Characterization of Drug Transport, ATP Hydrolysis, and Nucleotide Trapping by the Human ABCG2 Multidrug Transporter

MODULATION OF SUBSTRATE SPECIFICITY BY A POINT MUTATION*

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The overexpression of the human ATP-binding cassette half-transporter, ABCG2 (placenta-specific ABC transporter, mitoxantrone resistance-associated protein, breast cancer resistance protein), causes multidrug resistance in tumor cells. An altered drug resistance profile and substrate recognition were suggested for wild-type ABCG2 and its mutant variants (R482G and R482T); the mutations were found in drug-selected tumor cells. In order to characterize the different human ABCG2 transporters without possible endogenous dimerization partners, we expressed these proteins and a catalytic center mutant (K86M) in Sf9 insect cells. Transport activity was followed in intact cells, whereas the ATP binding and hydrolytic properties of ABCG2 were studied in isolated cell membranes. We found that the K86M mutant had no transport or ATP hydrolytic activity, although its ATP binding was retained. The wild-type ABCG2 and its variants, R482G and R482T, showed characteristically different drug and dye transport activities; mitoxantrone and Hoechst 33342 were transported by all transporters, whereas rhodamine 123 was only pumped by the R482G and R482T mutants. In each case, ABCG2-dependent transport was blocked by the specific inhibitor, fumitremorgin C. A relatively high basal ABCG2-ATPase, inhibited by fumitremorgin C, was observed in all active proteins, but specific drug stimulation could only be observed in the case of R482G and R482T mutants. We found that ABCG2 is capable of a vanadate-dependent adenine nucleotide trapping. Nucleotide trapping was stimulated by the transported compounds in the R482G and R482T variants but not in the wild-type ABCG2. These experiments document the applicability of the Sf9 expression system for parallel, quantitative examination of the specific transport and ATP hydrolytic properties of different ABCG2 proteins and demonstrate significant differences in their substrate interactions.

The multidrug-resistant phenotype of malignant cells can often account for the failure of chemotherapy in cancer pa-
tients. In many cases multidrug resistance is caused by the activity of ABC1 transporters, including MDR1/P-glycoprotein (1) and MRP1 (multidrug resistance-associated protein) (2). ABC transporters cause multidrug resistance by extruding their drug substrates from the resistant cells and maintaining the level of these cytotoxic agents below cell-killing concentrations.

The human ABC half-transporter, ABCG2 (placenta-specific ABC transporter/mitoxantrone resistance-associated protein/breast cancer resistance protein), has been identified recently (3–5) as a candidate protein responsible for tumor multidrug resistance. The overexpression of ABCG2 was found in several drug-selected cell lines (6–10), and in tumorous tissues of patients (11–13). The activity of the human ABCG2 was suggested to be the major cellular defense mechanism against the cytotoxic drug, mitoxantrone (6), but several other drugs have also been indicated as potential substrates of this drug pump (14).

The human ABCG2 protein belongs to the White (ABCG) subfamily of ABC transporters. The members of this subfamily are ABC half-transporters, i.e. they contain only one ABC and one transmembrane domain. The White protein, the Drosophila homolog of human ABCG2, forms heterodimers and transports different eye pigment precursors (15). However, the data in the literature (5, 16–19) suggest that ABCG2 can function as a homodimer.

It has been shown recently (20) that in drug-selected human tumor cells ABCG2 appears in three different forms, containing Arg, Gly, or Thr at amino acid position 482. It was suggested that these variants possess significant differences in their cross-resistance and drug transport patterns (20). Interestingly, in some drug-selected mouse cell lines, mutation of the equipositional amino acid in mouse ABCG2 occurred, causing altered drug resistance profiles for the mutant variants R482S and R482M (21).

According to the published consensus sequence in the human genome data base (GenBank™ accession number AF103796, see Ref. 3), Arg-482 is the wild-type form of ABCG2, and the other two variants may appear only during drug selection (20, 22). Data concerning the drug resistance patterns of mammalian cells expressing ABCG2 are somewhat contradictory. In one study (20) the confirmed wild-type form of ABCG2 produced significant mitoxantrone (MX) resistance and efflux, whereas in another report (22) the wtABCG2 was found to be inefficient in protecting cells from MX accumulation.

The baculovirus-Sf9 heterologous expression system has

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been shown to be an excellent tool for producing high level expression of biologically active ABC proteins, and isolated membranes from virus-infected cells are suitable for the investigation of the transport, ATPase, and nucleotide trapping activity of these ABC transporters (23–26). Previously, we demonstrated that the ABCG2–R482G multidrug transporter could also be expressed in insect cells, allowing the investigation of its membrane ATPase activity (18).

In the present study, we document the applicability of the baculovirus-sf9 expression system for measuring the ABCG2-dependent transport of different fluorescent compounds in intact insect cells. Notably, we also determined the kinetics of ABCG2-dependent Hoechst 33342 transport. In addition to the ATPase activity, the formation of a catalytic intermediate of the ATPase cycle is a characteristic feature of ABC transporter proteins (27–29). This fact allowed us to detect 8-azido-ATP binding and vanadate-dependent trapping by ABCG2, which, as documented here, required the presence of Co2+ cations.

We generated and expressed the human wild-type ABCG2 and its variants R482G and R482T in Sf9 cells, and we characterized their transport and ATP hydrolytic activity. As a control, we mutated a crucial amino acid in the catalytic center of ABCG2-R482G (Lys-86 was changed to Met) in hope of producing a non-functional transporter. Here we show that the expression of wtABCG2 and its variants could be achieved with a high yield. We compared the ABCG2-dependent transport of various fluorescent compounds. In parallel experiments, we investigated the ATP hydrolytic cycle of the ABCG2 protein in isolated membrane preparations. The full catalytic cycle was studied by measuring vanadate-sensitive, drug-stimulated ATPase activity. The formation of a catalytic intermediate of this reaction was examined by following vanadate- and Co2+-dependent adenine nucleotide trapping. The parallel investigation of transport activity, nucleotide trapping, and ATPase activity of wtABCG2 and its amino acid 482 variants allowed the comparison of the function of these proteins.

