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The human multidrug resistance protein MRP1 translocates sphingolipid analogs across the plasma membrane

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SUMMARY

Recently, we have provided evidence that the ABC-transporter MDR1 P-glycoprotein translocates analogs of various lipid classes across the apical plasma membrane of polarized LLC-PK1 cells transfected with *MDR1* cDNA. Here, we show that expression of the basolateral ABC-transporter MRP1 (the multidrug resistance protein) induced lipid transport to the exoplasmic leaflet of the basolateral plasma membrane of LLC-PK1 cells at 15°C. C_6-NBD-glucosylceramide synthesized on the cytosolic side of the Golgi complex, but not C_6-NBD-sphingomyelin synthesized in the Golgi lumen, became accessible to depletion by BSA in the basal culture medium. This suggests the absence of vesicular traffic and direct translocation of C_6-NBD-glucosylceramide by MRP1 across the basolateral membrane. In line with this, transport of the lipid to the exoplasmic leaflet depended on the intracellular glutathione concentration and was inhibited by the MRP1-inhibitors sulfinpyrazone and indomethacin, but not by the MDR1 P-glycoprotein inhibitor PSC 833. In contrast to the broad substrate specificity of the MDR1 P-glycoprotein, MRP1 selectively transported C_6-NBD-glucosylceramide and C_6-NBD-sphingomyelin, the latter only when it was released from the Golgi lumen by brefeldin A. This shows the specific nature of the lipid translocation. We conclude that the transport activity of MDR1 P-glycoprotein and MRP1 must be taken into account in studies on the transport of lipids to the cell surface.

Key words: Multidrug resistance (MDR), Lipid translocator, Basolateral, Epithelial, Glycolipid

INTRODUCTION

The human multidrug resistance protein MRP1 and the MDR1 P-glycoprotein (MDR1 Pgp) belong to the ATP-binding cassette (ABC) superfamily of membrane transporters. They actively transport a wide range of compounds with different structures and cellular targets across membranes out of the cytosol. MRP1 and MDR1 Pgp are highly expressed in a number of cancer cells, where they oppose chemotherapy by pumping drugs out of the cells, a phenomenon known as multidrug resistance (MDR, reviewed in Higgins, 1992). Whereas MRP1 expression is found in all major organs analyzed (Zaman et al., 1993), MDR1 Pgp is found in the brush border of proximal tubules of the kidney, in the bile canalicular membrane of hepatocytes, in the apical membrane of mucosal cells in the intestine, and in the luminal membrane of endothelial cells at blood-tissue barrier sites (Thiebaut et al., 1987; Cordon-Cardo et al., 1989).

Although both MDR1 Pgp and MRP1 are members of the ABC superfamily, they only display 15% amino acid identity (Cole et al., 1992). MDR1 Pgp was predicted to contain two similar halves, each containing six transmembrane-spanning domains (TMDs) and a nucleotide-binding domain. A different picture has emerged for MRP1. Based on antibody binding, limited proteolysis and epitope mapping experiments, several groups have demonstrated that MRP1 contains an amino-terminal hydrophobic domain containing five TMDs. This is followed by an MDR1 Pgp-like region containing the tandem repeat of six TMDs and a nucleotide-binding domain (Bakos et al., 1996; Hipfner et al., 1997; Kast and Gros, 1998).

Both MDR1 Pgp and MRP1 transport hydrophobic cytotoxic drugs; MDR1 Pgp transports compounds in an unmodified state. In contrast, MRP1 mainly transports substrates conjugated to glutathione, glucuronide and sulfate (reviewed in Deeley and Cole, 1997; Jedlitschky et al., 1997). In cases where MRP1 transports anti-cancer drugs of which no glutathione conjugates are known to exist, both in vitro and in vivo experiments have demonstrated that cytosolic glutathione is required for the transport (Versantvoort et al., 1995; Zaman et al., 1995; Loe et al., 1996). Experiments using embryonic stem cells with an inactivated *MRP1* gene strongly suggest that drugs are co-transported with glutathione (Rappa et al., 1997).

Human MDR3 Pgp (the homolog of mouse mdr2 Pgp), a protein that shows 80% amino acid homology to MDR1 Pgp

(van der Bliek et al., 1988), transports the phospholipid phosphatidylcholine (PC) across membranes (Ruetz and Gros, 1994; Smith et al., 1994). PC translocation is the physiological function of MDR3 Pgp in the liver canalicular membrane, where its presence is a prerequisite for the secretion of phospholipids into the bile (Smit et al., 1993). Recently it was demonstrated that MDR1 Pgp can also translocate lipids across membranes (van Helvoort et al., 1996; 1997; Bosch et al., 1997). Whereas MDR3 Pgp only translocates PC, MDR1 Pgp is more promiscuous in its specificity: besides analogs of PC it translocates phosphatidylethanolamine, sphingomyelin (SM) and glucosylceramide (GlcCer) molecules that carry a shortened fatty acid at the C2-position of the glycerol or sphingosine backbone (van Helvoort et al., 1996).

