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Fluorescent modified phosphatidylcholine floppase activity of reconstituted multidrug resistance-associated protein MRP1

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Abstract

Multidrug resistance-associated protein (MRP1) may function as a floppase in human red blood cells to translocate phosphatidylserine and/or phosphatidylcholine from inner membrane leaflet to outer leaflet. Here we report that the purified and reconstituted MRP1 protein into asolectin proteoliposomes is mainly in an inside–out configuration and possesses the ability to flop a fluorescent labeled *phosphatidylcholine* (NBD-PC) from outer leaflet (protoplasmic) to inner leaflet (extrocytoplasmic). The reconstituted MRP1 protein retains endogenous ATPase activity. ATP hydrolysis is required for the flopping since removal of ATP and/or Mg^{2+} inhibits the translocation of NBD-PC. Further evidence to support this conclusion is that the translocation of NBD-PC is inhibited by vanadate, which traps ATP hydrolysis product ADP in the nucleotide binding domains. In addition, the translocation of NBD-PC by proteoliposomes containing MRP1 protein is in a glutathione-dependent manner, similar to the process of translocating anticancer drugs such as daunorubicin. verapamil, vincristine, vinblastine, doxorubicin and oxidized glutathione partially inhibited the translocation of NBD-PC, whereas MK 571, an inhibitor of MRP1 protein, inhibited the translocation almost completely. Taken together, the purified and reconstituted MRP1 protein possesses the ability to flop NBD-PC from outer to inner leaflet of the proteoliposomes.

Keywords: MRP1; Floppase; NBD-PC

1. Introduction

Overexpression of P-glycoprotein (Pgp) and/or multidrug resistance-associated protein (MRP1) in cancer cells confers multidrug resistance [1–4]. Pgp is a 170 kDa membrane-bound glycoprotein with two transmembrane domains (TMD) and two nucleotide binding domains (NBD) [5–8]. MRP1 is a 190 kDa membrane-bound glycoprotein with similar core structure as Pgp, but with an extra TMD and a linker region at the N-terminus [4,9–11]. Therefore, both of them are members of the ATP binding cassette (ABC) superfamily of transport systems with two typical NBDs coupling ATP binding/hydrolysis to drug transport. Although the drug resistance profiles exerted by these two proteins are somewhat similar, they pump solutes out of cells in different fashions, i.e., Pgp transports hydrophobic compounds out of the cells directly [12–14], whereas MRP1 transports anionic conjugates, such as glutathione, glucuronate, or sulfate-conjugated aliphatic, prostanoid or heterocyclic compounds [15–23]. Common feature is that both of them actively transport a wide range of compounds with different structures and functions. One question has been asked is whether they translocate the components of the membrane bilayer where they reside. A bacterial ABC transporter, MsbA, a homolog of mammalian Pgp, had been proposed to transport nascent core-lipid A molecule across the inner membrane [24] and is responsible for the translocation of lipid A to the outer membrane [25]. Yeast ABC transporters, Pdr5p and Yor1p, are responsible for the translocation of a fluorescent-labeled phosphatidylethanolamine (PE) analogue, C6-NBD-PE (C6-2-(12-(7-nitrobenz-2-oxa-(1,3-diazol-4-yl)amino)dodecanoyl-1-hexade-canoyl-*sn*-glycero-3-phosphatidylethanolamine), since cells lacking the genes coding for these proteins showed increased levels of accumulation of the fluorescent derivatives [26]. Mouse Mdr1, human MDR1 and MDR3 can translocate short

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chain phosphatidylcholine (PC) [27]. Mdr2 Pgp in the mouse canalicular membrane plays an important role in the excretion of phospholipid into bile [28,29] since there is virtually no phospholipid secretion into canaliculus in Mdr2 knocked out mice [30]. Mouse Mdr2 expression in yeast secretory vesicles caused translocation of a fluorescent-labeled PC from outer to inner leaflet of the membrane [31]. All these results indicate that Pgp, no matter whether it comes from bacteria, yeast, mouse or human, can translocate the components of membrane bilayer where they reside. Direct evidence comes from the Pgp reconstituted into proteoliposomes in which it translocates a wide variety of NBD-lipids from the outer to the inner leaflet of the bilayer [32]. Whether MRP1 protein has a similar function as Pgp to translocate the components of the membrane bilayer where it resides has also been proved. MRP1 protein seems likely to translocate the fluorescent-labeled PC, phosphatidylserine, sphingomyelin and glucosylceramide from inner to outer leaflet across the plasma membrane of eukaryotic cells [33–36]. However, since those experiments were performed in intact cells, it was uncertain whether MRP1 alone or along with other factors had the ability to translocate the phospholipids. To test this, we have purified MRP1 protein, reconstituted it into proteoliposomes and investigated the translocation of a fluorescent-labeled PC analogue, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine (NBD-PC) by using the protocols established [32,37]. Our results indicated that the reconstituted MRP1 protein in proteoliposomes translocated NBD-PC from the outer leaflet to inner leaflet of the membrane bilayer in an ATP-, Mg^{2+}- and glutathione-dependent manner. Therefore, MRP1 alone appears to be able to translocate NBD-PC from one leaflet of the bilayer to the other.

2. Materials and methods

2.1. Materials

NBD-PC was purchased from Avanti Polar Lipids (Alabaster, AL). Asolectin, sodium dithionite, ATP, CHAPS, Triton X-100, vinblastine, vincristine, doxorubicin, daunorubicin and verapamil were purchased from Sigma Chemical Co. 3-([{3-(2-[7-chloro-2-quinolinyl] thenyl) phenyl}-{(3-dimethylamino-3-oxopropyl) thio}-methyl] thio) propanoic acid (MK-571) was provided generously by Dr. A.W. Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada). The other reagents were analytical grade.

2.2. Purification of MRP1 protein

A baby hamster kidney cell line stably expressing human MRP1/His was utilized for the purification of MRP1 protein [38]. Cells expressing MRP1/His were cultured at 37 °C in 5% CO_2 in the presence of 100 μM methotrexate. Membrane vesicles were prepared according to the published procedures [38]. Membrane vesicles were solubilized in a buffer A (20 mM Tris–HCl, pH 7.9, and 500 mM NaCl) containing 5 mM imidazole, 10 mM CHAPS and 20% (v/v) glycerol. The solubilized sample was sonicated and insoluble material was removed by centrifugation at 10,000 × *g* for 15 min [38]. The supernatant was applied on to a His.Bind Resin column, which was pre-equilibrated in buffer A, followed by three washes with six column volumes of buffers: first wash with buffer A containing 5 mM CHAPS and 25 mM imidazole; second wash with buffer A containing 5 mM CHAPS, 10% glycerol, and 40 mM imidazole; and the third wash with buffer containing 20 mM Tris–HCl (pH 7.4), 500 mM NaCl, 5 mM CHAPS and 40 mM imidazole. The protein was eluted with two column volumes of buffer containing 20 mM Tris–HCl (pH 7.4), 5 mM CHAPS, 0.05% β-mercaptoethanol and 300 mM imidazole. The eluate was dialyzed extensively against 50 volumes of buffer containing 5 mM Tris–HCl (pH 7.4), 5 mM CHAPS, 500 mM NH_4Cl and 0.05% β-mercaptoethanol in a cold room. The purified MRP1 protein was stored at −80 °C after being ultrafiltrated.

