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Identification of the Multidrug Resistance-Related P-Glycoprotein as a Cyclosporine Binding Protein

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SUMMARY

The immunosuppressive agent cyclosporine A has been shown to reverse multidrug resistance (MDR) in malignant cells. In the present study, a ^{3}H-cyclosporine diazirine analogue was used to photolabel viable MDR Chinese hamster ovary cells. The 170-kDa membrane P-glycoprotein, which functions as a drug efflux pump, was strongly labeled. The binding of ^{3}H-cyclosporine diazirine analogue to P-glycoprotein was competable by excess cyclosporine A and by the nonimmunosuppressive cyclosporine H. These results suggest that cyclosporine reverses the MDR phenotype by binding directly to P-glycoprotein and that this binding is not dependent on the immunosuppressive potential of the cyclosporine derivative. The identification of P-glycoprotein as a cyclosporine binding protein has obvious implications for cancer chemotherapy.

The development of MDR in malignant cells may be a major obstacle limiting successful chemotherapy (1–8). Gene transfection studies have provided compelling evidence that an increased expression of the 170-kDa plasma membrane P-glycoprotein is sufficient to cause MDR (9–11). P-glycoprotein likely functions as an energy-dependent drug efflux pump to mediate MDR in a variety of systems (12). The immunosuppressive agent CSA has been shown to be an effective chemosensitizing agent for reversing the MDR phenotype (13, 14). This finding poses the question of how chemosensitization is effected. In the present study, ^{3}H-PL-CS was used to photolabel viable MDR CHO cells.

Experimental Procedures

Materials. ^{3}H-PL-CS (Sandoz 212-122; provided by Dr. R. Wenger, Sandoz Basle) is a tritiated (4 μCi/μg) derivatized cyclosporine containing a diazirine group linked to the cyclosporine ring via an aminobutanoyl spacer, at position 8, (Fig. 1). This derivative binds cyclophilin and has immunosuppressive activity approximately 10% that of CsA.[2] CSA and CSH were dissolved at 10 μg/ml in ethanol. [^{3}H]Azidopine was purchased from Amersham (UK)(94 mCi/mg); verapamil, diltiazem, and colchicine were from Sigma.

Cell culture. Drug-sensitive parent CHO cells, AUXB1 cells, and the colchicine (10 μg/ml)-resistant CHO cell line, CH^{R}C5 were cultured in α-minimum essential medium (GIBCO) and subcultured as described (5).

Photolabeling. Cells were washed three times with PBS and suspended at 5 × 10^6/ml in PBS. Cells were incubated with 1 μg/ml ^{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, San Gabriel, CA), 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 lysate was centrifuged at 12,000 × g for 10 min at 4°. Supernatants were electrophoresed on 5.6% (w/v) acrylamide gels containing 1% SDS and 9 M urea (15). Gels were fixed in water/methanol/acetic acid (5:5:1), washed with water, soaked for 1 hr in 1 M sodium salicylate, dried, and exposed to Hyperfilm-MP (Amersham International) at −70.

Immunoprecipitation. Cells (25 × 10^6) were lysed in 200 μl of 0.05 M Tris, pH 7.4, 1% SDS, 2 mM PMSF, for 5 min on ice followed by sonication (3 × 15 sec; MSE sonicator). After the addition of 800 μl of 0.05 M Tris, 1.25% NP40, 150 mM NaCl, 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 of protein A-Sepharose (Pharmacia) before the addition of 9.4 μg of C219 antibody. After an overnight incubation at 4°, 25 μl of protein A-Sepharose were added (15 min at room temperature, followed by 105 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 bovine serum albumin, 2 mM PMSF, followed by a wash with 0.05 M Tris, pH 7.4, 150 mM NaCl. Precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described.

