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Transport of Methotrexate, Methotrexate Polyglutamates, and 17β-Estradiol 17-(β-D-glucuronide) by ABCG2: Effects of Acquired Mutations at R482 on Methotrexate Transport[1]

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ABSTRACT

ABCG2 is a plasma membrane efflux pump that is able to confer resistance to several anticancer agents, including mitoxantrone, camptothecins, anthracyclines, and flavopiridol. The antimetabolite methotrexate (MTX) was inferred recently to be an additional substrate of the pump based on the analysis of ABCG2-overexpressing cell lines. However, the transport characteristics of the pump with regard to this agent have not been determined. In addition, physiological substrates of ABCG2 have not been identified. Here we examine the *in vitro* transport properties of the pump using membrane vesicles prepared from HEK293 cells transfected with ABCG2 expression vector. In so doing it is shown that MTX is a high capacity low affinity substrate of the pump, with K_m and V_{max} values of 1.34 ± 0.18 mM and 687 ± 87 pmol/mg/min, respectively. Unlike previously characterized multidrug resistance protein family members, ABCG2 is also able to transport MTX diglutamate and MTX triglutamate. However, addition of even one more glutamyl residue is sufficient to completely abrogate ABCG2-mediated transport. By contrast with the wild-type protein (ABCG2-R482), two ABCG2 variants that have been identified in drug selected cell lines, R482T and R482G, were unable to transport MTX to any extent. Similarly, folic acid was subject to efflux by the wild-type protein but not by the two mutants. However, transport of the reduced folate leucovorin was not detected for either the wild-type or the mutant proteins. Finally, it is shown that ABCG2 is capable of transporting $E_2$17βG with K_m and V_{max} values of 44.2 ± 4.3 μM and 103 ± 17 pmol/mg/min, respectively. These results indicate that ABCG2 is a component of the energy-dependent efflux system for certain folates and antifolates, but that its transport characteristics with respect to polyglutamates and reduced folates are not identical to those of multidrug resistance protein family members. In addition, it is demonstrated that R482 mutations observed in drug-resistant cell lines have profound effects on the *in vitro* transport properties of the pump.

INTRODUCTION

ABCG2[3] is a plasma membrane efflux pump composed of a single nucleotide binding domain-appended NH_2-terminal to a series of approximately six transmembrane spanning helices (1–3). Transfection studies and analyses of drug-selected cell lines in which ABCG2 is overexpressed indicate that the pump is capable of conferring resistance to a variety of anticancer agents, including mitoxantrone, topotecan, anthracyclines, and flavopiridol (1, 2, 4–7). Analysis of resistant cell lines in which human *ABCG2* is overexpressed has also revealed that *ABCG2* is susceptible to the acquisition of mutations at amino acid position R482 and that these mutations affect the functional characteristics of the pump (4). This notion, which has been extended recently to murine *ABCG2* (8), has been inferred from studies showing that drug-resistant cell lines and transfected cells that have glycine or threonine (human ABCG2), or methionine or serine (murine ABCG2), at residue 482 of the protein have enhanced resistance toward anthracyclines, diminished resistance for topotecan, and enhanced cellular efflux of the dye rhodamine 123.

Whereas the cellular drug resistance capabilities of ABCG2 have been explored to some extent, important questions about the transporter remain, particularly with regard to MTX, a widely used antifolate for which only certain members of the MRP family are established efflux pumps (9–15). The possibility that ABCG2 is involved in MTX transport was suggested by a report showing that MCF7/MX, a mitoxantrone-resistant cell line in which *ABCG2* is amplified and overexpressed, exhibits 150-fold cross-resistance to MTX and an ATP-dependent accumulation defect for this agent, but does not have alterations in the expression levels of proteins that are known to be involved in MTX resistance (16). In accord with the notion that a pump distinct from MRP family members was responsible for MTX resistance in this cell line, MCF7/MX exhibited MTX resistance in standard (continuous drug exposure) growth assays, as opposed to the time-restricted drug exposure assays that are required for detecting the potent MTX resistance associated with overexpressed MRPs (12–14). However, a puzzling feature of this study was that MCF7 cells stably transfected with an ABCG2 cDNA, which has since been determined to have an R482T mutation, did not recapitulate MTX resistance. A potential explanation for these findings, namely, that the wild-type protein, but not residue 482 mutants, has the facility for mediating MTX resistance, was determined from a recent analysis of a panel of drug-resistant cell lines that overexpress ABCG2 (17). Whereas this report supports the notion that ABCG2 is able to confer MTX resistance, the transport characteristics of the wild-type and mutant protein with regard to MTX and its intracellular metabolites have not been determined.

Here we use membrane vesicles prepared from transfected cell lines to directly examine the ability of wild-type ABCG2 and its variants to transport MTX and related compounds. The results of these experiments show that wild-type ABCG2 is indeed capable of mediating the transport of MTX, but that neither the R482G nor the R482T mutants are able to transport this agent to any extent. In addition, it is shown that ABCG2 is not only able to transport MTX, but by contrast with MRP1, MRP2, MRP3, and MRP4 (9, 11), is also capable of transporting MTX-Glu_2 and MTX-Glu_3. However, addition of another glutamyl residue completely abrogates ABCG2-mediated transport.

