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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[§]

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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.

Among ABCG2 substrates are various anticancer agents, so the protein is one of the key players causing the so-called multidrug resistant phenotype of cancer cells, resulting in the failure of cancer chemotherapy.

ABCG2 has been documented to be located in cholesterol rich microdomains, the so-called rafts, and its direct interaction with caveolin-1, a marker of lipid rafts, has also been demonstrated (Storch et al., 2007). In accordance with this finding, high membrane cholesterol levels were found to significantly improve the function of ABCG2. We and others found that the enrichment of insect membranes with cholesterol greatly increases the ATPase and transport function of ABCG2 (Pal et al., 2007; Telbisz et al., 2007). A dramatic but reversible inhibition of ABCG2 function in mammalian cells upon depletion of cholesterol has also been shown (Storch et al., 2007; Telbisz et al., 2007). It is not known whether cholesterol is only a modulator of ABCG2 function or it is directly transported by the protein. Other members of the ABCG subfamily (ABCG1, ABCG4, and ABCG5/ABCG8) are involved in

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ABBREVIATIONS: ABCG2, ATP-binding cassette, subfamily G, member 2; BSEP, bile salt export pump; CA, cholic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EKI-785, *N*-[4-[(3-bromophenyl)amino]-6-quinazoliny]-2-butynamide; ESG, estradiol-glucuronide; GC, glycocholic acid; Hst, Hoechst 33342; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,6]pyrido[3,4-*b*]indole-3-propanoic acid 1,1-dimethylethyl ester; MRP2, multidrug resistance protein 2; MTX, methotrexate; PCR, polymerase chain reaction; RAMEB, randomly methylated β -cyclodextrin; R123, rhodamine 123; SBE, steroid-binding element; SN-38, 7-ethyl-10-hydroxy-camptothecin; TC, taurocholate; UDC, ursodeoxycholic acid; wt, wild type; ZD1839, *N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine, gefitinib, Iressa.

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 Sf9 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, in 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/MxxLxxL sequence or a shorter LxxL, 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 MxxLxxL motif; a Gly to Arg mutation at the beginning of this element causes loss of activity and sitosterolemia. Mutations in the LxxL motif in the homologous region of ABCG2, as a putative steroid-binding element (SBE), have been shown to cause failure in the progesterone and estradiol recognition of ABCG2 expressed in *Lactococcus lactis* (Velamakanni 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, tauroolithocholate 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., 2005). ABCG2-specific extrusion of a fluorescein-conjugated bile acid derivative cholyglycylamidofluorescein and also a low-level transport of cholic acid, glycocholic acid, taurocholic acid, and tauroolithocholic acid-3-sulfate have been suggested (Blazquez et al., 2012).

In our present study, we provide a detailed mutational analysis of the cholesterol-sensing capability of different ABCG2 R482 mutants as well as mutants carrying the L555A, L558A, or L555A/L558A point mutations. In addition, we present a comprehensive characterization of the interaction between ABCG2 and bile acids. We demonstrate the modulatory effects of these mutations on ABCG2 function and their contribution to the cholesterol/bile acid sensing capability of the protein. We also show that as compared with “professional” bile acid transporters ABCG2 has no significant bile acid transport capacity.

Materials and Methods

Unless indicated otherwise, all materials were purchased from Sigma-Aldrich (Budapest, Hungary). Cholesterol-RAMEB (cholesterol-loaded randomly methylated β -cyclodextrin) was kindly provided by CycloLab Ltd. (Budapest, Hungary). The BXP-21 monoclonal antibody (Maliepaard et al., 2001) and Ko143 [(3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[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 Rik Scheper, and from Dr. G. J. Koomen, respectively.

Expression Vectors. Generation of the baculovirus transfer vector (pAcUW21-L) harboring the cDNA for wild-type (wt) ABCG2 or the R482 and K86M mutants was described previously (Ozvegy et al., 2002; 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 CTC GCG GTC AAT CT and 5' AG ATT GAC CGC GAG ACC TGA A; L558A: 5' GT CTG TTG GTG AAT GCC ACA ACC ATT and 5' AAT GGT TGT GGC ATT CAC CAA CAG AC; L555/558A: 5' GT CTC GCG GTG AAT GCC ACA ACC ATT and 5' AAT GGT TGT GGC ATT CAC CGC GAG AC. The PCR fragments containing the mutant cDNAs were cloned between the NcoI-SacI sites of the pAcUW21-L/wtABCG2 vector. The base order of the constructs was confirmed by sequencing of the appropriate fragments. The R482G/L555/558A triple mutant was created by replacing the DNA fragment between the PstI-NcoI sites of the pAcUW21-L/R482G with that of derived from the pAcUW21-L/L555/558A vector.