2.3. Reconstitution of MRP1 protein

Purified MRP1 protein was reconstituted into proteoliposomes as described previously [32,39]. Briefly, 5 mg of asolectin and 15 μg of NBD-labeled PC (dissolved in a 4:1 (v/v) $CHCl_3$/MeOH mixture) were dispensed into a glass tube, dried under a nitrogen stream and further dried under vacuum for 2 h to remove the remaining trace of organic solvent. The dried lipids were then resuspended at a concentration of 20 mg/ml in the reconstitution buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol (DTT) and 1 mM $MgCl_2$. The lipid suspension was sonicated for 45 min in a bath-type sonicator at room temperature and then stored in aliquots at −80 °C. The purified MRP1 protein was reconstituted as following: 200 μl of the stored lipid aliquot (4 mg asolectin) and 100 μl 30% (w/v) CHAPS were mixed in an eppendorf tube and then 500 μg of the purified MRP1 protein in 700 μl of the reconstitution buffer containing 15 mM CHAPS was added to the lipid/detergent solution. After periodic mixing on ice for 1 h, CHAPS was removed by passing the mixture through a reconstitution buffer pre-equilibrated Sepharose CL-4B column (1 × 15 cm). Turbid fractions were collected and the reconstituted MRP1 proteoliposomes were harvested by centrifugation at 145,000 × *g* for 1 h at 4 °C. The proteoliposomes were resuspended in 200 μl of transport buffer (50 mM Tris–HCl, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$) for measurement of NBD-PC translocation. The final lipid/protein ratio of the reconstituted proteoliposomes should be in the range of 10:1 to 15:1 (w/w). Proteoliposomes were homogenized by passing through a

27.5-gauge needle five times and then stored in aliquots at − 80 °C.

2.4. Electron microscopy

To observe proteoliposomes by negative staining, 5 μl of the proteoliposomes (0.5 mg lipid/ml) with or without NBD-PC were adsorbed onto a carbon-coated copper grid. After 1 min, the droplet was removed with filter paper and for negative staining, 5 μl of 2% uranyl acetate, pH 4.5, applied to the grid. After 50 s, the droplet was adsorbed with filter paper. The grid was allowed to air dry and was examined in a PHILIPS TECNAI 20 electron microscope.

2.5. Measurement of ATPase activity

The ATPase activity of MRP1 was determined as described previously [40] by using a colorimetric method to measure the release of inorganic phosphate from ATP. Typically, 0.5–1.5 μg of the purified and reconstituted MRP1 protein was incubated at 37 °C in 0.1 ml assay buffer containing 50 mM Tris–HCl, pH 7.4, 2 mM ATP, 5 mM $MgCl_2$ for 4 h. The asymmetry of MRP1 incorporation was tested by measuring the ATPase activity difference before and after permeabilization with increasing concentrations of CHAPS, as described previously [32,40,41].

2.6. Fluorescence quenching assay

Experimental protocols [31,32,37] employed for measuring the phospholipid translocation were adopted. Briefly, samples were prepared by adding 20 μl of the proteoliposomes (50 μg/ml MRP1) to 430 μl of transport buffer (10 mM Tris–HCl, 250 mM sucrose, and 5 mM $MgCl_2$ (pH 7.4)) in 1.5 ml microfuge tubes. To initiate lipid transport, 50 μl of transport buffer containing ATP and ATP regeneration system [32] was added to the proteoliposome samples (final concentration was 1 mM ATP, 30 μg/ml creatine kinase and 3 mM creatine phosphate) and the reaction mixtures were incubated at 37 °C for the desired length of time. The sample was then transferred to a 1 ml quartz cuvette and the fluorescence emission was recorded at 22 °C at F-4010 fluorescence spectrophotometer, using excitation and emission wavelengths of 468 and 540 nm, respectively. After a stable baseline was established, 5 μl of sodium dithionite solution (final concentration of dithionite was 10 mM) in 1 M Tris–HCl (pH 10.0) was added to quench the NBD-PC in the outer leaflet of the proteoliposomes. When a new stable line was established, the proteoliposomes were permeabilized by adding 50 μl of 10% Triton X-100 (final concentration was 1%). The fluorescence quenching was followed until another new stable line was established. For control experiments, 50 μl of transport buffer, instead of ATP, was added.

The percentages of NBD-PC quenched by dithionite (outer leaflet) or protected from dithionite before the Triton X-100 treatment (inner leaflet) in the proteoliposomes were calculated by using the following equations.

$$\% \text{ Outer leaflet} = [(F_T - F_D)/(F_T - F_0)] \times 100$$

$$\% \text{ Inner leaflet} = [(F_D - F_0)/(F_T - F_0)] \times 100$$

Where F_T is the total fluorescence of the sample before addition of dithionite; F_D is the fluorescence of the sample following quenching with dithionite; and F_0 is the fluorescence of the sample following permeabilization with Triton X-100. F_T, F_D and F_0 values were derived from the average of 50 points within the respective stable lines.

2.7. Transport of daunorubicin by the reconstituted MRP1

In order to examine the relationship between the NBD-PC floppase activity and drug transport activity of the reconstituted MRP1, daunorubicin uptake into the proteoliposomes was determined. The experiments were performed as follows: 1 μg of the reconstituted MRP1 was mixed with daunorubicin (final concentration of 1 μM) and ATP (final concentration of 1 mM) plus an ATP regenerating system in a 500 μl of transport buffer mentioned above. After incubation at 22 °C for 40 min, 1 ml of ice-cold transport buffer was added to stop the reaction. The proteoliposomes were collected by ultracentrifugation at 145,000 × *g* for 20 min at 4 °C. After removal of the supernatant, the proteoliposomes were resuspended in 500 μl transport buffer and 0.1% Triton X-100 (final concentration) was added to release the daunorubicin entrapped in the proteoliposomes. The fluorescence intensities of the samples were measured immediately by using an F-4010 fluorescence spectrophotometer with an excitation and an emission wavelength of 490 and 590 nm at 22 °C.