Results and Discussion

Photolabeling of the multidrug-resistant CHO cell line CH^{R}C5 revealed the presence of two major cyclosporine binding proteins at 170 and 22 kDa and a minor component of 70 kDa

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ABBREVIATIONS: MDR, multidrug resistance; CHO, Chinese hamster ovary; PMSF, phenylmethylsulfonyl fluoride; CsA, cyclosporine A; SDS, sodium dodecyl sulfate; ^{3}H-PL-CS, ^{3}H-cyclosporine diazirine analogue; CsH, cyclosporine H; PBS, phosphate-buffered saline; NP40; Nonidet P-40.

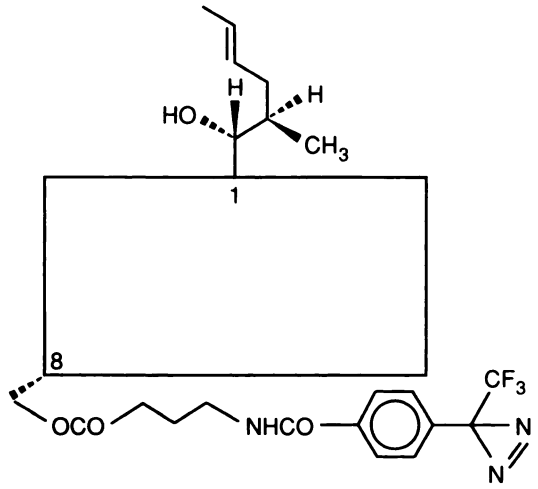

Fig. 1. Structure of photoactive cyclosporine derivative.

(Fig. 2A, *lane a*). Specificity of labeling was demonstrated by the lack of detectable labeling in the presence of a 20-fold excess of unlabeled CsA or in the absence of ^{3}H-PL-CS (Fig. 2A, *lanes b* and *c*). Similar studies performed on AuxB1, a drug-sensitive CHO cell line (Fig. 2A, *lane d*), and on the T leukemia cell line Jurkat (Fig. 2A, *lane e*) resulted in the labeling of only the 70- and 22-kDa proteins. Again, labeling of these proteins was inhibited by excess CsA (results not shown). These results indicated that the labeled 170-kDa cyclosporine binding protein was likely to be P-glycoprotein. This was confirmed by specific immunoprecipitation of the 170-kDa cyclosporine-labeled protein using the monoclonal antibody (C219) against P-glycoprotein (15) (Fig. 2B). The 22-kDa component is cyclophilin, the ubiquitous cyclosporine binding protein characterized previously (16, 17). This was also confirmed by immunoprecipitation.[2]

Photolabeling of CH^{R}C5 cells with ^{3}H-CS-PL in the presence of CsA and CsH (Fig. 3) showed that both compounds inhibited ^{3}H-PL-CS cross-linking to P-glycoprotein in a dose-dependent manner, whereas, as expected, cyclosporine labeling of the 22-kDa cyclophilin was less affected by CsH than by CsA. The 70-kDa component behaved in a manner similar to cyclophilin. These results suggest that binding of cyclosporines to P-glycoprotein is not related to the immunosuppressive potential of the cyclosporines. Both CsA and CsH were effective in reversing the drug resistance phenotype of CH^{R}C5, although CsH was slightly less active.[3] Taken together, these findings strongly suggest that the binding of cyclosporines to P-glycoprotein is biologically significant.

Previous studies have shown that photoactive analogues of ATP, vinblastine, and a calcium channel blocker, azidopine, were able to bind P-glycoprotein (18–21) in isolated membrane preparations. The current studies were performed on intact cells. Therefore, it was of interest to determine whether similar results are obtained. The calcium channel blockers verapamil and diltiazem reverse MDR (18–20). Both diltiazem (Fig. 4, lanes *a* and *b*) and verapamil (Fig. 4, lanes *c* and *d*) showed dose-dependent inhibition of cyclosporine labeling of the 170-kDa protein. However, colchicine in similar concentrations had no effect (Fig. 4, lanes *e* and *f*). The latter result correlates with the observation that colchicine does not inhibit the *in vitro* photolabeling of P-glycoprotein by a vinblastine analogue, whereas both verapamil and diltiazem were able to do so (18).

