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Single amino acid (482) variants of the ABCG2 multidrug transporter: major differences in transport capacity and substrate recognition

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Abstract

The human ABCG2 protein is an ATP binding cassette half-transporter, which protects our cells and tissues against various xenobiotics, while overexpression of ABCG2 in tumor cells confers multidrug resistance. It has been documented that single amino acid changes at position 482 resulted in altered drug resistance and transport capacity. In this study, we have generated nine Arg-482 mutants (G, I, M, S, T, D, N, K, Y) of ABCG2, and expressed them in insect cells. All ABCG2 variants showed cell surface expression and, in isolated membranes, an ABCG2-specific ATPase activity. When methotrexate accumulation was measured in inside-out membrane vesicles, this transport was supported only by the wild-type ABCG2. In intact cells, mitoxantrone was transported by all ABCG2 variants, except by R482K. Rhodamine 123 was extruded by most of the mutants, except by R482K, Y and by wild-type ABCG2. Hoechst 33342 was pumped out from cells expressing the wild-type and all Arg-482 variants, but not from those expressing R482K and Y. Our study demonstrates that the substrate specificity of the Arg (wild-type) form is unique and that amino acid replacements at position 482 induce major alterations in both the transport activity and substrate specificity of this protein.

Keywords: Multidrug half-transporter; Single amino acid mutant; Cell surface localization; Membrane ATPase; Vesicular transport; Fluorescent dye extrusion

1. Introduction

The human ABCG2 multidrug transporter (ABCP/BCRP/MXR) is a plasma membrane glycoprotein, which belongs to the ATP binding cassette (ABC) protein family. ABCG2 is a half transporter, possessing only one ATP binding and one transmembrane domain, and most probably acts as a homodimer [1,2]. The ABCG2 protein is present in several normal tissues [3,4], and its overexpression has also been documented in drug-resistant cell lines and tumors [1].

ABCG2 transports a wide variety of compounds, including cytotoxic agents (mitoxantrone, topotecan, flavopiridol, methotrexate), fluorescent dyes (e.g., Hoechst 33342) and different toxic compounds found in normal food (e.g., 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) or pheophorbide a) [4–8]. ABCG2 mediates the extrusion of these compounds towards the extracellular space, a process energized by ATP hydrolysis [1]. Transport function and tissue distribution of ABCG2 suggest its role in protection/detoxification against xenobiotics [3,9] and, indeed, ABCG2 has been shown to influence the intestinal topotecan absorption and its secretion into the bile [10,11].

There is little information about the amino acids responsible for the substrate specificity of ABCG2. The transmembrane domain of ABC transporters is thought to be responsible for the recognition of transported substrates. In

Abbreviations: ABC, ATP binding cassette; ABCP, placenta specific ABC transporter; BCRP, breast cancer resistance protein; β-gal., β-galactosidase; Hst, Hoechst 33342; MDR1, human multidrug resistance protein (P-glycoprotein, ABCB1); MRP, human multidrug resistance associated protein, ABCC1; MXR, mitoxantrone resistance protein; MTX, methotrexate; MX, mitoxantrone; R123, rhodamine 123; Sf9 cells, *Spodoptera frugiperda* ovarian cells; SN-38, 7-ethyl-10-hydroxy-camptothecin; TM, transmembrane; wt, wild-type

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the case of MDR1, deletion mutants containing only the transmembrane domains still retained their substrate binding capacity [12]. Moreover, different studies have identified amino acids in TM helices 4, 5, 6, 9, 10, 11 and 12 of MDR1, responsible for drug binding and thought to form the drug-binding domain of this protein [12–15]. In colchicine selected cells, mutation of Gly 185 to Val (found in the intracellular loop between TM helices 2 and 3) occurred in the overexpressed MDR1 protein [16]. The G185V mutant conferred altered basal ATPase activity and altered interaction with substrates, as well as with the inhibitor cyclosporin A [17,18]. In case of MRP1, 2 or 3, again amino acids found in the TM region were shown to influence substrate specificity [19–21].

In some drug-selected cell lines overexpressing human ABCG2 (or its mouse ortholog), a single amino acid change at position 482 (predicted to be situated in the third TM helix) occurred [22,23]. The mutants, containing R482G, T or M (R482M or S in the mouse abcg2), showed altered substrate specificity [22–24]. Previously, we have shown that the R482G and T mutants have increased ATP hydrolytic activity, therefore they are "gain of function" mutants in this regard [25]. However, the R482G and T mutants were unable to transport methotrexate, which is a substrate of the wtABCG2 [6,26]. A recent study by Miwa et al. [27] analyzed several mutant forms of ABCG2 in conferring resistance to mitoxantrone or SN38.

The aim of the present work was to analyze how different Arg-482 mutants influence the function of human ABCG2. Therefore, we have created seven additional ABCG2-R482 mutants (I, M, S, D, N, K and Y), representing various amino acid properties. We have expressed these mutants in the baculovirus-Sf9 insect cell expression system, which allows the investigation of both the transport and ATP-hydrolytic functions of ABCG2. Moreover, in this heterologous expression system there is no potential endogenous dimerization partner for the human ABCG2, thus the mutant variants function only as uniform homodimers.

The ATP hydrolytic capacity of the mutants was measured in isolated membrane vesicles, and the effect of potential substrates and inhibitors on this activity was determined. We also compared the MgATP-dependent methotrexate transport capacity of wtABCG2 and nine Arg-482 mutants using inside-out membrane vesicles. Additionally, we analyzed the transport of several fluorescent compounds (mitoxantrone, rhodamine 123 and Hoechst 33342) in intact cells expressing these mutants.

