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

Marcia L. Moss, Rick E. Palmer*, Petr Kuzmic, Brian E. Dunlap, William Henzel, James L. Kofron, William S. Mellon, Catherine A. Royer, and Daniel H. Rich

From the School of Pharmacy and Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706

A novel family of cyclosporin A (CsA) binding proteins was identified by using the biologically active, radioiodinated photoaffinity probe [D-Lys-N'-(4-azido-3-[125]iodophenyl)propionyl])'-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'(5-dimethylamino-1-naphthalenesulfonyl)]'-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'(5-dimethylamino-1-naphthalenesulfonyl)]'-CsA and [N'-t-butoxycarbonyl diaminebutyryl]'-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'(5-dimethylamino-1-naphthalenesulfonyl)]'-CsA and [N'-t-butoxycarbonyl diaminebutyryl]'-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) 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 (Cabantchik et al., 1988; Lin et al., 1991; Flanagan et al., 1991). This process was thought originally to result from the binding of the drug to cyclophilin (Handscharumacher et al., 1984). Subsequently, cyclophilin was discovered to be a peptidyl proline 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 (Brändts 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, 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]'-CsA (0.4% relative to CsA activity), strongly inhibits cyclophilin (K, 2 nM) whereas the moderately (30%) immunosuppressive analogue [MeBm2t]'-CsA (Aebi et al., 1990; Rich et al., 1990) poorly inhibits cyclophilin (K, 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 (Scalini 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 photoactive immunosuppressive CsA analogue, [D-Lys(A'IPP)]'-CsA 2 (Tung et al., 1989). The specific activity of the chemotrope of D-lysine with N-[3-(4-azido-3-[125]iodophenyl)propionyl]succinimide (A'IPP) 6 (Lowndes et al., 1987). The photoaffinity reagent 2 has a specific activity of

[22054]
2010 Cl/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 Freez. All column resins were obtained from Pharmacia LKB Biotechnology 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 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 phenylmethylsulfonyl 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 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 CaCl2. All purification steps were performed 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 MgCl2 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 from these sequencers. 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% of 7 M 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 Sphero column (2 × 100 mm, 4000 Å) from Synchrom by using a 50-min linear gradient of 0.1% trifluoroacetic acid to 70% 1-propanol at a flow rate of 0.2 ml/min.

**Photoaffinity Labeling of Studied Proteins**—Extracts from calf thymus and the human leukemic cell line Jurkat were treated separately with the photoactivatable [3H]-Diethylaminol-1-(4-N,N-diethylamino-1,2,4-triazethylamine)CsA reagent (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, 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). Diethylamino-1-[3H]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-AIPPP.

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<sup>129</sup>IPPS)]<sup>-</sup>-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<sup>129</sup>IPPS)]<sup>-</sup>-CsA.

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 the BPA assay. Cpm (*) indicate the amount of radioactivity as determined by γ counting incorporated into the 43-kDa protein after gel electrophoresis. B, autoradiogram of the gel with arrows indicating the positions of cyclophilin (18 kDa) and the 43-kDa protein.

The eluted proteins from the column were further separated by SDS-polyacrylamide gel electrophoresis. In addition to the 43-kDa CsA-binding protein was eluted near the end of the salt gradient (Fig. 3B). 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 XKAGRAGDPAVFPSIVGRPR 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<sub>r</sub> 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).
extracts using a CsA-affinity matrix. wurden eluted and run on a 12% SDS-polyacrylamide gel. The protein bands were detected by silver staining. The purified material; 49,500, 32,500, 27,500, and 18,500.

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.

Binding of Cyclosporin A to Actin-related Proteins—SSA1 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"IPP)]"-CsA 2 or the diethylamidine control photoreagent 7. SSA1 was labeled by [D-Lys(A"IPP)]"-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, diethylamine-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 the photoaffinity labeling reaction mixture prevented incorporation of the radioslabel 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"IPP)]"-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).

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)]"-CsA 5. This analogue, which binds tightly to cyclophilin (K, 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)]"-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. 5. Photoaffinity labeling of SSA1 with [D-Lys(A"IPP)]"-CsA in the presence or absence of purified G-actin. Samples were preincubated with [D-Lys(A"IPP)]"-CsA in 10 mM HEPES, pH 7.6, 0.5 mM dithiothreitol, 0.2 mM CaCl₂ for 2 h at 25 °C before photolysis. Carrier protein, soybean trypsin inhibitor (20 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. 6. Labeling of actin in the presence of cyclosporin A or [D-Dab(Boc)]"-CsA. Samples of actin purified through the Q-Sepharose column chromatography (4.3 μM) were preincubated for 2 h with nonradioactive competitors cyclosporin A or [D-Dab(Boc)]"-CsA and then treated with 200,000 cpm of [D-Lys(A"IPP)]"-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(Dns)]"-CsA in the presence of 0, 10, 20, 50, and 100 μM CsA; lanes 6–12, actin photolyzed with [D-Lys(A"IPP)]"-CsA and 0, 1, 2, 5, 10, 20, and 50 μM [D-Dab(Boc)]"-CsA.