Generation of His₆-Tagged ABCG2 Variants. His₆-ABCG2 was generated as described by Telbisz et al. (2013). The His₆-tagged L558A and L555/558A mutants were created by cloning the PstI-SacI site from pAcUW21-L/ABCG2-L558A or L555/558A into the pAcUW21-L/His₆-ABCG2.

Cell Culturing, Generation of Recombinant Baculoviruses, and Membrane Preparation. We cultured Sf9 cells and generated recombinant baculoviruses as described previously (Bakos et al., 2000; Ozvegy et al., 2001). Membrane preparation was performed as described by Ozvegy et al. (2001). Cholesterol loading of the membrane preparations was achieved by incubation with 2 mM cholesterol-RAMEB on ice for 30 minutes, as published previously (Telbisz et al., 2007).

Immunodetection of ABCG2. We suspended the Sf9 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 $\times$ dilution, and a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (10,000 $\times$ dilution, Jackson ImmunoResearch Laboratories, West Grove, PA).

Solubilization, Purification, and Reconstitution into Proteoliposomes. Solubilization of His₆-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⁺-nitrilotriacetate; 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 Sf9 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 Sf9 membrane vesicles were incubated in the presence or absence of 4 mM MgATP (or 4 mM MgATP + 1 μ M Ko143) in a buffer containing 40 mM 3-(N-morpholino) propanesulfonic acid-Tris (pH 7.0), 56 mM KCl, 6 mM MgCl₂, and 2 mM dithiothreitol, in a final volume of 140 μ l at 37°C for 5 minutes. The measurement was started by the addition of 100 μ M [³H]methotrexate (Moravsek Biochemicals and Radiochemicals, Brea, CA), 25 or 50 μ M [³H]estradiol-glucuronide (Perkin-Elmer Life and Analytical Sciences, Waltham, MA), 100 μ M [³H]glycocholate (Perkin-Elmer) or 100 μ M [³H]cholic acid (American Radiolabeled Chemicals, Inc., St. Louis, MO). The experiments were performed as described previously (Ozvegy-Laczka et al., 2005).

Cellular Dye Uptake and Calculation of ABCG2 Transport Activity. Measurement of pheophorbide A and rhodamine 123 extrusion by ABCG2 from intact insect cells was described earlier (Ozvegy-Laczka et al., 2008). Geometric mean fluorescence values measured in the absence (M_0) or presence (M_i) of inhibitor were determined, and the activity factor was calculated as follows: $(M_i - M_0)/M_i \times 100$.

Measurement of Hoechst 33342 Transport Activity. Hoechst 33342 transport was determined as described in Ozvegy-Laczka et al. (2004). Transport activity was calculated as $((F_{100} - F_0)/F_{100}) \times 100$, where F_{100} is fluorescence in the presence of 1 μ M Ko143, and F_0 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 Sf9 cell membranes has been characterized in detail previously (Pal et al., 2007; Telbisz et al., 2007). Briefly, human ABCG2 when expressed in cholesterol “poor” insect cells (5–8 μ g 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 μ g 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 Sf9 membranes (Pal et al., 2007; Telbisz 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 (Telbisz 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 Sf9 cell membranes increasing the membrane cholesterol levels did not significantly influence the activity of the R482G and R482T mutants (Telbisz et al., 2007), although experiments on purified ABCG2 reconstituted in proteoliposomes revealed that cholesterol is also essential for the function of the R482G variant (Telbisz 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 Sf9 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 Sf9 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 [3 H]methotrexate uptake in Sf9 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 Sf9 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 Sf9 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 Sf9 cells.

When the Sf9 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 Sf9 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 Sf9 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 Sf9 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

Fig. 1. ATPase and transport activity of the ABCG2-R482 mutants: effect of cholesterol. (A) Vanadate-sensitive ATP hydrolysis of wtABCG2 and its R482 mutant variants was determined in insect cell membranes (open bars) and in insect cell membranes loaded with cholesterol (black columns). The ratio of substrate-stimulated ATPase activity (measured in the presence of 100 μ M prazosin) and baseline ATP hydrolysis (no added substrate) is shown. Columns represent data obtained from two independent experiments with four parallels, bars $\pm$ S.D. values. * $P < 0.05$, Student's t test, significant difference. (B) Hoechst 33342 (1 μ M) uptake was determined in insect cells transfected with baculoviruses encoding different ABCG2-R482 mutants or the wild-type protein. Cellular fluorescence was determined via fluorescence spectrophotometer. Transport activity was calculated as $(F_{100} - F_0)/F_{100} \times 100$, where F_{100} is fluorescence in the presence of 1 μ M Ko143, and F_0 is fluorescence in the absence of the inhibitor. * $P < 0.05$, Student's t test, significant difference.