3. Results

3.1. Purification and reconstitution of MRP1

MRP1 protein was purified from BHK cells stably transfected with pNUT/MRP1/His [38] by using His.Bind Resin column. The purified and reconstituted MRP1 protein was subjected to SDS-PAGE (7.5% polyacrylamide gel) and detected by silver staining (Fig. 1, lanes 2 and 3) or Western blot analysis with MRPr1 antibody (Fig. 1, lanes 4 and 5). Greater than 90% of the purified proteins were MRP1 protein as judged by densitometry of a silver-stained SDS-PAGE and approximately 30% of MRP1 protein was recovered from the reconstitution. The results in lanes 4 and 5 proved that the silver-stained proteins in lanes 2 and 3 were indeed MRP1 protein detected by MRP1-specific antibody MRPr1.

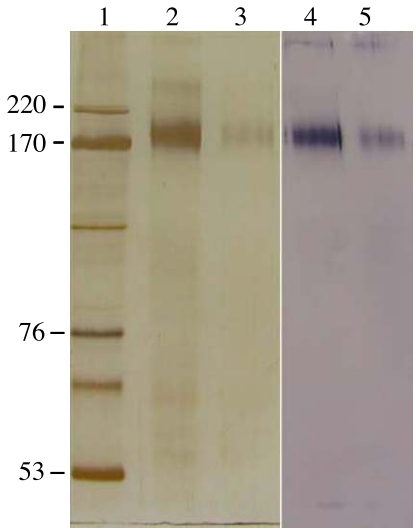


Fig. 1. Analysis of the purified and reconstituted MRP1 protein. MRP1 proteins in lanes 2 and 3 were detected by silver staining: lane 1, marker; lane 2, 1 μg of the purified MRP1 protein; lane 3, 0.2 μg of the reconstituted MRP1 protein in the proteoliposomes. Lanes 4 and 5, the purified and reconstituted MRP1 respectively, were detected by Western blot analysis using MRPr1 antibody.

3.2. Majority of MRP1 protein reconstituted into the proteoliposomes is in an inside–out configuration

Since the sealed lipid bilayer vesicle systems are impermeable to ATP [32,40–42], only the inside–out oriented MRP1 protein is expected to interact with ATP. Cryptic ATPase activity due to inside–in configuration of the Pgp (with ATP binding domains in the vesicle lumen) should be exposed to ATP only after detergent permeabilization. Accordingly, addition of increasing concentrations of the permeabilizing detergent CHAPS to proteoliposomes containing MRP1 protein resulted in a slight increase (5.6%) in ATPase activity (Fig. 2), implying that approximately 6% of the NBDs was prevented from contacting ATP. However, the treatment with the detergent CHAPS leads to the inactivation of the protein (Fig. 2), 6% increase of ATPase activity after treatment with CHAPS might be underestimated. When the activity curve for different CHAPS concentrations (Fig. 2) was extrapolated from higher to lower CHAPS concentrations, a higher percentage (than 6%) of inside–in oriented protein should be suggested. In any case, one can make a conclusion that majority of the reconstituted MRP1 protein in the proteoliposomes is in an inside–out configuration, which is consistent with previous reports [42].

3.3. Estimation of the NBD-PC distribution in outer and inner leaflets

The fluorescence quenching technique we used relies on the ability of dithionite to quench the fluorescence of NBD-labeled PC located only in the outer membrane leaflet, due to the membrane bilayer impermeability [31,32,43]. Initial experiments were carried out to optimize the dithionite concentration for efficient quenching of the fluorescence of NBD-PC in the outer leaflet without affecting the NBD-PC located in the inner leaflet. A stable baseline could not be established following addition of 20 mM dithionite, suggesting that dithionite at that concentration might disturb the membrane structure and penetrate the bilayer (data not shown). Stable baselines can be established by using either 2 or 10 mM dithionite. However, when the vesicle samples were analyzed in the presence of 2 mM dithionite and 1% Triton X-100, the fluorescence of the samples was not quenched completely (data not shown), indicating that 2 mM dithionite was not sufficient to quench the fluorescence of NBD-PC in the proteoliposomes. When the samples were treated with 10 mM dithionite and 1% Triton X-100, the fluorescence was quenched completely (to the background level), therefore, 10 mM dithionite was utilized in the following experiments.

The dithionite reduction technique depends on the *structural* integrity of the membrane system under study. To observe if the fluorescent NBD-PC lipids affects the integrity of the proteoliposomes, we analyzed and compared the structural integrity of MRP1 proteoliposomes with or without NBD-PC by electron microscope. The results indicated that both of the proteoliposomes had the same diameters in the range of 80–90 nm (Fig. 3). Furthermore, Fig. 4 shows that stable lines can be established within 10 min after dithionite treatment in the absence of Triton X-100, whereas this stable line cannot be established in the presence of 0.01% Triton X-100, implying that the NBD-PC located in the outer membrane leaflet was quenched completely within 10 min and dithionite cannot penetrate the membrane

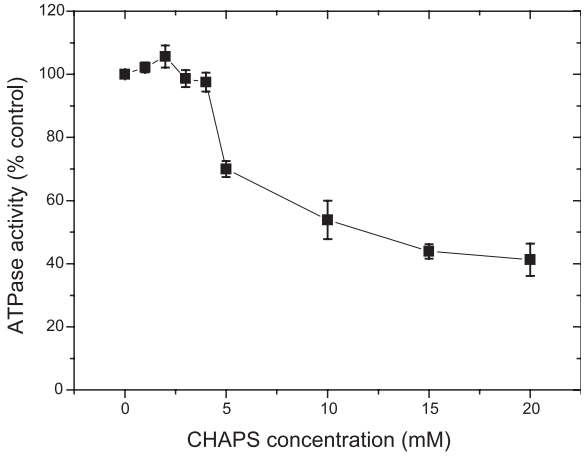


Fig. 2. MRP1 protein orientation in the proteoliposomes analyzed by ATPase activity before and after CHAPS treatment. ATPase assay was performed according to the method described in Materials and methods. Proteoliposomes were treated with increased concentrations of CHAPS indicated in the figure and ATPase activities were determined. Data (means ± S.E., $n = 3$) are expressed as the percentage of the control ATPase activity measured in the absence of CHAPS (100%).