The heterologous Sf9 expression system has the special advantage that insect ABC proteins probably do not form heterodimers with the human ABCG2 protein, because this latter component is expressed at a very high level in these cells. There-fore, this system allows a quantitative comparison of the substrate specificity and transport activity of homodimer-forming variants of the human ABCG2 multidrug transporter. The parallel transport and ATP hydrolysis studies presented below may help to understand the molecular mechanism of action of these ABCG2 proteins.

EXPERIMENTAL PROCEDURES

Materials—Mitoxantrone, doxorubicin, prazosin, verapamil, rhodamine 123, sodium orthovanadate, propidium iodide, and digitonin were purchased from Sigma. Hoechst 33342 was purchased from Molec-ular Probes. FTC was kindly provided by Lee M. Greenberger and was purchased from Mo-nomine 123, sodium orthovanadate, propidium iodide, digitonin, and ATP were purchased from Sigma. Hoechst 33342 was purchased from Molecular Probes. FTC was kindly provided by Lee M. Greenberger (Wyeth-Ayerst Research). Ko 143 was a generous gift from Drs. J. Allen and G. Koopen (Division of Experimental Therapy, The Netherlands Cancer Institute, and Laboratory of Organic Chemistry, University of Amsterdam, Amsterdam, The Netherlands). 8-Azido-[α-32P] ATP was purchased from ICN Biomedicals.

Generation of Transfer Vector Possessing Different Human ABCG2 cDNAs—pAcUW21-L/ABCG2 (R482C) was constructed as described (18). Wild-type ABCG2 (Arg-482) and its variants (R482T and R482M) were created using ABCG2-R482G cDNA as a template (4) by overlap extension PCR (30). Two internal complementary primer pairs were used with each containing the specific mutation as follows: the Arg-primer pairs were 5′-TTATACATGCGGATGAGGTACC-3′ and 5′-GGTACACTGGCGATGAGGTACC-3′; the R482T primer pairs were 5′-TTATACATGCGGATGAGGTACC-3′ and 5′-GGTACACTGGCGATGAGGTACC-3′; and the R482M primer pairs were 5′-TGAGGGCATGCTTGTGTTATT-3′ and 5′-TAATAACAGACAGATGCGTCCCA-3′, respectively. The two outer primer pairs were 5′-CTTGGGATATCTGACCAG-3′ and 5′-GGTACGAGGAGTGTGCTCA-3′ for the wild-type and the amine-acid 482 variants and 5′-GTATATGCTAAAAATACATTAC-CTG-3′ and 5′-GGTACATGCGGATGAGGTACC-3′ for the R482M mutant. The PCRs were performed as described previously (31). The PCR products containing the Arg-482 or R482T coding sequence were digested with PstI and MscI enzymes and ligated between the corresponding sites of the pAcUW21-L/ABCG2 vector. The PCR product coding for the R482M variant was digested with NoI and SpeI enzymes and ligated to the NoI and SpeI sites of the pAcUW21-L/ABCG2 (R482G) vector. The mutations were confirmed by sequencing the Pst-I-MscI or the NoI-SpeI fragments of the constructs, respectively.

Generation of Recombinant Baculoviruses—Recombinant baculoviruses, carrying the different human ABCG2 cDNAs, were generated with the Baculofold Transfection Kit (Pharmingen) according to the manufacturer’s instructions. Sf9 (Spodoptera frugiperda) cells were infected and cultured as described (32). Individual virus clones, expressing high levels of the different human ABCG2 variants, were obtained by endpoint dilution and subsequent amplification. ABCG2 protein expression was determined by immunoblotting and immunoflow cytometry (see below).

Cell Culturing and Tunicamycin Treatment—HL60 cells, infected with the retrovirus containing the cDNA of wtABCG2,2 were cultured in RPMI medium, supplemented with 10% fetal calf serum, 50 units/ml penicillin and streptomycin, and 100 μg mitoxantrone at 37 °C in 5% CO2. For inhibition of N-glycosylation, HL60/wtABCG2 cells were grown for 72 h in a medium containing 2.5 μg/ml tunicamycin.

Membrane Preparation and Immunodetection of ABCG2—After 3 days of virus infection, the Sf9 cells were harvested, and their membranes were isolated. Membrane protein concentrations were determined by the modified Lowry method as described (33). Immunoblotting was performed on membrane or cell extract samples as described in Ref. 32. Different ABCG2 proteins were detected by the monoclonal BXP-21 antibody (2000-fold dilution), which was kindly provided by Drs. George Scheffer and Rik Schepers (33). The protein-antibody interaction was visualized by the enhanced chemiluminescence technique (ECL, Amersham Biosciences) using anti-mouse horseradish pero-xidase-conjugated secondary antibody (20,000-fold dilution, Jackson Im-munoresearch). Immunoflow cytometry was performed by labeling the Sf9 cells also used for dye uptake studies; see below. Cells were cultured using the anti-ABCG2 monoclonal antibody 5D1 (eBioscience), which recognizes a cell-surface epitope on ABCG2. The antibody was used at a dilution of 1:500, and binding was visualized by the addition of a second, phycoerythrin-conjugated anti-mouse IgG (Immunotech), at a dilution of 1:250. Flow cytometric determination of the antibody reaction was carried out using a FACSCalibur cytometer (BD Biosciences).

Mitoxantrone or Rhodamine 123 Uptake in Intact Sf9 Cells—40 h after infection with the ABCG2 cDNA-containing baculoviruses, Sf9 cells were washed once and resuspended in HPMI medium (120 mM NaCl, 5 mM KCl, 400 μM MgCl2, 40 μM CaCl2, 10 mM Hepes, 10 mM NaHCO3, 10 mM glucose, and 5 mM Na2HPO4). Aliquots of the sus-pension were added to the intact Sf9 or Sf9 cells at 4°C in HPMI for 4 min and further incubated at 37 °C. Mitoxantrone or Rhodamine 123 solution. This dye becomes fluorescent only in a complex with DNA (34). Accumulation of Hoechst dye (Hst) was measured in a fluorescence spectrophotometer at 350 nm (excitation)/460 nm (emission), by using 530- and 670-nm bandpass filters, was used to deter-
ATPase Activity Measurement—Isolated membranes of Sf9 cells expressing ABCG2 were kept at −80 °C and were used within 2 months following supplementation. Drug-stimulated ATPase activity was measured by colorimetric detection of inorganic phosphate liberation, as described (18). Figures reflect the mean values of at least four independent experiments measured in two different membrane preparations for each type of ABCG2.

