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MDR1 P-Glycoprotein Is a Lipid Translocase of Broad Specificity, While MDR3 P-Glycoprotein Specifically Translocates Phosphatidylcholine

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Summary

The human MDR1 P-glycoprotein (Pgp) extrudes a variety of drugs across the plasma membrane. The homologous MDR3 Pgp is required for phosphatidylcholine secretion into bile. After stable transfection of epithelial LLC-PK1 cells, MDR1 and MDR3 Pgp were localized in the apical membrane. At 15°C, newly synthesized short-chain analogs of various membrane lipids were recovered in the apical albumin-containing medium of MDR1 cells but not control cells. MDR inhibitors and energy depletion reduced apical release. MDR3 cells exclusively released a short-chain phosphatidylcholine. Since no vesicular secretion occurs at 15°C, the short-chain lipids must have been translocated by the Pgps across the plasma membrane before extraction into the medium by the lipid-acceptor albumin.

Introduction

The human multidrug resistance MDR1 and MDR3 P-glycoproteins (Pgps) belong to the ubiquitous family of membrane transporters that contain an ATP-binding cassette (ABC–transporters) and consume ATP during translocation of a wide variety of molecules from the cytosol to the extracellular space (Higgins, 1992; Gottesman and Pastan, 1993; Gros and Buschman, 1993). The MDR1 Pgp is expressed in the apical membrane of many epithelial and endothelial cells, but it also occurs at the surface of many tumor cells. It has been found to extrude multiple drugs from the cell that have little in common but an amphipathic structure. One model for its mechanism of action proposes that MDR1 Pgp binds an amphipathic molecule in the cytoplasmic leaflet of the plasma membrane and flips its polar group across the plasma membrane to deliver the molecule to the exoplasmic leaflet of the plasma membrane (Higgins and Gottesman, 1992). Since the amphipathic molecule can now diffuse from the cell surface into the extracellular medium, the "flippase" action of MDR1 Pgp would effect a net removal of the substrate from the cytoplasm. Using ATP, the MDR1 Pgp can pump drugs against a concentration gradient, which in the flippase model is a gradient between the cytoplasmic and exoplasmic leaflet of the plasma membrane lipid bilayer.

No drug-pumping activity has been demonstrated thus far for the MDR3 Pgp (Gros and Buschman, 1993), which is highly homologous with MDR1 Pgp (van der Bliek et al., 1988). Human MDR3 and mouse mdr2 Pgp are present in high concentrations in the bile canalicular membrane. Homozygous disruption of the murine *mdr2* gene led to a complete absence of phospholipid from the bile (Smit et al., 1993), and MDR3 Pgp can functionally replace mdr2 Pgp in knock-out mice (A. Smith, P. Borst, and R. Oude Elferink, unpublished data). This has led to the suggestion that mdr2 and MDR3 Pgp may translocate the membrane phospholipid phosphatidylcholine (PC) towards the outer bilayer leaflet of the plasma membrane. Subsequently, it was concluded from experiments on membranes from yeast expressing the *mdr2* gene that mdr2 Pgp can translocate a PC with a short acyl chain and a fluorescent aromatic group in the 2-position of the glycerol, C_6-NBD-PC (N-6[7-nitro-2,1,3-benzoxadiazol-4-yl]-amino-hexanoyl- phosphatidylcholine), from the cytosolic leaflet to the exoplasmic leaflet of the lipid bilayer. The mouse homolog of human MDR1 Pgp, mdr1a Pgp (nomenclature as in Smit et al. [1994]), did not translocate C_6-NBD-PC in these experiments (Ruetz and Gros, 1994, 1995). Direct evidence that MDR3 Pgp can translocate endogenous PC has been obtained in fibroblasts from transgenic mice expressing the *MDR3* gene, where [^{3}H]choline-labeled PC became more rapidly accessible on the cell surface than in control fibroblasts (Smith et al., 1994).

In the present study, we have made stable transfectants of pig kidney epithelial (LLC-PK1) cells containing human MDR1 or MDR3 Pgp or mouse mdr1a Pgp and devised an assay to test whether the Pgps are capable of translocating membrane lipids from the cytosolic side to the exoplasmic leaflet of the plasma membrane. For this, we have made use of observations by Pagano et al. (1981; Lipsky and Pagano, 1985) that diacylglycerol with a short fluorescent acyl chain at the *sn*-2 position (C_6-NBD-diacylglycerol) is efficiently converted by cells to the homologous PC (C_6-NBD-PC) and phosphatidylethanolamine (C_6-NBD-PE), while C_6-NBD-ceramide yields the corresponding sphingomyelin (C_6-NBD-SM) and glucosylceramide (C_6-NBD-GlcCer). Synthesis of PC, PE, and GlcCer occurs on the cytosolic surface of the endoplasmic reticulum (ER) and Golgi apparatus (Kent, 1995; Burger et al., 1996). Because of their short acyl chain, the C_6-NBD-analogs of these lipids can freely exchange as monomers through the cytosol between their site of synthesis and the inner leaflet of the plasma membrane, possibly assisted by lipid transfer proteins. Translocation across the plasma membrane to its exoplasmic leaflet, the cell surface, can be measured by extracting short-chain lipids from the cell surface into

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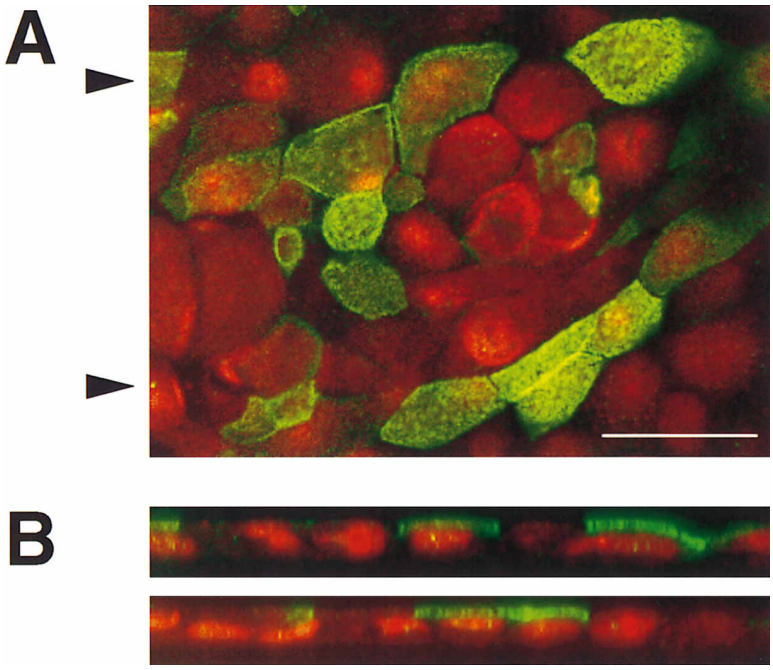

Figure 1. Immunolocalization of MDR3 Pgp in Transfected LLC-PK1 Cells by Confocal Laser Scanning Microscopy

Indirect immunofluorescence (FITC) picture with the anti-Pgp monoclonal antibody C219 on LLC-M3.4.44 cells is shown in green and nuclear counterstaining with propidium iodide in red.

(A) Top view of the cell layer. Bar, 25 μm.

(B) Optical sections perpendicular to the plane of the cell layer. Upper and lower arrowheads in (A) indicate the positions of the respective sections in (B).

medium containing excess albumin, a protein that binds short-chain lipids and in this case acts as a sink. Throughout the experiments with monolayers of LLC-PK1 cells, the apical medium remains separated from the basolateral medium underneath the filter because of the presence of tight junctions between the cells (van Meer et al., 1987; Schinkel et al., 1995). Arrival of lipids at the cell surface by the processes of exchange and translocation can be discriminated from delivery to the cell surface in membrane vesicles by lowering the temperature to 15°C, at which point vesicular lipid transport is blocked (van Genderen and van Meer, 1995).

