Phospholipid Flippase Activity of the Reconstituted P-Glycoprotein Multidrug Transporter†

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ABSTRACT: The P-glycoprotein multidrug transporter acts as an ATP-powered efflux pump for a large variety of hydrophobic drugs, natural products, and peptides. The protein is proposed to interact with its substrates within the hydrophobic interior of the membrane. There is indirect evidence to suggest that P-glycoprotein can also transport, or "flip", short chain fluorescent lipids between leaflets of the membrane. In this study, we use a fluorescence quenching technique to directly show that P-glycoprotein reconstituted into proteoliposomes translocates a wide variety of NBD lipids from the outer to the inner leaflet of the bilayer. Flippase activity depended on ATP hydrolysis at the outer surface of the proteoliposome, and was inhibited by vanadate. P-Glycoprotein exhibited a broad specificity for phospholipids, and translocated phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin. Lipid derivatives that were flipped included molecules with long, short, unsaturated, and saturated acyl chains and species with the NBD group covalently linked to either acyl chains or the headgroup. The extent of lipid translocation from the outer to the inner leaflet in a 20 min period at 37 °C was directly estimated, and fell in the range of 0.36−1.83 nmol/mg of protein. Phospholipid flipping was inhibited in a concentration-dependent, saturable fashion by various substrates and modulators, including vinblastine, verapamil, and cyclosporin A, and the efficiency of inhibition correlated well with the affinity of binding to Pgp. Taken together, these results suggest that P-glycoprotein carries out both lipid translocation and drug transport by the same path. The transporter may be a generic flippase for hydrophobic molecules with the correct steric attributes that are present within the membrane interior.

The development of resistance to multiple chemotherapeutic drugs in human cancers has been linked to the expression of P-glycoprotein (Pgp),[1] a member of the ABC superfamily (*1*, *2*). This efflux pump exports a wide range of hydrophobic natural products, drugs, and linear and cyclic peptides from the plasma membrane of eukaryotic cells, using the energy from ATP hydrolysis (reviewed in refs *3*−*5*). Pgp is believed to extract its substrates from the lipid bilayer, rather than the aqueous medium, and has been described as a "hydrophobic vacuum cleaner" (*6*). This proposal is supported by substantial experimental evidence (*7*), including the recent finding that the affinity of binding of drugs to reconstituted Pgp is modulated by their ability to partition into the membrane (*8*).

Two classes of Pgp genes exist. The class I proteins (exemplified by the human MDR1 gene product) are multidrug transporters, whereas the class III Pgp (the human MDR3 gene product) is known to be a phospholipid translocase, or flippase, specific for phosphatidylcholine (PC) (*9*, *10*). Class III Pgp is abundant in the hepatocyte canalicular membrane, and studies with knockout mice have shown that its role is to export PC into the bile (*11*). The high degree of sequence similarity (78%) between the human MDR1 and MDR3 proteins suggests that they may function by the same basic mechanism, but differ in substrate specificity. Recent domain switching experiments confirm this idea; substitution of three amino acid residues of MDR1 into transmembrane (TM) segment 6 in the N-terminal half of the protein allowed the transport of several drugs by an MDR3 backbone (*12*, *13*). A recent report indicates that the class III Pgp can also transport MDR spectrum drugs, and short chains PCs, although at a rate substantially slower than that observed for natural PC species (*14*). The multidrug transporting Pgps may thus operate as drug "flippases" (*6*), and it has been proposed that they remove their substrates from the cytoplasmic leaflet of the plasma membrane (*15*).

Several studies have suggested that the MDR1 multidrug transporting Pgp is also able to move phospholipid derivatives from the inner to the outer leaflet in intact cells. Human MDR1 in transfected polarized epithelial cells was reported to transport short chain fluorescent derivatives of PC, phosphatidylethanolamine (PE), sphingomyelin (SM), and glucosylceramide (GlcCer) (*16*−*18*). The level of accumulation of fluorescent derivatives of PC and PE was also reduced

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[1] Abbreviations: ABC, ATP-binding cassette; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DLS, dynamic light scattering; GlcCer, glucosylceramide; MDR, multidrug resistance; NB, nucleotide binding; NBD, nitrobenz-2-oxa-1,3-diazole; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Pgp, P-glycoprotein; SM, sphingomyelin; TM, transmembrane; TX-100, Triton X-100.

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in Pgp-expressing cells (*19*, *20*), implying that they are substrates for the transporter.

Other members of the ABC superfamily have been reported to be able to mediate the transbilayer movement of membrane lipids (reviewed in ref *21*). The glutathione-dependent multidrug transporter, MRP1, has been implicated in the outward transport of fluorescent PC, PS, SM, and GlcCer across the plasma membrane of eukaryotic cells (*22*−*25*). The novel ABC transporter, ABC1 (defective in Tangier disease), appears to mediate cholesterol efflux, and ABCR (defective in Stargardt's macular dystrophy) is likely an outwardly directed flippase for *N*-retinylidine-PE. A study of several yeast ABC multidrug transporters suggested that Pdr5p and Yor1p can transport C_6-NBD-PE, since cells lacking the genes for these proteins showed increased levels of accumulation of the fluorescent derivatives (*26*). In the only study to use reconstituted systems to date, the LmrA multidrug transporter of *Lactococcus lactis* was recently shown to mediate the transbilayer movement of a fluorescent phospholipid following purification and insertion into proteoliposomes (*27*).

Many studies of transmembrane phospholipid distribution have used fluorescent (often NBD-labeled) lipid derivatives. Short chain fluorescent analogues of phospholipids or fatty acids are able to insert spontaneously into the outer leaflet of the plasma membrane when added extracellularly. Two approaches have been developed for the assessment of their transmembrane distribution in intact cells. The first method involves back extraction of the short chain derivative from the outer leaflet of the plasma membrane with bovine serum albumin (see, for example, refs *16* and *28*). In the other technique, the impermeant quenching agent dithionite is added to the outer leaflet, and allows estimation of the transbilayer distribution at any point in time (*29*−*31*). The latter approach has been shown to be experimentally superior, and gives good time resolution with low errors (*29*).

To date, all of the studies implicating MDR1 Pgp in lipid flippase activity have used intact cells. This has restricted the lipids under study to short chain fluorescent lipid derivatives that spontaneously insert and can be back-extracted, so assessment of substrate specificity has been very limited. In addition, quantitative characterization of the translocation process has not been carried out, since the absolute amount of fluorescent lipid incorporated into the cells was unknown. Reconstituted systems, on the other hand, allow incorporation of a very wide range of fluorescent membrane phospholipids, including those with two long (C16 or C18) acyl chains, and those with the fluorescent labels incorporated into either the headgroup or the acyl chain. Since the amount of incorporated fluorescent lipid is well-defined, reconstituted systems also allow quantitative comparison of the transbilayer movement of the lipid. Finally, the use of intact cells necessarily gives rise to uncertainties as to nature of the flippase activity, and whether its relationship to Pgp is direct or indirect.

