Transbilayer Movement of NBD-Labeled Phospholipids in Red Blood Cell Membranes: Outward-Directed Transport by the Multidrug Resistance Protein 1 (MRP1)

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Received May 4, 1998; Revised Manuscript Received July 10, 1998

ABSTRACT: The outward movement (flop) of fluorescently labeled analogues of phosphatidylserine (PS) and phosphatidylcholine (PC) in human and murine red blood cells (RBC) was examined. 1-Oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl (C_6-NBD) analogues of PS and PC were incorporated in the inner leaflet of the plasma membrane through the action of aminophospholipid translocase or through equilibration upon prolonged incubation, respectively. After removal of noninternalized probe, externalization of C_6-NBD-PS or C_6-NBD-PC from the inner to outer leaflet was monitored by continuous incubation of the cells in the presence of bovine serum albumin. Flop rates for both probes in intact human RBC were virtually identical ($t_{1/2} \sim 1.5$ h), confirming earlier findings by Bitbol et al. [Bitbol, M., et al. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6783−6787] and Connor et al. [Connor, J., et al. (1992) *J. Biol. Chem. 267*, 19412−19417]. Flop activity in resealed RBC ghosts could only be found upon coinclusion of both ATP and oxidized glutathione (GSSG). Furthermore, flop in intact cells was sensitive to verapamil ($IC_{50} = 5-7\ \mu M$), vincristine ($IC_{50} = 20\ \mu M$), and indomethacin ($IC_{50} = 50\ \mu M$), suggesting the involvement of proteins conferring multidrug resistance (MDR). Experiments with RBC from knock-out mice for multidrug resistance P-glycoproteins (Mdr1a/1b-/- and Mdr2-/-) and multidrug resistance protein 1 (Mrp1-/-) revealed that Mrp1 is responsible for the observed flop of the fluorescent lipid analogues. We found no indications for outward transport of endogenous PS by any of these drug-transporting proteins as measured by a sensitive prothrombinase assay. Neither aminophospholipid translocase nor Ca^{2+}-induced lipid scramblase activities were affected in RBC of these knock-out mice. We conclude that lipid floppase activity, as detected with lipid probes, reflects the activity of MRP1 recognizing the modified lipid analogues as xenobiotics to be expelled from the cell.

The two leaflets of the plasma membrane of human red blood cells have a distinct phospholipid composition: cholinephospholipids, phosphatidylcholine (PC)[1] and sphingomyelin, primarily dominate the outer leaflet, whereas the inner leaflet consists mainly of phosphatidylinositol (PI) and the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE) (reviewed in ref 1). Phospholipid distributions across the membrane bilayer are presently thought to be regulated by at least four different proteins: (i) an ATP-dependent aminophospholipid translocase (*2*, *3*), responsible for the inward transport of aminophospholipids which contributes to the generation of membrane lipid asymmetry; (ii) a phospholipid scramblase (*4*−*6*), that can be activated to catalyze bidirectional movement of all phospholipid classes, resulting in a collapse of lipid asymmetry; (iii) a protein encoded by the multidrug resistance gene *Mdr2* (*7*), that facilitates ATP-dependent outward movement of PC and is known to be responsible for export of PC into the bile; and (iv) a protein which counteracts the aminophospholipid translocase by transporting lipids from the inner to outer leaflet albeit at a much slower rate than the translocase (*8*, *9*). This protein, also referred to as "floppase", was demonstrated to transport short-chain fluorescent analogues of PS, PC, and PE with similar kinetics from the inner to the outer leaflet of the erythrocyte membrane (*10*, *11*). It has been proposed that this protein-mediated outward translocation of lipids, in conjunction with aminophospholipid-specific inward transport, leads to an equilibrium status in which lipid asymmetry of the cell is maintained. In a previous study, we have shown that the outward movement of PS, PC, and PE was ATP-dependent and was blocked by oxidation of membrane sulfhydryls and by the histidine reagent bromophenacyl bromide (*10*). The aim of the present study was to further investigate the nature of this floppase activity. Evidence is presented that the multidrug resistance protein, MRP1, is responsible for the continuous outward migration of C_6-NBD-labeled lipids from inner to outer leaflet of the RBC membrane.

