Interaction of the P-Glycoprotein Multidrug Efflux Pump with Cholesterol: Effects on ATPase Activity, Drug Binding and Transport[†]

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ABSTRACT: Resistance to a broad spectrum of structurally diverse chemotherapeutic drugs (multidrug resistance; MDR) is a major impediment to the treatment of cancer. One cause of MDR is the expression at the tumor cell surface of P-glycoprotein (Pgp), which functions as an ATP-powered multidrug efflux pump. Since Pgp interacts with its substrates after they partition into the lipid bilayer, changes in membrane physicochemical properties may have substantial effects on its functional activity. Various interactions between cholesterol and Pgp have been suggested, including a role for the protein in transbilayer movement of cholesterol. We have characterized several aspects of Pgp−cholesterol interactions, and found that some of the previously reported effects of cholesterol result from inhibition of Pgp ATPase activity by the cholesterol-extracting reagent, methyl-β-cyclodextrin. The presence of cholesterol in the bilayer modulated the basal and drug-stimulated ATPase activity of reconstituted Pgp in a modest fashion. Both the ability of drugs to bind to the protein and the drug transport and phospholipid flippase functions of Pgp were also affected by cholesterol. The effects of cholesterol on drug binding affinity were unrelated to the size of the compound. Increasing cholesterol content greatly altered the partitioning of hydrophobic drug substrates into the membrane, which may account for some of the observed effects of cholesterol on Pgp-mediated drug transport. Pgp does not appear to mediate the flip-flop of a fluorescent cholesterol analogue across the bilayer. Cholesterol likely modulates Pgp function via effects on drug−membrane partitioning and changes in the local lipid environment of the protein.

Members of the ATP-binding cassette (ABC[1]) superfamily of proteins mediate the active transbilayer export or import of substrates coupled to the hydrolysis of ATP (*1, 2*). Most ABC family members are integral proteins that interact intimately with membrane lipids, and many of these transporters (especially the ABCA, ABCB and ABCG subfamilies) employ phospholipids and sterols among their substrates, and mediate their translocation (*3*−*5*). One such protein, the mammalian P-glycoprotein (Pgp; ABCB1), is a 170 kDa efflux pump for amphipathic molecules that has also been implicated in outwardly directed translocation (flipping) of membrane lipids (*6, 7*). Pgp comprises 6 membrane-spanning helices in each of two membrane-bound domains, and two cytosolic nucleotide-binding (NB) domains, and is thought to function physiologically as a protective mechanism against harmful hydrophobic xenobiotic compounds. However, upregulation of Pgp expression in tumor cells results in cross-resistance to a broad spectrum of chemotherapeutic drugs, a phenomenon termed multidrug resistance (MDR), which can be a major impediment to the successful clinical treatment of human cancers (*8*−*10*).

Substrates for Pgp are typically nonpolar, and show a high level of partitioning into the membrane (*11, 12*). The binding sites for at least two transport substrates are located within the cytoplasmic leaflet of the bilayer (*13, 14*), and there is substantial evidence suggesting that drugs are extracted by the protein from within the membrane. The physicochemical properties of the membrane would therefore be expected to influence the functional activity of Pgp (*15*). Indeed, we previously showed that the substrate binding (*11*), ATPase (*16*) and transport activities of the reconstituted protein (*17*) are modulated by the lipid environment in which it is embedded.

Reconstituted Pgp has been shown to translocate several fluorescent derivatives of phospholipids and simple glycosphingolipids (GSL) between bilayer leaflets in an ATP-dependent manner (*18, 19*). Recent work has also demonstrated that lipid-based drugs and platelet-activating factors bind to Pgp, modulate its ATPase activity and compete for drug transport and lipid flippase activity (*20*), further supporting the idea that Pgp interacts directly with lipids. A homologous protein, ABCB4, functions as a flippase in the liver canalicular membrane, exporting phosphatidylcholine (PC) into the bile (*21, 22*), and there is evidence that it also transports drugs at a low rate (*23*). These closely related proteins may thus function in a similar manner.

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[1] Abbreviations: ABC, ATP-binding cassette; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DHE, dehydroergosterol; DMPC, dimyristoyl-phosphatidylcholine; GSL, glycosphingolipid(s); H33342, Hoechst 33342; mβCD, methyl-β-cyclodextrin; MDR, multidrug resistance/resistant; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; NATA, *N*-acetyl-L-tryptophanamide; NB, nucleotide-binding; NBD, nitrobenzo-2-oxa-1,3-diazole; PC, phosphatidylcholine; Pgp, P-glycoprotein; R123, rhodamine 123; TNP, trinitrophenyl; TMR, tetramethylrosamine.

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There have been many observations over the years linking Pgp functional activity with cholesterol, which is a major component of mammalian plasma membranes. Pgp has been suggested to play a role in several cholesterol-related processes in the cell, including cholesterol esterification, transport/flipping and biosynthesis, and cholesterol has been proposed to regulate Pgp activity. However the results reported in the literature are contradictory and confusing. Pgp appeared to enhance cellular cholesterol esterification while the addition of Pgp inhibitors blocked the process (*24, 25*), suggesting the involvement of Pgp, possibly via a role in cholesterol transport. However, Issandou and Grand-Perret showed that the Pgp modulators typically employed were nonspecific, and also inhibited acyl CoA acyltransferase (*26*). In the presence of more specific Pgp inhibitors, acyl CoA acyltransferase was functional, Pgp was not, and cholesterol esterification was not inhibited. Several groups suggested that cholesterol removal from Pgp (*27, 28*) or reconstitution of Pgp into proteoliposomes lacking cholesterol (*29, 30*) inhibits ATPase activity, and addition of cholesterol stimulates activity (*27, 31*). There have also been reports that the presence of cholesterol decreases Pgp-mediated drug transport (*28, 31*), and that both increases and decreases in membrane cholesterol inhibit transport activity (*32*). It has been suggested that Pgp is involved in cholesterol uptake (*33*) or efflux (*27*), while others have shown that cholesterol efflux pathways themselves are upregulated in MDR cells (*34*). In mice, reports have agreed (*35*) or disagreed (*36*) with the hypothesis that mdrla/b Pgp is involved in cholesterol uptake. Finally, Wang et al. (*31*) suggested that cholesterol interacts with the daunorubicin binding site of Pgp, while Bucher et al. (*29*) implied that binding of verapamil and progesterone only occurs in the presence of cholesterol. However, these and many other drugs can bind to Pgp with high affinity in the absence of added cholesterol (*37*), and previous work in our laboratory suggests that cholesterol affects the binding of vinblastine but not daunorubicin or verapamil (*11*). Thus, it still remains to be established whether Pgp interacts directly with cholesterol, and regulates its transbilayer distribution by a flippase mechanism, as it can for phospholipids and GSL.

A better understanding of Pgp function and the factors influencing its activity (e.g., the presence of cholesterol) may allow development of treatments to circumvent Pgp-mediated MDR. Much of the past work focusing on the effects of cholesterol on Pgp function used whole cells or crude plasma membrane vesicle preparations. Thus, a systematic study of Pgp−cholesterol interactions employing purified protein is needed to clarify the role of this sterol in Pgp function. Reconstituted systems have also rarely been employed in the study of Pgp−cholesterol interactions, yet they are a powerful tool allowing for fine control of lipid composition and membrane biophysical properties. In the present work we have determined the effect of cholesterol on various functional activities of purified Pgp, and also investigated whether the protein plays a direct role in cholesterol transbilayer flip-flop. Results suggest that some of the effects reported in the literature may result from direct inhibition of Pgp by the cholesterol-extracting reagent, methyl-β-cyclodextrin (mβCD). The presence of cholesterol modulated the ATPase activity of reconstituted Pgp, the ability of drugs to bind to the protein, and its drug transport and lipid flippase functions. Cholesterol content was found to substantially alter the partitioning of hydrophobic drugs into the bilayer, accounting for some of the observed effects of cholesterol on Pgp function. However, Pgp does not appear to mediate the flip-flop of cholesterol in the membrane.