Indications for lipid translocation activity of the MDR Pgps would be as follows: first, increased depletion of C_6-NBD-lipids into the medium from transfected as compared with control cells; second, confinement of the difference to the apical cell surface where MDR Pgps are localized; third, sensitivity of the difference to typical MDR inhibitors and energy depletion; and fourth, specificity for defined lipid classes or species. As expected (Ruetz and Gros, 1994), MDR3 Pgp fulfilled all criteria and translocated C_6-NBD-PC but not the other lipids. Unexpectedly, human MDR1 and mouse mdr1a Pgp met the first through third requirements and translocated all lipids tested. We conclude that MDR1 and mdr1a Pgp may have a physiological function as flippases in translocating certain types of lipid molecules across the plasma membrane.

Results

Expression of Pgps in Transfected LLC-PK1 Cells

A protein blot analysis of Pgps in the cell line LLC-PK1 and the *MDR1*-, *MDR3*-, and *mdr1a*-transfected clones (as in Schinkel et al. [1995]) demonstrated comparable levels of expression of the human MDR1 and MDR3 Pgp and of the murine mdr1a Pgp in these cell lines (data not shown). The correct localization of MDR3 Pgp in the apical membrane of the transfected cell line LLC-M3.4.44 was verified in cells on filters by confocal laser scanning microscopy, using the Pgp-specific monoclonal antibody C219 (Georges et al., 1990). Approximately half of the cells displayed strong but heterogeneous staining (Figure 1). With MDR3-specific polyclonal antiserum α-REG1, identical results were obtained (data not shown). A perpendicular view of the cells confirmed that the MDR3 Pgp was exclusively localized apically (Figure 1B). No staining was observed in the untransfected cells (data not shown).

In the LLC-PK1 cells transfected with *MDR1* and *mdr1a* constructs, the Pgp activity has been localized in the apical membrane (Schinkel et al., 1995). Virtually all cells were positive for the Pgp by immunofluorescence (data not shown).

Apical Translocation of C_6-NBD-PC by *MDR3*-Expressing LLC-PK1 Cells and of C_6-NBD-PC and C_6-NBD-PE by *MDR1*-Expressing Cells

To generate C_6-NBD-PC and -PE in the cytosol, we incubated confluent monolayers of LLC-PK1 cells and *MDR* transfectants with C_6-NBD-PA complexed to bovine serum albumin (BSA) for 3 hr at 15°C (Pagano et al., 1981; van Helvoort et al., 1994). A small fraction of C_6-NBD-PA partitions into the plasma membrane and is dephosphorylated to C_6-NBD-diacylglycerol. Owing to the absence of a polar headgroup, this molecule can translocate across the plasma membrane and by monomeric exchange reach the choline- and ethanolaminephosphotransferase, probably at the ER, by which it is converted to C_6-NBD-PC and -PE even at 15°C (Figure 2). After 3 hr, only a small fraction of each lipid was found in the apical BSA medium of the control cells. Because

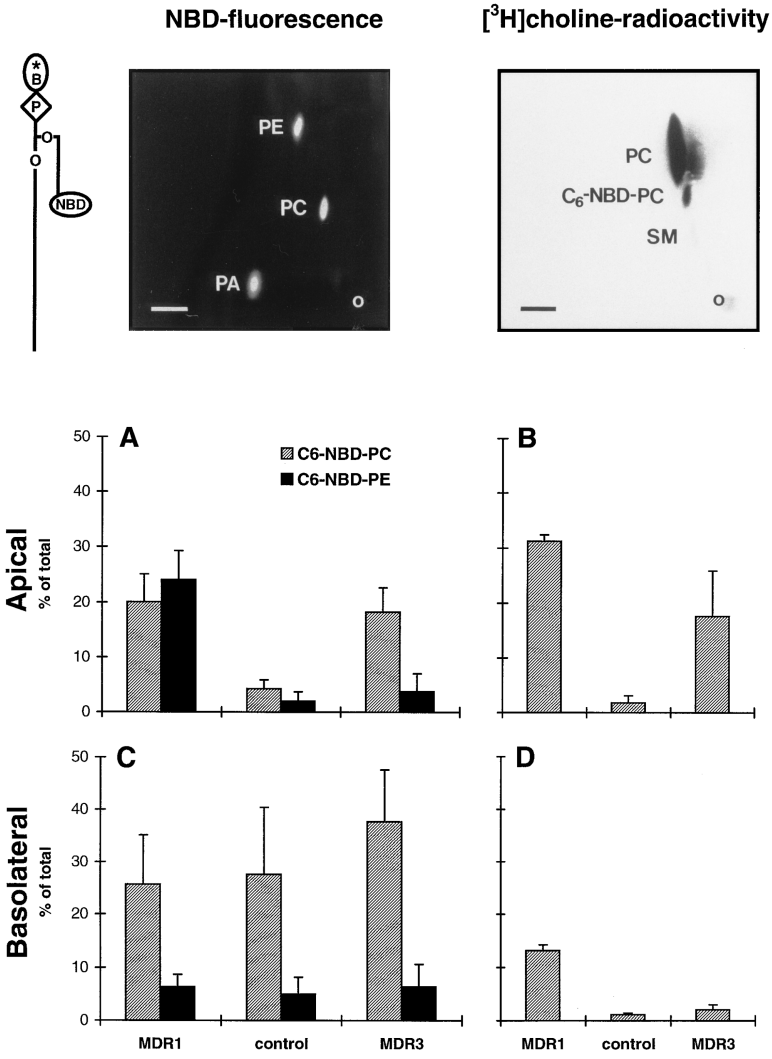

Figure 2. Translocation of C_6-NBD-Phospholipids across the Apical Plasma Membrane of *MDR*-Transfected LLC-PK1 Cells

Cell monolayers were incubated with 25 μM C_6-NBD-PA on both sides for 3 hr at 15°C. Fluorescent lipid products, C_6-NBD-PC (light bars) and C_6-NBD-PE (dark bars), appearing at the cell surface, were depleted into the BSA–containing medium. In the experiments in (B) and (D), the incubation was performed in the presence of [^{3}H]choline, and the availability of C_6-NBD-[^{3}H]PC for BSA depletion was assessed. Top panels illustrate TLC separation (10 × 20 cm^2) of cell lipids. O, origin; bar, 1 cm. C_6-NBD-diacyl- and triacylglycerol at the solvent front are not shown. Media contained much more C_6-NBD-PA but essentially no [^{3}H]PC. Lipids were quantitated as described in Experimental Procedures. Synthesis from 75 nmol C_6-NBD-PA was typically 15 pmol C_6-NBD-PC and 10 pmol C_6-NBD-PE per filter for all cells. Data in (A) and (C) are the mean of five independent experiments in duplicate (plus or minus standard deviation; n = 10). In (B) and (D), [^{3}H]choline incorporation in MDR1, control, and MDR3 cells into C_6-NBD-PC was 4 ×, 2 ×, and 1.5 × 10^3 dpm, while [^{3}H]PC contained 5 ×, 3.5 ×, and 2 × 10^5 dpm, respectively. Data are the mean of two experiments plus or minus difference from the mean. The structure formulas in the various figures (see Pascher, 1976) illustrate the position and length of the chains. B, base, choline in PC or ethanolamine in PE; NBD, fluorescent group; P, phosphate; asterisk, [^{3}H]radiolabel; "= O" and "−OH" have been omitted for clarity.

extraction from the membrane by BSA occurs down to 0°C (van Meer et al., 1987), this shows that transport to the cell surface is blocked at 15°C. Surprisingly, 20%–25% of both C_6-NBD-PC and -PE was found in the apical medium of *MDR1*-transfected cells, which suggests that MDR1 Pgp can translocate both C_6-NBD-PC and -PE across the apical membrane (Figure 2A). A portion (18%) of the C_6-NBD-PC but essentially no C_6-NBD-PE was recovered from the apical medium of the *MDR3*-transfected cells (Figure 2A), in line with a translocator activity for the MDR3 Pgp that is specific for C_6-NBD-PC as observed for the murine mdr2 Pgp (Ruetz and Gros, 1994, 1995). The lower level of transport of C_6-NBD-PC in the *MDR3*-transfected cells compared with the *MDR1*-transfectant will be due (partly) to the fact that approximately half of the MDR3 cells do not contain the Pgp (Figure 1), whereas the MDR1 cell line has a more homogeneous expression.