2. Materials and methods

2.1. Materials

ATP, methotrexate, mitoxantrone, Na-orthovanadate, prazosin, propidium iodide and rhodamine 123 were purchased from Sigma. Hoechst 33342 was purchased from Molecular Probes. Ko143 was a generous gift from Dr. G. Koomen (Division of Experimental Therapy, The Netherlands Cancer Institute, and Laboratory of Organic Chemistry, University of Amsterdam, Amsterdam, The Netherlands). [^{3}H]methotrexate was purchased from Moravek Biochemicals.

2.2. Generation of transfer vectors possessing different human ABCG2 cDNAs

pAcUW21-L/ABCG2 (wild-type, R482G, T or K86M/R482G) was constructed as described earlier [25]. In this study, we used the K86M-R482 single mutant, which was generated by cloning the *Not*I–*Spe*I fragment of pAcUW21-L/K86M-R482G [25] into the corresponding site of pAcUW21-L/R482. The seven additional Arg-482 variants were created using ABCG2-R482G cDNA as a template by overlap extension PCR [25,28]. The same outer primer pairs were used and the same cloning strategy was performed as described previously [25]. The two internal complementary primer pairs containing the specific mutation were: 5′-tta tta cca atg atc atg tta cc-3′ and 5-′gg taa cat gat cat tgg taa taa-3′ (R482I), 5′-tta tca gat cta tta ccc atg-3′ and 5′-gg taa cat cat cat ggg taa t-3′ (R482M), 5′-ta ccc atg tcg atg tta cca a-3′ and 5′-t tgg taa cat cga cat ggg ta-3′ (R482S), 5′-cc atg gac atg tta cca tcg att ata-3′ and 5′-tat aat cga tgg taa cat gtc cat gg-3′ (R482D), 5′-atg tta cca tcg att ata ttt acc-3′ and 5′-cc atg aat atg tta cca tcg att ata-3′ (R482N), 5′ -tta tta cct atg aag atg tta-3′ cc and 5′-gg taa cat ctt cat agg taa taa-3′ (R482K) and 5′-tta tta cct atg tac atg tta cc-3′ and 5′-gg taa cat gta cat agg taa taa-3′ (R482Y). The mutations were confirmed by sequencing the *Pst*I–*Msc*I fragments of the constructs.

2.3. Generation of recombinant baculoviruses

Recombinant baculoviruses carrying the different human ABCG2 cDNAs were generated as described [25,29]. ABCG2 protein expression was determined by immunoblotting and immunoflow cytometry (see below).

2.4. Membrane preparation and immunodetection of ABCG2

Virus-infected Sf9 cells were harvested after 72 h of infection. Membranes were isolated by differential centrifugation [30] and stored at −80 °C. The membrane protein concentrations were determined by the modified Lowry method [29]. Immunoblot detection was performed as described in Ref. [25]. The expression level of different ABCG2 mutants was determined by densitometry of the immunoblots (BioRad ChemiDoc).

Immunoflow cytometry was performed by labeling 2–5×10^5 Sf9 cells after 40 h of infection [25] at 37 °C, by using the anti-ABCG2 monoclonal antibody 5D3 (eBioscience), which recognizes a cell-surface epitope of human ABCG2 [4]. The antibody was used in a final concentration of 1 μg/ml, and binding was visualized by the addition of a

second, phycoerythrin-conjugated anti-mouse IgG (Immunotech), in 1 μg/ml final concentration. Flow cytometry determination of the antibody reaction was carried out at 488-nm excitation and 585-nm emission wavelengths using a FACSCalibur cytometer (Becton Dickinson).

2.5. Membrane ATPase measurements

ATPase activity was measured as described in Ref. [25] by determining the liberation of inorganic phosphate from ATP with a colorimetric assay. Normalized ATPase activities (A_n) were determined as follows: $A_n=((A_x-A_b)\times E_r)+A_b$. A_b: background ATPase activity (8 nmol P_i/min/mg protein) measured in membranes containing β-galactosidase, A_x: activity measured in the case of the ABCG2 mutants, E_r: relative expression level of the mutants compared to the wild-type, determined by densitometry (see above).

2.6. Measurement of [^{3}H]methotrexate transport by ABCG2

Sf9 membrane vesicles (90 μg) containing one of the Arg-482 mutants, wtABCG2 or the K86M mutant were prepared on the same day to ensure the same inside-out vesicle ratio of the different membranes. Membranes were incubated in the presence or absence of 4 mM MgATP (or 4 mM MgATP+1 μM Ko143) in a buffer containing 40 mM 3-(*N*-morpholino) propanesulfonic acid–Tris (pH 7.0), 56 mM KCl, 6 mM $MgCl_2$ and 2 mM dithiothreitol, in a final volume of 140 μl, at 37 °C for 5 min (or as indicated in Fig. 3A). The measurement was started by the addition of 10–3000 μM MTX. The reaction was carried out as described earlier [31].

2.7. Mitoxantrone or rhodamine 123 uptake in intact Sf9 cells

The uptake of mitoxantrone or rhodamine 123 was measured by using intact Sf9 cells overexpressing one of the ABCG2 mutants as described [25]. A FACSCalibur cytometer equipped with 488-nm argon and 635-nm red diode laser, and 530- and 670-nm bandpass filters, was used to determine the cellular fluorescence of rhodamine 123 and mitoxantrone, respectively. A total of 30 000 cells were counted; dead cell exclusion was based on propidium iodide staining.