As MDR1 Pgp and MRP1 have partially overlapping substrate specificities, the question arises whether MRP1 is able to translocate lipids across membranes. In the present study, we used a pig kidney-derived polarized cell line (LLC-PK1) stably transfected with *MRP1* cDNA. It has been shown before that MRP1 is located in the basolateral plasma membrane of these cells with only a small fraction of the MRP1 being present in intracellular membranes (Evers et al., 1996). To examine whether MRP1 can translocate short-chain analogs of membrane lipids from the cytoplasmic leaflet to the exoplasmic leaflet of the plasma membrane, we have applied an assay that has been used previously to demonstrate lipid translocation for MDR Pgps (van Helvoort et al., 1996). We show that MRP1 recognizes some of the lipids that are translocated by MDR1 Pgp, and that translocation by MRP1 is dependent on the intracellular glutathione concentration.

MATERIALS AND METHODS

Materials

Brefeldin A, bovine serum albumin fraction V (BSA), L-α-dioctanoyl phosphatidic acid (C_8C_8-PA), 1,2-diphenyl-4-(phenylsulfinylethyl)-3,5-pyrazolidinedione (sulfinpyrazone), DL-buthionine-[S,R]-sulfoximine (BSO) and γ-Glu-Cys-Gly-O-ethyl (GSH-ethylester) were purchased from Sigma (St Louis, MO). Indomethacin was purchased from ICN (Aurora, OH); PSC 833 was a kind gift of Sandoz Pharma bv (Uden, The Netherlands). 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoyl)-sphingosine (C_6-NBD-ceramide: C_6-NBD-Cer) was from Molecular Probes (Eugene, OR). C_6-NBD-phosphatidic acid (C_6-NBD-PA) was from Avanti Polar Lipids (Alabaster, AL). [^{3}H-methyl]choline chloride (3 TBq/mmol) and [^{3}H]C_6-Cer (825 GBq/mmol) were from DuPont NEN (Dordrecht, The Netherlands). Organic solvents were purchased from Riedel-de Haën (Seelze, Germany); silica TLC plates were from Merck (Darmstadt, Germany). Geneticin (G418) and cell culture media were from Gibco (Paisley, UK).

Cell culture

LLC-PK1 pig kidney epithelial cells were transfected with the mammalian expression vector pRC/RSV-MRP containing human *MRP1* cDNA, and a stable transfectant was isolated as described (Evers et al., 1996). LLC-PK1 cells overexpressing MDR1 Pgp were obtained from A. Schinkel, The Netherlands Cancer Institute, Amsterdam (Schinkel et al., 1995). Cells were cultured (mycoplasma-free) in M199 medium supplemented with 10% FCS as described. The MRP1 transfectants were cultured in the presence of G418 (800 μg/ml). For experiments 2×10^6 cells were seeded on 4.7 cm^2 filters glued to the bottom of plastic rings (Transwell, 0.4 μm pore diameter; Costar, Cambridge, MA) and were grown as monolayers for 4 days.

Transport incubations

C_6-NBD-Cer, [^{3}H]C_6-Cer

Ceramides were added to both sides of epithelial monolayers on filters for 3 hours at 15°C, as described before (van Helvoort et al., 1996). Ceramide was complexed to BSA by injection of 10 μl of an ethanolic solution of the precursor into 3 ml Hanks' balanced salt solution without bicarbonate, 10 mM Hepes, pH 7.4 (HBSS′) containing 1% (w/v) BSA (HBSS′+BSA) to yield a final concentration of 5 μM. During the incubation, newly synthesized short-chain GlcCer and SM appearing on the cell surface were depleted from the surface into the medium by the BSA. After 3 hours, the apical medium (1 ml) and basal medium (2 ml) were collected and the cells were washed in HBSS′+BSA for 30 minutes on ice. The lipids were extracted from media plus wash solutions and from filters (cells), and quantitatively analyzed (see below). Transport of each lipid class to the apical and basolateral cell surface was calculated as the percentage of that lipid being recovered in the apical and basal medium, respectively.

C_6-NBD-PA, C_8C_8-PA

Cell monolayers were preincubated in HBSS′ for 1 hour at 37°C to reduce intracellular choline pools and labeled for 1 hour at 37°C with 370 kBq/ml [^{3}H]choline in HBSS′+ BSA, with 0.5 ml on the apical side and 1 ml on the basolateral side of the filter. Subsequently, lipid precursor suspension was added (0.5 ml to the apical and 1 ml to the basal medium) for 3 hours at 15°C, resulting in a final concentration of 25 μM C_6-NBD-PA or 50 μM C_8C_8-PA. Lipid transport was measured as described above.

Lipid analysis

Lipids were extracted from cells and media by a two-phase extraction as before (van der Bijl et al., 1996). Lipid products from C_6-NBD-Cer and [^{3}H]C_6-Cer were separated in two dimensions by TLC as before (van der Bijl et al., 1996). In experiments with C_6-NBD-PA, the fluorescent C_6-NBD-[^{3}H]PC spot was marked and a film was applied to allow accurate separation of C_6-NBD-[^{3}H]PC from [^{3}H]PC with normal acyl chains (van Helvoort et al., 1996). Products from C_8C_8-PA were separated by one-dimensional TLC in chloroform/methanol/25% NH_4OH (65:35:4, v/v; van Helvoort et al., 1996). Fluorescent spots were quantitatively analyzed with a fluorimeter, and radiolabeled spots were detected by fluorography and quantified as before (van der Bijl et al., 1996) or, for [^{3}H]C_6-GlcCer, by scanning the film.

