**Special Section on Transporters in Toxicity and Disease**

**Regulation of the Function of the Human ABCG2 Multidrug Transporter by Cholesterol and Bile Acids: Effects of Mutations in Potential Substrate and Steroid Binding Sites**

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**ABSTRACT**

ABCG2 (ATP-binding cassette, subfamily G, member 2) is a plasma membrane glycoprotein that actively extrudes xenobiotics and endobiotics from the cells and causes multidrug resistance in cancer. In the liver, ABCG2 is expressed in the canalicular membrane of hepatocytes and excretes its substrates into the bile. ABCG2 is known to require high membrane cholesterol content for maximal activity, and by examining purified ABCG2 reconstituted in proteoliposomes we have recently shown that cholesterol is an essential activator, while bile acids significantly modify the activity of this protein. In the present work, by using isolated insect cell membrane preparations expressing human ABCG2 and its mutant variants, we have analyzed whether certain regions in this protein are involved in sterol recognition. We found that replacing ABCG2-R482 with large amino acids does not affect cholesterol dependence, but changes to small amino acids cause altered cholesterol sensitivity. When leucines in the potential steroid-binding element (SBE, aa 555–558) of ABCG2 were replaced by alanines, cholesterol dependence of ABCG2 activity was strongly reduced, although the L558A mutant variant when purified and reconstituted still required cholesterol for full activity. Regarding the effect of bile acids in isolated membranes, we found that these compounds decreased ABCG2-ATPase in the absence of drug substrates, which did not significantly affect substrate-stimulated ATPase activity. These ABCG2 mutant variants also altered bile acid sensitivity, although cholic acid and glycocholate were not transported by the protein. We suggest that the aforementioned two regions in ABCG2 are important for sterol sensing and may represent potential targets for pharmacologic modulation of ABCG2 function.

**Introduction**

Human ABCG2 (ATP-binding cassette, subfamily G, member 2) is a plasma membrane glycoprotein expressed in many tissues, especially in those with barrier or detoxifying functions (brain endothelium, placenta, gut, liver) (Robey et al., 2009). This protein is an active transporter, working as a homodimer, using the energy of ATP hydrolysis for the extrusion of various compounds across the plasma membrane. ABCG2 is a promiscuous transporter, and its physiologic role is the protection of the body and fetus against exogenous or endogenous toxic compounds.

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**ABBREVIATIONS:** ABCG2, ATP-binding cassette, subfamily G, member 2; BSEP, bile salt export pump; CA, cholic acid; CHAPS, 3-[(3-amino acids does not affect cholesterol dependence, but changes to small amino acids cause altered cholesterol sensitivity. When leucines in the potential steroid-binding element (SBE, aa 555–558) of ABCG2 were replaced by alanines, cholesterol dependence of ABCG2 activity was strongly reduced, although the L558A mutant variant when purified and reconstituted still required cholesterol for full activity. Regarding the effect of bile acids in isolated membranes, we found that these compounds decreased ABCG2-ATPase in the absence of drug substrates, which did not significantly affect substrate-stimulated ATPase activity. These ABCG2 mutant variants also altered bile acid sensitivity, although cholic acid and glycocholate were not transported by the protein. We suggest that the aforementioned two regions in ABCG2 are important for sterol sensing and may represent potential targets for pharmacologic modulation of ABCG2 function.

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cholesterol or sitosterol transport (Wang et al., 2008; Kerr et al., 2011).

We and others have recently shown that Arg 482 is important in cholesterol sensing of human ABCG2, as the function of ABCG2 mutants having Gly or Thr at position 482 was only slightly modified by cholesterol. We found that these mutants are fully active in S9 membranes with low cholesterol content or in mammalian cells partially depleted of cholesterol. In contrast, the ATPase and transport function of the wild-type ABCG2 greatly increases in insect membranes enriched with cholesterol. Still, isolated ABCG2 preparations even the R482G mutant required low levels of cholesterol for full function (Telbisz et al., 2013).

Structural analysis of steroid hormone (progesterone, androgen, estrogen, or glucocorticoid) receptors and other steroid-binding proteins such as oxysterol-binding protein-related proteins revealed a complex interaction between the ligand-binding domains and the steroid substrates. A conserved motif of these ligand-binding domains is an L/MLxxL sequence or a shorter LxxX, in which the leucines form Van der Waals bonds with the rings of steroid hormones (Williams and Sigler, 1998; Im et al., 2005). In the ABCG8 protein, which is involved in sterol transport by forming a heterodimer with ABCG5, the fifth transmembrane helix contains an MxLxxL motif; a Gly to Arg mutation at the beginning of this element causes loss of activity and sitosterolemia. Mutations in the LxxX motif in the homologous region of ABCG2, as a putative steroid-binding element (SBE), have been shown to cause failure in the proestrogen and estradiol recognition of ABCG2 expressed in Lactococcus lactis (Velamakkanni et al., 2008). Surprisingly, these mutants were similarly activated by cholesterol as the non-mutant protein.