8-Azido-[α-32P]ATP Labeling—In the 8-azido-[α-32P]ATP binding experiments, isolated Sf9 membranes (150 μg of protein) were incubated for 5 min on ice with 20 μM 8-azido-ATP (0.1 MBq radioactivity) in reaction buffer (50 mM Tris-HCl, 50 mM KCl, 100 μM EGTA Tris, and either 2 mM MgCl2 or CoCl2). The membranes were UV-irradiated and then washed twice with washing buffer (reaction buffer containing 10 mM Mg or CoATP).

In the nucleotide trapping experiments isolated Sf9 cell membranes (150 μg of protein) were incubated for 2–5 min at 37 °C in reaction buffer, with or without 1 mM sodium orthovanadate, 0.05 MBq of 2–5 μM 8-azido-[α-32P]ATP, and the compounds are described in the figure legends. Reactions were stopped by the addition of ice-cold washing buffer. After two washing steps the membranes were UV-irradiated on ice.

At the end of the binding and trapping experiments, the membranes were suspended in disaggregation buffer, run on 10% Laemmli-type gels, and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). Labeling was detected by quantitative autoradiography in a PhosphorImager (Bio-Rad). Labeling experiments were repeated two times, and the figures show the results of one representative experiment. The identity of the 32P-azido-nucleotide-labeled bands was confirmed by immunostaining the same blot with anti-ABCG2 BXP-21 antibody (see above).

RESULTS

Expression of Wild-type ABCG2 and Its Mutant Variants in Sf9 Cells—We have shown earlier that the human ABCG2 multidrug transporter can be expressed in its biologically active form in Sf9 insect cells (18) similarly to other ABC proteins (23–26). In the present study we utilized this expression system to produce the wild-type human ABCG2 protein and its variants, R482G and R482T, as well as a catalytic center mutant, K86M.

Site-directed mutagenesis was performed on a human ABCG2 cDNA, which possesses Gly at position 482 (4), to create the wild-type (Arg-482) and the R482T and K86M variants of the ABCG2 half-transporter. Fig. 1A shows the predicted membrane topology of the ABCG2 multidrug transporter and the localization of the mutated amino acids Lys-86 and Arg-482.

Sf9 cells were transfected with recombinant baculoviruses carrying the respective human ABCG2 cDNAs. The level of ABCG2 expression was detected by immunoblotting using the BXP-21 monoclonal antibody (33). Fig. 1B demonstrates that all four ABCG2 proteins were successfully expressed in Sf9 cells. The level of their expression was almost equal and about 60 times higher than that of the expression obtained by retroviral transduction of the wtABCG2 in HL60 cells. We have shown earlier that the human ABCG2 in Sf9 cells was expressed in an underglycosylated form. As documented in Fig. 1B, all the ABCG2 proteins expressed in Sf9 cells migrated at an apparently lower molecular weight (−60 kDa) than ABCG2 expressed in HL60 cells (about 70 kDa), but with the same apparent molecular weight as the lower (non-glycosylated) band in the tunicamycin-treated HL60 cells. Therefore, we conclude that wtABCG2 and its variants are expressed underglycosylated in the insect cells. Flow cytometry immuno-detection of cell surface expression of ABCG2, determined by the 5D3 monoclonal antibody, also showed similar expression levels for the wild-type ABCG2 and its mutant variants (see below).

Flow Cytometry Assay of Mitoxantrone and Rhodamine 123 Extrusion in Intact Sf9 Cells, Expressing wtABCG2 and Its Mutant Variants—In order to investigate the transport characteristics of the different ABCG2 proteins expressed in insect cells, we have developed appropriate assay conditions for detecting the extrusion of various fluorescent compounds by flow cytometry and/or spectrofluorimetry in intact Sf9 cells. Based on exploring a number of variables, we have established that 40 h after infection with the ABCG2 cDNA-containing baculoviruses, the Sf9 cells were suitable for intact cell assays. In this case heterologous membrane protein expression reached a uniform and near-maximum level, whereas cell destruction by baculovirus was limited to below 30%. We have also established that HPMI medium (see “Experimental Procedures”) adjusted to pH 7.4 (the optimal pH for growing Sf9 cells is 6.1) could be used in these transport experiments without significant cell destruction in a time frame of up to 2 h (data not shown in detail).

In order to assay fluorescent drug or dye uptake, Sf9 cells under the above conditions were incubated with the assayed compounds for 30 min at 37 °C, and then the cells were washed and resuspended in ice-cold HPMI for flow cytometric measurements. Staining of the samples with propidium iodide was used for gating out the dead cells (see “Experimental Procedures”). In each experiment the expression of the different ABCG2 proteins was determined by Western blotting of the same cell preparations, to ensure equal levels of the ABCG2s in all experiments. In parallel flow cytometric experiments, we have also measured the cell surface expression of ABCG2 protein in Sf9 cells by the 5D3 monoclonal antibody, detecting an extracellular epitope of human ABCG2 (35). We found equal expression levels for the wild-type, R482G, R482T, and K86M/R482G mutant variants (data not shown).

For blocking ABCG2-dependent drug or dye extrusion, we
used FTC, a specific inhibitor of this protein. As shown earlier, this agent strongly inhibits ABCG2 function in micromolar concentrations in a variety of cellular systems (36, 37), including the function of ABCG2 expressed in Sf9 cells (18). In order to ensure full blockade of ABCG2-dependent transport (see below), we have used 10 μM FTC or its powerful analog Ko 143 (see Ref. 38). These compounds have no measurable fluorescence in the wavelength ranges applied here and therefore could be used in these experiments.

