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Characterization of the Transport Properties of Human Multidrug Resistance Protein 7 (MRP7, ABCC10)

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ABSTRACT

Human multidrug resistance protein 7 (MRP7, ABCC10) is a recently described member of the C family of ATP binding cassette proteins (*Cancer Lett* **162:**181–191, 2001). However, neither its biochemical activity nor physiological functions have been determined. Here we report the results of investigations of the in vitro transport properties of MRP7 using membrane vesicles prepared from human embryonic kidney 293 cells transfected with MRP7 expression vector. It is shown that expression of MRP7 is specifically associated with the MgATP-dependent transport of 17β-estradiol-(17-β-D-glucuronide) ($E_2$17βG). $E_2$17βG transport was saturable, with K_m and V_{max} values of 57.8 $\pm$ 15 μM and 53.1 $\pm$ 20 pmol/mg/min. By contrast, with $E_2$17βG, only modest enhancement of LTC_4 transport was observed and transport of several other established substrates of MRP family transporters was not detectable to any extent. In accord with the notion that MRP7 has a bipartite substrate binding pocket composed of sites for anionic and lipophilic moieties, transport of $E_2$17βG was susceptible to competitive inhibition by both amphiphiles, such as leukotriene C_4 ($K_{i(app)}$, 1.5 μM), glycolithocholate 3-sulfate ($K_{i(app)}$, 34.2 μM) and MK571 ($K_{i(app)}$, 28.5 μM), and lipophilic agents such as cyclosporine A ($K_{i(app)}$, 14.4 μM). Of the inhibitors tested, LTC_4 was the most potent, in agreement with the possibility that it is a substrate of the pump. The determination that MRP7 has the facility for mediating the transport of conjugates such as $E_2$17βG indicates that it is a lipophilic anion transporter involved in phase III (cellular extrusion) of detoxification.

Investigations of members of the multidrug resistance family (MRP) have provided important insights into cellular resistance mechanisms associated with anticancer drugs, factors that influence drug distribution in the body, and cellular components that accomplish phase III detoxification (cellular extrusion) of compounds that are metabolized by the covalent addition of bulky anionic moieties. The MRP family consists of nine members (Bera et al., 2001; Hopper et al., 2001; Tammur et al., 2001; Yabuuchi et al., 2001), six of which have been characterized with regard to at least some of their functional properties (Borst et al., 2000; Kruh et al., 2001). MRP1, MRP2 (cMOAT), and MRP3 are MgATP-energized transporters of glutathione *S*-conjugates, such as leukotriene C_4 (LTC_4) and *S*-(2,4-dinitrophenyl)glutathione (DNP-SG), and glucuronate conjugates such as 17β-estradiol 17-(β-D-glucuronide) ($E_2$17βG) (Leier et al., 1994; Jedlitschky et al., 1996; Loe et al., 1996; Cui et al., 1999; Hirohashi et al., 1999; Kawabe et al., 1999; Zeng et al., 2000). However, differences in substrate range, subcellular localization, expression profiles and kinetic parameters of transport dictate distinct physiological functions for these three pumps. MRP1, which is widely expressed and localized at basolateral surfaces (Kruh et al., 1995; Flens et al., 1996; Evers et al., 2000), is distinguished from MRP2 and MRP3 by its higher affinity for LTC_4, a feature that is reflected in the specific role that MRP1 plays in mediating immune responses involving cellular export of this cystinyl leukotriene (Wijnholds et al., 1997; Robbiani et al., 2000). By contrast with MRP1, MRP2 is primarily expressed at canalicular (apical) surfaces of hepatocytes where it functions in the extrusion of endogenous organic anions such as bilirubin glucuronide and certain anticancer agents and in the provision of the biliary fluid constituent glutathione (Keppler and Kartenbeck, 1996). In

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ABBREVIATIONS: MRP, multidrug resistance protein (MRP1-MRP7, gene symbols ABCC1-ABCC6 and ABCC10); MOAT, multispecific organic anion transporter (MOAT-B, MOAT-C, MOAT-D and MOAT-E are alternative names for MRP4, MRP5, MRP3 and MRP6, respectively, and cMOAT is an alternative name for MRP2); LTC_4, leukotriene C4; DNP-SG, *S*-(2,4-dinitrophenyl)glutathione; $E_2$17βG, 17β-estradiol 17-(β-D-glucuronide); $E_2$3$SO_4$17βG, 17β-estradiol 3-sulfate-17-(β-D-glucuronide); ABC, ATP-binding cassette; HEK, human embryonic kidney; MK571, 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethyl-amino-3-oxopropyl)-thio}-methyl]thio)propanoic acid; PSC833, 3-oxo-4-butenyl-4-methyl-(Thr1)-(Val2)-cyclosporin.