The present study uses the dithionite quenching technique to unambiguously demonstrate that Pgp in reconstituted proteoliposomes mediates the transbilayer movement of a wide variety of NBD-labeled membrane phospholipids. Transport from the outer to the inner leaflet of proteoliposomes by inward-facing Pgp molecules took place in an ATP-dependent and vanadate-sensitive fashion. As well as investigating the lipid headgroup and acyl chain specificity of the Pgp flippase, we were also able to quantitate its activity. In addition, we show that phospholipid flipping was efficiently inhibited in a concentration-dependent, saturable fashion by various known Pgp substrates and modulators. Quantitation of the inhibition of flippase activity suggested that Pgp carries out both lipid translocation and drug transport by the same path.

MATERIALS AND METHODS

Materials. Egg PC and all NBD-labeled lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Sodium dithionite, sodium orthovanadate, AMP-PNP, ATP, CHAPS, Triton X-100, vinblastine, verapamil, pepstatin A, and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclosporin A was provided by Pfizer Central Research (Groton, CT). B. Sarkadi (National Institute of Haematology and Immunology, Budapest, Hungary) supplied reversin 121 (*32*).

Pgp Isolation and Reconstitution. Pgp was isolated from the plasma membrane of the highly drug resistant CH^RB30 cell line using a two-step detergent extraction as described previously (*33*, *34*), except that the resulting preparation, which contained >85% pure Pgp, was in 15 mM CHAPS buffer. Protein was assayed using the technique of Peterson (*35*), using bovine serum albumin (Sigma Chemical Co., crystallized and lyophilized) as a standard. The ATPase activity of reconstituted Pgp was quantitated using a colorimetric assay as described previously (*36*), using 1 mM ATP and an assay time of 20 min.

Pgp was reconstituted into proteoliposomes by a gel filtration technique (*34*, *37*, *38*). Briefly, 5 mg of egg PC and 15 μg of NBD-labeled lipid [dissolved in a 4:1 (v/v) $CHCl_3$/MeOH mixture] were dispensed into a glass tube, dried under a gentle stream of N_2, and pumped in vacuo for 30 min to remove traces of organic solvent. The lipid sample was then dissolved in 250 μL of 200 mM CHAPS in reconstitution buffer [50 mM Tris-HCl, 100 mM KCl, and 5 mM $MgCl_2$ (pH 7.4)] by warming to 37 °C, and 0.5 mg of Pgp in 15 mM CHAPS buffer was added. After periodic mixing on ice for 30 min, CHAPS was removed by passing the mixture through a column of Sephadex G-50 (1 cm × 10 cm), which was equilibrated and eluted with reconstitution buffer. Turbid fractions were collected and pooled (total volume of approximately 2.8 mL). The proteoliposomes were resuspended with a fine gauge needle and kept at 4 °C until they were ready to be used. The final lipid:protein ratio of the reconstituted proteoliposomes fell in the range of 10:1 to 15:1 (w/w). For experiments involving proteoliposomes lacking Pgp, the vesicles were prepared as outlined above, except that instead of purified Pgp, a comparable volume of 15 mM CHAPS buffer was added to the detergent/lipid mixture. The asymmetry of Pgp incorporation was tested for two different proteoliposome preparations, using measurements of ATPase activity following vesicle permeabilization, as described previously (*34*, *37*). Dynamic light scattering (DLS) measurements to determine the size profile of reconstituted proteoliposomes were carried out as described in previous studies (*34*, *39*). Liposome suspensions were transferred to a square cuvette (SO, Hellma, Concord, ON) and analyzed as previously described (*40*), using a BI9000AT

autocorrelator (Brookhaven Instruments Corp., Holtsville, NY). Intensity distributions were determined using a non-negative least-squares fitting routine (*41*).

Fluorescence Quenching Assay. Samples were prepared by adding 200 μL of proteoliposomes (lipid concentration of ~1.5 mg/mL) to 230 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. Each sample therefore contained approximately 0.3 mg of egg PC and 1.2−1.4 nmol of NBD-labeled lipid [0.3% (w/w) labeled lipid]. To initiate lipid transport, 50 μL of transport buffer containing ATP and an ATP regenerating system (*42*) was added to the proteoliposome sample (final concentrations were 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. Transport was terminated by adding 20 μL of sodium orthovanadate in transport buffer (final concentration was routinely 200 μM). The sample was then transferred to a 1 mL quartz cuvette (path length of 0.5 cm), and the fluorescence emission was recorded at 22 °C with a PTI Quantamaster C-61 steady state fluorimeter (Photon Technology International, London, ON), using excitation and emission wavelengths of 468 and 540 nm, respectively, and slit widths of 2 nm. After a baseline was established (approximately 5 min), 2 μL of sodium dithionite solution in 1 M Tris-HCl (pH 10.0) was added to quench the fluorescence of NBD-labeled lipids in the outer leaflet of the proteoliposomes (final concentration of dithionite of 2 mM). When a baseline was re-established (approximately 5 min), the proteoliposomes were permeabilized by adding 50 μL of 10% TX-100 (final concentration of 1%). The fluorescence trace was continued for an additional 5 min, or until a new baseline was apparent. For control experiments, 50 μL of transport buffer was added instead of ATP, and the vanadate was replaced with 20 μL of transport buffer.

The percent NBD-labeled lipid in the proteoliposomes that was accessible to dithionite (outer leaflet), or protected from dithionite (inner leaflet), was calculated using the following equations

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

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

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 TX-100. F_T, F_D, and F_0 were determined by averaging the last 10 data points (covering 10 s) of the recorded fluorescence traces.

The net percent NBD-labeled lipid translocated to the inner leaflet in the 20 min period was calculated from the difference in transbilayer distribution between Pgp proteoliposomes with and without ATP. Using the known amount of NBD-labeled phospholipid in the sample, this was converted into the net number of nanomoles translocated in the 20 min period per milligram of protein.

Competition between Lipid Flippase Activity and Drug Transport. Fluorescence traces were recorded essentially as outlined above, by initially mixing 200 μL aliquots of the proteoliposomes with 220 μL of transport buffer. Prior to initiation of lipid transport with ATP, a 10 μL aliquot of the desired drug (in 10% DMSO) or peptide (in 100% DMSO) was added to the proteoliposome sample. Control samples were treated with either 10 or 100% DMSO instead of a drug/peptide mixture, prior to initiation of lipid transport. The data were analyzed according to the median effect equation (*43*, *44*), which has previously been applied to Pgp-mediated drug transport (*45*, *46*). The basic median effect equation is

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m$$

where f_a is the fraction of the system that is affected at a concentration D (in this case, the fractional inhibition of NBD-PC translocation at 20 min), 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. Note that both f_a and f_u can vary between 0 and 1, and $f_a = 1 - f_u$. One transformation of the median effect equation is as follows

$$\log\left(\frac{f_a}{f_u}\right) = m \log D_m - m \log D_m$$

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

RESULTS

Incorporation of Fluorescent Lipid Probes into Reconstituted Pgp Proteoliposomes. Pgp was isolated from MDR Chinese hamster ovary cells using the detergent CHAPS and reconstituted into proteoliposomes of egg PC at a lipid:protein ratio in the range of 10:1 to 15:1 (w/w), using a gel filtration technique developed previously (*34*, *37*, *38*). Previous characterization of this reconstituted system using the synthetic phospholipid dimyristoylphosphatidylcholine (*34*) indicated that the proteoliposomes were sealed and largely unilamellar, with a relatively homogeneous size distribution. Pgp reconstituted in this fashion retains a high level of ATPase activity, and actively transports drugs and peptides (*37*, *47*). DLS measurements were carried out on proteoliposomes of egg PC containing Pgp, and protein-free liposomes of egg PC alone, prepared by the gel filtration method. The resulting size profile for protein-free liposomes of egg PC showed the existence of two populations of vesicles (Figure 1A). The majority of the vesicles had diameters in the range of 20−70 nm (mean ~ 33 nm), and the diameters of the larger subpopulation fell in the range of 150−300 nm (mean ~ 230 nm). Proteoliposomes containing Pgp exhibited a slightly different distribution (Figure 1B). The smaller vesicle subpopulation displayed a bimodal distribution, with mean diameters of ~30 and ~70 nm (the range for both was 20−100 nm), while the mean diameter of the larger subpopulation remained unchanged at ~230 nm.

