The reconstituted P-glycoprotein multidrug transporter is a flippase for glucosylceramide and other simple glycosphingolipids

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The Pgp (P-glycoprotein) multidrug transporter, which is linked to multidrug resistance in human cancers, functions as an efflux pump for non-polar drugs, powered by the hydrolysis of ATP at its nucleotide binding domains. The drug binding sites of Pgp appear to be located within the cytoplasmic leaflet of the membrane bilayer, suggesting that Pgp may function as a 'flippase' for hydrophobic compounds. Pgp has been shown to translocate fluorescent phospholipids, and it has been suggested that it may also interact with GlcCer (glucosylceramide). Here we use a dithionite fluorescence quenching technique to show that reconstituted Pgp can flip several NBD (nitrobenzo-2-oxa-1,3-diazole)-labelled simple glycosphingolipids, including NBD–GlcCer, from one leaflet of the bilayer to the other in an ATP-dependent, vanadate-sensitive fashion. The rate of NBD–GlcCer flipping was similar to that observed for NBD-labelled PC (phosphatidylcholine). NBD–GlcCer flipping was inhibited in a concentration-dependent, saturable fashion by various Pgp substrates and modulators, and inhibition correlated well with the K_d for binding to the protein. The addition of a second sugar to the headgroup of the glycolipid to form NBD–lactosylceramide drastically reduced the rate of flipping compared with NBD–PC, probably because of the increased size and polarity contributed by the additional sugar residue. We conclude that Pgp functions as a broad-specificity outwardly-directed flippase for simple glycosphingolipids and membrane phospholipids.

Key words: flippase, fluorescence quenching, glycosphingolipid, multidrug transporter, orthovanadate, P-glycoprotein.

INTRODUCTION

Pgp (P-glycoprotein; MDR1; ABCB1), a 170 kDa efflux pump for hydrophobic drugs, is believed to contribute to MDR (multidrug resistance) in human cancer. Pgp is a member of the ABC (ATP-binding cassette) superfamily of membrane proteins, which are implicated in the ATP-dependent transport of a variety of substrates into or out of cells, in organisms ranging from simple prokaryotes to humans [1,2]. Pgp couples the hydrolysis of ATP at its two NB (nucleotide binding) domains to the cellular efflux of hundreds of structurally diverse hydrophobic substrates, including a broad range of drugs, linear and cyclic peptides, and natural products [3].

The product of the closely related *MDR3* gene (ABCB4) is not a drug-resistance protein, but rather a PC (phosphatidylcholine)-specific phospholipid flippase [4,5]. The role of MDR3, which is found primarily in the hepatocyte canalicular membrane, is to export PC into the bile [6]. The high sequence identity (78 %) between these two classes of Pgps, coupled with a recent report indicating that MDR3 Pgp can transport MDR1 drug substrates at a low rate [7], suggests that MDR1 Pgp may function as a hydrophobic drug flippase, moving its substrates from the inner to the outer leaflet of the membrane, from which they can diffuse into the environment. Considerable evidence indicates that Pgp functions as a drug flippase. Pgp has been described as a 'hydrophobic vacuum cleaner', and is proposed to bind to its substrates within the membrane and export them from there to the extracellular medium [8]. Pgp substrates are generally hydrophobic, and their affinity of binding to the transporter is governed by their ability to partition into the membrane [9]. Using a FRET (fluorescence resonance energy transfer) approach, we recently showed that the binding sites for two fluorescent Pgp substrates, Hoechst 33342 [10] and LDS-751 [11], are situated within the cytoplasmic membrane leaflet.

Pgp also appears to function as a phospholipid flippase. Altered distributions of fluorescent PC, PE (phosphatidylethanolamine) and SM (sphingomyelin) derivatives were found in cells expressing recombinant Pgp [12–15] and in drug-selected cells overexpressing the protein [13,16]. In 2001, we showed directly for the first time that Pgp reconstituted into proteoliposomes was able to flip a variety of NBD (nitrobenzo-2-oxa-1,3-diazole)-labelled phospholipids in an ATP-dependent fashion [17]. Fluorescent lipids that were translocated included PC and phosphatidylserine with short or long acyl chains, and PE with NBD attached to either the headgroup or one of the acyl chains. In addition to MDR3, several other ABC proteins also appear to function as lipid flippases [18]. Among these, LmrA, a multidrug transporter and Pgp homologue in *Lactococcus lactis*, flips NBD–PE (NBD-labelled PE), but not NBD–PC, in a purified reconstituted system. The drug efflux pump MRP1 (multidrug resistance-associated protein 1; ABCC1) appears to flip several fluorescent membrane lipids in whole cells, and the yeast proteins Pdr5p and Yor1p can translocate NBD–PE.

In recent years, links have been established between Pgp and GSL (glycosphingolipids). Many cells expressing Pgp show elevated levels of GlcCer (glucosylceramide) and SM [19–23], and inhibitors of GlcCer synthase kill MDR cells [24]. Transfection of MDR1 also results in elevated GSL levels [25]. These observations led to the suggestion that Pgp may be able to flip GlcCer between membrane leaflets in both the plasma membrane and Golgi apparatus [15,25]. In the present study, we show for the first time that purified Pgp reconstituted into proteoliposomes

Abbreviations used: ABC, ATP-binding cassette; p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate; Cer, ceramide; GalCer, galactosylceramide; GlcCer, glucosylceramide; GSL, glycosphingolipid(s); LacCer, lactosylceramide; MDR, multidrug resistance/resistant; MRP, multidrug resistance-associated protein; NB domain, nucleotide binding domain; NBD, nitrobenzo-2-oxa-1,3-diazole; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pgp, P-glycoprotein; SM, sphingomyelin.

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can function as a flippase for a variety of simple GSL, including GlcCer, carrying the NBD fluorescent label. The rate of Pgp-mediated NBD–GlcCer flipping was similar to that for translocation of NBD–PC, and characterization of the two processes indicated that they displayed very similar features. The addition of a second sugar to the headgroup to form NBD–LacCer (lactosylceramide) drastically reduced the rate of flipping compared with NBD–PC, probably because of the increased size and polarity contributed by the additional sugar residue. We conclude from the present work and previous results that Pgp functions as a broad-specificity outwardly-directed flippase for membrane phospholipids and simple GSL.

EXPERIMENTAL

Materials

Egg PC, NBD–PC (16:0, 6:0) and NBD–C_6-SM were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). BSA, sodium dithionite (sodium hydrosulphite), sodium orthovanadate, ATP, ADP, p[NH]ppA (adenosine 5′-[β,γ-imido]triphosphate; 'AMP-PNP'), CHAPS, Triton X-100, verapamil, leupeptin, all NBD-labelled GSL and NBD–C_{12}-Cer were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cyclosporin A was provided by Pfizer Central Research (Groton, CT, U.S.A.). PSC-833 was supplied by Novartis Canada (Montreal, QC, Canada).

