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Functional Characterization of the Human Multidrug Transporter, ABCG2, Expressed in Insect Cells

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ABCG2 (also called MXR (3), BCRP (4), or ABCP (5)) is a recently-identified ABC half-transporter, which causes multidrug resistance in cancer. Here we report that the expression of the ABCG2 protein in Sf9 insect cells resulted in a high-capacity, vanadate-sensitive ATPase activity in isolated membrane preparations. ABCG2 was expressed underglycosylated, and its ATPase activity was stimulated by daunorubicin, doxorubicin, mitoxantrone, prazosin and rhodamine 123, compounds known to be transported by this protein. ABCG2-ATPase was inhibited by low concentrations of Na-orthovanadate, N-ethylmaleimide and cyclosporin A. Verapamil had no effect, while Fumitremorgin C, reversing ABCG2-dependent cancer drug resistance, strongly inhibited this ATPase activity. The functional expression of ABCG2 in this heterologous system indicates that no additional partner protein is required for the activity of this multidrug transporter, probably working as a homodimer. We suggest that the Sf9 cell membrane ATPase system is an efficient tool for examining the interactions of ABCG2 with pharmacological agents. © 2001 Academic Press

Key Words: **multidrug resistance; ABC half-transporter; ABCG2; MXR; BCRP; Sf9 cells; drug-stimulated ATPase activity.**

Abbreviations used: MXR, mitoxantrone resistance-associated protein; BCRP, breast cancer resistance protein; ABCP, placenta specific ABC transporter; ABC, ATP binding cassette; Sf9 cells, *Spodoptera frugiperda* ovarian cells; MDR1, multidrug resistance protein; MRP1, multidrug resistance-associated protein; TMD, transmembrane domain; TAP, transporter associated with antigen processing; Calcein-AM, calcein acetoxy-methylesther; NEM, *N*-ethylmaleimide; MX, mitoxantrone; FTC, fumitremorgin C; CsA, cyclosporin A.

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The multidrug resistant phenotype of malignant cells is a major obstacle in the efficient chemotherapy of patients suffering from cancer. It has been convincingly documented that several ABC proteins can cause drug resistance in cancer cells by actively extruding the clinically applied chemotherapeutic compounds. Two major groups of cancer multidrug resistance ABC transporters, the homologs of the MDR1/P-glycoprotein, as well as members of the MRP (multidrug resistance protein) family, have already been characterized in detail. Their membrane topology and basic mechanism of action has been explored in a variety of biochemical and cell biology studies (see 1, 2).

An ABC protein, causing cancer multidrug resistance, but having a different molecular architecture, has recently been identified. This protein is the product of the ABCG2 gene, and was named MXR (Mitoxantrone Resistance-associated protein (3)), BCRP (Breast Cancer Resistance Protein (4)) or ABCP (Placenta specific ABC transporter (5)) in the original descriptions. In accordance with the current ABC protein nomenclature, we use the term ABCG2 throughout this report.

The overexpression of ABCG2 has been detected in various cell types, showing multiple drug resistance without the expression of MDR1/Pgp or MRPs. These include cells derived from breast cancer (3, 6, 7), ovarian carcinoma (8), colon carcinoma (6), leukemia (9), and Ehrlich ascites tumor (10). It has also been shown that ABCG2 is overexpressed in the placenta (5). Data in the literature indicate that ABCG2 confers multidrug resistance by actively extruding cytotoxic compounds from the cells in which it is overexpressed (4, 11).

Characteristic protein domains in the ABC transporter family include the cytoplasmic ATP Binding Cassette (ABC) regions and six-helix transmembrane

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domains. In general, the ABC domains are responsible for nucleotide binding and hydrolysis, while the transmembrane domains (TMD) have been suggested to play a key role in the interaction with the transported substrates. In all ABC transporters studied so far, the interaction of at least two ABC units were required to obtain a functional transporter. The human MDR- and MRP-type proteins contain a tandem repeat of TMD + ABC domains within one polypeptide chain, both ABC units being C-terminally located from the TMDs (for review see 12).