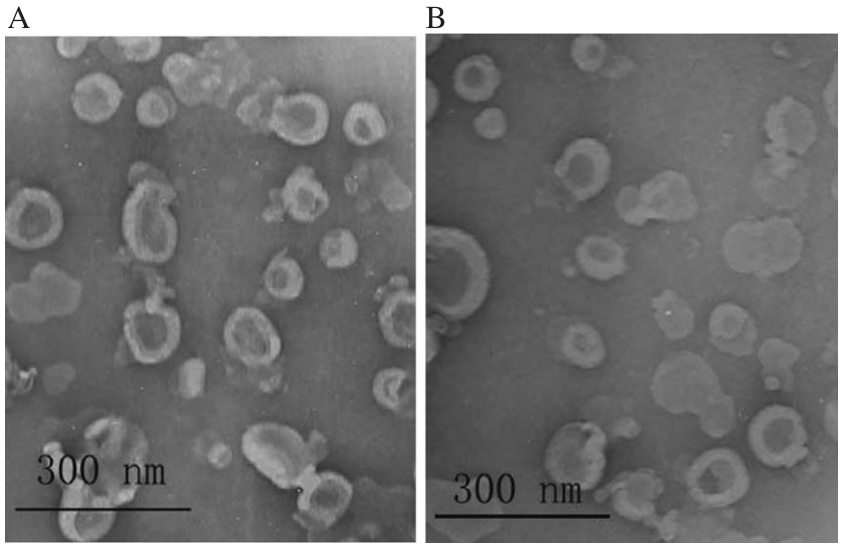


Fig. 3. Electron micrographs of negatively stained proteoliposomes. The protocols were described in Materials and methods. (A) MRP1 proteoliposomes without NBD-PC. (B) MRP1 proteoliposomes with NBD-PC. Bars = 300 nm.

bilayer under this condition. Fluorescence (62%) was quenched (calculated using the equations indicated in Materials and methods) under the condition without ATP, $MgCl_2$ and GSH, implying that 62% of the NBD-PC was localized in the outer leaflet and the other 38% in the inner leaflet in the control sample. However, there was a clear decrease in the amount of NBD-PC in the outer leaflet in the presence of ATP, $MgCl_2$ and GSH (Fig. 4), presumably the NBD-PC located in the outer leaflet was translocated to the inner leaflet by MRP1 in an ATP-dependent manner.

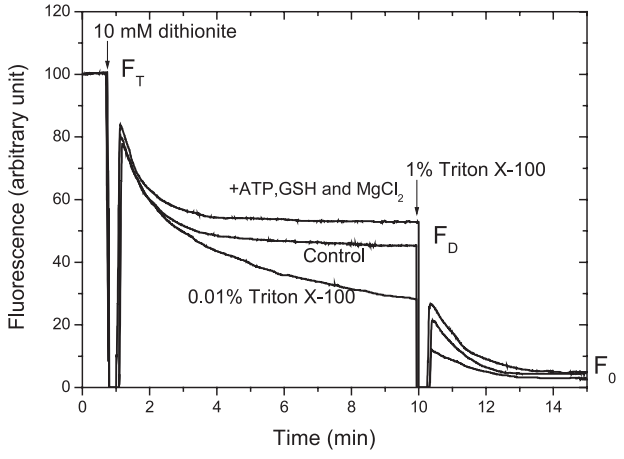


Fig. 4. NBD-PC located in the outer membrane leaflet is quenched completely within 10 min. Proteoliposomes containing MRP1 (1 μg in 500 μl transport buffer) and NBD-PC (20 ng) were incubated at 37 °C in the transport buffer with 1 mM ATP and ATP regenerating system for 40 min. The sample was then transferred to a cuvette and the fluorescence was measured over time at 22 °C, according to the procedures described in Materials and methods. After the establishment of the initial stable baseline (F_T), 10 mM dithionite (final concentration) was added to the reaction mixture. The samples were incubated at 22 °C until a new stable line was established, then Triton X-100 (final concentration of 1%, w/v) was added to make the vesicles permeable to dithionite. A stable line was established after 6–10 min incubation in the absence of detergent, whereas this stable line cannot be established if 0.01% Triton X-100 was added to the reaction mixture, indicating that NBD-PC located in the outer membrane leaflet was quenched completely within 10 min in the absence of Triton X-100. The fluorescence intensity of the baseline (F_T) was taken as 100%. F_D is the fluorescence intensity of the sample following quenching with dithionite. F_0 is the fluorescence intensity of the sample following the treatment with detergent Triton X-100.

Fig. 5 shows that the re-distribution of NBD-PC to establish equilibrium between the outer and inner leaflets by MRP1 in the presence of ATP, $MgCl_2$ and GSH takes approximately 40–50 min. The maximum amount of NBD-PC transported from outer to inner leaflet by MRP1 is approximately 9% (subtracting the amount translocated without ATP and GSH), which is consistent with the results

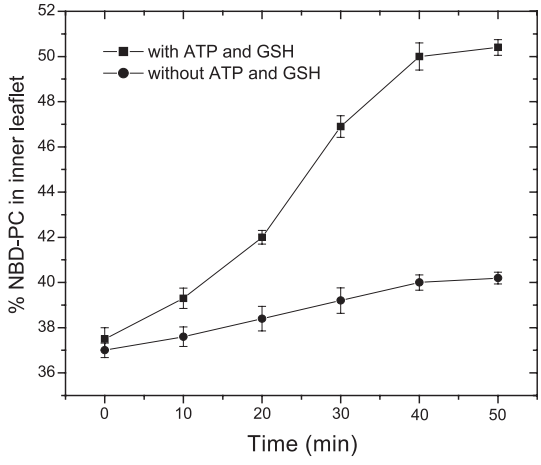
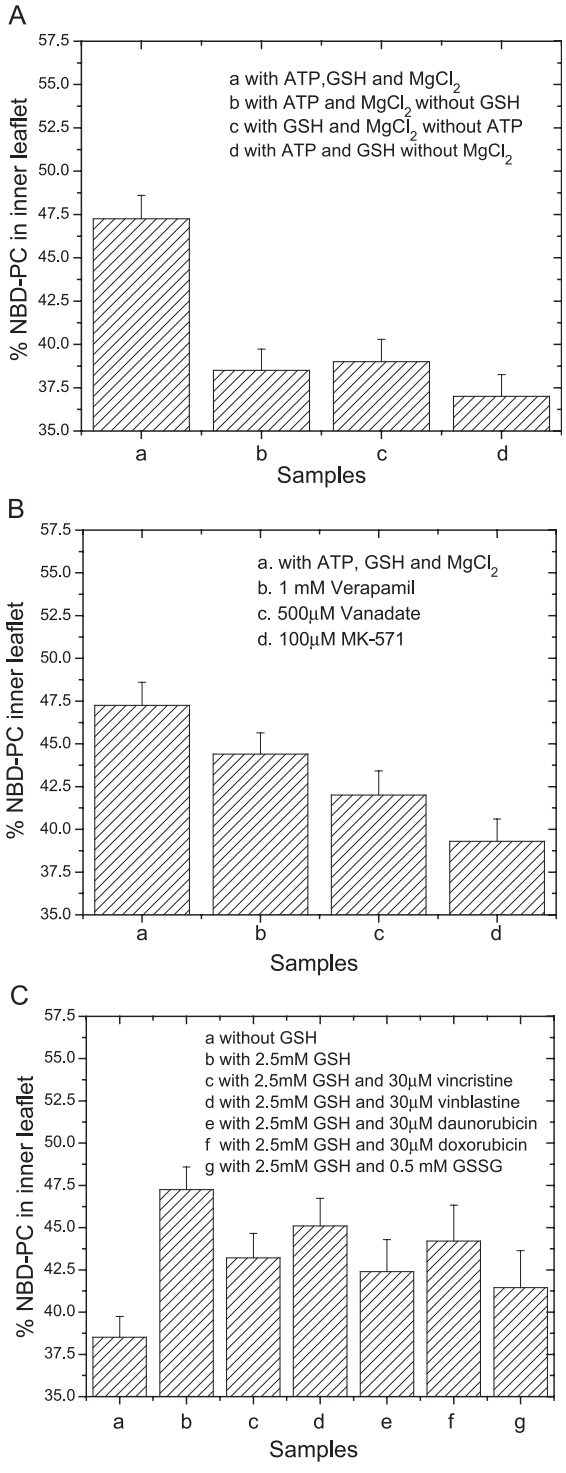