A significant amount of C_6-NBD-PC was found in the basolateral medium of all cells (Figure 2C). However, when the incubation was performed in the presence of [^{3}H]choline, virtually no C_6-NBD-[^{3}H]PC was found in the basolateral medium of control cells (Figure 2D). This shows that the C_6-NBD-PC on the basolateral surface is not derived from the intracellular pool but reflects C_6-NBD-PC synthesis on the basolateral cell surface, most likely by a sphingomyelin synthase (van Helvoort et al., 1994). In contrast, the C_6-NBD-PC in the apical media was [^{3}H]-labeled, so it was derived from the intracellular C_6-NBD-PC. This confirms the selective translocation of C_6-NBD-PC across the apical plasma membrane of both *MDR* transfectants (Figure 2B). Also, the basolateral medium of the MDR1 cells contained more C_6-NBD-[^{3}H]PC than that of untransfected cells. This may reflect the presence of some MDR1 Pgp at the basolateral surface of MDR1 cells (see below).

Owing to the continuous presence of precursor, synthesis of C_6-NBD-PC and -PE in MDR1 cells was linear with time for the first 5 hr at 15°C. The appearance of the lipids in the apical medium displayed a lag. After 2 hr, 12% of the C_6-NBD-PC and 19% of the C_6-NBD-PE was found in the apical medium, increasing to 25% for each lipid after 3 hr. Between 3 and 5 hr, the amount in the medium increased linearly in parallel with the synthesis, still equaling 25% after 5 hr. The same was observed for C_6-NBD-PC in MDR3 cells, with 10% in the apical

Table 1. Inhibition of the Appearance of C_6-NBD-PC and C_6-NBD-PE in the Apical Medium of *MDR1*-Transfected Cells by Verapamil and Energy Depletion

	C_6-NBD-PC (% of total in apical medium)			C_6-NBD-PE (% of total in apical medium)		
LLC-PK1 cells	Untreated	+ Verapamil (20 μM)	Energy depleted	Untreated	+ Verapamil (20 μM)	Energy depleted
MDR1	20.3 ± 4.1	6.7 ± 1.7	4.5 ± 3.9	21.3 ± 8.5	2.6 ± 1.5	3.7 ± 5.2
Control	4.5 ± 1.9	6.1 ± 1.1	6.3 ± 6.9	2.0 ± 1.5	0.4 ± 0.3	ND
MDR3	18.1 ± 3.8	6.0 ± 3.8	10.4 ± 0.2	4.2 ± 2.8	1.1 ± 1.1	ND

After a preincubation of 10 min at 37°C with 20 μM verapamil or in PBS plus 2 mM NaN_3 plus 50 nM deoxyglucose plus 2 mM KCN plus 20 mM KF, cell monolayers were incubated with 25 μM C_6-NBD-PA on both sides for 3 hr at 15°C in the presence of the inhibitors. Synthesis of C_6-NBD-PC was 1.0 ± 0.2 × control after verapamil and 0.6 ± 0.1 × control after energy depletion; for C_6-NBD-PE, this was 1.4 ± 0.3 × and 0.1 ± 0.1 × control, respectively, for all three cell lines. The quantity of C_6-NBD-PC in the basolateral medium was not reduced (less than 90% of control). Data are the mean of two independent experiments in duplicate and are followed by the standard deviation (n = 4). ND: could not be measured reliably because of reduced synthesis.

medium after 2 hr and 15% after 3 and 5 hr. The small percentage of C_6-NBD-PC and -PE in the apical medium of control cells, of C_6-NBD-PE in the apical medium of MDR3 cells, and of C_6-NBD-PE in the basolateral media of all cells displayed no lag. It was identical after 2, 3, and 5 hr, as was basolateral C_6-NBD-PC. The 3 hr timepoint reliably indicated the differences in translocation capacity of the cells and was selected for further experiments.

Apical Translocation of C_6-NBD-PC and C_6-NBD-PE Is Sensitive to the MDR Inhibitor Verapamil and to Energy Depletion

As a direct test for a functional involvement of MDR1 and MDR3 Pgps in the appearance of C_6-NBD-PC and -PE in the apical medium of the transfectants, we incubated the cells with C_6-NBD-PA in the presence and absence of verapamil, an inhibitor of the action of MDR1 (Gottesman and Pastan, 1993) and MDR3 Pgp (Ruetz and Gros, 1994). Verapamil reduced the fraction of C_6-NBD-PC and -PE in the apical medium of MDR1 cells to 33% and 12% of the control value, respectively, while the fraction of C_6-NBD-PC in the medium of MDR3 cells was reduced to 33% of the control (Table 1). Similar results were obtained on [^{3}H]choline-labeled C_6-NBD-PC (data not shown). Verapamil had no effect on the presence of C_6-NBD-PC in the basolateral medium, as expected for PC synthesized by an energy-independent headgroup transfer at the cell surface (see above). To assess whether the presence of C_6-NBD-PC and -PE in the apical medium was a consequence of active transport, we subjected cells to energy depletion before incubation with C_6-NBD-PA. Indeed, besides a reduced synthesis, such treatment resulted in a dramatic decrease in the fraction of both NBD products in the apical medium of MDR1 cells (Table 1). In MDR3 cells, energy depletion had less effect on the amount of apical C_6-NBD-PC (−40%). This possibly reflects the partial opening of the tight junctions, measured as a drop in electrical resistance (data not shown), by which some C_6-NBD-PC synthesized on the basolateral surface (see Figure 1) became available apically, masking the effect of energy depletion.

Apical Translocation of C_8C_8-PC and C_8C_8-PE by *MDR1*-Expressing LLC-PK1 Cells But Not by *MDR3*-Expressing Cells

To characterize further lipid translocation by MDR1 and MDR3 Pgp, we studied the behavior of PC and PE lacking the NBD moiety. Short chains were required for the use of BSA depletion as a translocation assay. When cells were incubated with C_8C_8-PA and [^{3}H]choline for 3 hr at 15°C, three products were formed. One of these comigrated with natural PC, a second with C_8C_8-PC, and a third migrated in between the others (Figure 3). The latter PC most likely originated from a reacylation event in which one C_8 chain of C_8C_8-PC had been exchanged for a C_{16} or C_{18} chain; when cells were incubated with C_8C_8-PA and [^{14}C]$C_{18:1}$, the two [^{14}C]products colocalized with natural PC and with the intermediate band (data not shown). To focus on the original product, reacylation was inhibited by the serine-esterase inhibitor phenylmethanesulfonyl fluoride, which in the concentration used (0.2 mM) had no effect on transport of C_6-NBD-PC into the apical medium of *MDR* transfectants. Efficient transport of C_8C_8-[^{3}H]PC was observed into the apical medium of MDR1 cells (28 ± 6%; n = 5), while only a small amount (9 ± 1%) was found in the basolateral medium and in the media of control cells (apical: 3.5 ± 0.3%; basolateral: 6 ± 3%). Remarkably, little transport of C_8C_8-[^{3}H]PC was observed to the apical medium of MDR3 cells (6 ± 2%; basolateral 5 ± 1%).