Various NBD-labeled phospholipids (PC, PE, and PS) were incorporated into the bilayers at 0.3% (w/w) of the total lipid (see below); this low level of tracer is expected to be

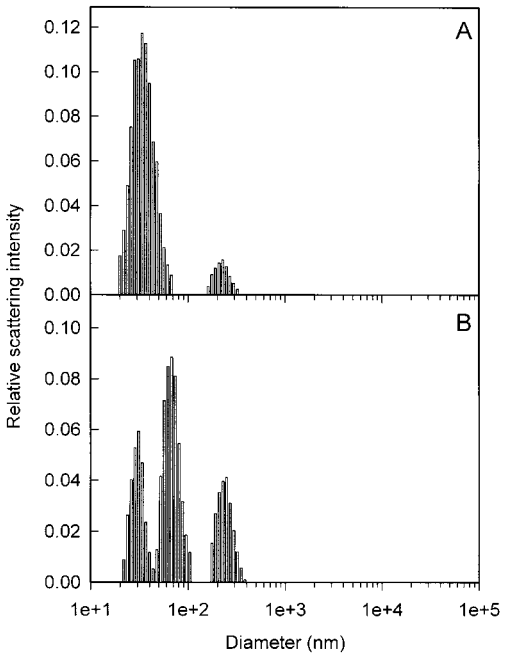

FIGURE 1: Size distribution of liposomes of egg PC alone and egg PC proteoliposomes containing reconstituted Pgp. DLS measurements were carried out to determine the size profile of liposomes of egg PC alone (A) and egg PC proteoliposomes containing Pgp (B) at a lipid:protein ratio of ~20:1 (w/w). Both were prepared via removal of detergent by gel filtration chromatography, as described in Materials and Methods. The scattering intensity of each peak is proportional to the lipid mass present.

minimally perturbing to the bilayer structure. Determination of the orientation of the reconstituted Pgp was carried out for two representative preparations, using the increase in ATPase activity following detergent permeabilization (see refs *34* and *37*). The results indicated that the majority of the Pgp molecules (70−80%) faced inward, with their NB domains exposed on the outer surface of the vesicle. A preference for the inward-facing orientation was previously noted for dimyristoylphosphatidylcholine proteoliposomes (*34*), and likely reflects a preference for location of the bulky NB domains on the exterior due to steric crowding at the internal surface of the vesicles, which will have significant curvature because of their relatively small diameter.

Optimization of the Dithionite Quenching Technique for Reconstituted Pgp Proteoliposomes. The fluorescence quenching technique we used relies on the ability of dithionite to quench the fluorescence of NBD-labeled lipids in only the outer membrane leaflet, due to bilayer impermeability (*29*). Initial experiments were carried out to optimize the dithionite concentration for efficient quenching of the fluorescence of NBD-labeled lipids in the outer leaflet, with no penetration into the inner leaflet. Experiments were performed at 37 °C to determine the minimum concentration of dithionite required to quench approximately 1 nmol of headgroup-labeled NBD-PE (di-16:0) incorporated into egg PC vesicles [0.3% (w/w) NBD-PE], which were generated by the same method as those containing Pgp. A stable baseline could not be established (fluorescence continued to decrease indefinitely) following addition of 20 or 10 mM dithionite, suggesting that at these concentrations, dithionite was permeating the vesicles. More stable baselines were established using 2 and 1 mM dithionite; however, if the vesicle samples were analyzed following addition of both 1 mM dithionite and 1% TX-100, all of the fluorescence of the sample was not quenched, suggesting that 1 mM dithionite was not sufficient to quench the fluorescence of 1 nmol of NBD-PE. However, If the samples were treated with 2 mM dithionite and 1% TX-100, the fluorescence was quenched to background levels; therefore, this dithionite concentration was used in future experiments.

To select the amount of NBD-labeled lipid to be used in the flippase assays, 2 mM dithionite and 1% TX-100 (final concentrations) were added to vesicle samples containing various quantities of headgroup-labeled NBD-PE (di-16:0), to determine the largest amount of NBD-labeled lipid that could be completely quenched by 2 mM dithionite. For these experiments, different quantities of egg PC vesicles were diluted in transport buffer to yield samples containing 0.1, 0.5, 1.0, and 1.5 nmol of NBD-PE. Fluorescence was completely quenched in all of these samples, indicating that 2 mM dithionite was sufficient to quench up to 1.5 nmol of NBD-labeled lipid. For all future assays, the reconstituted samples contained 1 nmol of NBD-labeled lipid, and the reactions were quenched with 2 mM dithionite and 1% TX-100.

The baselines in the fluorescence traces recorded at 37 °C were not completely flat following addition of 2 mM dithionite, indicating a small amount of dithionite penetration of the egg PC bilayers at this temperature. When reduction was performed at 22 °C, the baselines that were obtained were perfectly flat, indicating that at this temperature, 2 mM dithionite was unable to penetrate the bilayer. Therefore, all flippase reactions were performed at 37 °C, but the dithionite reduction was carried out at 22 °C; fluorescence traces were also recorded at this temperature. Similar optimization experiments were carried out with reconstituted proteoliposomes containing Pgp, with identical results.

The dithionite reduction technique depends on the structural integrity of the membrane systems under study. There is convincing evidence that the vesicles used in these experiments are not leaky. First, we have used identical reconstituted vesicles in transport experiments, where they take up drug into the lumen against a concentration gradient. Such a concentration gradient has been shown for colchicine (*37*), a linear tripeptide (*47*), and a rhodamine dye (*48*), and is dependent on well-sealed vesicles. To demonstrate the experimental results that would be observed in the case of leakiness, we used a much lower concentration of 0.01% Triton X-100 with Pgp proteoliposomes. As shown in Figure 2B, there was a slow decrease of the inner leaflet NBD fluorescence to zero over time, indicating slow permeation of dithionite into the vesicle interior. Identical results were obtained in the presence of 0.01% Triton X-100 and 100 μM vinblastine, which inhibits Pgp flippase activity (see below). In a second series of experiments, we added various CHAPS concentrations to the lipid vesicles before dithionite. Previous drug transport experiments have indicated that CHAPS concentrations of 1−4 mM are sufficient for permeabilization of Pgp proteoliposomes (*37*, *48*). When using 1 (see Figure 2C), 2, and 4 mM CHAPS, no stable baseline was obtained on addition of dithionite; all the NBD fluorescence was progressively quenched over a period of minutes, as the dithionite entered the vesicle lumen. The higher the CHAPS concentration, the more rapid this quenching. However, when a much lower CHAPS concen-