Fig. 2A shows MX accumulation studies in intact Sf9 cells, expressing either the wild-type ABCG2 or one of its mutant variants or β-galactosidase. As documented, MX uptake is relatively low in cells expressing the active ABCG2a, whereas FTC significantly increases this accumulation. In Sf9 cells, expressing the K86M mutant, MX uptake is significantly higher and reaches the level of MX accumulation found in the FTC-inhibited cells. This accumulation is similar to that found in mock (β-galactosidase)-infected Sf9 cells. These experiments indicate that wtABCG2 and the R482G or R482T variants actively extrude MX in the intact Sf9 cells, whereas the K86M mutant is inactive in this respect.

Prazosin, a known substrate of ABCG2 (16, 37), also increased the level of MX accumulation in insect cells expressing the active ABCG2 proteins, whereas it had no effect on the cells expressing the K86M mutant (see Fig. 2A). This observation indicates that prazosin is a substrate of the wtABCG2 and its amino acid 482 variants.

In order to compare the MX transport capacity of the wtABCG2 and its mutant variants, we applied a wide range of MX concentrations in similar experiments. Fig. 2, B and C, compiles the cellular fluorescence levels in these transport experiments by using 1–20 μM MX concentrations, with or without the addition of 10 μM FTC. As documented, at increasing MX concentrations, the wild-type (R482) ABCG2 was found to be somewhat less effective in protecting Sf9 cells against MX accumulation than the other two amino acid 482 variants; the accumulation of MX was about 15 ± 4% more in wtABCG2-expressing cells than in cells containing the R482G or R482T variants. In addition, the MX transport capacities of the amino acid 482 variants R482G and R482T were found to be similar in these experiments.

Next we performed similar transport studies for the fluorescent dye rhodamine 123, which was indicated to be a transported substrate of the ABCG2 variants R482G and R482T (20). As shown in Fig. 3, we found that insect cells expressing the wild-type ABCG2 or the K86M mutant accumulated significantly more rhodamine 123 than cells expressing the R482G or R482T variants. In contrast to that found for the wild-type ABCG2, in the R482G or R482T variants the addition of FTC greatly increased rhodamine 123 accumulation indicating an ABCG2-dependent extrusion of this compound.

These data support the fact that MX is a substrate for wtABCG2 and its amino acid 482 variants, although MX transport may be less efficient by the wild-type protein than by the R482G or R482T variants. In contrast, the wild-type ABCG2 is practically inactive for rhodamine 123 transport, whereas the R482G and R482T mutants actively extrude this fluorescent dye.

Kinetic Measurements of Hoechst 33342 Transport by wtABCG2 and Its Mutant Variants in Intact Sf9 Cells—The fluorescent dye Hoechst 33342 (Hst) has been described to be a transported substrate of ABCG2 present in mammalian cells (39). Moreover, stem cells expressing human ABCG2 showed a decreased Hoechst 33342 dye accumulation (35). In order to follow the kinetics of Hst dye accumulation in intact, ABCG2-expressing Sf9 cells, we have developed an assay system that allows the continuous detection of dye uptake. Hst dye penetrates cells passively and becomes fluorescent only upon its rapid and strong binding to DNA (34). Thus the increase in fluorescence intensity directly reflects the rate of dye accumulation. Active dye extrusion that prevents the increase of intracellular fluorescence can be continuously monitored in a spectrophotometer. By using the above-described cell preparation and incubation conditions, the Hst dye uptake assay could be applied for assessing the transport activity of the wtABCG2 and its variants in intact Sf9 cells.

Fig. 4A shows a typical Hst dye uptake experiment using intact Sf9 cells harvested post-40 h of recombinant baculovirus infection, expressing either ABCG2-R482G or its K86M mutant variant. After the addition of Hst (indicated by arrows), a fast initial increase of fluorescence intensity due to a rapid dye uptake and nuclear staining in dead cells can be observed. Further dye uptake of living cells, the result of Hst influx and its efflux by ABCG2, is reflected by a slower increase in fluorescence. As documented, Hst dye uptake is significantly slower in the Sf9 cells that express the ABCG2-R482G protein (Fig. 4A, line A) than in cells expressing the K86M/R482G mutant (line B). The latter rate is similar to that in control, β-galactosidase-infected Sf9 cells (not shown). The rate of Hst dye uptake in cells expressing ABCG2-R482G (Fig. 4A, line A) greatly increases upon the addition of the specific inhibitor FTC (or Ko 143, not shown). However, there is no change in the rate of Hst accumulation in cells expressing the K86M/R482G mutant (Fig. 4A, line B) or β-galactosidase (not shown). At the end of each experiment, for quantitation of the maximum cellular fluorescence, the cells were permeabilized by the addition of digitonin, and this maximum uptake was used as a correction factor in the quantitative estimations.

In order to compare the Hst transport activity of the wild-type ABCG2 and its mutant variants, by using the above-described technique, we determined the accumulation rate of 0.5–5 μM Hst into ABCG2-expressing Sf9 cells, both in the presence and in the absence of 10 μM FTC. Fig. 4, B and C, shows that the rate of Hst influx was low and was greatly increased by the inhibitor FTC in the wild-type ABCG2 (Fig. 4B) and also in the R482G and R482T variants (Fig. 4, B and C). The K86M mutant was found to be inactive at all Hst dye concentrations examined (Fig. 4C). Based on these quantitative estimations, we found no significant difference between the Hst transport capacity of the wtABCG2 and its amino acid 482 variants.

Membrane ATPase Activity of the Wild-type ABCG2 and Its Mutant Variants—In order to follow the catalytic rate of the wtABCG2 and its mutant variants, we measured vanadate-sensitive ATP hydrolysis in isolated Sf9 cell membrane preparations. The membrane fractions, containing equal amounts of the different ABCG2 proteins (see Fig. 1B), were assayed for ATPase activity, as described under “Experimental Procedures.”

We have shown earlier (18) that ABCG2-R482G expressed in Sf9 cells had a high level of vanadate-sensitive membrane ATPase activity, and this activity could be significantly increased by substrates and decreased by the inhibitors of this protein. In the present study we have compared the ATPase activity of the wild-type human ABCG2 and its variants (R482G, R482T, and K86M) in the presence and absence of a variety of potential ABCG2 substrates or inhibitors.