Bile acids are abundantly formed from cholesterol in hepatocytes, and bile acids and their derivatives are present in all tissue fluids. Due to their enterohepatic circulation, bile acid concentrations are especially high in the intestinal epithelial cells and in hepatocytes. The interaction between ABCG2 and bile acids has been extensively investigated, but these studies have yielded controversial data. Two groups have reported that there is no interaction with ABCG2 or direct transport of taurocholate, tauroholicholate sulfate, or tauroursodeoxycholic acid by this protein (Suzuki et al., 2003; Vaidya and Gerk, 2006). In contrast, two other laboratories described direct interaction between ABCG2 and bile acids (Imai et al., 2003; Janvilisri et al., 2006). ATPase activity of ABCG2 was measured in insect membranes enriched with 2 mM cholesterol-RAMEB on ice for 30 minutes, as published previously (Telbisz et al., 2007).

Immunodetection of ABCG2. We suspended the S9 membranes in Laemmli sample buffer containing 2% of the reducing agent β-mercaptoethanol. Western blot analysis was performed as described elsewhere (Ozvegy et al., 2002) by use of the BXP-21 monoclonal antibody in a 2000× dilution, and a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (10,000× dilution, Jackson ImmunoResearch Laboratories, West Grove, PA).

Solubilization, Purification, and Reconstitution into Proteoliposomes. Solubilization of His-tagged wtABCG2 and its SBE mutant variants was performed with 1% (w/v) DDM (dodecyl maltoside), 0.4% Escherichia coli lipid extract (100500; Avanti Polar Lipids, Inc., Alabaster, AL), as described (Telbisz et al., 2013). Purification and reconstitution were performed as described in our recent article (Telbisz et al., 2013). Briefly, Ni-NTA (Ni²⁺-nitritotriacetate; Sigma His-select, H-0537) was used for purification, and the purified ABCG2 protein (wild-type or its SBE mutants) was reconstituted in E. coli lipid extract supplemented with 0–0.4 mM cholesterol.

ATPase Activity Measurements. ATPase activity was measured on isolated membranes of S9 cells expressing wtABCG2 or its mutant variants by colorimetric detection of inorganic phosphate liberation, as previously described (Ozvegy et al., 2002). Figures represent the mean values of at least two independent experiments with four parallels, measured in two different membrane preparations for each type of ABCG2. ATPase activity of ABCG2 in proteoliposomes was determined as described previously (Telbisz et al., 2013).

Vesicular Uptake Measurements. The S9 membrane vesicles were incubated in the presence or absence of 4 mM MgATP or 4 mM MgATP + 1 μM Ko143 ([35S]6,12αS)-1,2,3,4,6,7,12,12α-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazinol[1′,2′:1,6][pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester) (Allen et al., 2002) were kind gifts from Drs. George Scheffer and Rick Scheper, and from Dr. G. J. Koomeen, respectively.

Expression Vectors. Generation of the baculovirus transfer vector (pAcUW21-L) harboring the cDNA for wild-type (wt) ABCG2 or the R482 and R86M mutants was described previously (Ozvegy-Laczka et al., 2005). The steroid-binding element mutants were created by site-directed polymerase chain reaction (PCR) mutagenesis using the following complementary primer pairs: L555A: 5′ T TCA GGT GTC CTC GTC AAT CT and 5′ AGT ATT GAC GCC GAG ACC TGA A; L558A: 5′ GTG CTG TTG GTG AAT GCC ACA ACC ATT and 5′ AAT GGT TGT GGC ATT CAC CAA CAC AC; L555/58A: 5′ GTT CTC GCG GTG AAT GCC ACA ACC ATT and 5′ AAT GGT TGT GGC ATT CAC CAG AC; L555/58A: 5′ GTC ATT GCC GAG ACC ATT and 5′ AAT GGT TGT GGC ATT CAC CAG AC. The PCR fragments containing the mutant cDNAs were cloned between the BglII-SacI sites of the pAcUW21-L/wtABCG2 vector. The base order of the constructs was confirmed by sequencing of the appropriate fragments. The R482G/L555/58A triple mutant was created by replacing the DNA fragment between the Pst-I-Sacl sites of the pAcUW21-L/R482G with that of derived from the pAcUW21-L/L555/58A vector.

Generation of His₆-Tagged ABCG2 Variants. His₆-ABCG2 was generated as described by Telbisz et al. (2013). The His₆-tagged L555A and L555/58A mutants were created by cloning the Pst-I-Sacl site from pAcUW21-L/ABCG2-L555A or L555/58A into the pAcUW21-L/His₆-ABCG2.

Cellular Dye Uptake and Calculation of ABCG2 Transport Activity. Measurement of pheophorbide A and rhodamine 123 extrusion by ABCG2 was determined as described previously (Ozvegy-Laczka et al., 2008). Geometric mean fluorescence values measured in the absence (M₀) or presence (Mₘ) of inhibitor were determined, and the activity factor was calculated as follows: (Mₘ − M₀)/M₀ * 100.
Measurement of Hoechst 33342 Transport Activity. Hoechst 33342 transport was determined as described in Ozvegy-Laczka et al. (2004). Transport activity was calculated as (F_{100} - F_{0}) * 100, where F_{0} is fluorescence in the presence of 1 μM Ko143, and F_{100} is fluorescence in the absence of the inhibitor.