Isolation and reconstitution of Pgp

Pgp was isolated from the plasma membrane fraction of the MDR CH^RB30 cell line using a detergent extraction procedure described previously [26]. Protein was assayed by the method of Peterson [27] using a BSA standard. Only protein samples with ATPase specific activity of greater than 0.5 μmol of P_i released/min per mg of protein were used for flippase measurements. Pgp was usually reconstituted into egg PC proteoliposomes containing approx. 0.3 % (w/w of total lipid) NBD-labelled lipid. Typically, 5 mg of the desired lipid mixture was solubilized in 250 μl of 200 mM CHAPS buffer, and 1 ml of 15 mM CHAPS buffer containing 0.5 mg of protein was added. The final lipid/protein ratio was approx. 10:1 (w/w). Reconstitution was carried out using gel filtration as described previously [17], with the following modifications. CHAPS detergent was removed by passage through a 1 cm × 15 cm Sephadex G-50 column, and the resulting yellow-coloured fractions were pooled to give a total volume of 3 ml. For time-course experiments up to 90 min using NBD–PC (16:0, 6:0), two samples of proteoliposomes were prepared in parallel from the same batch of protein, and the appropriate fractions from each column were pooled to give a total volume of 6 ml. We also employed preformed vesicles with reconstituted Pgp where NBD–PC (6:0, 16:0) was inserted into the vesicles by addition from an ethanol stock solution. Vesicles were centrifuged to remove excess soluble NBD–PC and resuspended in buffer lacking the fluorescent probe.

Flippase assay using NBD-labelled lipids

The ability of reconstituted Pgp to flip NBD–PC, NBD–C_{12}-Cer, NBD–C_6-SM or NBD–GSL was determined as described previously for NBD-labelled phospholipids [17], with some modifications. Proteoliposome samples were kept on ice and equilibrated at 22 °C for 5 min before transfer to 37 °C and initiation of the translocation assay by the addition of ATP and an ATP-regenerating system. Translocation was terminated by the addition of sodium orthovanadate at a final concentration of 200 μM, and the samples were then equilibrated at 22 °C for 5 min before commencing fluorescence measurements. A PTI Quantamaster C-61 steady-state fluorimeter (Photon Technology International, London, ON, Canada) was employed, using the excitation and emission wavelength maxima determined for each reconstituted NBD–lipid, which ranged from 464 to 468 nm for excitation (bandwidth 2 nm) and from 534 to 540 nm for emission (bandwidth 2 nm). Sodium dithionite was added 3 min after initiation of fluorescence measurements. Triton X-100 was added after 7 min, and the fluorescence intensity was monitored for an additional 3 min. Total fluorescence due to NBD-labelled lipid in both the inner and outer leaflets, fluorescence due to only the inner leaflet, and background fluorescence after quenching all NBD–lipids were determined by averaging the last 20 data points (20 s) before addition of dithionite, addition of Triton X-100 and termination of the experiment respectively.

NBD–GSL-concentration-dependence of flippase activity

The dependence of Pgp-mediated flippase activity on the concentration of the substrate NBD–C_6-GlcCer was determined as described above, except that Pgp was reconstituted into egg PC vesicles in the presence of 0.1–1.0 % (w/w of total lipid) NBD–C_6-GlcCer. For experiments involving concentrations higher than 0.3 % (w/w) NBD–lipid, 4 mM dithionite (final concentration) was used as the quenching agent to ensure that all of the NBD fluorophore was quenched.

Nucleotide-dependence of flippase activity

Nucleotide-dependence experiments were carried out as described above, with the following changes. Final ATP concentrations were in the range 0–10 mM, with the usual concentrations of ATP-regenerating system reagents. For experiments involving p[NH]ppA, the nucleotide was added at a concentration of 1 mM in the absence of ATP, but in the presence of the regenerating system. In the case of ADP, the nucleotide was added at 1 mM, and the regenerating system contained phosphocreatine but no creatine kinase, to prevent the formation of ATP from ADP.

Vanadate inhibition of flippase activity

Vanadate stock solution was prepared in transport buffer and added as a 10 μl aliquot to the proteoliposome samples to a final concentration of 0–500 μM, prior to initiation of lipid transport by the addition of ATP and the regenerating system. Flippase activity was assessed in the usual way, and the experiment was terminated in each case with an additional 200 μM vanadate.

Inhibition of lipid flippase activity by drugs and modulators

Fluorescence traces were recorded as described above with the following modifications. At 5 min prior to initiation of lipid transport by the addition of ATP and the regenerating system, a 10 μl aliquot of the desired drug in 100 % (v/v) DMSO was added (final concentration 2 %, v/v). Control samples were treated either with 100 % DMSO (positive control; 100 % flippase activity) or with the highest drug concentration used but without the addition of ATP and the regenerating system (negative control; 0 % flippase activity). The data were analysed according to the median effect equation [28], which has been used previously in the analysis of both Pgp-mediated drug transport [29,30] and phospholipid flippase activity [17]:

$$f_a/f_u = (D/D_m)^m$$

where f_a is the fraction of the system that is affected (in this case, the fractional inhibition of NBD–C_6-GlcCer translocation at

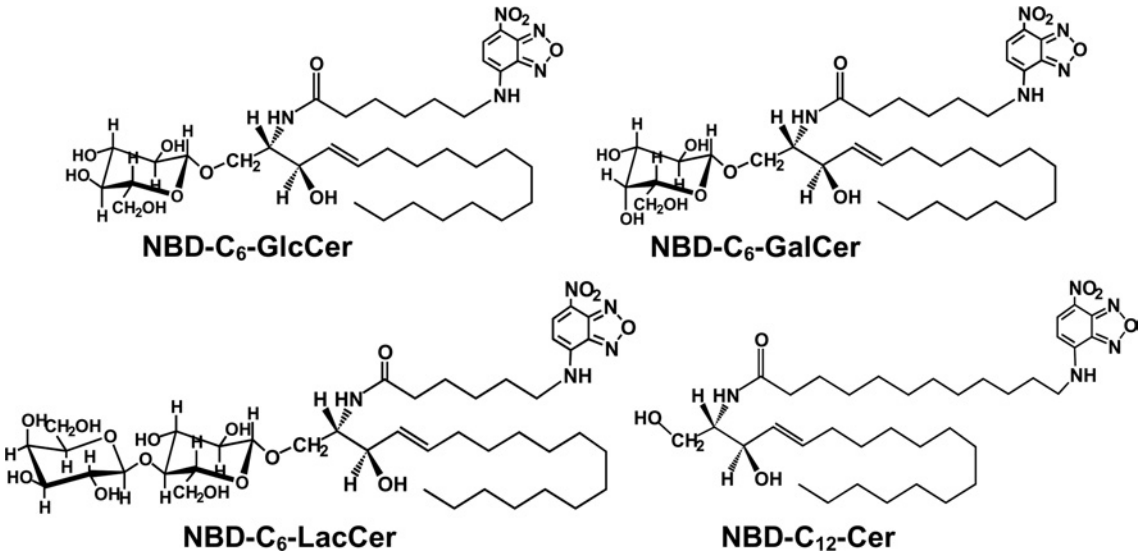

Figure 1 Structures of some of the NBD-labelled GSL used in this study

NBD–C_6-GlcCer, NBD–C_6-GalCer, NBD–C_6-LacCer and NBD–C_{12}-Cer are shown. NBD–C_{12}-LacCer has the NBD moiety attached via a 12-carbon chain, rather than the 6-carbon chain shown for NBD–C_6-LacCer.

