Cholesterol Potentiates ABCG2 Activity in a Heterologous Expression System: Improved in Vitro Model to Study Function of Human ABCG2


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ABSTRACT

ABCG2, a transporter of the ATP-binding cassette family, is known to play a prominent role in the absorption, distribution, metabolism, and excretion of xenobiotics. Drug-transporter interactions are commonly screened by high-throughput systems using transfected insect and/or human cell lines. The determination of ABCG2-ATPase activity is one method to identify ABCG2 substrate and inhibitors. We demonstrate that the ATPase activities of the human ABCG2 transfected Sf9 cell membranes (MXR-Sf9) and ABCG2-overexpressing human cell membranes (MXR-M) differ. Variation due to disparity in the glycosylation level of the protein had no effect on the transporter. The influence of cholesterol on ABCG2-ATPase activity was investigated because the lipid compositions of insect and human cell lines are largely different from each other. Differences in cholesterol content, shown by cholesterol loading and depletion experiments, conferred the difference in stimulation of basal ABCG2-ATPase of the two cell membranes. Basal ABCG2-ATPase activity could be stimulated by sulfasalazine, prazosin, and topotecan, known substrates of ABCG2 in cholesterol-loaded MXR-Sf9 and MXR-M cell membranes. In contrast, ABCG2-ATPase could not be stimulated in MXR-Sf9 or in cholesterol-depleted MXR-M membranes. Moreover, cholesterol loading significantly improved the drug transport into inside-out membrane vesicles prepared from MXR-Sf9 cells. MXR-M and cholesterol-loaded MXR-Sf9 cell membranes displayed similar ABCG2-ATPase activity and vesicular transport. Our study indicates an essential role of membrane cholesterol for the function of ABCG2.

ABCG2 (also referred to as breast cancer resistance protein and MXR) is one of the most important efflux transporters in endothelial and epithelial cells, modulating absorption distribution metabolism excretion properties of drugs and other xenobiotics (for review, see Mao and Unadkat, 2005). ABCG2 exhibits a broad substrate specificity because it transports hydrophobic, anionic, and cationic drugs (Mao and Unadkat, 2005). Therefore, it is widely believed that ABCG2 plays an important role in intestinal absorption (Polli et al., 2004) and secretion of xenobiotics and metabolites (Ebert et al., 2005), secretion of sulfate conjugates in the liver (Zamek-Gliszczynski et al., 2006), and prevention of penetration of drugs into the brain (Breedveld et al., 2005). ABCG2 may play a pivotal role in placenta as a defensive barrier to drugs as well as reducing steroid levels in fetal tissues (Jonker et al., 2000). ABCG2 knockout mice models shed light on some special functions of ABCG2, such as secretion of xenobiotics into the milk (Jonker et al., 2005) and protection of stem cells from hypoxia-induced protoporphyrin accumulation and damage (Krishnamurthy and Schuetz, 2005).

ABCG2 function is commonly studied using high-throughput cellular (Robey et al., 2004) or membrane-based assays (Ozvegy et al., 2001; Janvilisri et al., 2003) (for review, see Glavinas et al., 2004; http://www.solvo.com/). For most applications, the transporter is expressed in insect cell lines (e.g., Sf9), taking advantage of the robust baculovirus insect cell...
system (Ozvegy et al., 2001). In contrast, membranes can be prepared from human cell lines selected for drug resistance overexpressing the transporter (Han and Zhang, 2004). More recently, it has turned out that during the selection process, the protein acquired a mutation in the position of amino acid 482 (Honjo et al., 2001). The R482G/T mutants display a substrate specificity different from the wild-type (WT) protein (Honjo et al., 2001; Ozvegy et al., 2002). More strikingly, unlike the ATPase function of the R482G/T mutants, the ATPase activity of the WT protein could not be stimulated by prazosin, a known ABCG2 substrate (Xiao et al., 2006), when expressed in insect cell membranes (Ozvegy et al., 2001; Ishikawa et al., 2003). We have shown that this nonresponsiveness is not an inherent property of the transporter because vanadate-sensitive ATPase activity of human cell membranes expressing similar amounts of ABCG2 can be stimulated by substrate of the transporter (H. Glavinás, E. Kis, A. Pál, R. Kovács, M. Jani, E. Vágó, É. Molnár, S. Bánszági, Z. Kele, T. Janáky, G. Bátóri, O. von Richter, G.-J. Koomen, and P. Krajcsi, unpublished data). Likewise, ATPase activity of ABCG2-expressing membranes isolated from Lactococcus lactis was shown to be stimulated by similar substrates (Janvilsíri et al., 2003).