As shown in Fig. 5A, the basal, vanadate-sensitive ATPase activity was significantly higher in membranes containing any of the wtABCG2 or its amino acid 482 variants than in those containing ABCG2-K86M/R482G or β-galactosidase (not shown here). Despite the similar ABCG2 expression levels, this
Functional Characterization of ABCG2 Variants

Basal ATPase activity was ~1.5-fold higher in case of the R482G variant (71 ± 10.8 nmol of P_\text{i}/mg of membrane protein/min) than in case of the wild-type ABCG2 or the R482T variant (45 ± 8.85 and 46 ± 9.04 nmol of P_\text{i}/mg of membrane protein/min, respectively) and negligible in the ABCG2-K86M mutant (5 ± 0.5 nmol of P_\text{i}/mg of membrane protein/min). This latter vanadate-sensitive ATPase activity was similar to that observed in the β-galactosidase-containing Sf9 cell membranes (4.2 ± 0.8 nmol of P_\text{i}/mg of protein/min). In wtABCG2 and in R482G and R482T proteins FTC (or Ko 143) powerfully inhibited the vanadate-sensitive ATPase activity.

When examining the substrate stimulation of the ABCG2 ATPase activity, we found that this stimulation was significantly different in the three active proteins (Fig. 5, B–D). In case of the R482G and R482T mutants, vanadate-sensitive ATPase activity could be greatly stimulated by several potential ABCG2 substrates (e.g. prazosin, mitoxantrone, adriamycin, rhodamine 123, benzamil, and camptothecin), corresponding to the transport activity of these proteins. However, in case of the wild-type protein, we found no measurable stimulation of the ATPase activity by any of these compounds, including known wild-type ABCG2 substrates (e.g. mitoxantrone and prazosin). Interestingly, Hoechst 33342, a transported substrate of all active ABCG2 transporters (see above), did not stimulate the ATPase activity of any of these proteins, rather, the addition of Hst to the membranes decreased the ATPase activity in a concentration-dependent manner (see Fig. 5, B–D). It is worthwhile to note that Hst had no effect on the ATPase activity of Sf9 membranes containing ABCG2-K86M or β-galactosidase (data not shown).

We found that the effect of different ABCG2 substrates on the ATPase activity of variants R482G and R482T was almost similar. There was no significant difference either in the relative extent of their drug stimulation or in the drug concentration causing half-maximal activation (K_{act} value). Adriamycin stimulated their ATPase activity about 40%, with K_{act} values of 5 and 6.8 μM, respectively. Rhodamine 123, a specific substrate of the amino acid 482 mutants, gave a maximum of 30% stimulation, and the K_{act} values were 4.5 and 4 μM, respectively, for the R482G and R482T mutants. The maximum extent of stimulation of the R482G and R482T-ATPases by prazosin was 100 and 70%, and the K_{act} values were 7 and 5 μM. Mitoxantrone showed a maximum of 50% stimulation of the ATPase activity of the R482G and R482T variants, with K_{act} values of 1 and 0.8 μM, respectively.

FTC was found to be an effective inhibitor for the vanadate-sensitive ABCG2-ATPase activity both for the wild-type enzyme (76% inhibition at 10 μM) and the R482G variant (74% inhibition at 10 μM); however, its inhibitory effect was smaller and required higher FTC concentrations in the case of the R482T variant (37% inhibition at 10 μM) (see Fig. 5). The concentration of FTC causing half-maximal inhibition of the ABCG2-ATPases was 0.4 μM for the wild-type enzyme, 0.5 μM for the R482G variant, and 0.7 μM for the R482T mutant.

The lack of drug stimulation for the ATPase activity of the wild-type ABCG2 was surprising in the light of its high efflux mutants. Sf9 cells were incubated for 30 min at 37 °C with (−) or without (−) the addition of 10 μM MX, 10 μM MX + 10 μM FTC (−), or 10 μM MX + 10 μM prazosin (−). After incubation, the cells were washed and suspended in an ice-cold buffer containing propidium iodide, for the recognition of dead cells. Flow cytometry was performed as described under “Experimental Procedures.” B and C, concentration dependence of MX accumulation in Sf9 cells, expressing wtABCG2 and its mutant variants. The experiments were performed as described at A, by using 1–20 μM MX. Intracellular mean fluorescence values, obtained as a function of MX concentration during the uptake experiment, are shown. The figure shows the average ± S.D. of three measurements.
Procedures.

as described under dead cells. Flow cytometry was performed with propidium iodide, for the recognition of pended in an ice-cold buffer containing incubation the cells were washed and sus-

(- - -) or absence (---) of 10 μM FTC. After incubation the cells were washed and sus-
pended in an ice-cold buffer containing

Fig. 3. Rhodamine 123 accumulation in Sf9 cells expressing the wild-type (Arg-482), R482G, R482T, or K86M/R482G variants of ABCG2. Sf9 cells were incubated for 30 min at 37 °C with 2 μM rhodamine 123 in the presence (---) or absence (---) of 10 μM FTC. After incubation the cells were washed and sus-
pended in an ice-cold buffer containing propidium iodide, for the recognition of dead cells. Flow cytometry was performed as described under “Experimental Procedures.”

ciency drug and dye extrusion present in the intact Sf9 cells. We speculate that this may be the result of unknown ABCG2 substrates, present in the membrane preparations or ATPase assay media. In order to explore these possibilities, we have examined the effects of salts and buffering solution, antioxidants (e.g. dithiothreitol), as well as protease inhibitors present in the membrane preparations and the ATPase assay. We found no significant effect of any of these conditions on the ATPase activity of the wild-type ABCG2 or the R482G variant; neither their basal activity nor the effects of drugs were changed. Washing the membranes with KCl (0.1 M) or albu-
mamin (2% bovine serum albumin) also had no significant effect on the ABCG2-ATPase or its drug stimulation (data not shown).