EXPERIMENTAL PROCEDURES

Materials. Acrylamide was purchased from BioRad (Mississauga, ON). ATP, bovine serum albumin, concanavalin A-Sepharose 4B, cholesterol, daunorubicin, sodium deoxycholate, mβCD, *N*-acetyl-L-tryptophanamide (NATA), dehydroergosterol (DHE), progesterone, rhodamine 123 (R123), Sephadex G50, sodium dithionite (sodium hydrosulfite), sodium ortho-vanadate, Triton X-100, verapamil and vinblastine were purchased from Sigma Chemical Co. (Oakville, ON). CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) was obtained from MP Biochemicals (Aurora, OH). Hoechst 33342 (H33342), NBD-cholesterol (22-(*N*-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol), tetramethylrosamine (TMR) and the Amplex Red cholesterol assay kit were obtained from Molecular Probes (Eugene, Oregon). Dimyristoylphosphatidylcholine (DMPC), egg PC, and NBD-PC (16:0, 6:0) were purchased from Avanti Polar Lipids (Alabaster, AL). All phospholipid stocks were prepared in 4:1 (v/v) $CHCl_3$-MeOH and stored at −20 °C.

Pgp Purification and Reconstitution. Pgp was isolated from plasma membrane preparations of MDR CH^RB30 cells using a CHAPS extraction procedure described previously (*38*), modified as described (*20*). CHAPS-extracted Pgp (~70% pure) was used directly for some experiments, or purified further using concanavalin A-Sepharose 4B affinity chromatography, as described (*38*). Protein content was assayed by a modification of the Lowry method (*39*) using bovine serum albumin as the standard. CHAPS-extracted Pgp was reconstituted into proteoliposomes of either egg PC/0.3% (w/w) NBD-lipid (flippase experiments) or DMPC (ATPase activity, drug binding in the presence of cholesterol, and transport experiments). Typically, 5 mg of the desired lipid mixture was solubilized in 250 μL of 200 mM CHAPS buffer, and 0.5 mg of Pgp in 1 mL of 15 mM CHAPS buffer was added. Reconstitution was carried out by Sephadex G50 gel filtration chromatography, as described previously (*19, 20*). The final lipid:protein ratio was ~10:1 (w/w). Where cholesterol was included in the bilayer of the proteoliposomes, it made up 0−30% (w/w) of the lipid component of the membrane. Proteoliposomes of egg PC, NBD-PC and Pgp were diluted in reconstitution buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, pH 7.4) to a final lipid concentration of 0.069 mg/mL.

Cholesterol Content. The cholesterol content of samples was determined either by a chemical analysis method described previously (*40, 41*), or enzymatically with cholesterol oxidase using the Amplex Red cholesterol assay kit. Both methods produced comparable results.

Lipid−Water Partitioning of Drugs. The partitioning of a variety of Pgp substrates (1 mg/mL) into 10 mg/mL egg PC containing 0−20% (w/w) cholesterol was determined at 22 °C as described previously (*11*), except that absorbance values were measured for 300 μL of standards and samples in 96-well UV plates using a plate-reader at the following

wavelengths: vinblastine, 270 nm; verapamil, 278 nm; H33342, 350 nm; daunorubicin, 484 nm; R123, 500 nm; and TMR, 514 nm. The distribution coefficient, K_{lip}, was calculated using the following equation:

$$K_{lip} = \frac{(C_T - C_W)V_1}{C_W V_2} \cong \frac{(C_T - C_W)W_1}{C_W W_2} \quad (1)$$

where C_T is the initial aqueous concentration of drug, C_W is the final aqueous concentration of drug in the supernatant, V_1 and W_1 are the volume and weight of aqueous phase, respectively, and V_2 and W_2 are the volume and weight of the lipid, respectively.

ATPase Activity. ATPase specific activity was determined for plasma membrane vesicles, purified Pgp in CHAPS buffer and Pgp reconstituted into proteoliposomes as described previously (*42*), using 1 mM ATP and a 20 min assay time at 37 °C. Control samples were treated identically but contained heat-inactivated protein, and these values were used to correct the experimental data. mβCD was prepared as 100 mM or 500 mM stocks in ATPase buffer and heated to 37 °C for 5 min to aid in solubilization. Drugs were added to samples and controls as DMSO solutions and preincubated for 5 min before ATP addition. Final DMSO concentrations did not exceed 10%, which had no effect on Pgp ATPase activity. For experiments involving removal of mβCD by gel filtration chromatography, a 1 mL sample of purified Pgp in 50 mM mβCD was applied to a 30 cm Sephadex G50 column equilibrated with 2 mM CHAPS buffer. The column was eluted at a rate of 1 drop/10 s, and 0.5 mL fractions were collected. The protein-containing fractions were pooled and assayed for ATPase activity. For experiments involving deoxycholate delipidation, ATPase buffer at a pH of 8.1 (rather than 7.4) was used to maintain the solubility of deoxycholate. Control samples for deoxycholate experiments also used ATPase buffer at a pH of 8.1. CHAPS-extracted Pgp was incubated with 2 mM deoxycholate for 10 min at 4 °C before addition of lipid, and the ATPase assay was carried out as usual.

Substrate Binding Affinity by Fluorescence Quenching. Binding affinity was determined for ATP and a variety of Pgp drug substrates at 20 °C by quenching of the intrinsic Trp fluorescence of purified Pgp reconstituted into DMPC proteoliposomes containing varying concentrations of cholesterol, essentially as described previously (*20, 38*). We used a PTI QuantaMaster C-61 or QM-8/2005 steady-state fluorimeter (Photon Technology International, London, ON), with a 2 nm bandwidth for excitation ($\lambda_{ex} = 290$ nm) and emission ($\lambda_{em} = 330$ nm). Reconstituted Pgp was at a concentration of 100 μg/mL in reconstitution buffer. In experiments to determine the effect of cholesterol on binding, drugs were added as solutions in DMSO. Drug titrations with lipid blanks were performed using unilamellar vesicles of 0.5 mg/mL DMPC with 0−30% (w/w lipid) cholesterol, produced by extrusion 20 times through 100 nm polycarbonate filters. To determine if mβCD was a Pgp substrate, the compound was dissolved in 2 mM CHAPS buffer and titrated with purified Pgp in 2 mM CHAPS buffer (2 mM CHAPS, 50 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, pH 7.5). To determine the binding of cholesterol, the sterol was solubilized in absolute ethanol (<10% ethanol final concentration in the samples, which had no effect on Pgp fluorescence). Fluorescence data were collected after equilibration of samples at 20 °C. Data were corrected for dilution, lipid scattering, and inner filter effect (*43*−*45*), and fitted to an equation describing binding to a single site:

$$\left(\frac{\Delta F}{F_0} \times 100\right) = \frac{\left(\frac{\Delta F_{max}}{F_0} \times 100\right) \times [S]}{K_d + [S]} \quad (2)$$

where $(\Delta F/F_0 \times 100)$ is the percent fluorescence quenching (percent change in fluorescence relative to the initial value, F_0), following addition of substrate at a concentration [S], and K_d is the dissociation constant for binding to Pgp. Fitting was carried out using nonlinear regression (SigmaPlot, Systat Software, Chicago, IL), and values of K_d and the maximum percent fluorescence quenching, $(\Delta F_{max}/F_0 \times 100)$, were extracted. Control titrations were performed with 30 μM NATA to assess the nonspecific quenching of Trp fluorescence by substrates.

Quenching of Pgp Intrinsic Fluorescence by Acrylamide. A solution of freshly prepared 5 M acrylamide in 2 mM CHAPS buffer was added in 2 μL aliquots to 250 μL of 100 μg/mL purified Pgp in 2 mM CHAPS buffer, in the presence of 0.5 mg/mL lipid consisting of DMPC and 0−30% (w/w lipid) cholesterol. After incubation at 20 °C, fluorescence emission was measured at 330 nm following excitation at 290 nm (bandwidths of 2 nm). Fluorescence intensities were corrected for dilution and scattering. Parallel experiments were carried out using 30 μM NATA to assess acrylamide quenching of Trp fluorescence in aqueous solution. Quenching data were analyzed using the Stern−Volmer equation,

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and presence of acrylamide, respectively, [Q] is the acrylamide concentration, and K_{SV} is the Stern−Volmer quenching constant. For a collisional quenching mechanism, a plot of F_0/F vs [Q] gives a line with a slope of K_{SV}.