Because it is of pivotal importance that in vitro models closely mimic the physiological phenotype, we have decided to study the underlying differences between the ABCG2-ATPase when the transporter was expressed in Sf9 and in human cell membranes. We report here that differences in glycosylation of ABCG2 in the two expression systems do not affect the ATPase activity of the transporter. In contrast, the low-cholesterol content of the insect membranes profoundly affects drug-stimulated ATPase activity of the expressed ABCG2 protein because cholesterol loading decreases the basal ABCG2-ATPase activity in MXR Sf9 membranes to a degree similar to the native, cholesterol-rich human MXR-M membranes. The modulation of ABCG2 activity by cholesterol may have implications on the localization of the ABCG2 transporter in distinct compartments of the plasma membrane because two other multidrug resistance protein ABC transporters ABCB1 (Bacso et al., 2004) and ABCC2 (Tietz et al., 2005) have been shown to localize in the cholesterol-rich raft microdomains of cell membranes.

Materials and Methods

Chemicals and Biochemicals. [3H]Methotrexate was purchased from Moravek Biochemicals (Brea, CA). [3H]Estrone-3-sulfate and [3H]prazosin were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). Topotecan was purchased from LKT Laboratories (St. Paul, MN). The antibody against ABCG2 was purchased from Abcam (Cambridge, UK). Recombinant baculoviruses encoding wild-type human ABCG2 were kind gifts from Prof. B. Sarkadi (National Medical Center, Budapest, Hungary). Randomly methylated-β-cyclodextrin (RAMEB) and cholesterol complex of RAMEB (cholesterol@RAMEB; Piel et al., 2006; cholesterol content, 4.74%) was provided by Cyclolab (Cyclodextrin Research and Development Laboratory, Budapest, Hungary). Koi134 and Koi13 (Allen et al., 2002) were kind gifts of Prof. G. J. Koomen (National Cancer Institute, Amsterdam, The Netherlands). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise in the text.

Membrane Preparation. Human membrane vesicle preparations (MXR-M) as well as membrane vesicle preparations obtained from insect cells expressing ABCG2 (MXR-Sf9) were obtained from SOLVO Biotechnology (Budapest, Hungary; http://www.solvo.com/). Both membranes are overexpressing the wild-type version of ABCG2. The insect membrane vesicle preparations were produced using recombinant baculoviruses encoding ABCG2 (Ozvegy et al., 2002). Sf9 cells were cultured and infected with recombinant baculovirus stocks as described earlier (Sarkadi et al., 1992). Purified membrane vesicles from baculovirus-infected Sf9 cells were prepared essentially as described previously (Sarkadi et al., 1992). Membrane protein content was determined using the BCA method (Pierce Biotechnology, Rockford, IL).

ABCG2 Deglycosylation. Enzymatic deglycosylation was done using peptide-N-glycosidase F (Sigma-Aldrich). One microliter of the enzyme at 500 U/ml was added to 50 μl of membrane suspension (5 mg/ml protein) in TMEP (50 mM Tris, 50 mM mnanitol, 2 mM EGTA, 8 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml phenylmethysulfonyl fluoride, and 2 mM dithiothreitol, pH 7.0) vigorously mixed and incubated at 37°C for 10 min. Deglycosylation was detected by Western blotting.

Western Blotting. The proteins were separated using a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) at 350 mA in a transfer buffer composed of 25 mM Tris, 192 mM glycine, and 15% (v/v) methanol, pH 8.3. The membrane was treated with blocking buffer (5% nonfat dry milk powder and 0.5% bovine serum albumin in phosphate-buffered saline with 0.05% Tween 20) for 2 h at room temperature. The membrane was then incubated with the primary antibody, a mouse anti-ABCG2 monoclonal antibody BXP-21 (Abcam), diluted 1:1000 in blocking buffer for 2 h at room temperature. The membrane was washed for 3 × 10 min with phosphate-buffered saline@0.05% Tween 20 at room temperature. It was then incubated with the secondary antibody, anti-mouse IgG-HRP, a horseradish peroxidase-conjugated species-specific whole antibody (Sigma-Aldrich) diluted 1:5000 in blocking buffer for 1 h at room temperature. The membrane was subsequently washed as described above, and immunoreactive bands were visualized with ECL Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

ABCG2-ATPase Activity. ATPase activity was measured as described earlier (Sarkadi et al., 1992). In brief, membrane vesicles (20 μg/well) were incubated in ATPase assay buffer (10 mM MgCl2, 40 mM MOPS-Tris, pH 7.0, 50 mM KCl, 5 mM dithiothreitol, 0.1 mM EGTA, 4 mM sodium azide, 1 mM ouabain), 5 mM ATP, and various concentrations of test drugs for 40 min at 37°C. ATPase activities were determined as the difference of inorganic phosphate liberation measured with and without the presence of 1.2 mM sodium orthovanadate (vanadate-sensitive ATPase activity). In the experiments presented in Fig. 5, the PREDEASY ABCG2-ATPase Kit (SB-MXR-HAM-PREDEASY-ATPase Kit; SOLVO Biotechnology, Szeged, Hungary) was used for the determination of ABCG2-ATPase activity according to the manufacturer’s suggestions.