2.8. Hoechst 33342 dye accumulation assay

Accumulation of Hoechst dye (Hst) was performed by using intact Sf9 cells overexpressing one of the ABCG2 mutants, in a fluorescence spectrophotometer (Perkin Elmer LS 50B) at 350 nm (excitation)/460 nm (emission) as described [25]. The increase in cellular fluorescence due to Hoechst accumulation was determined in the absence (F_0) or presence of 1 μM Ko143 (F_{100}). Transport activity of ABCG2 protein variants was calculated as $((F_{100}-F_0)/F_{100}))\times 100$.

3. Results

3.1. Expression of ABCG2-R482 mutants in Sf9 cells

In the present study, we have generated seven Arg-482 mutants (I, M, S, D, N, K and Y) and expressed them in Sf9 cells using recombinant baculoviruses. Expression of the ABCG2 mutants was detected by immunoblotting, using the BXP-21 monoclonal antibody [3]. Fig. 1A demonstrates that all ABCG2-R482 mutants were successfully expressed in Sf9 cells, although with some variations, at about equal protein levels. The expression levels of the R482I and the R482D variants were usually somewhat lower than those for the other mutants.

To find out whether the mutants were properly expressed and localized in insect cells, we performed flow cytometry by using the 5D3 monoclonal antibody, which recognizes an extracellular epitope and thus detects cell surface expression of ABCG2 [4]. Fig. 1B shows that all ABCG2-R482 mutants were clearly recognized by the 5D3 antibody in intact insect cells, indicating that all of these proteins localize to the plasma membrane. Similar membrane localization was found for the inactive K86M human ABCG2 mutant [25] in the insect cells. In the control Sf9 cells, or in those expressing the MDR1 protein, there was no measurable labeling by the 5D3 antibody.

3.2. ATPase activity measurements in isolated insect cell membranes

We have previously shown that the overexpression of human ABCG2 in insect cells results in high ATPase activity without exogenously added substrates, and this basal activity is approximately three times higher than that of human MDR1 [32]. The high basal ATPase activity of ABCG2 is sensitive to Na-orthovanadate and to specific ABCG2 inhibitors (e.g., Fumitremorgin C and Ko143).

To find out whether the seven newly expressed Arg-482 mutants were active, we determined their ATPase activity by using membrane vesicles prepared from insect cells expressing these proteins. Fig. 2 demonstrates that all seven, new ABCG2-R482 mutants showed a significantly higher, vanadate-sensitive, basal ATPase activity than the K86M mutant. In order to compare the ATP hydrolytic capacity of the mutants with that of wtABCG2, we normalized the ATPase activity of the different Arg-482 mutants to their actual expression levels (determined by densitometry, see Section 2) in each experiment, and the measurements were repeated three times for all variants. The slight variations in the expression levels were most probably due to the differences in actual culturing conditions and/or the differences in the titers of the recombinant baculoviruses.

Comparing the normalized ATPase activities, we have found that the G, S, T and N mutants had higher, the I, M, K and Y had similar, while the D variant had a lower basal ATPase activity than wtABCG2 (see Fig. 2).