Fig. 5. Time course of translocation for NBD-PC in proteoliposomes containing MRP1. The proteoliposomes were incubated for various times with 5 mM $MgCl_2$ and 1 mM ATP (and a regenerating system) as well as 2.5 mM GSH, or without ATP and GSH, followed by quenching with dithionite to determine the transbilayer distribution of the NBD-PC. Data points represent mean values (± S.E.) of three independent determinations.

derived from Fig. 4. Since it takes approximately 40 min to establish the equilibrium between the outer and inner leaflets, the amounts of NBD-PC translocated within 40 min, for example, 10 min incubation, may be over-estimated due to the NBD-PC in the outer leaflet cannot be completely quenched immediately and might be continuously transported to the inner leaflet during the quenching. However, after 40 min incubation, the distribution of NBD-PC reached equilibrium, and this time, incubation is used in the following experiments.



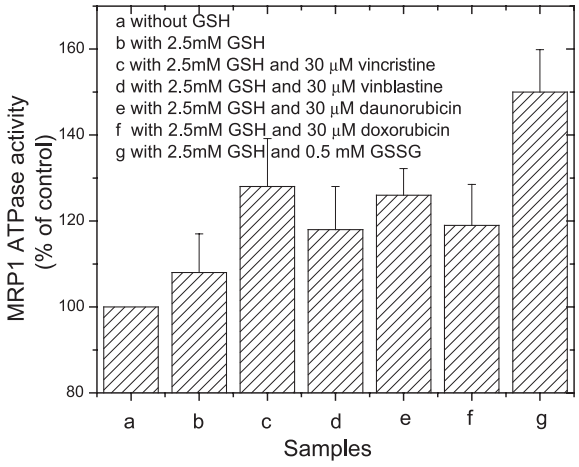


Fig. 7. Effects of the drugs on the reconstituted MRP1 ATPase activity. The ATPase activity of MRP1 in proteoliposomes was assayed in a reaction mixture containing 50 mM Tris–HCl, pH 7.4, 2 mM ATP, 5 mM $MgCl_2$ and various drugs, by measuring the release of inorganic phosphate from ATP, using a colorimetric method adopted from Ref. [39]. The amount of ATP hydrolyzed without GSH was considered as 100%.

3.4. Translocation of NBD-PC by MRP1 is ATP-, Mg^{2+}- and GSH-dependent

Fig. 4 shows that 38% of NBD-PC was located in the inner leaflet of the bilayer in the absence of ATP, $MgCl_2$ and GSH, however, the amount of NBD-PC in the inner leaflet was significantly increased in the presence of these compounds. We were curious whether the translocation of NBD-PC by MRP1 required all three compounds or only one of them. Fig. 6A(a) shows that the amount of NBD-PC in the inner leaflet of the bilayer was increased from 38% to 47% in the presence of all three compounds, whereas the translocation of NBD-PC in the absence of either GSH (Fig. 6A(b)), ATP (Fig. 6A(c)) or $MgCl_2$ (Fig. 6A(d)) was inhibited completely. In addition, 86% of the translocation was inhibited by 100 μM MK571 (Fig. 6B(d)), a potent MRP1 inhibitor [44]; 56% by 500 μM vanadate (Fig. 6B(c)), a non-specific ATPase inhibitor which traps the ATP hydrolysis product ADP at the nucleotide binding site;

Fig. 6. The effects of ligands, substrates and inhibitors on the translocation of NBD-PC. Transbilayer distribution of NBD-PC in the reconstituted MRP1 proteoliposomes was determined by fluorescence quenching with dithionite under the conditions indicated in the figure. Experiments were performed in triplicate and the data represent the mean values (± S.E.). A(a), 1 mM ATP (and a regenerating system), 2.5 mM GSH and 5 mM $MgCl_2$; (b), 1 mM ATP (and a regenerating system) and 5 mM $MgCl_2$, without 2.5 mM GSH; (c), 2.5 mM GSH and 5 mM $MgCl_2$, without ATP; (d), 1 mM ATP and 2.5 mM GSH, without $MgCl_2$. B(a), 1 mM ATP (and a regenerating system), 2.5 mM GSH and 5 mM $MgCl_2$; as well as 1 mM verapamil (b), 500 μM vanadate (c), or 100 μM MK571 (d). C, 1 mM ATP (and an ATP regenerating system), 5 mM $MgCl_2$, and 2.5 mM GSH (b) as well as the indicated compounds (a, c, d, e, f, g).

and 29% by 1 mM verapamil (Fig. 6B(b)), a compound that inhibited LTC4 transport by MRP1 [45]. To test whether other substrates, such as anticancer drugs, should also inhibit the translocation of the NBD-PC, the experiments were performed in the presence of ATP, $MgCl_2$, GSH and individual substrate. Fig. 6C shows that the translocation of NBD-PC in the presence of 30 μM vincristine (Fig. 6C(c)), 30 μM vinblastine (Fig. 6C(d)), 30 μM daunorubicin (Fig. 6C(e)), 30 μM doxorubicin (Fig. 6C(f)) and 500 μM oxidized glutathione (GSSG) (Fig. 6C(g)) was inhibited 45%, 24%, 54%, 34% and 64%, respectively. Whether those inhibitions are competitive or non-competitive are not known yet. However, when the MRP1 ATPase activities were analyzed under the same conditions in the presence of 30 μM vincristine (c), 30 μM vinblastine (d), 30 μM daunorubicin (e), 30 μM doxorubicin (f) and 500 μM GSSG (g), the ATPase activities were increased 28%, 18%, 26%, 19% and 50%, respectively (Fig. 7). The trend of translocation inhibition by these compounds is consistent with the trend of ATPase activity stimulation, implying that one MRP1 molecule cannot translocate an anticancer drug molecule and an NBD-PC lipid at the same time.