Results

Effect of Cholesterol on the Function of ABCG2 R482 Mutant Variants

Effect of Cholesterol on the ATPase Activity of the ABCG2 R482 Mutants. The cholesterol dependence of human ABCG2 in S9 cell membranes has been characterized in detail previously (Pal et al., 2007; Telbísz et al., 2007). Briefly, human ABCG2 when expressed in cholesterol “poor” insect cells (5–8 μg cholesterol/mg membrane protein) has well-measurable ATPase and transport activity. However, increasing the cholesterol content of the insect membranes by cholesterol-loaded randomly methylated β-cyclodextrin (cholesterol-RAMEB) up to 40–60 μg cholesterol/mg membrane protein results in 4- to 20-fold activation of the transport function of ABCG2. Accordingly, substrate stimulation of the ATPase activity becomes more pronounced in cholesterol-enriched S9 membranes (Pal et al., 2007; Telbísz et al., 2007). Moreover, in our recent study we demonstrated that cholesterol is essential for ABCG2 function: purified ABCG2 is only active when the proteoliposomes contain 20–30 mol % cholesterol (Telbísz et al., 2013). Despite the crucial role of cholesterol in ABCG2 function, the cholesterol-sensing sites in ABCG2 are yet to be defined.

The Arg482 residue, residing in or near the third transmembrane helix of ABCG2, has been documented to significantly alter the substrate interactions of the transporter. Several of the R482 variants gain a rhodamine 123 transport function, while all of them lose methotrexate (MTX) transport capacity (Ozvegy et al., 2002; Ozvegy-Laczka et al., 2005). We showed earlier that Arg482 also has a critical role in the cholesterol sensitivity of human ABCG2. In contrast to the wild-type protein, in S9 cell membranes increasing the membrane cholesterol levels did not significantly influence the activity of the R482G and R482T mutants (Telbísz et al., 2007), although experiments on purified ABCG2 reconstituted in proteoliposomes revealed that cholesterol is also essential for the function of the R482G variant (Telbísz et al., 2013).

To examine how the characteristics of amino acid 482 influence the cholesterol-sensing capability of ABCG2, we have analyzed seven additional R482 mutants (R482D, I, M, N, S, Y, and K). These ABCG2-R482 variants were expressed in S9 insect cells at similar level to the wild-type protein (data not shown); as documented earlier, all these mutants were active, showing a measurable basal ATPase activity that was sensitive to Ko143, a specific inhibitor of ABCG2 (Ozvegy-Laczka et al., 2005). In our present study, the ATPase activity of the mutant variants was measured in “control” (native, cholesterol-poor) and cholesterol-enriched S9 membranes. In cholesterol-loaded membranes, the basal ATPase activity of most of these variants increased (see Supplemental Table 1), but the increase in membrane cholesterol levels did not change the relative substrate stimulation of the R482D, G, N, S, and T variants (Fig. 1A). In contrast, in the case of the R482I, K, M, and Y variants, similarly to the wild-type ABCG2, cholesterol enrichment significantly improved the ratio of substrate stimulation, as examined after the addition of prazosin (Fig. 1A). We also observed a similar effect of other substrates (e.g., quercetin) on the ATPase activity of these mutants (data summarized in Supplemental Table 1).

Effect of Cholesterol on the Transport Activity of the ABCG2 R482 Mutants. To find out whether membrane cholesterol has a direct effect on the transport activity of the R482 mutants, we measured the [3H]methotrexate uptake in S9 inside-out membrane vesicles containing different R482 variants either in the presence or absence of excess cholesterol. As shown in Supplemental Fig. 1A, in contrast to wild-type ABCG2, none of the R482 mutants exhibited significant methotrexate transport, either in the control or in the cholesterol-loaded membrane vesicles. These findings suggest that cholesterol enrichment of the membranes did not alter this particular substrate interaction of the mutant ABCG2 variants.

Intact S9 cells, which transiently express ABC transporters, are suitable for fluorescent dye accumulation measurements in which the transport activity of the human ABCG2 can be determined (Ozvegy et al., 2002). In the following experiments, intact S9 cells were harvested 24–40 hours after transfection, when approximately 60–70% of the cells are viable; accumulation of different ABCG2 substrates was measured in control or cholesterol-enriched S9 cells.

When the S9 cells were loaded with cholesterol by incubating them with 2 mM cholesterol-RAMEB, we found that this treatment did not influence cell viability, at least during the period of the transport experiments. The cellular fluorescence of Hoechst 33342 (Hst) and rhodamine 123 (R123) was analyzed by fluorometry and flow cytometry, respectively. The activity factors for the different ABCG2 variants were calculated based on the intracellular fluorescence detected in the presence or absence of the specific inhibitor Ko143. The actual expression levels of the ABCG2 variants were examined by Western blot analysis, and they showed similar expression levels of the transporter (data not shown).

Similar to earlier findings, there was a well-measurable Ko143-sensitive Hst dye transport both in the cells expressing wtABCG2 and in those expressing most R482 mutants, with only very low activity in the case of the R482K and R482Y variants (Fig. 1B). When the cells were loaded with cholesterol, this Hst dye uptake was significantly improved in the wild-type and the R482I and M variants. Moreover, significant Hst transport activity occurred in the case of the R482K and Y variants. In contrast, we did not observe a significant effect of cholesterol on the Hst transport by the R482D, G, N, S, and T variants. Note that these cholesterol-mediated alterations in the Hst transport capacity of ABCG2 correspond to the effect of cholesterol on the ABCG2-ATPase activity.