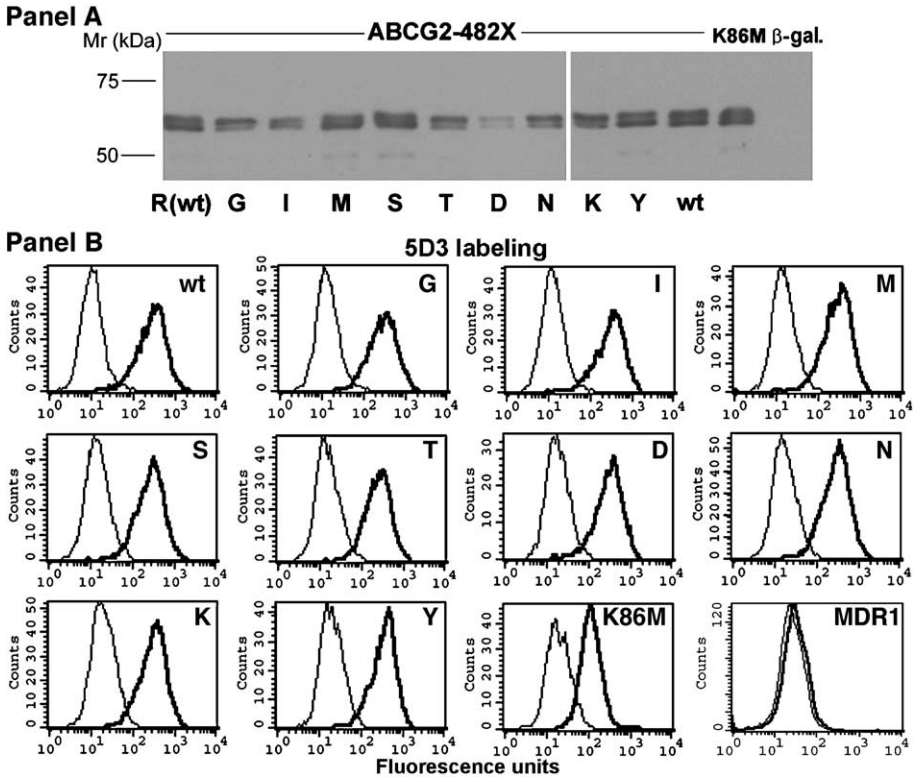

Fig. 1. Expression of ABCG2 482 mutants in insect cells. Panel A: Immunoblot detection of ABCG2 variants, expressed in Sf9 cells. Sf9 membranes were prepared and dissolved in disaggregation buffer. Proteins were separated by SDS-PAGE on 7.5% gels, electrotransferred to PVDF membranes and analyzed by immunodetection with the BXP-21 antibody. The amounts of the total membrane proteins loaded on the gel were 2 μg. Experiments were performed three times, the figure shows the result of one representative experiment. Panel B: Immunodetection of cell surface expression of the ABCG2 mutants. Sf9 cells infected with baculoviruses containing the cDNA of the ABCG2 variants, or MDR1, were collected after 40 h of infection. Labeling was performed with 1 μg/ml 5D3 antibody (heavy solid line) or 1 μg/ml isotype control (solid line) and 1 μg/ml phycoerythrin conjugated anti-mouse IgG. Fluorescence was detected in a FACSCalibur cytometer. Dead cells were excluded based on propidium iodide staining. Experiments were performed twice for each variants, the figure shows one representative experiment.

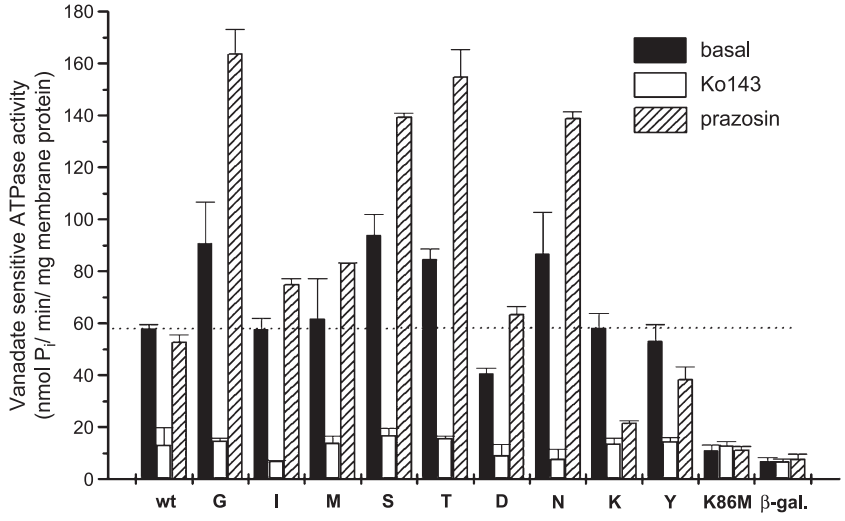

Fig. 2. Vanadate-sensitive ATPase activity measured in membranes of Sf9 cells, expressing the wild-type ABCG2 or its R482 or K86M mutants. ATPase activity of isolated Sf9 membranes was determined by measuring vanadate-sensitive inorganic phosphate liberation, using 3.3 mM MgATP in the absence of added compounds (black columns), with 100 μM prazosin (shaded columns), or with 1 μM Ko143 (blank columns). The data were normalized based on the difference in the expression level of the various ABCG2 forms (see Section 2). Data points represent the mean±standard deviation (S.D.) values of at least four measurements, performed with two different membrane preparations.

Ko143 is a specific inhibitor of ABCG2 [33]. When tested in the ATPase assay, this compound was found to inhibit the basal ATPase activity of all Arg-482 mutants (Fig. 2). This observation indicates that all Arg-482 mutants are active, since all show a specific, ABCG2-inhibitor-sensitive ATPase activity. When testing different Ko143 concentrations, we could not detect any major difference between the ABCG2 mutants with respect to their Ko143 sensitivity (i.e., the Ko143 concentrations producing 50% inhibition of the basal ATPase activities were between 20 and 100 nM for each variant; data not shown).

Prazosin is a substrate of the wtABCG2, R482G and R482T [34], and it stimulates the ATPase activity of R482G and T mutants, but it has no major effect on the ATPase activity of wtABCG2 [25]. When tested in the ATPase assay, prazosin was found to stimulate the activity of the R482I, M, S, D and N mutants by 1.3- to 1.6-fold. In contrast, the basal ATPase activity of R482K and Y mutants was rather inhibited than stimulated by prazosin