ABCG2, a 655-amino acid membrane protein, belongs to the ABCG/white subfamily. Members of this subfamily contain only one ABC and one TMD unit within one polypeptide, the ABC domain being N-terminally located from the TMD (see Fig. 1A). Proteins containing only one ABC and TMD domain are called ABC half-transporters, as most probably they can function only as dimers or oligomers. A homolog of the ABCG2 protein, the product of the *Drosophila white* gene forms a heterodimer with one of its homologs, *brown* or *scarlet*, and the different dimers transport different eye pigment precursor molecules (13). Thus it is most likely that these proteins function as obligate heterodimers. Other ABC half-transporters, although with a different ABC + TMD arrangement, like TAP1 and TAP2, were also demonstrated to act as heterodimers, when functioning as peptide translocators (15).

In the case of the ABCG2 protein previous studies have suggested the formation of an active homodimer. Experiments in ABCG2-overexpressing multidrug-resistant cells showed an exclusive amplification of the ABCG2 gene (16), and a human breast cancer cell line became resistant upon transfection with solely the ABCG2 cDNA (4, 11, 28). However, ABCG2 expression level in mammalian cells is relatively low, and these cells may contain additional ABC half-transporter proteins, forming a functional heterodimer with ABCG2.

In order to characterize the function of ABCG2 and to examine the question of its homo- or heterodimerization, we used a heterologous expression system. Here we provide evidence that the human ABCG2, when expressed in Sf9 insect cells, shows a membrane ATPase activity with similar substrate stimulation specificity and inhibitor sensitivity to those of the ABCG2 protein expressed in mammalian cells. These results indicate that ABCG2 can function as a homodimer. Also, we suggest that the Sf9 cell expression system provides an efficient research tool for the functional investigation of ABCG2.

MATERIALS AND METHODS

Materials. Daunorubicin, doxorubicin, prazosin, rhodamine 123, cyclosporin A, calcein-AM, verapamil, Na-orthovanadate and NEM were from Sigma; mitoxantrone was obtained from Lederle Laboratories. Fumitremorgin C was provided by Dr. Lee M. Greenberger (Wyeth-Ayerst Research).

Generation of the human ABCG2 containing transfer vector. pAcUW21-L/ABCG2 was constructed by removing the full-length human ABCG2 cDNA (G482 variant) (3) from pcDNA3.1(−)/ABCG2 with *Sac*I digestion, and ligating the resulting fragment to the SacI site of the modified baculovirus transfer vector, pAcUW21-L (17).

Generation of recombinant baculoviruses. Recombinant baculoviruses, carrying the human ABCG2 cDNA were generated with the BaculoGold Transfection Kit (Pharmingene), according to the manufacturer's suggestions. Sf9 cells were infected and cultured as described (18). Individual clones expressing high levels of the human ABCG2 were obtained by end-point dilution and subsequent amplification. The clone producing the highest yield of the ABCG2 protein was selected by immunoblotting (see below). Sf9 cell membranes were isolated as described (19).

Cell culturing and tunicamycin treatment. MCF-7/MX cells (6) were cultured in IMEM medium, supplemented with 10% fetal calf serum, 50 U/ml penicyllin and streptomycin, and 10 nM mitoxantrone, at 37°C in 5% CO_2. For inhibition of N-glycosylation, MCF-7/MX cells were grown for 60 h in a medium containing 5 μg/ml tunicamycin.

Membrane preparation and immunoblotting. Three days after virus transfection, the Sf9 cells were harvested, their membranes were isolated, and the membrane protein concentrations were determined by the modified Lowry method. Proteins of isolated Sf9 membranes were separated on 10% Laemmli-type SDS-gels and the proteins were electroblotted onto PVDF membranes. Immunoblotting was performed as described earlier (18), by using the anti-MXR 405 polyclonal antibody (11), in 2000× dilution, and an anti-rabbit HRP-conjugated secondary antibody (10,000× dilution, Jackson Immunoresearch). HRP-dependent luminescence was developed by the enhanced chemiluminescence technique (ECL, Amersham).

ATPase activity measurements. Membrane ATPase activity was measured by colorimetric detection of inorganic phosphate liberation as described (19), with minor modifications. The reaction mixture contained 40 mM Mops–Tris (pH 7.0), 50 mM KCl, 2 mM dithiothreitol, 500 μM EGTA–Tris, 5 mM Na-azide, 1 mM oubain and 5–20 μg membrane protein. The reaction was started with addition of 3.3 mM MgATP. The vanadate sensitive fraction was determined in the presence of 1 mM Na-orthovanadate.