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[3] The abbreviations used are: ABCG2, gene symbol for breast cancer resistance protein/mitoxantrone resistance protein/placenta-specific ATP-binding cassette gene, BCRP/MXR/ABCP; MTX, methotrexate; MRP, multidrug resistance protein; FA, folic acid; FTC, fumitregmorgin C; $E_2$17βG, 17β-estradiol 17-(β-D-glucuronide).

Finally, it is demonstrated that both FA and $E_2$17βG are transport substrates of the pump.

Three conclusions derive from these findings: (*a*) ABCG2 is a component of the efflux system for MTX and certain folates; (*b*) mutations at residue 482 have a profound effect on the ability of the pump to transport the latter compounds *in vitro*; and (*c*) ABCG2, which has been reported to be expressed in the placenta and canalicular membranes (18), may contribute to the hepatobiliary excretion of endogenous steroids and/or glucuronides, and may similarly protect the fetus from these compounds.

MATERIALS AND METHODS

Materials and Cell Lines. [^{3}H]MTX (23 Ci/mmol), [^{3}H]MTX-Glu_2 (15 Ci/mmol), [^{3}H]MTX-Glu_3 (17 Ci/mmol), [^{3}H]MTX-Glu_4 (20 Ci/mmol), [^{3}H]FA (42 Ci/mmol), and [^{3}H]N^5-formyltetrahydrofolic acid (leucovorin, 16.6 Ci/mmol) were purchased from Moravek (Brea, CA). Unlabeled MTX and MTX polyglutamates were purchased from Schircks Laboratories (Jona, Switzerland). [^{3}H]$E_2$17βG (40.5 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Unlabeled N^5-formyltetrahydrofolic acid (leucovorin), FA, $E_2$17βG, creatine phosphokinase, phosphocreatine, ATP, and AMP were purchased from Sigma Chemical Co. (St. Louis, MO) and Fumitremorgin C was synthesized by Thomas McCloud (Developmental Therapeutics Program, NIH, Bethesda, MD). HEK293 cells transfected with ABCG2-R482, ABCG2-R482G, and ABCG2-R482T were generated using the respective cDNAs cloned into pcDNA3.[4] LLC/PK1 cells transfected with MRP2 expression vector, and HEK293 cells transfected with MRP3 expression vector were described previously (19, 20). Cells were maintained in DMEM (HEK293 cells) or M199 (LLC/PK1 cells), supplemented with 10% bovine serum, penicillin/streptomycin, and glutamine.

Immunoblot Analysis. Membrane vesicle preparations were separated by 8% SDS-PAGE, and proteins were electrotransferred to nitrocellulose filters using a wet transfer system as described previously (21). ABCG2 was detected using monoclonal antibody BXP-21 at 1:500 dilution (Kamiya Biomedical, Seattle, WA) and alkaline phosphatase-conjugated secondary antibody.

Preparation of Membrane Vesicles and Transport Experiments. Membrane vesicles were prepared by the nitrogen cavitation method as described previously (22). Experiments on transport into inside-out membrane vesicles were performed using the rapid filtration method essentially as described (23), and carried out in medium containing membrane vesicles (10 μg), 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 4 mM ATP or 4 mM AMP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and radiolabeled substrate ± unlabeled substrate, in a total volume of 50 μl. Reactions were carried out at 37°C and stopped by the addition of 3 ml ice-cold stop solution [0.25 M sucrose, 100 mM NaCl, and 10 mM Tris-HCl (pH 7.4)]. Samples were passed through 0.22 μm Durapore membrane filters (Millipore, Bedford, MA) under vacuum. The filters were washed three times with 3-ml ice-cold stop solution and dried at room temperature for 30 min. Radiolabel that bound to the filters in the absence of membrane vesicles was usually <5–10% of the total radioactivity and was not subtracted from the time-dependent uptake experiments shown in "Results." Radioactivity was measured by the use of a liquid scintillation counter.

RESULTS

Analysis of MTX Transport by Wild-Type ABCG2, ABCG2-R482G, and ABCG2-R482T. The ability of wild-type ABCG2 (R482) to mediate the transport of MTX was analyzed using density fractionated membrane vesicles prepared from HEK293 cells transfected with either parental plasmid or with ABCG2 expression vector. Membrane vesicles prepared from transfected HEK293 cells were a rich source of ABCG2 protein, as indicated by the intensely immunoreactive band of M_r ~70,000, which was detected in preparations of cells transfected with ABCG2 expression vector (Fig. 1, *Lane 2*), but not in membranes prepared from parental vector-transfected control cells (Fig. 1, *Lane 1*). Robust MgATP-dependent uptake of 100 μM MTX into inside-out membrane vesicles was readily detected for wild-type ABCG2 (Fig. 2*A*). In medium containing MgATP the initial uptake rate for ABCG2-enriched membrane vesicles was 82 pmol/mg/min. By comparison, the uptake rates for the same membranes in medium containing MgAMP, or for control membranes in the presence of either MgATP or MgAMP, were only 41 pmol/mg/min, 42 pmol/mg/min, and 41 pmol/mg/min, respectively.