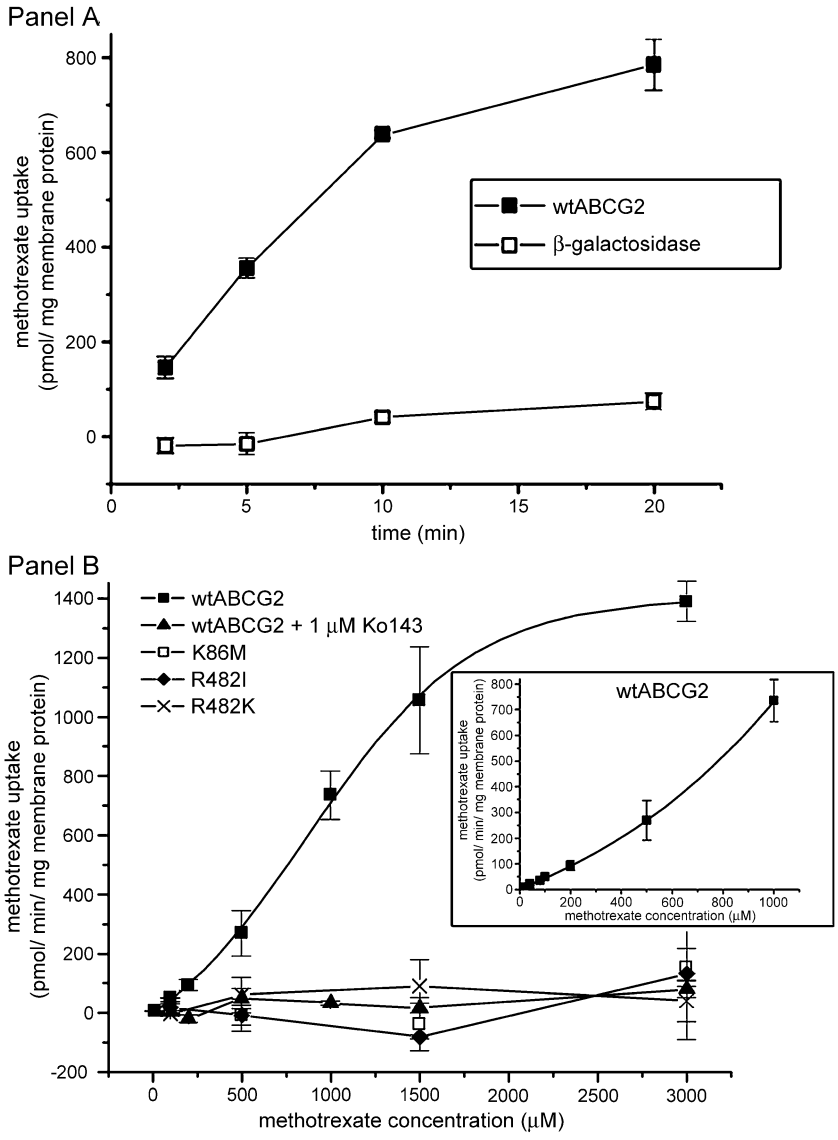

Fig. 3. [^{3}H]methotrexate transport characteristics of wtABCG2 and its mutant variants. Panel A: Time course of methotrexate transport. Sf9 membrane vesicles (90 μg) containing wtABCG2 or β-galactosidase were incubated with 100 μM MTX, containing [^{3}H]MTX, in the presence or absence of 4 mM MgATP, in a final volume of 150 μl, at 37 °C for different time periods. ATP-dependent transport was calculated by subtracting the values obtained in the absence of ATP from those in the presence of ATP. Panel B: Concentration dependence of MTX uptake by wtABCG2, R482I, R482K and K86M. Sf9 membrane vesicles (90 μg) containing wtABCG2 (solid square), K86M (open square), R482I (diamond), and R482K (cross) were incubated in the presence or absence of 4 mM MgATP, with (up-triangle) or without 1 μM Ko143, with different MTX concentrations (10–3000 μM in a final volume of 150 μl) at 37 °C for 5 min. ATP-dependent [^{3}H]MTX uptake was calculated by subtracting the values obtained in the absence of ATP from those in the presence of ATP. Inset: Concentration dependence of MTX uptake by wtABCG2 below 1000 μM. Values shown are means of at least four independent experiments±standard deviation (S.D.) values.

(see Fig. 2). In case of the latter two mutants, all ABCG2 substrates tested (e.g., mitoxantrone, rhodamine 123; not shown here) produced an inhibition of the ABCG2-ATPase activity.

3.3. Characterization of [^{3}H]methotrexate transport by ABCG2 mutants

It has been shown earlier that methotrexate is a transported substrate of the wtABCG2 but not of R482G or T [6,26]. In order to analyze the methotrexate (MTX) transport characteristics of wtABCG2, expressed in Sf9 cells, we determined the time and concentration dependence of [^{3}H]methotrexate transport in isolated membrane vesicles. Fig. 3, panel A shows that a significant, MgATP-dependent MTX uptake by the wtABCG2 into membrane vesicles could be observed, which was practically linear for about 5 min. On the other hand, MTX accumulation measured in membranes from cells expressing β-galactosidase (Fig. 3A) or ABCG2-K86M (see below) was very low, and did not increase during this time period.

Fig. 3B documents that, when measuring the MTX concentration dependence of MTX uptake by wtABCG2, this transport followed a sigmoidal curve (see insert in Fig. 3B), with an approximate V_{max} of 1389±69 pmol/min/mg membrane protein. MTX transport by ABCG2 was found to be fully inhibited by Ko143, down to the level of that seen in the presence of the ABCG2-K86M mutant (see Fig. 3B).