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[1] Abbreviations: C_6-NBD-PS, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine; NBD-PC, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; RBC, red blood cell(s); MDR, multidrug resistance; MRP1, multidrug resistance protein; BSA, bovine serum albumin; GSSG, oxidized glutathione.

10.1021/bi981011w CCC: $15.00
Published on Web 10/01/1998

MATERIALS AND METHODS

Materials. C_6-NBD-PS, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine, and NBD-PC, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine, were obtained from Avanti Polar Lipids, Alabaster, AL. Verapamil, indomethacin, vincristine, oxidized glutathione (GSSG), and BSA were purchased from Sigma (St. Louis, MO). Coagulation factors Xa, Va, and prothrombin were purified from bovine blood as described before (*11*). Chromogenic substrate for thrombin, S2238, was from AB Kabi Diagnostica (Stockholm, Sweden). All other reagents were of the highest grade commercially available.

Isolation of RBC and Preparation of Resealed Ghosts. Whole blood was obtained from healthy volunteers by venipuncture, using acid citrate dextrose (ACD: 0.18 M glucose, 0.08 M trisodium citrate, 0.052 M citric acid) as anticoagulant in a final ratio of 1:5 (v/v). RBC were collected and washed with a Hepes buffer (136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 0.2 mM EGTA, 10 mM glucose, adjusted at pH 7.5). Cells were finally resuspended in the same buffer and were used within 1 day.

Red blood cell ghosts were prepared by hypotonic shock in ice-cold lysis buffer (13.6 mM NaCl, 0.27 mM KCl, 2 mM $MgCl_2$, 1 mM Hepes, 0.2 mM EGTA, pH 7.5). After 25 min on ice and addition of Mg-ATP (10 mM) or other compounds, tonicity was restored by addition of 0.1 volume of 9% NaCl, followed by incubation at 37 °C for 5 min. Resealed ghosts were collected by centrifugation at 2250*g* for 20 min and subsequently washed 3 times in Hepes buffer containing 10 mM $MgCl_2$ and 2 mM glucose, pH 7.5, at 4 °C.

Labeling of RBC and Resealed Ghosts with C_6-NBD-Phospholipids. Washed human or murine RBC were resuspended in Hepes buffer to a cell concentration of 10^8 cells/mL and loaded with 0.4 μM C_6-NBD-PS, which corresponds to approximately 1% of the endogenous phospholipids. Translocation of the probe proceeded for 1 h at 37 °C, resulting in 75−95% internalization. For labeling with C_6-NBD-PC, human red cells were incubated overnight with 0.8 μM C_6-NBD-PC, resulting in about 20% internalization of the probe. To measure outward movement, residual C_6-NBD-lipid remaining in the cells' outer monolayer was removed by washing for 5 min with ice-cold 0.5% BSA prior to the experiment.

For measuring flop in resealed ghosts, ghosts were prepared from RBC that were prelabeled with C_6-NBD-PS or C_6-NBD-PC. Alternatively, in the case of C_6-NBD-PS experiments, ghosts with included ATP were labeled by 1 h incubation in which the aminophospholipid translocase activity ensured sufficient accumulation of probe in the inner leaflet.

Translocation of Lipids from the Outer-to-Inner and Inner-to-Outer Leaflet. Inward movement of C_6-NBD-PS was measured using the BSA back-exchange procedure as described by Connor et al. (*10*). Briefly, 200 μL aliquots from the cell suspension were removed at the indicated time intervals and placed on ice for 5 min in the presence and absence of 1% BSA. Pellets obtained after 3 min of centrifugation at 12000*g* were solubilized in 2 mL of 1% (w/v) Triton X-100, and the amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after back-exchange.

Outward movement of C_6-NBD-PS and C_6-NBD-PC was measured by incubating the cells at 10^8/mL at 37 °C in the absence or presence of 1% BSA (continuous extraction). Aliquots of cells were taken at the indicated time intervals, and the fraction of lipid remaining associated with the cells was determined as described above. Similar procedures were followed to measure transbilayer migration of C_6-NBD-lipids in resealed ghosts. The fluorescence intensities were measured (λ_{ex} 472 nm, λ_{em} 534 nm) on a Shimadzu RF-5001PC spectrofluorometer (Shimadzu Europe, Duisburg, Germany).