Having determined that wild-type ABCG2 is capable of mediating the MgATP-energized uptake of MTX into membrane vesicles (Fig. 2*A*), we next examined the impact of R482 mutations on the pump. Membrane vesicles were prepared from HEK293 cells transfected with ABCG2-R482G and ABCG2-R482T, so as to analyze both types of R482 mutations that have been identified in drug-resistant cell lines (4). The two ABCG2 mutants were expressed in membrane vesicles at levels that were comparable with the wild-type protein (Fig. 1, *Lanes 3* and *4*). However, by contrast with ABCG2-R482, neither of the mutants were able to transport [^{3}H]MTX to any extent, in that MgATP-dependent uptake of this agent by ABCG2-R482G and ABCG2-R482T-enriched vesicles was indistinguishable from uptake by the same vesicles in the presence of MgAMP, or uptake by negative control vesicles in the presence of either MgATP or MgAMP (Fig. 2, *B* and *C*).

Transport of MTX Polyglutamates by ABCG2. MTX, a monoglutamate, is metabolized to γ-linked polyglutamates in the cell. To assess the consequences of polyglutamylation on MTX transport by ABCG2, the ability of membrane vesicles prepared from HEK293 cells transfected with ABCG2-R482 to mediate the transport of 100 μM concentrations of MTX-Glu_2, MTX-Glu_3, and MTX-Glu_4 was analyzed. These experiments showed that of the three polyglutamate species assayed, ABCG2 was competent in the transport of MTX diglutamate and MTX triglutamate (Fig. 3, *A–C*). However, the rates and extent of uptake of the MTX-Glu_2 and MTX-Glu_3 were reduced by comparison with the corresponding values for the parent compound. In striking contrast, uptake of MTX-Glu_4, when measured by the same procedure on the same membrane preparations, was not detected to any extent, and was indistinguishable from uptake observed for control membranes in the presence of either MgATP or MgAMP (Fig. 3*D*). As expected, transport of MTX polyglutamates was not detected for either of the ABCG2 mutants (data not shown).

These experiments suggested that the MTX transport properties of ABCG2 differ from those of several previously analyzed MRPs in that whereas the latter are able to transport MTX, they are unable to transport MTX diglutamate or MTX triglutamate (9, 11). To directly compare ABCG2 to an MRP family member that has been determined previously to be competent in the transport of MTX but not lower

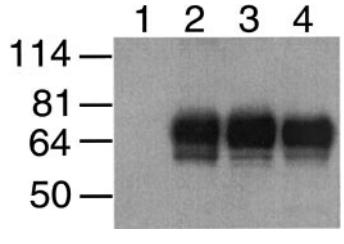

Fig. 1. Immunoblot detection of ABCG2 in membrane vesicles prepared from transfected cells. Membrane vesicles were prepared from HEK293 cells transfected with parental vector (*Lane 1*), ABCG2-R482 (*Lane 2*), ABCG2-R482G (*Lane 3*), and ABCG2-R482T (*Lane 4*). Protein (10 μg/lane) was resolved by SDS-PAGE on 8% gels, electrotransferred to nitrocellulose membranes and incubated with polyclonal ABCG2 antibody. The sizes of molecular weight standards (in kDa) are indicated. This experiment was repeated three times, and a representative blot is shown.

[4] R. W. Robey, *et al.*, Mutations at amino acid 482 in the ABCG2 gene affect substrate and antagonist specificity, submitted for publication.

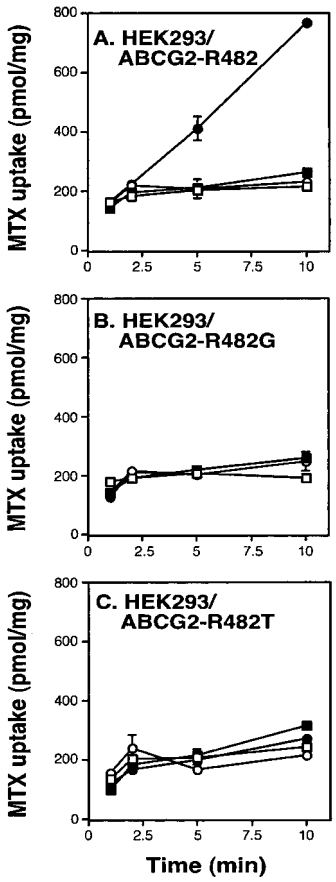

Fig. 2. Time course of ATP-dependent uptake of [^{3}H]MTX by ABCG2-R482, ABCG2-R482G, and ABCG2-R482T. Membrane vesicles (10 μg) prepared from HEK293 cells transfected with ABCG2-R482 (*A*), ABCG2-R482G (*B*), and ABCG2-R482T (*C*), and from parental plasmid-transfected control cells, were incubated at 37°C in uptake medium containing 100 μM [^{3}H]MTX and 4 mM MgATP (*solid symbols*) or 4 mM MgAMP (*open symbols*). *Circles* indicate ABCG2-enriched membrane vesicles, and *squares* indicate control (parental plasmid) membrane vesicles. Values shown are means of a measurement performed in duplicate; *bars,* ± SE. This experiment was performed at least three times, and a representative experiment is shown.

polyglutamyl MTX species, and to rule out the possibility that the measured transport of [^{3}H]MTX-Glu_2 and [^{3}H]MTX-Glu_3 by ABCG2 was the consequence of contamination of the latter compounds with unmodified [^{3}H]MTX, uptake experiments were performed simultaneously on membrane vesicles prepared from HEK293 cells transfected with ABCG2 or MRP3. These experiments supported the conclusion that transport of lower polyglutamyl MTX species is a property of ABCG2 but not MRP3. As shown in Fig. 4, uptake of [^{3}H]MTX by ABCG2 was comparable with that of MRP3 (Fig. 4*A*). However, by contrast with ABCG2, uptake of neither [^{3}H]MTX-Glu_2 nor [^{3}H]MTX-Glu_3 was detected for MRP3 (Fig. 4, *B* and *C*). As expected, uptake of [^{3}H]MTX-Glu_4 was not detected for either membrane vesicle preparation (Fig. 4*D*).