Vesicular Transport Assay. Inside-out membrane vesicles were incubated in the presence or absence of 4 mM ATP. For methotrexate vesicular transport, the measurements were carried out in 7.5 mM MgCl2, 40 mM MOPS-Tris, pH 7.0, 70 mM KCl at 37°C for 12 min. The transport was stopped by addition of cold wash buffer (40 mM MOPS-Tris, pH 7.0, 70 mM KCl).

For prazosin vesicular transport, 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 10 mM MgCl2 containing buffer was incubated at 37°C for 20 min. The transport was stopped by addition of cold wash buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 100 mM NaCl).

For estrone-3-sulfate vesicular transport, 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 10 mM MgCl2 containing buffer were incubated at 32°C for 1 min. The transport was stopped by addition of cold wash buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 100 mM NaCl).

The incubation mix was then rapidly filtered through class B glass fiber filters (pore size, 0.1 μm). Filters were washed with 5 × 200 μl of ice-cold wash buffer, and radioactivity retained on the filter was
measured by liquid scintillation counting. ATP-dependent transport was calculated by subtracting the values obtained in the absence of ATP from those in the presence of ATP.

**Determination of Cholesterol Content.** The cholesterol content of the membranes was determined using the cholesterol oxidase method (Contreras et al., 1992). Ten microliters of reaction mix (500 mM MgCl₂, 500 mM Tris buffer, 100 mg Triton X-100, pH 7.4) was mixed with 10 µl of cholesterol oxidase enzyme at 1 mg/ml (Roche Diagnostics, Basel, Switzerland) and 50 µl of membranes. The solution was incubated at 37°C for 30 min. The reaction was stopped by adding 100 µl of a methanol/ethanol solution (50% v/v) and incubated at 0°C for an additional 30 min. After 5 min of centrifugation at 700g, 25 µl of the supernatant was analyzed by high-performance liquid chromatography. For chromatographic separation, high-performance liquid chromatography (1100 Series set; Agilent Technologies, Santa Clara, CA) and C18 reverse-phase chromatographic column (3 µM, 100 × 2 mm Luna; Phenomenex, Torrance CA) were used. Analytes were separated using 1% v/v acetic acid/methanol as mobile phase at a flow rate of 0.3 ml/min. The oxidized cholesterol was detected using an UV detector at 241 nm. To quantify the amount of cholesterol, external cholesterol calibration standards were used.

**Cholesterol Loading and Depletion.** For optimization of cholesterol@RAMEB, concentrations used for cholesterol loading, MXR-Sf9, and MXR-M membrane preparations were diluted 10 times in ATPase assay buffer containing cholesterol@RAMEB complex at concentrations indicated (Fig. 3, A and D). After a 1-h incubation at 37°C, membrane vesicles were washed with ATPase assay buffer and suspended in the reaction medium. For optimization of cholesterol loading time, the incubation was carried out in the presence of 2 mM cholesterol@RAMEB at 37°C for time periods indicated in Fig. 3C.

Cholesterol loading of MXR-Sf9 membranes used in Figs. 4 to 8 was carried out for 30 min at 37°C in the presence of 1 mM cholesterol@RAMEB complex. In cholesterol depletion studies (Fig. 3B), MXR-M and MXR-Sf9 membranes were diluted 10 times in ATPase assay buffer containing various concentrations of RAMEB and incubated for 30 min at 37°C. Membrane vesicles then were washed with ATPase assay buffer and suspended in the reaction medium.

For cholesterol depletion of vesicles used in experiments shown in Figs. 4 and 7, MXR-M membranes were incubated in ATPase assay buffer containing 8 mM RAMEB for 30 min at 37°C. Then, membrane vesicles were washed with ATPase assay buffer and suspended in the reaction medium. Control (nontreated) membranes were incubated and washed similarly in ATPase reaction buffer without the presence of RAMEB or cholesterol@RAMEB complex. The cholesterol-loaded transporter-containing membrane preparations are the proprietary technology of SOLVO Biotechnology.