([α-32P]8-azido-ATP Binding and Nucleotide Trapping by wtABCG2 and Its Mutant Variants in Isolated Sf9 Cell Membranes—It has been reported (28) that the steps of the catalytic cycle (ATP binding and the transition state formation during ATP hydrolysis) of various ABC proteins can be investigated by using the radioactive ATP analog 8-azido-[α-32P]ATP in a complex with Mg2+, Co2+, or Mn2+ cations. ATP binding can be followed by labeling under non-hydrolytic conditions, whereas the formation of an inhibitor (vanadate and fluoro-aluminate)-sensitive reaction intermediate is examined under hydrolytic, “nucleotide trapping” conditions (26–29). In order to establish proper labeling conditions, we tested Mg-8-azido-ATP in the membrane ATPase activity measurement, and we found that it is a good energy donor substrate for the ABCG2 ATPase. In the presence of 3.1 mM Mg-8-azido-ATP, the vanadate-sensitive ATPase activity of ABCG2-R482G was 77% compared with the activity measured in the presence of a similar concentration of MgATP (data not shown).

To characterize the ATP binding activity of the wild-type and mutant ABCG2 proteins, we performed photoaffinity labeling in isolated Sf9 membranes. Membranes containing the different ABCG2 variants were incubated on ice with 20 μM 8-azido-[α-32P]ATP and either 2 mM MgCl2 or 2 mM CoSO4 for 5 min. After UV irradiation, washing, gel electrophoresis, and electro-

blotting, the radioactivity in the ABCG2 protein bands (con-

firmed by immunoblotting) was quantitated.

As shown in Fig. 6A, we found that there was no significant ATP binding to ABCG2 when an Mg2+-complex of azido-ATP was used. However, the addition of Co2+ to the media resulted in highly detectable 8-azido-ATP binding to ABCG2 (Fig. 6B), which was competed in the presence of 8 mM unlabeled CoATP. Therefore, in further labeling experiments we used the cobalt complex of 8-azido-[α-32P]ATP. We found that 8-azido-ATP binding was similar in the wild-type and in the R482G, R482T, and K86M mutant variants under these conditions (Fig. 6B). ATP binding was abolished in all variants in the presence of EGTA (chelating all divalent cations used here, not shown).

In order to analyze the transition state formation of the ABCG2 transporters, Sf9 membranes containing the different ABCG2 variants were incubated at 37 °C for 2–5 min in the presence of 5 μM 8-azido-[α-32P]ATP, either 2 mM MgSO4 or CoSO4, and with or without 1 mM sodium orthovanadate. Again, there was no significant labeling of ABCG2 proteins when Mg2+ was used in these experiments (see Fig. 7A), neither in the absence or presence of vanadate nor in the presence of 1 mM AlF4− or 1 mM BeF4− used as trapping anions (data not shown).

As shown in Fig. 7B, in the presence of 1 mM vanadate and 5 μM Co-8-azido-[α-32P]ATP, ABCG2 (R482G) showed a high level of nucleotide trapping (lane 3). The radioactive band, corresponding to the nucleotide trapping by the ABCG2 pro-

tein, was specific for this protein (confirmed by immunoblot-

ting), and there was no nucleotide occlusion observed when the reaction mixture did not contain sodium orthovanadate (Fig. 7B, lane 1) or when 4 mM Na-EDTA was present in the reaction mixture (lane 2). The ABCG2-specific radioactive band was absent in the membranes of β-galactosidase-expressing Sf9 cells (Fig. 7B, lane 4). As documented in Fig. 7B, the function-

ally inactive ABCG2-K86M/R482G did not show any nucleotide trapping activity under these conditions (lane 5).

It has been shown in the case of well characterized ABC multidrug transporters, MDR1 and MRP1, that transported drugs significantly increase the rate of nucleotide trapping (26, 29). In order to explore the effect of drugs on the nucleotide
trapping in the wtABCG2 and its amino acid 482 variants, we examined such effects in the above-described conditions and in the presence of various concentrations of possible ABCG2 substrates.

As shown in Fig. 8, we found significant differences in the adenine nucleotide trapping of the wild-type, R482G, and R482T ABCG2 transporters. When we analyzed these data with a quantitative PhosphorImager, in the absence of added drugs the wild-type ABCG2 (Fig. 8, lane 4) showed a more pronounced nucleotide trapping activity, $2.6 \pm 0.03$- and $2.05 \pm 0.5$-fold of the R482G and R482T variants (lanes 7 and 10), respectively. The addition of prazosin, an activator of the ABCG2-ATPase of the R482G and R482T variants, significantly stimulated nucleotide trapping in the same variants (see Fig. 8, lanes 6 and 9, the stimulations were $2.0 \pm 0.22$- and $1.7 \pm 0.04$-fold, respectively). However, prazosin had an inhibitory effect ($50 \pm 6\%$ decrease in the labeling) on the nucleotide incorporation in the wild-type ABCG2 (lane 3). Mitoxantrone, also a substrate of all three active ABCG2 transporters, had a similar effect on the nucleotide trapping of the ABCG2 variants (not shown). The specific ABCG2 inhibitor, FTC, eliminated nucleotide trapping by all ABCG2 variants (Fig. 8, lanes 2, 5, and 8). Verapamil is not a substrate of the ABCG2 multidrug transporters, and this compound had no effect on the trapping of the active protein variants (not shown). We confirmed by immunoblotting that the amount of ABCG2 proteins used in the labeling experiments were approximately equal (see Fig. 8). As a further control, the experiments were repeated three times, and similar results were found when both 2 or 5 $\mu M$ 8-azido-ATP was used.

**DISCUSSION**

In this paper we describe the expression and detailed functional analysis of the wild-type human ABCG2 multidrug transporter and its mutant variants R482G, R482T, and K86M. This protein has been demonstrated to provide drug resistance in tumor cells by an active extrusion of various cytotoxic compounds (5, 16). ABCG2, which may also have important physiological functions, for example, providing xenobiotic resistance in the placenta and stem cells (3, 35, and 39), is a so-called ABC half-transporter, which most probably requires dimerization partner(s) for its transport activity (14). Three human ABCG2 protein sequences have been published (3–5), which differed in their amino acid position 482. According to the established sequence in the human genome data base (GenBank™ accession number AF103796), the wild-type ABCG2 contains arginine at this position, whereas the other two variants R482G and R482T most probably were generated during drug selection in tumor cell lines (20, 22).