addition to the transport of glutathione and glucuronate conjugates, MRP3 has the additional capability of mediating the transport of monoanionic bile acids (Hirohashi et al., 2000; Zeng et al., 2000). The latter feature, in combination with its induction at basolateral surfaces of hepatocytes and cholangiocytes under cholestatic conditions (Donner and Keppler, 2001; Soroka et al., 2001, and references therein), support the notion that it functions as a compensatory backup mechanism to eliminate from these cells potentially toxic compounds that are ordinarily excreted into the bile. With regard to drug-resistance capabilities, MRP1, MRP2, and MRP3 are able to confer cellular resistance to natural product agents to varying extents, and all three pumps are potent methotrexate resistance factors under conditions in which drug exposure is restricted to the first few hours of a 3- or 4-day growth assay (Borst et al., 2000; Kruh et al., 2001). Recent investigations of MRP4 and MRP5 indicate that they have the facility for mediating the transport of cyclic nucleotides, a property that has implicated the two pumps in the regulation of intracellular levels of these second messengers as well as in the cellular extrusion of cAMP involved in intercellular signaling (Jedlitschky et al., 2000; Chen et al., 2001; van Aubel et al., 2002). MRP4 also has the ability to transport conjugates such as $E_2 17\beta G$ and methotrexate (Chen et al., 2001, 2002). In accord with their capacity to transport cyclic nucleotides, MRP4 and MRP5 have the facility for conferring resistance to certain antiviral and anticancer nucleotide analogs but do not seem to be capable of effluxing natural product agents (Schuetz et al., 1999; Wijnholds et al., 2000; Chen et al., 2001; Lai and Tan, 2002). MRP6, whose hereditary deficiency results in pseudoxanthoma elasticum (Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000; Struk et al., 2000), a disease that affects elastic tissues in the skin, eyes, and cardiovascular system, has recently been determined to be competent in the transport of glutathione conjugates and the cyclic pentapeptide BQ123 (Madon et al., 2000; Belinsky et al., 2002; Ilias et al., 2002). However, the physiological transport substrate involved in the pathogenesis of pseudoxanthoma elasticum remains to be elucidated.

Recently, we characterized the predicted protein and expression pattern of MRP7 and determined, on the basis of amino acid sequence comparisons, that it is a member of the C branch of ABC transporters (Hopper et al., 2001), a family of proteins that includes both lipophilic anion pumps and regulators of ion channels. Phylogenetic analysis indicates that MRP7 is about as related to lipophilic anion pumps as it is to proteins involved in the regulation of ion channels (Hopper et al., 2001; Tammur et al., 2001), but nothing is currently known about the functional properties of the protein. Herein, we examine the biochemical activity of MRP7 by the analysis of MRP7-mediated transport in membrane vesicles prepared from transfected HEK293 cells. In so doing, it was demonstrated that MRP7 was able to catalyze the MgATP-energized transport of the glucuronide $E_2 17\beta G$. By comparison with $E_2 17\beta G$, only modest transport was observed for LTC_4, and transport of a range of other compounds that are established substrates of other MRP family members was not detected to any extent. The determination that MRP7 has the facility for mediating the transport of $E_2 17\beta G$ indicates that it is a lipophilic anion pump and a component of the energy-dependent efflux system involved in the cellular extrusion of lipophilic compounds that are metabolized by the covalent attachment of bulky anionic moieties.

Materials and Methods

Materials. [^{3}H]$E_2 17\beta G$ (40.5 Ci/mmol) and [^{3}H]LTC_4 (130 Ci/mmol) were purchased from PerkinElmer Life Science Products (Boston, MA). [^{3}H]cGMP (6.8 Ci/mmol), [^{3}H]cAMP (21.9 Ci/mmol), [^{3}H]methotrexate (21.2 Ci/mmol), and [^{3}H]folic acid (20.2 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [^{14}C]Glycocholic acid (0.056 Ci/mmol) was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). [^{3}H]Taurocholic acid (2.0 Ci/mmol) was purchased from PerkinElmer Life Sciences. Creatine kinase, creatine phosphate, ATP, AMP, $E_2 17\beta G$, LTC_4, cGMP, and cAMP were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). DNP-SG and [^{3}H]DNP-SG were synthesized from 1-chloro-2,4-dinitrobenzene and unlabeled or labeled [^{3}H]glycine-2-glutathione (44.8 Ci/mmol; PerkinElmer Life Sciences) as described previously (Awasthi et al., 1981). HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamine.

Preparation of MRP7-Transfected HEK293 Cells. The MRP7 coding sequence (GenBank accession number BAA9227; Hopper et al., 2001) was inserted into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA), and MRP7 expression vector and parental plasmid were introduced into HEK293 cells by electroporation. Individual colonies were selected in medium containing G418 (1000 μg/ml) and expanded for further analysis. Two colonies in which MRP7 protein was detected by immunoblot analysis were employed in the present study.

Generation of MRP7 Polyclonal Antibody and Immunoblot Analysis. A cDNA fragment encoding amino acids 2676 to 2982 of MRP7 was inserted into PGEX2T (Amersham Biosciences, Piscataway, NJ) and the resulting glutathione *S*-transferase fusion protein was purified using glutathione beads according to the manufacturer's recommendations. Rabbits were immunized with the purified recombinant protein, and the specificity of the resulting antiserum was confirmed in immunoblots of lysates prepared from insect cells expressing the full-length MRP7 protein.