**Data Analysis.** Assays were run in duplicates. Data are presented as means ± S.D. The potencies of drugs to alter ATPase activity were obtained from plots of the rate of ATP hydrolysis as a function of logarithm of drug concentration by nonlinear regression of the following equation:

\[
v = \frac{V_{\text{max}}}{1 + 10^{\log EC_{50} - \log [A] \times x}}
\]

where \(v\) is enzyme activity (nanomoles of Pi per minute per milligram), \(V_{\text{max}}\) is maximal ATPase activity (nanomoles of Pi per minute per milligram), \(K_m\) is the Michaelis-Menten constant for the tested substrate (nanomolar) and [S] substrate concentration (nanomolar).

**Results**

**Known Substrates Show Different ATPase Profiles in ABCG2-Expressing Insect and Human Membranes.** To investigate the relevance of the heterologous baculovirus-insect cell expression system, the WT human ABCG2 protein was expressed in Sf9 cells. The membranes purified from the ABCG2-overexpressing insect cells (MXR-Sf9 membranes) were studied along with membranes purified from ABCG2-overexpressing human cells (MXR-M membranes; http://www.solvo.com). Using prazosin, a known substrate of the WT human ABCG2 (Xiao et al., 2006), as well as sulphasalazine, a substrate of the mouse breast cancer resistance protein 1 (Zaher et al., 2006) and a substrate of the human protein (van der Heijden et al., 2004), a clear difference was seen in the ATPase stimulation profile of ABCG2 expressed in these membranes. Sulphasalazine and prazosin stimulated the different types of curves, an equation that is a combination of two sigmoid responses was used:

\[
v = \frac{Dip + \frac{V_{\text{min}1} - Dip}{1 + 10^{\log EC_{501} - \log [A] \times x}} + \frac{V_{\text{min}2} - Dip}{1 + 10^{\log EC_{502} - \log [A] \times x}}}{2}
\]

where \(v\) is response (nanomoles of Pi per minute per milligram), \(V_{\text{min}1}\) and \(V_{\text{min}2}\) are minimal responses, Dip is maximal response, \(EC_{501}\) and \(EC_{502}\) are ligand concentrations producing 50% of maximal response (efficacy), [A] is the actual test drug concentration, and Hill slope 1 and Hill slope 2 are the constants of cooperativity.

The Michaelis-Menten parameters of maximal velocity (\(V_{\text{max}}\)) and drug affinity (\(K_m\)) were obtained from plots of the ATPase activity as a function of test drug concentration by nonlinear regression of the following equation:

\[
v = \frac{V_{\text{max}} \times [S]}{K_m + [S]}
\]
ABCG2-ATPase activity (3.9- and 2.7-fold, respectively) when expressed in human cell membrane (Fig. 1). In contrast, in the Sf9 system, neither sulfasalazine nor prazosin did significantly modulate the ABCG2-ATPase activity (Fig. 1). The observed differences could not be attributed to differences in expression levels because the proteins were overexpressed at similarly high levels in the two systems (Fig. 2A).

**The Difference in Glycosylation of ABCG2 in Sf9 versus Human Membranes Does Not Confer the Difference in ATPase Activation Profiles.** The ABCG2 content

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**Fig. 2.** Deglycosylation does not affect kinetics of MXR-M ATPase. A, representative Western blot of ABCG2 in MXR-M and MXR-Sf9 membranes. Membrane fractions from ABCG2-overexpressing human cells were loaded as a reference for the fully glycosylated protein (lane 1) and fully deglycosylated protein after peptide-N-glycosidase (0.5 U) treatment (lane 2). Native MXR-Sf9 membrane was loaded as control (lane 3). B, inhibition of basal ABCG2-ATPase activity of deglycosylated MXR-M membrane (●) and untreated control (▲) by the ABCG2-specific inhibitor Ko134. C, activation of deglycosylated MXR-M membrane (●) and untreated control (▲) by estrone-3-sulfate. Data represent mean ± S.D. of duplicates.