Incubation of cells with C_8C_8-PA and [^{14}C]ethanolamine resulted in the production of three [^{14}C]products. As in the case of C_8C_8-PC, these represented natural PE, C_8C_8-PE, and reacylated C_8C_8-PE. The addition of phenylmethanesulfonyl fluoride resulted in the production of appreciable amounts of C_8C_8-[^{14}C]PE. Like C_8C_8-[^{3}H]PC, C_8C_8-[^{14}C]PE was selectively recovered from the apical medium of MDR1 cells (12 ± 1%; n = 4) and not control (2 ± 1%) or MDR3 cells (2 ± 1%), nor from the basolateral media (2 ± 1% in all cells), demonstrating the ability of MDR1 Pgp to translocate phospholipids with short chains but lacking the NBD moiety (Figure 3).

Apical Translocation of C_6-NBD-SM and C_6-NBD-GlcCer by *MDR1*-Expressing Cells and Not by *MDR3*-Expressing Cells

To study the backbone specificity of MDR1 and MDR3 Pgp, we incubated the cells with C_6-NBD-ceramide to synthesize the sphingolipids sphingomyelin (SM), which has the same phosphocholine headgroup as PC but a ceramide backbone instead of a diacylglycerol, and glucosylceramide (GlcCer). C_6-NBD-SM is synthesized in the lumen of the Golgi but has no access to the cytosol and reaches the cell surface by vesicular traffic but not

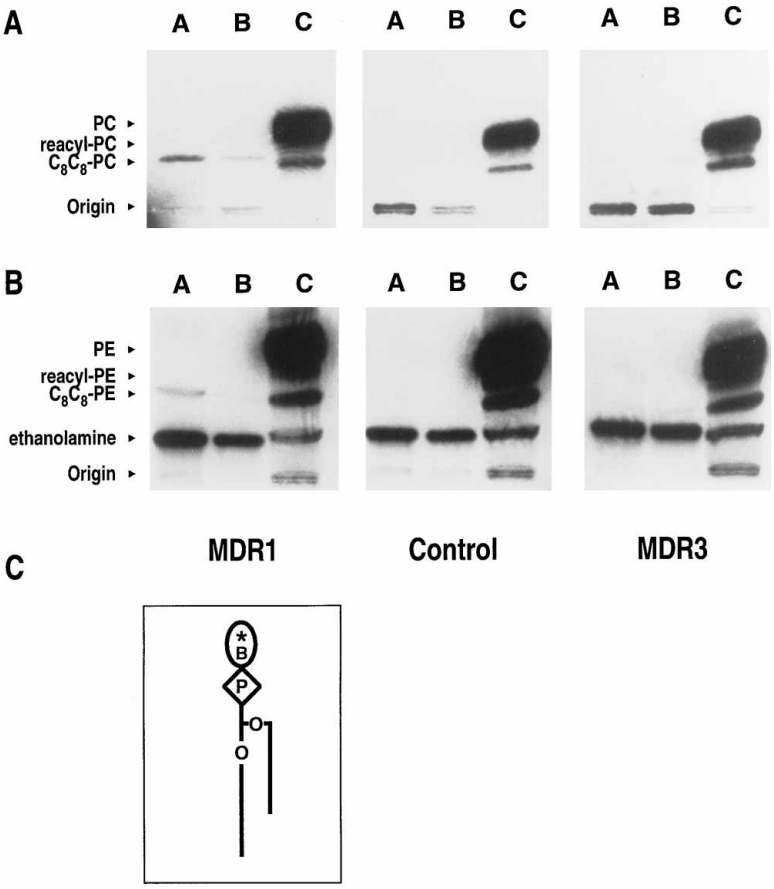

Figure 3. Translocation of C_8C_8-[^{3}H]PC and C_8C_8-[^{14}C]PE across the Apical Plasma Membrane of *MDR1*-Transfected Cells

After a choline/ethanolamine depletion in HBSS for 1 hr at 37°C, cells were preincubated with [^{3}H]choline (A) or [^{14}C]ethanolamine (B) for another 1 hr at 37°C, after which C_8C_8-PA (50 μM) and 0.2 μM phenylmethanesulfonyl fluoride were added to the media. After 3 hr at 15°C, lipids from media and cells were extracted and separated by one-dimensional TLC, all as described in Experimental Procedures. Lanes A, apical medium; lanes B, basolateral medium; lanes C, cells. Incorporation of radiolabel in MDR1, control, and MDR3 cells, respectively: [^{3}H]PC: 8 ×, 3 ×, and 1 × 10^5 dpm; C_8C_8-PC 10% of the natural PC in each cell line; [^{14}C]PE: 4 ×, 6 ×, and 2 × 10^4 dpm; C_8C_8-PE 5% of the natural PE in each cell line. Structure (C): B, choline in PC or ethanolamine in PE; P, phosphate; asterisk, [^{3}H]radiolabel in PC or [^{14}C]radiolabel in PE.

at 15°C (van Genderen and van Meer, 1995). No C_6-NBD-SM was found in the apical media. In contrast, C_6-NBD-GlcCer, like PC and PE, is synthesized on the cytosolic surface of an intracellular membrane, in this case the Golgi, from where it can freely equilibrate with the inner leaflet of the plasma membrane owing to its short chain (see Burger et al., 1996). C_6-NBD-GlcCer was found in the apical medium of MDR1 cells exclusively (Figure 4A). At temperatures greater than or equal to 20°C, significant amounts of both C_6-NBD-GlcCer and -SM appeared in apical and basolateral media owing to the contribution of vesicular membrane transport (data not shown; see van Genderen and van Meer, 1995).

The situation was different when the cells were pretreated with brefeldin A. Now, not only C_6-NBD-GlcCer but also C_6-NBD-SM was recovered from the apical medium of MDR1 cells (Figure 4B). Brefeldin A has been shown to induce retrograde transport of Golgi enzymes to the ER (Klausner et al., 1992), where lumenal lipids have access to the cytosolic side (see Burger et al., 1996; Buton et al., 1996). The selective translocation of C_6-NBD-SM into the apical medium of MDR1 cells (and not MDR3 cells) demonstrates that C_6-NBD-SM in the presence of brefeldin A had access to the cytosolic surface of the plasma membrane and cannot be translocated by MDR3 Pgp. The C_6-NBD-SM in the basolateral medium of all cells most likely originated from the action of an SM synthase on the basolateral cell surface (van Helvoort et al., 1994), the presence of which was evident from the production of C_6-NBD-PC but not -PE on the basolateral surface (see Figure 2).

A functional involvement of MDR1 Pgp in apical translocation of C_6-NBD-GlcCer at 15°C was confirmed by the effect of the MDR inhibitors verapamil and PSC 833. While verapamil reduced apical transport by 75%, transport was virtually blocked by PSC 833. Also, energy depletion efficiently inhibited transport (Figure 5).

Apical Translocation of C_6-GlcCer and C_8C_8-GlcCer by *MDR1*- But Not *MDR3*-Expressing Cells

To demonstrate that the NBD moiety was not essential for translocation of GlcCer, cells were incubated with [^{14}C]C_6-ceramide or [^{3}H]C_8C_8-ceramide. Synthesis of short-chain GlcCer and SM and distribution over cells and media were measured after 3 hr at 15°C (Figure 6). Indeed, both types of GlcCer were translocated into the apical medium of MDR1 cells but not of control or MDR3 cells (data not shown). The corresponding SMs were not recovered in any of the apical media, in line with the observation that SM after synthesis in the lumen of the Golgi has no access to the cytosolic surface of the Golgi (see above). The fact that some basolateral transport of C_6-NBD-[^{3}H]PC and the various GlcCers was observed in cells transfected with *MDR1*, and that it was sensitive to PSC 833, suggests that some MDR1 Pgp molecules were present on the basolateral surface.

A much larger fraction of C_8C_8-GlcCer than of C_6-GlcCer was recovered in the 15°C medium. This probably reflects the fact that the former analog owing to its two short chains is essentially water-soluble (Karrenbauer et al., 1990) and after synthesis has more rapid access to the inside of the plasma membrane.