To examine the potential effects of cholesterol on the substrate specificity of the mutant variants, we also measured ABCG2-dependent R123 extrusion both in the control and cholesterol-loaded S9 cells, expressing the human ABCG2 variants (Supplemental Fig. 2B). As noted earlier, the wtABCG2 had no R123 transport activity, whereas several mutant variants acquired such a transport function. Cholesterol loading significantly increased R123 extrusion in cells expressing the R482I and M variants, while there was no measurable effect in the D, G, N, S, or T variants. In the case of the wild-type protein and the R482K and Y variants, there was no detectable R123 extrusion in either the absence or presence of cholesterol.

Effect of Cholesterol on the Function of ABCG2-SBE Mutant Variants

A predicted steroid-binding element (SBE, LxxL) of ABCG2 is located in the potential fifth transmembrane helix of the transporter (see Introduction). The motif was reported to alter ABCG2-mediated recognition of progesterone and estradiol, two steroid hormones closely related to cholesterol (Velamakanni et al., 2008). We have generated the Leu to Ala mutations L555A, L558A, and L555A/L558A in this motif. The mutants were expressed in S9 cells at equal levels to wtABCG2 (Fig. 2A), and the effect of cholesterol on the ATP hydrolysis and transport activity of the mutants was investigated using membrane vesicles or transporter-expressing intact S9 cells.

Effect of Cholesterol on the ATPase Activity of the SBE Mutant ABCG2. As shown in Fig. 2B, the L555A, L558A, and L555A/L558A mutants exhibited a well-measurable vanadate-sensitive ATPase
activity. However, given the similar expression levels of the wild-type and the mutant proteins, we found that L555A and L555A/L558A had only about one-third the basal ATPase activity as compared with wtABCG2. Moreover, in the case of the L555A and L555A/L558A mutants, ATPase turnover in the presence of quercetin was also well below that measured for the wild-type protein or the L558A mutant.

When analyzing the effect of cholesterol loading on the quercetin-stimulated ATP hydrolysis, we found no significant change in ATPase turnover in any of the three SBE mutants (see Fig. 2B).

Next, we studied the effect of numerous wtABCG2 substrates on the ATPase activity of the SBE mutants in both control and cholesterol-enriched Sf9 cell membranes. Several activators of wtABCG2 ATPase (doxorubicin, flavopiridol, SN-38 [7-ethyl-10-hydroxy-camptothecin], and topotecan) did not stimulate ATP hydrolysis of the mutants even in cholesterol-enriched membranes (not shown). However, we found a few substrates (nilotinib, prazosin, quercetin, and EKI-785 (N-[4-[[3-bromo-phenyl]amino]-6-quinazolyl]-2-butyramidine) that enhanced ATP hydrolysis in these mutants as well. To investigate the effect of cholesterol on substrate-stimulated ATPase activity of the SBE mutants, we have measured the effect of these “activators” in membranes loaded with different amounts of cholesterol (by applying 0.025–2 mM cholesterol-RAMEB for cholesterol loading). We found that increasing the cholesterol content of the Sf9 cell membranes did not significantly enhance the substrate stimulation of the ATPase activity in the LxxL mutants. These effects of nilotinib and quercetin in such membrane preparations are shown in Supplemental Fig. 2; prazosin and EKI-785 gave similar results (data not shown).

**Effect of Cholesterol on the Transport Activity of the SBE Mutant ABCG2.** In these experiments, we examined the effect of cholesterol on the [3H]methotrexate ([3H]MTX) and [3H]estradiolglucuronide ([3H]ESG) transport activity of ABCG2 L555A, L558A, and L555A/L558A mutant variants expressed in Sf9 insect cells. As discussed earlier, cholesterol enrichment of the Sf9 inside-out membrane vesicles greatly increases MTX and ESG transport by wtABCG2 (see the previous discussion and Telbisz et al., 2007). To test whether cholesterol has any effect on the transport function of the SBE mutants, Sf9 membrane inside-out vesicles containing the SBE mutants were analyzed in membranes loaded with cholesterol, we found only a nonsignificant increase in this transport activity (Supplemental Fig. 3A). In the ESG vesicular transport experiments, again none of the three mutants showed ABCG2-specific activity, and cholesterol did not improve their ESG transport function (Supplemental Fig. 3B).

To further analyze the effect of cholesterol on the activity of the SBE mutants, we measured the accumulation of different fluorescent ABCG2 substrates in intact Sf9 insect cells. Accumulation of Hoechst 33342 or pheophorbide A, but in contrast to the wild-type protein, vesicular transport experiments, again none of the three mutants showed ABCG2-specific activity, and cholesterol did not improve their dye extrusion capacity (Fig. 2, C and D).

**Effect of Cholesterol on the Function of Isolated and Reconstituted ABCG2-SBE Mutants**

It was shown earlier that an increase in the cholesterol levels of the insect membranes did not significantly modulate the function of the ABCG2 R482G mutant, which would imply its apparent cholesterol insensitivity. However, reconstitution of the purified ABCG2 R482G variant revealed that the presence of cholesterol was also essential for the function of this mutant variant; however, lower cholesterol levels (amounts that are most probably present in native insect membranes) were sufficient to achieve its full activity as compared with wtABCG2 (Telbisz et al., 2013). To analyze the cholesterol sensing of the purified ABCG2 variants, we have generated N-terminally His-tagged versions of the L558A and L555/558A variants. The His tag of L558A and His tag of L555A/L558A ABCG2 mutants were successfully expressed in Sf9 cells, and we also found that tagging did not alter their functionality (data not shown).