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**Fig. 3.** Cholesterol content modulates ABCG2-ATPase activity in membrane preparations from MXR-Sf9 and MXR-M. A, MXR-Sf9 (▲) and MXR-M (●) membranes were loaded with cholesterol by treatment with cholesterol@RAMEB complex. Membrane cholesterol content was determined by the cholesterol oxidase method. Concentrations on x-axis represent the total cholesterol concentration in the incubation medium. B, cyclodextrin-mediated cholesterol depletion of MXR-Sf9 (▲) and MXR-M (●) membranes was carried out by incubating the membranes for 30 min at 37°C in the presence of RAMEB at concentrations indicated in the figure. C and D, optimization of cholesterol loading. Basal (●) and sulfasalazine-stimulated (▲) activities were monitored after completing the loading procedure. For optimizing the incubation time (C), MXR-Sf9 membranes were incubated in the presence of cholesterol@RAMEB (2 mM total cholesterol in the incubation medium) for times indicated in the figure. For optimization of cholesterol loading (D), MXR-Sf9 membranes were incubated in the presence of various cholesterol concentrations for 1 h. ABCG2-ATPase activity data represent mean ± S.D. of duplicate measurements.
and glycosylation level of the membrane preparations were analyzed by Western blotting with BXP-21, an ABCG2-specific antibody (Fig. 2A). MXR-M membranes (Fig. 2A, lane 1) and MXR-Sf9 membrane preparations (Fig. 2A, lane 3), both overexpressing ABCG2, displayed strong bands with apparent molecular masses of 75 to 80 and 60 to 65 kDa, respectively. ABCG2 is a glycosylated protein (Diop and Hrycyna, 2005; Mohrmann et al., 2005), and it has been shown that in Sf9 cells, the protein is severely underglycosylated because inhibition of glycosylation in a human cell line using tunicamycin yielded nonglycosylated species with a mobility corresponding to a molecular mass of 60 to 65 kDa that is similar to the mobility of ABCG2 expressed in Sf9 membranes (Ozvegy et al., 2001). However, in accordance with published data (Ozvegy et al., 2001), tunicamycin did not yield complete unglycosylation (data not shown); therefore, we used peptide-N-glycosidase F to deglycosylate ABCG2 expressed in MXR-M membranes. The enzymatic cleavage resulted in a complete deglycosylation of ABCG2 (Fig. 2A, lane 2).

Ko134, a known specific inhibitor of ABCG2, fully inhibited the transporter-dependent vanadate-sensitive basal activity of the glycosylated and deglycosylated forms of the protein with equal potency (Fig. 2B). Likewise, no difference was observed in the estrone-3-sulfate-stimulated ATPase activation profile of the fully glycosylated and deglycosylated forms (Fig. 2C). Therefore, we conclude that differences in the glycosylation of ABCG2 in the Sf9 and the human membranes do not explain the observed differences in drug-stimulated ATPase activity of the protein.

**Cholesterol Loading Potentiates Drug-Induced ATPase Stimulation of ABCG2-Expressing MXR-Sf9 Membranes.** It has been known that lipid composition of insect and mammalian membranes significantly differ. One of the major difference is the cholesterol content of Sf9

![Fig. 4](image-url)
plasma membrane being approximately 5- to 10-fold lower than that of human plasma membrane (Gimpl et al., 1995). As expected, the cholesterol contents of the MXR-Sf9 and MXR-M vesicles containing ABCG2 protein were markedly different, with MXR-Sf9 displaying an approximately 4- to 5-fold lower cholesterol level (6.5 versus 28.99 μg/mg protein (Fig. 3A) and 6.26 versus 23.68 μg/mg protein (Fig. 3B)). Cholesterol loading of both membranes using cholesterol@RAMEB treatment resulted in approximately a 15-fold increase of cholesterol content in MXR-Sf9 vesicles and 3-fold increase in MXR-M vesicles yielding ~90 μg/mg protein final cholesterol content in both membranes (Fig. 3A). On the other hand, RAMEB treatment removed the cholesterol from both membranes very efficiently (Fig. 3B).

For further experiments, cholesterol loading of ABCG2-expressing Sf9 membranes has been optimized for ATPase activity with respect to exposure time (Fig. 3C) and loading concentration of cholesterol (Fig. 3D). For experiments shown in Figs. 4 to 7, 30 min and 1 mM cholesterol@RAMEB were chosen as optimal time and concentration. The membrane cholesterol content after treatment is around 60 μg/mg protein, approximately 2-fold greater than in the MXR-M membrane. We selected these values because under these conditions the membrane cholesterol content was relatively insensitive for either parameter (Fig. 3, C and D); therefore, it allowed for reproducible production of cholesterol-loaded membranes. For cholesterol depletion of MXR-M membranes, a 30-min treatment using 8 mM RAMEB was selected, yielding membrane preparations with an average cholesterol content of 3.5 μg/mg protein.

