ABCG2 Transports Sulfated Conjugates of Steroids and Xenobiotics*

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The mechanism for the cellular extrusion of sulfated conjugates is still unknown. In the present study, we investigated whether human wild type ABCG2 transports estrone 3-sulfate (E1S) using membrane vesicles from cDNA-transfected mouse lymphoma cell line (P388) cells. The uptake of [3H]E1S into ABCG2-expressing membrane vesicles was stimulated by ATP, and the Km value for [3H]E1S was determined to be 16.6 μM. The ABCG2-mediated transport of [3H]E1S was potently inhibited by SN-38 and many sulfate conjugates but not by glucuronide and glutathione conjugates or other anionic compounds. Other sulfate conjugates such as [3H]dehydroepiandrosterone sulfate (DHEAS) and [35S]4-methylumbelliferone sulfate ([35S]4-MUG) were also transported by ABCG2. Although [3H]methotrexate, [3H]17β-estradiol-17β-3-sulfate, [3H]2,4-dinitrophenyl-glutathione, and [14C]4-methylumbelliferone glucuronide were transported by ABCG2, this took place to a much lesser extent compared with [3H]E1S. It was suggested that ABCG2 preferentially transports sulfate conjugates and that E1S and DHEAS are the potential physiological substrates for this transporter.

Human ABCG2, also referred to as placenta-specific ABC transporter/breast cancer resistance protein/mitoxantrone resistance-associated protein, belongs to the ATP-binding cassette (ABC) transporter family (1–3). The structure of ABCG2 differs from that of MDR1 P-glycoprotein and multidrug resistance-associated protein (MRP/ABCC) family proteins, which are two major groups of cancer multidrug resistance ABC transporters. Although MDR1/ABCB1 contains two tandem repeats of transmembrane and ABC domains, and many of the MRP family proteins (such as MRP1–3/ABCC1–3) contain the additional third transmembrane domain prior to the two tandem repeats of transmembrane and ABC domains, ABCG2 consists of only one ABC and one transmembrane domain and, therefore, is referred to as a half-sized ABC transporter (1–3). ABCG2 was initially identified as an mRNA expressed in placenta (2) and as a non-MDR1 and non-MRP type resistance factor from cell lines which were selected in the presence of anthracyclines and mitoxantrone (1, 3). Although most of the half-sized ABC transporters are located on the intracellular organelle membrane, immunohistochemical studies revealed that ABCG2 was expressed on the plasma membrane (4). The fact that the intracellular concentration of substrate anticancer drugs is reduced in ABCG2-expressing cells (5) is consistent with its localization on the plasma membrane.

Although the function of ABCG2 has been studied extensively in terms of multidrug resistance, the physiological and/or pharmacological functions of this transporter have not been clarified yet. In normal human tissues, ABCG2 has been found to be expressed on the apical membrane of trophoblast cells in placenta, the apical membrane of enterocytes, the bile canalicular membrane of hepatocytes, and the apical membrane of lactiferous ducts in the mammary gland (6). These results suggest that ABCG2 may play an important role in protecting these tissues against the exposure to xenobiotics by extruding them across the apical membrane. In addition, ABCG2 has been demonstrated to be expressed in a wide variety of stem cells and to be a molecular determinant of the side-population phenotype (7). Recently, it was demonstrated that the disruption of the ABCG2 gene results in the loss of the number of side-population cells in the bone marrow and skeletal muscle (8). Moreover, ABCG2 (−/−) hematopoietic cells were more sensitive to mitoxantrone in the drug-treated transplanted mice, suggesting its protective role against cytotoxic substrates (8). It was recently demonstrated that ABCG2 (−/−) mice were more sensitive to phophorhobide, a breakdown product of chlorophyll, resulting in the phototoxic lesions on the light-exposed skin (9). The function of ABCG2 in determining the drug disposition has been investigated by examining the disposition of topotecan, a substrate for ABCG2 and MDR1 P-glycoprotein, in mdr1a/1b (−/−) mice (10). Jonker et al. (10) reported that the oral absorption of topotecan was increased by the administration of GF120918, an ABCG2 inhibitor. GF120918 has been demonstrated to alter the disposition of topotecan by preventing intestinal (re)absorption and/or biliary excretion (10).