20 min) at concentration D, f_u is the fraction of the system that is unaffected at concentration D, D_m is the compound concentration causing 50 % inhibition, and m is a parameter indicating the sigmoidicity of the dose–effect curve. Rearrangement of the median effect equation yields:

$$\log (f_a/f_u) = m\log D - m\log D_m$$

A plot of $\log (f_a/f_u)$ against $\log D$ produces a straight line with slope m and an x-intercept of $\log D_m$.

RESULTS

Pgp mediates translocation of NBD–GlcCer in a reconstituted system

Several reports indicate that Pgp may be able to mediate transbilayer movement of GlcCer in intact cells. We investigated the ability of Pgp to translocate NBD–GSL and related species in reconstituted proteoliposomes, which lack the ambiguities of whole-cell systems. Some of the lipids used in the present study are shown in Figure 1, and include NBD–C_6-GlcCer, NBD–C_6-GalCer (galactosylceramide), NBD–C_6-LacCer and NBD–C_{12}-Cer. Related sphingolipid species such as NBD–C_6-SM and NBD–C_{12}-LacCer were also employed. To provide a benchmark for comparison, each experiment was normalized to the flippase activity with NBD–PC (16:0, 6:0) measured for the same batch of protein. We found significant batch-to-batch variability in the ability of Pgp to flip this NBD–PC, and only preparations with relatively high ATPase activity (> 0.5 μmol of P_i released/min per mg of protein) were used for flippase experiments. In general, preparations with high ATPase activity also had good flippase activity; a quantitative comparison was not possible, since initial rates of flipping were not determined. Proteoliposomes of egg PC containing Pgp (lipid/protein ratio $\sim$10:1, w/w) and $\sim$0.3 % (w/w total lipid) of the desired NBD-labelled lipid species were prepared by gel filtration chromatography. A previous study characterized Pgp proteoliposomes prepared by this method [17]. The vesicles showed a trimodal size distribution, and 70–80 % of the Pgp molecules faced inwards, with the NB domains exposed on the exterior.

To assess the ability of reconstituted Pgp to flip NBD–lipids, a dithionite reduction technique was used. Dithionite rapidly reduces the nitro group of the NBD fluorophore to a non-fluorescent amine [31]. Under the conditions used in this study, dithionite is membrane impermeant, and therefore can only quench the fluorescence of NBD-labelled lipids present in the outer leaflet of the proteoliposome [17]. To assess ATP-dependent, Pgp-mediated changes in the distribution of the NBD–lipid over time, we used dithionite quenching in both the absence and the presence of permeabilizing detergent, after incubation with or without ATP and an ATP-regenerating system (Figure 2). For NBD–PC (16:0, 6:0) (Figure 2A), more NBD–lipid was present in the inner membrane leaflet following incubation with ATP for 20 min. In previous work, NBD–phospholipid flippase activity was followed for 20 min [17]. We now measured the flippase activity of Pgp for NBD–PC (16:0, 6:0) over time periods up to 90 min, to ascertain whether the transbilayer distribution of labelled lipid would reach a steady state over longer times (Figure 2B). The Pgp-mediated translocation of NBD–PC to the inner leaflet increased rapidly in the first 20 min, and reached a maximum after 60 min at 37 °C. To verify that ATP was not limiting during the course of the experiment, proteoliposomes were incubated in the presence of ATP and the regenerating system at 37 °C for 45 min, after which additional ATP (2 mM) and regenerating system were added, and the sample was incubated for a further 45 min. The quantity of NBD–PC translocated was similar to that observed after a 90 min incubation with 1 mM ATP, indicating that ATP was not limiting over the course of the reaction (Figure 2B).

We observed a large increase in fluorescence due to NBD–C_6-GlcCer in the inner leaflet of proteoliposomes after incubation at 37 °C in the presence of ATP and the regenerating system for 20 min, compared with a sample incubated in the absence of ATP, or a sample incubated with ATP for 0 min (Figure 2C). We noted an inner/outer leaflet ratio for NBD–C_6-GlcCer in Pgp proteoliposomes of approx. 25:75 in the absence of ATP, which represents a slightly higher proportion present in the inner leaflet than that observed for NBD–PC both in the present work

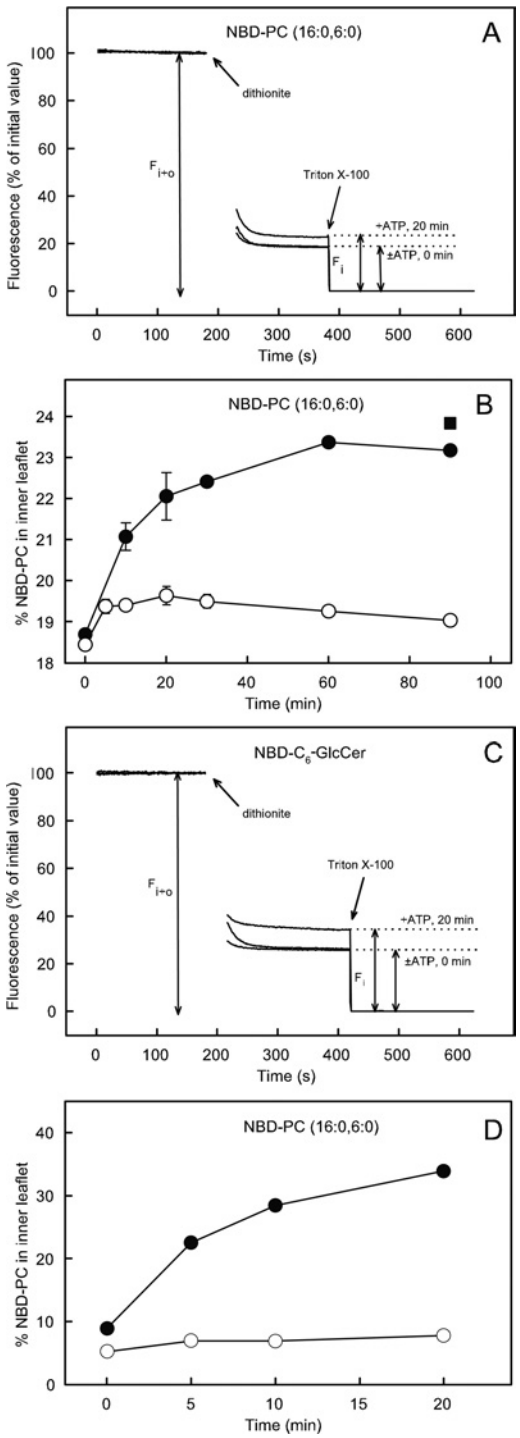

Figure 2 Translocation of NBD-labelled lipids by Pgp in reconstituted proteoliposomes

