Biochemical Pharmacology, Vol. 43, No. 5, pp. 953–960, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
Pergamon Press plc

COVALENT BINDING OF CYCLOSPORINE INHIBITS IRREVERSIBLY T-LYMPHOCYTE ACTIVATION

BERNHARD RYFFEL,* GAËTANE WOERLY, VALÉRIE F. J. QUESNIAUX, HOLGER HUSI and BRIAN M. J. FOXWELL†

Sandoz Pharma Ltd, CH-4002 Basel, Switzerland

(*Received* 17 *September* 1991; *accepted* 2 *December* 1991)

Abstract—A diazirine derivative of cyclosporine (PL-CS) was used to photolabel recombinant human cyclophilin (rhCyp), the cytosolic receptor for the immunosuppressant cyclosporine. The affinity of PL-CS for rhCyp and the immunosuppressive activity were 10-fold reduced as compared to cyclosporine A. Whereas cyclosporine immunosuppression was fully reversible, UV cross-linking of PL-CS resulted in permanent inhibition of lymphocyte activation as shown by proliferation of anti-CD3 stimulated human peripheral lymphocyte, interleukin (IL)-2 gene transcription and IL-2 synthesis in the human T-leukemia cell line Jurkat. *In vivo* photolabeling of viable Jurkat cells revealed that a 21-kDa complex was the major radiolabeled product which was identified as a cyclophilin–cyclosporine complex. In addition, cyclophilin B (25 kDa) and proteins of an unidentified nature at 40, 46 and 60 kDa were observed in Jurkat cells. The cyclosporine-resistant human fibroblast cell line MRC5 displayed a different labeling pattern: cyclophilin B (25 kDa) and a 65-kDa protein were the major labeled products, while the 46- and 60-kDa components were not detectable and cyclophilin was only faintly labeled. In summary, covalent cyclosporine binding caused irreversible lymphocyte inactivation and revealed in addition to cyclophilin other specifically labeled proteins in lymphoid cells. The role and identity of these proteins is presently unknown.

The immunosuppressant cyclosporine (cyclosporine A, CSA‡) has been shown to inhibit lymphocyte activation at the level of IL-2 and other cytokine gene transcription [1–4] acting primarily on T-lymphocytes. Uptake and intracellular concentration of CSA have been demonstrated by radiolabeled CSA in T-lymphocytes and other cells [5–7]. Two proteins have been proposed as molecular targets for CSA: cyclophilin and calmodulin. Cyclophilin, an 18-kDa protein which has been shown recently to have rotamase activity [8, 9], had been identified earlier as an abundant intracytoplasmic binding protein [10, 11]. On the other hand, Colombani *et al.* [12] suggested CSA binding to calmodulin and inhibition by CSA of calmodulin-dependent enzyme activation. Recent investigations into the properties of CSA binding to these purified proteins indicated that calmodulin did not bind CSA [13–16] whereas cyclophilin bound specifically to residues of the CSA molecule known to be necessary for its immunosuppressive action [17, 18]. In addition, it could be shown that all immunosuppressive analogues of CSA did bind to cyclophilin *in vitro*, whereas derivatives unable to bind cyclophilin were devoid of immunosuppressive activity, suggesting that binding to cyclophilin could be a prerequisite for this activity. However, the abundant and ubiquitous distribution of cyclophilin would hardly account for the high selectivity of CSA for lymphocytes [19–21]. To reconcile these findings it could be hypothesized that cyclophilin plays a role only in the intracellular accumulation of CSA. This in turn would imply that the primary molecular targets of CSA expressed selectively in T-lymphocytes and involved in its immunosuppressive activity remain to be identified.

In order to address these questions, an immunosuppressively active, tritiated diazirine CSA derivative ([^{3}H]PL-CS) was developed for the photolabeling of viable human cells of lymphoid and non-lymphoid origin. We show that this derivative binds to rhCyp and cytosolic cyclophilin after labeling of viable lymphocytes. Covalent binding of PL-CS after UV cross linking resulted in irreversible immunosuppression. In addition to cyclophilin, other proteins were specifically labeled with PL-CS. Finally, the pattern of labeled cyclosporin-binding proteins differed substantially between a lymphocytic and a fibroblastic cell line.

MATERIALS AND METHODS

Materials. [^{3}H]PL-CS (SDZ 212-122, Fig. 1, described previously [22, 23]) CSA and CSH were obtained from Sandoz (Basle, Switzerland) and dissolved in ethanol at 10 mM. Antibodies to cyclophilin were provided by Dr M. Harding (Yale University School of Medicine, New Haven, CT, U.S.A.) and sheep antibodies against calmodulin were obtained from Polyscience (Warrington, PA, U.S.A.)). rhCyp was obtained from Dr H. P. Kocher (Sandoz Pharma), bovine calmodulin was purchased

* Corresponding author: Institute of Toxicology, ETH, CH-8603 Schwerzenbach-Zürich, Switzerland.

† Present address: Charing Cross Sunley Research Center, Lurgan Avenue, London W6 8LW, U.K.

‡ Abbreviations: CSA, CSH, cyclosporine A, H; PL-CS, photolabile cyclosporine; rhCyp, recombinant human cyclophilin; PBMC, peripheral blood mononuclear cells; IL-2, interleukin 2; PBS, phosphate-buffered saline; NP40, Nonidet P40; PMSF, phenylmethyl sulfonyl fluoride; BSA, bovine serum albumin; mAb, murine, monoclonal antibody.

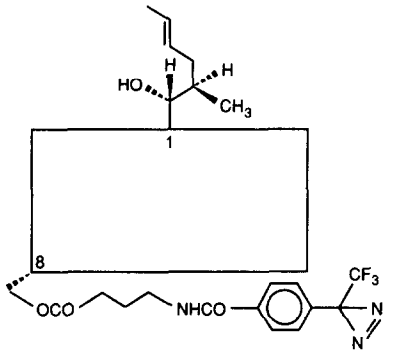

Fig. 1. Structure of PL-CS. A diazyrine group is coupled to tritiated lysine-8 cyclosporine.

from Amersham International (Amersham, U.K.) and murine mAb OKT3 from Ortho Diagnostics (Raritan, NY, U.S.A.).