Kinetics of MTX Transport and Inhibition by Fumitremorgin C. The substrate concentration dependence of MgATP-energized [^{3}H]MTX uptake by membrane vesicles prepared from HEK293 cells transfected with wild-type ABCG2 approximated Michaelis-Menten kinetics (Fig. 5). When measured over a broad range of concentrations, the initial rates of MgATP-dependent uptake exhibited saturation kinetics. The results of four independent experiments yielded mean K_m and V_{max} values of 1.34 ± 0.18 mM and 687 ± 87 pmol/mg/min, respectively.

FTC, a mycotoxin, is one of the most active agents identified for reversing ABCG2-conferred cellular resistance to mitoxantrone, anthracyclines, and topotecan (24). As an additional test of the specificity of the measured transport for ABCG2, and to assess the potency of this agent as an *in vitro* inhibitor of the pump, the ability of FTC to inhibit MTX transport was analyzed. FTC was indeed a potent competitive inhibitor of [^{3}H]MTX transport by membranes prepared from ABCG2-transfected HEK293 cells (Fig. 5, *inset*). The K_i value determined from three independent experiments was 0.30 ± 0.03 μM.

Analysis of Transport of FA and Leucovorin by ABCG2. We reported previously that several members of the MRP family that are capable of transporting MTX are also competent in mediating the

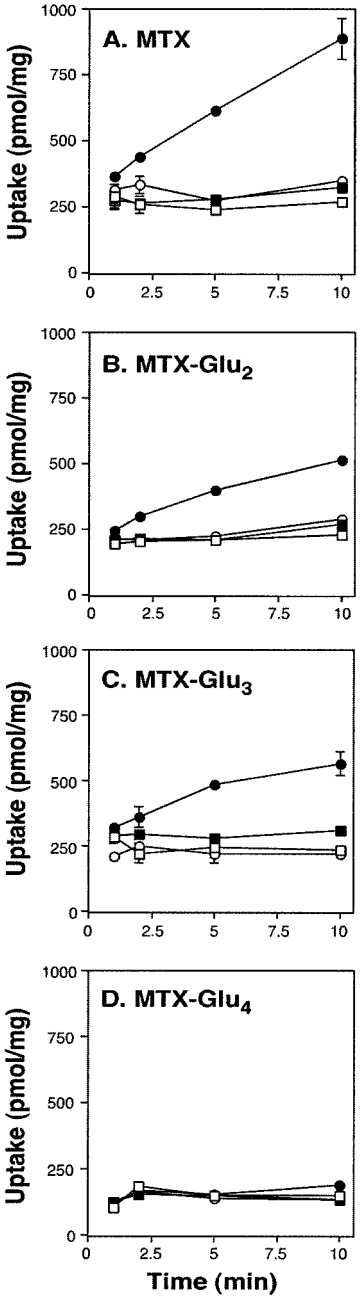

Fig. 3. Time course of ATP-dependent uptake of polyglutamylated MTX by ABCG2-R482. Membrane vesicles (10 μg) prepared from ABCG2-R482-transfected HEK293 cells (*circles*) or parental plasmid-transfected HEK293 cells (*squares*) were incubated at 37°C in uptake medium containing 100 μM [^{3}H]MTX (*A*), 100 μM [^{3}H]MTX-Glu_2 (*B*), 100 μM [^{3}H]MTX-Glu_3 (*C*), or 100 μM [^{3}H]MTX-Glu_4 (*D*). *Closed symbols,* uptake from medium containing 4 mM MgATP; *open symbols,* uptake from medium containing 4 mM MgAMP. Values shown are means of a measurement performed in duplicate; *bars*, ±SE. This experiment was performed at least three times, and a representative experiment is shown.