(**A**) Proteoliposomes of egg PC containing Pgp and 0.3 % (w/w) NBD–PC (16:0, 6:0) were incubated at 37 °C with 1 mM ATP and the regenerating system for various times. After addition of 0.2 mM vanadate, fluorescence emission ($\lambda_{ex} = 468$ nm, $\lambda_{em} = 540$ nm) was monitored at 22 °C until a stable baseline was achieved. After 3 min, 2 mM dithionite was added (indicated by an interruption in the trace), and after a stable baseline was again reached, 1 % (w/v) Triton X-100 was added. Fluorescence traces were obtained at time zero with or without ATP (± ATP, 0 min) and 20 min after adding ATP (+ ATP, 20 min). Traces were normalized to the fluorescence intensity recorded just prior to dithionite addition, which was taken as 100 %. The vertical arrows represent the total fluorescence of NBD–PC in both the inner and outer leaflets (F_{i+o}), and the fluorescence of NBD–PC in the inner leaflet in the absence and presence of ATP (F_i). (**B**) Time course for the translocation of NBD–PC (16:0, 6:0) in proteoliposomes of egg PC containing reconstituted Pgp. Proteoliposomes were incubated for 0–90 min either with (●) or without (○) 1 mM ATP and the regenerating system, or with 1 mM ATP for the first 45 min, after which more ATP was added (2 mM final concentration) and the sample was incubated for a further 45 min (■). Data points represent the means for duplicate determinations; error bars represent the range (where not visible, they fall within the symbols). (**C**) Proteoliposomes containing Pgp and 0.3 % (w/w) NBD–C_6-GlcCer were treated as in (**A**). (**D**) Translocation of NBD–PC (16:0, 6:0) inserted asymmetrically into the outer leaflet of egg PC proteoliposomes containing Pgp, with (●) or without (○) 1 mM ATP and the regenerating system.

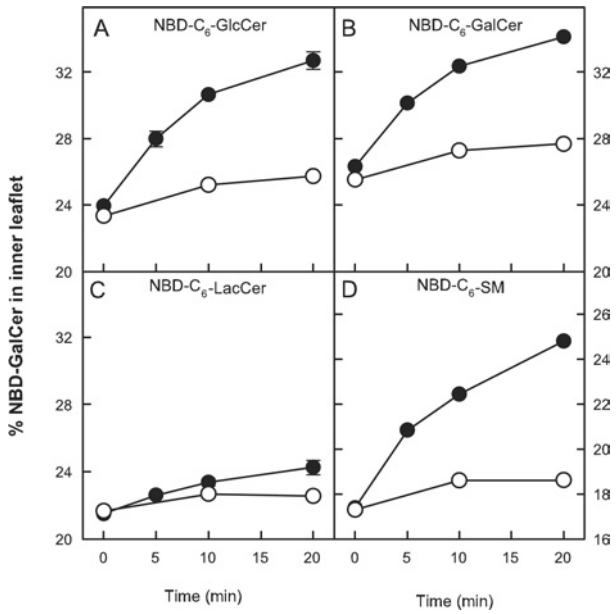

Figure 3 Time course for the translocation of three NBD-labelled GSL and NBD–SM in proteoliposomes of egg PC containing reconstituted Pgp

Proteoliposomes were incubated either with (●) or without (○) 1 mM ATP and the regenerating system. The transbilayer distribution of the NBD–lipid was measured using dithionite quenching: (**A**) NBD–C_6-GlcCer, (**B**) NBD–C_6-GalCer, (**C**) NBD–C_6-LacCer and (**D**) NBD–C_6-SM. Data points represent the means ± range for duplicate determinations; where not visible, the error bars fall within the symbols.

and reported previously [17]. The time course for translocation of NBD–C_6-GlcCer was determined for incubation times up to 20 min with and without ATP (Figure 3A). There was a substantial increase in the proportion of NBD–C_6-GlcCer in the inner leaflet of the proteoliposomes in the presence of ATP and the regenerating system, compared with control samples in the absence of ATP, at each incubation time. The net amount of NBD–C_6-GlcCer translocated over a 20 min period was essentially identical to the value observed for NBD–PC (16:0, 6:0) with the same batch of protein (Table 1).

We also measured the translocation of NBD–PC (16:0, 6:0) after its insertion into preformed egg PC proteoliposomes containing reconstituted Pgp. Over 90 % of the NBD–PC was in the outer leaflet, as assessed by dithionite quenching (Figure 2D). We observed rapid ATP-dependent translocation of NBD–PC to the inner leaflet over a 20 min period (Figure 2D), confirming the results obtained with symmetrically incorporated probe. Several factors made this approach unsuitable for quantification of flippase activity. Much of the probe remained in the soluble supernatant, and vesicles were lost upon centrifugation, so that the amount of NBD–PC inserted was not easily controlled or quantified. Spontaneous movement of NBD–PC to the inner leaflet is a factor in assays carried out after some time has elapsed. Finally, only PC derivatives with a least one short acyl chain

Table 1 GSL flippase activity of Pgp reconstituted into egg PC proteoliposomes

The extent of translocation was determined as the difference between proteoliposomes with and without ATP and an ATP-regenerating system, and was normalized to the flippase activity measured for NBD–PC (16:0, 6:0) using the same Pgp preparation, which was taken as 100 %. Data represent means ± S.D. for 2–3 experiments, each carried out in duplicate. ND, not determined due to very rapid spontaneous flip-flop of this derivative in the membrane.

NBD-labelled lipid	Extent of NBD–lipid translocation in 20 min (%)
NBD–C_6-GlcCer	102 ± 11
NBD–C_6-GalCer	120 ± 40
NBD–C_6-LacCer	27 ± 0.4
NBD–C_{12}-LacCer	24 ± 6
NBD–C_6-SM	170 ± 13
NBD–C_{12}-Cer	ND

are water-soluble and insert readily into bilayers, whereas the symmetrical reconstitution approach works for all long- or short-chain probes.

Dependence of flippase activity on the bilayer concentration of NBD–GlcCer

Initial experiments used 0.3 % (w/w) NBD–C_6-GlcCer to measure Pgp-mediated flippase activity, because this amount gave an adequate fluorescence signal. We determined the dependence of the GlcCer flippase activity of Pgp on the substrate concentration in the bilayer, where NBD–C_6-GlcCer ranged from 0.1 % to 1.0 % (w/w) of the total lipid (Figure 4A). For experiments using greater than 0.3 % (w/w) NBD–C_6-GlcCer, the dithionite added to the samples was increased from 2 to 4 mM (final concentration) to ensure that all of the NBD fluorescence was quenched after addition of Triton X-100. The proteoliposomes were not permeable to either 2 or 4 mM dithionite at 22 °C, as indicated by stable fluorescence emission baselines (due to inner-leaflet NBD–C_6-GlcCer) upon addition of the quencher. The extent of Pgp-mediated flipping of NBD–C_6-GlcCer increased from 1.02 to 2.10 nmol/mg of protein over 20 min as the concentration of labelled GlcCer increased from 0.1 % to 0.4 % (w/w) of the total lipid present. The maximum activity occurred when the fluorescent lipid was present in the membrane at 0.6 % (w/w) of the total lipid, and was nearly 3-fold the value observed at a concentration of 0.3 % (w/w). A further increase to 1.0 % (w/w) of the total lipid resulted in a modest decrease in the extent of flipping from the maximum value at 0.6 % (w/w). For further experiments, we used a concentration of the NBD-labelled species of 0.3 % (w/w) of the total lipid for consistency, and because this amount gave a good fluorescence signal and an easily measurable extent of flipping for both NBD–C_6-GlcCer and NBD–PC (16:0, 6:0).