Cell culture. PBMC were obtained by Ficoll separation from human volunteers. PBMC and the human T-leukemia cell line Jurkat (subline K16) were cultured in complete RPMI 1640 medium containing 10% fetal calf serum, and the human fibroblast cell line MRC5 (obtained from ATCC, Bethesda, U.S.A.) in DMEM medium containing 10% fetal calf serum. PBMC were activated by 2.5 ng/mL murine mAb OKT3 (Ortho) in the presence of increasing amounts of CSA and derivatives (up to 1 μg/mL). Proliferation was assessed after a 6-hr [^{3}H]thymidine pulse (0.5 μCi/well) at 72 hr of culture; incorporated radioactivity was measured by liquid scintillation counting (Packard, Tricarb).

IL-2 assay. The murine, IL-2-dependent HT-2 cells were used to assess IL-2 in the supernatant of primary culture as described elsewhere [24]. Briefly, serial dilutions of supernatants were incubated with 10^4 HT-2 cells in 100 μL complete RPMI 1640 medium containing 10% fetal calf serum in 96 microtitre plates for 24 hr. Proliferation was assessed after a 6-hr [^{3}H]thymidine pulse by measuring cell-associated radioactivity as described above. For the quantification a recombinant human IL-2 standard was used and the results were quantified as units per mL [24].

Northern blot analysis. After a 4-hr culture Jurkat cells were frozen and the RNA extracted on cesium chloride ($CsCl_2$) gradient [25]. Total RNA (10 μg) was size fractionated on 1% agarose-formaldehyde gel and transfered on a nylon membrane (Gene Screen Plus, NEN-Research, Boston, MA, U.S.A.). The filter was processed according to the manufacturer's instruction, prehybridized and hybridized with ^{32}P-labeled human IL-2 probe [26].

Photolabelling. Viable cells were washed three times with PBS at pH 7.2 and suspended at 5×10^6/mL in PBS. Cells were incubated with 1 μg/mL of [^{3}H]PL-CS in a 24-well Costar plate (Gibco) for 30 min at room temperature in the dark. After exposure to UV light (UV lamp, model B-1004, Blak-Ray, CA, U.S.A.) at a distance of 10 cm on ice for 5 min, the cells were washed and lysed in 50 μL of PBS containing 1% NP40, 2 mM PMSF and 1 mM EDTA for 30 min on ice. The cell lysate was centrifuged at 12,000 *g* for 10 min at 4° The supernatants were mixed 1:1 with gel sample buffer (20% glycerin, 2% SDS, 62.5 mM Tris–HCl, 0.0004% bromophenolblue, pH 6.8 in distilled water) containing 5% 2-mercaptoethanol and heated at 97° for 3 min. Supernatants were electrophoresed on 15% (w/v) polyacrylamide gel according to Laemmli [27]. The gels were fixed in water/methanol/acetic acid (5:5:1), washed in water and soaked for 1 hr in 1 M sodium salicylate, and dryed and exposed to Hyperfilm-MP (Amersham) at −70°.

For the direct comparison of cell lines, protein concentrations were determined [28] and identical amounts of protein were loaded on the gels.

Western blotting. Western transfers were performed as described previously [7]: in brief, gels were presoaked in Tris (25 mM), glycine (150 mM) buffer at pH 7.2 containing 2% methanol for 30 min and transferred on to nitrocellulose membrane by the use of the Biometra fast blot system. After blockade in 1% casein and 1% BSA in PBS the membrane was incubated either with murine anti-CSA mAb (3 μg/mL, [29, 30]) or anti-cyclophilin serum from rabbit (obtained from Dr M. Harding, 1:5000, [11, 17]). Subsequently, the membranes were incubated with biotinylated sheep anti-mouse immunoglobulin (1:200, Amersham) or anti-rabbit immunoglobulin, followed by horse radish peroxidase–streptavidin complex (Amersham, 1:500). The reactive proteins were developed with the substrate diaminobenzidine (0.5 mg/mL) and cobalt chloride (0.3 mg/mL) in PBS.

Solid phase immunoassay for cyclophilin. The binding of cyclosporine to cyclophilin was determined in a competitive solid phase immunoenzymatic assay as described previously [17]. Briefly, cyclosporine coupled to BSA was coated on ELISA plates. Cyclophilin (100 ng/mL) was incubated without or with increasing concentrations of free CSA (0.001–10 μg/mL) overnight at 4°. After washing, cyclophilin bound to the CSA conjugate coated on the solid phase was detected by rabbit anti-cyclophilin antiserum (1:10,000) followed by anti-rabbit immunoglobulin goat antibodies coupled to alkaline phosphatase (Sigma Chemical Co., St Louis, MO, U.S.A., 1:1000). The absorbance at 405 nm was measured after hydrolysis of the enzymatic substrate *p*-nitrophenylphosphate.

Immunoprecipitation. Photolabeled cells (25×10^6) were lysed in 1 mL 0.05 M Tris, 1% NP40, 150 mM NaCl, 1% BSA and 2 mM PMSF. The lysate was centrifuged at 12,000 *g* for 10 min at 4°. The supernatant was incubated twice at 4° with 100 μL protein A Sepharose (Pharmacia) before addition of 5 μL of anti-cyclophilin, 5 μL, rabbit anti-mouse (Sigma) or 5 μL sheep anti-calmodulin (Polysciences) antibodies. After an overnight incubation at 4°, 25 μL protein A Sepharose was added (15 min at room temperature, followed by 60 min at 4°). The Sepharose was washed four times with 0.05 M Tris pH 7.4, 0.1% NP40, 0·03% SDS, 150 mM NaCl, 1 mg/mL BSA and 2 mM PMSF followed by a wash with 0.05 M Tris pH 7.4–150 mM NaCl. Precipitated

proteins were analysed by SDS–PAGE as described. Both anti-cyclophilin and anti-calmodulin were able to precipitate 18-kDa proteins from [^{35}S]methionine-labeled Jurkat cells using the above technique (data not shown).