The membrane isolation as well as the purification and reconstitution of the L558A variant were successful. However, though...
the expression level of the His6-L555A/L558A variant in the Sf9 cells was comparable to that of the other variants, the purification yielded a much lower amount of this mutant (data not shown).

We analyzed the ATPase activity of the purified L558A variant, reconstituted in E. coli lipids in the absence and in the presence of cholesterol. Interestingly, this ABCG2 mutant had a negligible ATP hydrolysis in cholesterol-free E. coli lipids, similar to the wild type, while increasing cholesterol concentrations greatly accelerated both the basal (Fig. 3A) and the substrate-stimulated (data not shown) ATPase activities, again in harmony with the wild type.

Surprisingly, the L555/558A mutant lost its functionality upon purification. There was no ATPase activity in proteoliposomes, regardless the amount of cholesterol applied. When we investigated the dimerization state of the double mutant, we found that in contrast to the wild-type protein, the disulphide bridge linked homodimer (corresponding to the physiologic state of ABCG2) could not be detected in the case of the double mutant after purification (Fig. 3B). Therefore, the inactivity of the double mutant can be due to its impaired dimerization capability.

### Effect of Bile Acids on the Function of Human Wild-Type ABCG2 Expressed in Sf9 Cell Membranes

Bile acids are continuously present in all tissue fluids, including blood plasma, where after a meal free bile acid levels can reach 100 μM. Bile acids participate in continuous enterohepatic circulation, and...
their concentrations inside intestinal epithelial cells or in hepatocytes are considerably higher, especially in the bile-secreting canalicular membranes (Li and Chiang, 2012). The critical micelle concentrations of bile acids are in the range of 2–10 mM (Simonović and Momirović, 1997), and hepatic bile acid secretion occurs by the formation of mixed micelles with phospholipids and cholesterol. In the next set of experiments, we determined whether, similar to cholesterol, bile acids modulate the function of ABCG2.

**Effect of Bile Acids on the ATPase Activity and Transport Function of Wild-Type ABCG2.** To compare the effect of cholesterol and bile acids, we performed studies with these sterols alone or in combination. In the control S9 cell membranes, there is no stimulation of ATPase activity with prazosin (a known transported substrate of ABCG2), whereas quercetin (also a transported substrate of the protein) has a measurable stimulatory effect even in these conditions. When the cholesterol content of the membranes is increased, significant activation of ATP hydrolysis by prazosin occurs, and an increase in quercetin-stimulated ATPase activity is also observed (see Fig. 4A). When cholic acid (CA, 1 mM) is added to the control membranes, a significant reduction in the baseline ABCG2-ATPase (measured in the absence of added substrates) and a slight stimulatory effect of prazosin are observed (the effect of quercetin is unaltered; Fig. 4B, left columns). However, the most pronounced effect of CA is observed in cholesterol-stimulated ATPase activity (Fig. 4B, right columns); baseline ATPase activity is strongly reduced (almost to the level of S9 membranes expressing the inactive ABCG2-K86M mutant; see Fig. 2B), while drug-stimulated ATPase activity is unchanged. Thus, relative drug-stimulated ATPase activity (ATP hydrolysis with drug substrates/basal ATP hydrolysis) is increased up to 6- to 8-fold.

To characterize these phenomena in more detail, we examined the concentration-dependent effects of bile acids on human wtABCG2-ATPase activity in cholesterol-loaded S9 cell membranes, closely reflecting the plasma membrane environment of mammalian cells. It has to be emphasized that bile acids were used under their critical micelle concentration values, thus a direct membrane effect was unlikely to occur.

As shown in Fig. 4C, all the investigated bile acids—glycocholate (GC), taurocholate (TC) cholic acid (CA) and ursodeoxycholic acid (UDC) and the synthetic derivative CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate), which has a critical micelle concentration of 6–10 mM—effectively reduced the baseline ATPase activity of wtABCG2, while substrate-stimulated ATPase was unaltered. Due to these effects, the relative drug-stimulated ATPase activity (measured in the presence of quercetin) was increased, and especially UDC produced a high ratio of drug-stimulated and basal ABCG2-ATPase activity (Fig. 4D).

We examined drug-stimulated ATPase activity for several other known ABCG2 substrates and found that bile acids did not increase the Vmax of ATPase; however, due to the decreased baseline ATP hydrolytic activity, relative drug stimulation was increased. These effects for several substrates are documented in Table 1. It should also be noted that the effect of the ABCG2 inhibitor Ko143 remained unaltered in the presence of bile acids.

In the following experiments, we examined the effect of various bile acids on the transport function of ABCG2 in vesicular uptake assays. In these studies, we used radiolabeled MTX and ESG as established ABCG2 substrates and added various bile acids to the medium. We found that at low concentrations (25–50 μM) bile acids had no effect on ABCG2-dependent substrate transport, but higher bile acid concentrations (above 250 μM) inhibited both MTX (data not shown) and ESG transport activity (Supplemental Fig. 4). Because in the vesicular transport measurements we used low substrate concentrations, a high ratio of bile acid/transported substrate may explain these latter results (see Discussion).

It had been documented by others that bile acids can stimulate the transport activity of MRP2 (Bodo et al., 2003). This was also observed by us when measuring ATP-dependent ESG transport by MRP2—the addition of 250 μM cholic acid doubled the ESG transport activity in the case of MRP2, and glycocholate also increased this transport by 25% (Supplemental Fig. 4).

