Human P-glycoprotein Transports Cyclosporin A and FK506*  
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Cyclosporin A, a cyclic undecapeptide, and FK506 are efficient immunosuppressive agents. They also attract attention as effective P-glycoprotein modulators that inhibit P-glycoprotein from binding to anticancer drugs and overcome multidrug resistance. Cyclosporin A itself interacts with a common binding site of P-glycoprotein to which Vinca alkaloids and verapamil bind. We were interested to determine whether cyclosporin A and FK506 are substrates for P-glycoprotein to transport, and we studied their transcellular transport. In LLC-PK1 cells, derived from porcine kidney proximal tubule and forming a highly polarized epithelium, cyclosporin A was transported in a saturable manner. LLC-GA5-COL300, a transformant cell line derived by transfecting LLC-PK1, with human MDRI cDNA isolated from normal adrenal gland, expresses P-glycoprotein specifically on the apical surface and shows a typical multidrug-resistant phenotype. LLC-GA5-COL300 cells showed increased transport of cyclosporin A from the basal to the apical side. Kinetic analysis showed that this transport was a typical saturable transport with the calculated apparent Michaelis constant ($K_M$) and the maximum flux ($V_{max}$) as 8.4 $\mu$M and 2.4 nmol/mg protein/h, respectively. LLC-GA5-COL300 also showed increased transport of FK506 from the basal to the apical side. These results indicate that P-glycoprotein transports the immunosuppressive agents cyclosporin A and FK506.

The development of multidrug resistance (MDR) is a major obstacle in cancer chemotherapy. Typical MDR is against various anticancer drugs that have no structural similarity. P-glycoprotein is a 170-kDa membrane protein coded by the MDRI gene in humans and is believed to be involved in MDR. P-glycoprotein binds various anticancer drugs including Vinca alkaloids, anthracyclines, and actinomycin D (1–3), and pumps them out of the cell using the energy from ATP hydrolysis (4–6). P-glycoprotein also interacts with peptides. Overproduction of human P-glycoprotein confers resistance against the peptide ionophore antibiotics gramicidin D (7) and valinomycin. Chinese hamster and human P-glycoproteins were reported to be capable of transporting a tripeptide, N-acetyl-leucyl-leucyl-norleucinal, which is an inhibitor of various intracellular proteinases (9). It was also reported that the mouse mdr3 gene but not the human MDRI gene could complement yeast STE6, which is a homologue of mammalian mdr genes and mediates export of a-factor mating peptide in Saccharomyces cerevisiae (9, 10).

In in vitro experiments, some lipophilic compounds are able to reverse the MDR phenotype. These compounds include natural alkaloids, some calcium channel blockers, protein kinase inhibitors, and some immunosuppressive agents. They are thought to modulate MDR by interacting with drug-binding site(s) of P-glycoprotein competitively (11). Among them, cyclosporin A, a cyclic undecapeptide with highly immunosuppressive effects, efficiently prevents P-glycoprotein from binding with anticancer drugs and overcome MDR, and attracts a great deal of attention as an effective P-glycoprotein modulator (12–14). A photoreactive analogue of cyclosporin A was shown to label P-glycoprotein as well as cyclopellin in living cells, and this labeling was inhibited by cyclosporin A, cyclosporin H, diltiazem, and verapamil, but not by colchicine (15). A binding study using membrane vesicles of MDR cells indicated that cyclosporin A competitively interacts with a common drug-binding site of P-glycoprotein, to which Vinca alkaloids and verapamil bind (16). It was also reported that MDR Chinese hamster cells accumulated less cyclosporin A than the corresponding drug-sensitive cells (16, 17), but it remains to be answered if cyclosporin A is a substrate for human P-glycoprotein to transport. This question is important not only for understanding the exact mechanism by which effective modulators reverse MDR, but also for understanding the pharmacokinetics of immunosuppressive agents.