Pgp-mediated NBD–GlcCer flipping is ATP-dependent and vanadate-sensitive

From the experiments described above, ATP was clearly required to observe Pgp-mediated flipping of NBD–GlcCer from one leaflet to the other. To characterize further the nucleotide dependence, we reconstituted Pgp with NBD–C_6–GlcCer into egg PC vesicles and measured flipping in the presence of 1 or 2 mM ATP, or in the absence of ATP (Figure 4B). Both 1 and 2 mM ATP allowed significant flipping of fluorescent GlcCer over the 20 min incubation period, whereas, as expected, no significant translocation of lipid occurred in the absence of ATP. No significant flipping of NBD–C_6-GlcCer was observed in the presence of 1 mM ADP (Figure 4B), suggesting that ATP hydrolysis, rather than simply nucleotide binding, was required for NBD–C_6-GlcCer translocation to occur. Similarly, the non-hydrolysable ATP analogue p[NH]ppA did not support GlcCer translocation, and trapping with ATP and vanadate to abolish the ATPase activity of

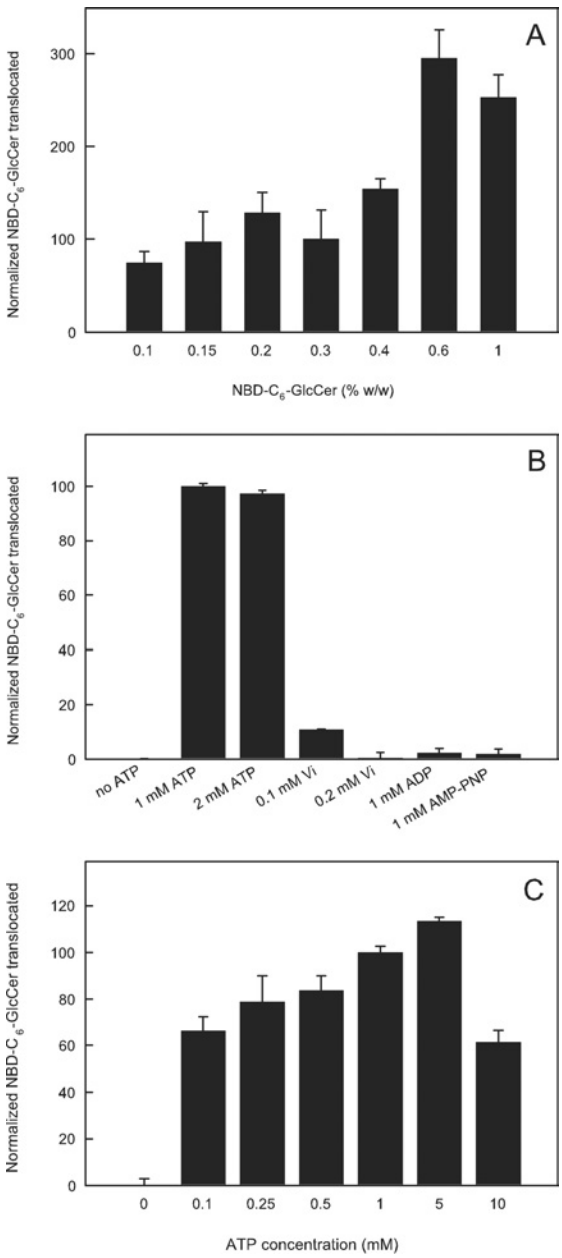

Figure 4 Characterization of Pgp-mediated translocation of NBD–C_6-GlcCer

(**A**) Dependence of translocation on the concentration of NBD–C_6-GlcCer in the bilayer. Reconstituted proteoliposomes of egg PC containing 0.1–1.0 % (w/w) NBD–C_6-GlcCer were incubated for 20 min at 37 °C, and the extent of translocation was determined. Data were normalized to the extent of NBD–C_6-GlcCer translocated with 0.3 % (w/w) NBD–lipid, which was taken as 100 %, and are represented as the means ± range for duplicate determinations. (**B**) Nucleotide dependence of NBD–C_6-GlcCer translocation. Egg PC proteoliposomes were incubated in the presence of 0, 1 or 2 mM ATP and the regenerating system, in the presence of 0.1 or 0.2 mM vanadate (Vi), 1 mM ADP or p[NH]ppA (AMP-PNP), and the transbilayer distribution of NBD–C_6-GlcCer was determined. The data were normalized to the value with 1 mM ATP, which was taken as 100 %. Data points represent means ± range for duplicate determinations. (**C**) ATP dependence of NBD–C_6-GlcCer translocation. Egg PC proteoliposomes were incubated at 37 °C for 20 min in the presence of 0–10 mM ATP and the regenerating system. The transbilayer distribution of NBD–C_6-GlcCer was determined, and the data were normalized to the value with 1 mM ATP, which was taken as 100 %. Data points represent means ± range for duplicate determinations.

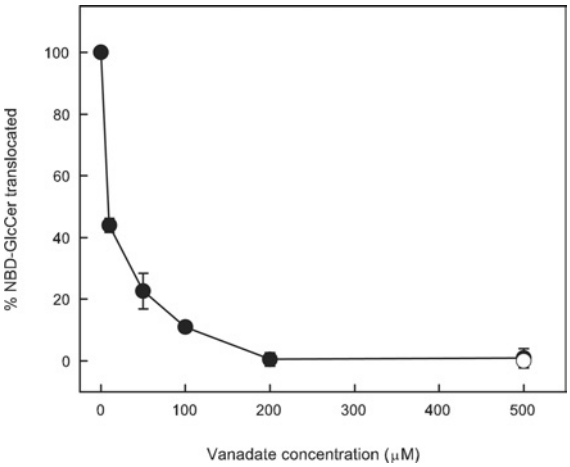

Figure 5 Inhibition of Pgp-mediated NBD–C_6-GlcCer translocation by vanadate

Egg PC proteoliposomes were incubated in the presence of 1 mM ATP and the regenerating system plus 0–500 μM vanadate (●), or in the absence of ATP and the presence of 500 μM vanadate (○), and the transbilayer distribution of NBD–C_6-GlcCer was determined. Data were normalized to the value in the presence of 1 mM ATP but in the absence of vanadate, which was taken as 100 %. Data points represent means ± range for duplicate determinations; where not visible, error bars fall within the symbols.

the protein also completely eliminated flippase activity (Figure 4B).

The ATP concentration was increased in the presence of the same concentration of the regenerating system, so that ATP regeneration would not be limiting (Figure 4C). The flippase activity of Pgp for NBD–C_6-GlcCer reached a maximum at approx. 5 mM ATP, and declined significantly at 10 mM ATP. At 0.1 mM ATP, there was still significant translocation (two-thirds of the activity observed at 1 mM ATP). The K_m of Pgp for catalysis of ATP hydrolysis is in the range 0.2–0.4 mM [32,33]. However, here we are monitoring the total amount of NBD–lipid translocated over a 20 min period rather than an initial rate of translocation. When we measured Pgp-mediated NBD–C_6-GlcCer flipping with 1 mM ATP and the regenerating system in the presence of various concentrations of vanadate (Figure 5), we observed that a vanadate concentration as low as 10 μM was sufficient to inhibit more than 50 % of the translocation. At vanadate concentrations over 200 μM, flipping of GlcCer was completely abolished.