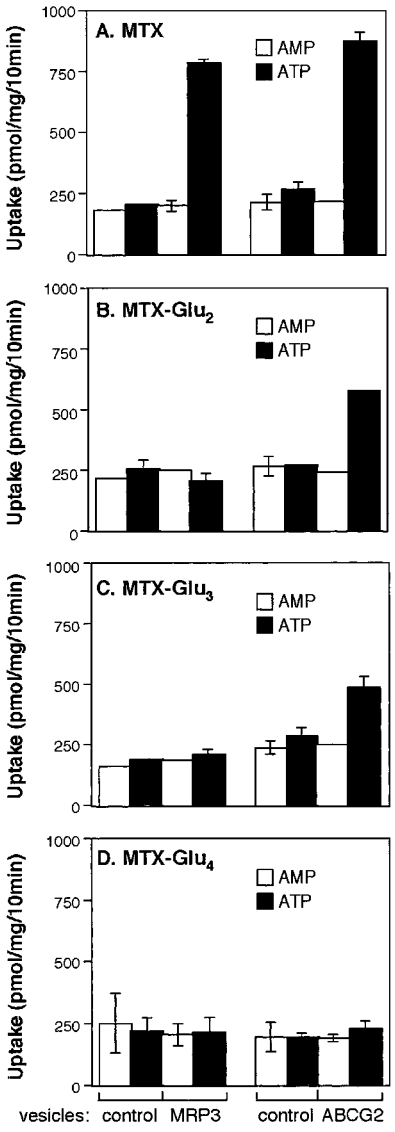

Fig. 4. Uptake of [^{3}H]MTX and [^{3}H]MTX polyglutamates by ABCG2-R482 and MRP3. Membrane vesicles (10 μg) prepared from MRP3-transfected HEK293 cells, the corresponding parental plasmid-transfected HEK293 cells, ABCG2-R482-transfected HEK293 cells, and the corresponding parental plasmid-transfected HEK293 cells, were incubated at 37°C for 10 min in uptake medium containing 100 μM [^{3}H]MTX (*A*), 100 μM [^{3}H]MTX-Glu$_2$ (*B*), 100 μM [^{3}H]MTX-Glu$_3$ (*C*), or 100 μM [^{3}H]MTX-Glu$_4$ (*D*). ■, uptake from medium containing 4 mM MgATP; □, uptake from medium containing 4 mM MgAMP. The vesicle preparations that correspond to the bars are indicated at the *bottom* of the figure. Values shown are means of a measurement performed in duplicate; *bars*, ±SE. This experiment was performed at least three times, and a representative experiment is shown.

transport of FA and the a reduced 1 carbon-bearing folate leucovorin (9, 11). Therefore, we determined whether ABCG2 is also competent in the transport of these two folates. The results of experiments in which uptake of FA was examined were in complete accord with the properties of ABCG2 as determined from the MTX transport experiments, in that whereas membrane vesicles prepared from wild-type ABCG2-transfected cells were able to catalyze the uptake of 100 μM [^{3}H]FA, uptake was not detected for R482G or R482T (Fig. 6, *A–C*). The initial uptake rate for the wild-type protein in the presence of MgATP was 87 pmol/mg/min, a value that was comparable with rates observed for MTX (Fig. 2*A*).

By contrast with [^{3}H]FA, uptake of 100 μM [^{3}H]leucovorin by ABCG2-R482 was not observed (Fig. 6*D*). In positive control experiments transport of [^{3}H]leucovorin was apparent for membranes prepared from HEK293 cells transfected with MRP3 (Fig. 6*E*), as reported previously (9), and for LLC/PK1 cells transfected with MRP2 (Fig. 6*F*), an MRP family member whose capacity to transport physiological folates has been inferred previously from experiments on canalicular membranes prepared from MRP2-deficient rats (25).

Transport of $E_2$17βG by ABCG2. Physiological substrates of ABCG2 have not been identified. Knowing that ABCG2 has been reported to be expressed in hepatocyte canalicular membranes (18), that experiments using canalicular membranes prepared from rats that are hereditarily deficient in MRP2 indicate that another unknown pump contributes to the hepatobiliary excretion of certain glucuronate conjugates (26, 27), and that transport of the prototypical glucuronide $E_2$17βG is a property of MRP family members that are able to transport MTX (10, 28–31), we examined the possibility that ABCG2 might be involved in the hepatobiliary extrusion of physiological glucuronides by determining whether $E_2$17βG is a transport substrate.

As shown in Fig. 7*A*, [^{3}H]$E_2$17βG was indeed subject to MgATP-dependent transport by wild-type ABCG2. Although MgATP-dependent uptake was also detected for the control membranes, an increment consequent on ABCG2 expression was observed consistently. In the presence of medium containing MgATP, uptake of 1.0 μM [^{3}H]$E_2$17βG by ABCG2-enriched membranes was 3.3 pmol/mg/min, whereas uptake by control membranes in the same medium was only 1.8 pmol/mg/min. By contrast, negligible uptake was observed in medium containing MgAMP for membranes prepared from either ABCG2-transfected HEK293 cells or parental vector-transfected HEK293 cells.

The concentration dependence of [^{3}H]$E_2$17βG transport by ABCG2 was analyzed. MgATP-dependent uptake for ABCG2-enriched membrane vesicles and control membrane vesicles, enumerated as the difference between uptake in medium containing MgATP and uptake in medium containing MgAMP, exhibited saturation kinetics (Fig. 7*B*). Nonlinear least squares fitting of the data to the Michaelis-Menten equation for four independent determinations yielded K_m and V_{max} values for ABCG2-enriched membranes and control membranes