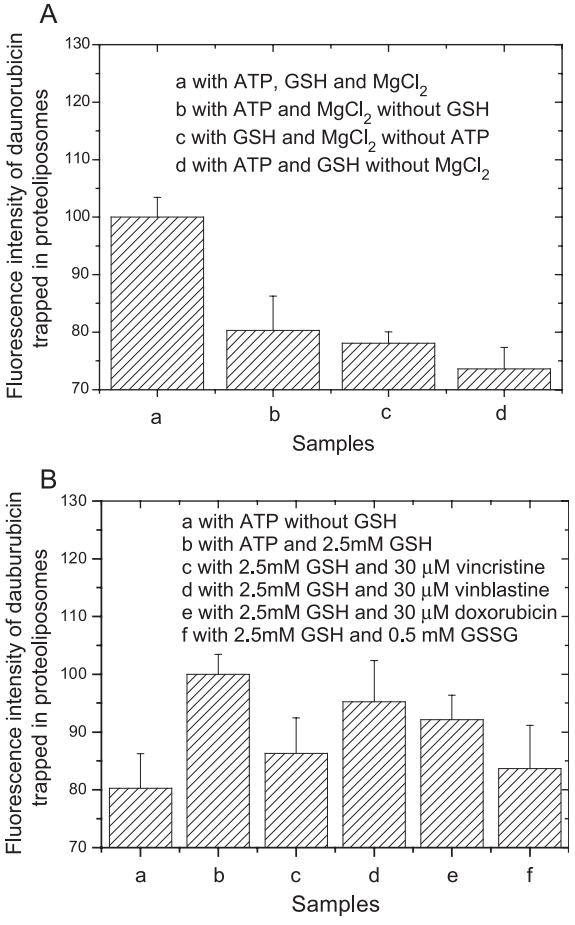


Fig. 8. Daunorubicin transported by the reconstituted MRP1 proteoliposomes. The experiments were performed according to the protocol described in Materials and methods. Data represent the mean values (± S.E.) of triplicate determinations and the amount of daunorubicin transported in the presence of ATP and GSH was considered as 100%. A(a), 1 mM ATP with an ATP regenerating system, 2.5 mM GSH and 5 mM $MgCl_2$; (b), 1 mM ATP with an ATP regenerating system and 5 mM $MgCl_2$, without GSH; (c), 2.5 mM GSH and 5 mM $MgCl_2$, without ATP; (d), 1 mM ATP with an ATP regenerating system and 2.5 mM GSH, without $MgCl_2$. B, 1 mM ATP with an ATP regenerating system and 5 mM $MgCl_2$, as well as the compounds indicated in the figure (a, c, d, e, f, g).

3.5. Translocation of daunorubicin by MRP1 is facilitated by ATP, Mg^{2+} and GSH

To test whether the translocation of an anticancer drug by the reconstituted MRP1 in the proteoliposomes is also ATP-, Mg^{2+}- and GSH-dependent, the experiments were performed in the presence or absence of either ATP, Mg^{2+} or GSH. The fluorescence intensity of daunorubicin transported by the reconstituted MRP1 in the presence of ATP (with an ATP regenerating system), 2.5 mM GSH and 5 mM $MgCl_2$ was taken as 100% (Fig. 8A(a)). The fluorescence intensities of daunorubicin transported into the proteoliposomes in the absence of GSH, ATP or $MgCl_2$ decreased to 80% (Fig. 8A(b)), 78% (Fig. 8A(c)) and 74% (Fig. 8A(d)), respectively. This 74–80% translocation in the absence of either GSH, ATP or $MgCl_2$ might reflect the hydrophobic compound daunorubicin inserted into the proteoliposomes. Therefore, the insertion of daunorubicin into the MRP1-reconstituted proteoliposomes is facilitated by the presence of ATP, Mg^{2+} and GSH. This ATP-dependent daunorubicin transport can be partially inhibited by other MRP1 substrates, such as vincristine (Fig. 8B(c)), vinblastine (Fig. 8B(d)), doxorubicin (Fig. 8B(e)) or GSSG (Fig. 8B(f)).

4. Discussion

MRP1 protein is ubiquitously expressed in normal tissues [46]. What is the function of the MRP1 protein in those tissues? MRP1 protein has been found to transport anionic conjugates, including glutathione-, glucuronate- or sulfate-conjugated aliphatic, prostanoid or heterocyclic compounds [15–23]. These hydrophobic aliphatic, prostanoid or heterocyclic compounds may come from food, herbicides, tobacco-related products or chemotherapeutic agents and are toxic to the cells. The active efflux of these potentially toxic compounds by MRP1 protein is generally an important aspect of cellular detoxification [47]. Does MRP1 protein have another common function in all these normal tissues? MRP1 has been postulated to function as a floppase in cells to translocate the phospholipids in membrane bilayer [36]. Red blood cells derived from knockout mice for Mrp1 (Mrp −/−) lost the ability to translocate fluorescent-labeled phospholipids NBD-PC and NBD-PS [33], hinting that MRP1 protein is responsible for the observed flop of the fluorescent lipid analogues. The inhibitory effects of various anticancer drugs, glutathione-conjugates and oxidized GSSG on the flop of the fluorescent-labeled NBD-PS closely correlated with those reported for active