RESULTS AND DISCUSSION

Expression of ABCG2 in Sf9 cells. We have cloned the ABCG2 cDNA into a baculovirus vector and infected Sf9 insect cells with the recombinant virus. The immunoblot presented in Fig. 1B demonstrates that the ABCG2 protein was efficiently expressed in the baculovirus-infected Sf9 cells. The expression level of ABCG2, as recognized by the MXR-specific antibody, in Sf9 cells (lane 3) was found to be approximately ten times higher than that in the MCF-7/MX mitoxantrone-selected, highly multidrug resistant (6, 20) breast cancer cells (lane 1). The doublet protein bands, corresponding to the immunoreactive ABCG2 in the Sf9 cell membranes, were also visible by Coomassie blue staining of the gels (not shown).

As shown in Fig. 1B, ABCG2 expressed in Sf9 cells migrated at a lower apparent molecular mass (as a doublet around 60 kDa) than the ABCG2 in MCF-7/MX cells (a wide band at about 70 kDa). Membrane proteins expressed in insect cells are underglycosylated

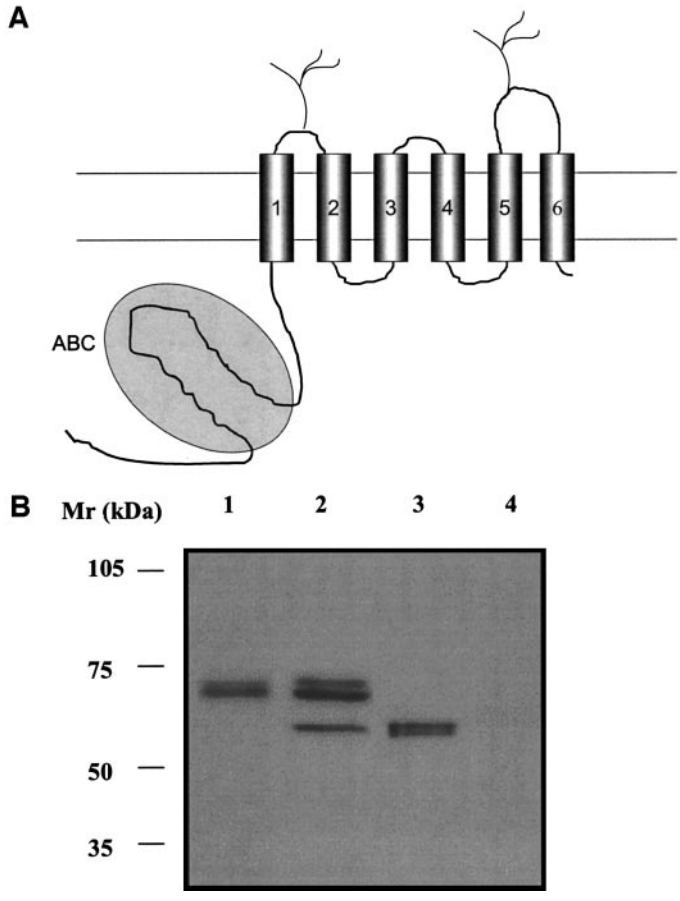

FIG. 1. (A) Membrane topology model of ABCG2. The numbers indicate the predicted transmembrane helices. Predicted N-glycosylation sites are also indicated. (B) Immunoblot detection of the ABCG2-multidrug transporter expressed in Sf9 insect cells. Whole cell lysates, dissolved in dissagregation buffer, were subjected to electrophoresis on 10% Laemmli-type gels and blotted to PVFD membranes, followed by immunodetection with anti-MXR 405 antibody, as described under Materials and Methods. Lane 1, MCF-7/MX, 10 μg; lane 2, MCF-7/MX treated with 5 μg/ml tunicamycin, 15 μg; lane 3, ABCG2-expressing Sf9 cells, 1 μg; lane 4, β-galactosidase-expressing Sf9 cells, 10 μg.