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 of 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-bromophenyl)amino]-6-quinazolinyl]-2-butynamide) 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 [3 H]methotrexate ([3 H]MTX) and [3 H]estradiol-glucuronide ([3 H]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 for their [3 H]MTX and [3 H]ESG transport activity.

We found that despite their comparable expression level to wtABCG2, the L555A and L555A/L558A mutants did not show any detectable

vesicular transport activity for MTX in either control or cholesterol-rich membranes. Even in the case of the L558A mutant, which showed high ATPase activity, we could detect only very low MTX transport activity, similar to that observed in the R482 mutants (Supplemental Figs. 1A and 3A). When we analyzed [3 H]MTX transport by the L558A mutant 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 was determined by fluorometry or flow cytometry, respectively, in both control cells and cells loaded with 2 mM cholesterol-RAMEB. The actual expression levels of ABCG2 variants were examined in each experiment by Western blot analysis (data not shown). We found that the SBE mutants were able to transport both Hoechst 33342 and pheophorbide A, but in contrast to the wild-type protein, 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 SBE mutants, we have generated N-terminally His₆-tagged versions of the L558A and L555/558A variants. The His₆-L558A and His₆-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

Fig. 2. Expression and ATPase activity of ABCG2 SBE mutants in Sf9 cells. (A) Western blot detection of the SBE mutants in insect membranes using the BXP-21 antibody. Samples were loaded in equal amounts to the gel. (B) ATP hydrolysis of the SBE mutants in control and cholesterol-loaded membranes. ATPase activity was determined in the absence of added substrates (open bars) or in the presence of 1 µM quercetin (black columns) or 10 µM Ko143 (striped bars). Data were compiled from two independent experiments with four parallels, and the average ± S.D. values are indicated. (C and D) Effect of cholesterol on the transport activity of wtABCG2 and the SBE mutant variants in insect cells. Fluorescent dye uptake was determined in insect cells transfected with baculoviruses encoding different ABCG2-SBE mutants or wild-type protein. Cellular fluorescence was determined in a FACsCalibur cytometer (2 µM pheophorbide A) or in a fluorescence spectrophotometer (1 µM Hoechst 33342). Transport activity was calculated as described in the experimental procedures or in Fig. 1. For pheophorbide A uptake, data were normalized to the activity measured in the absence of added cholesterol. Average ± S.D. values of three independent experiments are shown.

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

Fig. 3. (A) ATPase activity of purified wtABCG2, ABCG2-L558A, and L555/558A in proteoliposomes. N-terminally His₆-tagged ABCG2 was isolated, purified, and reconstituted in 1 mg/ml *E. coli* lipid extract plus various amounts of cholesterol, as indicated on the x-axis. ATPase activity was measured in the absence of added substrates. Data points show the average of three independent experiments $\pm$ S.D. values. (B) Lack of dimerization of the purified the L555/558A mutant. Purified wtABCG2 or L555/558A was treated with 4 mM oxidized dithiothreitol (DTT) for 5 minutes at 37°C, and then dissolved in sample buffer (62.5 μ M Tris HCl pH 6.8, 2% SDS, 10 μ M EDTA-Na pH 6.8, 10% glycerol, 2 M urea, 0.14 mg/ml bromophenol blue). Electrophoresis and Western blotting have been performed as described in *Materials and Methods* section. ABCG2 was detected by the BXP-21 antibody.

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 Sf9 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 drug-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-loaded membranes (Fig. 4B, right columns): baseline ATPase activity is strongly reduced (almost to the level of Sf9 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 Sf9 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 $V_{\max}$ 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 μ M) bile acids had no effect on ABCG2-dependent substrate transport, but higher bile acid concentrations (above 250 μ M) 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 μ M 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

Fig. 4. Effect of bile acids on ABCG2 ATPase function. (A) Vanadate-sensitive ATPase activity in Sf9 membrane vesicles containing wtABCG2 was determined in either the absence (A) or the presence (B) of 1 mM cholic acid both in control and 2 mM cholesterol-RAMEB-treated membranes. Open columns represent baseline ATPase activity (no added compound); black or striped columns show ATP hydrolysis in the presence of 100 μ M prazosin or 5 μ M quercetin, respectively. Average of two independent experiments, each with two parallels $\pm$ S.E. values. (C and D) Relative ATPase activity of wtABCG2 in cholesterol-loaded Sf9 membranes: effect of increasing amounts of bile acids. (C) ATP hydrolysis in cholesterol-loaded ABCG2-containing Sf9 membranes is shown as a relative value compared with the activity measured in the absence of added substrates and bile acids. (D) ATP hydrolysis in the presence of 5 μ M quercetin compared with the baseline ATPase activity (no drug-substrate, no bile acid added). Measurements were performed in quadruplicate; mean $\pm$ S.E. values are shown.