**Examination of the Transport of Bile Acids by ABCG2.** As described earlier, bile acids decrease the basal ATPase activity of
ABCG2. However, this does not necessarily mean that they are not transported molecules (e.g., Hoechst 33342, a known transported ABCG2 substrate, inhibits the ATPase function; Ozvegy et al., 2002). To explore this question, we examined the transport of several bile acids in transporter-expressing inside-out Sf9 membrane vesicles. ATP-dependent uptake of different radiolabeled bile acids was measured in ABCG2-containing, cholesterol-loaded, inside-out insect membrane vesicles (showing maximum transport activity, such as for ESG). In parallel experiments, we also examined similar bile acid transport by using membrane vesicles containing multidrug resistance protein 2 (MRP2, ABCC2) or BSEP (bile salt export pump, ABCB11), two known bile acid transporters. In all cases, the transport of labeled GC, CA, ESG, or MTX was examined in concentrations already documented to provide well-measurable uptake in the case of these transporters.

As documented in Fig. 5, significant, ATP-dependent, GC uptake was measured in the case of MRP2 and BSEP, whereas ABCG2 showed no transport activity. CA was efficiently transported by BSEP but not by MRP2 or ABCG2. These direct transport measurements also indicate that bile acids in the applied concentrations (100 μM) have no pronounced effect on the stability of the inside-out Sf9 membrane vesicles used in these experiments. In the case of ESG, MRP2 had the highest transport activity, and MTX was best transported by ABCG2. These results indicate that ABCG2 is most probably not a professional bile acid transporter.

Effect of Bile Acids on the Function of ABCG2 R482 and SBE Mutants

We documented that mutations in amino acid position 482 and the replacement of leucines to alanines in the potential steroid-binding element (SBE) of ABCG2 strongly influence the cholesterol-sensing capability of the protein. To explore the effect of bile acids, we performed these experiments in cholesterol-loaded Sf9 cell membranes by examining selected representatives of the R482 variants. These were the R482G and R482S variants, which are fully active already at low membrane cholesterol levels, and the R482K and R482I mutants, which show similar cholesterol-sensing capability to the wtABCG2 (see earlier). We also examined the effect of bile acids on the LxxL mutants (see Fig. 6).
TABLE 1

<table>
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<tr>
<th>Compound</th>
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<th>Relative ATPase Activity Compared with Baseline</th>
</tr>
</thead>
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<tr>
<td></td>
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<td>Control</td>
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<tr>
<td>ABCG2 substrates</td>
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<td>Quercetin</td>
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<td>ZD1839</td>
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<tr>
<td>Flavopiridol</td>
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</tr>
<tr>
<td>Sulfasalazine</td>
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<tr>
<td>Prazosin</td>
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<td>1.8</td>
</tr>
<tr>
<td>ABCG2 inhibitor</td>
<td>Ko143</td>
<td>1</td>
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</table>

When measuring the effect of bile acids on ABCG2-ATPase activity in isolated Sf9 membranes, we found that, in contrast to the wild-type protein (Figs. 4, C and D, and 6A), in the case of the R482G or S variants low concentrations of CA did not significantly alter ABCG2-ATPase activity; however, when we used higher bile acid concentrations (above 0.5 mM), both baseline and substrate-stimulated ATPase activities decreased (see Fig. 6B for the R482G mutant; R482S is not shown). Thus, relative substrate stimulation was not increased by bile acids in these variants (see also Fig. 6D). In the case of the R482K and I mutants, a variable alteration in the substrate stimulation was observed for different bile acids (Fig. 6D). GC had no effect on either baseline or substrate-stimulated activity of the L558A mutant (Supplemental Fig. 5).

Discussion

Human ABCG2 has an important role in detoxification. Therefore better understanding of the regulation of its function may allow the modulation of ADME-Tox (absorption, distribution, metabolism, excretion and toxicity) properties thus may improve anticancer therapies. Membrane cholesterol has been shown to modulate the activity of ABCG2 (Pal et al., 2007; Storch et al., 2007; Telbisz et al., 2007); however, the protein sites for cholesterol recognition have not yet been identified.

To achieve a better understanding of the interaction between sterols and ABCG2, we generated several mutant variants of the transporter, expressed them in insect cells, and characterized their sterol sensitivity. Previously, Arg482 was found to be critical in cholesterol-sensing of ABCG2 (Telbisz et al., 2007). Here, we investigated R482 mutants with various amino acid side-chain characters. Based on our present results, the cholesterol-sensing capability of these variants could be grouped into two clusters.

Members of the first cluster, also including the wild-type protein (R), contain large (hydrophobic or positively charged) amino acids, represented by R482L, M, K, and Y. The function of these ABCG2 protein variants is highly cholesterol-dependent; they are significantly activated by increased membrane cholesterol levels (Fig. 1, A and B). Interestingly, the activating effect of cholesterol is the most pronounced in the case of the R482K and Y mutants, as these variants are practically unable to transport Hoechst 33342 unless high levels of cholesterol are present in the cell membranes (Fig. 1B).

In contrast, R482 mutants represented by relatively small (polar, uncharged, or negatively charged) amino acids formed another cluster regarding cholesterol sensing. The basal ATP hydrolysis in these variants was slightly accelerated by increased membrane cholesterol levels (Supplemental Table 1), and their substrate stimulation and transport function was practically unaltered by cholesterol loading (Fig. 1).