(see 21, 22), which could explain the lower molecular mass of the Sf9-expressed ABCG2-protein. In order to examine this possibility, we have cultured MCF-7/MX cells in the presence of 5 μg/ml tunicamycin, a known inhibitor of N-glycosylation. In samples obtained from tunicamycin treated MCF-7/MX cells (Fig. 1B, lane 2) two different faster migrating forms of ABCG2 were observed, and the non-glycosylated ABCG2 migrated with a similar apparent molecular mass as the Sf9-expressed protein. In experiments not shown here, the isolated MCF-7/MX cell membranes were treated with *N*-glycosydase. In this case a strong reduction in the apparent molecular mass of the ABCG2 protein was observed, and the deglycosylated protein comigrated with ABCG2, expressed in Sf9 cells (Litman *et al.*, manuscript in preparation).

The membrane topology model of ABCG2 (Fig. 1A, see Refs. 3, 5) predicts two possible N-glycosylation sites. All the above data suggest that both glycosylation sites are active in the mammalian cells, while only a partial, core glycosylation of the ABCG2 is performed by the Sf9 cells. The doublet bands on Fig. 1B, lane 3 probably represent the non-glycosylated and the core-glycosylated forms of ABCG2, respectively, in Sf9 cells. Glycosylation may have a role in the routing or processing of ABCG2. However, it has been convincingly demonstrated that the function of the ABC multidrug transporters MDR1 and MRP1 are unaffected by glycosylation (23, 24). Also, several studies documented the expression of human ABC-transporters in insect cells in a biologically active form, in spite of the lack of their glycosylation (19, 21, 22).

ABCG2-dependent ATPase activity and its modulation in isolated Sf9 cell membranes. Multidrug resistance ABC transporters utilize the energy of ATP for their drug transport activity. In the case of MDR1 and MRP proteins both their drug transport activity and the related ATP cleavage are inhibited by Na-orthovanadate, and by SH-group modifying agents, like N-ethylmaleimide (NEM). The function of these transporters, when expressed in Sf9 cells, has been successfully studied by measuring their vanadate-inhibited and substrate-stimulated ATPase activity (19, 26, 27). A membrane ATPase activity, related to the overexpression of ABCG2 in mammalian cells, has already been demonstrated (10).

As shown in Fig. 2A, when ABCG2 was expressed in Sf9 cells, in the isolated membranes we observed the appearance of a high capacity (about 70 nanomoles/mg protein/min), vanadate-sensitive ATPase activity. Such an ATPase activity was absent in control, β-galactosidase expressing membranes, while the vanadate-insensitive membrane ATPase had a similar low-level as found in the control membranes (not shown). Vanadate-inhibition of the ATPase activity occurred with a K_i value of about 20 μM Na-orthovanadate. NEM also inhibited the ATPase activity at micromolar concentrations (K_i NEM was 10 μM, data not shown in detail). The MgATP concentration producing half-maximum membrane ATPase activity was 0.3 mM (see below). All these values for the ABCG2-ATPase are in a similar range as those measured earlier for the MDR1-ATPase activity (18, 19, 32).

In the following experiments, in order to characterize the ATPase activity produced by ABCG2 expression, we examined the effects of several, previously described ABCG2 substrates and inhibitors. We have also compared the ATPase activity of ABCG2-containing Sf9 membranes with those containing the human MDR1 protein.

As shown in Fig. 2A, ABCG2-containing membranes had a basal ATPase activity of about 3–5 times higher