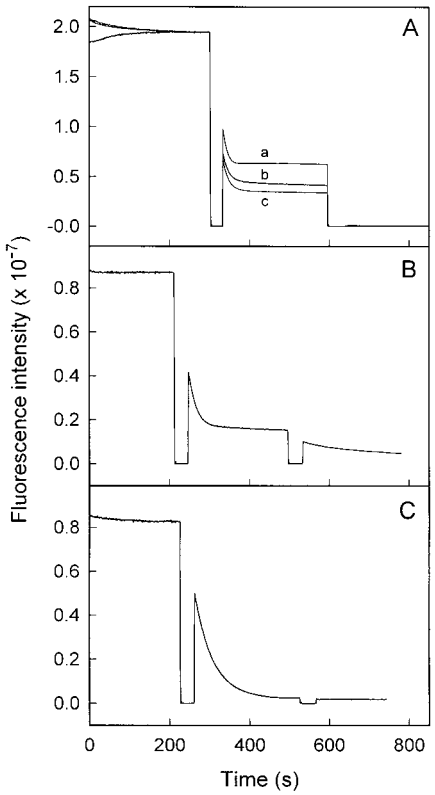

FIGURE 2: Reaction of dithionite with reconstituted proteoliposomes containing Pgp and NBD-PC. (A) Proteoliposomes of egg PC containing Pgp (curves b and c) or liposomes of egg PC alone (curve a) containing 0.3% (w/w) fluorescently labeled phospholipid NBD-PC (16:0, 6:0) were incubated at 37 °C in buffer with 1 mM ATP and a regenerating system for the desired time, as indicated in Materials and Methods. After termination of Pgp transport activity by addition of vanadate, the sample was transferred to a cuvette, and the fluorescence emission at 540 nm was monitored at 22 °C using excitation at 468 nm, until a stable baseline was achieved. At 300 s, dithionite was added to a final concentration of 2 mM (indicated by an interruption in the trace), and the sample was incubated until a stable baseline was again reached. At 600 s, Triton X-100 [final concentration of 1% (w/v)] was added to make the vesicles permeable to dithionite. The fluorescence trace obtained for liposomes with no Pgp after incubation for either 0 or 20 min with ATP and a regenerating system is indicated by curve a. Curves b and c show the fluorescence traces for reconstituted Pgp proteoliposomes 20 min after adding ATP and immediately after adding ATP (time zero), respectively. Traces were normalized to the fluorescence intensity just prior to addition of dithionite. (B) A similar experiment was carried out using Pgp proteoliposomes in the absence of ATP, but adding a much lower Triton X-100 concentration of 0.01% (w/v). The progressive decrease in fluorescence arises from slow leakage of dithionite into the vesicle lumen. (C) A similar experiment was carried out using Pgp proteoliposomes in the absence of ATP, but adding 1 mM CHAPS (a permeabilizing concentration) prior to dithionite. No stable baseline is achieved, indicating complete quenching of NBD-labeled lipids in the inner leaflet.

tration of 0.04 mM was added (drug transport experiments indicate that this does not induce leakiness), two-step quenching of outer and inner leaflet lipids was observed. The addition of 100 μM vinblastine together with 0.04 mM CHAPS did not alter this behavior. The Pgp proteoliposomes always displayed a highly stable baseline after dithionite addition, and never showed this type of slow decrease in fluorescence, indicating that they are nonleaky both in the absence and in the presence of drug substrates.

Estimation of the Transbilayer Distribution of NBD-Labeled Phospholipids. The fluorescent phospholipid NBD-PC (16:0, 6:0) was incorporated into liposomes of egg PC alone at 0.3% (w/w). Addition of 2 mM dithionite led to an immediate quenching of the fluorescence of NBD-PC in the outer leaflet, until a stable baseline was reached (Figure 2, curve a). The remaining fluorescence, which represents the inner leaflet lipid, was completely quenched by dithionite on subsequent addition of TX-100 (Figure 2, curve a). Calculation of the distribution of NBD-PC between the outer and inner leaflet based on the relative fluorescence intensities indicated that 68% of the lipid was localized in the outer leaflet, and 32% in the inner leaflet (see Table 1). Packing geometry considerations based on the radius of curvature of small vesicles result in more lipid being located in the outer leaflet (*29*). The expected ratio of outer to inner leaflet lipid [estimated using the equation of Thomas and Poznansky (*49*)] for vesicles 33 nm in diameter is 64:36, which is in good agreement with the experimental results.

In the next series of experiments, proteoliposomes containing Pgp were incubated in the presence of ATP and a regenerating system, activity was stopped by addition of vanadate, and dithionite reduction was carried out as described above (Figure 2, curve c). The transbilayer distribution of NBD-PC was altered in reconstituted proteoliposomes, with 83% of the NBD-PC in the outer leaflet and 17% in the inner leaflet. This shift of fluorescent lipid into the outer leaflet was observed for proteoliposomes containing all the NBD-labeled phospholipids tested in this study (see Table 1). Since the Pgp is also oriented asymmetrically, so that most of the NB domains are on the vesicle exterior, this effect may arise from specific interactions of the fluorescent derivatives with domains of the Pgp molecule in either the outer leaflet or the cytoplasmic face of the protein. The presence of 100 μM vinblastine did not alter the transbilayer distribution of the NBD-labeled lipids in the presence of Pgp. After incubation with ATP and a regenerating system for 20 min to allow lipid flippase activity, there was a clear decrease in the amount of NBD-PC in the outer leaflet, and a corresponding increase in the inner leaflet (Figure 2, curve b), indicating ATP-dependent transfer of the lipid from the outer to the inner leaflet. If the NBD-labeled lipid were being somehow buried within the protein in the bilayer, it would not be completely accessible to dithionite quenching.

Characterization of Pgp-Mediated Phospholipid Flippase Activity. The transbilayer distribution of NBD-PC (16:0, 6:0) was determined after incubation of Pgp proteoliposomes for 20 min under various conditions. As shown in Figure 3, substantially more fluorescent phospholipid was present in the inner leaflet in the presence of ATP, compared to an identical sample incubated in the absence of ATP. Increasing the Pgp content of the proteoliposomes resulted in a proportionate increase in the amount of NBD-labeled lipid translocated to the inner leaflet. For example, a proteoliposome sample containing 18 μg of Pgp displayed a net translocation of NBD-PC (16:0, 12:0) of 0.6 $\pm$ 0.1% after 20 min, whereas a sample with the same amount of lipid and 36 μg of protein translocated 1.6 $\pm$ 0.3% of the labeled lipid in the same time period. This flippase activity was progressively inhibited by increasing concentrations of vanadate, a known inhibitor of Pgp ATPase activity (*50*).