Glutathione depletion

Intracellular reduced glutathione (GSH) levels of cell monolayers on filters were depleted by growing the cells in M199 medium containing 25 μM BSO during 24 hours prior to the transport incubations. For two out of four filters, intracellular GSH levels were restored prior to the transport incubations by extracellular addition of 5 mM GSH-ethyl-ester in HBSS′+BSA for 4 hours at 37°C. During the transport incubations BSO, or BSO plus GSH ethyl ester, were present in the incubation medium.

Glutathione determination

Cell monolayers on filters were washed with phosphate-buffered saline and scraped in 10% perchloric acid. Precipitated protein was removed by centrifugation and the supernatant was neutralized by the addition of 0.4 volumes of 0.5 M MOPS/5 M KOH. The concentration of total glutathione (oxidized and reduced) was determined according to the recycling method of Tietze et al. (1969).

RESULTS

Translocation of C_6-NBD-GlcCer to the basolateral surface of LLC-PK1 cells transfected with MRP1

Confluent monolayers of LLC-PK1 cells, and LLC-PK1 cells

transfected with *MRP1* or *MDR1* cDNAs (LLC-MRP1 and LLC-MDR1 cells, respectively) were incubated with C_6-NBD-Cer for 3 hours at 15°C. Because of its short acyl chain C_6-NBD-Cer can diffuse across aqueous phases and because it has no polar headgroup it rapidly diffuses over membranes. Thus, C_6-NBD-Cer partitions between the various cellular membranes. It is converted to C_6-NBD-GlcCer on the cytosolic surface of the Golgi and to C_6-NBD-SM in the Golgi lumen. Consequently, C_6-NBD-GlcCer should equilibrate with the cytosolic surface of the plasma membrane (Jeckel et al., 1992; van Helvoort et al., 1996). Any C_6-NBD-GlcCer translocated to the exoplasmic leaflet of the plasma membrane is extracted by BSA in the medium, which binds this short-chain lipid and acts as a sink. Arrival of lipids on the surface by exchange through the cytosol and translocation is discriminated from delivery to the plasma membrane via vesicular transport by lowering the temperature: at 15°C the traffic of membrane vesicles is blocked (Fries and Lindström, 1986). In addition, in a number of experiments the cells were pretreated with brefeldin A (BFA), which independently blocks vesicular traffic of lipids and proteins from the ER and Golgi to the plasma membrane (see Klausner et al., 1992; van Helvoort et al., 1997).

After incubation for 3 hours at 15°C only a small fraction (less than 5%) of both C_6-NBD-GlcCer and C_6-NBD-SM was found in the apical and basal medium of the LLC-PK1 cells (Fig. 1). Because BSA extracts short-chain lipids from the membrane at 15°C, this confirms that in LLC-PK1 cells transport to the cell surface is essentially blocked at this temperature (van Genderen and van Meer, 1995). In contrast, 44% of C_6-NBD-GlcCer was recovered in the apical medium of the LLC-MDR1 cells, while a small increase was observed in the fraction of C_6-NBD-GlcCer in the basal medium. This is in accordance with our previous results (van Helvoort et al., 1996) and demonstrates the translocation activity of MDR1 Pgp in the apical membrane. In contrast, in the case of cells overexpressing *MRP1*, 25-30% of C_6-NBD-GlcCer was recovered in the basal medium with a small fraction of the C_6-NBD-GlcCer in the apical medium. This suggests the possibility that the basolaterally localized MRP1 protein translocated C_6-NBD-GlcCer from the cytoplasmic to the exoplasmic leaflet of the basolateral membrane. Part of the 70% of the C_6-NBD-GlcCer that remained in the cell might have been translocated to the lumen of Golgi or endosomes by MRP1 residing in these membranes. However, only a little MRP1 has been observed inside the transfected LLC-PK1 cells (Evers et al., 1996).