Lipid Flippase Activity. The ability of reconstituted Pgp to flip NBD-lipids was determined essentially as described previously (*19*). Briefly, proteoliposome samples were incubated for varying times at 37 °C in the presence or absence of 1 mM ATP and an ATP-regenerating system, and translocation was terminated by the addition of sodium ortho-vanadate at a final concentration of 200 μM. An excitation wavelength of 466 nm and an emission wavelength of 536 nm were used (bandwidths of 2 nm). Sodium dithionite (2 mM final concentration) 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. For samples containing varying concentrations of cholesterol, proteoliposomes of egg PC, 0.3% (w/w lipid) NBD-PC and 0−30% (w/w lipid) cholesterol were prepared as described above. For experiments involving extraction with mβCD, proteoliposomes containing 30% (w/w) cholesterol were incubated for 1 h at 37 °C with varying concentrations of mβCD. Proteoliposomes were collected by centrifugation for 10 min and resuspended in fresh buffer using a 26 G needle. For experiments involving NBD-cholesterol, egg PC liposomes were prepared in the absence of protein, and contained 0.3%

(w/w) NBD-PC, 0.3% (w/w) NBD-cholesterol, or 0.15% (w/w) NBD-PC plus 0.15% (w/w) NBD-cholesterol.

Inhibition of Lipid Flippase Activity in the Presence of Cholesterol. Inhibition of lipid flippase activity was determined using the NBD-lipid assay as described using egg PC proteoliposomes containing 0−30% (w/w) cholesterol. At 5 min prior to initiation of lipid transport by ATP and the regenerating system, a 10 μL aliquot of the desired concentration of vinblastine in DMSO was added (final DMSO concentration was 2%, v/v). The data were analyzed according to the median effect equation, as described previously (*19, 20, 46*):

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m \quad (4)$$

where f_a is the fraction of the system that is affected (in this case, the fractional inhibition of NBD-PC translocation by vinblastine at 20 min) at a vinblastine concentration D, f_u is the fraction of the system that is unaffected at concentration D, D_m is the vinblastine concentration causing 50% inhibition, and m is a parameter indicating the sigmoidicity of the dose−effect curve. Rearrangement of the median effect equation yields

$$\log\left(\frac{f_a}{f_u}\right) = m \log D - m \log D_m \quad (5)$$

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

TMR and H33342 Transport by Pgp. Initial rates of transport of TMR or H33342 into DMPC proteoliposomes containing Pgp and 0−30% (w/w lipid) cholesterol were determined essentially as described previously (*20*). Proteoliposomes composed of DMPC and 0−30% (w/w lipid) cholesterol were prepared. Fluorescence was monitored at 27 °C with excitation and emission wavelengths of 550/575 nm for 1 μM TMR, or 355/450 nm for 5 μM H33342, respectively, with bandwidths of 1.75 nm for excitation and 3 nm for emission. The initial rate of TMR or H33342 transport was calculated using the first 20 or 50 s, respectively, after the addition of ATP and the regenerating system. For samples containing mβCD, proteoliposomes of DMPC and 20% (w/w lipid) cholesterol were incubated at 37 °C with the desired mβCD concentration for 5 min before beginning the assay.

RESULTS

Cholesterol Extraction by Cyclodextrin. mβCD is a cyclic polysaccharide with a hydrophobic central binding site that can be occupied by cholesterol, phospholipids or other hydrophobic molecules (*47*). It can be used to extract cholesterol from, or deliver cholesterol to, lipid membranes, and has been employed in many studies to determine the effects of cholesterol on Pgp ATPase activity and/or drug transport (*27, 28, 30, 32*). Indeed, when increasing concentrations of mβCD were added to plasma membrane vesicles from CH^RB30 cells overexpressing Pgp, the cholesterol content of the membranes was progressively reduced, along with the ATPase activity (Figure 1A), which is primarily attributable to Pgp (*48*). At 50 mM mβCD, the cholesterol was essentially completely removed from the membrane, and only 30% of Pgp ATPase activity remained.

Verapamil is a Pgp modulator known to stimulate ATPase activity in a biphasic manner at micromolar concentrations. We determined the ATPase specific activity of Pgp in CH^RB30 plasma membrane vesicles in the presence of 0−1 mM verapamil and 0, 1 and 10 mM mβCD (Figure 1B). In the absence of mβCD, verapamil gave a typical biphasic stimulation profile, where the ATPase activity of Pgp increased by ~40% in the 10−100 μM concentration range. As the concentration of mβCD was increased, the relative degree of stimulation by verapamil increased significantly, with a >2-fold increase in relative ATPase activity at 10 mM mβCD (Figure 1B), even as the absolute basal activity of the protein was greatly reduced (see Figure 1A and 1B inset). The maximal absolute level of ATPase activity achieved with verapamil was similar in the absence and presence of mβCD (Figure 1B inset).

We reconstituted CHAPS-extracted Pgp into proteoliposomes of DMPC alone, and DMPC containing 30% (w/w) cholesterol, and treated the proteoliposomes with 0.01 and 10 mM mβCD. The basal ATPase specific activity of reconstituted Pgp was progressively reduced by increasing concentrations of mβCD, with ~13% and ~26% of the initial ATPase activity remaining at 10 mM mβCD for liposomes of DMPC-cholesterol and DMPC alone, respectively (Figure 1C). Thus the deleterious effect of mβCD on Pgp function appeared to be independent of the presence of cholesterol in the membrane. Cyclodextrins are not exclusively specific for cholesterol (*47*); they can also accommodate lipophilic drugs as guest molecules and have been reported to extract phospholipids from cell membranes and liposomes, thus solubilizing and disrupting them (*49, 50*). We investigated the possible involvement of phospholipid depletion and consequent liposome disruption in the observed loss of Pgp ATPase activity. When DMPC proteoliposomes containing Pgp were treated with mβCD, they could be sedimented in the usual fashion by centrifugation, indicating that their integrity was preserved. After resuspension in fresh buffer lacking mβCD, Pgp ATPase activity was restored (Figure 1C), suggesting that the presence of mβCD itself was responsible for the loss of function.

The concentrations of DMPC and cholesterol in the cholesterol-containing proteoliposome sample were ~2.72 and 1.29 mM, respectively, and the residual CHAPS remaining in the sample was estimated to be <0.009 mM (*51*). Thus, 10 mM mβCD is expected to be sufficient to remove all of cholesterol from the bilayer (plus residual CHAPS and phospholipid, as well, if this were to occur). At 0.01 mM mβCD, however, where ATPase activity was reduced by 20% (Figure 1C), the mβCD concentration is not sufficient to extract 20% of the cholesterol in the sample, again suggesting that cholesterol extraction is not solely responsible for the loss of activity. Pgp solubilized in 2 mM CHAPS in the absence of added lipids also possesses ATPase activity, and interestingly, increasing concentrations of mβCD also inhibited its activity, with <15% of the initial activity remaining at 10 mM mβCD (Figure 1D).

Pgp is an ATP-dependent drug transporter and lipid flippase, and the presence of mβCD also affected these activities of the protein (Figure 2). Pgp was reconstituted into proteoliposomes of egg PC and cholesterol containing tracer amounts of the fluorescent lipid NBD-PC. Exposure to mβCD and subsequent removal of the compound by