Prothrombinase Measurements. Surface exposure of phosphatidylserine was determined by measuring the ability of RBC to enhance the rate of conversion of prothrombin to thrombin by the enzyme complex consisting of factors Xa and Va, as described previously for platelets (*12*). Since the basal prothrombinase activity of RBC is extremely low (*13*), high cell counts were used in the assay. The conditions were as follows (final concentrations): 10^8 RBC/mL were incubated in Hepes buffer containing 0.5 mg/mL human serum albumin and 3 mM $CaCl_2$ with 3 nM factor Xa and 6 nM factor Va for 2 min at 37 °C. Subsequently, prothrombin was added at a concentration of 4 μM, and thrombin was formed for 2 min. Aliquots from this incubation mixture were analyzed for the amount of thrombin present using a thrombin-specific substrate, S2238.

This prothombinase assay was also used to measure Ca^{2+}-induced lipid scramblase activity, as described previously (*13*).

Knockout Mice. A double knockout for P-glycoprotein Mdr1a/1b-/- and a knockout for Mdr2 and their corresponding controls were in FVB background and were generously provided by A. Schinkel and J. Smit, respectively. The Mrp1 knockout and its corresponding control were in a 50% Ola 129 and 50% FVB background and were kindly donated by J. Wijnolds. All three knockouts have been previously described (*14*, *7*, *15*, respectively).

RESULTS

Outward Movement of C_6-NBD-Phospholipids in Human RBC and Resealed Ghosts. To measure outward movement of lipids, RBC were first incubated with C_6-NBD-PS or C_6-NBD-PC. While the C_6-NBD-PS is rapidly internalized by the aminophospholipid translocase, an overnight incubation was required to allow sufficient accumulation of C_6-NBD-PC in the cells' inner leaflet. To ensure that at the start of the floppase measurement all probe was localized in the inner leaflet, cells were back-exchanged with BSA. Flop of NBD-lipid was then measured by continuous BSA extraction as described under Materials and Methods. Figure 1A (closed symbols) confirms earlier findings showing that C_6-NBD-PS and C_6-NBD-PC were externalized with similar kinetics (*10*). When outward movement of C_6-NBD-lipids was examined in resealed ghosts, it was found that upon further dilution during the lysis step the amount of probe that became accessible to extraction by BSA gradually diminished (Figure 1A, open symbols). Resealed ghosts, obtained from cells that were lysed in a 10-fold volume of hypotonic buffer, almost completely lost floppase activity. To investigate whether floppase was abrogated because of shortage of ATP,

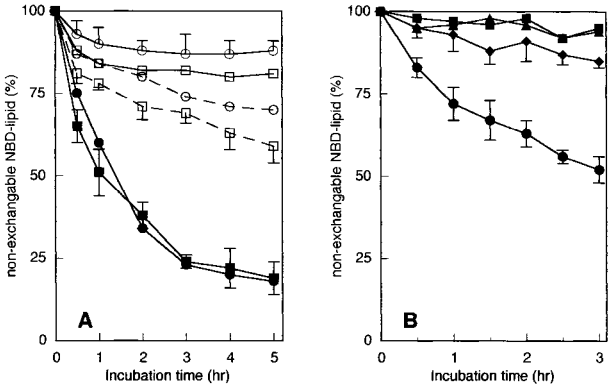

FIGURE 1: Outward movement of fluorescent lipid analogues in human RBC. (A) Outward transport of C_6-NBD-PS (circles) and C_6-NBD-PC (squares) in intact cells (closed symbols) and resealed ghosts obtained from cells lysed in a 3-fold (open symbols, dashed lines) and in a 10-fold volume (open symbols, solid lines) of lysis buffer. (B) Outward transport of C_6-NBD-PS in ghosts obtained from C_6-NBD-PS-labeled cells, lysed in a 10-fold volume of lysis buffer, and subsequently resealed in the absence (■) or presence of 10 mM Mg-ATP (◆), 5 mM GSSG (▲), or 10 mM Mg-ATP + 5 mM GSSG (●). Shown are the mean values of three experiments. All standard deviations are less than 12%. Error bars are only shown when they do not interfere with the clarity of the figure.