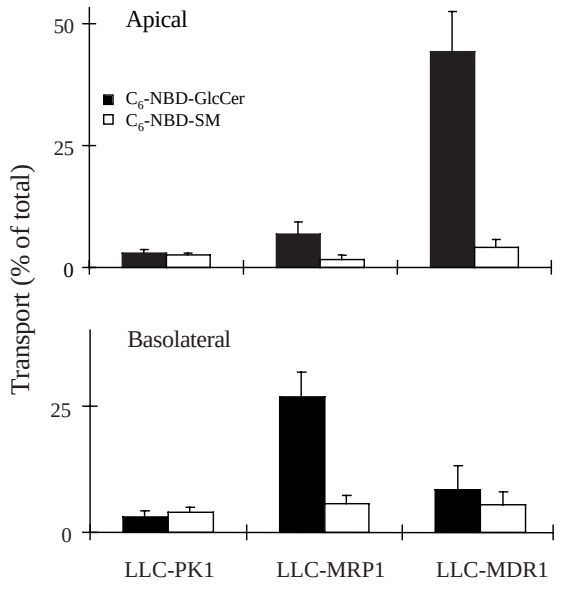


Fig. 1. Translocation of C_6-NBD-glucosylceramide across the plasma membrane of LLC-PK1 cells transfected with *MRP1* or *MDR1*. Cell monolayers on filters were incubated with 5 μM C_6-NBD-Cer on both sides for 3 hours at 15°C. The fluorescent lipid products, C_6-NBD-GlcCer (filled bars) and C_6-NBD-SM (open bars), when appearing at the cell surface, were depleted into the BSA-containing medium. Lipids were quantified as described in Materials and methods. Transport is expressed as percentage of the fluorescent lipid recovered in each medium. Synthesis from 15 nmol C_6-NBD-Cer was typically 40 pmol C_6-NBD-GlcCer for the LLC-MRP1 and LLC-MDR1 cells and 70 pmol for the untransfected cells. C_6-NBD-SM synthesis was typically 60 pmol in LLC-MRP1 cells and 125 pmol in LLC-MDR1 cells and untransfected LLC-PK1 cells. Data on LLC-MRP1 and -MDR1 are the means of 7 independent experiments (±s.d.; n=10-14).

In both LLC-MDR1 and LLC-MRP1 cells, essentially no C_6-NBD-SM entered the medium (Fig. 1), suggesting that C_6-NBD-SM cannot reach the exoplasmic leaflet of the plasma membrane under these conditions (van Genderen and van Meer, 1995; van Helvoort et al., 1996). This is in line with the fact that C_6-NBD-SM is synthesized in the lumen of the Golgi and has no access to the cytosol (see Jeckel et al., 1992), and that vesicular traffic is inhibited at 15°C (Fries and Lindström, 1986). However, the situation is different after treatment of the cells with the drug BFA, which induces retrograde transport of Golgi enzymes to the ER (Klausner et al., 1992). Sphingomyelin has been observed to translocate readily across the ER membrane (Herrmann et al., 1990). Indeed, after such redistribution, C_6-NBD-SM has access to the cytoplasmic leaflet of the plasma membrane, which is evident from the fact that it was translocated across the plasma membrane by MDR1 Pgp (van Helvoort et al., 1996, 1997).

In cells pretreated with 1 μg/ml BFA for 30 minutes at 37°C, transport of newly synthesized C_6-NBD-GlcCer to the apical and basolateral surface of the various cells at 15°C displayed the same characteristics as in untreated cells (Figs 1 and 2). However, the situation was very different for C_6-NBD-SM. While still essentially no C_6-NBD-SM was found in the medium of non-transfected cells, nearly 20% of the C_6-NBD-SM was recovered in the apical medium of BFA-treated LLC-MDR1 cells (Fig. 2; van Helvoort et al., 1996). Under these conditions, also nearly 20% of total C_6-NBD-SM reached the basal medium in the BFA-treated LLC-MRP1 cells (Fig. 2). These results suggest that C_6-NBD-SM under these conditions had access to the cytosolic surface of the plasma membrane and that this short-chain SM is a substrate for MRP1.

MRP1-mediated lipid translocation displays specificity for C_6-NBD-sphingolipids

To further characterize lipid translocation by MRP1, we studied the transport of the short-chain lipid C_6-GlcCer. This MDR1 Pgp substrate lacks the fluorescent moiety on the acyl chain. After incubation with [^{3}H]C_6-Cer, [^{3}H]C_6-GlcCer did reach the apical medium of LLC-MDR1 cells at 15°C, like [^{14}C]C_6-GlcCer (van Helvoort et al., 1996). However, it was not recovered in the basal medium of LLC-MRP1 cells (Table

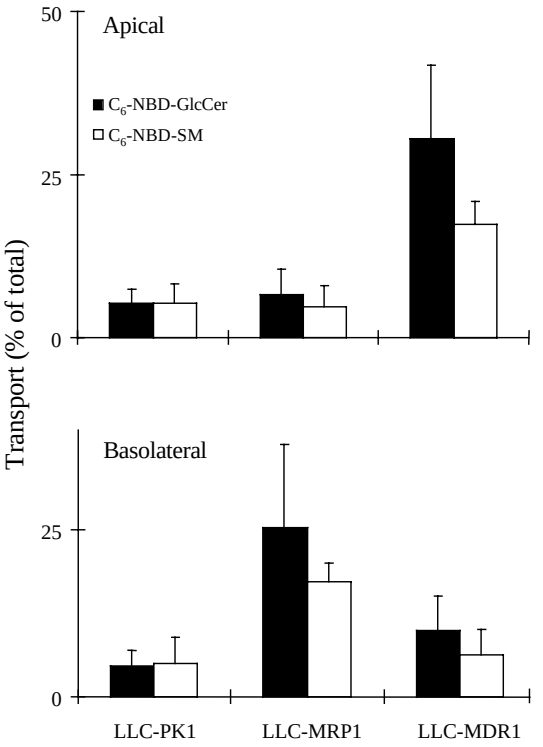


Fig. 2. Translocation of C_6-NBD-SM across the plasma membrane of LLC-MRP1 and LLC-MDR1 cells. Cells were preincubated for 30 minutes at 37°C with 1 μg/ml BFA. Subsequently, the transport experiment was performed as described in the legend to Fig. 1. Synthesis was typically 30 pmol for C_6-NBD-GlcCer and 120 pmol for C_6-NBD-SM. Data are the means of 5 independent experiments (±s.d.; *n*=5-7).