Differences in Cholesterol Content Confer the Difference in ATPase Stimulation of ABCG2-Expressing MXR-Sf9 and MXR-M Membranes. The ATPase activity of WT ABCG2-expressing Sf9 membranes was refractory to...
stimulation upon treatment with sulfasalazine (Fig. 4A). Cholesterol loading significantly potentiated sulfasalazine-induced activation of ABCG2-ATPase (Fig. 4A). In contrast, ABCG2 activity of MXR-M membranes was stimulated by sulfasalazine in a concentration-dependent manner (Fig. 4B) without requiring cholesterol treatment. Depletion of cholesterol of the MXR-M membranes by means of RAMEB treatment impaired the ABCG2-ATPase response (Fig. 4B). Both the concentration dependence and the general biphasic shape of the cholesterol-loaded MXR-Sf9 ABCG2-ATPase closely resembled that of the MXR-M ATPase activation (Fig. 4, A and B). Moreover, the low-cholesterol versions of both membranes (native MXR-Sf9 and cholesterol-depleted MXR-M) were also similar (Fig. 4, A and B) because ABCG2-ATPase was not efficiently activated by sulfasalazine in either membrane. Likewise, topotecan and prazosin both significantly stimulated ABCG2-ATPase in cholesterol-loaded MXR-Sf9 (Fig. 4, C and E) and MXR-M membranes (Fig. 4, D and F), whereas untreated MXR-Sf9 (Fig. 4, C and E) and cholesterol-depleted MXR-M membranes (Fig. 4, D and F) were non-responsive. Kinetic data on the ABCG2-ATPase are summarized in Table 1. The data show good correlation between $V_{\text{max}}$ values obtained with the cholesterol-loaded MXR-Sf9 membranes and MXR-M membranes. However, the $K_{m}$ values observed for the substrates were approximately 1.5 to 3.0 times greater with the cholesterol-loaded MXR-Sf9 membranes.

Using the PREDEASY ABCG2-ATPase assay kit, cholesterol-loaded MXR-Sf9 membranes were tested for ATPase activation and inhibition. In the inhibition assay, the effect of substrates and inhibitors on the sulfasalazine (10 μM)-stimulated cholesterol-loaded MXR-Sf9 ABCG2-ATPase were monitored. All substrates, sulfasalazine, prazosin, and topotecan (Fig. 5, A–C), but Hoechst 33342 (Fig. 5D) stimulated the basal ATPase activity and inhibited the activated ATPase in a concentration-dependent manner. Hoechst 33342, a known substrate of WT human ABCG2, however, displayed a profile similar to Ko134 (Fig. 5E), a known specific inhibitor of ABCG2, because both compounds inhibited the basal as well as the stimulated ATPase activity of ABCG2.

Cholesterol Loading Significantly Enhances ABCG2-Mediated Vesicular Transport into MXR-Sf9 Inside-Out Vesicles. The membrane assay system allows for transport studies as the transported substrates accumulate in the inside-out vesicles in an ATP-dependent manner. To study whether cholesterol loading affects transport kinetics of ABCG2-expressing MXR-Sf9 vesicles, we monitored the transport of a known ABCG2 substrate, methotrexate, commonly used in VT studies (Fig. 6A). To evaluate the cholesterol effect, $V_{\text{max}}$ and $K_{m}$ have been calculated. Cholesterol loading dramatically increased maximal velocity of the transport (2144 versus 367 pmol/mg/min) without affecting $K_{m}$ (1068 versus 935 μM). We also studied prazosin transport. Prazosin, although a known ABCG2 substrate (Xiao et al., 2006), is not commonly used in VT studies. Indeed, little ATP-dependent transport (85 pmol/mg/min) was seen in the MXR-Sf9 vesicles (Fig. 6B). On the contrary, a very significant transport was seen in the cholesterol-loaded MXR-Sf9 vesicles with a maximal velocity of 702 pmol/mg/min (Fig. 6B).