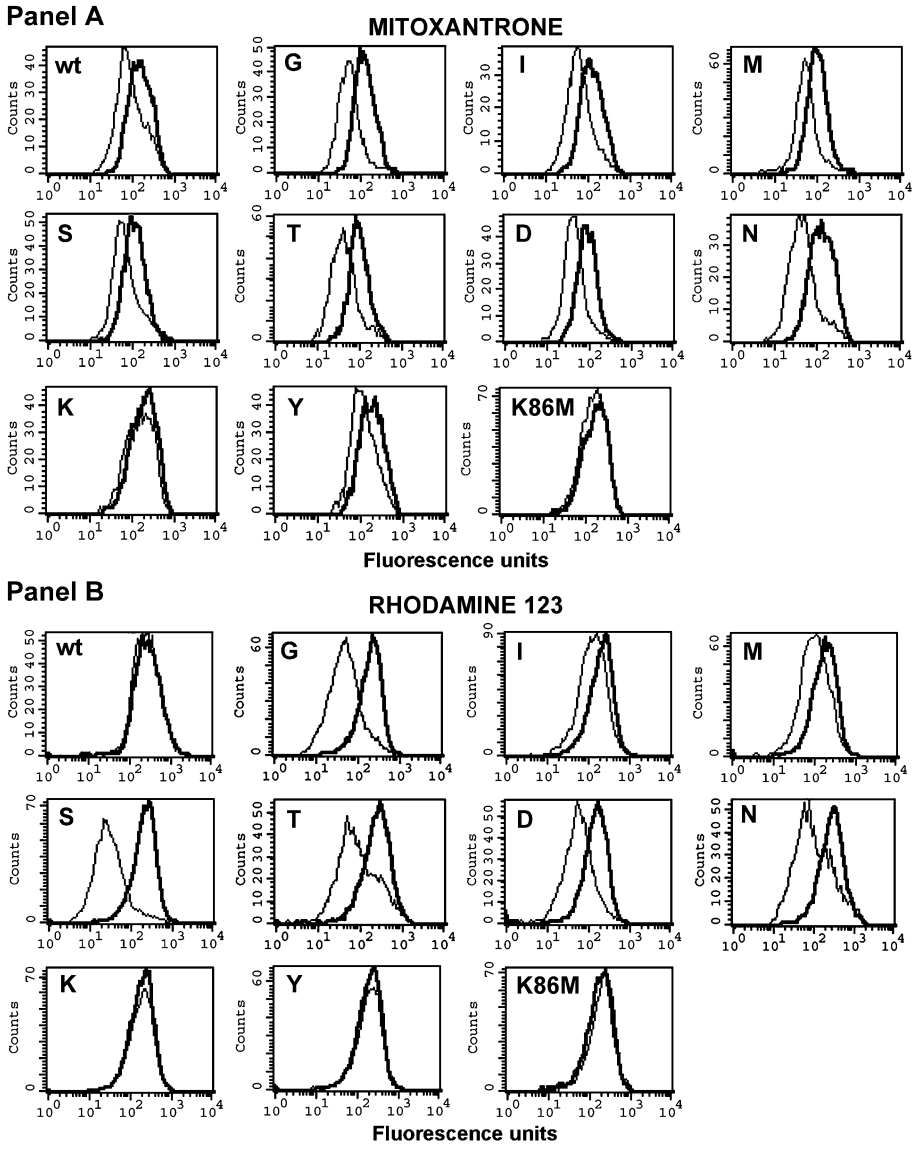

Fig. 4. Mitoxantrone and rhodamine 123 accumulation in Sf9 cells expressing wild-type ABCG2 (Arg-482), the R482X or K86M mutants. Sf9 cells were incubated for 30 min at 37°C with 20 μM MX (panel A) or 2 μM rhodamine 123 (panel B), in the presence (heavy solid line) or in the absence (solid line) of 1 μM Ko143. Flow cytometry was performed as described in Section 2. Dead cells were excluded based on propidium iodide staining. Measurements were repeated at least three times. Figures show the result of one representative experiment.

In order to test whether the various ABCG2-R482 mutants were also able to transport MTX, we measured [^{3}H]methotrexate accumulation at various MTX concentrations (100–3000 μM) in inside-out Sf9 cell membrane vesicles, for 5 min at 37 °C. We made sure that the different membrane preparations contained similar percent of inside-out vesicles, and that the expression levels of the mutants were comparable with that of the wild-type ABCG2. We found that none of the Arg-482 mutants had any measurable MTX uptake, as compared to the inactive ABCG2-K86M mutant (see Fig. 3B for R482I and for R482K; the data for the other variants are not shown).

These results indicate that wtABCG2, as expressed in insect cells, can transport methotrexate, while the Arg-482 mutants are inactive in this regard. Interestingly, we found that the MTX-transport characteristics of wtABCG2 expressed in insect cells, i.e., the S-shape curve indicating a complex interaction of this transporter with MTX (see Fig. 3B inset), have not been observed in vesicles prepared from mammalian cells [26,35].

3.4. Flow cytometry assay of mitoxantrone and rhodamine 123 extrusion from intact Sf9 cells expressing wtABCG2 and its Arg-482 mutants

It has been documented earlier that mitoxantrone (MX) is a transported substrate both of the wtABCG2 and its R482G or T mutants, while rhodamine 123 (R123) is transported only by the R482G and R482T variants [22,25]. Both MX and R123 are fluorescent, therefore we could directly analyze their accumulation in ABCG2-expressing intact cells, using flow cytometry [22,25].

In order to characterize the transport of MX or R123 by the Arg-482 mutants, we have used intact insect cells expressing one of the nine 482 mutants, the wtABCG2, or the K86M mutant (as a negative control). The transport rate of these fluorescent compounds was determined by flow cytometry, as described in Section 2. The expression level of the ABCG2 variants in each experiment was confirmed by Western blotting.

Fig. 4A shows the accumulation of mitoxantrone in insect cells, expressing one of the ABCG2 variants. We found that cells containing wtABCG2 or R482G, I, M, S, T, D, N, and Y mutants accumulate less mitoxantrone than cells expressing the inactive K86M mutant. However, in each case, when ABCG2 function was blocked by Ko143, MX accumulation increased to the level observed in the ABCG2-K86M expressing cells. These data indicate that most of the ABCG2-R482 mutant variants can actively extrude mitoxantrone, while the R482K mutant is inactive in this regard.

Fig. 4B demonstrates rhodamine 123 accumulation in the ABCG2-expressing insect cells. We found that while the R482K, R482Y, the wtABCG2, and the inactive K86M mutant had no R123 extrusion activity, several ABCG2 variants were highly active in R123 extrusion. R123 transport by all these active forms was inhibited by Ko143, down to the level of the K86M mutant.