1). The results on the LLC-MDR1 cells imply that the newly synthesized lipid did reach the cytoplasmic leaflet of the plasma membrane. Apparently, MRP1 cannot translocate [^{3}H]C_6-GlcCer across the plasma membrane.

To study whether short-chain analogs of the glycerophospholipid PC are also translocated by MRP1, LLC-PK1 cells were incubated with C_6-NBD-phosphatidic acid. In the plasma membrane this lipid is hydrolyzed to C_6-NBD-diacylglycerol (DAG), which is converted to C_6-NBD-PC on the cytosolic surface of the ER. As part of the C_6-NBD-PC is synthesized on the basolateral cell surface, [^{3}H]choline was added to the 15°C incubation. It labels specifically the intracellularly synthesized C_6-NBD-PC (van Helvoort et al., 1996). The appearance of C_6-NBD-[^{3}H]PC in the medium was monitored. Similarly, we studied translocation of C_8C_8-[^{3}H]PC. While MDR1 Pgp translocated both lipids to the apical surface in LLC-MDR1 cells, little MRP1-mediated translocation to the basolateral exoplasmic plasma membrane was observed in the LLC-MRP1 cells (Table 1).

Specific inhibition of translocation by inhibitors of MRP1-mediated drug transport

As a direct test for the involvement of MRP1 in the translocation of C_6-NBD-GlcCer to the basolateral surface of LLC-MRP1 cells, the transport experiments were repeated in the presence of sulfinpyrazone and indomethacin, both reported to inhibit MRP1 (Evers et al., 1996; Holló et al., 1996; Draper et al., 1997) and PSC 833, an inhibitor of MDR1 Pgp (Boesch et al., 1991; Twentyman and Bleehen, 1991; van Helvoort et al., 1996) but not of MRP1 (Leier et al., 1994). Both sulfinpyrazone and indomethacin reduced the fraction of C_6-NBD-GlcCer and -SM recovered from the basal medium of LLC-MRP1 cells to the level of transport in the untransfected cells (Figs 2, 3), with little or no effect on the fraction of C_6-NBD-lipids in the apical medium of LLC-MDR1 cells. In contrast, PSC 833 had little effect on the appearance of C_6-NBD-GlcCer on the basolateral plasma membrane surface in the LLC-MRP1 cells, but strongly inhibited its appearance at the apical surface of the LLC-MDR1 cells (Fig. 3). These results confirm the notion that the basolateral transport observed in the LLC-MRP1 cells was caused by MRP1.

