one of a plethora of biological activities exhibited by CsA, and little is known about the biochemical events leading to nephrotoxicity and graft vessel disease caused by chronic rejection, two major side effects produced by this drug (Borel *et al.*, 1989). It is possible that cyclosporin produces these effects through binding to other as yet unidentified proteins in a variety of tissues. To examine this issue, we have synthesized analogue **2**, a highly radioactive, biologically active, photoaffinity probe. In our previous report, we demonstrated that photoaffinity reagent **2** selectively labeled cyclophilin, one of the CsA binding proteins thought to be essential to immunosuppression (Tung *et al.*, 1989). In this paper we describe the identification and partial characterization of a novel family of CsA binding proteins.

Novel CsA binding proteins (43, 50–55 kDa) were detected in bovine calf thymus and in Jurkat extracts. The assay conditions were optimized to maximize specific binding of reagent **2** to the 43-kDa protein and used to purify the 43-kDa protein present in calf thymus, a source chosen for its ready availability. Sequence analysis of the purified protein established that the 43-kDa protein is γ-cytosolic actin. When only partially purified material was affinity-labeled and sequenced, profilin copurified with γ-cytosolic actin. Profilin itself was not labeled, which indicates that CsA and profilin bind to different sites on actin. These experiments were performed at high enough protein concentrations (4–20 μM) so that most of the actin would be tied up in the profilin-actin complex (K_d 2 μM-10 μM; Lal and Korn (1985)). Also, profilin and actin cochromatographed with the specific 43-kDa binding activity of the photoaffinity label, indicating that under certain experimental conditions, the label **2** may have greater affinity for the profilin-actin complex than for actin alone.

In order to compare the relative affinities of the newly identified proteins for CsA with those previously reported for other receptors (Foxwell *et al.*, 1988 1989; Palaszynski *et al.*, 1991), we devised a new fluorescence displacement assay. The fluorescent analogue [D-Lys(Dns)]^8-CsA **4** was synthesized and used in a competition experiment as a mixture with the spectroscopically invisible ligands CsA and [D-Dab(Boc)]^8-CsA **5**. Statistical analysis of the data by using a recursive form of the equilibrium equation (Kuzmic *et al.*, 1992) made it possible to extract the binding constant for the analog [D-Dab(Boc)]^8-CsA **5** to actin.

Actin clearly binds CsA analogues that contain hydrophobic side chains in the 8 position with higher affinity than cyclosporin A. [D-Lys(Dns)]^8-CsA **4** and [D-Dab(Boc)]^8-CsA **5** bind to actin with dissociation constants of 60 ± 33 and 570 ± 380 nM, respectively. The binding of CsA itself to actin is much weaker, with an estimated dissociation constant greater than 1 μM. It is intriguing that like actin, cyclophilin prefers these 8-position analogues compared to the parent drug, cyclosporin A, even though these proteins are not known to be structurally related. Both [D-Lys(Dns)]^8-CsA **4** (K_d 5 nM, Kuzmic *et al.*, 1992) and [D-Dab(Boc)]^8-CsA **5** (K_i 2 nM, Kofron *et al.*, 1991) bind to cyclophilin an order of magnitude tighter than CsA (K_d 30 nM, Kuzmic *et al.*, 1992). The lower immunosuppressive activity of the Dab(Boc) analog (10% the activity of CsA) is most likely due to its higher affinity for actin, which because of its high cellular concentration (5–20% of the total protein) can compete with cyclophilin for binding to cyclosporin A and its analogues. Actin is important for a variety of cellular processes including mRNA transport and localization (Sundell and Singer, 1991), cell growth, and motility. We stress, however, that it is unclear at this time whether the biological effects produced by CsA are a result of the interaction of this cyclic peptide with actin.

The fact that the tertiary structure of the nucleotide binding domain of actin is closely related to the tertiary structure of the ATPase domain of the heat shock cognate 70 (HSC 70)

(Kabsch *et al.*, 1990) prompted us to test if CsA or the photoaffinity labeling analogue **2** would bind to proteins belonging to the 70-kDa heat shock protein family. SSA1, the cytosolic Hsp 70 protein from yeast, was found to be labeled by the AIPP derivative **2** after photolysis. Quantitative analysis of the relative binding by using the fluorescence displacement assay established that **4** binds approximately equally well to actin (K_d 60 nM) and to SSA1 (K_d 53 nM). Because the binding experiments with actin and SSA1 were performed under slightly different buffer conditions (ATP and calcium were used to stabilize the G actin and were present in the actin binding assay, whereas SSA1 was provided to us as a stock solution in 10% glycerol and 50 mM KCl) the two dissociation constants may not be exactly comparable. Since the ATP binding sites of actin and Hsp 70 are structurally related, but not identical, it is possible that the phenyl ring of photoreagent **2** or the naphthalene ring of the dansyl derivative **4** occupy the ATP binding site in the groove between the two subdomains of actin. The Hsp 70 family is believed to catalyze protein folding *in vivo* (Rothman, 1989), and it will be important to determine if other Hsp 70s exist that will bind to cyclosporin A analogues under physiological conditions.

When a mixture of actin and a 1.4-fold excess of SSA1 were photolyzed in the presence of CsA **2**, only actin was labeled. Similarly, photoaffinity experiments on cell homogenates labeled actin but not Hsp 70. These results are surprising because the binding studies indicate that the affinity of the closely related DNS-CsA derivative **4** for these proteins is nearly identical so that we would have expected to detect at least some labeling of SSA1 (or Hsp 70) in either experiment. One possible explanation for this discrepancy is that photolysis results and the binding assays are not directly comparable (for the reasons cited above) and that the efficiency of labeling SSA1 by photolabel **2** may further be affected by differences in labeling efficiencies between proteins for aryl azides (Ruoho *et al.*, 1984). Consequently, the relative labeling of these proteins by the cyclosporin affinity-labeling reagent may not reflect their relative binding affinities. However, an alternative and more provocative explanation is that actin is binding to SSA1 so as to prevent access of CsA **2** to its binding site, and thus provides some information about the nature of the complex. If this occurs, it happens both in the purified protein and in the cell homogenate experiments. Further work is needed to test this hypothesis.

CsA binding proteins with M_r of approximately 45,000 and 50,000 have been reported previously in Jurkat extracts (Foxwell *et al.*, 1988; Palaszynski *et al.*, 1991). We have purified and identified one of these components as actin by CsA affinity purification followed by Western analysis. These findings again establish that actin recognizes and binds to the parent drug, cyclosporin A. The characterization of the 55-kDa protein labeled by **2** has not yet been completed, but several observations merit reporting at this time. While this work was in progress, Hsp 56 was identified as an FK 506 binding protein and in addition was found to have some sequence homology to FKBP 12 and 13 (Yem *et al.*, 1992). Hsp 56, Hsp 70, and Hsp 90 are components of the inactive steroid-receptor protein complexes. In the absence of ligand, steroid receptors are designated as inactive, because they are unable to regulate transcriptional events through binding to specific response elements on DNA. The glucocorticoid and progesterone receptors have been proposed to exist as inactive complexes in the cytoplasm, and upon activation through binding to their respective ligands, migrate to the nucleus where they become competent to bind DNA. We have established that one member of the inactive steroid receptor complexes, Hsp 70, has affinity for immunosuppressive CsA derivatives. Work is in progress to determine if the 55- and 90-kDa proteins we have purified from Jurkat extracts are related to other steroid receptor binding proteins.

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