RESULTS

Binding of PL-CS to cyclophilin

The ability of PL-CS to bind rhCyp after the introduction of the diazirine group at position 8 was tested directly on an SDS–PAGE gel and by ELISA. As shown in Fig. 2A, exposure of rhCyp (18 kDa) to PL-CS resulted in the formation of a novel component at 21 kDa as shown by silver staining (lane b) which was radiolabeled on exposure on X-ray film (lane c) confirming that the new component is a product of photocross-linked [^{3}H]PL-CS and cyclophilin. The specificity of binding was demonstrated by the competition of cyclophilin labeling by an excess of CSA or unlabeled PL-CS (lane d, e), but not by the inactive CSH (lane f). Finally, neither bovine serum albumin (lane g) nor calmodulin (lane h) were labeled.

The relative affinity for cyclophilin was determined in a competitive solid phase immunoenzymatic assay [17]. As shown in Fig. 2B the binding activity of the diazirine derivative PL-CS was 10-fold reduced as compared to CSA. UV cross-linking of PL-CS to rhCyp (Fig. 2A) may result in long lasting inactivation of its rotamase activity, which may be important for cellular activation.

Inhibition of lymphocyte activation

The immunosuppressive properties of CSA, PL-CS and the inactive CSH were compared on anti-CD3-induced human T-lymphocyte proliferation (Fig. 3A). The immunosuppressive activity of PL-CS paralleled the cyclophilin binding activity (Fig. 2B) and was about 10-fold reduced as compared to CSA, but still full inhibition of proliferation was obtained between 500 and 1000 nM. The IC_{50} was 10 nM for CSA, 100 nM for PL-CS and >1000 nM for CSH.

The immunosuppressive effects of cyclosporines were shown previously to be fully reversible. Thus, after a 1-hr pre-incubation the immunosuppressants could be removed by repeated washing. However, if the 1-hr preincubation was followed by a three min UV exposure on ice, a treatment which did not affect cell viability, cells exposed to PL-CS were blocked irreversibly (Fig. 3B). Thus, UV light caused covalent binding and inactivation of cyclophilin *in vivo*. Furthermore, UV cross-linking of PL-CS on the human T-leukemia cell line Jurkat caused irreversible inhibition of IL-2 gene transcription (Fig. 3C) and IL-2 synthesis (Fig. 3D).

These results indicate that PL-CS upon exposure to UV light engaged the cyclosporine receptor protein cyclophilin irreversibly resulting in a blockage of cell activation. We next tried to confirm the *in vivo* labeling of the receptor.

Identification of cyclophilin and other minor CSA binding proteins after in vivo *photoaffinity labeling of lymphocytes*

Viable Jurkat cells were incubated with [^{3}H]PL-CS, exposed to UV light (3 min), lysed in detergents and cellular proteins were separated on SDS–PAGE gel (15%). The [^{3}H]PL-CS cross-linked proteins were detected using fluoroautoradiography (Fig. 4). The major component was a 21-kDa protein complex (lane a), which was inhibited by CSA (lane d) but not inactive CSH (lane b) or by the macrolide immunosupressant FK506 (lane c). This 21-kDa component was identified unequivocally as cyclophilin by Western blot analyses (lane e) and immunoprecipitation using rabbit anti-cyclophilin antibody (lane h). Finally, immunoprecipitation of radiolabeled Jurkat cells with anti-calmodulin antibody was negative (lane i). In addition to cyclophilin (21-kDa component), there were protein complexes at 25, 40, 46 and 60 kDa, which were also inhibited by excess CSA, but not by CSH. Photocross-linking of Jurkat cells over a broad concentration range indicated that for all labeled proteins 50% saturation was in the 0.3 μM range (data not shown).

The results identify directly cyclophilin as the major cyclosporin binding protein *in vivo*. However, in addition to this ubiquitous and abundant cytosolic binding protein additional receptor proteins have been identified, which may have a role in the immunosuppressant activity of cyclosporine. These proteins are most likely localized in the membranes (H. Husi, in preparation). The 25-kDa protein was identified as cyclophilin B by Western blot analysis and immunoprecipitation (preliminary data obtained in collaboration with Dr G. Spik, Nantes, France). Cyclophilin B has been reported previously to function as a CSA binding protein in the endoplasmatic reticulum [31–33]. The CSA–protein complex at 60 kDa gave a broad signal; it is possible that this complex is composed of calcineurin A, which has been suggested recently to be a target of CSA [34] and of a second protein at 65 kDa, which was mainly found in the CSA-resistant cell line (see below).

The specificity of CSA receptor proteins was assessed further by comparison of the photolabeling patterns of a cyclosporine-sensitive and -resistant cell line. So far, the generation of a cyclosporine-resistant lymphoid cell line has been elusive. We thus used the human fibroblast cell line MRC5 which is reportedly cyclosporine-resistant after cell activation [35].