transport by MRP1 protein and thus it was postulated that MRP1 protein functions as a floppase on the plasma membrane of the erythrocyte [34]. LLC-PK1 pig kidney epithelial cells transfected with human MRP cDNA gained the ability to specifically translocate C6-NBD-glucosylceramide and C6-NBD-sphingomyelin [35], also implying that MRP1 protein translocates those lipids. However, all these results derived from intact cells provide indirect evidences showing that MRP1 protein may function as a floppase to translocate the phospholipids from inner to outer leaflet. Here we proved that the majority of the purified MRP1 protein was reconstituted into proteoliposomes in an inside–out configuration (Fig. 2) and translocated the fluorescent-labeled PC from outer to inner leaflet (Figs. 4 and 5), which is consistent with the results derived from intact cells that translocated the fluorescent-labeled lipids from inner to outer leaflet [34,35]. Therefore, we provide direct evidence that the purified and reconstituted human MRP1 protein functions as a floppase to translocate the fluorescent-labeled NBD-PC. The translocation requires ATP and $MgCl_2$ (Fig. 6A), indicating that MRP1 protein couples ATP binding/hydrolysis to the translocation of the phospholipid analogue. Vanadate, a non-specific ATPase inhibitor which traps ATP hydrolysis product ADP at the ATP binding site, inhibits the translocation of the NBD-PC (Fig. 6B), further supporting the conclusion that translocation of NBD-PC requires ATP hydrolysis. In addition, the translocation is GSH-dependent (Fig. 6A(b)), sharing the same mechanism to transport anticancer drugs [18]. Whether NBD-PC occupies the same solute binding site as those anticancer drugs is not clear yet. Although those anticancer drugs, such as vincristine, vinblastine, daunorubicin and doxorubicin, more or less inhibit the translocation of NBD-PC (Fig. 6C), the results do not prove directly that NBD-PC and the anticancer drugs occupy the same binding site. However, since translocations of both anticancer drugs and phospholipids are GSH- and ATP hydrolysis-dependent (Figs. 6 and 8), no matter whether they occupy the same or different binding sites, the transport of those anticancer drugs should more or less inhibit the translocation of the phospholipids. Consistent with this conclusion, the MRP1 substrate GSSG [48] also inhibits the translocation of NBD-PC (Fig. 6C). In addition, MK-571, a potent MRP1 inhibitor known to specifically reverse MRP1-mediated anticancer drug resistance [44], inhibits the NBD-PC translocation almost completely (Fig. 6B(d)). All these results support the notion that MRP1 protein possesses the ability to translocate the PC analogue in the membrane bilayer where the protein resides and this may be another important physiological function of MRP1 protein to re-distribute the phospholipids in the plasma membrane bilayer. However, since the substrate we used in the experiments is a fluorescent-labeled phosphatidylcholine analogue, it might not reflect that of the native lipids, but might merely reflect the recognition of NBD-PC as xenobiotics by MRP1 protein. It has been noticed that the PC analogue, NBD-PC, is more easily transferred between membranes than native PC [31,37,43,49]. However, NBD-phosphatidylethanolamine, another NBD-labeled phospholipid, is not translocated by MRP1 protein in the same MRP1-reconstituted proteoliposomes (data not shown), implying that MRP1 protein recognizes the phosphatidylcholine backbone rather than the NBD. Therefore, although NBD-PC is not a native phospholipid, our results do prove that MRP1 protein recognizes phospholipid backbone and translocates them to another leaflet.

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References

[1] R.L. Juliano, V. Ling, A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants, Biochim. Biophys. Acta 455 (1976) 152–162.
[2] S.E. Mirski, J.H. Gerlach, S.P. Cole, Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin, Cancer Res. 47 (1987) 2594–2598.
[3] Y.N. Chen, L.A. Mickley, A.M. Schwartz, E.M. Acton, J.L. Hwang, A.T. Fojo, Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein, J. Biol. Chem. 265 (1990) 10073–10080.
[4] S.P. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M. Duncan, R.G. Deeley, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line, Science 258 (1992) 1650–1654.
[5] C.J. Chen, J.E. Chin, K. Ueda, D.P. Clark, I. Pastan, M.M. Gottesman, I.B. Roninson, Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells, Cell 47 (1986) 381–389.
[6] J.H. Gerlach, J.A. Endicott, P.F. Juranka, G. Henderson, F. Sarangi, K.L. Deuchars, V. Ling, Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance, Nature 324 (1986) 485–489.
[7] P. Gros, J. Croop, D. Housman, Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins, Cell 47 (1986) 371–380.
[8] E. Georges, T. Tsuruo, V. Ling, Topology of P-glycoprotein as determined by epitope mapping of MRK-16 monoclonal antibody, J. Biol. Chem. 268 (1993) 1792–1798.
[9] E. Bakos, T. Hegedus, Z. Hollo, E. Welker, G.E. Tusnady, G.J. Zaman, M.J. Flens, A. Varadi, B. Sarkadi, Membrane topology and glycosylation of the human multidrug resistance-associated protein, J. Biol. Chem. 271 (1996) 12322–12326.
[10] D.R. Hipfner, K.C. Almquist, E.M. Leslie, J.H. Gerlach, C.E. Grant, R.G. Deeley, S.P. Cole, Membrane topology of the multidrug resistance protein (MRP). A study of glycosylation-site mutants reveals an extracytosolic NH2 terminus, J. Biol. Chem. 272 (1997) 23623–23630.
[11] G.E. Tusnady, E. Bakos, A. Varadi, B. Sarkadi, Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters, FEBS Lett. 402 (1997) 1–3.