In a parallel experiment, azidopine photolabeling of intact CH^{R}C5 cells resulted in the labeling of the 170-kDa P-glycoprotein as well as producing a smear of labeled products of 40–120 kDa, indicating that this agent binds cellular proteins other than P-glycoprotein (Fig. 5). The presence of both CsA and the nonimmunosuppressive CsH totally abolished labeling in the

[3] V. Quesniaux, manuscript in preparation.

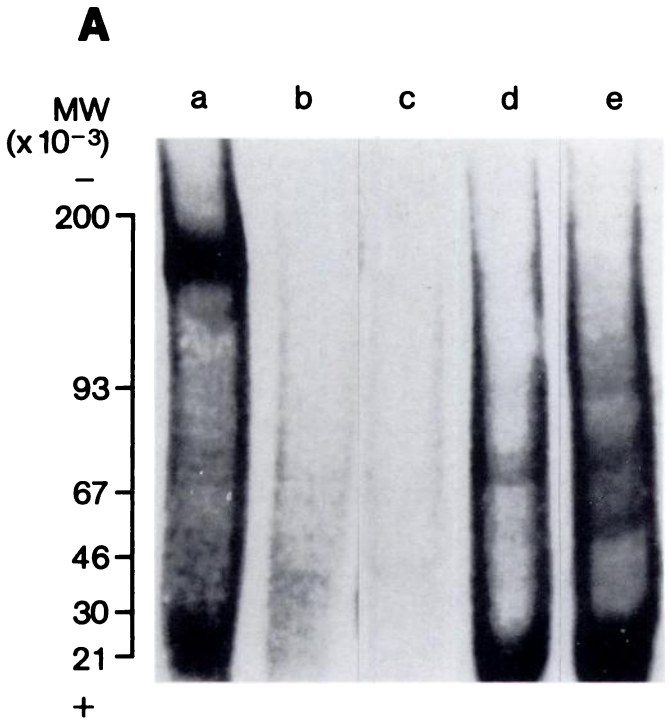

Fig. 2. A, Cyclosporine photolabeling followed by electrophoresis and autoradiofluorographic analysis of CHO cells. *Lane a*, colchicine-resistant cell line CH^{R}C5 photolabeled with ^{3}H-PL-CS; *lane b*, CH^{R}C5 photolabeled with ^{3}H-PL-CS in the presence of 20 μg/ml CsA; *lane c*, CH^{R}C5 exposed to UV light without ^{3}H-PL-CS; *lane d*, photolabeled drug-sensitive cell line AuxB1; *lane e*, photolabeled T leukemic cell line Jurkat. B, Immunoprecipitation with anti-P-glycoprotein antibody C219. *Lane a*, colchicine-resistant cell line CHRC5; *lane b*, drug-sensitive cell line Aux B1.