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

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)]⁴-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)]⁴-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)]⁴-CsA 5 (1 μM) and [D-Lys(Dns)]⁴-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 is the fluorescence at zero protein concentration, ΔF is the asymptotic fluorescence change, Kd is the dissociation constant, and [R] is 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 Kd for [D-Dab(Boc)]⁴-CsA 5 is 570 ± 380 nM. Cyclosporin A, in contrast, could not displace the [D-Lys(Dns)]⁴-CsA (200 nM) at concentrations as high as 2 μM which means that the Kd for CsA is greater than or equal to 1 μM.

\[
F = F_o + \Delta F \frac{[R]}{[R] + K_d}
\]

\[
[R]^3 + [R][K_a + K_d + [L]_o + [L]_i - [R]_o + [R][K_a + K_d + K_m(L)_o + K_m(L)_i - [R][K_a + K_d + K_m] + K_m[R]_o] = 0
\]

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)]⁴-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 Kd 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 calcmodulin-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-cytoosolic actin. When only partially purified material was affinity-labeled and sequenced, profilin copurified with γ-cytoosolic 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 (Kd 2 μM/10 μM; Lai 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 (Poxwill et al., 1988 1989; Palaszynski et al., 1991), we devised a new fluorescence displacement assay. The fluorescent analogue [D-Lys(Dns)]⁴-CsA 4 was synthesized and used in a competition experiment as a mixture with the spectroscopically invisible ligands CsA and [D-Dab(Boc)]⁴-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 analogue [D-Dab(Boc)]⁴-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)]⁴-CsA 4 and [D-Dab(Boc)]⁴-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)]⁴-CsA 4 (Kd 5 nM, Kuzmic et al., 1992) and [D-Dab(Boc)]⁴-CsA 5 (Kd 2 nM, Kofron et al., 1991) bind to cyclophilin an order of magnitude tighter than CsA (Kd 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 CaA 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 (Kd 60 nM) and to SSA1 (Kd 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 analogues under physiological conditions, no labeling of SSA1 (or Hsp 70) in either experiment. One possible explanation for this discrepancy is that photoaffinity labeling experiments on cell homogenates and in vivo will be important to determine if other Hsp 70s exist that will bind to cyclosporin A analogues under physiological conditions.

Acknowledgments—We thank Dr. George Flenkie for characterization of the synthesis of the cyclosporin A IPP photoaffinity label, Dr. Claude B. Klee for antibodies to calcineurin and samples of purified calcineurin A and B, and Dr. Elizabeth Craig for samples of SSA1.