Concerning the cellular extrusion of xenobiotics and/or their metabolites, the role of MDR1 and MRP family proteins has...
been documented (11–15). It has been established that MDR1 preferentially extrudes hydrophobic cationic and/or neutral compounds, whereas MRP family proteins preferentially transport organic anions, including conjugated metabolites (11–15). Indeed, it is well known that MRP family proteins act synergistically with conjugative enzymes to detoxify xenobiotics (11–13, 15, 16). For example, the glucuronide and glutathione conjugates formed in hepatocytes by UDP-glucuronosyl transferases and glutathione-S-transferases, respectively, are excreted into the bile via MRP2, an apically located efflux transporter (11–13, 15, 16). Although the substrate specificity of UDP-glucuronosyl transferases and sulfotransferases resemble each other, limited information is available for the cellular extrusion of sulfated conjugates. Our results indicated that xenobiotic sulfates are not significantly transported by MRP2 but, rather, stimulated the function of MRP2 (17, 18). MRP1, a basolaterally located efflux transporter, has been shown to transport estrone 3-sulfate (E1S), and this transport is extensively stimulated in the presence of reduced glutathione (GSH) (19). Very recently, it was demonstrated that dehydroepiandrosterone sulfate (DHEAS) is extruded from MRP2-expressing cells (20). In the present study, we examined whether ABCG2 transports sulfated conjugates using membrane vesicles from wild type human ABCG2-expressing mouse lymphoma (P388) cells.

EXPERIMENTAL PROCEDURES

Materials—[3H]E1S (43.1 Ci/mmol), [3H]17β-estradiol-17β-glucononide (E17βG, 55.0 Ci/mmol), [3H]lutein C4 (LTC4, 136.9 Ci/mmol), [3H]DHEAS (60 Ci/mmol), and [3H]taurocholate (2.00 Ci/mmol) were purchased from PerkinElmer Life Science, Inc. (Boston, MA).

FIG. 1. Western blot analysis of ABCG2 in membrane vesicles. Membrane vesicles isolated from ABCG2-transfected (P388-HA) and control P388 cells (25 μg of protein) were separated on an 8.5% polyacrylamide gel containing 0.1% SDS. The proteins transferred to the polyvinylidene difluoride membrane by electroblotting were detected by monoclonal antibodies against human ABCG2.

FIG. 2. Time profiles for [3H]E1S uptake by membrane vesicles. Membrane vesicles (5 μg of protein) prepared from ABCG2-transfected P388 cells (circles) or vector-transfected P388 cells (squares) were incubated at 37 °C in medium containing 500 nm unlabeled and 52 nm [3H]-labeled E1S in the presence (closed symbols) and absence (open symbols) of 5 mM ATP. Each point and vertical bar represents the mean ± S.E. of triplicate determinations (closed symbols) or the mean values of two determinations (open symbols). Where vertical bars are not shown, the S.E. is within the limits of the closed symbols.
TABLE I
Inhibition of ABCG2-mediated [3H]E1S transport by sulfated conjugates and anticancer drugs

| Unlabeled | Percentage of control | Maximum concentration tested | μM | μM |
|-----------|----------------------|------------------------------|----|----|
| DHEAS     | 55                   |                              | 20 | 50 |
| E1S       | 77                   | 100                          | 1  | 1  |
| 4-MUS     | 14                   | 100                          | 6  | 150|
| E3040S    | 6                    | 150                          | 10 | 600|
| PNPS      | 53                   | 200                          | 37 | 75 |
| TLC-S     | 37                   | 250                          | 61 | 500|
| SL-S      | 22                   | 200                          | 59 | 80 |
| Mitoxantrone | 61                 | 200                          | 37 | 500|
| Daunomycin| 37                   | 200                          | 59 | 80 |

Approximate IC50 values were estimated from the data shown in Fig. 4. 3H-E1S uptake by membrane vesicles prepared from ABCG2-transfected cells and vector-transfected P388 cells was determined at 37°C for 2 min in medium containing 500 nM unlabeled and 33 nM 3H-labeled E1S, with or without the compounds at the indicated concentrations. The uptake values were calculated by subtracting the values for control membrane vesicles from those for ABCG2-expressing membrane vesicles in the presence of 5 μM ATP.