In earlier studies (Velamakanni et al., 2008), a potential steroid-binding element (SBE, aa 555–558) in ABCG2 was suggested to be responsible for sterol sensing in this transporter. In their study, Velamakanni et al. (2008) found that the ABCG2-L555A/L558A mutant does not have an altered cholesterol sensing, but progesterone and estradiol binding as well as transport were abolished. In our present work, we expressed and analyzed in detail the SBE (or LxxL motif) mutants L555A, L558A, and L555S/L558A of human ABCG2. We found that the mutants are active, showing a vanadate and Ko143 (an ABCG2-specific inhibitor) sensitive ATPase activity and are able to transport several established wtABCG2 substrates (Fig. 2). Additionally, though the SBE mutants were active, they showed altered substrate specificity, as compared with the wtABCG2 protein (see Effect of Cholesterol on the Function of ABCG2-SBE Mutant Variants).

When examining the effect of cholesterol on their function, we found that although a slight increase in the baseline ATP hydrolysis of the L555A and L558A mutants occurred in cholesterol-enriched membranes (fold activation was 1.2 ± 0.1 and 1.5 ± 0.1, respectively), their relative substrate stimulation (ratio of ATP hydrolysis in the presence and absence of substrates) did not change (Fig. 2B and Supplemental Fig. 2). Accordingly, in direct transport experiments we found that all three SBE mutants were already fully active without excess cholesterol (Fig. 2, C and D). Moreover, the L555A/L558A mutant was absolutely insensitive to cholesterol loading in the ATPase-activity measurements (Fig. 2B).
Figure 6. Effect of bile acids on the ATPase activity of wtABCG2 and its mutant variants. (A–C) Increasing amounts of cholic acid were added to insect cell membranes previously loaded with cholesterol. Basal (■) or substrate-stimulated ATP hydrolysis (▲), measured in the presence of 1 μM quercetin (A and B), or 1 μM nilotinib (C) was determined. The figure shows the average of at least two independent measurements, four parallels ± S.E. values. (D) Effect of bile acids on the substrate stimulation of the ATPase activity of wtABCG2 and its mutant variants. Membranes were incubated with or without 1 mM bile acid; the ratio of ATP hydrolysis in the presence of 1 μM quercetin (wt, R482G, S, K, and I) or 1 μM nilotinib (SBE mutants) and the baseline ATPase activity were calculated. Columns represent an average of at least four data points, and bars show S.D. values.

The effect of sterols has been studied in detail in the case of the ABCB1/MDR1 protein (Garrigues et al., 2002). Increased cholesterol accelerates the basal ATPase activity of this multidrug transporter, while sterols have no major effect on substrate-stimulated ATP hydrolysis. It has also been shown that membrane cholesterol differentially modulates the effect of MDR1 substrates, depending on the size of these molecules. Kimura et al. (2007a) found that the presence of cholesterol increased the recognition of small-molecular-weight MDR1 substrates (molecular mass below 800 Da) but had no impact on the transport of larger molecules (molecular mass higher than 900 Da), and cholesterol itself was not transported by MDR1. Based on these findings, a review suggested a cholesterol fill-in model (Kimura et al., 2007b)—that is, in the case of larger substrates, the binding pocket of MDR1 is completely filled, and there is no room for cholesterol. However, in the case of smaller substrates, an empty space remains in the binding pocket, which can be filled up with cholesterol.

In the case of wtABCG2, we tested a large number of substrates and found that cholesterol increased the activation in all cases. When analyzing the size of these compounds, we found that all these molecules belong to the “smaller” category, so the effect of cholesterol found in the case of ABCG2 may be in harmony with the cholesterol fill-in model. Larger compounds (e.g., cyclosporin A) mostly behave as inhibitors of ABCG2, so this question is difficult to answer. Based on our current results, we suggest that in the case of ABCG2, larger side chains at position aa 482 and 555 or 558 may keep the gate of the substrate binding pocket clogged, and cholesterol is required to widen the channel for the entry of the substrates.
In the next series of experiments, we investigated the modulatory effect of bile acids on the function of wtABCG2. We found that bile acids and their derivatives, much below their critical micelle concentrations, strongly reduce the basal ABCG2-ATPase activity of the wild-type protein without significantly affecting its substrate-stimulated ATPase (Fig. 4C). Moreover, we observed that when membranes were loaded with cholesterol, CA decreased the baseline ATP hydrolysis down to the background level, that is, to ATP hydrolysis in membranes expressing the inactive mutant ABCG2-K86M (see Fig. 2B). As a consequence, in the presence of bile acids the relative substrate stimulation was greatly increased (Fig. 4D).

A key advantage of the insect cell overexpression system is that isolated membrane preparations contain a much higher level of ABCG2 than membranes isolated from mammalian overexpression systems (Özvegy et al., 2001), thus providing an efficient tool for examining the modulation of ABCG2 ATPase or transport activity (Hegedus et al., 2009). Based on our present observations, we suggest that to obtain maximum activation with ABCG2 substrates, cholesterol-loaded insect membranes should be used in the presence of bile acids. This provides an improved ATPase assay for testing the potential substrate interactions of ABCG2.