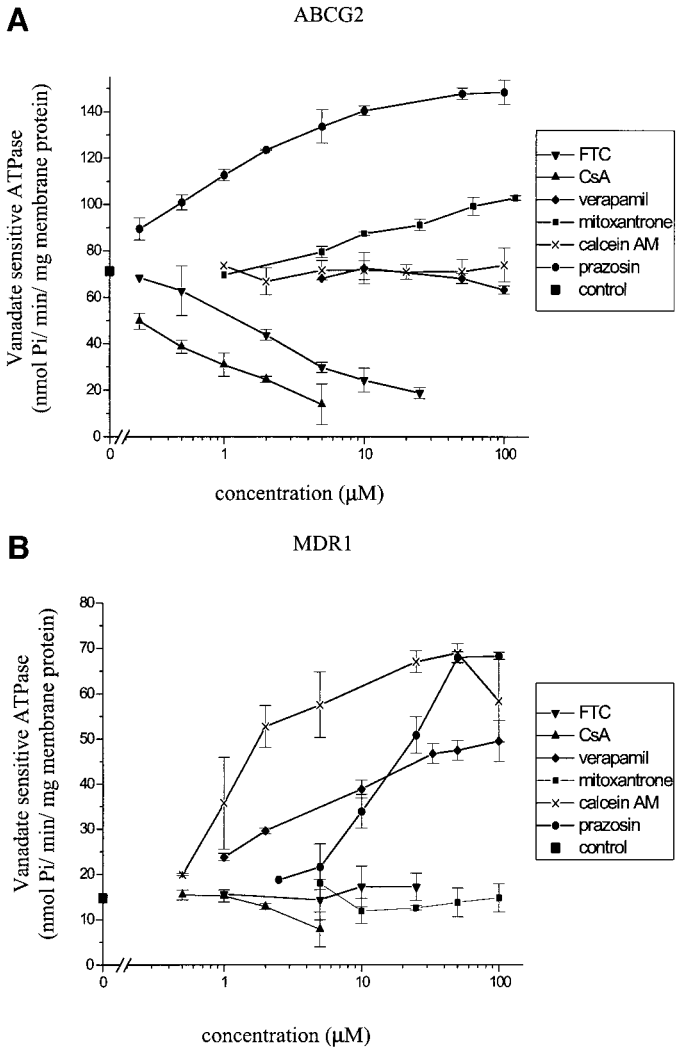

FIG. 2. Comparison of the effects of various compounds on the vanadate sensitive ATPase activity in isolated Sf9 membranes of ABCG2 (A) or MDR1 (B) expressing Sf9 cells. ATPase activity of isolated Sf9 membranes was determined by measuring vanadate sensitive inorganic phosphate liberation, using 3.3 mM MgATP, as described under Materials and Methods. Data points indicate the mean ± SD values of at least four measurements, performed in two or three different membrane preparations. Control values show the activity measured in the absence of added compounds.

than that seen in the MDR1-containing membranes (Fig. 2B). Addition of mitoxantrone (MX), a well established substrate drug for ABCG2 (3, 6, 7, 10) stimulated the ABCG2-ATPase activity in a concentration dependent manner. In cancer cells MDR1 expression did not evoke significant mitoxantrone resistance (30) and, indeed, mitoxantrone had no significant effect on the MDR1-ATPase activity (Fig. 2B). Prazosin, a vasodilatator agent, has been shown to be actively extruded from various multidrug resistant cells (11, 31). As shown in Fig. 2, prazosin significantly stimulated the ATPase activity of both ABCG2 and MDR1, although the K_{act} value of prazosin in the case of ABCG2 was about 1 μM, while this value in the case of MDR1 was an order of magnitude higher (about 15 μM).

Verapamil has been shown to be an excellent substrate of MDR1, and it significantly stimulates the MDR1-ATPase activity (19). In contrast, the multidrug resistance caused by ABCG2 expression was reported to be only slightly sensitive to verapamil (10, 28). In the present experiments we found no verapamil stimulation of the ABCG2-ATPase activity (only a slight inhibition was observed at higher verapamil concentrations, see Fig. 2A). In contrast, as also documented earlier, we observed a 3.3-fold stimulation of the MDR1-ATPase by low concentrations of verapamil (Fig. 2B).

Calcein-AM is an excellent MDR1 substrate (32) and in the present experiments it stimulated the Sf9 membrane MDR1-ATPase 4.5-fold, with a K_{act} of about 1 μM. In contrast, as shown in Fig. 2A, Calcein-AM had no effect on the ABCG2-ATPase activity. This latter finding is in accordance with results showing no measurable Calcein-AM extrusion from ABCG2 overexpressing, drug-resistant cells (11).