Evidence for unique binding proteins by comparison of cyclosporine-sensitive and -resistant cell lines

A direct comparison of photolabeled proteins in the cyclosporin-sensitive Jurkat cell and in the resistant human fibroblast cell line MRC5 was performed. As shown in Fig. 5 the fibroblast cell line MRC5 displayed photolabeled proteins at 25 and 65 kDa; the 21-kDa component (cyclophilin) showed only faint labeling and the 46- and 60-kDa proteins observed in Jurkat cells were not present. The reduced labeling of cyclophilin in the resistant cell was surprising, since equal amounts of cyclophilin were found in both cells by ELISA [21]. This finding raises the question as to whether cyclophilin is not accessible to CSA in MRC5 fibroblasts. The prevalence of the 25-kDa protein in MRC5 fibroblasts is interesting and preliminary evidence suggests that

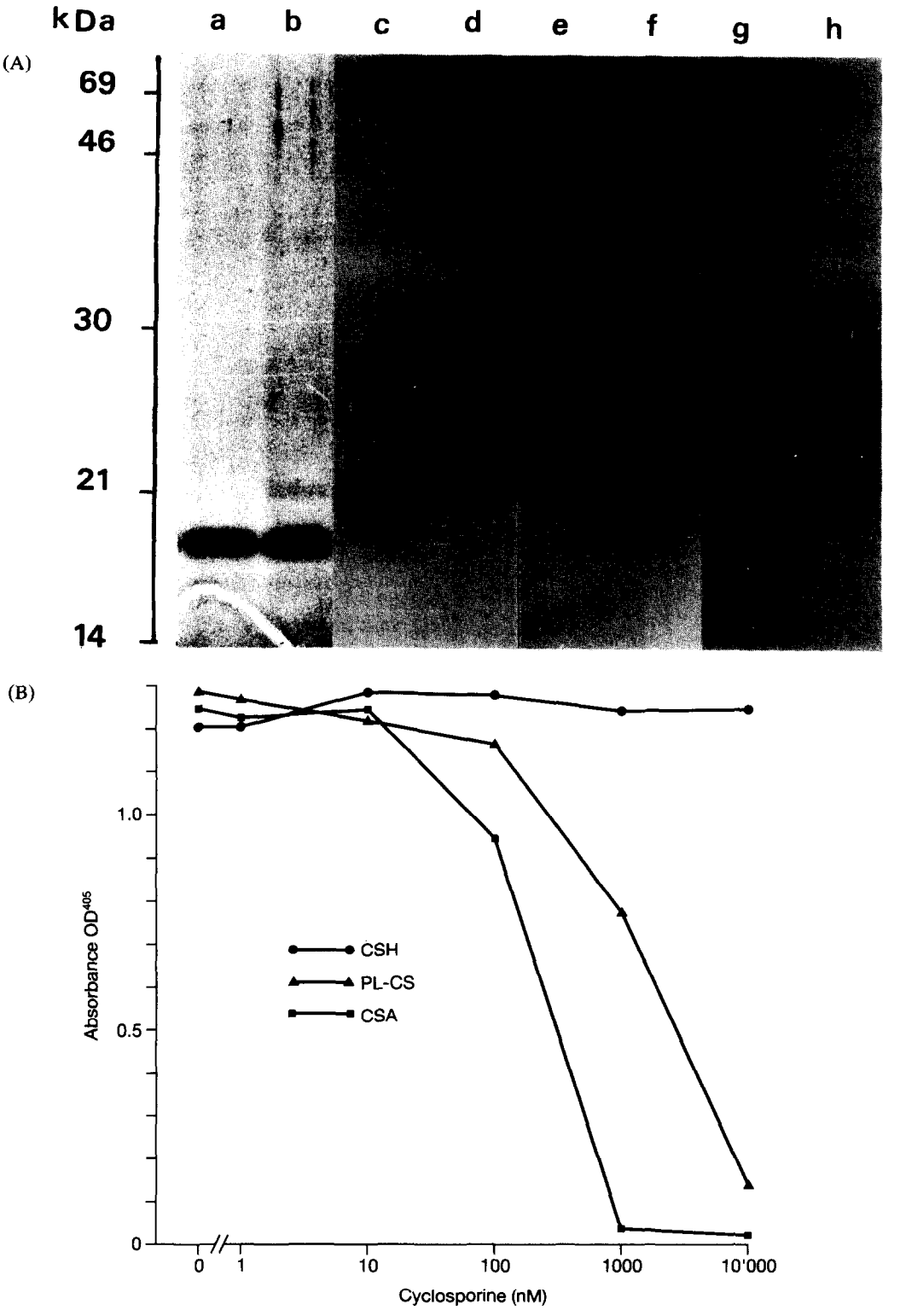

Fig. 2. Binding of PL-CS to rhCyp. (A) Photoaffinity labeling of cyclophilin. rhCyp (1 μg) was incubated with 1 μM [^{3}H]PL-CS (b–f) in the absence (a–c) or presence of a 10-fold molar excess of unlabeled PL-CS (d), CSA (e) or CSH (f), followed by UV exposure (b–f). In addition, 1 μg BSA (g) and calmodulin (h) were incubated with [^{3}H]PL-CS, exposed to UV light and served as specificity control. The samples were size separated on different SDS–polyacrylamide gels (7.5%) and the proteins visualized either by silver staining (a, b) or by autoradiofluorography (c–h). (B) Competition for cyclophilin binding. Cyclophilin (100 ng/mL) was incubated with increasing concentrations of competitor (0–1000 ng/mL) on CSA-coated ELISA plates as described previously [17]; bound cyclophilin was detected by rabbit anti-cyclophilin antibody followed by goat anti-rabbit alkaline phosphatase.