Table 1: Transbilayer Phospholipid Distribution and Phospholipid Flippase Activity for Proteoliposomes Containing Reconstituted Pgp

fluorescent phospholipid	location of fluorophore[a]	λ_{ex} (nm)	λ_{em} (nm)	outer:inner leaflet ratio[b]		net lipid translocation[c] (nmol/mg of protein)
				without Pgp	with Pgp	
NBD-PC (16:0, 6:0)	acyl chain	466	564	68:32	83:17	1.03 ± 0.11
NBD-PC (18:1, 6:0)	acyl chain	468	542	70:30	81:19	1.63 ± 0.05[d]
NBD-PC (16:0, 12:0)	acyl chain	468	542	69:31	83:17	0.47 ± 0.07
NBD-PE (di-16:0)	headgroup	468	535	49:51	68:32	0.58 ± 0.03
NBD-PE (di-18:1)	headgroup	459	597	35:65	61:39	0.65 ± 0.13
NBD-PE (16:0, 6:0)	acyl chain	468	540	61:39	77:23	0.47 ± 0.09
NBD-PS (16:0, 6:0)	acyl chain	468	542	65:35	79:21	0.48 ± 0.10
NBD-PS (16:0,12:0)	acyl chain	468	543	59:41	75:25	0.36 ± 0.13[d]
NBD-SM (6:0)	acyl chain	468	540	75:25	85:15	1.83 ± 0.37[d]

[a] The NBD fluorophore is covalently linked to either the headgroup (PE) or the C_6 or C_{12} acyl chain. [b] The transbilayer distribution was calculated by the dithionite quenching technique as described in Materials and Methods, for liposomes of egg PC alone or proteoliposomes containing reconstituted Pgp. [c] The net translocation of NBD-labeled phospholipid in a 20 min period, calculated as the difference between proteoliposomes with and without ATP. [d] $n = 2$; for all other determinations, $n = 4$.

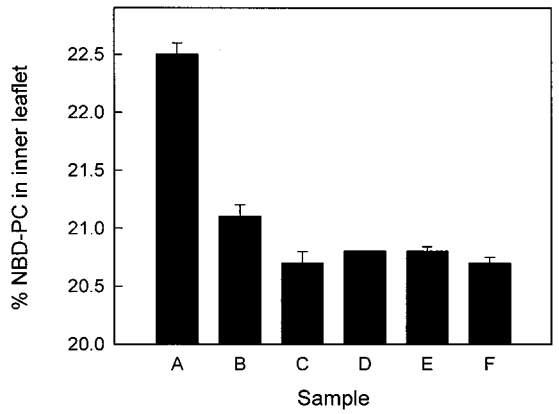

FIGURE 3: Transbilayer distribution of NBD-PC (16:0, 6:0) in reconstituted Pgp proteoliposomes determined after incubation for 20 min under the following conditions: (A) 1 mM ATP, (B) 1 mM ATP with 100 μM vanadate, (C) 1 mM ATP with 200 μM vanadate, (D) 1 mM ATP with 500 μM vanadate, (E) 1 mM AMP-PNP, and (F) no ATP. Data points represent the means for duplicate determinations.

At a concentration of 100 μM, vanadate inhibited NBD-PC translocation by ~75%, and 200 and 500 μM vanadate reduced flippase activity to background levels. Hydrolysis of ATP was required, since the nonhydrolyzable analogue AMP-PNP did not support fluorescent lipid translocation. These characteristics are shared by the drug transport activity of reconstituted Pgp (*37*, *51*).

Translocase Activity for Various NBD-Labeled Phospholipids. Various NBD-labeled phospholipids were incorporated into liposomes of egg PC alone, or egg PC containing reconstituted Pgp. These included species with different acyl chain lengths and unsaturation, various headgroups (PC, PE, and PS), and a sphingosine backbone (SM). In addition, the site of covalent linkage of the NBD group was either at the end of the acyl chain (C_6 or C_{12}) or in the headgroup region. The outer:inner leaflet distribution ratio for these labeled lipids in protein-free liposomes was generally similar to that seen for NBD-PC (16:0, 6:0) described above, in the approximate range of 60:40 to 70:30. These NBD-labeled lipids therefore appear to distribute randomly across the bilayer, with the outer:inner leaflet ratios as expected on the basis of the curvature of the vesicles. However, a few fluorescent lipids exhibited an altered distribution; substantially more of the di-16:0 and di-18:1 headgroup-labeled PEs preferred the inner leaflet (ratios of 49:51 and 35:65, respectively; see Table 1). An NBD-labeled PE was previously noted to show a preference for the inner leaflet in contrast to the behavior of the unlabeled lipid (*31*). Labeled SM showed a preference for the outer leaflet, displaying the largest outer:inner leaflet ratio of 75:25. There have been no reports of the transbilayer distribution of this fluorescent sphingolipid in synthetic bilayers to date. Asymmetric transbilayer distributions have been noted for various unlabeled lipid species in highly curved small vesicles, and presumably reflect molecular shape and packing considerations. It seems likely that the chemical nature of the lipid species making up the host lipid bilayer will also have an effect on the observed asymmetry.

The time course of lipid flippase activity was investigated for NBD-PC (16:0, 6:0). Over a 20 min incubation in the presence of ATP, there was a substantially increased rate of movement of the fluorescent lipid from the outer to the inner leaflet, compared to the control in the absence of ATP (Figure 4A). The first data point, formally time zero, reflects the finite time taken to mix the sample components, and then add vanadate, which also does not inhibit instantaneously (one round of ATP hydrolysis must take place). Thus, the zero time point probably reflects 1 min of flippase activity so that the time zero data points in the presence and absence of ATP have diverged slightly during this time period. In general, the small differences between the zero time points with and without ATP were somewhat variable in independent experiments using the same lipid, with panels B and C of Figure 4 showing differences at the low and high end of the range, respectively. The transbilayer redistribution process slowed over a 20 min time period, and appeared to approach a new equilibrium. ATP is not expected to be limiting for flippase activity, since an ATP regenerating system is present. However, to investigate whether ATP depletion was a factor, a second aliquot of ATP was added after 20 min, and flippase activity was followed for an additional 10 min. No additional NBD-labeled lipid was translocated to the inner leaflet relative to an identical sample without additional ATP, or a sample to which buffer was added instead of extra ATP. Reconstituted Pgp also exhibited substantial flippase activity with other NBD-labeled PCs (Table 1). A high rate of translocation was observed for a species with one long unsaturated acyl chain and one short saturated chain, NBD-PC (18:1, 6:0; Figure 4B), and a species with one long and one medium-length saturated chain, NBD-PC (16:0, 12:0) was also flipped at a substantial rate. The range of NBD-labeled phospholipids located in the inner leaflet after 20

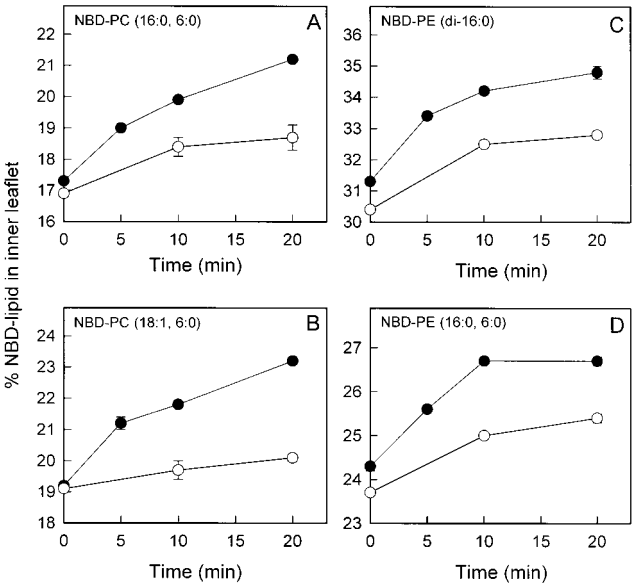

FIGURE 4: Time course of translocation for four different NBD-labeled phospholipids in proteoliposomes of egg PC containing reconstituted Pgp. Proteoliposomes were incubated for various times either with (●) or without (○) 1 mM ATP, followed by quenching with dithionite to determine the transbilayer distribution of the NBD-labeled phospholipid: (A) NBD-PC (16:0, 6:0), (B) NBD-PC (18:1, 6:0), (C) NBD-PE (di-16:0), and (D) NBD-PE (16:0, 6:0). Data points represent the means for duplicate determinations; where error bars are not visible, they fall within the symbols.