Inhibition of Pgp-mediated NBD–GlcCer translocation by drugs and modulators

If GSL occupy the same binding sites as Pgp drug substrates, and are translocated via the same basic mechanism, the presence of high-affinity substrates and modulators should inhibit Pgp-mediated flippase activity in a concentration-dependent manner. We examined a variety of Pgp substrates, including cyclic and linear peptides, cytotoxic agents, and the modulators verapamil and PSC-833 (Table 2), for their ability to inhibit translocation of NBD–GlcCer. van Helvoort et al. [12] found that verapamil reduced the apical movement of NBD–C_6-GlcCer by ~75 % in whole cells expressing Pgp that were provided with the precursor NBD–C_6-Cer, while movement was almost completely blocked by PSC-833. NBD–C_6-GlcCer translocation was inhibited by each of the Pgp substrates and modulators in a concentration-dependent, saturable fashion (Figures 6 and 7). At sufficiently high concentrations of each substrate or modulator, NBD–C_6-GlcCer translocation was inhibited to the background values observed for

Table 2 Inhibition of Pgp NBD–C_6-GlcCer flippase activity by substrates and modulators

The ability of various Pgp substrates and modulators to inhibit ATP-driven flippase activity was determined by median effect analysis of the inhibition data shown in Figures 6 and 7.

Compound	Type	Substrate or modulator	D_m (μM)
Colchicine	Cytotoxic agent	Substrate	248
Leupeptin	Linear peptide	Substrate	45.0
Verapamil	Cytotoxic agent	Modulator	9.23
Cyclosporin A	Cyclic peptide	Modulator	1.47
PSC-833	Cyclic peptide	Modulator	0.145

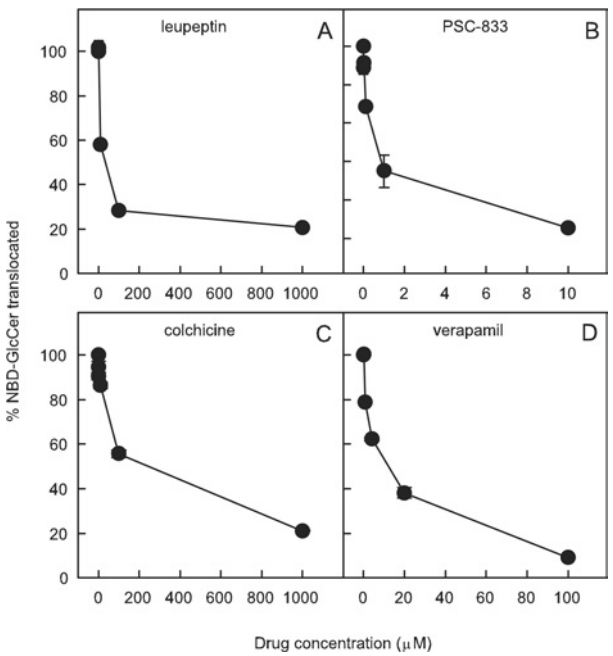

Figure 6 Inhibition of ATP-dependent NBD–C_6-GlcCer translocation by various Pgp transport substrates and modulators

The net translocation of NBD–C_6-GlcCer was assessed in the presence of various drugs, relative to a control without drug (taken as 100 %), and a control with drug but without ATP (taken as 0 %). Proteoliposomes containing Pgp were treated with (**A**) leupeptin, (**B**) PSC-833, (**C**) colchicine or (**D**) verapamil, and translocation of NBD–C_6-GlcCer was assessed after 20 min at 37 °C. Data points represent means ± range for duplicate determinations; where not visible, error bars fall within the symbols.

proteoliposomes in the absence of ATP. Control samples were treated with the highest concentration of each drug in the absence of ATP. Stable baselines were seen after addition of dithionite in each case, indicating that the integrity of the proteoliposomes was maintained.

The median effect equation was used previously in analysis of inhibition of Pgp-mediated drug transport [29,30] and phospholipid flipping [17]. We analysed the inhibition data using the median effect equation to determine D_m, the substrate or modulator concentration causing 50 % inhibition of NBD–C_6-GlcCer translocation after 20 min (Table 2; see Figure 7 for analysis using data for cyclosporin A). The value of D_m varied from 0.145 μM for the high-affinity modulator PSC-833 to 248 μM for the low-affinity substrate colchicine (Table 2). The efficiency of inhibition of NBD–C_6-GlcCer translocation, as indicated by the values of D_m for the substrates and modulators tested, correlated well with their K_d values for binding to Pgp (measured using fluorescence quenching), which span over four orders of magnitude [34,35]

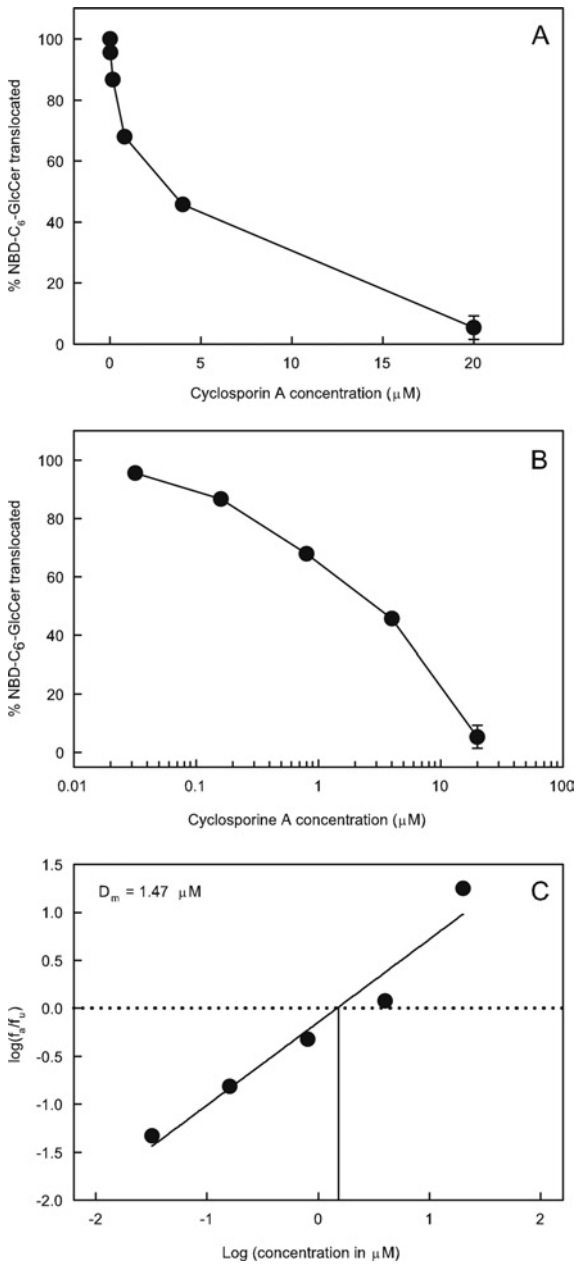

Figure 7 Quantification of inhibition of NBD–C_6-GlcCer translocation by cyclosporin A

(**A**) Net translocation over a 20 min time period was assessed using proteoliposomes containing Pgp in the presence of cyclosporin A, as in Figure 6. (**B**) Data from (**A**) displayed with a logarithmic scale for the independent variable. (**C**) Median effect plot of log (f_a/f_u) against log [cyclosporin A] (see the Experimental section for theory). D_m is determined from the intercept on the *x* axis, where log (f_a/f_u) = 0, as indicated on the plot. Data points represent means ± range for duplicate determinations; where not visible, error bars fall within the symbols.