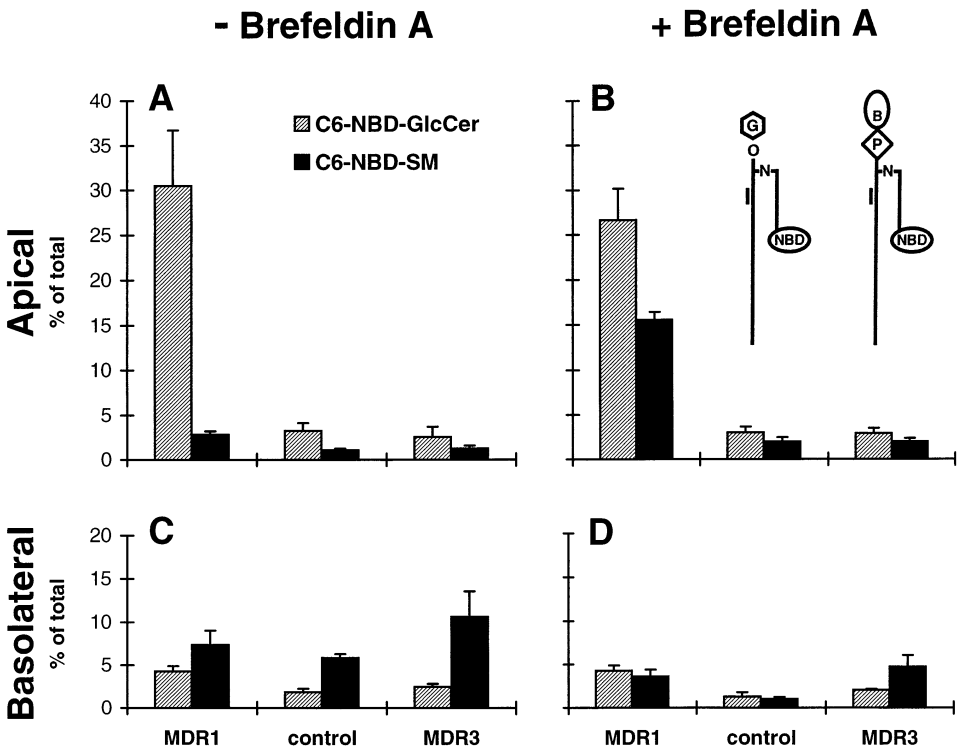

Figure 4. Translocation of C_6-NBD-SM and C_6-NBD-GlcCer across the Apical Plasma Membrane of *MDR1*-Transfected Cells

Cells were incubated with 5 μM C_6-NBD-ceramide for 3 hr at 15°C, after which C_6-NBD-GlcCer (shaded bars) and C_6-NBD-SM (solid bars) in cells and media were quantitatively analyzed as described in Experimental Procedures. In (B) and (D), cells were pretreated with 1 μg/ml of brefeldin A for 30 min at 37°C, after which the incubation at 15°C was carried out in the presence of brefeldin A. Synthesis in (A) and (C) was 68 ± 16 pmol (n = 30) for C_6-NBD-GlcCer and 153 ± 29 pmol (n = 30) for C_6-NBD-SM per filter for all three cell lines. Data are the mean of five independent experiments in duplicate (plus or minus standard deviation; n = 10). Synthesis of C_6-NBD-GlcCer was not affected by brefeldin A, whereas C_6-NBD-SM synthesis increased two to four times. Data in (B) and (D) are the mean of two independent experiments in duplicate (plus or minus standard deviation; n = 4). Structure: B, choline in SM; G, glucose in GlcCer; NBD, fluorescent group; P, phosphate.

A Mouse Homolog of Human MDR1 Pgp, mdr1a Pgp, Translocates C_6-NBD-PC, -PE, -GlcCer, and -SM

Evidence has been presented suggesting that mouse mdr1a Pgp, in contrast to mouse mdr2 Pgp, cannot translocate C_6-NBD-PC across the membrane (Ruetz and Gros, 1994). Since these Pgps are the homologs of the human MDR1 and MDR3 Pgp, respectively (see Smit et al., 1994), and since MDR1 Pgp does translocate C_6-NBD-PC, we tested the translocation activity of mdr1a Pgp in *mdr1a*-transfected LLC-PK1 cells. When the cells were incubated with C_6-NBD-PA or -ceramide for 3 hr at 15°C, a large fraction of the C_6-NBD-PC, -PE, and -GlcCer, but not of the C_6-NBD-SM, was recovered in the apical medium (Figure 7). Except for the PC (surface synthesis), only small amounts were found in the basolateral medium. In cells treated with brefeldin A, C_6-NBD-SM also appeared in the apical medium. We conclude that mdr1a Pgp expressed at the same level as MDR1 Pgp displays a comparable lipid translocation activity. It is unclear why Ruetz and Gros (1994) did not observe translocation of C_6-NBD-PC in their experimental system.

In the Absence of BSA, MDR1 Pgp Translocates C_6-NBD-GlcCer to the Outer Leaflet of the Plasma Membrane Bilayer

When MDR1 cells and MDR3 cells were incubated with C_6-NBD-ceramide in the presence of the minimal concentration of BSA needed to complex the ceramide (0.03%; w/v), after 3 hr at 15°C the apical medium of the MDR1 cells contained 7 ± 1% of the C_6-NBD-GlcCer. Subsequently, two washes for 30 min on ice with 1% BSA released an additional 31 ± 3% of the total C_6-NBD-GlcCer. If BSA was present in the 15°C incubation medium (compare with Figure 4), this contained 92 ± 3% (n = 2) of the total amount of C_6-NBD-GlcCer that was recovered in incubation medium plus washes. As expected (see Figure 4), the combined media and washes from MDR3 cells contained only 5 ± 1% of the total C_6-NBD-GlcCer. Of the C_6-NBD-SM, 2 ± 1% was found in the combined apical media and 8 ± 1% in the combined basolateral media of both cell types (n = 4). Obviously, in MDR1 cells, 30%–40% of total C_6-NBD-GlcCer had been translocated to the outer leaflet of the apical plasma membrane during 3 hr at 15°C, to be released into the medium only in the presence of BSA. This experiment demonstrates that a substrate translocated by MDR1 Pgp indeed accumulates in the outer leaflet of the plasma membrane.

Discussion

Human MDR3 Pgp, a PC Translocase

After the original suggestion that the function of the mouse mdr2 Pgp in the bile canalicular membrane might be translocation of PC across the plasma membrane to

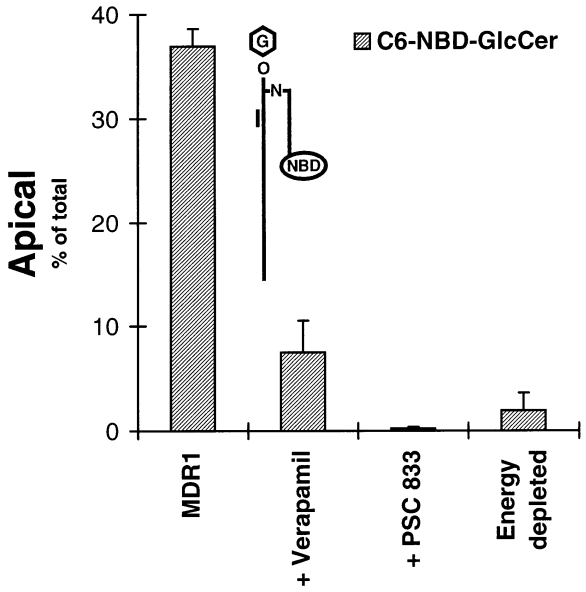

Figure 5. Inhibition of the Appearance of C_6-NBD-GlcCer in the Apical Medium of *MDR1*-Transfected Cells by MDR Inhibitors and Energy Depletion