TABLE II
Effect of organic anions including glucuronide and glutathione conjugates on ABCG2-mediated [3H]E1S transport

| Percentage of control | Maximum concentration tested | μM | μM |
|-----------------------|------------------------------|----|----|
| 35S-4-MUS             | 100                          | 1  | 1  |
| 4-MUS                 | 75                           | 500| 500|
| 3H-4-MUG              | 200                          | 2  | 2  |
| E17βG                 | 75                           | 75 | 75 |
| 4-MUG                 | 500                          | 500| 500|
| E3040G                | 250                          | 250| 250|
| Estrene               | 2                            | 2  | 2  |
| LTC4                  | 2                            | 2  | 2  |
| DNP-SG                | 80                           | 80 | 80 |

*Significantly lower than the control (p < 0.01).
**Significantly lower than the control (p < 0.05).

Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) for 1 h. Subsequently, the membrane was rinsed four times with PBS containing 0.1% Tween 20 for 5 min. The enzyme activity was assessed by using 5000-fold diluted Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, Inc.) with an Odyssey infrared imaging system (LI-COR, Inc., Lincoln, NE).

The transport studies were performed using a rapid filtration technique (25, 26). Briefly, 15 μl of transport medium (10 μl Tris-HCl, 250 μm sucrose, 10 μM MgCl2, pH 7.4) containing radiolabeled compounds, with or without unlabeled substrate, was preincubated at 37°C for 3 min and then rapidly mixed with 5 μl of membrane vesicle suspension (5–10 μg of protein). The reaction mixture contained 5 μM ATP or other nucleotides, along with the ATP-regenerating system (10 μM creatine phosphate and 100 μg/ml creatine phosphokinase). The transport reaction was terminated by the addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 0.1 mM NaCl, and 10 mM Tris-HCl (pH 7.4). The stopped reaction mixture was filtered through a 0.45-μm membrane filter (GVWP, Millipore Corp., Bedford, MA) and then washed twice with 5 μl of stop solution. Radioactivity retained on the filter was determined in a liquid scintillation counter (LSC-3500, Aloka Co., Tokyo, Japan). The ATP-dependent uptake of ligands was calculated by subtracting the ligand uptake in the absence of ATP from that in its presence.

RESULTS

Expression of ABCG2—The expression level of the wild type human ABCG2 in a mouse lymphoma cell line was determined by Western blot analysis using anti-human ABCG2 (BXP-21) monoclonal antibody. As shown in Fig. 1, ABCG2 was only detectable in the transfected cells, and its molecular mass was ~72 kDa, which is consistent with the previously reported molecular mass (24). The Western blot analysis with an antibody against MRP1 (MRP1) revealed that the strength of the band at 175 kDa is the same between control and ABCG2-transfected cells (data not shown). Although we could detect the band in the Western blot analysis of crude membrane fraction from mouse liver and intestine, but not that of the membrane vesicles from the parental P388 cells, with BXP-21 monoclonal antibody (data not shown), we cannot deny the possibility that the presence of an unknown amount of related mouse transporter may significantly influence the results of the transport studies. It should be noted that the use of mouse lymphoma cells has disadvantages due to this possibility.

Uptake of 3H-E1S into Membrane Vesicles—The time profiles for the uptake of 3H-E1S by membrane vesicles from P388 cells are shown in Fig. 2. The uptake of 3H-E1S into membrane...
vesicles from ABCG2-transfected P388 cells, but not into that from vector-transfected cells, was markedly stimulated by ATP (Fig. 2). Indeed, the uptake of $[^3H]E_1S$ into ABCG2-expressing membrane vesicles at 2 min was more than 20-fold higher than that into control membrane vesicles (Fig. 2). We also confirmed that the vesicle-associated $[^3H]E_1S$ represents uptake into the intravesicular space, rather than binding to the vesicle surface, by confirming the osmotic sensitivity (data not shown).

Characterization of ABCG2-mediated Transport of $[^3H]E_1S$—The ATP-dependent uptake of $[^3H]E_1S$ into ABCG2-expressing membrane vesicles was saturable (Fig. 3A). Nonlinear regression analysis of the ATP-dependent uptake of $[^3H]E_1S$ revealed that the uptake can be described by a single saturable component with a $K_m$ of 16.6 ± 3.4 $\mu$M and a $V_{max}$ of 2.34 ± 0.24 nmol/min/mg of protein, respectively. Kinetic analysis revealed that the $K_m$ of ATP was 1.23 ± 0.20 mM (Fig. 3B), which is higher than the previously reported value for the ATP concentration producing the half maximum membrane ATPase activity (0.3 mM) in the presence of 100 $\mu$M prazosin or producing the half maximal velocity of SN-38 transport (~0.8 mM) (27, 28).