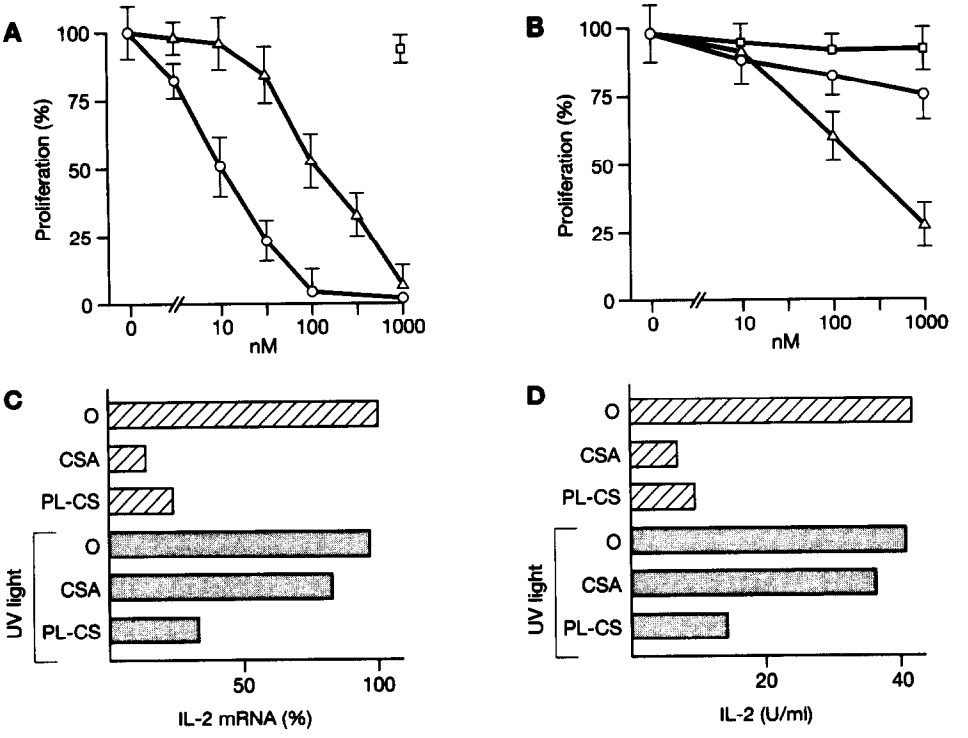

Fig. 3. Cross-linking of PL-CS by UV light inhibits irreversibly T-lymphocyte activation. (A) Inhibition of anti-CD3 antibody-stimulated lymphocyte proliferation. Lymphocytes (10^5) were stimulated with 2.5 ng/mL OKT3 mAb in the presence of CSA, CSH or PL-CS. [^{3}H]Thymidine incorporation for the last 6 hr was measured after a 72-hr culture period. (○) CSA, (△) PL-CS, (□) CSH. (B) UV cross-linking causes irreversible inhibition of lymphocyte proliferation. Lymphocytes were incubated as above (A) with CSA, PL-CS or CSH for 30 min followed by a short UV exposure (3 min on ice). Lymphocytes were washed extensively in a medium containing 20% fetal calf serum and 10 μg/mL anti-CSA mAb. After six washes the cells were resuspended in complete medium and stimulated with 2.5 ng/mL OKT3 mAb. [^{3}H]Thymidine incorporation for the last 6 hr was measured after a 72-hr culture period. (○) CSA, (△) PL-CS, (□) CSH. (C) Inhibition of IL-2 production by Jurkat cells. The T-leukemia cell line Jurkat was stimulated with PHA (1 μg/mL) and PMA (10 ng/mL; for 48 hr in the absence or presence of CSA (100 nM) or PL-CS (300 nM). IL-2 synthesis was assessed in the supernatant after 48 hr culture using the murine IL-2-dependent HT2 cell as a proliferation assay. Duplicate cultures were exposed to UV light and extensive washing. (D) UV cross-linking of PL-CS inhibits IL-2 gene transcription in Jurkat Cells. Jurkat cells were treated as above and total cellular RNA was extracted, size fractionated and hybridized with IL-2 specific RNA probe.

it is identical to cyclophilin B [32]. Although the identity of the 46- and 60-kDa proteins (only in Jurkat cells) and the 65-kDa protein (observed only in MRC5 fibroblasts) has not yet been defined, these results demonstrate distinct molecular targets of CSA in addition to cyclophilin in these two cell lines which differ in cyclosporine sensitivity.

DISCUSSION

A biologically active, photoaffinity derivative has been used to identify the intracellular molecular targets of the immunosuppressant cyclosporine. Photolabeling of rhCyp resulted in the formation of a 21-kDa band which was most likely to have been composed of a PL-CS–protein complex at 1:1 ratio, since there was no evidence of any intermediate product (i.e. doublets). The fact that the cyclosporine–cyclophilin complex was found at 21 instead of 19.5 kDa (PL-CS has a M_r of 1.5 kDa) may be due to the slightly altered migratory properties of complexed cyclophilin in the gel. Labeling of cyclophilin was specific for immunosuppressive cyclosporines, since only CSA and not the immunosuppressively inactive CSH was able to inhibit the photolabeling. Whereas the inhibitory effect of CSA on lymphocyte activation was reversible, photocross-linking of PL-CS to viable human cells, resulted in irreversible inhibition of lymphocyte activation as shown by proliferation, IL-2 gene transcription and IL-2 protein synthesis.

Photolabeling *in situ* of viable Jurkat cells gave—either by immunoblotting with anti-CSA monoclonal antibodies or fluoroautoradiography—a major labeled 21-kDa product, which was shown to contain cyclophilin by immunoprecipitation. Attempts to precipitate photolabeled calmodulin complexes were unsuccessful and correlate with the inability to