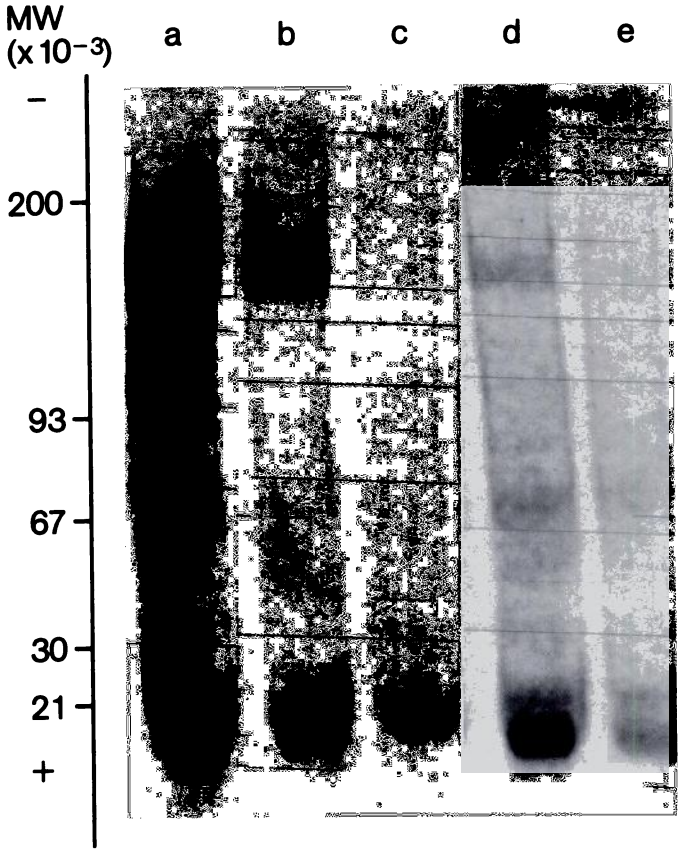

Fig. 3. Effect of CsA and CsH on the cyclosporine photolabeling of CH^RC5 cells. Cells (5×10^6) were photolabeled with 1 μg/ml ^{3}H-PL-CS in the presence of no additions (*lane a*), 3 μg/ml CsA (*lane b*), 10 μg/ml CsA (*lane c*), 3 μg/ml CsH (*lane d*), or 10 μg/ml CsH (*lane e*).

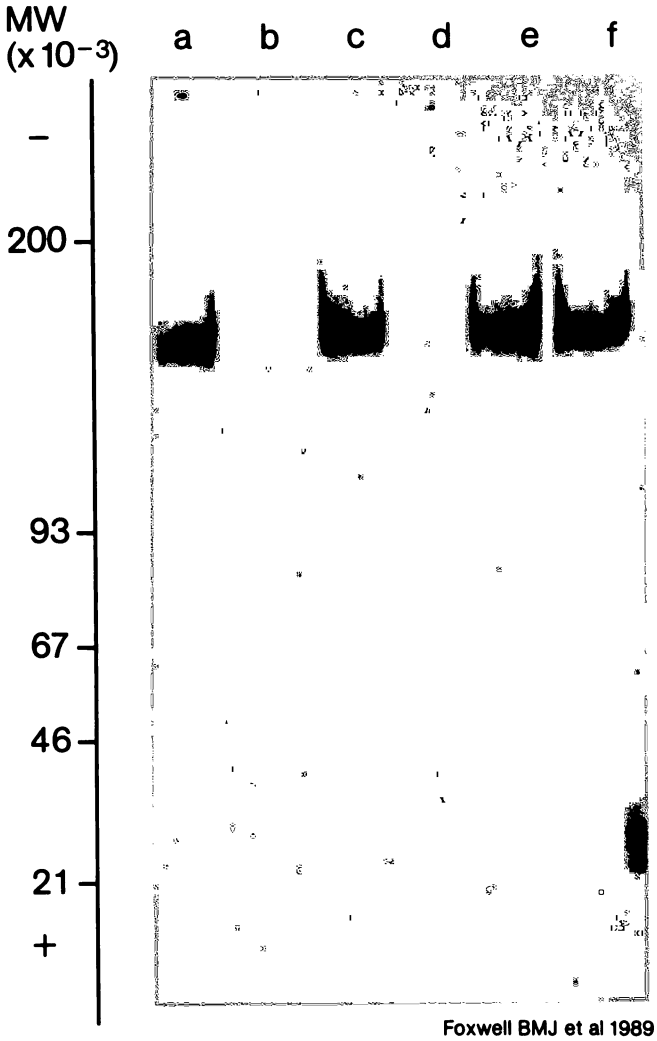

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Fig. 4. Effect of calcium channel blockers on cyclosporine photolabeling of CH^RC5. CH^RC5 (5×10^6) cells were photolabeled with 1 μg/ml ^{3}H-PL-CS in the presence of 3 μM diltiazem (*lane a*), 30 μM diltiazem (*lane b*), 3 μM verapamil (*lane c*), 30 μM verapamil (*lane d*), 3 μM colchicine (*lane e*), or 30 μM colchicine (*lane f*).