Effects of Cholesterol on ATPase and VT Measurements Correlate. To study the effect of membrane cholesterol levels on ABCG2-ATPase activity and vesicular transport, estrone-3-sulfate was used as a substrate (Fig. 7). Cholesterol loading increased $V_{\text{max}}$ of transport of estrone-3-sulfate by more than 20-fold (3408 versus 122.7 pmol/mg/min) with relatively little change in $K_{m}$ (14.11 versus 27.58 μM) (Fig. 7A). In the MXR-M membranes, a similar effect of membrane cholesterol level was observed because cholesterol depletion decreased $V_{\text{max}}$ by approximately 5-fold (455.5 versus 99.02 pmol/mg/min) with little change in $K_{m}$ (5.49 versus 7.89 μM) (Fig. 7B). The increased transport rate was paralleled in the ATPase assay because low-cholesterol membranes (MXR-Sf9 and cholesterol-depleted MXR-M) displayed an impaired activation upon estrone-3-sulfate treatment (Fig. 7, C and D). Moreover, the $K_{m}$ and $V_{\text{max}}$ values for estrone-3-sulfate-induced ABCG2-ATPase activity showed good correlation between the cholesterol-loaded MXR-Sf9 and MXR-M membranes (Table 1). The correlation between membrane cholesterol content of MXR-Sf9 vesicles loaded with different concentrations of cholesterol@RAMEB complex and initial rate of $[^{3}H]$estrone-3-sulfate (1 μM) transport was excellent (Fig. 8). The correlation between membrane cholesterol content and initial rate of sulfasalazine (10 μM)-induced ATPase activity was reasonably good (Fig. 8).
ABCG2 protein is one of the most important multidrug transporters involved in drug absorption distribution metabolism excretion. Many assays used to study the effects of drug transporters are based on membranes purified from Sf9 insect cells into which the respective transporter gene is introduced by means of baculoviral infection. Utilization of any heterologous expression systems should warrant thorough correlation and validation studies. However, few studies address directly this correlation. One way of assay validation is cross-validation with other assays. For ABCB1, daunomycin EC\textsubscript{50} values in the MDR1-Sf9 ATPase assay showed an acceptable correlation with the reversal of daunomycin resistance in the human cell line (2 and 5 \textmu M, respectively) (for review, see Litman et al., 2001). The correlation of transport was even better for the ABCC1/MRP1-mediated LTC4 transport (K\textsubscript{m} in HeLa-MRP1 and MRP1-Sf9 cells, 97 versus 67 nM; Leier et al., 1994; Gao et al., 1996, respectively) and rat Abcb11/Bsep-mediated taurocholate transport in the Sf9 system and rat canalicular membranes (K\textsubscript{m} in rat liver canalicular membranes and BsepSf9 membranes were 2.1 versus 5.0 \textmu M) (Stieger et al., 2000).

There was good correlation between the ATP dependence of the ABCG2-mediated methotrexate transport and ABCG2-ATPase profiles in plasma membrane preparation from Sf9 cells transfected with ABCG2 and plasma membranes from human cells overexpressing the transporter (Fig. 1) (Glavinas et al., unpublished data). We hypothesized that either the altered glycosylation and/or the different membrane composition of Sf9 and of human plasma mem-

**Discussion**

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**Table 1**

Comparison of ABCG2-ATPase kinetics of sulfasalazine, estrone-3-sulfate, and topotecan obtained in cholesterol-loaded MXR-Sf9 and native MXR-M membranes

<table>
<thead>
<tr>
<th>Test Drug</th>
<th>MXR-Sf9 Cholesterol Loaded</th>
<th>MXR-M</th>
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<tr>
<td></td>
<td>V\textsubscript{max}\textsuperscript{*}</td>
<td>K\textsubscript{m}\textsuperscript{†}</td>
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\textsuperscript{*} nanomoles of Pi per milligram of protein per minute.

\textsuperscript{†} Micromolar.

**Fig. 7.** Effect of cholesterol on ABCG2 vesicular transport and ABCG2-ATPase correlates in both the MXR-Sf9 and MXR-M membranes. Estrone-3-sulfate transport was carried out at 32°C for 1 min and monitored by measuring the amount of membrane-associated [\textsuperscript{3}H]estrone-3-sulfate (A and B). In estrone-3-sulfate-stimulated ABCG2-ATPase activity measurements, membranes were incubated for 40 min at 37°C with estrone-3-sulfate at concentrations indicated in the figure (C and D). Cholesterol-loaded (●) and control (▲) MXR-Sf9 membranes (A and B) and cholesterol-depleted (●) and control (▲) MXR-M membranes (B and C) were studied. Data represent mean ± S.D. of duplicates.

**Fig. 8.** Correlation of membrane cholesterol content and ABCG2 activity in MXR-Sf9 membranes. Cholesterol-loaded membranes were prepared by the treatment of MXR-Sf9 with various concentrations of cholesterol-@RAMEB. The cholesterol concentration of cholesterol-@RAMEB-treated membranes is shown in the x-axis. Transport rate of estrone-3-sulfate (1 \textmu M) measured at 32°C for 1 min and sulfasalazine (10 \textmu M)-stimulated ATPase activity measured at 37°C for 40 min is shown in the y-axis. Data are plotted as mean ± S.D. of duplicates.

The table shows the comparison of ABCG2-ATPase kinetics of sulfasalazine, estrone-3-sulfate, and topotecan obtained in cholesterol-loaded MXR-Sf9 and native MXR-M membranes. The data includes V\textsubscript{max} and K\textsubscript{m} values for each test drug in both membrane types. The values are expressed in nanomoles of Pi per milligram of protein per minute for V\textsubscript{max} and micromolar for K\textsubscript{m}.