Several studies have already investigated the interaction between the ABCG2 transporter and bile acids, although with contradictory results (see Introduction). In our experiments, we could not detect direct transport of the investigated bile acids in membrane vesicles (Fig. 5), but we found an inhibitory effect of higher bile acid concentrations on ABCG2-dependent vesicular uptake (Supplemental Fig. 4). A direct effect of bile acids on membrane vesicle integrity was clearly excluded by the finding that under the same conditions bile acids significantly increased MRP2-dependent vesicular ESG transport (Bodo et al., 2003) (Supplemental Fig. 4), and both MRP2- and BSEP-containing vesicles performed efficient bile acid uptake (Fig. 5).

Our data thus do not support the findings of some earlier reports, but can be explained by the different expression systems and experimental conditions used. Even Blazquez et al. (2012) suggested that because the expression of this protein in the liver is significantly lower than that of the “professional” bile acid transporter BSEP, the role of ABCG2 in this function is questionable (while placental bile acid transport by ABCG2 may be relevant). Accordingly, Abcg2−/− mice do not develop the symptoms of cholestasis (Mennone et al., 2010); thus, ABCG2 may have only a moderate effect on bile acid transport. Based on our results, we suggest that although bile acids modulate basal ATP hydrolysis by ABCG2, they are not exported from the cells at a significant rate by this transporter. A low-affinity, low-transport-activity type interaction of ABCG2 with bile acids may explain this phenomenon.

Interestingly, we found that R482 and the L555-L558 positions are also crucial for bile acid recognition of ABCG2 (Fig. 6, B–D; Supplemental Fig. 5). Mutations at these sites removed the major modulatory effect of bile acids. We found that sterols may also modulate ABCG2 transcription and expression levels through interactions with nuclear receptors such as peroxisome proliferator-activated receptor (PPARγ), pregnane X receptor (PXR), and constitutive androstane receptor (CAR) (Jigorel et al., 2006).

Based on our present results, we suggest a model describing complex posttranslational regulation of ABCG2 function in the liver (Fig. 7). Although cholesterol and bile acids are mostly transported by professional active ABC transporters—that is ABCG5/G8, BSEP, and MRP2—into the bile, the drug-transport function of ABCG2 is significantly modulated by sterols. Based on these findings, we also suggest that the amino acid positions characterized in this study may serve as potential targets for pharmacologic modulation of ABCG2 function.

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Authorship Contributions
Participated in research design: Telbisz, Hegedüs, Özvegy-Laczka, Sarkadi.
Conducted experiments: Telbisz, Hegedüs, Özvegy-Laczka.
Performed data analysis: Telbisz, Hegedüs, Özvegy-Laczka.
Wrote or contributed to the writing of the manuscript: Hegedüs, Özvegy-Laczka, Várdai, Sarkadi.

References


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Regulation of the function of the human ABCG2 multidrug transporter by cholesterol and bile acids: effects of mutations in potential substrate- and steroid-binding sites

Ágnes Telbisz, Csilla Hegedüs, András Váradi, Balázs Sarkadi and Csilla Özvegy-Laczka

Supplemental Table: Effect of cholesterol loading on substrate stimulated ATPase activity of wtABCG2 and the R482 mutants

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<tr>
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Figure 1: Effect of cholesterol on the transport activity of the R482 mutants. Panel A: 
[^3]H]methotrexate uptake was measured in control or cholesterol-loaded insect inside-out membrane vesicles. ATP-dependent uptake is shown. Columns represent mean ± SD values of data obtained from two independent experiments.

Panel B: Rhodamine 123 (2 μM) fluorescence was determined in insect cells expressing wtABCG2 or one of the R482 mutants by fluorescent cell sorting. Dead cells were excluded by propidium iodide staining. Transport activity was calculated as described in the Experimental procedures. White columns show transport in control cells, black columns stand for activity in 2 mM cholesterol-loaded cells. Mean ± SD values are shown. *: Student’s t-test, significant difference, p < 0.05
Figure 2: Effect of cholesterol on substrate stimulation of wtABCG2 and SBE mutants. Insect membranes were incubated with different amounts of cholesterol and baseline ATPase activity and that obtained in the presence of 1 μM nilotinib (Panel A) or 1 μM quercetin (Panel B) were determined. Substrate stimulation is shown as a ratio of ATP hydrolysis in the presence of substrates and in the absence of added compounds (panels A and B). Experiments were performed in quadruplicates; average ± SD values are shown.
Figure 3: Effect of cholesterol on the transport activity of the SBE mutants. ATP-dependent [3H]methotrexate (Panel A) or [3H]estradiol-glucuronide (Panel B) uptake was determined in control or cholesterol-loaded insect inside-out membrane vesicles. Mean ± SD values of two independent experiments are shown.
Figure 4: Effect of bile acids on \[^3\text{H}\]estradiol-glucuronide uptake. \[^3\text{H}\]ESG uptake (25 \(\mu\)M) was measured in cholesterol-loaded inside out vesicles containing wtABCG2 or MRP2 in the absence (control) or in the presence of 250 \(\mu\)M bile acids (glycocholate (GC), taurocholate (TC) or cholic acid (CA)). Measurement was done in triplicates, mean ± SD values are shown.
Figure 5: Effect of bile acids on the ATPase activity of the L558A mutant. Cholesterol-loaded Sf9 membranes were incubated with increasing amounts of bile acids (cholic acid, CA, taurocholate, TC or glycocholate, GC) with the addition of 1 µM nilotinib. Experiments were carried out in duplicates, mean ± SD values are shown.