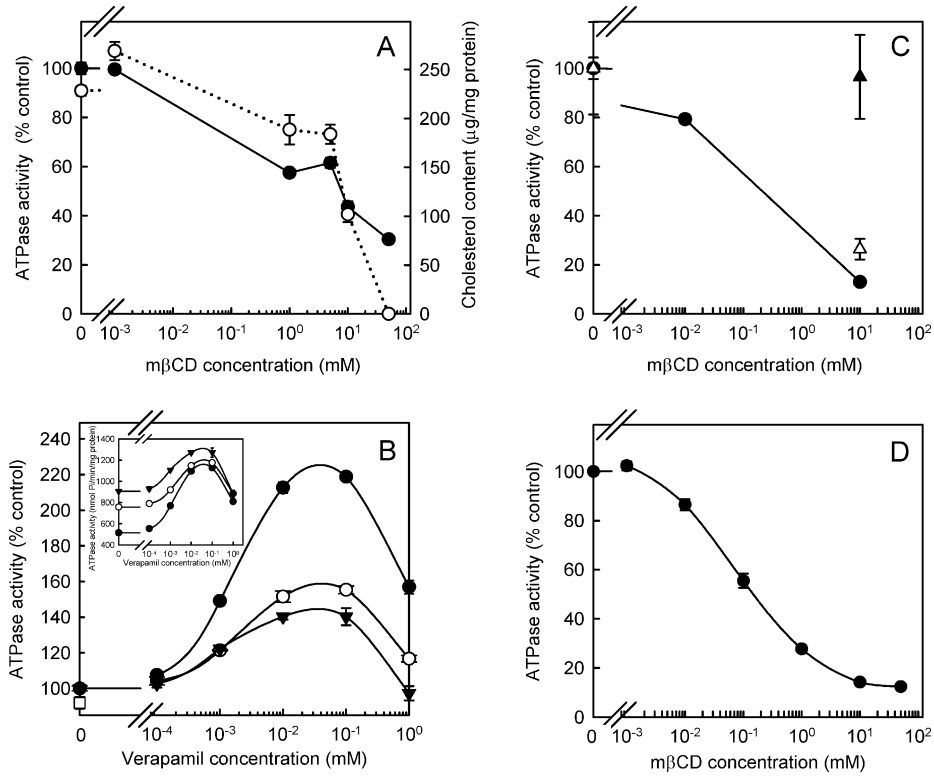

FIGURE 1: mβCD modulates Pgp ATPase activity. (A) Effect of increasing concentrations of mβCD on ATPase specific activity (●) and cholesterol content (○) in CH^RB30 plasma membrane vesicles. (B) Stimulation of the ATPase activity of Pgp in membrane vesicles by verapamil in the absence (▼) and presence of 1 mM (○) or 10 mM (●) mβCD. Control activity in the absence of DMSO is shown by □. Activity is expressed relative to a control to which no mβCD was added (the inset shows a plot of absolute ATPase activity). (C) Effect of increasing concentrations of mβCD on Pgp ATPase activity in DMPC proteoliposomes lacking (△) or containing (●) 30% (w/w) cholesterol. DMPC proteoliposomes were subjected to mβCD treatment, followed by centrifugation and resuspension in buffer lacking mβCD (▲). (D) Effect of mβCD on the ATPase activity of Pgp in CHAPS. Data points represent the mean ± SEM ($n = 3$), and where error bars are not visible, they fall within the symbols. Activity is expressed relative to a control to which no mβCD was added.

centrifugation resulted in inhibition of NBD-PC flippase activity at 10 mM mβCD to ~45% of the initial value (Figure 2A). After centrifugation and resuspension of the treated proteoliposomes, the fluorescence intensity was unchanged, indicating that mβCD had not extracted a measurable amount of NBD-PC. Thus, under these conditions, the inhibitory effect of cyclodextrin on flippase activity was not due to depletion of the fluorescent phospholipid. When the experiment was repeated without centrifugation of the proteoliposomes to remove mβCD, flippase activity was even more strongly inhibited, to ~27% of the initial value (data not shown).

mβCD also inhibited the transport of two fluorescent drug substrates into the interior of Pgp proteoliposomes composed of DMPC-cholesterol. We found previously that optimal drug transport occurred with 1 μM TMR and 5 μM H33342 (*20*). Transport of 1 μM TMR was essentially abolished by 1 mM mβCD, and transport of 5 μM H33342 was almost completely inhibited by 5 mM mβCD (Figure 2B and 2C). However, we noted that a portion of a 10 μM TMR sample mixed with mβCD was eluted from a gel filtration column in the void volume, likely in association with cyclodextrin aggregates, suggesting that sequestration of the substrate by mβCD may contribute to the decreased rate of drug transport that was observed.

The results obtained with membrane vesicles and reconstituted Pgp showed that ATPase activity, ATPase stimulation by Pgp substrates, flipping of NBD-PC and drug transport were all modulated in the presence of mβCD, and that mβCD decreased the cholesterol content of membranes in parallel. This suggested at first that the activity of the protein might be controlled by the cholesterol content of the lipid bilayer. However, Pgp reconstituted into synthetic PC bilayers possesses high levels of ATPase activity, and high levels of both lipid flippase and drug transport are observed using reconstituted protein in the complete absence of added cholesterol (*17*−*20, 20, 52, 53*). In addition, the observation that mβCD inhibited the activity of Pgp in detergent solution (in the absence of added lipid) suggested that this compound may have a direct inhibitory effect on Pgp, independent of its ability to deplete cholesterol. This proposal was supported by the observation that, when lipid flippase experiments were carried out in the presence of mβCD, the activity was inhibited to a greater extent than when its concentration was significantly lowered by centrifugation. To test this idea, we added 50 mM mβCD to Pgp in CHAPS, and then removed it by gel filtration chromatography. ATPase measurements were carried out for Pgp in CHAPS in the absence and presence of mβCD, and for a protein sample collected after application to the gel filtration column. As shown in Figure 2D, mβCD significantly inhibited the ATPase activity of Pgp, and this activity was essentially completely restored by removal of mβCD. These results suggest that mβCD directly inhibits the catalytic activity of Pgp, rather than exerting its effect by extracting cholesterol from the membrane. We attempted to use mβCD to deliver cholesterol to membrane vesicles previously depleted in cholesterol, but did not observe restoration of ATPase activity (not shown). We

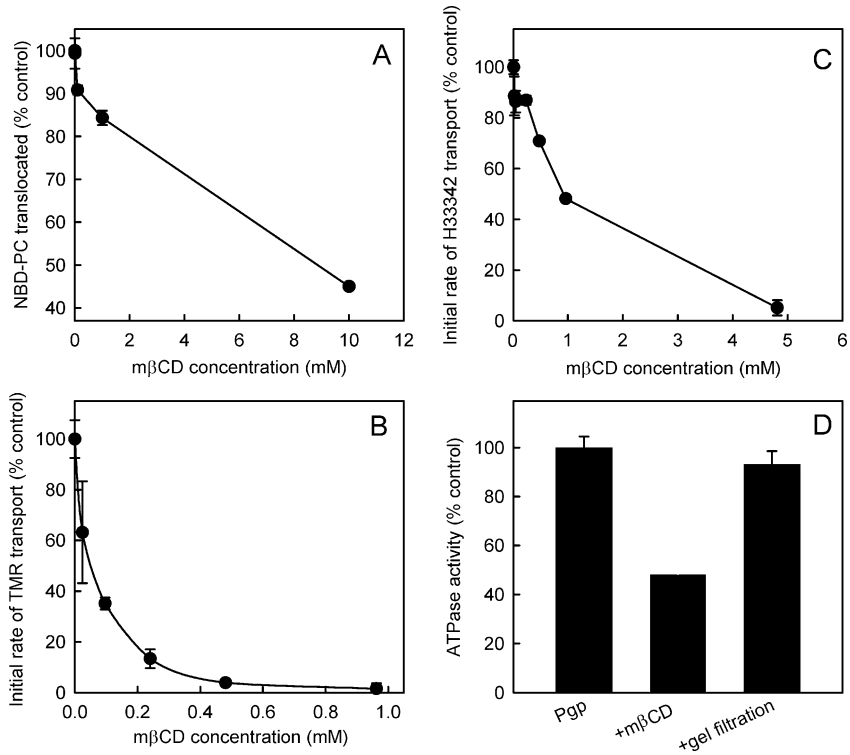

FIGURE 2: Effects of mβCD on the lipid flippase and substrate transport activities of reconstituted Pgp. (A) Effect of increasing concentrations of mβCD on the NBD-PC flippase activity at 37 °C of Pgp in egg PC proteoliposomes containing 30% (w/w) cholesterol. (B) Effect of mβCD on the TMR transport activity at 27 °C of Pgp in DMPC proteoliposomes containing 20% (w/w) cholesterol. (C) Effect of mβCD on H33342 transport activity at 27 °C of Pgp in DMPC proteoliposomes containing 20% (w/w) cholesterol. Data points represent the mean ± range ($n = 2$), and where error bars are not visible, they fall within the symbols. (D) Removal of mβCD restores ATPase activity to Pgp. Pgp in CHAPS was treated with 50 mM mβCD, which was then removed by gel filtration chromatography in CHAPS buffer, and ATPase activity was measured. Data points represent the mean ± SEM ($n = 3$), and where error bars are not visible, they fall within the bars.

believe that repletion of cholesterol did not result in an increase in ATPase activity due to the presence of an inhibitory concentration of mβCD.