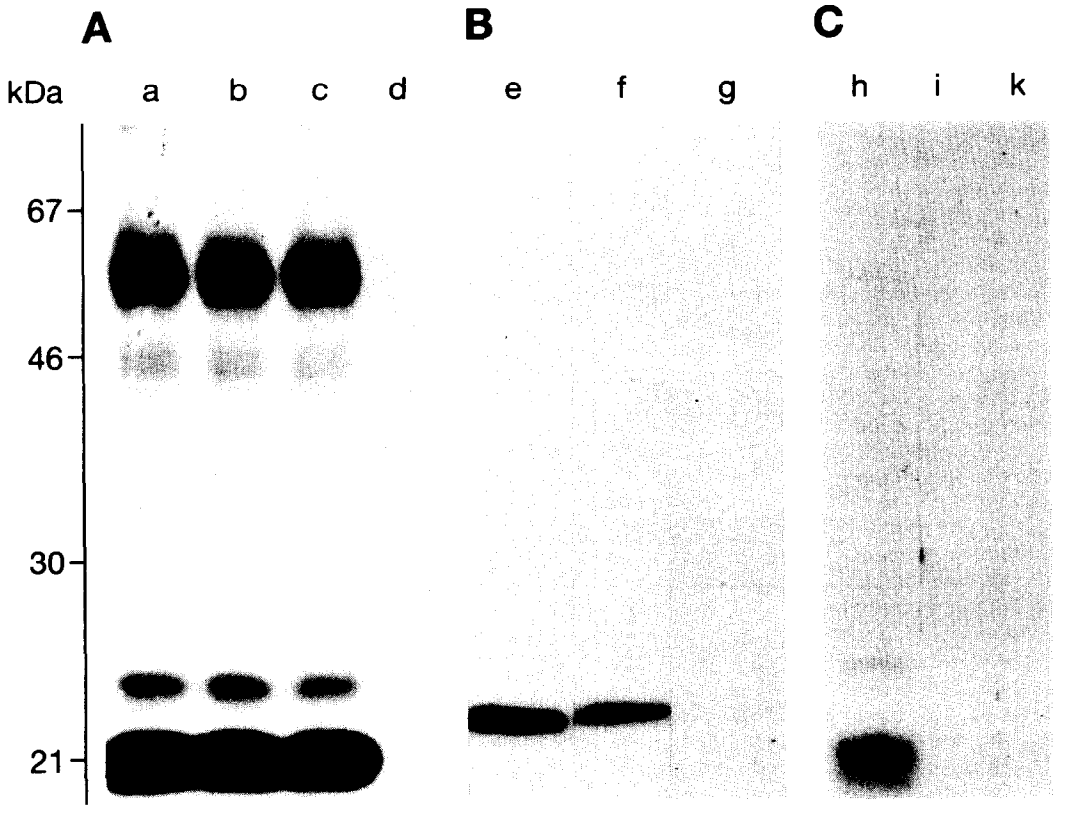

Fig. 4. A photocross-linking of cyclosporin on the human T-leukemia cell line Jurkat. Jurkat cells (5×10^6/mL) were incubated in the dark with [^{3}H]PL-CS at 1 μM in the absence (a) or presence of a 10-fold excess of CSH (b), FK 506 (c) or CSA (d) for 30 min, followed by UV exposure on ice (3 min). The cell lysate was separated on a 15% SDS–polyacrylamide gel and the labeled proteins detected by autoradiofluorography. (B) Immunoblot analysis of photoaffinity labeled proteins. Photolabeled proteins (Jurkat, 5×10^6/mL, 1 μM PL-CS) were transferred on a nitrocellulose membrane and detected by cyclophilin rabbit serum (e), CSA murine mAb (f) or a rabbit control serum (g). (C) Immunoprecipitation of photolabeled proteins. The labeled lysate proteins were incubated with cyclophilin rabbit serum (h), calmodulin sheep antiserum (i) or a rabbit control serum (k).

photolabel purified calmodulin; the absence of CSA binding to calmodulin agrees with results from previous studies [13–16, 18]. In addition to cyclophilin, other proteins were photolabeled. Jurkat cells showed labeling of 25-, 30-, 40-, 46- and 60-kDa proteins. The labeling of these proteins was—as for cyclophilin—inhibited totally by excess CSA, but only slightly reduced by the inactive CSH.

The present results demonstrate for the first time the *in vivo* labeling of cyclophilin. Cyclophilin has been cloned from various species [37–41] and identified previously as a CSA acceptor protein *in vitro* [10, 11]. The cyclosporine complex at 25 kDa was identified as cyclophilin B, a recently cloned cyclosporine binding protein which has high homology to cyclophilin and contains a membrane signal peptide (Refs 31–33 and unpublished data). Although our previous studies showed that both Jurkat and MRC5 cells contain similar levels of cyclophilin in the 2 μg/mg range [21], [^{3}H]PL-CS labeling gave a major band at 25 kDa in MRC5 cells, whereas cyclophilin was only faintly labeled. In addition, a major 65-kDa protein was labeled in MRC5 cells whereas the 46- and 60-kDa proteins were absent.

These results are intriguing since the fine specificity of cyclophilin–CSA binding [17, 18, 42, 43] as well as the lymphoid cell specificity of cyclosporine has been at odds with the ubiquitous and abundant occurrence of this protein. However, the present data suggest that intracellular cyclophilin is not accessible to CSA and/or cyclophilin's binding capacity may differ among various cells. Another explanation could be that the 25-kDa protein is more abundant in MRC5 cells and thus reduces cyclophilin photolabeling. The role of the other proteins in CSA immunosuppression is unclear.

The family of immunophilins, a term coined by Schreiber [44] for low molecular weight acceptor proteins for cyclosporine and macrolide structured immunosuppressants, is growing and their physiological role has not yet been identified. In addition to cyclophilin and cyclophilin B, various other cyclosporine acceptor sites have been reported (reviewed in Ref. 46).