MDR1 cells were incubated with 5 μM C_6-NBD-ceramide under the conditions for inhibition of MDR1 Pgp or for energy depletion described in the legend to Table 1. The concentration of PSC 833 was 2 μM. C_6-NBD-GlcCer in the basolateral medium was about 5% of the total. C_6-NBD-GlcCer synthesis was 1.3 $\pm$ 0.2 $\times$ control (verapamil), 1.5 $\pm$ 0.1 $\times$ control (PSC 833), and 0.6 $\pm$ 0.1 $\times$ control (energy depletion). All data are from two experiments in duplicate (n = 4). Structure: G, glucose; NBD, fluorescent group.

the outer leaflet (Smit et al., 1993), experimental evidence has been provided that mdr2 Pgp can translocate the PC analog C_6-NBD-PC across membranes (Ruetz and Gros, 1994, 1995) and that the human homolog MDR3 Pgp can translocate natural PC (Smith et al., 1994). In the present study, we have devised a much simpler assay for the translocation activity of MDR3 Pgp that will allow systematic testing of inhibitors and definition of substrate specificity. At 15°C, where vesicular lipid transport from the Golgi to the cell surface is inhibited, first, cells transfected with *MDR3* but not control cells exposed intracellularly synthesized C_6-NBD-[^{3}H]PC on the cell surface (Figure 2); second, translocation was limited to the apical surface (Figure 2), where the MDR3 Pgp was located (Figure 1); third, transport was sensitive to the MDR inhibitor verapamil and was energy-dependent (Table 1), showing that MDR3 Pgp is not the putative energy-independent PC translocator in the bile canalicular membrane (Berr et al., 1993); and fourth, translocation by the MDR3 Pgp was selective for C_6-NBD-PC. A lipid with the same backbone, C_6-NBD-PE (Figure 2), and a lipid with the same headgroup, C_6-NBD-SM (Figure 4), were not translocated. The specificity of MDR3 Pgp is even more stringent since, as well, a PC with shorter acyl chains C_8C_8-PC was hardly translocated (Figure 3). Possibly, this PC was not inserted into the membrane well enough to be recognized by the MDR3 Pgp. Each lipid must have been able to reach the inner leaflet of the plasma membrane, since all were translocated by MDR1 Pgp (Figures 2–4).

Human MDR1 Pgp and Mouse mdr1a Pgp, Broad-Specificity Lipid Translocases

Surprisingly, MDR1 and mdr1a Pgps are as efficient as MDR3 Pgp in translocating C_6-NBD-PC across the apical surface (Figures 2 and 7). This is in conflict with the conclusion by Ruetz and Gros (1994) from a different assay system in which mdr1a (termed Mdr3) Pgp expressed in yeast secretory vesicles did not translocate NBD-PC. The reason for this is unclear.

Translocation by MDR1 and mdr1a Pgp was not selective, since C_6-NBD-PE, -GlcCer, and -SM were translocated as well (Figures 4 and 7). MDR1 Pgp also translocated the more natural versions of these lipids lacking the NBD moiety (Figures 3 and 6). First of all, this finding has implications for the mechanism of action of MDR1 Pgp. Since the concentration of the C_6-NBD-lipids in the aqueous phase is low, the fact that they are a substrate for the MDR1 Pgp suggests that they were able to reach the substrate binding site from within the membrane. In the absence of a suitable acceptor, C_6-NBD-GlcCer ended up in the outer leaflet of the plasma membrane of MDR1 cells (see Results), suggesting a lipid flippase action of the MDR1 Pgp (see Devaux and Zachowski, 1994). This provides support for the flippase hypothesis for the mechanism of the multidrug transport by the MDR1 Pgp (Higgins and Gottesman, 1992).

Implications of Short-Chain Lipid Translocation by MDR1 Pgp for Studies on Intracellular Lipid Transport

Short-chain lipid analogs have been widely applied to study the topology of synthesis and the intracellular transport of membrane lipids (see Pagano, 1990; Trotter and Voelker, 1994; van Helvoort and van Meer, 1995). Our present finding (Figures 4 and 6) that newly synthesized C_6-NBD-GlcCer, C_6-GlcCer, and C_8C_8-GlcCer can reach the cell surface by the action of MDR1 Pgp requires a reassessment of studies in which transport of these lipids to the cell surface at 37°C has been discussed in terms of vesicular traffic (Lipsky and Pagano, 1985; Karrenbauer et al., 1990; our own studies summarized in van der Bijl et al. [1996]).

Our interpretation that the preferential delivery of C_6-NBD-GlcCer over C_6-NBD-SM to the apical surface of epithelial cells might be due to lipid sorting in a vesicular pathway (van Meer et al., 1987) is compatible with the observation that C_6-NBD-GlcCer can translocate towards the lumenal leaflet of the Golgi membrane (Burger et al., 1996) and reach the cell surface on the inside of carrier vesicles (see van Helvoort and van Meer, 1995). However, the higher concentration of C_6-NBD-GlcCer on the apical surface can also be explained by the fact that C_6-NBD-GlcCer is a substrate for MDR1 Pgp (Figure 4) and that MDR1 Pgp is exclusively present in apical membranes (Gros and Buschman, 1993). This would not apply to C_6-NBD-SM, since it has no access to the MDR Pgp (Figure 4). In preliminary experiments, transport of C_6-NBD-lipids to the apical surface of MDCK cells was reduced by MDR inhibitors, while transport of α-hydroxy-C_6-sphingolipids (van der Bijl et al., 1996) was virtually unaffected (K. N. J. Burger and G. van Meer, unpublished data). To define the mechanism of lipid sorting in epithelial cells, it must be determined whether

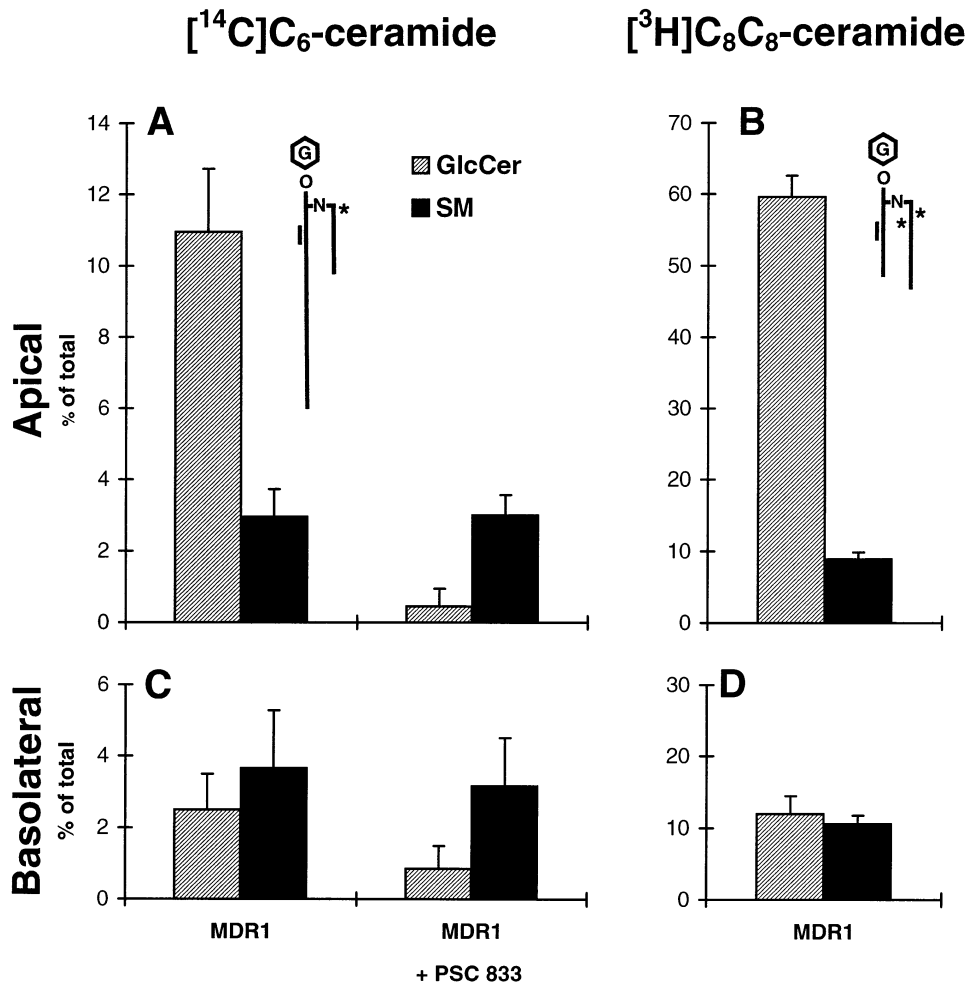

Figure 6. Translocation of [^{14}C]C_6-GlcCer and [^{3}H]C_8C_8-GlcCer across the Apical Plasma Membrane of *MDR1*-Transfected Cells