The possibility remains that mβCD might inhibit the activity of CHAPS-solubilized Pgp by sequestering some of the detergent. Gel filtration experiments showed that when monomeric CHAPS (4 mM, below the critical micelle concentration) was mixed with 10 mM mβCD, the leading edge of the detergent peak eluted somewhat earlier than normal, suggesting that the cyclodextrin may bind some CHAPS. To explore this further, we first treated CHAPS-solubilized Pgp with 10 mM mβCD and then added back increasing concentrations of CHAPS (0−20 mM). We found no restoration of Pgp ATPase activity, suggesting that the inhibitory effect of mβCD was not due to removal of CHAPS.

mβCD is a hydrophilic cyclic oligosaccharide, unlike most Pgp substrates, which tend to be relatively hydrophobic, however the possibility remains that it may behave as a "substrate" and interact with the drug-binding pocket of Pgp. To address this, we determined the effect of increasing mβCD concentrations on the intrinsic Trp fluorescence of Pgp. mβCD did not significantly quench the fluorescence of the protein at concentrations up to 3 mM (not shown). Since Pgp substrates typically have K_d values in the nM to μM range, there is no evidence that mβCD interacts with the drug-binding pocket of the protein.

Effect of Bilayer Cholesterol on the ATPase Activity of Pgp. The possibility remains that the presence of cholesterol in the bilayer may have effects on the catalytic activity of Pgp. We measured the ATPase activity of CHAPS-extracted Pgp in the presence of varying concentrations of cholesterol (solubilized in absolute ethanol), and observed no change in the ATPase activity over the concentration range tested (Figure 3A). However, adding "solubilized" cholesterol to Pgp in detergent is not an ideal system to study its effects on a membrane-bound protein, and "solubilized" cholesterol may be present as microaggregates, so that the exact concentrations available to the protein are uncertain. Therefore, to better assess the effect of cholesterol on Pgp, the protein was reconstituted into DMPC vesicles with cholesterol content varying from 0−30% (w/w). We found that ATPase specific activity was slightly increased in the presence of 20% (w/w) cholesterol (Figure 3B). However, overall, the activity of reconstituted Pgp is affected to only a small extent by large changes in the cholesterol content of the membrane.

Verapamil stimulation was more pronounced for reconstituted Pgp compared to Pgp in membrane vesicles, showing an increase in activity of ~220% in bilayers of DMPC alone (Figure 3C vs Figure 1B). When Pgp was reconstituted into DMPC proteoliposomes with increasing cholesterol content, we observed a slight decrease in Pgp ATPase stimulation to ~170% in the presence of 30% (w/w) cholesterol. Thus, the cholesterol content of the membrane has a modest influence on both the basal and drug-stimulated ATPase activity of Pgp.

Early work by our group on Pgp−lipid interactions showed that a variety of lipids were important in preserving the ATPase activity of Pgp. Removal of protein-associated

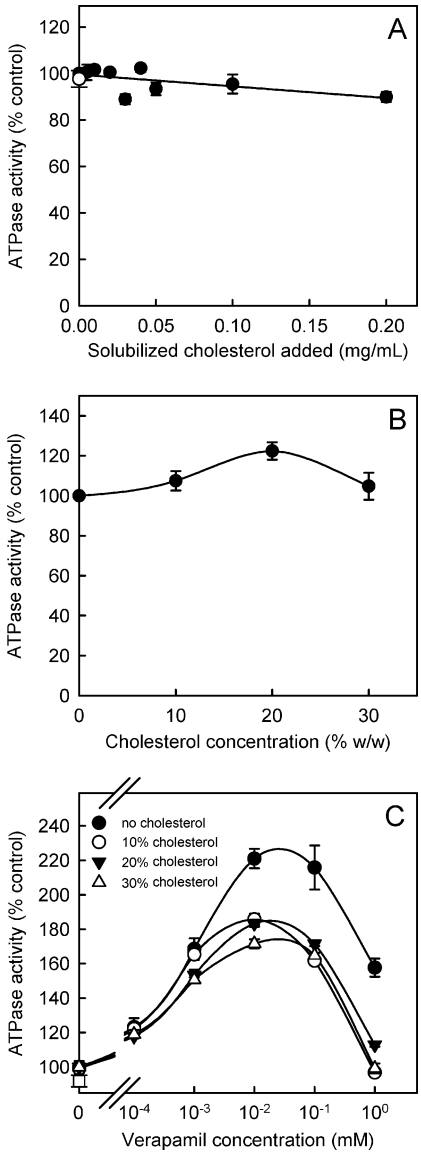

FIGURE 3: Effect of cholesterol on Pgp ATPase activity. (A) ATPase activity of Pgp in CHAPS solution in the presence of increasing concentrations of cholesterol added in ethanol. The control value in the absence of ethanol is shown by ○, while activity in the absence of cholesterol and the presence of ethanol is given by ● at the 0 mg/mL data point. (B) Relative ATPase activity of reconstituted Pgp in DMPC proteoliposomes containing 0−30% (w/w) cholesterol. (C) Verapamil stimulation of Pgp ATPase activity in DMPC proteoliposomes containing 0−30% (w/w) cholesterol, normalized to values in the absence of verapamil for each proteoliposome preparation. The control value in the absence of DMSO is shown by □. Data points represent the mean ± SEM ($n = 3$), and where error bars are not visible, they fall within the symbols.

phospholipids by treatment with deoxycholate greatly reduced Pgp ATPase activity, which was restored by addition of various phospholipids, such as egg PC (*54*). We used a similar approach to determine if cholesterol is also able to restore Pgp ATPase activity after delipidation. Deoxycholate treatment reduced the catalytic activity of Pgp to <45% of control. Subsequent treatment with 1 mg/mL egg PC restored the activity to 73% of the initial value, while addition of 0.5 mg/mL cholesterol resulted in some recovery of activity, to 54% of the initial value (data not shown). However, lower concentrations of cholesterol were unable to restore activity, with 0.25 mg/mL cholesterol having no effect. A combination of cholesterol and egg PC resulted in the best recovery of function, with an average of 81% of the initial ATPase activity restored.

Effect of Cholesterol on Pgp-Mediated Drug Transport and Lipid Flippase Activity. The initial rate of TMR transport for proteoliposomes with 0−20% (w/w) cholesterol varied in a biphasic fashion. For 5% and 10% cholesterol, the transport activity was elevated by about 40%, but reduced at 20% cholesterol (Figure 4A). Maximal H33342 transport of 160−170% of control was also observed in proteoliposomes of 10% (w/w) cholesterol, however, in this case the transport rate remained elevated in the presence of 20% cholesterol (Figure 4B). Since the ATPase activity of Pgp is not greatly stimulated by the presence of cholesterol, the increase in transport rate observed in the presence of cholesterol may result from alterations in the membrane environment, and indirect effects on parameters such as drug partitioning into the bilayer.

Pgp-mediated lipid flippase activity in proteoliposomes was also modulated by varying cholesterol content. We observed a modest decrease in NBD-PC flippase activity at 20−30% cholesterol (Figure 4C). Previous work by our group showed that Pgp-mediated flipping of fluorescent lipids is inhibited by the presence of Pgp substrates, and the parameter D_m represents the drug concentration causing 50% inhibition of flippase activity (*18, 19*). There is probably competition by these compounds for transport by Pgp, suggesting that lipids and drugs are transported by the same pathway in the protein. In the absence of cholesterol, vinblastine inhibits the transport of NBD-PC with a D_m of 12.6 μM (Figure 4D). As increasing concentrations of cholesterol were incorporated into egg PC proteoliposomes containing Pgp, the D_m for inhibition of flipping of NBD-PC by vinblastine decreased to 4.7 μM at 30% (w/w) cholesterol (Figure 4D). Thus cholesterol decreases the net flippase activity while enhancing the ability of vinblastine to compete with lipids for transport.