Ziegler *et al.* [45] reported on the photoaffinity

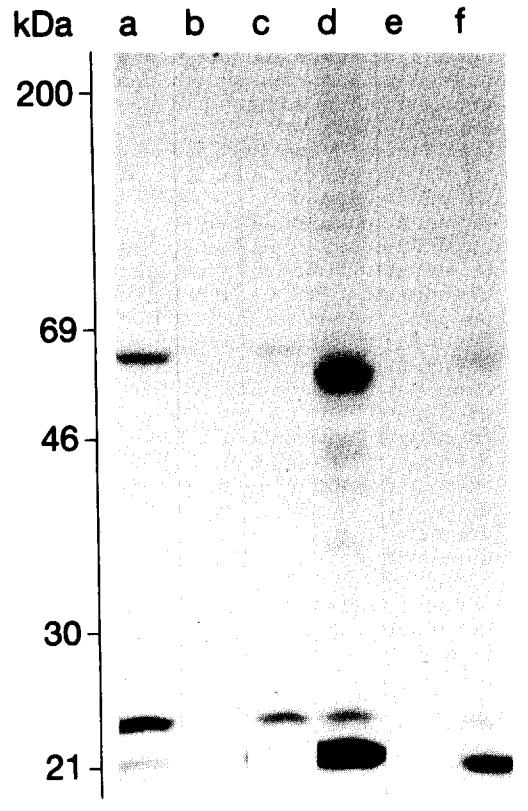

Fig. 5. Photoaffinity labeling of the fibroblast cell line MRC5 shows distinct differences in CSA binding protein. MRC5 fibroblast (a, b, c) and Jurkat cells (d, e, f) incubated with 1 μM [^{3}H]PL-CS were UV cross-linked as described above. Identical amounts of proteins were loaded on the SDS–polyacrylamide gel (500 μg). Photolabeling was performed in the absence (a, d) or in the presence of a 20-fold excess of CSA (b, d) or CSH (c, e).

labeling of cyclosporine binding proteins on renal and hepatic membranes. In the renal brush border membrane labeling of a 75-kDa protein which is identical to the Na^+-D-glucose cotransporter has been identified [46]. We identified recently another membrane protein on multidrug resistant tumor cell lines, the gp170, as an acceptor of cyclosporine [23]. Cyclosporine binding to the gp170 membrane protein, which functions as a drug efflux pump, reverses multidrug resistance. Previously, investigators have suggested that cyclosporine could bind to various sites including the nucleus, findings which have not been substantiated by direct experimental evidence (summarized in Ref. 46).

In summary, using a photolabeling analogue of cyclosporine, cyclophilin was shown to constitute the major but not unique cyclosporine binding protein in viable lymphoid cells. Covalent binding of PL-CS resulted in irreversible inhibition of lymphocyte activation. Other cyclosporine protein complexes at 25 (cyclophilin B), 40, 46 and 60 kDa were identified in Jurkat cells. In CSA-resistant MRC5 fibroblasts cyclophilin photolabeling was greatly reduced and superseded by the 25-kDa protein as the major labeled component. Thus, the intracellular targets of CSA may vary considerably in drug-sensitive and -resistant cell types, which helps to explain the lymphoid cell specificity of CSA. The role of cyclophilin and cyclophilin B as well as the higher molecular weight cyclosporin binding proteins in the immunosuppressive and toxic effect of the drug is presently unknown.

REFERENCES

1. Kroenke M, Leonard W, Depper J and Greene W, Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proc Natl Acad Sci USA* **81**: 5214, 1984.
2. Elliot J, Lin Y and Mizel S, Induction of interleukin 2 messenger RNA inhibited by cyclosporin A. *Science* **226**: 1439–1441, 1984.
3. Granelli-Piperno A, Inaba K and Steinman RM, Stimulation of lymphokine release from T lymphoblasts. *J Exp Med* **160**: 1792–1802, 1984.
4. Foxwell B, and Ryffel B, The mechanisms of action of cyclosporine. *Immunol Allergy Clin North Am* **9**: 79–93, 1989.
5. Merker M and Handschumacher R, Uptake and nature of the intracellular binding of cyclosporin A in a A murin tymoma cell line BW5147. *J Immunol* **132**: 3064–3070, 1984.
6. Ryffel B, Goetz U and Heuberger B, Cyclosporin receptors on human lymphocytes. *J Immunol* **129**: 1978–1982, 1982.
7. Foxwell B, Frazer G, Winters M, Hiestand P, Wenger R and Ryffel B, Identification of cyclophilin as the erythrocyte cyclosporin-binding protein. *Biochim Biophys Acta* **938**: 447–455, 1988.
8. Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T and Schmid FX, Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**: 476–473, 1989.
9. Takahashi N, Hayano T and Suzuki M, Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337**: 473–475, 1989.
10. Handschumacher R, Harding M, Rice J and Drugge R, Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* **226**: 544–547, 1984.
11. Harding M, Handschumacher R and Speicher D, Isolation and amino acid sequence of cyclophilin. *J Biol Chem* **261**: 8547–8555, 1986.
12. Colombani P, Robb A and Hess A, Cyclosporin A binding to calmodulin: a possible site of action on T lymphocytes. *Science* **228**: 337–339, 1985.
13. Foxwell B, Wong WC, Borel JF and Ryffel B, A comparison of cyclosporin binding by cyclophilin and calmodulin. *Transplant Proc* **21**: 873–875, 1989.
14. Foxwell B, Hiestand P, Wenger R and Ryffel B, A comparison of cyclosporin binding by cyclophilin and calmodulin and the identification of a novel 45 KD cyclosporine-binding phosphoprotein in Jurkat cells. *Transplantation* **46**: 35S–40S, 1988.
15. LeGrue SJ, Turner R, Weisbrodt N and Dedman JR, Does the binding of cyclosporin to calmodulin result in immunosuppression? *Science* **234**: 68–71, 1986.
16. Hait WN, Harding MW and Handschumacher RE, Calmodulin, cyclophilin and cyclosporin A. *Science* **233**: 987–988, 1986.
17. Quesniaux V, Schreier M, Wenger R, Hiestand P, Harding M and Van Regenmortel M, Cyclophilin binds to the region of cyclosporin involved in its immunosuppressive activity. *Eur J Immunol* **17**: 1359–1365, 1987.
18. Quesniaux V, Schreier M, Wenger R and Van