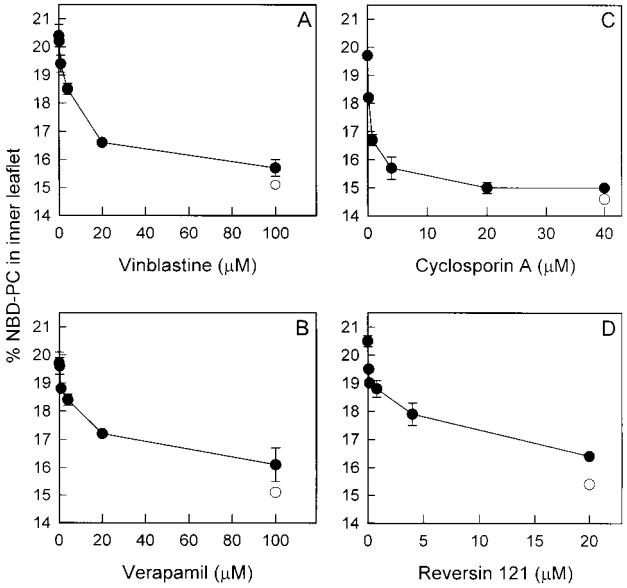

FIGURE 5: Inhibition of ATP-dependent NBD-labeled phospholipid translocation by various Pgp transport substrates and modulators. Net translocation of NBD-PC (16:0, 6:0) in a 20 min time period, relative to a control with no added ATP, was assessed in the presence of various drugs. Proteoliposomes containing Pgp were treated with increasing concentrations of (A) vinblastine, (B) verapamil, (C) cyclosporin A, and (D) reversin 121, and translocation of NBD was assessed after a 20 min incubation in either the presence (●) or absence (○) of 1 mM ATP. Data points represent the means for duplicate determinations; where error bars are not visible, they fall within the symbols.

min at 37 °C was generally within 1% of the total lipid, e.g., 20−21% for NBD-PC (16:0, 6:0) (six independent experiments), 26−27% for NBD-PE (16:0, 6:0) (two independent experiments), and 41−42% for NBD-PE (di-18:1) (two independent experiments). Thus, Pgp can translocate PCs of varying chain length and unsaturation, where the NBD fluorophore is located on one of the acyl chains. Since the amount of NBD-PC in the bilayer is known, it is possible to express this level of activity in terms of the net number of nanomoles of lipid transported per milligram of protein in 20 min, relative to control proteoliposomes in the absence of ATP (see Table 1). This gives a semiquantitative estimate of the relative rates of translocation of the fluorescent phospholipids.

To test the importance of the location of the fluorescent tag within the phospholipid molecule, flippase experiments were conducted with NBD-PE (di-16:0) and NBD-PE (di-18:1), where the NBD group is covalently linked to the headgroup. Good levels of translocation activity were noted for these lipids as well (Figure 4C and Table 1); thus, Pgp can translocate lipids with two full-length acyl chains. Another PE species with labeled acyl chains, NBD-PE (16:0, 6:0), was flipped at a similar rate (Figure 4D and Table 1). Reconstituted Pgp was also able to translocate two fluorescent PS species, NBD-PS (16:0, 6:0) and NBD-PS (16:0, 12:0), although at a lower rate, as well as a labeled sphingomyelin, SM (6:0), the latter at a relatively high rate. Thus, Pgp appears to be able to flip a variety of NBD-labeled phospholipids with different headgroups and backbone structures. The amount of lipid translocated in a 20 min period is in the range of 0.4−1.8 nmol/mg of protein, which is within the order of magnitude observed for transport of drugs by Pgp in vesicle systems. An exact comparison is difficult, since the concentration dependence for transport of NBD-labeled phospholipids has not been investigated, so that the lipid may not be at saturating concentrations within the bilayer, and Pgp transport data have been reported for substrate concentrations that vary from 1000-fold below saturation (*37*) to close to saturating (*47*).

Competition between Drug Transport and Phospholipid Flippase Activity. It is not clear whether the phospholipid flippase activity of Pgp is directly related to its drug transport function; in other words, do drugs and phospholipids occupy the same substrate binding site within the protein? To address this question, we investigated the ability of various Pgp transport substrates and modulators to compete with phospholipid flippase activity. We observed that a variety of structurally diverse substrates (both peptides and nonpeptides), including vinblastine (Figure 5A), verapamil (Figure 5B), cyclosporin A (Figure 5C), and reversin 121 (Figure 5D), inhibited translocation of NBD-PC (16:0, 6:0) in a concentration-dependent manner. In each case, flippase activity at high concentrations was reduced to the background level seen in the absence of ATP. These results suggest that drug transport and fluorescent phospholipid translocation take place via the same path.

For each competing substrate, the NBD-PC flippase activity can be expressed as the percent control relative to an identical proteoliposome sample without ATP (see Figure 6A for vinblastine). To quantitate the competition process, the data were analyzed according to the median effect equation, which was previously applied to competition for Pgp-mediated drug transport (*45*, *46*). A median effect plot is shown for vinblastine in Figure 6B, and allows estimation of D_m, the concentration of competing substrate that causes 50% inhibition of NBD-PC translocation. D_m was determined

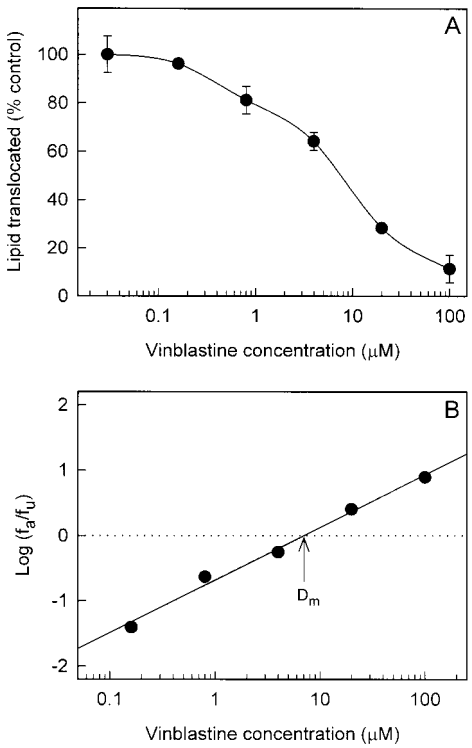

FIGURE 6: Characterization of inhibition of lipid translocation by drugs and modulators. The net amount of NBD-PC (16:0, 6:0) translocated in a 20 min time period, relative to a control with no added ATP, was measured in the presence of increasing concentrations of various drugs. A sample set of data is shown for vinblastine. (A) Plot of percent inhibition of lipid translocation with increasing drug concentrations. Data points represent means for duplicate determinations. (B) Median effect plot of $\log(f_a/f_u)$ vs log[drug] (see Materials and Methods for theory). D_m is determined from the intercept on the x axis, where $\log(f_a/f_u) = 0$, as indicated by the arrow.