Effect of Cholesterol on the Binding of Pgp Substrates. Drug and ATP binding to Pgp can be measured by fluorescence quenching of Pgp labeled with an extrinsic fluorescent probe (2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; MIANS), or by quenching of its intrinsic Trp fluorescence (*38, 43*). Our laboratory previously employed MIANS-labeled Pgp fluorescence quenching to determine the effects of cholesterol on binding of substrates and modulators to the protein. Of the three compounds tested, only vinblastine showed altered binding to Pgp in the presence of varying cholesterol concentrations, with the K_d decreasing 10-fold at 20% (w/w) cholesterol (*11*). We determined the K_d for binding of vinblastine to purified Pgp reconstituted into DMPC proteoliposomes containing 0−30% (w/w) cholesterol, using quenching of the intrinsic Trp fluorescence (Table 1). In the absence of cholesterol, the vinblastine-induced quenching of Pgp fluorescence can be fitted to a binding equation to yield a K_d value of 0.68 μM. The binding affinity of vinblastine decreased roughly 6-fold to 3.8 μM at 30% (w/w) cholesterol (Table 1). This effect was also observed for progesterone, which showed a 3.7-fold decrease in binding affinity at 30% cholesterol. However, other substrates did not show this pattern. R123 showed little change in K_d value up to 20% (w/w) cholesterol and an increase in binding affinity at 30% (w/w) cholesterol, while daunorubicin showed no change in K_d up to 30%

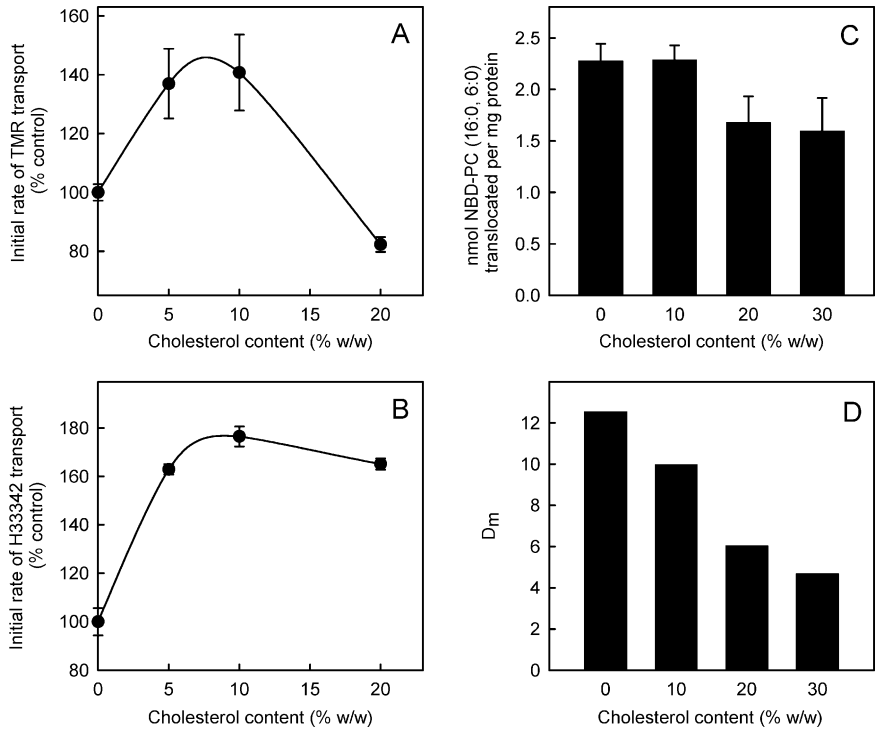

FIGURE 4: Effect of cholesterol on the lipid flippase and substrate transport activities of reconstituted Pgp. (A) Effect of cholesterol on the TMR transport activity of Pgp at 27 °C in DMPC proteoliposomes containing 0−30% (w/w) cholesterol. (B) Effect of cholesterol on the H33342 transport activity of Pgp at 27 °C in DMPC proteoliposomes containing 0−30% (w/w) cholesterol. (C) NBD-PC flippase activity of Pgp at 37 °C, determined in egg PC proteoliposomes containing 0−30% (w/w) cholesterol. Data points represent the mean ± range (n = 2), and where error bars are not visible, they fall within the symbols. (D) Effect of cholesterol on the D_m for inhibition of NBD-PC flipping by vinblastine. The net translocation of NBD-PC was assessed in the presence of varying concentrations of vinblastine in Pgp proteoliposomes of egg PC containing 0−30% (w/w) cholesterol. Translocation was measured after 20 min at 37 °C relative to controls without vinblastine (taken as 100%), and controls with vinblastine but without ATP (taken as 0%). D_m was determined from the x-intercept of a plot of log (f_a/f_u) vs log vinblastine concentration as described in Experimental Procedures.

Table 1: Effect of Cholesterol on ATP and Drug Binding to Reconstituted Pgp

	dissociation constant, K_d[a] (μM)				
bilayer cholesterol content % (w/w)	ATP	daunorubicin	progesterone	R123	vinblastine
0	790 ± 140	23 ± 2.9	0.89 ±0.16	65 ± 8.7	0.68 ± 0.17
10	1030 ± 290	27 ± 3.8	0.58 ± 0.07	54 ± 5.8	1.2 ± 0.42
20	910 ± 11	22 ± 2.6	1.9 ± 0.39	62 ± 10	1.9 ± 0.29
30	1980 ± 74	21 ± 2.4	3.3 ± 0.60	23 ± 3.1	3.8 ± 0.89

[a] Dissociation constants for ATP and drug binding were obtained by fitting Pgp intrinsic fluorescence quenching data to an equation for a single binding site (see eq 2), given as values ± SEM for fitting.

cholesterol (Table 1). The affinity of Pgp for binding ATP was reduced ~2.5-fold when 30% (w/w) cholesterol was included in the bilayer (Table 1).

Cholesterol Flippase Activity of Pgp. We investigated whether Pgp can maintain an altered bilayer leaflet distribution of NBD-cholesterol using a fluorescence-based flippase assay. Using this method we can determine the distribution of the NBD-lipid between the inner and outer leaflets of reconstituted Pgp-proteoliposomes before and after incubation with ATP. When NBD-PC was included in the liposome membrane, addition of dithionite produced a stable baseline when the outer leaflet portion was quenched, due to the inaccessibility of the fluorescent PC in the inner leaflet of well-sealed vesicles (Figure 5). However, when NBD-cholesterol was used, we were unable to measure a transbilayer gradient of the lipid after dithionite treatment, in liposomes of egg PC alone (Figure 5), or in proteoliposomes containing Pgp (not shown). This behavior could be due to (1) leaky vesicles that allow dithionite to quench the fluorescence in both leaflets, or (2) localization of NBD-cholesterol solely in the outer leaflet, or (3) rapid flip-flop of NBD-cholesterol. To investigate the possibility that the vesicles were not well-sealed, we prepared egg PC liposomes incorporating both 0.15% (w/w) NBD-PC and 0.15% (w/w) NBD-cholesterol. We were able to measure a stable baseline of fluorescence after addition of dithionite, indicating that the vesicles were in fact well sealed (Figure 5). The fluorescence intensity attained after dithionite addition indicated that all of the NBD-cholesterol and the outer leaflet portion of NBD-PC were quenched in these vesicles. This suggests that NBD-cholesterol flip-flops more rapidly between the inner and outer leaflets of the bilayer than the time resolution of the experiment. We observed a longer lag period between addition of dithionite and complete quenching of NBD-cholesterol fluorescence when the experiment was performed at lower temperatures (5−10 °C), but a stable fluorescence baseline could still not be achieved. We previously noted similar behavior for an NBD-labeled ceramide, which, like cholesterol, lacks a large polar headgroup (*19*).

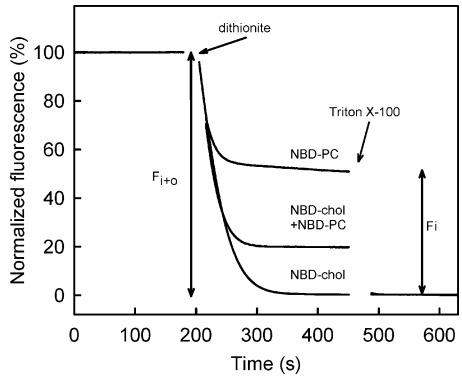

FIGURE 5: Quenching of NBD-PC and NBD-cholesterol fluorescence in egg PC liposomes by dithionite. Liposomes of egg PC containing 0.3% (w/w) NBD-PC, 0.3% (w/w) NBD-cholesterol, or 0.15% (w/w) NBD-PC plus 0.15% (w/w) NBD-cholesterol were equilibrated at 22 °C for 5 min. Fluorescence emission (λ_{ex} = 466 nm, λ_{em} = 536 nm) was then 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 reached again, 1% (w/v) Triton X-100 was added (second interruption in the trace). Data 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-lipid in both the inner and outer leaflets (F_{i+o}), and the fluorescence of NBD-lipid in the inner leaflet after quenching of the fluorescence due to outer leaflet lipids (F_i).