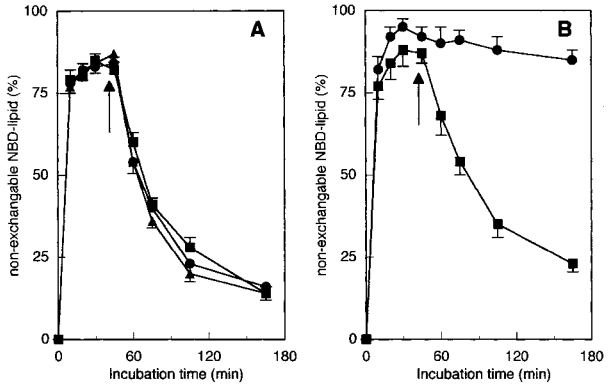

FIGURE 2: Transbilayer movement of C_6-NBD-PS in intact RBC from knockout mice. Washed RBC were incubated for 45 min with C_6-NBD-PS, and samples were analyzed for probe internalization by BSA back-exchange. After 45 min (arrow), BSA was added to the cell suspensions to start the continuous extraction, to prevent C_6-NBD-PS which is transported to the outer leaflet from being pumped back to the inner leaflet. Aliquots are taken at different time intervals, and residual fluorescence intensity remaining in the cells was analyzed as described under Materials and Methods. Panel A: RBC from Mdr1a/1b-/- double knockout (●), Mdr2-/- (▲) knockouts, and corresponding wild-type (■) mice. Panel B: RBC from Mrp1-/- (●) knockout and corresponding wild-type (■) mice. Shown are the mean values ± SD obtained from four experiments.

Table 1: Inhibition of Inside-to-Outside Movement (Flop) of C_6-NBD-PS in Human RBC[a]

inhibitor	IC_{50} (μM)
verapamil	5−7
vincristine	17−22
indomethacine	44−55

[a] Washed RBC were incubated with different concentrations of inhibitor for 60 min, followed by incubation with C_6-NBD-PS for 45 min. Flop of internalized C_6-NBD-PS was measured as described under Materials and Methods. Inhibition was calculated as a percentage of the amount of NBD-PS flop in absence of inhibitors and plotted as a function of the inhibitor concentration. From these titrations, IC_{50} values were determined. Titrations were performed in triplicate; data are the range of values found in these experiments.

the ghosts were resealed in the presence of 10 mM Mg-ATP. Although we could confirm that this amount of ATP was sufficient to sustain aminophospholipid translocase activity (*2*) (data not shown), floppase activity was not recovered (Figure 1B, triangles). Floppase activity in ghosts was found to be present, however, when Mg-ATP (10 mM) and oxidized glutathione (GSSG, 5 mM) were both included during the resealing step (Figure 1B, circles). Inclusion of GSSG without ATP did not reveal floppase activity (Figure 1B, diamonds). The outward movement of C_6-NBD-PC was also dependent on the simultaneous presence of ATP and GSSG (data not shown).

The finding that GSSG plus ATP stimulated NBD-lipid flop suggested that this activity might be related to that of the multidrug resistance protein 1 (MRP1), also known as the glutathione-conjugate transporter (GS-X transporter) (*16*−*18*). Therefore, a number of known inhibitors of MRP were tested for their effect on the outward movement of C_6-NBD-PS. As shown in Table 1, verapamil, vincristine, and indomethacine indeed inhibited the flop of C_6-NBD-PS in RBC with IC_{50} values ranging from 5 to 50 μM. Similar values were obtained for the effects of these inhibitors on C_6-NBD-PC outward transport (data not shown). Under the same conditions, no appreciable inhibition of aminophospholipid translocase activities was observed.

NBD-Lipid Movement in Murine RBC Deficient in Mdr1a and Mdr1b, Mdr2, or Mrp1. Since the above-mentioned inhibitors are not specific for extrusion of drugs mediated by the MDR P-glycoproteins or by MRP, we used RBC from different knockout mice in an attempt to identify the transporter for the outward movement of fluorescent lipid analogues: (i) Mdr1a/1b-/- is a double knockout mouse for *Mdr1a* (also called *Mdr3*) and *Mdr1b* (also called *Mdr1*) (nomenclature according to Smit et al., ref *19*). These two genes encode for the P-glycoproteins that are involved in multidrug resistance in mice and are known to translocate a wide variety of short-chain lipid analogues (*20*). These two proteins together fulfill the same functions as the single human MDR1 P-glycoprotein (*14*). (ii) Mrp1-/- is a knockout of a gene different from *Mdr*, but also encoding for a protein belonging to the ABC (ATP Binding Cassette) protein family involved in expelling of drugs; and (iii) a knockout of Mdr2 (MDR3 in human), known as the phosphatidylcholine transporter in the bile canalicular system (*7*), was used. All three mice strains have been previously described (*7*, *14*, *15*). Figure 2 shows the inward and outward transport of C_6-NBD-PS in RBC obtained from these knockout mice and from their corresponding wild types. While inward transport of C_6-NBD-PS seems unaffected in all three knockout mice, outward movement of this PS analogue was virtually absent only in the Mrp1-/- RBC. The other two knockouts exhibited outward transport kinetics indistinguishable from those of corresponding controls.