(Figure 8). These results indicate that GSL translocation and drug transport are likely to occur via the same pathway.

Pgp-mediated flippase activity for NBD–GSL of differing structures

We assessed the ability of reconstituted Pgp to flip several commercially available NBD–GSL (see Figure 1) in an ATP-dependent manner. The chosen GSL had headgroups with one or two sugar residues, and the NBD fluorophore was attached via either a C_6 or a C_{12} acyl chain. This allowed us to assess the effects

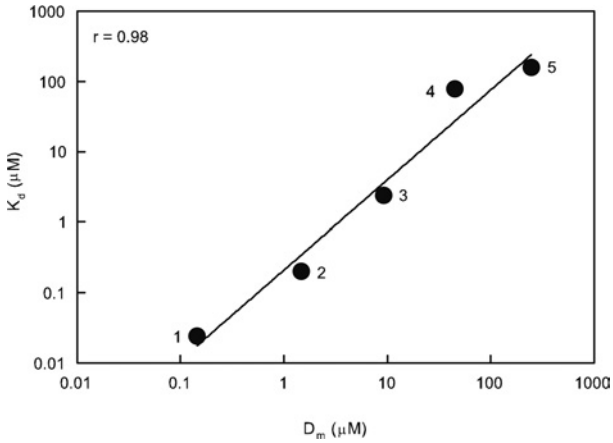

Figure 8 Correlation between inhibition of NBD–C_6-GlcCer flippase activity and affinity of binding to Pgp for several transport substrates and modulators

Agents used were as follows: 1, PSC-833; 2, cyclosporin A; 3, verapamil; 4, leupeptin; 5, colchicine. The D_m values for the compounds are plotted on a log–log scale against their measured K_d values for binding to purified Pgp labelled with MIANS [2-(4-maleimidoanilino)-naphthalene-6-sulphonic acid].

of different structural attributes on the ability of Pgp to mediate GSL translocation. The NBD–C_6-GalCer derivative had a slightly higher inner/outer leaflet ratio than NBD–C_6-GlcCer (Figure 3B), and the extent of translocation was somewhat higher than that of NBD–C_6-GlcCer (Table 1). The addition of a second sugar residue to the headgroup to give LacCer drastically reduced the ability of Pgp to flip this derivative, with the NBD fluorophore attached via either a C_6 (Figure 3C) or a C_{12} (results not shown) chain. Extension of the time of incubation showed that the extent of Pgp-mediated flipping of LacCer reached a maximum at approx. 20 min, and did not increase further up to a time of 90 min (results not shown). Normalized flippase activity for both LacCer derivatives was less than 30 % of the value for NBD–PC (Table 1), indicating that the size and hydrophilicity of the headgroup have a major effect on the ability of Pgp to flip GSL. Further experiments showed that Pgp can flip the fluorescently labelled sphingolipid NBD–C_6-SM, which has a phosphocholine headgroup, to a significantly greater extent than NBD–PC over a period of 20 min (Figure 3D and Table 1).

Interaction of Pgp with NBD–C_{12}-Cer (ceramide)

Pgp is clearly able to flip GSL with a variety of sugar headgroups. Translocation activity is diminished as the size of the headgroup increases (compare GlcCer and GalCer with LacCer; Table 1), and it is greatly increased when the headgroup is changed from a sugar residue to choline (compare GlcCer and GalCer with SM; Table 1). It was, therefore, of interest to see if Pgp could translocate NBD–Cer, which contains the backbone of both SM and GSL, but lacks a choline or sugar residue headgroup. Pgp was reconstituted into egg PC proteoliposomes containing ~0.3 % (w/w) NBD–C_{12}-Cer, and we attempted to monitor the transbilayer distribution by dithionite quenching. At room temperature we observed rapid and complete quenching of NBD–lipid fluorescence (Figure 9), regardless of the length of incubation or the presence or absence of ATP. The presence of this small amount of NBD–Cer should be minimally perturbing to the structure of the proteoliposomes, but it is possible that it caused them to become permeable to dithionite. To assess whether this was a possibility, we prepared egg PC liposomes (lacking Pgp) with 0.15 % (w/w) NBD–C_{12}-Cer and 0.15 % (w/w) NBD–PC (16:0, 6:0). Thus ~50 % of

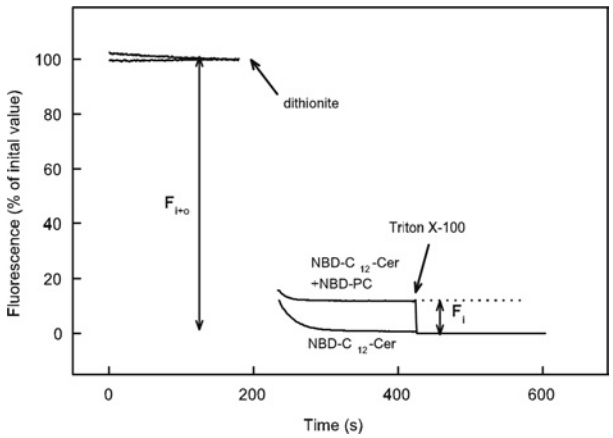

Figure 9 Dithionite quenching of NBD–C_{12}-Cer in proteoliposomes

The bilayer distribution of egg PC proteoliposomes containing Pgp and 0.3% (w/w) NBD–C_{12}-Cer was determined at 22°C using dithionite quenching as described in the legend to Figure 2. A stable plateau due to the fluorescence of inner-leaflet NBD–C_{12}-Cer could not be established. The bilayer distribution was also determined for egg PC liposomes lacking reconstituted Pgp, but including 0.15% (w/w) NBD–PC (16:0, 6:0) and 0.15% (w/w) NBD–C_{12}-Cer. Traces were normalized to the fluorescence intensity recorded just prior to the addition of dithionite. The vertical arrows represent the total fluorescence due to NBD-labelled lipids in both the inner and outer leaflets (F_{i+o}) and the fluorescence due to NBD–lipids in the inner leaflet (F_i).

the fluorescence of the liposomes was due to NBD–PC (16:0, 6:0) and ~50% was due to NBD–C_{12}-Cer. Upon quenching with dithionite, a stable baseline due to the fluorescence of inner-leaflet NBD-labelled lipids was achieved (11.8% of the total NBD–lipid fluorescence; see Figure 9), which indicates that NBD–Cer did not render the liposomes permeable to the quenching agent. The outer/inner leaflet ratios of liposomes lacking Pgp are slightly different compared with those containing Pgp, but these results suggest that all of the NBD–C_{12}-Cer, plus the normal outer-leaflet portion of NBD–PC (16:0, 6:0), was quenched upon addition of dithionite.