Cells were incubated for 3 hr at 15°C with [^{14}C]C_6-ceramide or [^{3}H]C_8C_8-ceramide. GlcCer (shaded bars) and SM (solid bars) in media and cells were quantitated as described in Experimental Procedures. The MDR1 inhibitor PSC 833 was used at 2 μM. Synthesis was 1,500 dpm for [^{14}C]C_6-GlcCer and 40,000 dpm for [^{3}H]C_8C_8-GlcCer. Data are from two experiments in duplicate. Structure: G, glucose; asterisk, [^{3}H] or [^{14}C]radiolabel.

endogenous GlcCer is a substrate for MDR1 Pgp, in which case the relative contribution of the vesicular and MDR pathways to the delivery of lipids to each cell surface must be defined.

In a second series of studies, short-chain analogs of PS and PE have been inserted into the exoplasmic leaflet of the plasma membrane of various cell types to study the kinetics of ATP-dependent translocation to the inner leaflet by the aminophospholipid translocator (see Devaux and Zachowski, 1994; Menon, 1995; Pomorski et al., 1996). This is not the MDR1 Pgp (Auland et al., 1994; Hanada and Pagano, 1995; Tang et al., 1996). Our finding that short-chain PE is a substrate for ATP-dependent translocation in the opposite direction implies that if MDR1 Pgp was present in the plasma membrane of the cells studied, the previous studies have measured the combined action of both transport systems.

Does MDR1 Pgp Translocate Natural Lipids?

Identification of the MDR1 and mdr1a Pgp as flippases or translocators for short-chain analogs of endogenous membrane lipids of very different structure, from a glycerophospholipid like PC to a glycosphingolipid like GlcCer, brings up the question of whether the translocation of lipids is a physiological function of these proteins. Endogenous short-chain lipids occur in living cells and could be substrates for MDR1 Pgp: in the first place, the prime example is the bioactive lipid platelet-activating factor (PAF), which is a C_2-PC. The mechanism by which PAF is secreted at sites of inflammation is unclear, but translocation across the plasma membrane may be involved (Prescott et al., 1990; Vallari et al., 1990; Bratton, 1993). Our results (Figure 2) suggest that PAF may be a substrate for both MDR1 and MDR3 Pgp, and especially from the clinical point of view it will be interesting to see whether MDR1 or MDR3 Pgp are actually involved in PAF release. In the second place, a second class of lipids with a short-chain at the *sn*-2 position consists of oxidatively fragmented membrane phospholipids. These toxic lipids, mostly PC in which an unsaturated acyl chain has been peroxidized and shortened (Tanaka et al., 1993), have a PAF–like activity (see Heery et al.,

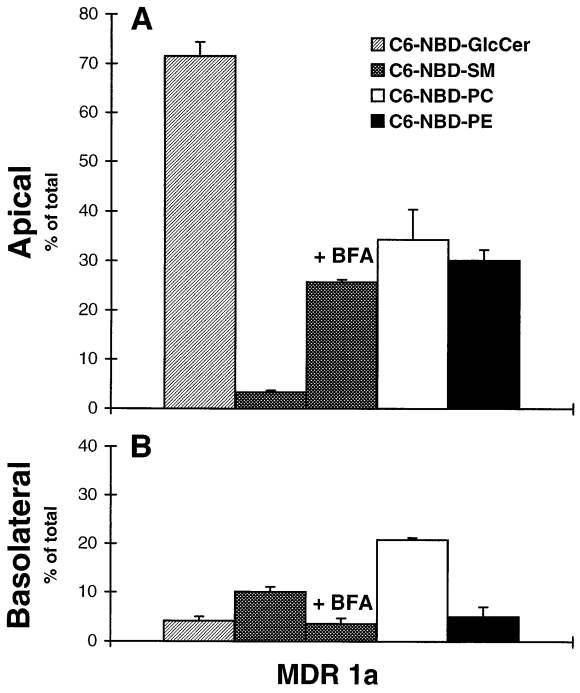

Figure 7. Translocation of C_6-NBD-PC, -PE, -GlcCer, and -SM across the Apical Plasma Membrane of *mdr1a*-Transfected Cells

Cells were incubated for 3 hr at 15°C with 25 μM C_6-NBD-PA or 5 μM C_6-NBD-ceramide as in Figures 2 and 4 (two experiments in duplicate, n = 4). In cells treated with brefeldin A (BFA) as in Figure 4, transport of C_6-NBD-GlcCer was identical to that in untreated cells. Synthesis of C_6-NBD-lipid/filter: GlcCer, 16 ± 2 pmol; SM, 36 ± 5 pmol; PC, 12 ± 1 pmol; PE, 7 ± 1 pmol. Brefeldin A did not affect synthesis of C_6-NBD-GlcCer but increased synthesis of C_6-NBD-SM 3.1 times. For lipid structures see Figures 2 and 4.

1995). MDR1 Pgp may assist in their removal from the body by translocation across the apical membranes of the cells lining the gastrointestinal and urinary tracts. In the third place, possibly, lysophospholipids are also substrates for Pgps. Lyso-PC displays a half-time of translocation across liposomes and plasma membranes of hours (see Bhamidipati and Hamilton, 1995). During signal transduction, translocation may serve to remove newly generated lysophospholipids, lyso-PA and lyso-PC, from the inner leaflet of the plasma membrane. Fourth, finally, short-chain glycosphingolipids have been reported to occur in cultured cells (Sandvig et al., 1994). Their physiological relevance is presently unclear.

It seems unlikely that MDR1 and mdr1a Pgp can translocate endogenous long-chain membrane lipids. Although both mouse mdr2 (Ruetz and Gros, 1994, 1995) and mdr1a Pgp (Figure 7) can translocate C_6-NBD-PC, mdr1a Pgp appears unable to compensate for the absence of mdr2 Pgp in transporting PC into the bile of the *mdr2* knock-out mouse (Smit et al., 1993). Furthermore, the occurrence of both a broad-specificity translocase and the aminophospholipid translocase (Devaux and Zachowski, 1994) working in opposite directions in one membrane would lead to a futile cycle of utilizing ATP to pump PE back and forth. Of course, it remains possible that these translocators are differentially and coordinately regulated (see Gaffet et al., 1995). Whatever the role of MDR1 and mdr1a Pgp in lipid translocation, the importance of the latter Pgp for protecting the organism against xenotoxins has been directly and convincingly demonstrated (Schinkel et al., 1994; 1995).

Conclusion

The capability of MDR1 Pgp to translocate a wide variety of short-chain lipid molecules across the plasma membrane may radically change our views on the physiological function of the protein. Besides pumping xenotoxins out of cells, it may serve a function in organizing natural lipids, be it short-chain or long-chain. Many different lipid translocation events occur in cellular membranes, and many of those must be protein-mediated (Menon, 1995). The involvement of the ABC–transporters Pat1 and Pat2 in peroxisomal import of long-chain fatty acids (Hettema et al., 1996), the present identification of MDR1 and mdr1a Pgp as lipid translocators of broad specificity, and the identification of MDR3 and mdr2 Pgp as specific translocators of PC suggest that a systematic assessment of lipid-translocating properties of ABC–transporters may open a new chapter in the search for lipid flippases.