In a previous analysis, Honjo et al. (20) described significant differences in the substrate specificity of the three different ABCG2 proteins found in drug-selected cell lines or transiently expressed in mammalian cells. However, the corresponding results in different publications were somewhat inconsistent, for example MX resistance was almost absent in cells expressing the confirmed wild-type ABCG2 in one study (22), whereas such a resistance and active MX extrusion were found to exist in another study (20). Relatively low expression levels, detected for ABCG2 in mammalian cells, hindered a detailed biochemical characterization, and the possible involvement of endogenous ABCG2-related proteins in these cells may significantly modify the transport function of this dimerizing protein.

The aim of the present study was to provide a quantitative characterization both for the transport and the ATP hydrolytic activity of the wtABCG2 and its variants R482G and R482T. For this purpose we applied the Sf9-baculovirus expression system, which has already been established as a suitable system for the expression of ABC transporters (23–25), including ABCG2 (18).

As documented under “Results,” we obtained a uniformly high level expression of the wtABCG2 and its amino acid 482 variants, and as a negative control also expressed a Walker A Lys mutant of this protein (K86M). We introduced the K86M mutation into the R482G variant of ABCG2, because we expected that the mutation of the key Lys will abolish the function of ABCG2 regardless the amino acid found in position 482. Similar mutations, even if present only in one catalytic domain,
ATPase activity measured in membranes of Sf9 cells expressing the wild-type, R482G, R482T, or K86M/R482G variants of human ABCG2. ATPase activity of isolated Sf9 membranes was determined by measuring vanadate-sensitive inorganic phosphate liberation, using 3.3 mM MgATP. Data points represent the mean ± S.D. values of at least four measurements, performed in two or three different membrane preparations. A, ATPase activity in the absence of added compounds (control), with 100 μM prazosin, or with 10 μM FTC. B–D, effects of different concentrations of compounds on the vanadate-sensitive ATPase activity in isolated membranes of Sf9 cells expressing different ABCG2 variants. ATPase activity determined in membranes of wtABCG2- (B), R482G- (C), or R482T (D)-expressing Sf9 cells. Control indicates the ATPase activity measured in the absence of added compounds. The background ATPase activity measured in Sf9 cells expressing β-galactosidase is indicated on the figures with dashed lines.
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**Fig. 6.** [α-32P]-8-azido-ATP binding of the wild-type and R482G, R482T, or K86M/R482G mutant ABCG2 proteins. SF9 membranes containing the different ABCG2 variants or β-galactosidase were incubated on ice for 5 min with 20 μM 8-azido-[α-32P]ATP and 2 mM Mg2+(A) or 2 mM Co2+(B). Labeling and detection were performed as described under “Experimental Procedures.” The position of human ABCG2 is indicated by an arrow. β-gal, β-galactosidase.

**Fig. 7.** [α-32P]-8-azido-ATP trapping of the R482G and K86M/R482G mutant ABCG2 proteins. SF9 membranes containing ABCG2-R482G (see A, lane 2, and B, lanes 1–3), ABCG2-K86M (B, lane 5), or β-galactosidase (β-gal) (see A, lane 1 and B, lane 4) were incubated for 5 min at 37°C with 5 μM 8-azido-[α-32P]ATP, 1 mM sodium orthovanadate (except for lane 1) and 2 mM Mg2+(A) or 2 mM Co2+(B) as described under “Experimental Procedures.” Lane 2 represents when 4 mM NaEDTA was added to the reaction. Labeling and detection were performed as described under “Experimental Procedures.” The position of the ABCG2 multidrug transporter is indicated by an arrow.

inactivated all known ABC multidrug transporter proteins examined so far (32, 40, 41). The K86M mutant of ABCG2/R482G was already investigated for Hoechst 33342 transport in mammalian cells (35), and it was found to be inactive. On the other hand the R482G variant could be easily investigated in all four assays as described under “Results.”

In order to follow the transport activity and the molecular mechanism of action of the wtABCG2 and its mutant variants, we developed suitable assays. First, we showed that intact SF9 cells expressing the different ABCG2 transporters can be used for measuring the uptake of the fluorescent drug MX and the fluorescent dye rhodamine 123 by flow cytometry. Second, we developed a quantitative assay for ABCG2-dependent Hst extrusion to follow the kinetics of Hoechst 33342 (Hst) dye uptake, a known substrate of the MDR1 and ABCG2 multidrug transporters (35, 39, 42). Finally, in order to trace the catalytic steps related to the transport activity of ABCG2, we utilized isolated SF9 cell membranes, and we found the appropriate conditions to detect the specific ATP-binding and adenine nucleotide trapping that is characteristic of many related ABC transporters.

Comparing the transport activity of the wtABCG2 and its mutant variants, we found that the wild-type and the two amino acid 482 variants actively exported mitoxantrone, whereas rhodamine 123 extrusion could only be observed in cells expressing the R482G and R482T proteins. We found that wild-type ABCG2, although expressed at similar levels in the same cell type, was somewhat less effective in MX extrusion than the other two amino acid 482 variants. Our MX-transport results are in accordance with data presented by Honjo et al. (20), whereas they contradict the lack of MX transport activity found for the wild-type ABCG2 by Komatani et al. (12). When measuring the Hst transport activity of the different ABCG2 proteins in intact cells, we found that there was no significant difference in the Hst transport capacity of the wtABCG2 and its amino acid 482 variants. This study provides the first comparative data regarding the Hst transport activity of different ABCG2 variants and the MX-transport capacity of these variants expressed in the same amount in the same cell type. Our direct transport experiments fully support that ABCG2 forms an active homodimer.