Table 2: Inhibition of Lipid Flippase Activity by Transport Substrates and Modulators[a]

compound	D_m (μM)	compound	D_m (μM)
colchicine	300	vinblastine	7.07
pepstatin A	52.3	reversin 121	1.86
verapamil	14.5	cyclosporin A	0.54

[a] The ability of various Pgp substrates and modulators to inhibit ATP-driven flippase activity was determined by median effect analysis of the inhibition data (see Figure 6).

for several different Pgp substrates and modulators, including natural products, cyclic peptides, and linear peptides (see Table 2). The values of D_m varied from 0.54 μM for the high-affinity modulator, cyclosporin A, to 300 μM for the low-affinity substrate, colchicine. These D_m values correlated well with K_d values for binding of the compounds to purified Pgp, as measured by quenching of MIANS-Pgp (*52*−*54*) (Figure 7). This again suggests that the lipid flippase activity of Pgp is intimately associated with its drug transport function.

DISCUSSION

Substantial indirect evidence suggests that the MDR1 Pgp, like the MDR3 isoform, is involved in transbilayer movement of glycerophospholipids and sphingolipids. In the study presented here, we directly demonstrate that Pgp reconstituted into lipid vesicles is in fact able to act as a translocase or

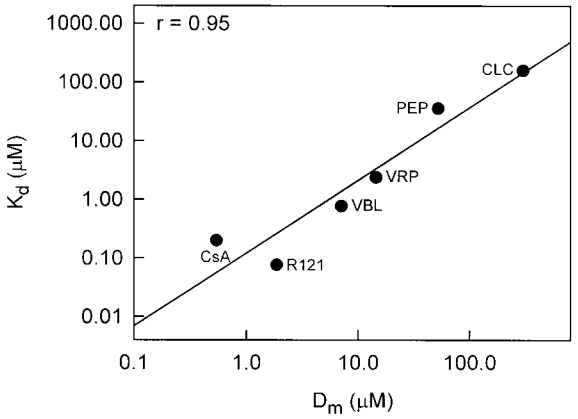

FIGURE 7: Correlation between inhibition of lipid flippase activity and affinity of binding to Pgp for several transport substrates and modulators. The D_m values for the compounds shown in Table 2 are plotted on a log−log scale vs their measured K_d values for binding to purified MIANS-labeled Pgp (see refs *52*−*54*).

flippase for various phospholipids. We used tracer, nonperturbing amounts of fluorescent NBD-labeled lipids incorporated into a lipid bilayer of egg PC as substrates for the transporter. A dithionite quenching technique was employed to quantitate the transbilayer distribution of these lipids in the vesicles, both in the absence and in the presence of reconstituted Pgp. The advantage of this method is that it provides an accurate, direct estimate of the amount of fluorescent tracer lipid in the outer and inner leaflets of the bilayer, and can readily be adapted to kinetic analysis on a time scale of minutes.

Proteoliposomes containing reconstituted Pgp and tracer NBD-labeled lipid displayed several interesting features. Pgp inserted into the bilayers asymmetrically so that the majority of the protein faced inward, with the NB domains at the outside surface of the proteoliposomes. Since the vesicles were relatively small and thus highly curved, this arrangement is likely adopted to relieve steric packing problems at the luminal surface. For the most part, the NBD-labeled lipids distributed themselves randomly between the outer and inner leaflets (as dictated by the curvature of the vesicles) in the absence of Pgp. A preference for the inner leaflet was observed for some PE species, and for the outer leaflet with SM, again, likely related to packing issues. However, there was a shift of all the NBD-labeled lipids to the outer leaflet when Pgp was incorporated into the bilayer so that outer: inner leaflet ratios were typically around 80:20. The shift in transbilayer distribution when Pgp is included in the bilayer may arise from specific interactions of the NBD-labeled phospholipids with the regions of the transporter closest to the cytoplasmic face of the plasma membrane, perhaps the substrate-binding sites, which are proposed to be located there (*55*).

Direct incorporation of the NBD-labeled lipid substrates into the bilayer allowed detailed characterization of the flippase process. Substantial translocation took place over a 20 min time frame, in an ATP-dependent fashion. We found that flippase activity shared many important characteristics with Pgp-mediated drug transport, including a requirement for ATP hydrolysis and inhibition by orthovanadate.

Translocation of NBD proceeded rapidly after addition of ATP, typically reaching a maximum of around 4−5% of the total labeled lipid, when the rate of transfer plateaued and a

new transbilayer equilibrium was approached. Ruetz and Gros previously used the dithionite quenching technique to examine the flippase activity of the class III Pgp, expressed in yeast secretory vesicles (*9*), and observed a similar phenomenon, with maximal translocation of 3.5−4%. We speculate that not all of the lipid is translocated for exactly the same reason that not all drug is moved to the vesicle lumen in transport experiments. Pgp pumps substrates up a concentration gradient into the vesicle lumen, but they diffuse back at a relatively high rate by passive diffusion so a steady state is reached when uphill pumping into the vesicle is exactly balanced by downhill diffusion out of the vesicle (*42*, *48*, *54*). Pgp-mediated translocation will result in a higher concentration of NBD-labeled lipid in the inner leaflet relative to the outer leaflet, essentially creating a concentration gradient across the two membrane leaflets. This unequal distribution will drive movement of the NBD-labeled lipid back to the outer leaflet, eventually balancing out the rate of inward flipping by Pgp. Another important factor is likely to be the extra packing stress produced in the inner leaflet by inserting NBD-labeled lipids into it, resulting in compensating movement of lipids the other way. The class III Pgp was able to translocate ~20 pmol of NBD-labeled lipid per milligram of membrane protein over the time course of an experiment (*9*). If we assume that 2% of the protein in the yeast secretory vesicles is Pgp, then this translates into ~1 μmol of NBD-PC translocated per milligram of Pgp, comparable to the values observed in the work presented here. In addition, in these experiments, Pgp is likely not "saturated" with substrate, since the affinity for lipid is probably quite low compared to those of drugs (see Figure 7), and the NBD-labeled lipid makes up only 0.3% of the bilayer.

We were also able to directly address the issue of lipid specificity. The MDR3 Pgp is highly specific for the export of PC into the bile (*11*), and was shown to act as a flippase for PC in yeast secretory vesicles (*9*), fibroblasts from transgenic mice (*10*), and transfected LLC-PK1 cells (*16*). The MDR1 Pgp, on the other hand, has been reported to be involved in translocation of a wider variety of short chain lipids, including PC, PE, SM, and GlcCer (*16*, *17*). We found high levels of translocation with NBD derivatives of SM and PC, lower levels with PE, and the poorest activity with PS. Interestingly, Bosch and Croop reported that C_{12}-NBD derivatives of PC and PE, but not PS, behaved as substrates for the transporter (*19*). The reason for this difference remains unknown. However, the experiments of Bosch et al. were carried out in intact cells using donor liposomes, a situation very different from the purified reconstituted systems used in this work. One might speculate that the failure to observe a reduced level of uptake of NBD-PS might have resulted from a cellular process, perhaps a different mode of uptake of donor liposomes containing anionic PS. We used a wide variety of NBD-labeled lipids, including those with one long chain and one short chain, one long chain and one medium-length chain, and two long chains. Reconstituted Pgp exhibited good activity with all of these species as substrates, with only 3-fold differences between species with different acyl chains. Pgp also did not appear to show any great preference for unsaturated or saturated chains, with both being transported well.