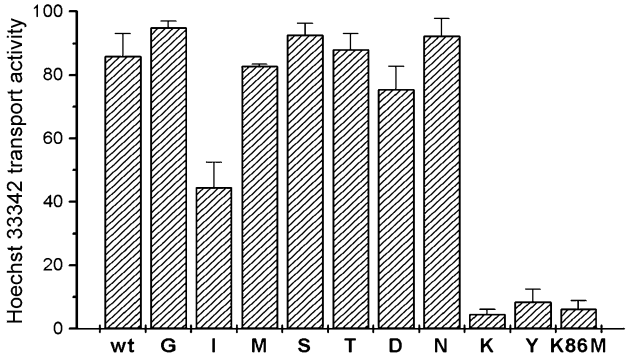

Fig. 5. Hoechst 33342 (Hst) accumulation in Sf9 cells expressing different ABCG2 variants. A total of 2–5×10^5 Sf9 cells with different ABCG2 variants were incubated with Hoechst 33342 (2 μM) for 10 min at 37 °C, then 1 μM Ko143 was added. Hoechst fluorescence was measured in a fluorescence spectrophotometer. Transport activity of ABCG2 protein variants was calculated as described in Section 2.

3.5. Measurement of Hoechst 33342 transport by the ABCG2-R482 mutants

It has been shown that Hoechst 33342 is a transported substrate of the wtABCG2 protein, as well as of its R482G and T mutant variants. The transport kinetics of the Hoechst dye can be determined by spectrofluorometry [25] in insect cells, expressing either wtABCG2 or Arg-482 mutants. This assay allows a quantitative comparison of the transport capacities of the various ABCG2 forms.

In order to compare the Hoechst 33342 (Hst) transport capacity of the seven new Arg-482 mutants, we measured Hst transport kinetics in intact insect cells, expressing one of the mutants. The expression levels of the different ABCG2 variants were determined and quantitated by densitometry on Western blots.

Fig. 5 shows the calculated transport activities for the different Arg-482 variants. We found that the R482G, M, S, T, D and N mutants were active in Hoechst transport, and there was no significant difference between the Hst-transport kinetics of these Arg-482 mutants and the wtABCG2. The R482I mutant was also active in this assay, but it had a decreased Hst-transport activity when compared to the wild-type protein, which may be due to a lower expression level of this mutant (not shown). On the other hand, mutants R482K and Y showed no Hst-transport capacity, although their expression levels were similar to that of the wild-type ABCG2 (not shown).

4. Discussion

Human ABCG2 was shown to confer tumor cell resistance to various clinically important compounds, e.g., mitoxantrone, methotrexate, topotecan, SN38, flavopiridol

[1,2]. Understanding the substrate recognition of ABCG2 could promote the development of specific inhibitors and modulators of this protein. Additionally, it could help to predict whether ABCG2 plays a role in the absorption and/or secretion of a given compound with potential pharmacological importance. There is only limited information available about amino acids influencing the substrate specificity of ABCG2.

The aim of this study was to investigate the 482 position of ABCG2, namely how different amino acids at this position influence the activity of the protein. We have generated Arg-482 mutants that represent different amino acid characters (I, D, N, Y), together with the mutant that occurred in the mouse ortholog, abcg2 (S), and the mutant found both in human ABCG2 and mouse abcg2 (M). We have also replaced Arg-482 with another positively charged amino acid (K) in order to determine the functional role of a positive charge in this position.

All Arg-482 variants (including R482G, T, wild-type, and K86M, as a negative control) were expressed in insect cells, which produce high levels of ABCG2 and allow the detailed characterization of the function of the protein. Also, there is no potential endogenous dimerization partner for ABCG2 which could significantly alter the observed features of the mutant variants.

Recently, Miwa et al. [27] have published the expression of 15 different Arg-482 mutants (Y, N, C, M, S, T, V, A, G, E, W, D, Q, H and K) in murine fibroblasts, and analyzed these mutants mostly for their drug resistance activities. The present study is a significant extension of these experiments, providing a detailed cell biology and biochemical characterization of the mutant proteins.

In this paper, we show that all Arg-482 mutants expressed in insect cells show cell surface localization (Fig. 1A and B). This finding suggests that Arg-482 has no crucial role in the correct folding and membrane insertion of ABCG2, although ABC proteins may fold differently in different expression systems and at different culturing temperatures [36].

When we studied the ABCG2-specific ATPase activity in isolated membranes, all Arg-482 mutants were found to show a high basal ATPase activity, characteristic for ABCG2, when expressed in insect cells. These ATPase activities were sensitive to Ko143, a specific inhibitor of ABCG2, again confirming the functionality of the mutants (Fig. 2). In addition, the maximum activities of the mutants were quite variable: a three- to fourfold variation in the maximum, Ko143-sensitive ATPase activities was observed.

We have shown previously that the transported substrates could not stimulate the ATPase activity of the wtABCG2, while they stimulated the ATPase activity of the R482G and T mutants (26). In order to find out the effects of substrates in the newly generated Arg-482 mutants, we have examined the effects of various substrates on their ATPase activities. We found that most of the new Arg-482 mutants (I, M, S, D and N) were clearly distinguished from wtABCG2, i.e., their ATPase activity was stimulated by, e.g., prazosin (Fig. 2). On the other hand, mutants R482K and Y showed inhibition in the presence of prazosin, as well as in the presence of all other potential substrates tested (e.g., mitoxantrone, rhodamine 123; not shown here).