When investigating the catalytic cycle of the human ABCG2 protein, we compared the vanadate-sensitive ATPase activities of the wtABCG2 and its mutant variants and the possible modulation of this activity by transported substrates and inhibitors. We have already described a high level, vanadate-sensitive ATPase activity, stimulated by several transported compounds, for the human ABCG2-R482G variant in isolated SF9 cell membranes (18). In the present experiments a relatively high basal ATPase activity was found in case of the wild-type ABCG2 and the R482T variant, too. However, stimulation of the ABCG2-ATPase activity was observed only in SF9 membranes containing the R482G or R482T variants and not the wild-type ABCG2.
Despite this difference, similarities were found for the wtABCG2 and its amino acid 482 variants. The selective transport inhibitors, FTC and Ko 143, inhibited the ATPase activity of all these proteins. Also, Hoechst 33342, a transported ABCG2 substrate, did not stimulate ATPase activity but produced a concentration-dependent inhibition of the ATPase activity both of the wild-type and the ABCG2–482 mutants.

A possible explanation for the above described findings in the ATPase activity measurements may be a partial and variable uncoupling of the ABCG2-ATPase activity from the transport activity in these isolated membranes. However, this would make it difficult to explain how the substrate drugs and inhibitors reduce this ATPase activity in a concentration-dependent manner. Alternatively, and more likely, a significant stimulation of the ABCG2-ATPase can be caused by (yet unknown) substrates present in these membrane preparations. In case of the R482G and R482T variants, this endogenous stimulation (caused by endogenous activators, producing what we observe as a high base-line ATPase) is only partial, and further stimulation by the transported compounds is observed. In case of the wild-type ABCG2, endogenous stimulation reaches a maximum level; therefore, upon addition of substrates either no effect or only the inhibition of the ATPase activity is seen when the transported substrates compete with the endogenous activators. A third possibility is that human ABCG2 expressed in insect cells could be lacking post-translational modifications (e.g. glycosylation and phosphorylation) necessary for complete function. However, the results of our transport experiments did not indicate an altered function of the wild-type ABCG2 expressed in insect cells. Additional experiments, involving mammalian cell expression systems, will be needed to explore these issues further.

In order to investigate further the mechanistic properties of the wtABCG2 transporter and its variants, we have studied both specific ATP binding and nucleotide trapping of these proteins. We used 8-azido-ATP and found that neither ATP binding nor nucleotide trapping could be observed in ABCG2 in the absence of divalent cations or in the presence of Mg2+ ions. However, significant nucleotide binding and trapping could be detected in the presence of Co-8-azido-ATP. The requirement of Co-8-azido-ATP for labeling may be the result of a spatial arrangement of the reactive groups in the ABCG2 protein, allowing its covalent interaction with only Co-8-azido-ATP. A Co-ATP complex has already been used in studying the MDR1-ATPase reaction. In that case both Mg2+ and Co2+ ions were found to be suitable to obtain a trapped nucleotide, although an increased stability (and decreased hydrolysis) of the Co-ATP complex was observed (28). The use of Co-ATP complex in the present studies opened the door for a detailed characterization of the ATP hydrolytic cycle of the ABCG2 protein.

We have documented that the 8-azido-ATP labeling of the wtABCG2 and its amino acid 482 variants and that of the K86M mutant was similar, which is to say that they seem to have similar ATP binding capacities. However, we found significant differences in the vanadate-dependent nucleotide trapping, reflecting the transition state intermediate formation of the wtABCG2 and its variants. Clearly, the R86M mutant was unable to form the transition state intermediate, in agreement with the inactivity of this mutant in the ATPase and transport measurements. The formation of a similar lysine residue in a single ABC domain of MDR1 or MRPI fully inhibits their ATP hydrolysis and vanadate-induced transition state formation (32, 40, 41).

We found that the nucleotide trapping characteristics of the R482G or R482T variants were different from the wild-type ABCG2. Whereas this transition state formation in variants R482G and R482T could be significantly stimulated by various ABCG2 substrates (prazosin and mitoxantrone), the transition state formation of the wild-type ABCG2 could not be stimulated but rather was inhibited by these compounds. In all cases transition state formation was eliminated by the specific inhibitor, FTC. These results are in close correlation with the ATPase activity data; both the drug stimulation of the ABCG2 ATPase activity and nucleotide trapping showed similar behavior in the ABCG2 variants. The possible sources of these findings have already been discussed above.

It is important to note that the inhibitory effect of FTC was almost 100% in transport and trapping experiments; however, in the ATPase assay, FTC inhibited the activities only partially. The mechanism of action of FTC is as yet unknown, but our nucleotide trapping experiments indicate that it decreases the formation of the transition state intermediate. The assays described in this paper use varying conditions (e.g. different assay media, Mg- or Co-ATP, ATP, or 8-azido-ATP) and investigate different aspects of the activity of ABCG2, making it difficult to compare the effect of FTC on the different functions examined.

In summary, in this study we have documented the applicability of the Sf9 expression system for a direct, quantitative examination of the transport properties of wtABCG2 and its variants. Our results support the idea that ABCG2 functions as a homodimer and show that this heterologous expression system provides an appropriate tool for selectively studying ABCG2 mutants.

We demonstrated key differences in the substrate interactions of these variants in intact cells, which were also reflected when studying the steps of ATP hydrolysis. However, in several cases the substrate stimulation of the ABCG2-ATPase could not be directly correlated with the actual transport processes. Therefore, these results raise the possibility that membrane-bound substrates or partial uncoupling may significantly and differently influence the catalytic mechanism of the amino acid 482 variants of this ABC half-transporter. The detailed investigation of these phenomena in various expression systems may help in explaining these discrepancies.

Altogether, the transport methods (especially the fluorimetric dye uptake assay) described here for intact Sf9 cells expressing human ABC transporters and the ATPase assay may serve as an important basis for developing high throughput methods for the evaluation of drug interactions with multidrug transporters.

The significant variations found in the substrate handling of the wtABCG2 and its mutant variants and the better understanding of their effects on the function of ABCG2 may play an important role in modulating chemotherapy in ABCG2-expressing tumors. In addition, as we have recently shown,2 the mutant ABCG2 variants with altered substrate specificity may be successfully applied as drug-specific selectable markers in human gene therapy approaches.

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