We have reported about transcellular transport by human P-glycoprotein expressed in LLC-PK1 cells, derived from the epithelial cells of porcine kidney proximal tubule (18, 19). Human P-glycoprotein was expressed specifically on the apical surface, and transported a cardiac glycoside, digoxin (19), vinblastine, and the steroid hormones aldosterone and cortisol (18). Because this transcellular transport system is useful to investigate transport by P-glycoprotein without annoyance by nonspecific binding of lipophilic compounds, we attempted to show if cyclosporin A and the more potent immunosuppressive drug FK506 (20) are transported by P-glycoprotein. Here we report that cyclosporin A and FK506 were substrates for P-glycoprotein to transport and P-glycoprotein reduced the accumulation of cyclosporin A and FK506 in cells.

EXPERIMENTAL PROCEDURES  
MATERIALS—[3H]Cyclosporin A (433 GBq/mmol, 37 MBq/ml) was obtained from Amerham Corp. [3H]FK506 (473 MBq/mmol) was provided by Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. Unlabeled cyclosporin A was provided by Sandoz Pharmaceuticals, Ltd., Tokyo, Japan. [3H]Sucrose (148 MBq/ml) was from Du Pont-New England Nuclear. Microplates with bottom-filtered cups (Transwell 3414, 24.5 mm in diameter polycarbonate filter with tissue culture treatment, 3.0-$\mu$m pore size) was from Costar. Medium 199 was from Gibco.

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1 The abbreviation used is: MDR, multidrug resistance.
2 Y. Fuji, H. Konishi, K. Ueda, and T. Komano, unpublished observation.
Cell Lines—LLC-PK₁ is a cell line derived from porcine kidney proximal tubules. LLC-PK₁ cells were transfected with human MDR1 cDNA (21), and a clone designated as LLC-GA5-COL300 was isolated by selecting with 300 ng/ml colchicine (18). LLC-GA5-COL300 cells express P-glycoprotein specifically in the apical membrane.

Transcellular Transport of Cyclosporin A and FK506—LLC-PK₁ cells were plated on bottom-filtered cups at a density of 4 × 10⁶ cells/cm². LLC-GA5-COL300 cells were plated at a density of 5 × 10⁶ cells/cm², and colchicine was added at a final concentration of 300 ng/ml. Cells were incubated over 3 nights, and media were changed for fresh and colchicine-free medium 6 h before experiments. For measurement of transcellular transport, the medium of either the basal or apical side of the monolayer was replaced with medium containing 3.7 kBq/ml [³H]cyclosporin A and 3.7 kBq/ml (24.8 μM) [¹⁴C]sucrose, or 2.9 kBq/ml (6.2 μM) [¹⁴C]FK506. The cells were incubated at 37 °C. Aliquots (25 μl) of the medium of the donor and the other side were taken at 1, 2, and 3 h, and the radioactivities were measured. The paracellular fluxes were monitored by measuring the appearance of [¹⁴C]sucrose in the other side. After the 3-h sampling, cells were washed with phosphate-buffered saline twice. The filter was cut out of the cup, and cells were lysed in 1 ml of 0.3 N NaOH. Then protein and cyclosporin A accumulated in the cells were measured.

Kinetic Study of Transcellular Transport—Media of the basal side of LLC-PK₁ and LLC-GA5-COL300 monolayers were replaced with media containing 3.7 kBq/ml [³H]cyclosporin A and various concentrations of unlabeled cyclosporin A. Transcellular transport was measured after 3 h. Because the amount of cyclosporin A passed across LLC-GA5-COL300 monolayer increased linearly with time within 3 h (see Fig. 1A), we used the transport rate at 3 h as the initial rate.