Although these ATPase activities may not directly answer the exact substrate specificities of these ABCG2 variants, they can be highly informative regarding the maximum turnover (transport and ATP hydrolysis) rates, as well as for interactions with wide range of potential substrates. If the basal ATPase activity of ABCG2 is indeed due to the presence of a low affinity endogenous substrate in insect cell membranes, this endogenous substrate differently affects the ATPase activity of the ABCG2 variants examined here. While the wtABCG2, the R482K and R482Y mutants are already fully activated, and prazosin either does not affect or reduces the ATPase activity, the other variants can be further stimulated by exogenously added substrates.

In order to characterize the transport properties, we performed direct transport studies with the expressed ABCG2 variants. Methotrexate uptake was examined in inside-out membrane vesicles, while mitoxantrone, rhodamine 123 and Hoechst 33342 transport was examined in intact Sf9 cells.

When we measured the uptake of [^{3}H]methotrexate into Sf9 vesicles (see Fig. 3), we found a specific, ATP-dependent and Ko143-sensitive transport of MTX in membranes containing wtABCG2. The concentration dependence of MTX uptake by wtABCG2 showed a sigmoidal curve that suggests a complex interaction between ABCG2 and methotrexate. This finding indicates that ABCG2, similarly to other ABC transporters [37], has multiple binding and/or transport sites of MTX, and the endogenous substrates in Sf9 membranes may have a heterotropic effect on MTX transport. As shown in Fig. 3, none of the Arg-482 mutants showed any MTX transport, similarly to the entirely inactive K86M mutant and the R482G or T mutants [6,26].

Methotrexate is an anionic drug, and it is feasible that the positively charged Arg in the wtABCG2 plays a crucial role in the acceptance of this compound at the transport site. However, the 482 Lys mutant, in spite of having the same positive charge, had no methotrexate transport activity, indicating that Arg-482 is absolutely critical in handling of methotrexate by ABCG2.

When measuring the accumulation of mitoxantrone, rhodamine 123 or Hoechst 33342 in intact, ABCG2-expressing Sf9 cells (see Figs. 4 and 5), we found that most of the Arg-482 mutants (R482G, I, M, S, T, D and N) were able to actively extrude these compounds. The exceptions were the R482Y mutant, effective only in mitoxantrone transport, and the R482K mutant, showing no transport activity with any of these substrates.

The above ATPase and transport experiments seem to define two major groups of Arg-482 mutants of ABCG2. One group (G, I, M, S, T, D and N) contains amino acids

with small, hydrophobic, nucleophilic, acidic or amide side chains. These are characterized with high ATPase capacity, mitoxantrone, rhodamine 123 and Hoechst 33342 transport activity, and methotrexate transport inability. The other group of mutants is represented by the aromatic Tyr and the basic Lys. These mutants resemble wtABCG2 regarding the level of their relatively low basal ATPase activity, and the absence of rhodamine 123 transport. On the other hand, they are distinguished from wtABCG2 because of the lack of methotrexate and Hoechst 33342 transport. Actually, the R482K mutant showed no measurable transport activity in any of the assays applied here, while the R482Y was found to be active only in the whole-cell mitoxantrone extrusion assay (see Fig. 4A).

The above data indicate that the substrate specificity of the wild-type, Arg-482 form of ABCG2 is unique, which may have important consequences regarding the interaction of this protein with natural substrates or various toxic agents. This finding may explain why the Arg form of ABCG2 was conserved during evolution, in spite of its relatively lower transport and ATPase capacity.

Arg-482 is found in the third TM helix of ABCG2. It is feasible that some of the Arg-482 mutants alter the substrate specificity of ABCG2 by altering the structure of the transmembrane domain of this protein. However, our cell surface localization data (Fig. 1B) argue against major changes in the membrane topology of the ABCG2 mutants because the conformation sensitive anti-ABCG2 5D3 antibody recognized all mutant variants. Since structural data are only available for certain bacterial ABC transporters [38,39], which are not closely related to ABCG2, we could not perform a detailed homology analysis in this regard.

When comparing our results to those of Miwa et al. [27], expressing Arg-482 mutants of ABCG2 in murine fibroblast cells, we can analyze their drug resistance and our transport biochemistry data in these protein variants. Mutants R482G, M, S, T, D and N were shown to confer higher resistance against mitoxantrone (MX) and doxorubicin than the wtABCG2 [22]. Indeed, these mutants showed well measurable mitoxantrone transport capacities in our assay. We found that the R482Y mutant is a very weak mitoxantrone transporter (see Fig. 4A), and Miwa et al. [27] demonstrated that this mutant confers decreased resistance in murine cells against MX. Only low level of expression of the R482K mutant was achieved in murine cells, nevertheless, the cells were sensitive to MX. We could generate insect cells expressing this mutant in high quantity (see Fig. 1) and found that it possesses no mitoxantrone transport capacity (see Fig. 4A). As a summary, the results obtained in the two entirely different experimental systems are in harmony, and further demonstrate the key role of the side-chain position 482 in substrate handling.

In addition to their relevance of structure-function studies in ABCG2, our studies may have important applications in the field of stem cell-based gene therapy. As shown recently, ABCG2-R482G has been successfully applied as a selectable marker protein in ex vivo gene transfer experiments. Based on the relatively small cDNA, natural expression in stem cells, and significantly altered transport properties, some of the Arg-482 variants of human ABCG2 may be ideal candidates for providing selective in vitro and in vivo advantages of modified stem cells in medical gene therapy [40].

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