The fluorescence of the cholesterol analogue DHE is not quenched by dithionite, however, it can be quenched by a trinitrophenyl (TNP) group via a resonance energy transfer mechanism (*55, 56*). It has been reported that the transbilayer distribution of DHE can be measured in the presence of TNP-labeled amino acids (*55*). Therefore, we attempted to measure the transbilayer distribution of DHE in vesicles in the presence of increasing concentrations of TNP-Asp, TNP-Gly and TNP-Glu, on either the exterior or interior of the vesicles. We found that increasing concentrations of TNP-amino acids progressively quenched the fluorescence of DHE, which reached zero at sufficiently high concentrations (data not shown). We were again unable to observe a stable fluorescence baseline arising from DHE in one leaflet. Addition of ATP to proteoliposomes containing Pgp and DHE did not result in a change in fluorescence at any concentration of TNP-Asp used. Thus, it appears that this method is also unable to selectively quench the fluorescence of only the outer leaflet DHE. Garrigues et al. used oxidation of cholesterol by cholesterol oxidase to monitor the effect of Pgp on the cholesterol transbilayer distribution (*27*). When we added cholesterol oxidase to CH^RB30 plasma membrane vesicles, we noted a progressive decrease in free cholesterol content, due to oxidation of the cholesterol present in the outer membrane leaflet. Following incubation with ATP, we observed no significant difference in the cholesterol content of the membrane compared to a control with no ATP, contrary to what would be expected if Pgp translocated sterol to the inner leaflet where it would be protected from the action of cholesterol oxidase. Taken together, these results suggest that Pgp is not involved in cholesterol redistribution between leaflets of the membrane.

Effect of Cholesterol on Pgp Conformation. Pgp does not seem to be involved directly in cholesterol translocation, however, cholesterol does affect the drug binding, ATPase activity and drug transport properties of the protein. These effects might arise from cholesterol-mediated alterations in the structure of Pgp. To assess the influence of cholesterol on Pgp conformation, we measured acrylamide quenching of its intrinsic Trp fluorescence in the presence of DMPC vesicles containing 0−30% (w/w) cholesterol. Acrylamide quenching is an indicator of the accessibility of protein Trp residues to solvent, and changes in their accessibility (and thus changes in protein conformation) under varying conditions. The Stern−Volmer plot of F_0/F vs acrylamide concentration was linear, suggesting that only one class of Trp residues is quenched by acrylamide, and they are all equally accessible to quencher, as previously reported (*38*). In the absence of cholesterol, the Stern−Volmer quenching constant (K_{SV}) had a value of 1.8 M^{-1} (Table 2). When 10 or 20% cholesterol was added, the value of K_{SV} remained unchanged, indicating that the accessibility of Trp residues (and therefore Pgp conformation) was unaffected. In the presence of 30% cholesterol, there was an increase in the value of K_{SV} (Table 2), suggesting that at very high cholesterol concentrations, there is an increase in Trp accessibility. The water-soluble Trp analog NATA gave a Stern−Volmer quenching constant of ~26 M^{-1} in the absence of cholesterol, over 10-fold greater than that observed for Pgp, indicating that the change in Pgp conformation at 30% cholesterol (ΔK_{SV} of <0.6 M^{-1}) is relatively small.

Table 2: Acrylamide Quenching of Pgp Intrinsic Fluorescence in the Presence of Cholesterol

cholesterol content[a] (% w/w)	Stern−Volmer quenching constant[b] K_{SV} (M^{-1})
0	1.833 ± 0.015
10	1.825 ± 0.015
20	1.853 ± 0.019
30	2.451 ± 0.038

[a] Quenching of the intrinsic Trp fluorescence of CHAPS-solubilized Pgp by acrylamide was determined at 20 °C in the presence of DMPC vesicles containing increasing concentrations of cholesterol. [b] The K_{SV} was determined from linear Stern−Volmer plots using eq 3, given by values ± SEM for fitting.

Effect of Cholesterol on Membrane Partitioning of Drugs. Some of the effects of cholesterol on Pgp function could arise from effects of the sterol on the physicochemical properties of the membrane. We previously reported that, for a series of PC lipids, the Pgp binding affinity for a particular drug increased as partitioning of drug into the bilayer increased (*11*). The concentration of drug in the membrane is important for interaction with the transporter, and is likely a major factor influencing the apparent binding affinity. Membrane partitioning of drugs, and thus Pgp binding affinity, could be altered by the presence of cholesterol in the membrane. We determined the membrane−water distribution coefficient, K_{lip}, for a series of Pgp substrates, using egg PC vesicles containing 0−20% (w/w) cholesterol (Figure 6). These drugs were chosen because they had measurable UV−visible absorption for quantitation. For vinblastine, partitioning was highest in the absence of cholesterol and decreased progressively as the cholesterol content increased; at 20% cholesterol, K_{lip} was reduced by about 6-fold. This agrees well with the decreased Pgp binding affinity noted for vinblastine in the presence of cholesterol (Table 1). Other drugs showed a similar reduction in partitioning, with daunorubicin and verapamil experiencing

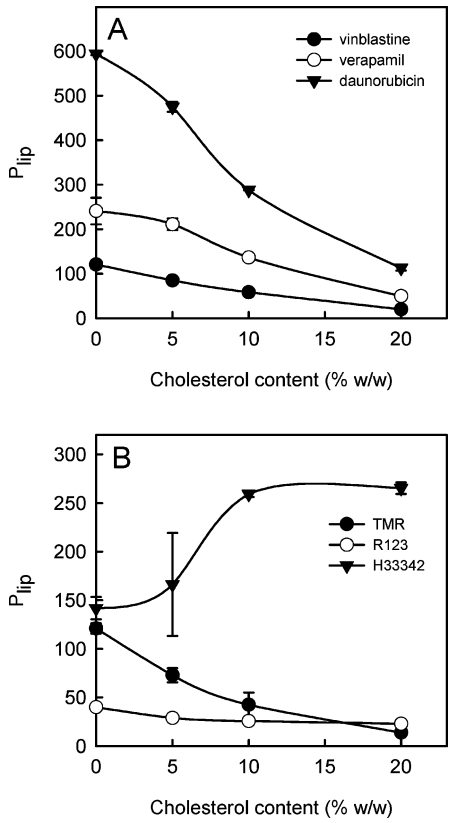

FIGURE 6: Partitioning of Pgp drug substrates, (A) vinblastine, verapamil and daunorubicin, and (B) TMR, R123 and H33342 into egg PC liposomes containing 0−20% (w/w) cholesterol. K_{lip} values were determined as described in Experimental Procedures. Symbols represent the mean ± SD ($n = 3$), and where error bars are not visible, they fall within the symbols.

about a 5-fold reduction in K_{lip} at 20% cholesterol, and TMR showing a reduction of ~8-fold. R123 showed only a ~2-fold reduction in partitioning at 20% cholesterol, which agrees well with the lack of effect of cholesterol on its binding affinity. However, the binding affinity of daunorubicin was not greatly affected by the presence of cholesterol. In contrast to the other drugs, H33342 lipid partitioning increased ~2-fold in the presence of 20% cholesterol (Figure 6B). H33342 transport was also greatly increased in the presence of cholesterol (Figure 4B), possibly as a result of the enhanced ability of the drug to enter the membrane in the presence of cholesterol.