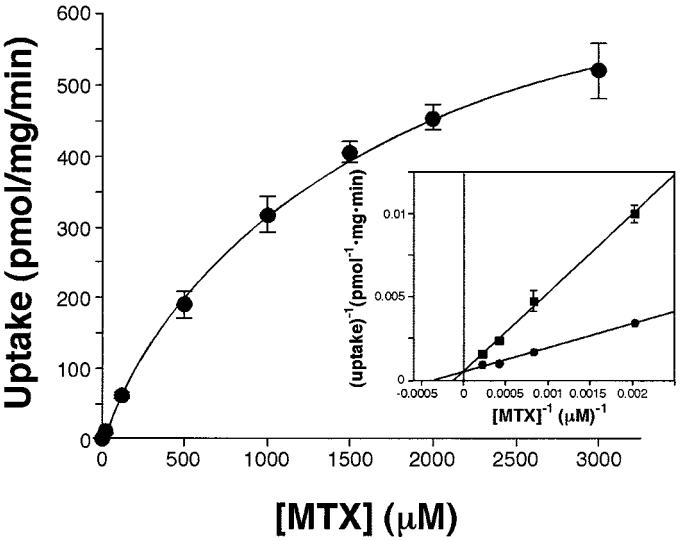

Fig. 5. Concentration dependence of [^{3}H]MTX uptake by ABCG2-R482 and inhibition by Fumitremorgin C. Concentration dependence of [^{3}H]MTX uptake: the rates of MgATP-dependent uptake of [^{3}H]MTX into membrane vesicles prepared from HEK293 cells transfected with ABCG2-R482 vector were measured for 10 min at 37°C in uptake medium containing 4 mM MgATP or 4 mM MgAMP. Values shown (means) are rates measured in the presence of MgATP minus rates measured in the presence of MgAMP for an experiment performed in duplicate; *bars*, ±SE. The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (50). *Inset,* inhibition of [^{3}H]MTX uptake by 1 μM Fumitremorgin C (square symbols). The experiments were repeated at least three times, and representative experiments are shown.

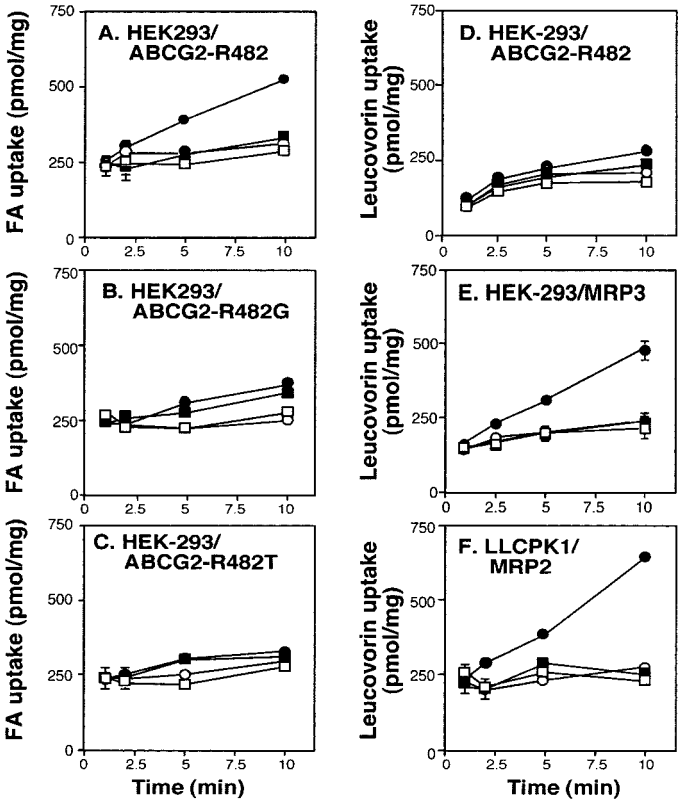

Fig. 6. Time course of ATP-dependent uptake of [^{3}H]FA and [^{3}H]leucovorin by ABCG2. Membrane vesicles (10 μg) prepared from HEK293 cells transfected with ABCG2-R482 (*A* and *D*), ABCG2-R482G (*B*), ABCG2-R482T (*C*), and MRP3 (*E*), from LLC/PK1 cells transfected with MRP2 (*F*), and from the respective parental plasmid-transfected counterparts, were incubated at 37°C in uptake medium containing 100 μM [^{3}H]FA (*A–C*) or 100 μM [^{3}H]leucovorin (*D–F*), and 4 mM MgATP (*solid symbols*) or 4 mM MgAMP (*open symbols*). The *circles* indicate ABCG2- (*A–D*), MRP3- (*E*), and MRP2-enriched (*F*) membrane vesicles, and the *squares* indicate control membranes prepared from the respective parental vector transfected cells. Values shown are means of a measurement performed in duplicate; *bars*, ±SE. This experiment was repeated at least three times, and a representative experiment is shown.

of 31.2 ± 6.1 μM and 119 ± 15 pmol/mg/min, and 18.2 ± 5.6 μM and 32.8 ± 3.2 pmol/mg/min, respectively. MgATP-dependent uptake attributable to ABCG2 was enumerated as the difference in uptake in medium containing MgATP between ABCG2-enriched membranes and control membrane (Fig. 7*C*). This yielded K_m and V_{max} values of 44.2 ± 4.3 μM and 103 ± 17 pmol/mg/min for ABCG2.