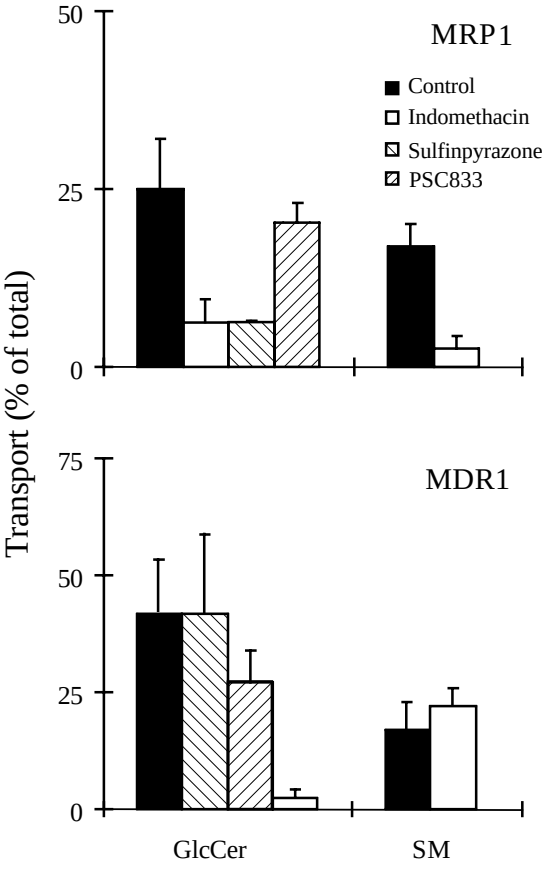


Fig. 3. Specific effects of MRP1 and MDR1 inhibitors on C_6-NBD-sphingolipid translocation in LLC-MRP1 and LLC-MDR1 cells. Monolayers of LLC-MRP1 cells or LLC-MDR1 cells were pre-incubated for 10 minutes at 37°C with 20 μM indomethacin, 2 mM sulfinpyrazone or 5 μM PSC 833, followed by an incubation with 5 μM C_6-NBD-Cer on both sides of the filter for 3 hours at 15°C in the presence of the inhibitor. Transport of C_6-NBD-lipid to the basolateral surface of the LLC-MRP1 cells and apical surface of the LLC-MDR1 cells was assessed as described under Materials and methods. Synthesis in the control cells was 40 pmol C_6-NBD-GlcCer and 130 pmol C_6-NBD-SM. The inhibitors had no effect on C_6-NBD-SM synthesis, but did affect the synthesis of C_6-NBD-GlcCer: indomethacin, 125%; sulfinpyrazone, 75%; and PSC 833, 130% of control. In order to assess transport of C_6-NBD-SM, cells were pretreated with BFA as in Fig. 2 (*n*=3-7). C_6-NBD-GlcCer transport was measured ± BFA (*n*=4-21).

MRP1-mediated lipid translocation depends on the glutathione concentration

MRP1 can transport glutathione-conjugates out of the cell, and transport of a number of other molecules is stimulated by reduced glutathione (GSH) in the cytosol. To test whether GSH plays a role in the translocation of C_6-NBD-GlcCer by MRP1, the GSH concentration was lowered by BSO, an inhibitor of the enzyme catalyzing the first step in GSH synthesis, γ-glutamylcysteine synthetase. Incubation of LLC-MRP1 cell monolayers with 25 μM BSO for 24 hours prior to the transport incubation reduced intracellular GSH levels from 15±4 to 7±2 nmol/mg protein (*n*=2; in LLC-MDR1 cells these numbers were 48±3 and 12±1 nmol/mg protein, respectively). In LLC-MRP1 cells incubated with BSO the appearance of C_6-NBD-GlcCer in the basal medium was reduced to 37% of control values, whereas BSO treatment did not affect transport into the apical medium by MDR1 Pgp (Fig. 4). Cellular GSH levels can be restored by addition of GSH-ethylester, which is taken up by cells and hydrolyzed intracellularly to GSH (Griffith and Meister, 1979). Incubation of BSO-treated monolayers for 4 hours with 5 mM GSH-ethylester in the presence of 25 μM BSO increased the intracellular GSH level in LLC-MRP1 cells to 200% of control, 33±3 nmol/mg protein (24±4 nmol/mg protein in LLC-MDR1 cells). Under these conditions, the appearance of C_6-NBD-GlcCer on the basolateral surface of LLC-MRP1 cells was restored to 120% of control, with essentially no effect in LLC-MDR1 cells (Fig. 4). This showed that BSO did not directly inhibit the activity of MRP1 and that the translocation of C_6-NBD-GlcCer by MRP1 was stimulated by intracellular GSH. A transport assay in the presence of BFA, in order to give C_6-NBD-SM access to MRP1 (Fig. 2), showed that in the LLC-MRP1 cells transport of C_6-NBD-SM to the basolateral surface was also affected by BSO, whereas SM transport by MDR1 Pgp was BSO-insensitive.

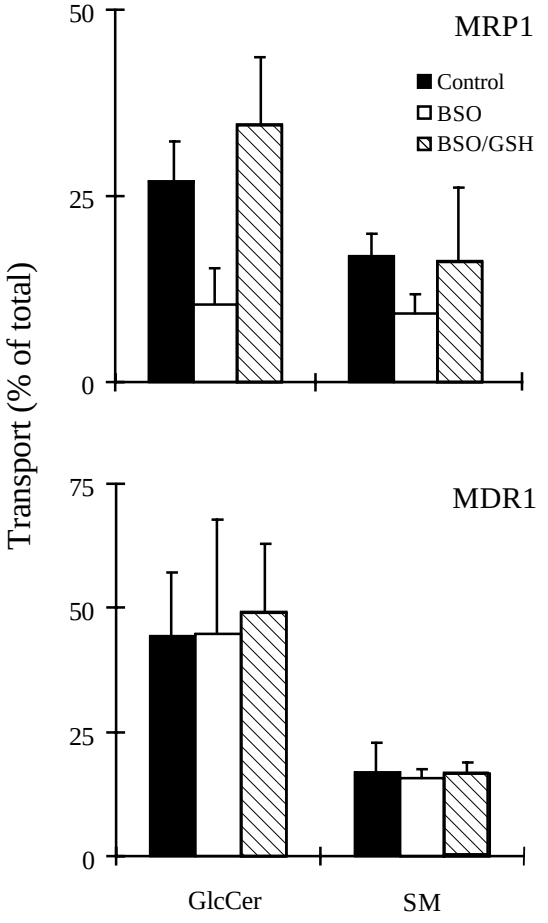
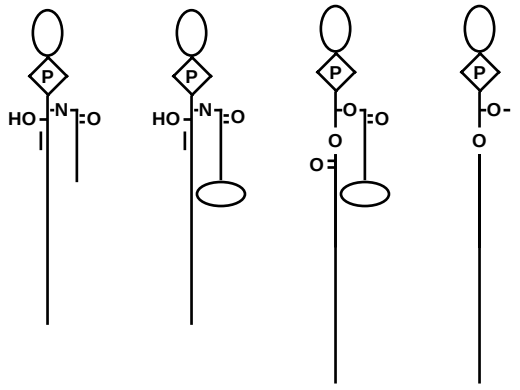