GTP and UTP also stimulated the uptake of $[^3H]E_1S$ to the same extent as ATP, whereas the stimulatory effect of CTP was ~50% that of ATP. In contrast, nonhydrolyzable analogues such as ATPγS or GTPγS, along with AMP, GMP, or UMP, did not support the ABCG2-mediated transport of $[^3H]E_1S$. However, from the present results, we cannot discuss details on the ATPase activity of ABCG2, which has been demonstrated by Ozvegy et al. (27, 28).

To further characterize the ABCG2-mediated transport of $[^3H]E_1S$, we examined the effect of inhibitors. Sulfated conjugates (DHEAS, $E_2S$, TLC-S, PNPS, 4-MUS, and E3040S) significantly inhibited the ABCG2-mediated transport of $[^3H]E_1S$ (Fig. 4 and Table I). The inhibitory effect of anti-tumor drugs (such as SN-38, mitoxantrone, and daunomycin), along with that of estrone, was also observed (Fig. 4 and Tables I and II). In contrast, glutathione conjugates (LTC$_4$ and DNP-SG), glucuronide conjugates ($E_2$17βG, 4-MUG, and E3040G), or non-conjugated organic anions (pravastatin, methotrexate, and taurocholate) did not potently reduce the uptake of $[^3H]E_1S$ (Table II). We also found that the ABCG2-mediated transport of $[^3H]E_1S$ was not affected by 10 mM dithiothreitol (DTT), 10 mM DTT and 4 mM GSH, and 10 mM DTT and 4 mM nonreducing S-methyl-GSH (data not shown).

**Determination of the Substrate Specificity of ABCG2—**In ad-
tion to [3H]E1S, the transport of other organic anions was examined in ABCG2-expressing membrane vesicles. As shown in Fig. 5, [3H]DHEAS, a steroid sulfate conjugate, was accepted by ABCG2 as a substrate. Furthermore, the uptake of E3040G was examined at 2.5 μM, and it was found that the uptake of E3040G (900 pmol/mg/5 min) was much higher than that of E3040G (12.5 pmol/mg/5 min) (Fig. 5). Sulfate-preferential transport was also observed for 4-MU, although we cannot directly compare the absolute values of the uptake between 4-MUS and 4-MUG, because the medium concentrations for 4-MUS and 4-MUG were 5.0 and 4.1 μM, respectively (Fig. 5). 4-MUS and 4-MUG were extensively transported by ABCG2 under the present experimental conditions (Fig. 5).

Kinetic analysis was also performed for ABCG2-mediated transport of [35S]4-MUS and [35S]E3040G. As shown in Fig. 6, the K_m values for [35S]4-MUS and [35S]E3040 were 12.9 ± 2.1 μM and 26.9 ± 4.0 μM, respectively.

DISCUSSION

We examined the function of ABCG2 by using membrane vesicles prepared from the wild type human ABCG2-transfected mouse lymphoma cells (P388 cells) and suggested that the sulfated conjugates of steroids and xenobiotics are preferentially transported by this transporter. However, the use of mouse lymphoma cells has disadvantages, because the presence of an unknown amount of related mouse proteins may significantly influence the results.

In the present study, it was suggested that E1S and DHEAS are the potential endogenous substrates for ABCG2 (Figs. 1 and 5), although in vivo experiments are required to clarify its physiological significance. In addition, sulfate conjugates of xenobiotics are also preferentially transported by ABCG2 (Fig. 5).

If we consider the fact that MRP1, another transporter capable of transporting sulfated conjugates, is highly expressed on testicular Leydig cells, which is the major site of sulfate conjugation of estrogen in the testis, it is possible that MRP1 protects the testis from exposure to cytotoxic compounds by extruding them after conversion into sulfate conjugates (19). ABCG2 may also play a host defensive role by being expressed in placenta, liver, and intestine, where sulfotransferase activity is very high with minimal MRP1 expression (6, 14, 29). Moreover, the apical localization of ABCG2 is in marked contrast to the basolateral localization of MRP1 (6, 11). Collectively, it is possible that the apically located ABCG2 in placenta, liver, and intestine may be responsible for protection of the fetus, biliary excretion, and prevention of xenobiotic absorption, respectively, whereas basolaterally localized MRP1 in testis is responsible for protecting the germline (19).