170 kDa region, without having any apparent effect on any other labeled protein, which finding again shows the specificity of cyclosporine for P-glycoprotein.

There is ample evidence that cyclosporines function as chemosensitizing agents in reversing the MDR phenotype in malignant cells (13, 14, 22–24).[4] Based on our studies, cyclosporine appears to mediate its effect on MDR by binding the P-glycoprotein drug transporter. Furthermore, P-glycoprotein binding of cyclosporines seems not to discriminate between immunosuppressive and nonimmunosuppressive derivatives of the drug, unlike the other identified cyclosporine binding protein cyclophilin (17). The observation that the Ca^{2+} channel blockers prevent cyclosporine binding of P-glycoprotein and vice versa indicates that all these compounds bind similar or interactive sites on the P-glycoprotein molecule; however, allosteric effects cannot be excluded. The precise mechanism by which binding to P-glycoprotein results in inhibited transport is as yet unclear. There appears to be no obvious relationship between the compounds that inhibit MDR, although several Ca^{2+} channel blockers can mediate this effect. Their ability to block calcium channels does not define this activity (8), and cyclosporines do not inhibit Ca^{2+} influx during T lymphocyte activation (25). Regardless of the mechanism, nontoxic analogues of cyclosporines may be useful for sensitizing some clinically chemoresistant tumors.

Of particular interest is the observation that P-glycoprotein is normally expressed in the kidney and liver (8), sites of cyclosporine toxicity (26). It is possible that the drug's interference with the normal function of P-glycoprotein could be responsible for a wide spectrum of biological effects associated with compound's clinical usage. Further studies will be required to delineate some of these.

[4] F. Loor and V. Quesniaux, manuscript in preparation.

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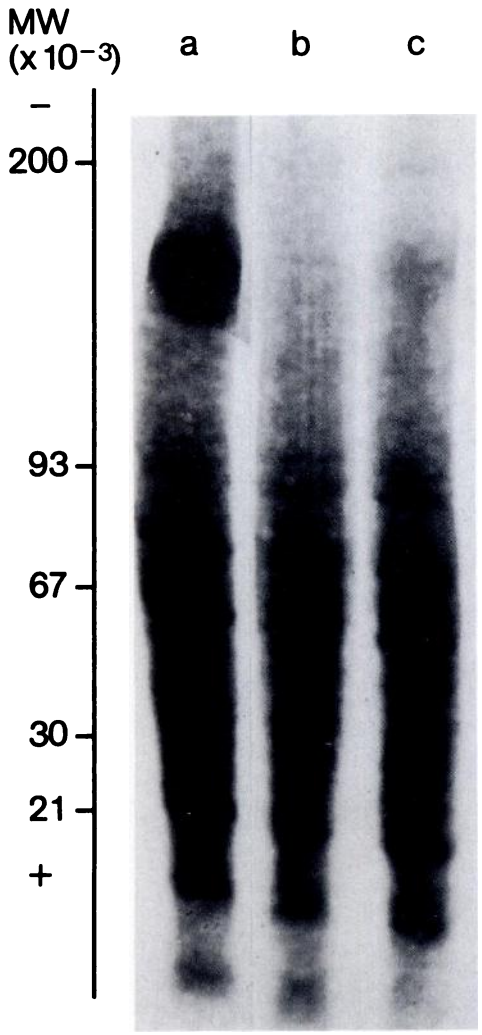

Fig. 5. Effect of cyclosporines on [^{3}H]azidopine photolabeling of CH^{R}C5 cells. PBS-washed cells (5 × 10^6/ml) were incubated with 250 pg/ml [^{3}H] azidopine (94 mCi/mg; Amersham) for 30 min without additions (*lane a*), with 20 μM CsA (*lane b*), or with 20 μM CsH (*lane c*). Cells were exposed to a single flash of UV light using an Isomess photoflash apparatus (West Germany). Cells were analyzed as described in Experimental Procedures.

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