We speculated either that all of the NBD–Cer is present in the outer leaflet (or the NBD label was in some other manner accessible to dithionite on the outside of the vesicles), or that NBD–Cer flip-flops between the two leaflets much more rapidly than the time required to measure its distribution by this technique (~200 s). If rapid flip-flop is occurring, it may be slowed down or prevented by decreasing the temperature. To explore this effect, liposomes containing a 1:1 ratio of NBD–PC (16:0, 6:0)/ NBD–C_{12}-Cer were quenched by dithionite at 22°C, 15°C and 5°C. It took longer to form a stable baseline as the temperature decreased; however, the calculated bilayer leaflet distribution of NBD–lipids remained essentially the same (results not shown). We have noted similar behaviour for NBD-labelled cholesterol in which the NBD replaces much of the cholesterol alkyl chain (P. D. W. Eckford and F. J. Sharom, unpublished work). Thus it appears that NBD–lipids lacking a hydrophilic headgroup flip too rapidly to allow assessment of their bilayer leaflet distributions.

DISCUSSION

We reported previously that Pgp flipped NBD-labelled phospholipids with a variety of headgroups, acyl chain lengths, and fluorophore locations [17]. The present work shows for the first time that reconstituted Pgp can function as a GSL flippase, translocating the simple glycolipids NBD–C_6-GlcCer and NBD–C_6-GalCer to a similar extent as NBD–PC (16:0, 6:0). Pgp also translocated NBD–C_6-SM, a phosphosphingolipid, at a high rate. The addition of a second sugar to the headgroup to form NBD–LacCer drastically reduced the rate of flipping compared with NBD–PC, probably because of the increased size and hydrophilicity contributed by the additional sugar residue. We anticipate that Pgp will be unable to flip GSL derivatives with headgroups larger than two sugar residues. The transbilayer distribution and ability of Pgp to translocate NBD–Cer could not be determined, probably because flipping of this lipid occurs very rapidly. Several ABC superfamily members are known to be involved in lipid translocation [36], and one normal physiological function of Pgp may be as a flippase for endogenous lipids. van Meer and colleagues [37] recently suggested that outward movement of natural PC in intact mammalian cells is probably mediated by an ABC transporter. One limitation of using fluorescent lipids is the possibility that the modifications in structure may result in activity that is not present in the corresponding natural lipid. The NBD group alone is unlikely to be a substrate for Pgp [17]; in fact, because of its hydrophilicity, it may in fact lower the flippase activity of Pgp towards these GSL analogues compared with the unmodified membrane lipids. Measuring flip-flop of natural membrane lipids relies on methods that involve large-scale enzymatic degradation of lipids in the outer leaflet, coupled to inner-to-outer-leaflet flip-flop [38]. Unfortunately, such methods are very difficult to apply to an ABC protein that requires ATP in the vesicle lumen to achieve such translocation.

Both membrane transporters [39] and transmembrane peptides [40] can accelerate flip-flop of phospholipids, and it was proposed that the membrane-spanning domains of integral proteins can mediate this process merely by their presence in the membrane [40]. In the case of Pgp, the GSL flip-flop process appears to be intimately associated with its active drug transport function, in that flipping of NBD–C_6-GlcCer was dependent on both the concentration of NBD–lipid in the bilayer and the concentration of ATP. Hydrolysis of ATP is required, as neither ADP nor p[NH]ppA could substitute for ATP. Trapping of Pgp in a stable (but inactive) transition-state analogue by vanadate also inhibited translocation, further supporting the view that flipping was totally dependent upon ATP hydrolysis. Several Pgp substrates and modulators inhibited flipping of NBD–C_6-GlcCer in a concentration-dependent, saturable fashion, and the efficiency of inhibition of flip-flop was highly correlated with the affinity of the drugs for binding to Pgp. This suggests that the drug binding/transport and GSL flippase activities of Pgp occur via the same pathway, rather than the flip-flop process being promoted by the membrane-spanning regions of the protein. This conclusion lends further weight to the proposal that Pgp is a broad-specificity flippase for drugs, membrane phospholipids and simple glycolipids.

Pgp-mediated flip-flop of lipids to the inner leaflet approached a maximum after approx. 60 min of incubation with ATP at 37°C. This phenomenon is similar to the establishment of a steady-state drug concentration gradient observed in transport experiments in proteoliposomes [41,42]. Further movement of NBD-labelled lipid from the outer to the inner leaflet is probably prevented by packing constraints in the inner leaflet, which would lead to more rapid spontaneous flip-flop of the probe back to the outer leaflet.

Some chemotherapeutic drugs (e.g. daunorubicin) kill their target cells by inducing apoptosis, mediated by Cer production [43,44]. One mechanism of resistance to chemotherapy involves up-regulation of GlcCer synthase [45], which forms GlcCer from Cer. This decreases the pool of Cer generated and therefore inhibits Cer-mediated signalling processes, such as apoptosis. GlcCer synthase is present in the Golgi apparatus, and synthesis

of GlcCer occurs at the cytosolic leaflet of this organelle. This orientation is noteworthy, because GlcCer is a precursor for LacCer and all other GSL, and the active sites of the enzymes synthesizing these lipids are oriented towards the Golgi lumen. This implies that there must be a mechanism to flip newly made GlcCer from its site of synthesis on the cytosolic leaflet to the lumenal Golgi leaflet, where it can be metabolized further. Some of the cellular pool of Pgp is present in the Golgi apparatus [25], and the present results suggest that it may be involved in this flip-flop process. Buton et al. [46] reported that natural flip-flop of GlcCer and GalCer in lipid vesicles was slow (half-time of 2–5 h at 20 °C), whereas in rat liver endoplasmic and Golgi, flip-flop was rapid and ATP-independent, with a half-time of 3 min. Pgp may operate in concert with this energy-independent flippase.

The sphingolipid composition of several MDR cell lines is altered from that of normal cells (reviewed in [47]). In particular, GlcCer, GalCer and SM levels are elevated in Pgp-overexpressing cells compared with their drug-resistant parent. Cer exerts its signalling function in the inner leaflet of the plasma membrane, and the pool of SM from which Cer is generated must be present in this leaflet. If Pgp were to translocate SM, GlcCer or Cer from the inner to the outer leaflet of the plasma membrane, Cer-mediated signalling, including apoptosis, would be inhibited. The Pgp modulator PSC-833 restored the normal membrane distribution of SM in KG1a cells [48], and re-established the production of Cer from SM in MDR HL-60 cells [49]. In ovarian carcinoma cells, a variety of GSL, including GlcCer, increased sensitivity to doxorubicin, implying that these lipids inhibit the drug transport function of Pgp [50]. Other work with intact drug-resistant cells and cells expressing recombinant Pgp also suggests that simple GSL, and GlcCer in particular, may be flipped by Pgp [12,15,25]. However, whole-cell systems are highly complex, with many different pathways for the movement of lipids, and a direct interaction of Pgp with simple GSL such as GlcCer has not been conclusively demonstrated until now.

Based on the results of the present work, and previous data, we conclude that Pgp functions as an outwardly-directed flippase with broad specificity for simple GSL and membrane phospholipids. This lipid translocation process shares many of the fundamental characteristics of drug transport, such as dependence on ATP hydrolysis and vanadate sensitivity, and drugs compete with lipid flippase activity, suggesting that membrane lipids probably follow the same path through the Pgp molecule as drug substrates. Further investigation of the route and mechanism of lipid translocation may help to shed light on the process of drug transport.

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