The inhibitory effect of several compounds on the ABCG2-mediated transport of E1S needs to be discussed in relation to their chemical structure. No potent inhibitory effect was observed up to 80 and 2 μM for DNP-SG and LTC4, respectively, on ABCG2-mediated E1S transport (Table II). In addition, E17βG and E3040G did not potently inhibit ABCG2 function up to 75 and 250 μM, respectively (Table II). In contrast to the much lower sensitivity toward glutathione and glucuronide conjugates, many sulfated conjugates reduced ABCG2-mediated transport (Fig. 4 and Table I). The approximate IC_{50} values of DHEAS, PNPS, 4-MUS, and E3040G were 55, 53, 6, and 10 μM, respectively (Fig. 4 and Table I). Collectively, it appears that ABCG2 preferentially recognizes sulfated conjugates.

In addition to E1S, DHEAS was also transported by ABCG2 (Fig. 5). It is noteworthy that 4-MUS and E3040S, but not 4-MUG or E3040G, were extensively transported by ABCG2 (Fig. 5 and 6), and the IC_{50} values of 4-MUS and E3040G for the ABCG2-mediated transport of E1S were 6 and 10 μM, respectively (Fig. 4 and Table I), whereas 4-MUG or E3040G did not potently inhibit this transport up to 500 and 250 μM, respectively (Table II). Although TLC-S inhibited the ABCG2-mediated transport with an IC_{50} of less than 50 μM (Fig. 4 and Table I), TLC-S was not significantly transported by ABCG2 under the present experimental conditions (Fig. 5). These results may be accounted for by assuming that ABCG2 has minimal V_{max} value for TLC-S. We also found ABCG2-mediated transport of MRP substrates, such as methotrexate and E17βG, although these compounds are transported to a much lesser extent compared with E1S (Figs. 1 and 5). Indeed, their affinity for ABCG2 was quite low, because 600 μM methotrexate or 75 μM E17βG did not significantly inhibit the ABCG2-mediated transport of E1S (Table II).

The affinity of anti-tumor drugs for ABCG2 should be discussed in relation to resistance to this anti-tumor drug because of the previous finding that ABCG2-expressing cells acquired resistance against these drugs (1, 30, 31). Concerning the resistance of ABCG2-overexpressing cells against SN-38, it has been demonstrated that ABCG2 transports this drug with a K_m value of 4.0 μM (32). The finding that IC_{50} value of SN-38 on ABCG2-mediated transport of E1S is ~1.6 μM (Fig. 4 and Table I) is consistent with this previously reported K_m value (32). It was also suggested that daunomycin, mitoxantrone, and doxorubicin have low affinity for ABCG2 (Fig. 4 and Table I). Such transport characteristics mediated by ABCG2 should also be discussed in relation to the mutation in its gene structure. It has been reported that the amino acid at position 482 plays an important role in determining the substrate specificity of ABCG2 (33). For example, ABCG2 containing a Thr or Gly residue at position 482 transports rhodamine 123 and the arachacyclines (doxorubicin and daunorubicin), whereas wild type ABCG2 containing an Arg at this position does not (33). In contrast, all the ABCG2 variants transport mitoxantrone (33). Moreover, the overexpression of ABCG2 containing an Arg residue at position 482, but not those containing Thr or Gly at this position, results in the acquisition of resistance against methotrexate by extruding this compound from the cells (34). Because the amino acid at position 482 in our clone was Arg, the present results showing that Arg-482 ABCG2 transports methotrexate (Fig. 5) is consistent with the previous findings by Volk et al. (34).

It would be noteworthy to point out the fact that the effective concentration of methotrexate to reduce the growth of ABCG2 overexpressing MCF/MX cells was much lower than the affinity of this drug toward ABCG2 (Table II); Volk et al. (34) reported that the IC_{50} values of methotrexate to reduce the cell growth were 0.10 and 11.3 μM for parental MCF7/WT and MCF7/MX cells, respectively.

In conclusion, ABCG2 was found to efficiently transport sulfated conjugates of steroids and xenobiotics such as E1S, DHEAS, and 4-MUS. ABCG2 also accepts substrates for MRP1 and 2 (such as methotrexate, E17βG, and DNP-SG) to a much lesser extent compared with E1S. It was suggested that ABCG2 preferentially transports sulfated conjugates and that E1S and DHEAS are the potential physiological substrates for this transporter.

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