[12] M. Horio, M.M. Gottesman, I. Pastan, ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 3580–3584.
[13] A.H. Schinkel, P. Borst, Multidrug resistance mediated by P-glycoproteins, Semin. Cancer Biol. 2 (1991) 213–226.
[14] F.J. Sharom, R. Liu, Y. Romsicki, Spectroscopic and biophysical approaches for studying the structure and function of the P-glycoprotein multidrug transporter, Biochem. Cell. Biol. 76 (1998) 695–708.
[15] M. Muller, C. Meijer, G.J. Zaman, P. Borst, R.J. Scheper, N.H. Mulder, E.G. de Vries, P.L. Jansen, Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 13033–13037.
[16] I. Leier, G. Jedlitschky, U. Buchholz, S.P. Cole, R.G. Deeley, D. Keppler, The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates, J. Biol. Chem. 269 (1994) 27807–27810.
[17] M. Muller, E.G. de Vries, P.L. Jansen, Role of multidrug resistance protein (MRP) in glutathione S-conjugate transport in mammalian cells, J. Hepatol. 24 (Suppl. 1) (1996) 100–108.
[18] D.W. Loe, R.G. Deeley, S.P. Cole, Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione, Cancer Res. 58 (1998) 5130–5136.
[19] B.D. Stride, C.E. Grant, D.W. Loe, D.R. Hipfner, S.P. Cole, R.G. Deeley, Pharmacological characterization of the murine and human orthologs of multidrug-resistance protein in transfected human embryonic kidney cells, Mol. Pharmacol. 52 (1997) 344–353.
[20] M. Gao, D.W. Loe, C.E. Grant, S.P. Cole, R.G. Deeley, Reconstitution of ATP-dependent leukotriene C4 transport by co-expression of both half-molecules of human multidrug resistance protein in insect cells, J. Biol. Chem. 271 (1996) 27782–27787.
[21] D. Keppler, I. Leier, G. Jedlitschky, R. Mayer, M. Buchler, The function of the multidrug resistance proteins (MRP and cMRP) in drug conjugate transport and hepatobiliary excretion, Adv. Enzyme Regul. 36 (1996) 17–29.
[22] D. Lautier, Y. Canitrot, R.G. Deeley, S.P. Cole, Multidrug resistance mediated by the multidrug resistance protein (MRP) gene, Biochem. Pharmacol. 52 (7) (1996) 967–977.
[23] D.W. Loe, K.C. Almquist, S.P. Cole, R.G. Deeley, ATP-dependent 17 beta-estradiol 17-(beta-D-glucuronide) transport by multidrug resistance protein (MRP). Inhibition by cholestatic steroids, J. Biol. Chem. 271 (1996) 9683–9689.
[24] A. Polissi, C. Georgopoulos, Mutational analysis and properties of the msbA gene of Escherichia coli, coding for an essential ABC family transporter, Mol. Microbiol. 20 (1996) 1221–1233.
[25] Z. Zhou, K.A. White, A. Polissi, C. Georgopoulos, C.R. Raetz, Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis, J. Biol. Chem. 273 (1998) 12466–12475.
[26] A. Decottignies, A.M. Grant, J.W. Nichols, H. de Wet, D.B. McIntosh, A. Goffeau, ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p, J. Biol. Chem. 273 (1998) 12612–12622.
[27] A. van Helvoort, A.J. Smith, H. Sprong, I. Fritzsche, A.H. Schinkel, P. Borst, G. van Meer, MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine, Cell 87 (1996) 507–517.
[28] J.J. Smit, A.H. Schinkel, R.P. Oude Elferink, A.K. Groen, E. Wagenaar, L. van Deemter, C.A. Mol, R. Ottenhoff, N.M. van der Lugt, M.A. van Roon, Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease, Cell 75 (1993) 451–462.
[29] R.P. Oude Elferink, R. Ottenhoff, M. van Wijland, J.J. Smit, A.H. Schinkel, A.K. Groen, Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse, J. Clin. Invest. 95 (1995) 31–38.
[30] C.M. Frijters, R. Ottenhoff, M.J. Van Wijl, C. Van Nieuwkerk, A.K. Groen, R.P. Oude Elferink, Influence of bile salts on hepatic mdr2 P-glycoprotein expression, Adv. Enzyme Regul. 36 (1996) 351–363.
[31] S. Ruetz, P. Gros, Phosphatidylcholine translocase: a physiological role for the mdr2 gene, Cell 77 (1994) 1071–1081.
[32] Y. Romsicki, F.J. Sharom, Phospholipid flippase activity of the reconstituted P-glycoprotein multidrug transporter, Biochemistry 40 (2001) 6937–6947.
[33] D.W. Dekkers, P. Comfurius, A.J. Schroit, E.M. Bevers, R.F. Zwaal, Transbilayer movement of NBD-labeled phospholipids in red blood cell membranes: outward-directed transport by the multidrug resistance protein 1 (MRP1), Biochemistry 37 (1998) 14833–14837.
[34] D. Kamp, C.W. Haest, Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane, Biochim. Biophys. Acta 1372 (1998) 91–101.
[35] R.J. Raggers, A. van Helvoort, R. Evers, G. van Meer, The human multidrug resistance protein MRP1 translocates sphingolipid analogs across the plasma membrane, J. Cell. Sci. 112 (1999) 415–422.
[36] A. Sokal, L. Pulaski, B. Rychlik, A. Fortuniak, G. Bartosz, Is the glutathione S-conjugate pump a flippase? Biochem. Mol. Biol. Int. 44 (1998) 97–105.
[37] H. Suzuki, M. Kamakura, M. Morii, N. Takeguchi, The phospholipid flippase activity of gastric vesicles, J. Biol. Chem. 272 (1997) 10429–10434.
[38] X.B. Chang, Y.X. Hou, J.R. Riordan, ATPase activity of purified multidrug resistance-associated protein, J. Biol. Chem. 272 (1997) 30962–30968.
[39] Q. Mao, R.G. Deeley, S.P. Cole, Functional reconstitution of substrate transport by purified multidrug resistance protein MRP1 (ABCC1) in phospholipid vesicles, J. Biol. Chem. 275 (2000) 34166–34172.
[40] F.J. Sharom, X. Yu, C.A. Doige, Functional reconstitution of drug transport and ATPase activity in proteoliposomes containing partially purified P-glycoprotein, J. Biol. Chem. 268 (1993) 24197–24202.
[41] Y. Romsicki, F.J. Sharom, Interaction of P-glycoprotein with defined phospholipid bilayers: a differential scanning calorimetric study, Biochemistry 36 (1997) 9807–9815.
[42] L. Manciu, X.B. Chang, J.R. Riordan, J.M. Ruysschaert, Multidrug resistance protein MRP1 reconstituted into lipid vesicles: secondary structure and nucleotide-induced tertiary structure changes, Biochemistry 39 (2000) 13026–13033.
[43] J.C. McIntyre, R.G. Sleight, Fluorescence assay for phospholipid membrane asymmetry, Biochemistry 30 (1991) 11819–11827.
[44] V. Gekeler, W. Ise, K.H. Sanders, W.R. Ulrich, J. Beck, The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance, Biochem. Biophys. Res. Commun. 208 (1995) 345–352.
[45] D.W. Loe, R.G. Deeley, S.P. Cole, Verapamil stimulates glutathione transport by the 190-kDa multidrug resistance protein 1 (MRP1), J. Pharmacol. Exp. Ther. 293 (2000) 530–538.
[46] M.J. Flens, G.J. Zaman, P. van der Valk, M.A. Izquierdo, A.B. Schroeijers, G.L. Scheffer, P. van der Groep, M. de Haas, C.J. Meijer, R.J. Scheper, Tissue distribution of the multidrug resistance protein, Am. J. Pathol. 148 (1996) 1237–1247.
[47] E.M. Leslie, R.G. Deeley, S.P. Cole, Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters, Toxicology 167 (2001) 3–23.
[48] I. Leier, G. Jedlitschky, U. Buchholz, M. Center, S.P. Cole, R.G. Deeley, D. Keppler, ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump, Biochem. J. 314 (1996) 433–437.
[49] J.W. Nichols, Kinetics of fluorescent-labeled phosphatidylcholine transfer between nonspecific lipid transfer protein and phospholipid vesicles, Biochemistry 27 (1988) 1889–1896.