Ruetz and Gros (*9*) previously reported that the mouse MDR1 protein did not result in translocation of (presumably C_6) NBD-PC across the membrane of yeast secretory vesicles. However, these results have been questioned recently by Angeletti and Nichols (*56*), who found that the secretory vesicles were highly permeable to dithionite at 25 °C, which would confound the determination of membrane leaflet distribution. MRP1 apparently acts as an outward flippase (i.e., floppase) for C_6-NBD-labeled PC and PS with similar rates in human red blood cells (*22*, *23*), and also transports C_6-SM and C_6-GlcCer across the plasma membrane of transfected LLC-PK1 cells in a glutathione-dependent fashion (*24*). LmrA apparently transported fluorescent derivatives of PE but not PC (*27*), and indirect accumulation experiments implicated Yor1p and Pdr5p in the transport of a labeled PE (*26*).

Several results of the study presented here, taken together, suggest that Pgp flips NBD-labeled lipids by the same path as that used to transport drugs. First, the amount of lipid translocated is relatively high (0.36−1.83 nmol per milligram of protein in 20 min), of the same order of magnitude as the amount of drug transported into the lumen of inside-out vesicle systems (*37*, *42*). Second, a variety of structurally diverse drugs and modulators effectively inhibited lipid translocation in a concentration-dependent manner, suggesting that these compounds compete directly for the transport process. Last, characterization of the competition process indicated that the efficiency with which drugs compete with lipid translocation is well correlated to their affinity for direct binding to purified Pgp. This correlation is strikingly similar to the reported correlation between K_d and inhibition of colchicine transport for many different Pgp substrates (*52*−*54*). It has been reported recently that the human class III MDR3 Pgp is also able to transport several drugs, but at a relatively low rate, explaining why this isoform does not appear to confer drug resistance (*14*). Thus, it appears that the two Pgp isoforms, which are closely related and likely arose from a gene duplication event, have each evolved to transport a specific group of substrates.

How likely is it that MDR1 Pgp can transport natural long chain membrane phospholipids? Since MDR1 is unable to compensate for the lack of MDR3 in knockout mice, the rate of lipid translocation must be slow compared to that of MDR3 Pgp. However, other evidence (see below) suggests that transport of natural membrane lipids may take place at a low rate. Although it may not transport unmodified long chain membrane lipids rapidly, MDR1 Pgp may be involved in transport or flipping of physiologically important short chain lipid analogues or lipid metabolites. For example, we have recently demonstrated that platelet activating factor is a substrate for purified Pgp, binding to the transporter in a saturable fashion with a K_d of 15 μM (R. Liu and F. J. Sharom, unpublished data).

It seems possible that a fluorescent group is required for a high rate of translocation, to make the lipid "look" more like a drug. However, we noted that the site of covalent attachment of the NBD group did not appear to affect the ability of Pgp to translocate a lipid; two PE species with the label on the headgroup were translocated at a rate similar to that of the acyl chain-labeled species. Thus, if Pgp is recognizing the fluorescent label rather than the lipid backbone, its depth in the membrane is flexible, and NBD

groups can be recognized at positions C_{12} and C_6, and in the headgroup. In addition, the NBD group is not inherently very lipophilic, unlike most Pgp transport substrates. We tested to see whether two water-soluble NBD conjugates were able to stimulate Pgp ATPase activity, which would indicate that they are Pgp substrates. NBD-Cl covalently linked to ethanolamine or dithioerythritol had no effect on Pgp ATPase activity over a concentration range up to 5-fold higher than the NBD-labeled lipid concentration used in flippase experiments, suggesting that the NBD group per se is not a Pgp substrate.

The ability of natural lipid molecules to interact with Pgp may help to explain the observed modulation of its drug transport activity by the lipids in the host membrane. We previously hypothesized that the observed changes in the affinity of binding of drugs to reconstituted Pgp in response to alterations in the host lipid matrix might reflect "competition" between the host lipid molecules and the drug molecule for interaction with the substrate binding site(s) of the protein (*8*). For example, if the lipid making up the matrix has a relatively low affinity for interaction with Pgp, it would be easily displaced from the substrate binding site, and the drug would have a high apparent affinity for binding to Pgp. On the other hand, if the lipid making up the matrix has a higher affinity for Pgp, a higher drug concentration would be required to displace it, giving an apparently lower drug binding affinity. In this way, the nature of the lipids surrounding Pgp might have substantial effects on the binding affinity of drugs and modulators.

One puzzling aspect of Pgp has been its ability to hydrolyze ATP constitutively, in the absence of drug substrates (*36*, *57*, *58*). This activity is observed following reconstitution of purified Pgp into bilayers of purely synthetic phospholipids (*38*), and so cannot be attributed to contamination of the lipid preparation with lipophilic compounds, as suggested by Borgnia et al. (*59*). Since many drugs and modulators stimulate this basal ATPase activity, the transporter can best be described as partially uncoupled. Krupka has proposed that the constitutive ATPase activity of Pgp may be explained by a half-coupled ATPase mechanism (*60*). It was suggested that a half-coupled mechanism could originally have evolved in a flippase, since uncoupled ATP hydrolysis would be precluded by immersion of such a transporter in its membrane lipid substrate. The scheme presented by Krupka for the catalytic cycle relies on the presence within the membrane of an endogenous substrate or inhibitor of the transporter that is able to occupy the drug binding site and allow ATP turnover. Thus, it seems possible that the basal ATPase activity observed for Pgp is due to the presence of membrane lipids that also act as substrates. Since the surrounding bilayer lipid is present at a very high concentration, membrane lipids could produce significant ATP turnover even if they bound with low affinity, or were transported at a low rate, compared to drugs. The constitutive ATPase activity would thus not be strictly uncoupled. The idea that membrane lipids might be responsible for the basal ATPase activity of Pgp is supported by previous observations that delipidation of the protein resulted in complete loss of ATPase activity, which was restored by addition of exogenous lipids (*58*, *61*−*63*), and that phospholipids stimulated ATPase activity of purified Pgp in a concentration-dependent manner (*36*).

There appears to be little structural similarity between Pgp and other membrane proteins with phospholipid translocase activity (*64*, *65*), although it is not clear if they share functional attributes. The lipid flippase first identified, and still the best known from the point of view of biochemical activity, is the aminophospholipid translocase (*66*), whose gene was recently identified as the founding member of a new class of P-type ATPases (*67*). In addition, a bidirectional Ca^{2+}-dependent lipid scramblase exists, which appears to be a single membrane-spanning protein unrelated to the ABC superfamily. It will be interesting to compare the structural features of these proteins, as they are characterized molecularly, with those of Pgp and other members of the ABC superfamily involved in lipid translocation.

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