The figures illustrate the effect of cholesterol on ABCG2 vesicular transport and ABCG2-ATPase activity. Figure 7 shows the correlation of estrone-3-sulfate transport and ABCG2-ATPase activity in both MXR-Sf9 and MXR-M membranes. Figure 8 demonstrates the correlation of membrane cholesterol content and ABCG2 activity in MXR-Sf9 membranes, showing the transport rate of estrone-3-sulfate and sulfasalazine-stimulated ATPase activity as a function of cholesterol concentration.
brane vesicles could be responsible for the differences observed in the activation profile of ABCG2-ATPase. Deglycosylation of human WT ABCG2 expressed in human MXR-M membranes did not affect the ATPase activity (Fig. 2, B and C). Therefore, the effect of cholesterol on the ABCG2-ATPase activation was studied because insect cells harbor much less cholesterol in their membrane than human cells (Gimpl et al., 1995). Indeed, cholesterol loading specifically improved the rate of drug-stimulated ABCG2-ATPase and maximal velocity of transport for the substrates studied in vesicular transport experiments (Figs. 3–8). Cholesterol loading of MXR-S9 membranes makes their ATPase (Table 1; Figs. 4 and 7, C and D) similar to the MXR-M membranes that contain high amounts of endogenous cholesterol. Therefore, cholesterol-loaded ABCG2-overexpressing insect cell membranes are suitable models to study ABCG2 function. Our results have two major implications on data generated earlier, using the native, low-cholesterol MXR-S9 membranes. Cholesterol loading mainly affected enzyme activity with relatively little effect on the affinity to ABCG2. Therefore, vesicular transport data generated in the past using plasma membranes prepared from insect cells with the aim of identifying ABCG2 substrates as well as substrate specificities are not affected. However, ABCG2-ATPase data using the MXR-S9 system may have given false negatives especially for high-affinity substrates. This supports the notion that cholesterol-loaded MXR-S9 membranes are the test systems of choice to study drug-ABCG2 interactions at high throughput.

Importantly, the effect of cholesterol loading inhibited the transport activity of endogenous calcium ATPase in the S9 membranes (data not shown). No significant change was observed in permeability of membranes for the substrates used in the vesicular transport studies (data not shown). Therefore, the observed potentiating effect of cholesterol on transport rate by ABCG2 is not a nonspecific event due to increased vesicular size or decreased passive permeability of substrates.

A few studies addressing the effect of cholesterol on ABC transporters have been published so far (Garrigues et al., 2002; Arima et al., 2004). ABCB1 is the only ABC transporter where the effect of cholesterol on transporter activity has been investigated in detail (Garrigues et al., 2002). Likewise, to our observations (data not shown), most studies have reported an elevation of basal ABCB1 ATPase activity (Wang et al., 2000; Garrigues et al., 2002; Gayet et al., 2005). One study has attributed this increase in ABCB1 ATPase activity to ABCB1-mediated cholesterol flipping (Garrigues et al., 2002). In some studies, cholesterol depletion resulted in an increased stimulation of ABCB1 ATPase activity upon substrate treatment (Garrigues et al., 2002) or increased transport rate (Wang et al., 2000; Gayet et al., 2005). It has been suggested that cholesterol is an ABCB1 substrate, a conclusion challenged lately (Le Goff et al., 2006).

The mechanism of the stimulating effect of cholesterol on ABCG2-ATPase function is not clear. The elevated ABCG2-ATPase baseline levels may imply that cholesterol is an ABCG2 substrate. This would not be unprecedented in the ABCG family because other members, ABCG1 and ABCG4 (Wang et al., 2004), as well as the ABCG5/ABCG8 dimer (Yu et al., 2002) have been implicated in cholesterol transport. However, we have not observed any signs of inhibition of ABCG2 transport exerted by cholesterol in our studies, questioning the substrate nature of cholesterol. Alternatively, cholesterol may act as an allosteric regulator for ABCG2-function. Further studies are required to elucidate the mechanism of cholesterol-mediated potentiation of ABCG2-ATPase activity and vesicular transport.

Cholesterol forms separate domains (rafts or caveolae) in mammalian membranes. Interestingly, several reports found multidrug transporters localized in the raft/caveolar regions (Bacso et al., 2004; Tietz et al., 2005). One may speculate that the cholesterol sensitivity of the ABCG2 protein might be related to a raft/caveolar localization of the transporter.

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