Fumitremorgin C, a fungicide, was described as a powerful inhibitor of the ABCG2-mediated multidrug resistance (28) or ATPase activity (Robey *et al., Biochim. Biophys. Acta*, in press). As shown in Fig. 2, in isolated Sf9 cell membranes Fumitremorgin C strongly inhibited the ABCG2-ATPase, while it had no significant effect either on the basal or the verapamil (33 μM) stimulated MDR1-ATPase activity. Cyclosporin A (CsA) has been shown to act only as a weak inhibitor of ABCG2-dependent drug resistance (4) but decreased the ATPase activity measured in an ABCG2-overexpressing mammalian cell line (10). In the present study we found that CsA inhibited both the ABCG2- and the MDR1-ATPase (see Table I and below). The

TABLE I

Effects of Different Drugs on the Vanadate Sensitive ATPase Activity in Membranes of ABCG2-Expressing Sf9 Cells

Compound	K_{act} (μM)	K_i (μM)	Maximum stimulation (%)[a]	Maximum inhibition (%)[a]
Mitoxantrone	7	—	40	—
Prazosin	1	—	100	—
Doxorubicin	5	—	30	—
Daunorubicin	2.5	—	50	—
Rhodamine 123	4.5	—	20	—
Cyclosporin A	—	0.5	—	80
Fumitremorgin C	—	1.3	—	74

Note. Values in the Table were estimated by the determination of the vanadate sensitive ABCG2 ATPase activity in two sets of experiment, and by using at least five different concentrations for each drug.

[a] Relative to the ATPase activity measured in the absence of compound.

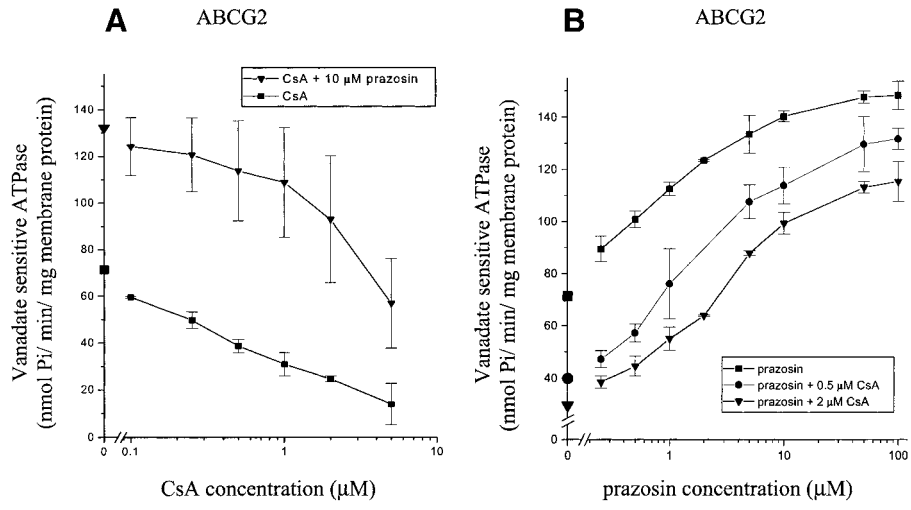

FIG. 3. (A) Effect of cyclosporin A on the basal and the prazosin stimulated ATPase activity in ABCG2 expressing Sf9 cells. CsA concentration was varied at a constant (10 μM) prazosin concentration. The data points show the mean values of at least four determinations. (B) Effect of prazosin on the ABCG2-ATPase activity in the presence of cyclosporin A. Prazosin concentration was varied at constant (0.5 or 2 μM) CsA concentrations. The data points show the mean values of at least four determinations.

kinetic parameters obtained for the effects of the above and some other compounds on the ABCG2-ATPase activity are compiled in Table I. It is important to note that all these agents had practically no effect on the low level ATPase activity measured in control, β-galactosidase expressing Sf9 cells.

All the above detailed experiments clearly demonstrate that the expression of the human ABCG2 induces a high-capacity membrane ATPase activity, with a distinct substrate-stimulation and inhibitor sensitivity, as compared to those of other human multidrug transporters, like the MDR1 protein. Moreover, the effects of substrates and inhibitors on ABCG2-dependent membrane ATPase activity in all cases showed a close correlation with the effects of these compounds in ABCG2-overexpressing mammalian cells.