Fig. 4. Glutathione dependent C_6-NBD-sphingolipid translocation by LLC-MRP1 but not by LLC-MDR1 cells. LLC-MRP1 and LLC-MDR1 cell monolayers were incubated 24 hours prior to the transport incubation without (control) or with 25 μM BSO (BSO and BSO/GSH), after which the filters were incubated for 4 hours at 37°C in HBSS′ (control), HBSS′ plus BSO (BSO) or with 5 mM GSH-ethylester in HBSS′ plus BSO (BSO/GSH). The subsequent incubation with 5 μM C_6-NBD-Cer on both sides of the filter for 3 hours at 15°C was carried out in the presence of BSO (BSO) or in the presence of BSO and GSH-ethylester (BSO/GSH). Synthesis was typically 35 pmol C_6-NBD-GlcCer. The presence of BSO or GSH-ethylester in the incubation medium did not influence the synthesis of C_6-NBD-GlcCer in the two cell lines. In order to assess transport of C_6-NBD-SM, cells were pretreated with BFA as in Fig. 2 (*n*=2-7). C_6-NBD-GlcCer transport was measured ± BFA (*n*=4-21).

DISCUSSION

Recently, we and others have reported that the multidrug transporter MDR1 Pgp is able to translocate a number of membrane lipid analogs across the cellular plasma membrane, the apical plasma membrane of epithelial cells. Here we report that, in contrast, the multidrug resistance protein MRP1 induced lipid transport to the basolateral surface in the absence of vesicular traffic. The localization of MRP1 in the basolateral plasma membrane, the sensitivity of the transport process to



	C_6-SM	C_6-NBD-SM	C_6-NBD-PC	edelfosine
MRP1	–	+	–	+
MDR1	+	+	+	+

Fig. 5. Recognition of phosphocholine-containing membrane lipid analogs by MRP1 and MDR1 Pgp. While C_6-NBD-SM and edelfosine (1-O-octadecyl-2-O-methyl-*sn*-glycero-3-phosphocholine; ET-18-OCH_3) are substrates for MRP1 (Fig. 2; Ruetz et al., 1997), this is not found to be the case for C_6-NBD-PC (Table 1). C_6-SM was found not to be translocated to the basolateral cell surface by LLC-MRP1 cells, while it was efficiently translocated to the apical surface of LLC-MDR1 cells, both as compared to untransfected LLC-PK1 cells, in two experiments at 37°C in the presence of BFA (data not shown).

MRP1 inhibitors and to cytosolic depletion of reduced glutathione (GSH) suggest that the transport reflects direct translocation of the lipids across the basolateral membrane by MRP1. MRP1 displayed a more stringent substrate specificity than MDR1 Pgp.

Mechanism of action

The fact that both MDR1 Pgp and MRP1 can translocate the sphingolipid analogs C_6-NBD-GlcCer and C_6-NBD-SM from the cytoplasmic to the exoplasmic leaflet of the plasma membrane, suggests that the two ABC-transporters share a common mode of action. C_6-NBD-sphingolipids have a very low water solubility. In the absence of lipid binding proteins they are stably integrated in the membrane. Therefore, it seems most likely that both transporters bind these lipids in the cytoplasmic leaflet of the plasma membrane lipid bilayer, and move them across, into the exoplasmic leaflet, by a flippase mechanism (van Helvoort et al., 1996). The other known substrates of MDR1 Pgp and MRP1, all lipophilic in nature, are presumably translocated across the plasma membrane by the same mechanism as was suggested for MDR1 Pgp before (Higgins and Gottesman, 1992).

The translocation of the C_6-NBD-sphingolipids by MRP1 was reduced to one third by halving the concentration of GSH in the cytosol (Fig. 4). It has been reported that transport of a number of drugs by MRP1 depends on cytosolic GSH. GSH depletion by BSO in cells overexpressing *MRP1* led to reversal of the resistance to doxorubicin, daunorubicin and vincristine and a decreased efflux of daunorubicin (Versantvoort et al., 1995; Zaman et al., 1995). We do not know the exact role of GSH in the transport of NBD-lipids by MRP1, but it is not unlikely that, like cytotoxic drugs, these compounds are co-transported with GSH (Rappa et al., 1997; R. E. and P. Borst, unpublished data). In our lipid analyses we found no indications for the formation of covalent glutathionyl-lipid conjugates: C_6-NBD-lipids in the medium were unmodified, as judged by 2D-TLC analysis.