RESULTS AND DISCUSSION

LLC-PK₁ cells derived from epithelial cells of porcine kidney proximal tubule form an epithelium consisting of a monolayer of highly polarized cells. Some lipophilic compounds, such as vinblastine, and some sterols can pass across the epithelium by simple diffusion, and the amount passed from the basal to the apical side and that from the apical to the basal side are almost equal (18). LLC-GA5-COL300 is a transformant cell line that was derived by transfection with human MDR1 cDNA isolated from normal adrenal gland (21) and by selection with colchicine. LLC-GA5-COL300 expresses P-glycoprotein specifically on the apical surface and shows a typical MDR phenotype. We demonstrated that in LLC-GA5-COL300 cells the amounts of vinblastine, aldosterone, cortisol (18), and digoxin (19) passed from the basal to the apical side were increased and the amounts from the apical to the basal side were decreased, indicating that these compounds are substrates for P-glycoprotein to transport. Using this system, we measured the time course of transcellular transport of cyclosporin A (Fig. 1A). After 3 h, the amount passed across the LLC-PK₁ epithelium from the basal to the apical side and that from the apical to the basal side were 4.5 and 3.5%, respectively. These amounts are quite low compared to that from the apical to the basal side were 4.5 and 3.5% (Fig. 1A). This increased amount of cyclosporin A passed across the epithelium was suppressed by adding a 1000-fold excess of unlabeled cyclosporin A (Fig. 1D). These results suggest that P-glycoprotein transports cyclosporin A and that this transport could be saturable. The amount of [¹⁴C]sucrose moved across the LLC-GA5-COL300 monolayer was significantly higher than that in LLC-PK₁ (Fig. 1A, lower panel), indicating that the tight junction of LLC-GA5-COL300 cells was slightly loose for a small aqueous solute compared to that of LLC-PK₁ cells. However, it might not be the case with lipophilic substances, because almost equal amounts of [³H]nitrendipine, which is a calcium channel blocker and was reported not to bind to the membrane vesicles of MDR cells (22), were transported in host and transformant cells in both directions (Fig. 1B). Furthermore, the difference in the amount of transported [³H]staurosporine, a protein kinase inhibitor, between LLC-GA5-COL300 cells and LLC-PK₁ cells was smaller than [¹⁴C]sucrose and was less than 1%/h in both directions (Fig. 1C). Because the molecular weight of cyclosporin A (1201) is significantly higher than that of staurosporine (495), the paracellular leak across LLC-GA5-COL300 monolayer is expected to be lower than that of staurosporine. These results suggest that the tight junction of LLC-GA5-COL300 cells was as tight for lipophilic substances such as cyclosporin A as that of host cells and that it is worth comparing the transport of lipophilic substances across LLC-GA5-COL300 monolayers with that across LLC-PK₁ monolayer.

Cells were lysed in 0.3 N NaOH after 3 h, and the amounts of cyclosporin A accumulated in cells were measured (Fig. 2). When cyclosporin A was added to the apical side, the amount of cyclosporin A accumulated in LLC-GA5-COL300 was one-sixth of that in LLC-PK₁, although no decrease of the apical
to-basal transport in transformant cells was detected in transcellular transport experiments (Fig. 1A). This is probably because the amount passed from the apical to the basal side of host cells was very low and the decrease from that was undetectable. On the other hand, when cyclosporin A was added to the basal side, the difference in accumulation between the transformant and the host cells was not significant, although LLC-GA5-COL300 cells accumulated less cyclosporin A than LLC-PKI. These results could be explained as follows. When cyclosporin A was added to the apical side, P-glycoprotein localized in the apical membrane efficiently caught cyclosporin A during the process of entering cells, and pumped it back to the apical medium. When cyclosporin A was added to the basal side, considerable amount of cyclosporin A was adsorbed by cellular components before it reached to the apical membrane. These results indicate that P-glycoprotein mediates the efflux of cyclosporin A from cells, and are consistent with the polarized localization of P-glycoprotein in the apical plasma membrane of LLC-GA5-COL300.

We further investigated the mode of interaction of cyclosporin A with P-glycoprotein by a kinetic study. Various concentrations of cyclosporin A were added in the media of basal side, and the radioactivities appeared in the apical side were measured (Fig. 3A). Transport of cyclosporin A across LLC-PK1, as well as LLC-GA5-COL300 monolayer was saturated at the concentration of 20-40 nM.