REFERENCES

Aebi, J. D., Deyo, D. T., Sun, C., Guillaume, D., Dunlap, B., and Rich, D. H. (1990) J. Med. Chem. 33, 999-1009
Borel, J. (1983) in Transplantation Proceedings, Suppl. 1 and 2, First International Congress on Cyclosporin, 2218-2229
Borel, J. F., Di Padova, F., Mason, J., Quemerais, X., Ryffel, B., and Wenger, K. (1989) J. Pharmacol. Exp. Ther. 241, 423-431
Brandza, J. D., Halfvorsen, H. R. and Brennan, M. (1975) Biochemistry 14, 4953-4963
Cobucci, W. J., Tung, R. D., Petri, J. A., and Rich, D. H. (1990) J. Org. Chem. 55, 2855-2863
Cebraa, G. R. (1989) Science 243, 355-360
Dumont, F. J., Starch, M., Manders, S. L., Melino, M. R., and Sigal, N. H. (1990) J. Immunol. 144, 251-258
Fischer, G., Bang, H., Berger, E., and Schellenberger, A. (1984) Biochem. Biophys. Res. Commun. 121, 85-97
Flanagan, W. M., Corteschy, Blaise, Bram, R. J., and Cebraa, G. R. (1991) Nature 352, 860-867
Foxwell, B. M. J., Hiestand, P. C., Wenger, R., and Ryffel, B. (1988) Transplantation (Baltimore) 46, 365-368
Foxwell, B. M. J., Mackie, A., Ling, V., and Ryffel, B. (1989) Mol. Pharmacol. 36, 543-549
Goldschmidt-Clermont, P. J., and Janmey, P. A. (1991) Cell 66, 419-421
Hendrichsmacher, R. E., Harding, M. W., Rice, J., Drage, R. J., and Speicher, D. W. (1984) Science 226, 544-547
Her, D., Rodrigues, H., and Uren, S. (1987) J. Chromatogr. 404, 415-422
Hess, A. D., Colombani, P. M., Donnenberg, A. D., Fischer, A. C., and Ryffel, B. (1983) Transplantation Proc. XVII, 1419-1427
Inglesse, J., Blaschzy, B. A., and Benkovsz, S. J. (1989) J. Med. Chem. 32, 937-940
Kabsch, W., Mannherz, H. G., Ouck, S., Pui, E. F., and Holmes, K. C. (1990) Nature 347, 37-44
Klee, C. B., Drasuta, G. F., and Hubbard, M. J. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 149-200
Kock, M., Kessler, H., Seebach, D., and Thaler, A. (1992) J. Am. Chem. Soc. 114, 2676-2680
Koffron, J. L., Kuznicz, P., Kishore, V., Colon-Bonilla, E., and Rich, D. H. (1991) Biochemistry 30, 6127-6134
Koffron, J. L., Kuznicz, P., Kishore, V., Gremmeke, G., Fesik, S. W., and Rich, D. H. (1992) J. Am. Chem. Soc. 114, 2676-2680
Kuznicz, P., Moss, M. L., Kofron, J. L., and Rich, D. H. (1992) Biochem. Biophys. Res. Commun. 505, 63-69
Lal, A. A., and Korn, E. D. (1985) J. Biol. Chem. 260, 10132-10138
Lin, C. S., Bolts, M. C., Siekerka, J. J., and Sigal, N. H. (1991) Cell Immunol. 133, 289-294
Liu, J., Farmer, D. J., Jr., Lane, W. S., Friedman, J. H., Weissman, I., and Schreiber, S. L. (1991) Cell 66, 807-815
Lowndes, J. H., Keon Havelaar, E., and Ruoho, A. E. (1988) Analyt. Biochem. 180, 2849-2853
Marguardt, D. W. (1983) J. Soc. Ind. Appl. Math. 11, 431-444
Palaszynski, E. W., Donnelly, J. G., and Solidin, S. J. (1991) Clin. Biochem. 24, 63-70
Rich, D. H., Sun, C., Plenk, K. J., Aebi, J. D., Dunlap, B. E., Durette, P. L., Dumont, F., and Starch, M. J. (1990) in Peptides: Chemistry, Structure and Biology (Rivier, J. E., and Marshall, G. R., eds) pp. 49-52, ESCOM Press, Leiden, The Netherlands
Rothman, J. E. (1989) Cell 59, 591-601
Roth, R. A., Rashidbaifi, A. R. and Roeder, P. E. (1984) in Membrane, Detergents, and Receptor Solidification (Venter, J. C., and Harrison, L. C., eds) pp. 119-160, Alan R. Liss, Inc., New York
Sorvari, F., Sandri, S., Savoldi, S., Catinelli, L., and Maio, R. (1987) Transplant. Proc. XIX, 1745-1748
Sigel, N. H., Dumont, F., Durette, P., Siekerka, J. K., Peterson, L., Rich, D. H., Dunlap, B. E., Starch, M. J., Melino, M. R., Koprak, S. L., Williams, D., Witzel, B., and Pianko, J. M. (1991) J. Exp. Med. 173, 619-628
Sundell, C. L., and Singer, S. J. (1984) Proc. Natl. Acad. Sci. USA 81, 1181-1184
Tung, R., Dunlap, B., Aebi, J. D., Meillon, W., Ruoho, A. E., Dhanasekaran, N., and Rich, D. H. (1980) in Synthetic Peptides: Approaches to Biological Problems (Tam, J., and Kasper, E. E., eds) UCLA Symposium on Molecular and Cellular Biology, New Series, Vol. 86, pp. 321-335, Alan R. Liss, Inc., New York
Yem, A. W., Tombassi, A. G., Heinrighs, R. M., Zurcher-Neeley, H., Ruff, V. A., Johnson, R. A., and Deibel, M. R., Jr. (1992) J. Biol. Chem. 267, 2688-2691.

Downloaded from http://www.jbc.org/ on guest on March 24, 2020
Identification of actin and HSP 70 as cyclosporin A binding proteins by photoaffinity labeling and fluorescence displacement assays.
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

*J. Biol. Chem.* 1992, 267:22054-22059.

Access the most updated version of this article at http://www.jbc.org/content/267/31/22054

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/267/31/22054.full.html#ref-list-1