Substrate specificity

In contrast to MDR1 Pgp, which can translocate a wide variety of short-chain analogs (Table 1; van Helvoort et al., 1996), MRP1 translocates a much narrower range of lipids. In the present test system only C_6-NBD-GlcCer and C_6-NBD-SM were translocated. As the other lipids also reached the cytoplasmic leaflet of the plasma membrane, evident from their translocation by MDR1 Pgp (Table 1; van Helvoort et al., 1996), this result can only be explained by the substrate preference of MRP1. The C_6-NBD-Cer backbone seems essential as, remarkably, two close analogs of C_6-NBD-SM with a different backbone were not translocated to a significant extent: C_6-SM, which has a ceramide backbone but lacks the NBD-moiety, and C_6-NBD-PC, which has the C_6-NBD-fatty acid but has a diacylglycerol backbone (Table 1; Fig. 5). Interestingly, Ruetz et al. (1997) recently reported evidence suggesting that a synthetic PC with an ether-linked C18 at the C1 position and a methyl group at the C2 position of the glycerol, edelfosine (Fig. 5), may be translocated by MRP1 and by MDR1 Pgp, whereby the methyl group at position sn-2 was not required. At this moment, a common structural determinant in the lipid molecule that is required for translocation by MRP1 is not forthcoming, nor is it evident what natural lipids might be physiological substrates. Importantly, the MRP1 knock-out mouse is normal apart from an increased sensitivity to etoposide and a decreased sensitivity to a stimulus of inflammation (Lorico et al., 1997; Wijnholds et al., 1997), neither of which seems to be related to lipid translocation.

A high degree of lipid specificity has also been reported for the PC-translocator MDR3 Pgp. The absence of PC from the bile of an MDR3 knock-out mouse suggested that MDR3 Pgp is involved in translocating PC across the bile canalicular membrane (Smit et al., 1993). An in vitro study later confirmed that natural PC is a substrate for MDR3 Pgp (Smith et al., 1994). Also C_6-NBD-PC serves as a substrate for MDR3 Pgp-mediated translocation across the membrane (Ruetz and Gros, 1994; van Helvoort et al., 1996; Bosch et al., 1997). However, unexpectedly, C_8C_8-PC was not transported by MDR3 Pgp, whereas it was translocated by MDR1 Pgp (van Helvoort et al., 1996), which again illustrates the complexity of the recognition process.

Table 1. Lipid substrates for MRP1 and MDR1 Pgp*

	% of the lipid in the medium after 3 hours at 15°C			
Substrate	LLC-PK1 (basolateral)	LLC-MRP1 (basolateral)	LLC-PK1 (apical)	LLC-MDR1 (apical)
C_6-NBD-GlcCer‡	4±2 (7)	27±8 (15)	3±1 (7)	41±13 (21)
C_6-NBD-SM§	4±4 (5)	17±3 (5)	5±2 (5)	17±3 (8)
[^{3}H]-C_6-GlcCer	3±2 (2)	4±1 (2)	3±2 (2)	16±4 (2)
C_6-NBD-[^{3}H]PC	3±1 (2)	7±5 (4)	2±0 (2)	32±5 (2)
C_8C_8-[^{3}H]PC	5±1 (2)	8±2 (2)	4±1 (2)	28±2 (2)

*To establish the specificity in lipid translocation by the human MRP1 protein, several lipids were tested in the translocation assay in untransfected cells and cells transfected with MRP1 or MDR1 Pgp, as described under Materials and methods. Data are presented as percentage of total±s.d. (*n*), (or ±range when *n*=2).

‡Transport assay performed after pretreatment of the cells±BFA (data from Figs 1 and 2).

§Transport assay performed after pretreatment of the cells with BFA (data from Fig. 2).

Studies on cellular lipid transport using short-chain lipid analogs

The membranes of the various cellular organelles possess different lipid compositions. The generation and maintenance of these differences requires that intracellular transport of lipid molecules displays specificity. What provides this specificity is presently unclear. MDR1 Pgp and MRP1 are expressed in cells in vivo and in cell lines, and both can translocate many of the short-chain lipids that have been used in the published work. This is true for the short-chain sphingolipids used by ourselves and others to study sphingolipid transport and sorting (see van Helvoort et al., 1996; van IJzendoorn and Hoekstra, 1998 and the references therein), but also most studies on the translocation of aminophospholipids have relied on spin-labeled and fluorescently labeled short-chain analogs (see Zwaal and Schroit, 1997). While the inward-directed aminophospholipid translocase has now been identified as a P-type ATPase (Tang et al., 1996) and while a bidirectional phospholipid scramblase has been cloned (Zhou et al., 1997), recent data show that MRP1 also contributes to the movement of short-chain phosphatidylserine by outward translocation across the membrane of erythrocytes (Dekkers et al., 1998; Kamp and Haest, 1998). The observations that MDR Pgps and

MRP1 can translocate C_6-NBD-lipids make it necessary to (1) re-evaluate the earlier results for the potential involvement of these (and other) translocators, and (2) to establish how these processes apply to the natural long-chain lipids.

Conclusion

Like the MDR1 Pgp, the multidrug resistance protein MRP1 can translocate certain analogs of membrane lipids across the plasma membrane. On the one hand, this observation may provide us with tools to study the mechanism of action of these drug transporters. On the other hand, it confronts us with the intriguing question of whether and how these and other transporters translocate lipids across apical and basolateral plasma membrane domains. The elucidation of the complex interplay between the translocators that work at different sites and in opposite directions will help us to understand the purpose of regulating the transmembrane distribution of the various lipids.

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