Net transport, calculated by subtracting the amount of cyclosporin A passed across the LLC-PK1 monolayer from that across the LLC-GA5-COL300 monolayer (Fig. 3A), representing the P-glycoprotein-mediated transcellular transport, was plotted in Fig. 3B. This subtraction should cancel the nonspecific transport, including the simple diffusion and the paracellular leak, provided that these effects are similar between two kind of cells. The transport of cyclosporin A associated with P-glycoprotein showed a typical pattern of saturable transport. The double-reciprocal plot of the rate of transport against the cyclosporin A concentration at the basal side clearly showed a linear relationship with the correlation coefficient of 0.99 (Fig. 3C). The apparent Michaelis constant (Kp) and the maximum transport (Vmax) were calculated as 8.4 μM and 2.4 nmol/mg protein/h, respectively. These parameters reveal the overall characteristics of transcellular transport, which was accelerated by P-glycoprotein because we used the concentration at the basal side of media instead of intracellular concentration. It has been reported that the concentration of vincristine necessary to inhibit the growth of multidrug-resistant K562/ADM cells was shifted from 680 nM to 84 nM and to 15 nM in the presence of 3 μM and 10 μM cyclosporin A, respectively, whereas that of the drug-sensitive parent K562 cells was 0.8 nM (20). Kp of 8.4 μM for the transcellular transport of cyclosporin A seems to be reasonable, if the overall characteristics of cyclosporin A transcellular transport and the effect of cyclosporin A to overcome MDR phenotype can be directly compared. Although the values are apparent, our data above do indicate that P-glycoprotein transports cyclic peptide cyclosporin A by a saturable mechanism.

Because cyclosporin A, an efficient and clinically used immunosuppressive agent, was found to be a substrate for P-glycoprotein to transport, we were interested to find if another potent immunosuppressive agent, FK506, is also transported by P-glycoprotein. It was reported that FK506 modulated P-glycoprotein functions more effectively than cyclosporin A (20). FK506 overcame resistance to vincristine and doxorubicine of various MDR cells in vitro and increased the chemotherapeutic effect of vincristine for MDR tumor cell-bearing mice in vivo (20). The amount of FK506 passed from the basal to the apical side and that from the apical to the basal side of LLC-PK1 epithelium were 27 and 26%, respectively (Fig. 4A), indicating that FK506 passed across LLC-PK1 monolayer probably by simple diffusion. The amount passed across LLC-GA5-COL300 monolayer from the basal to the apical side was increased to reach 48% in 3 h, and the amount from the apical to the basal side was decreased to 10%, representing the typical pattern of oriented transport by P-glycoprotein observed in our previous study (18, 19). Accumulated FK506 in LLC-GA5-COL300 after 3 h decreased to one-fourth and one-fifth of that in LLC-PK1, when the donor side was the basal and the apical side, respectively. These results indicate that P-glycoprotein mediated transport of FK506.
FK506, which are clinically useful immunosuppressive agents, are substrates for P-glycoprotein to transport. Because cyclosporin A was reported to interact with the common drug-transport site of P-glycoprotein (16), we examined the inhibitory effect of verapamil or vinblastine on the transport under our conditions is that 8.6 μM is a substrate for P-glycoprotein to transport. Because cyclosporin A (data not shown). We could not use higher concentrations of verapamil and vinblastine, because such causes remarkable paracellular leaks. Alternatively, the competition of photoaffinity labeling may not reflect the competition of the transport by P-glycoprotein. We reported that cortisol, which scarcely inhibited azidopine photoaffinity labeling, was transported by P-glycoprotein, and that progesterone, which efficiently inhibited azidopine binding, was not transported by P-glycoprotein (18). Sawa et al. (23) also reported that efficiency in competing with azidopine photoaffinity labeling of mutant P-glycoprotein did not par

![Figure 4: Transcellular transport and accumulation of FK506](image-url)

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