Regenmortel M, Molecular characteristics of cyclophilin-cyclosporine interaction. *Transplantation* **46**: 23S–27S, 1988.
19. Koletsky A, Harding M and Handschumacher R, Cyclophilin: distribution and variant properties in normal and neoplastic tissues. *J Immunol* **137**: 1054–1059, 1986.
20. Ryffel B, Foxwell B, Gee A, Greiner B, Woerly G and Mihatsch MJ, Cyclosporine-relationship of side effects to mode of action. *Transplantation* **46**: 90S–96S, 1988.
21. Ryffel B, Woerly G, Greiner B, Haendler B, Mihatsch MJ and Foxwell B, Distribution of the cyclosporin binding protein cyclophilin in human tissues. *Immunology* **72**: 399–404, 1991.
22. Wenger RM, Cyclosporin: conformation and analogues as tools for studying its mechanism of action. *Transplant Proc* **20**: 313–318, 1988.
23. Foxwell B, Mackie A, Ling V and Ryffel B, Identification of the multi-drug resistance related, *p*-glycoprotein as a cyclosporine binding protein. *Mol Pharmacol* **36**: 543–546, 1989.
24. Foxwell B, Simon J, Herrero J, Taylor D, Woerly G, Cantrel D and Ryffel B, Anti-CD3 antibody-induced expression of both p55 and p75 chains of the high affinity interleukin-2 receptor on human T lymphocytes is inhibited by cyclosporin A. *Immunology* **69**: 104–109, 1990.
25. Maniatis T, Fritsch E and Sambrook J, *Molecular Cloning*. Cold Spring Laboratory, 1982.
26. Brantschen S, Gauchat J, de Weck A and Stadler B, Regulatory effect of recombinant interleukin IL3 and IL4 on cytokine gene expression of bone marrow and peripheral blood mononuclear cells. *Eur J Immunol* **19**: 2017–2023, 1989.
27. Laemmli U, Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature* **227**: 680–682, 1970.
28. Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–253, 1974.
29. Quesniaux V, Tees R, Schreier M, Wenger R and Van Regenmortel M, Fine specificity and cross-reactivity of monoclonal antibodies to cyclosporine. *Mol Immunol* **24**: 1159–1168, 1987.
30. Quesniaux V, Tees R, Schreier M, Maurer G and Van Regenmortel M, Fine specificity and cross-reactivity of monoclonal antibodies to improve therapeutic monitoring of cyclosporine. *Clin Chem* **33**: 32–37, 1987.
31. Price ER, Cidowski LD, Jin M, Baker CH, McKion FD and Walsh CHT, Human cyclophilin B, a second cyclophilin gene encodes a pepti-prolyl isomerase with a signal sequence. *Proc Natl Acad Sci USA* **88**: 1903–1907, 1991.
32. Spik G, Haendler B, Delmass O, Mariler Ch, Chamou M, Stettmann K, Kocher HP, Keller R, Hiestand PC and Movva NR, A novel secreted cyclophilin-like protein (SCYLT). *J Biol Chem* **266**: 10735–10738, 1991.
33. Caroni P, Rotenflueh A, McGlynn E and Schneider C, S-cyclophilin, new member of the cyclophilin family associated with the secretary pathway. *J Biol Chem* **266**: 10739–10742, 1991.
34. Liu J, Farmer JD, Lane WS, Friedman J, Weissman I and Schreiber SL, Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP–FK506 complexes. *Cell* **66**: 807–815, 1991.
35. Gunter K, Irvine S, Zipfel P and Kelly K, Cyclosporin in A-mediated inhibition of mitogen-induced gene transcription is specific for the mitogenic stimulus and cell type. *J Immunol* **142**: 3286–3291, 1989.
36. Ziegler K, Frimmer M and Koepsell H, Photoaffinity labeling of membrane proteins from rat liver and pig kidney with cyclosporin diazirine. *Transplantation* **46**: s15–s20, 1989.
37. Tropschug M, Nicholson D, Hartl F, Koeler H, Pfanner N, Wachter E and Neupert W, Cyclosporin A-binding protein (cyclophilin) of *Neurospora crassa*. *J Biol Chem* **263**: 14433–14440, 1988.
38. Haendler B, Hofer-Warbinek R and Hofer E, Complementary DNA for human T-cell cyclophilin. *EMBO* **6**: 947–950, 1987.
39. Haendler B, Keller R, Hiestand P, Kocher H and Wegmann G, Yeast cyclophilin: isolation and characterization of the protein, cDNA and gene. *Gene* **83**: 39–46, 1989.
40. Danielson P, Forss-Petter S, Brow M, Calavetta L, Douglass J, Milner R and Sutcliffe J, p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. *DNA* **7**: 261–267, 1988.
41. Liu J, Albers MW, Chen CM, Schreiber SL and Walsh CT, Cloning expression and purification of human cyclophilin in *Escherichia coli* and assessment of the catalytic role of cysteines by site-directed mutagenesis. *Proc Natl Acad Sci USA* **87**: 2304–2308, 1990.
42. Sigal N, Dumont F, Turette T, Siekierka J, Peterson L, Rich D, Staruk M, Melino M, Koprak S, Williams D, Witzl D and Bisano J, Is cyclophilin involved in the immunosuppressive and nephrotoxic mechanism of action of cyclosporin A? *J Exp Med* **173**: 619–628, 1991.
43. Hsu V, Heald S, Harding M, Handschumacher R and Armitage I, Structural elements pertinent to the interaction of cyclosporin A with its specific receptor protein, cyclophilin. *Biochem Pharmacol* **40**: 131–140, 1990.
44. Schreiber S, Chemistry and biology of the immunophilines and their immunosuppressive ligands. *Science* **251**: 283–287, 1991.
45. Ziegler K, Frimmer M, Fritzsch G and Koepsell H, Cyclosporin binding to a protein component of the renal Na^+-D-glucose cotransporter. *J Biol Chem* **265**: 3270–3277, 1990.
46. Ryffel B, Cellular activation: regulation of intracellular events by cyclosporin. *Pharmacol Rev* **41**: 407–421, 1989.