DISCUSSION

In the present work and previous studies, we observed high levels of ATPase activity and drug transport for Pgp reconstituted into synthetic PC proteoliposomes devoid of cholesterol. Substrate transport (*17, 52, 57*), lipid flippase activity (*18, 19*) and binding of drugs (*11*) also take place in bilayers lacking added cholesterol. Thus it appears that cholesterol is not strictly required for Pgp function. Membrane cholesterol content is highly regulated in the cell, and the absolute cholesterol content of the plasma membrane varies very little. However, some regions of the membrane (such as lipid rafts and caveolae) are enriched in cholesterol, and if Pgp moved from regions of low to high cholesterol content under various conditions, this could be a mechanism to modulate its activity. Evidence suggests that Pgp may exist in detergent-resistant membrane microdomains in some cell types (*32, 41, 58*).

mβCD is a reagent that is frequently employed to extract cholesterol from membranes. At sufficiently high concentrations, mβCD can efficiently extract essentially all the cholesterol from membranes (see, for example, Figure 1A). While mβCD does not appear to bind directly to Pgp (as assessed by intrinsic fluorescence quenching), the present work suggests that it inhibits Pgp catalytic activity, independent of its ability to extract membrane cholesterol. We observed that mβCD inhibited Pgp ATPase activity, transport of TMR and H33342, and flipping of NBD-PC. As the absolute ATPase activity decreased with increasing mβCD concentrations, there was greater relative stimulation of the ATPase activity in the presence of drug substrates, however, the maximal level of ATPase activity induced by verapamil was similar before and after cholesterol depletion. The inhibitory effect of mβCD was reversible, and Pgp activity was restored when it was removed. An alternative view of the observed mβCD effects is that this molecule may sequester drug or NBD-PC in transport/flippase experiments. Under the conditions of our experiments, mβCD did not disrupt or solubilize the proteoliposomes, neither did it extract NBD-PC from the bilayer. Its effects on detergent-solubilized Pgp also did not appear to arise from CHAPS sequestration. The cyclodextrin did seem to show some TMR binding, which may contribute to its observed inhibition of Pgp-mediated transport. Taken together, our results make it clear that mβCD is unsuitable for use in studies of Pgp. Several reports in the literature regarding the effect of cholesterol on Pgp have employed mβCD (*27, 28, 30, 32*), complicating interpretation of the results. In further work reported here, we altered the cholesterol content directly by reconstituting Pgp into defined lipid bilayers, thus avoiding any effects of reagents such as mβCD.

Inclusion of cholesterol in the membrane had a modest stimulatory effect on Pgp ATPase activity, and also reduced verapamil stimulation of activity. However, the concentration of verapamil causing maximum stimulation was unaltered in the presence of cholesterol, suggesting no major change in the protein−drug interaction. We observed no effect of cholesterol on the activity of Pgp in detergent solution, but this may be because cholesterol cannot interact closely with the protein under these conditions, as it does in membranes.

The presence of cholesterol in the bilayer modulated the transport and lipid flippase activities of Pgp. While flippase activity was moderately reduced by cholesterol, transport of the fluorescent drugs TMR and H33342 was greatly stimulated at 5−10% cholesterol. The presence of other transport substrates can stimulate the transport of TMR in proteoliposomes (*17*), indicating that cholesterol could potentially be a Pgp substrate. However, a more likely explanation is that cholesterol alters the physicochemical properties of the bilayer, which in turn affect drug transport (*17*). The measured rate of TMR transport is the difference between Pgp-mediated transport of the drug up a concentration gradient, which results in self-quenching in the proteoliposome interior, and passive diffusion of TMR down the concentration gradient. Any change in the ability of TMR to cross the membrane would change the apparent transport rate. Dos Santos et al. used 1,6-diphenylhexa-1,3,5-triene fluorescence anisotropy measurements, as well as pyrene-

eximer formation, to show that cholesterol reduces membrane fluidity in the acyl chain region (*32*). Decreased membrane fluidity would likely reduce the rate of passive outward diffusion of TMR, and thus the apparent net accumulation of the dye inside proteoliposomes would increase. In agreement with these ideas, we observed a decrease in TMR partitioning into lipid in the presence of cholesterol. H33342 transport was greatly increased in the presence of cholesterol. This compound is weakly fluorescent in solution and highly fluorescent in the hydrophobic core of the membrane. The H33342 transport assay depends heavily on the enhanced fluorescence of the drug within the membrane (*59*). We found that H33342 partitions better into bilayers containing cholesterol than into bilayers of PC alone. Therefore, the drug concentration in the membrane will be higher in the presence of cholesterol, resulting in a higher transport rate.

The lipid flippase activity of Pgp is much less affected by the presence of cholesterol than the drug transport activity. In this assay, NBD-PC is present at the same concentration in the membrane regardless of the cholesterol content. It is interesting, however, that the D_m for inhibition of Pgp-mediated lipid flipping by vinblastine is greatly reduced as the cholesterol content is increased, suggesting that vinblastine competes much better with NBD-lipid for binding to Pgp in the presence of cholesterol. However, the ability of vinblastine to partition into lipid membranes is greatly reduced in the presence of cholesterol, as is the apparent Pgp binding affinity. There is currently no satisfactory explanation for the decrease in the value of D_m.

The binding affinity of Pgp for ATP and some drugs was altered by the presence of cholesterol. We previously reported that ATP binding affinity was dependent on membrane fluidity, with a higher K_d measured in the gel phase than in the fluid liquid-crystalline phase (*16*). This is in agreement with the results presented here, in which we observe a lower ATP binding affinity in the presence of increasing cholesterol concentrations. For some drug substrates, such as vinblastine and progesterone, binding affinity was lower in the presence of cholesterol, while for others, such as daunorubicin and R123 at <20% cholesterol, it was essentially unaltered. This suggests that the effects of cholesterol are specific for each drug, and may be related to physical parameters such as drug−lipid partitioning. In contrast to the report of Kimura et al. (*30*), we found no relationship between drug molecular weight and the effect of cholesterol on binding affinity. Vinblastine (a large drug) displayed a large reduction in binding affinity at higher cholesterol levels, whereas daunorubicin (a small drug) showed very little change in binding affinity with increasing cholesterol.

We found that cholesterol at 20% (w/w) had a large effect on lipid partitioning of Pgp substrates, reducing the K_{lip} of all drugs except H33342 by 2- to 9-fold. Since Pgp is proposed to interact with its substrates within the membrane, its drug binding and transport properties are expected to be modulated by the presence of cholesterol at concentrations sufficient to reduce drug-membrane partitioning. The presence of cholesterol would thus be expected to greatly alter the transport rate, as was observed in this work. Cholesterol also reduced the affinity of some drugs for binding to Pgp, which is in accordance with its effect on their membrane partitioning, however for other drugs there was little effect of cholesterol on binding. Wang et al. reported that cholesterol interacted with the daunorubicin binding site of Pgp and increased ATPase activity (*31*). We observed a ~5-fold reduction in partitioning of daunorubicin into lipid containing cholesterol, but no effect on K_d, which is in agreement with previous work using MIANS-labeled Pgp (*11*). However, Wang et al. employed a water-soluble cholesterol derivative, polyoxyethylcholesteryl sebacate, which has a large polar group that would likely greatly alter the molecular properties compared to native cholesterol. Polyoxyethyl-based compounds, such as pluronic block copolymers, have also been implicated in modulation of Pgp activity (*60, 61*) and thus the conclusions drawn from the study of Wang et al. are uncertain.

Cholesterol does not appear to require a transport protein to move between bilayer leaflets, since its flip-flop rate is quite fast (reviewed in ref *62*). Our results using NBD-cholesterol confirm that the intrinsic flip-flop rate of cholesterol is faster than the time resolution of the assay. We also attempted to measure flipping of cholesterol by making use of its accessibility to cholesterol oxidase, with similar conclusions. It is possible that Pgp plays an indirect role in the maintenance of cholesterol distribution between bilayer leaflets. We previously showed that Pgp can act as a flippase for sphingomyelin and simple GSL (*19*). Cholesterol has an affinity for sphingomyelin (*47*), and may "follow it" in the membrane, so if Pgp alters the sphingomyelin distribution, this may control the distribution of cholesterol indirectly.

Pgp has an annulus of ~53 phospholipids surrounding it, and it is not currently known whether cholesterol is also present in this boundary lipid layer. Our work suggests that this is not likely. The ATPase activity of delipidated Pgp was restored to approximately the same extent by egg PC-cholesterol as egg PC alone, and cholesterol alone did not restore ATPase activity. Cholesterol also had essentially no effect on Pgp conformation, as assessed by Trp accessibility to acrylamide quenching, further suggesting that there is little direct interaction with the sterol. Finally, it was not possible to cross-link a radiolabeled photoactive cholesterol analogue (7-azi-5-α-cholestan-3β-ol [3,5,6-^{3}H]) to purified Pgp under conditions where the sterol-binding protein, NPC1, showed strong binding (R. Liu, P. Lu, J. W. K. Chu, and F. J. Sharom, unpublished results). Overall, the work presented here suggests that the indirect effects of cholesterol likely play the dominant role in modulating Pgp activity, rather than a direct interaction with the protein itself.

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