Experimental Procedures

Materials

Vincristine, verapamil, and BSA fraction V were purchased from Sigma (St. Louis, MO). PSC 833 was a gift of Dr. D. Cohen at Sandoz (NJ). [*Methyl*-^{3}H]-choline chloride (3.0 GBq/μmol) was from DuPont NEN (Dordrecht, The Netherlands) and [2-^{14}C]-ethanolamine hydrochloride (2.1 GBq/mmol) from Amersham (Little Chalfont, UK). Geneticin (G418) and cell culture media were from Gibco (Paisley, UK), and phenylmethanesulfonyl fluoride and silica thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Federal Republic of Germany). Organic solvents were from Riedel-de Haën (Seelze, Federal Republic of Germany).

1-Hexadecanoyl-2-(C_6-NBD)-*sn*-glycero-3-phosphocholine (C_6-NBD-PC) and C_8C_8-PA were from Avanti (Alabaster, AL). C_6-NBD-phosphatidic acid was prepared from C_6-NBD-PC using phospholipase D (van Helvoort et al., 1994). C_6-NBD-ceramide was from Molecular Probes (Eugene, OR). [1-^{14}C]C_6-ceramide (0.15 MBq/μmol) was synthesized as before (van der Bijl et al., 1996). [2,3-^{3}H]Octanoyl-D-erythro-C_8-sphingosine (C_8C_8-ceramide; 1.85 GBq/μmol) synthesized as in Karrenbauer et al. (1990) was a gift of Dr. F. Wieland (Heidelberg, Federal Republic of Germany).

Cells

LLC-PK1 pig kidney epithelial cells were obtained from American Type Culture Collection (Rockville, MD) and cultured (mycoplasma-free) in M199 medium supplemented with 10% fetal calf serum as described (Schinkel et al., 1995). For experiments, 2×10^6 cells were seeded on 4.7 cm^2 filters, 0.4 μm pore diameter, glued to the bottom of plastic rings ("Transwell," Costar, Cambridge, MA) so as to be able to separate apical and basolateral medium (van Meer et al., 1987), and grown as monolayers for 4 days.

Transfection with Human *MDR1* and *MDR3* and Mouse *mdr1a*

Human *MDR1* and mouse *mdr1a* cDNA were transfected as the plasmids pFRCMVMDR1.1 and pFRCMVmdr1a into LLC-PK1 cells as described (Schinkel et al., 1995). Mouse *mdr1a* (*mdr3-S*) cDNA was a gift of Dr. P. Gros (McGill University, Montreal, Canada). The transfectants were maintained at 640 nM vincristine selection.

A mixed cDNA/genomic DNA *MDR3* gene (Smit et al., 1994) was cloned into the mammalian expression vector pRc/RSV (Invitrogen, Leek, The Netherlands). The resulting construct (10 μg) with 10 μg of salmon sperm carrier DNA was used to transfect LLC-PK1 cells by calcium phosphate coprecipitation. Transfected cells were selected

with G418 (0.8 mg/ml). Colonies that arose were picked after 3 weeks and expanded, and MDR3 Pgp expression was determined by protein blot analysis of cell lysates using MDR3 Pgp-specific polyclonal antiserum AVLCL2 (Smit et al., 1994). Mock-transfected cells did not give rise to colonies, and colonies resulting from transfection with an empty pRc/RSV vector did not express MDR3 Pgp. A stable transfectant with a high level of MDR3 Pgp expression was subcloned to yield the LLC-M3.4.44 cell line used in this study.

Immunocytochemistry

LLC-PK1 and LLC-M3.4.44 cells (2×10^6) were seeded on a 3 μm pore-size 4.7 cm^2 Transwell filter (Costar) and grown for 3 days. The cells were washed in phosphate-buffered saline (PBS), fixed in 70% ethanol ($-20°C$) for 15 min, and dried in air at room temperature. The filters were blocked in 2% (w/v) BSA in PBS and washed in PBS, and MDR3 Pgp was stained using either MDR3-specific rabbit polyclonal antiserum α-REG1 (1:20 in PBS; Smit et al., 1994) or Pgp-specific mouse monoclonal antibody C219 (10 μg/ml in PBS). As secondary antibodies, FITC–labeled goat anti-rabbit IgG and goat anti-mouse IgG (Nordic, Tilburg, The Netherlands) were used, respectively. After staining, the cells were overlaid with Vectashield (Vector Labs, Burlingame, CA) containing propidium iodide (1 μg/ml) and examined with an MRC-600 laser scanning confocal fluorescence imaging system (BioRad, Hertfordshire, UK) coupled to a Nikon microscope.

Transport Incubations

C_6-NBD-PA, C_6-NBD-Ceramide, or [^{14}C]C_6-Ceramide

Lipid precursors were added to both sides of epithelial monolayers on filters, as before (van Helvoort et al., 1994), but at 15°C for 3 hr. For this, precursor lipids were complexed to BSA by injection of 3 μl of an ethanolic solution of the precursors into 3 ml of Hank's balanced salt solution without bicarbonate, 10 mM HEPES (pH 7.4) (HBSS) containing 1% (w/v) BSA (HBSS plus BSA), to yield the following final concentrations: 25 μM C_6-NBD-PA, 5 μM C_6-NBD-ceramide, or 33 μM [^{14}C]C_6-ceramide. During the incubation, short-chain lipid products appearing on the cell surface were depleted into the medium by the BSA. After 3 hr, the apical medium (1 ml) and basolateral medium (2 ml) were collected, and the cells were washed in HBSS plus BSA for 30 min on ice. The apical and basolateral washes were pooled with the corresponding 15°C media, and the filter was cut from its ring, after which the lipids from media and cells were analyzed.

[^{3}H]C_8C_8-Ceramide

Cells were incubated with 150 KBq [^{3}H]C_8C_8-ceramide in 0.5 ml HBSS on the apical side and 300 KBq in 1 ml basolaterally, for 3 hr at 15°C, followed by lipid analysis. Addition of 30 nmol/ml unlabeled C_8C_8-ceramide had no effect on the fraction of each lipid found in the medium.

C_6-NBD-[^{3}H]PC, C_8C_8-[^{3}H]PC, and C_8C_8-[^{14}C]PE

Cell monolayers were preincubated in HBSS for 1 hr at 37°C to reduce cellular choline and ethanolamine pools and prelabeled for 1 hr at 37°C with 370 KBq/ml [^{3}H]choline or 37 KBq/ml [^{14}C]ethanolamine in HBSS, with 0.5 ml on the apical side and 1 ml on the basolateral side of the filter. At $t = 60$ min, lipid precursor suspension was added to the radiolabel medium for 3 hr at 15°C as above, 0.5 ml to the apical medium and 1 ml to the basolateral medium, to achieve a final concentration of 25 μM C_6-NBD-PA or 50 μM C_8C_8-PA.

Lipid Analysis

Lipids were extracted and analyzed essentially as in van Genderen and van Meer (1995). Lipid products from C_6-NBD-PA, C_6-NBD-ceramide, and [^{14}C]C_6-ceramide were separated in two dimensions by borate–TLC, but over 20 cm in the first dimension. In the case of C_6-NBD-[^{3}H]PC, fluorescence and autoradiography were used to discriminate C_6-NBD-[^{3}H]PC from [^{3}H]PC with normal acyl chains. Media contained little of the natural PC (less than 0.1%). Products from C_8C_8-PA were separated by TLC in chloroform/methanol/25% NH_4OH (65:35:4, v/v). Products from [^{3}H]C_8C_8-ceramide were extracted from cells and media using reversed-phase columns and analyzed by TLC (Karrenbauer et al., 1990). Fluorescent spots were quantitatively analyzed in a fluorimeter, and radiolabeled spots were detected by fluorography and quantified as before (van der Bijl et al., 1996).

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