Flop of C_6-NBD-PC in RBC from knockout mice could not be measured, because murine RBC do not accumulate sufficient amounts of C_6-NBD-PC in the inner leaflet to allow reliable measurement of outward movement. This was found for RBC from wild type as well as Mdr2-/- and Mrp1-/- mice. Presumably, the rate of efflux of C_6-NBD-PC by Mdr2 in the Mrp1-/- mice and conversely by Mrp1 in the Mdr2-/-

Table 2: Prothrombinase Activities of Murine Erythrocytes[a]

mouse strain	nonstimulated RBC, 10^8/mL	ionophore-treated RBC, 10^7/mL
Mdr+/+	43 ± 4	508 ± 32
Mdr1a/1b-/-	40 ± 10	375 ± 55
Mdr2-/-	34 ± 13	445 ± 38
Mdr1+/+	53 ± 19	514 ± 43
Mrp1-/-	50 ± 14	422 ± 37

[a] Washed murine RBC were incubated with coagulation factors as described under Materials and Methods. Nonstimulated RBC were measured at a cell count of 10^8/mL. Incubation with ionomycin (5 μM) was carried out for 30 min in the presence of 1 mM Ca^{2+}. Prothrombinase activities of these ionophore-treated cells were measured at a cell count of 10^7/mL. Prothrombinase assays were performed in triplicate on three different blood samples of all mice strains. Results are presented as the mean value ± SD of all measurements and expressed as nanomolar thrombin formed per minute.

mice may be too high in comparison to the spontaneous inward movement, precluding sufficient incorporation in the inner leaflet.

Measurement of Prothrombinase Activity of RBC from MDR and MRP1 Knockout Mice. Figure 2B shows that the uptake of C_6-NBD-PS in the Mrp1-/- RBC seems to exceed that of the control, supporting the notion that net uptake is a result of the balance between aminophospholipid translocase and floppase activities. This phenomenon was repeatedly observed in the Mrp1-/- knockout RBC, and raised the question whether such an altered distribution of the C_6-NBD-PS is representative for the distribution of endogenous PS. To address this question, we have used the prothrombinase assay. The conversion of prothrombin to thrombin by the enzyme complex consisting of factors Xa and Va is strongly catalyzed by the presence of an anionic phospholipid surface. Hence, the prothrombinase assay is a very sensitive method to detect traces of PS in a membrane surface. We have investigated whether the knockout mice had an altered PS distribution of their RBC membranes. However, as shown in Table 2, no significant differences in the prothrombinase activities of these RBC could be discerned. In addition, murine RBC challenged with Ca^{2+}-ionophore exhibit rapid scrambling of phospholipids while no appreciable difference in rate (data not shown) and extent (Table 2) of PS exposure could be observed in erythrocytes from the three knockout mice and their corresponding controls, suggesting that scramblase activity is unrelated to the Mdr P-glycoproteins or Mrp1.