DISCUSSION

The susceptibility of MTX to intracellular polyglutamylation is crucial to its activity as a cytotoxic agent (32–34). In a reaction catalyzed by folylpoly-γ-glutamate synthetase, the condensation of successive glutamate residues to the γ-carboxyl group of MTX, a monoglutamate, yields MTX-Glu_{2-7} derivatives (35, 36). Polyglutamylated MTX is effluxed poorly from the cell, by comparison with the parent compound, which is subject to efflux via an energy-dependent process (37, 38). As a consequence, polyglutamylated MTX, which is at least as active as the parent compound in inhibiting folate requiring enzymes (39), exhibits prolonged intracellular retention and an associated massive enhancement in cytotoxicity. Therefore, the precise mechanism of efflux of this agent is of fundamental importance to its potency, and also has a bearing on the cellular physiology of endogenous folates in that their retention is similarly dependent on folylpoly-γ-glutamate synthetase-mediated polyglutamylation. Reports on several members of the MRP family provided the first insights into the long-standing issue of the identities of the cellular components responsible for efflux of MTX. The ability of certain MRPs to efflux this agent was inferred from studies showing that MRP2-deficient rats have reduced hepatobiliary clearance of MTX (25), and that cell lines transfected with MRP1, MRP2, MRP3, and MRP4 are resistant to this agent (12–14, 20). We investigated recently the MTX transport characteristics of these pumps in detail, and determined that they each satisfy a cardinal biochemical feature of previously characterized MTX efflux systems by demonstrating that whereas they are able to transport the parent compound, they have little or no activity toward polyglutamates (9, 11). The effect of polyglutamylation was profound in that MTX transport was completely abrogated by the addition of even one glutamyl residue.

In the experiments described here, we investigated the *in vitro* transport characteristics of ABCG2, a pump that is not a member of

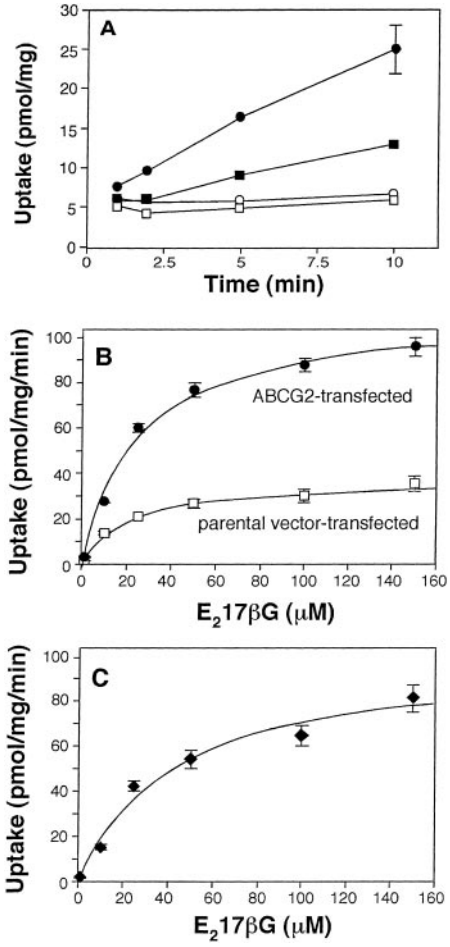

Fig. 7. Transport of $E_2$17β-G by ABCG2. *A,* time course of ATP-dependent uptake of [^{3}H]$E_2$17β-G by ABCG2-R482. Membrane vesicles (10 μg) prepared from HEK293 cells transfected with wild-type ABCG2 were incubated at 37°C in uptake medium containing 1 μM $E_2$17β-G and 4 mM MgATP (*solid symbols*) or 4 mM MgAMP (*open symbols*). *Circles* indicate ABCG2-enriched membrane vesicles, and *squares* indicate control (parental plasmid) membrane vesicles. The values shown are means of a measurement performed in duplicate; *bars*, ±SE. *B,* concentration dependence of [^{3}H]$E_2$17β-G uptake by ABCG2-enriched and control membrane vesicles. The rates of MgATP dependent uptake of [^{3}H]$E_2$17β-G into membrane vesicles prepared from ABCG2-transfected HEK293 cells (●) or vesicles prepared from parental plasmid-transfected cells (□) were measured for 5 min at 37°C in uptake medium containing 4 mM MgATP or 4 mM MgAMP. The values shown (means) are rates measured in the presence of MgATP minus rates measured in the presence of MgAMP for a measurement performed in triplicate; *bars*, ±SE. *C,* concentration dependence of $E_2$17β-G transport by ABCG2. The values shown are derived from *B* and represent rates measured in the presence of MgATP for ABCG2-transfected cells minus rates measured in the presence of MgATP for parental plasmid-transfected cells. The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (50). The experiments were repeated at least four times and representative experiments are shown.