In the Sf9 cell membranes the expression levels of the ABCG2 and MDR1 proteins, based on Coomassie-stained gel-electrophoretograms, were found to be similar (not shown), and the maximum, drug-stimulated ATPase activities of the two transporters were also comparable (in the present experiments 140 vs 70 nanomoles/mg membrane protein/min, for the ABCG2 and the MDR1 protein, respectively). This finding indicates that, similarly to MDR1, ABCG2 is a high-activity, ATP-dependent drug transporter, and its turnover rate greatly exceeds e.g. that of the members of the MRP family (see 27).

The effects of various substrates may be somewhat different in different clones of the human ABCG2 protein, with established sequence variations. Indeed, the ABCG2 proteins containing different amino acids at position 482 (Gly, as in the construct used by us, Arg, or Thr) have slightly different substrate specificities (S. E. Bates, personal communication). Studying the ATPase activity of these ABCG2 variants, when expressed in Sf9 cells separately or together, should help to investigate these questions. Also, possible functional dimerization partners of ABCG2 can be co-expressed and studied in this system.

As shown in Fig. 2, in the ABCG2-expressing Sf9 cell membranes, in contrast to that seen for MDR1, we found a relatively high-level basal ATPase activity. This finding may suggest an endogenous stimulation of the transporter (e.g., by the presence of certain lipids or lipid-derivatives in these membranes), or a partial uncoupling, caused e.g. by the presence of improperly folded ABCG2 molecules.

In order to explore the nature of this phenomenon, we measured the MgATP-concentration dependence for the basal and the drug-stimulated ABCG2-ATPase (not shown here in detail). We used 100 μM prazosin as an ABCG2-ATPase stimulating agent. The MgATP-dependence of the ATPase activity under these conditions was similar, although the K_M ATP value without an added substrate was 0.6 mM, while in the presence of drug-substrate this value decreased to about 0.3 mM. The K_i value for vanadate inhibition was about 20 μM both in the absence and presence of prazosin. All these data showed a close similarity in the characteristics of the basal and drug-stimulated ABCG2-ATPase activities, and argued against the presence of a misfolded ABCG2 population in the Sf9 cell membrane preparations.

In order to further explore the nature of this phenomenon, in the following experiments we measured the ABCG2-ATPase activity in the presence of increasing concentrations of Cyclosporin A (CsA), both in the absence and presence of 10 μM prazosin (Fig. 3A). In

another set of experiments we varied prazosin concentration in the presence of constant (0.5 or 2 μM) CsA concentrations (Fig. 3B).

We found that CsA inhibited both the basal and the prazosin-stimulated ABCG2-ATPase activity, with K_i values of about 0.5 and 1.5 μM, respectively (Fig. 3A). As shown in Fig. 3B, 0.5 or 2 μM Cyclosporin A reduced the basal ATPase activity in ABCG2-containing membranes by about to 45 and 60%, respectively. However, increasing concentrations of prazosin in the presence of CsA still could stimulate the ABCG2-ATPase activity, up to the levels observed in the absence of CsA. There was a significant shift (from 1 to about 3 μM) in the prazosin concentration producing half-maximum ATPase activation by the presence of 0.5 μM CsA. All these experiments can be interpreted to mean that CsA is a competitive inhibitor of the substrate-stimulated ATPase activity of ABCG2, and the baseline ATPase activity of ABCG2 is induced by a relatively low affinity substrate, present in the isolated membranes. This higher baseline activity may explain the relatively smaller magnitude of additional drug-stimulation of ABCG2-ATPase than that found for MDR1.

Taken together, our current experiments indicate that the ABCG2 multidrug transporter can be efficiently expressed in Sf9 cells and its transport ATPase characteristics studied in isolated membranes. Since the ABCG2 protein is an ABC half-transporter, requiring the formation of functional dimers, most probably such a dimerization occurs when the protein is inserted into the Sf9 cell membranes. In this heterologous expression system the presence of a partner ABC half-transporter, with similarly high expression levels can be convincingly excluded, thus ABCG2 most probably functions here as a homodimer (or homooligomer).

Our results presented here suggest that the Sf9 cell membrane ATPase system is an efficient tool for the investigation of the catalytic mechanism of the ABCG2 multidrug transporter, as well as for studying its interactions with anticancer drugs or other pharmacological agents. The application of this assay system may significantly help the rapid identification of novel substrates and inhibitors of ABCG2, with potential use at the cancer clinics.

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