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 μ M)

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

Effect of cholic acid on the stimulation or inhibition of the ABCG2-ATPase in cholesterol-loaded, isolated Sf9 membranes by various ABCG2-interacting compounds.

Compound	Concentration	Relative ATPase Activity Compared with Baseline	
		Control	With 1 mM Cholic Acid
	<i>μM</i>		
ABCG2 substrates			
Quercetin	5	2	5.4
ZD1839	1	1.7	2.7
Nilotinib	0.1	1.6	3.1
Topotecan	100	1	1.7
Flavopiridol	50	0.8	1.8
Sulfasalazine	5	2	2.8
Prazosin	50	1.8	6
ABCG2 inhibitor			
Ko143	1	0.2	0.3

ZD1839, *N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine, gefitinib, Iressa.

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).

Figure 6C shows that CA does not influence ATP hydrolysis of the L555A/L558A mutant—that is, both basal and substrate-stimulated activities remained unaltered. GC and TC also did not influence the activity of the L555A/L558A mutant (data not shown). Similarly, the activity of the L555A was not altered by the presence of bile acids. In the case of the L558A mutant, a similar effect of bile acids was observed as in the case of the R482G variant: CA and TC inhibited both basal and substrate-stimulated ATPase activity (Supplemental Fig. 5), but the relative substrate activation was practically unchanged

(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 R482I, 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 L555/558A 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). This apparent cholesterol independence of the L555A/L558A

Fig. 5. ATP-dependent uptake of different bile acids and ABCG2-substrates into cholesterol-loaded Sf9 vesicles. Inside-out membrane vesicles containing wtABCG2, MRP2, or BSEP were incubated with [^3H]-labeled glycocholate (GC, 100 μM), cholic acid (CA, 100 μM), methotrexate (MTX, 100 μM), or estradiol-glucuronide (ESG, 50 μM). Mean values of ATP-dependent uptake obtained from at least two independent experiments with four parallels $\pm$ S.D. values are shown.

Fig. 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 μ M quercetin (A and B), or 1 μ M nilotinib (C)) was determined. The figure shows the average of at least two independent measurements, four parallels $\pm$ 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 μ M quercetin (wt, R482G, S, K, and I) or 1 μ M 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.

mutant contradicted the results described by Velamakanni et al. (2008), which may be due to the fact that they investigated a triple mutant of ABCG2, which had R482G besides the L555A/L558A mutation, whereas we performed our experiments using the wild-type ABCG2 (482R) as a background. As demonstrated elsewhere and in this report as well, the R482G variant is already fully active in cholesterol-deficient Sf9 membranes. To solve this contradiction, we also generated the triple mutant R482G/L555A/L558A of ABCG2 and expressed this protein in insect cells. Still, this variant was also not activated by cholesterol (data not shown).

Because even very low levels of membrane sterols may affect ABCG2 function, we have purified and reconstituted the L558A and L555A/L558A mutants in cholesterol-free liposomes. Surprisingly, we found that the L558A mutant also needs cholesterol for its full activity. Unfortunately, cholesterol sensing of the double mutant could not be defined as it became inactive during purification. In summary, we suggest that the aforementioned two regions, both localized to the transmembrane domain, modulate both cholesterol sensing and substrate recognition of ABCG2.

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, though 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 effects of bile acids on basal ATPase activity, thus suggesting a site-specific modulatory effect of bile acids.

Sterols may significantly affect the function of ABCG2, especially in the liver. In hepatocytes, ABCG2 is abundantly expressed and is a key transporter for xenobiotics into the bile. Cholesterol significantly activates the transport function of ABCG2; therefore, an increased cholesterol level may promote enhanced detoxification. Currently we cannot exclude whether cholesterol (albeit with low efficiency) is also a transported substrate of this protein. Our present data reveal that bile acids, although probably not efficiently transported by ABCG2, also significantly modulate its function. In the liver, where a large amount of bile acids is formed from cholesterol, bile acids may cause a reduction in futile ATP consumption by ABCG2, represented as baseline ATPase activity. In addition to the functional regulation,

Fig. 7. Schematic representation of a complex regulation of ABCG2 function in hepatocytes.

although not examined in our present report, it should be mentioned that sterols may also modulate ABCG2 transcription and expression levels through interactions with nuclear receptors such as peroxisome proliferator-activated receptor (PPAR γ), 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, Váradi, Sarkadi.

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