DISCUSSION

Mammalian cells possess an energy-dependent aminophospholipid translocase which ensures sequestration of PS and PE in the inner leaflet of the plasma membrane. A few studies have suggested the existence of an ATP-dependent floppase that facilitates outward movement of lipids (*8*, *10*). It has been proposed that the synchronous and cooperative action of the aminophospholipid translocase and floppase activities contributes to the generation and maintenance of membrane phospholipid asymmetry (*10*). While aminophospholipid translocase has been suggested to involve a 110 kDa Mg-ATPase (*21*, *22*), the identity of the transporter responsible for outward lipid movement is less clear. It was conceivable, in fact, that floppase activity was an intrinsic property of the aminophospholipid translocase (*11*). In this study, we demonstrate that inward and outward movement of short-chain fluorescent lipid analogues is mediated by distinct proteins, because (i) flop of NBD-lipids requires the simultaneous presence of ATP and GSSG, whereas translocase only requires ATP (*2*), (ii) flop of NBD-lipids is inhibited by drugs interfering with multidrug resistance proteins, without inhibiting translocase, and (iii) virtually complete absence of flop of C_6-NBD-PS was found in RBC from Mrp1 knockout mice, while translocase activity in these cells was fully active. This finding proves that outward transport of NBD-lipids in RBC is catalyzed by the multidrug resitance protein MRP1, which is in line with recent observations showing outward transport of NBD-sphingomyelin and NBD-glucosylceramide in LLC-PK1 cells transfected with human MRP1 (R. Raggers, A. van Helvoort, R. Evers, and G. van Meer, personal communication).

Several studies have implicated that MRP1 is a so-called GS-X pump, that transports drugs as glutathione conjugates (*16*−*18*) and is present in many, if not all, mammalian cells, including erythrocytes (*23*). Indeed, export of drugs from cells by MRP1 was demonstrated to specifically require glutathione, in contrast to expelling of drugs by the P-glycoprotein MDR1, although it has been suggested that MRP1 can also transport compounds such as daunorubicin in unmodified form (*17*). We have found no evidence suggesting that the NBD-lipid probes might be transported as glutathione conjugates; all cell-associated fluorescence, as well as the fluorescence of the supernatants after 6 h incubation of human RBC in BSA, was from unmodified NBD-lipids as judged by TLC analysis (*10*). Moreover, flop of NBD-lipids in resealed ghosts required the presence of oxidized glutathione and not its reduced form, thus making conjugation of the lipid probe unlikely. Like P-glycoproteins, MRP is a tandemly duplicated ABC transporter, that may contain two or more drug binding sites with different specificities: one for conjugated drugs and another for unconjugated drugs (*24*). Our present findings are in line with this model: GSSG, being a glutathione-*S*-conjugate of glutathione and a known substrate for MRP1 (*25*), stimulates export of the unconjugated NBD-lipid.

MDR1 P-glycoprotein can transport a variety of lipids, including the C_6-NBD-analogues (*20*, *26*, *27*). As the Mrp1-/- RBC show no appreciable C_6-NBD-PS flop, this would imply either a low number or a low activity of Mdr1a/1b P-glycoproteins in murine RBC. Indeed, low levels of expression of Mdr1a and Mdr1b were found in murine RBC (A. J. Smith, personal communication). A similar conclusion may be drawn for human RBC based on the observation that efflux of the fluorescent lipid analogues is dependent on GSSG, a compound not required for transport by P-glycoproteins.

The finding that MRP1 is responsible for outward transport of C_6-NBD-PS and C_6-NBD-PC raises the question whether these modified lipids are merely considered as xenobiotic compounds that need to be rapidly expelled, or are reliable reporters of the endogenous phospholipids. The second possibility would implicate that drugs are expelled by MRP in symport with endogenous phospholipids including PS, which is then rapidly pumped back to the inner leaflet by the aminophospholipid translocase. Although we observed a more extreme accumulation of C_6-NBD-PS in the inner leaflet of RBC from Mrp1 knockout mice (cf. Figure 2), the

assessment of PS exposure by the prothrombinase assay did not lend support to the possibility that Mrp1 affects endogenous PS distributions. The present findings suggest that caution should be exercised in extrapolating data obtained with lipid analogues used to study transbilayer migration of lipids, as they may not under all circumstances reflect transbilayer movement of endogenous lipids. In any event, aminophospholipid translocase activities measured with aminophospholipid probes are likely underestimated in cells containing an active MRP1. Conversely, experiments designed to measure MDR using NBD-lipid probes are subject to the influence of the activity of transporters for endogenous lipids that also recognize these probes.

ACKNOWLEDGMENT

We acknowledge Drs. G. van Meer (Academic Medical Center, Amsterdam) and A. van Helvoort (The Netherlands Cancer Institute, Amsterdam) for helpful discussion. We thank Drs. J. Wijnolds, A. H. Schinkel, J. Smit, and P. Borst from The Netherlands Cancer Institute (Amsterdam) for making available the different knockout mice for this study. Finally, we thank Dr. A. van Helvoort for critically reading the manuscript.

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BI981011W