the MRP family but of which the ability to transport MTX was inferred recently from studies on drug-resistant cell lines that overexpress ABCG2 (16, 17). In so doing, it has been determined that ABCG2 indeed satisfies the requirements predicted for components of this system. We show that ABCG2 has the facility for mediating the MgATP-energized transport of MTX with an affinity (K_m = 1.3 mM) that is comparable with the K_m values we reported for MRP1 and MRP3 (2.2 and 0.62 mM, respectively; Refs. 9, 11), and that, like the latter pumps, its activity is attenuated by polyglutamylation. However, the properties of ABCG2 are not exactly the same as those of characterized MRPs in at least two regards. Firstly, our results suggest that ABCG2 differs from MRP1, MRP2, MRP3, and MRP4, in that whereas MRPs are competent in the transport of FA and the reduced 1-carbon bearing folate leucovorin (9, 11), ABCG2 was able to transport FA but not leucovorin under the conditions used in our assays. Secondly, whereas MRPs are unable to efflux MTX diglutamate to any appreciable extent (9, 11), AGCG2 has the capacity to transport MTX species having up to three glutamate residues. Although efflux of lower polyglutamyl species of MTX has been described in a few reports (39–41), this property of ABCG2 is perhaps surprising in that despite these expanded *in vitro* capabilities for polyglutamate transport compared with MRPs, the pump appears to be a weak MTX resistance factor (2–3-fold), at least in continuous drug exposure assays (17),[5] and in that the cellular extrusion of MTX polyglutamates is not a generally accepted phenomenon. A potential explanation for the latter could be that studies on the cellular efflux of MTX polyglutamates did not use cells in which ABCG2 is endogenously expressed (and we note in passing that ABCG2 is expressed in at least two of the cell types in which efflux of lower polyglutamyl species has been reported, MCF7 cells[6] and hepatocytes; Ref. 18). Nevertheless, the facility of ABCG2 for affecting the cellular accumulation and efflux of polyglutamates requires detailed analysis.

The notion that R482 plays an important role in the substrate selectivity of ABCG2 was initially inferred from investigations of the properties of ABCG2-overexpressing cell lines (4). The effects of mutation of the wild-type R482 residue to threonine or glycine in human ABCG2, or to serine or methionine in the murine protein, appear to be similar: increased facility for conferring resistance to anthracyclines and for effluxing the fluorescent dye rhodamine 123, and reduced capacity to mediate resistance to camptothecins (4, 8). More recently this residue was implicated as a factor that determines the facility of the pump for mediating MTX resistance (17). The analysis of *in vitro* transport by wild-type and R482 variants described here provide direct support for the notion that this residue is important for substrate recognition by demonstrating that two R482 mutations completely abrogate transport of both MTX and FA. Interestingly, our results concerning MTX and FA, in combination with the results of studies on the phenotypic effects of these mutations, suggest that the mutations at R482 may result in reduced affinity of the pump for negatively charged substrates (*e.g.*, MTX and topotecan) and enhanced capabilities for compounds that are mildly positive (*e.g.*, doxorubicin, daunorubicin, and rhodamine 123). Given that the mutations involve substitutions of an uncharged amino acid (glycine, threonine, serine, or methionine) for a basic residue (arginine), it is tempting to speculate that this apparent shift in preference from anionic substrates to mildly positive substrates is consequent to the involvement of R482 in direct substrate binding.

The determination that ABCG2 is also able to mediate the transport of the glucuronide $E_2$17βG, together with a previous report indicating that it is also able to transport SN38-glucuronide (42), indicates that the substrate selectivity of the pump is unexpectedly broad with regard to amphipathic anions. In addition, $E_2$17βG transport, in combination with the capacity of the pump to transport anthracyclines, which are presumed to be substrates based on the drug resistance capabilities of the pump and the ability of doxorubicin to stimulate ABCG2 ATPase activity *in vitro* (43), indicates that substrate selectivity of ABCG2 has a considerable degree of overlap with MRP family members such as MRP1 and MRP2, for which $E_2$17βG, MTX, and anthracyclines are established substrates (44). The facility of ABCG2 for transporting $E_2$17βG, which is the first physiological substrate of the pump to be identified, may also provide insights into the functions of ABCG2 in the body. One proposed function of ABCG2 is protection of the fetus from xenobiotics present in the maternal circulation (45, 46). Analogous reasoning with regard to ABCG2-mediated transport of $E_2$17βG suggests the possibility that ABCG2 may function to protect the fetus from at least some types of endogenous steroids. That ABCG2 may be able to transport other steroids is suggested by a report showing that drug resistance in ABCG2-overexpressing cell lines can be modulated by estrone and 17β-estradiol (47). The ability of ABCG2 to transport $E_2$17βG, a compound that is extruded into the bile, in combination with a report showing that the pump is expressed on canalicular membranes of hepatocytes (48), also implicates ABCG2 in efflux of $E_2$17βG into the bile. However, ABCG2 probably does not play a major role in the hepatobiliary excretion of this particular compound, in that it has been inferred from studies on MRP2-deficient rats that MRP2 is the predominant canalicular pump for $E_2$17βG (49). Our measurements showing that the affinity of ABCG2 for $E_2$17βG (K_m = 44.2 μM) is ~6-fold lower than the value reported for human MRP2 (7.2 μM; Ref. 31) may provide one explanation for the apparently limited contribution of ABCG2 to this process. It is also possible that ABCG2 expression in canalicular membranes is low by comparison with MRP2. However, ABCG2 may contribute to the hepatobiliary excretion of other glucuronides whose extrusion into bile is not exclusively attributable to MRP2, and several such glucuronides have been described (26, 27). The identification of additional physiological substrates of ABCG2, as well as the analysis of ABCG2-deficient mice should help to determine whether the pump subserves these and/or other functions in the body.

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[5] M. G. B and G. D. K. unpublished observations.
[6] Z-S. C. and G. D. K., unpublished observations.

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