Membrane vesicles preparations were analyzed by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, as described previously (Laemmli, 1970). Proteins were transferred to nitrocellulose filters using a wet transfer system as described previously (Towbin et al., 1979). MRP7 was detected using polyclonal antibody (1:500) 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 (Cornwell et al., 1986). Transport experiments were performed using the rapid filtration method essentially as described previously (Leier et al., 1994). Transport experiments were 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, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and radiolabeled substrate $\pm$ unlabeled substrate, in a total volume of 50 μl. Reactions were carried out at 37°C and stopped by the addition of 3 ml of 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 GVWP filters (Millipore, Bedford, MA) under vacuum. The filters were washed three times with 3 ml of ice-cold stop solution and dried at room temperature for 30 min. Radioactivity was measured by the use of a liquid scintillation counter. Rates of net ATP-dependent transport were determined by subtracting the values obtained in the presence of 4 mM AMP from those obtained in the presence of 4 mM ATP. Uptake rates were linear for up to 5 min and rates for concentration dependence experiments were measured at 5 min.

Data Analysis. Kinetic parameters were computed by nonlinear least-squares analysis (Marquardt, 1963) using the Ultrafit computer software (BioSoft, Ferguson, MO).

Results

Expression of Recombinant MRP7 in Membrane Vesicles Prepared from Transfected HEK293 Cells. HEK293 cells were transfected with MRP7 expression vector and two G418 resistant colonies in which the recombinant protein was detected, HEK-MRP7-C17 and HEK-MRP7-C18, were selected for characterization of MRP7 transport activity. MRP7-dependent transport activity was assayed on density-fractionated membrane vesicles prepared from these two cell lines and from HEK293 cells transfected with parental plasmid. As determined by immunoblot analysis, HEK-MRP7-C17 and HEK-MRP7-C18 membranes were a rich source of MRP7 protein, which migrates as an M_r ~171,000 electrophoretic species (Fig. 1). This apparent molecular mass is larger than the calculated molecular mass of MRP7 (~162 kDa) and the apparent molecular mass of the in vitro synthesized protein (M_r ~158,000) (Hopper et al., 2001), as would be expected for a glycosylated transmembrane protein.

Transport of $E_2$17βG and LTC_4 by MRP7. Glucuronate and glutathione conjugates are established substrates of several MRP family members (Borst et al., 1999; Kruh et al., 2001). To determine whether conjugates are substrates of MRP7, transport of $E_2$17βG, LTC_4 and DNP-SG, prototypical glucuronate and glutathione conjugates, were selected as model test compounds. Of these three compounds, robust uptake was observed only for $E_2$17βG (Fig. 2, A and B). When measured at an initial concentration of 5.0 μM and at the 5-min time point of the assay, [^{3}H]$E_2$17βG was taken up by HEK-MRP7-C17 and HEK-MRP7-C18 membranes at rates of 8.8 pmol/mg/min and 10.9 pmol/mg/min, respectively, from media containing MgATP, and at rates of only 4.4 and 3.7 pmol/mg/min from media containing MgAMP. Uptake rates of less than 3.8 pmol/mg/min from media containing either MgATP or MgAMP were observed for membranes prepared from HEK293 cells transfected with parental plasmid.

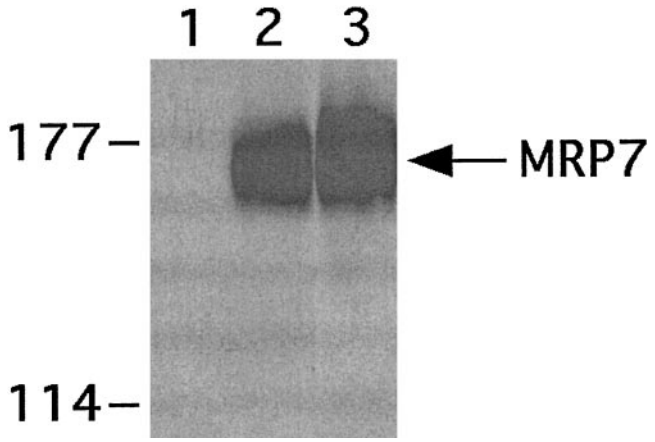

Fig. 1. Immunoblot detection of MRP7 in membrane vesicle preparations. Membrane vesicles were prepared from HEK293 cells transfected with parental plasmid (lane 1) or HEK-MRP7-C17 and HEK-MRP7-C18 (lanes 2 and 3, respectively). Protein (20 μg/lane) was resolved by SDS-polyacrylamide gel electrophoresis on 8% gels, electrotransferred to nitrocellulose membranes, and incubated with polyclonal MRP7 antibody. The sizes of molecular mass standards (in kilodaltons) are indicated. The arrow indicates MRP7 protein.

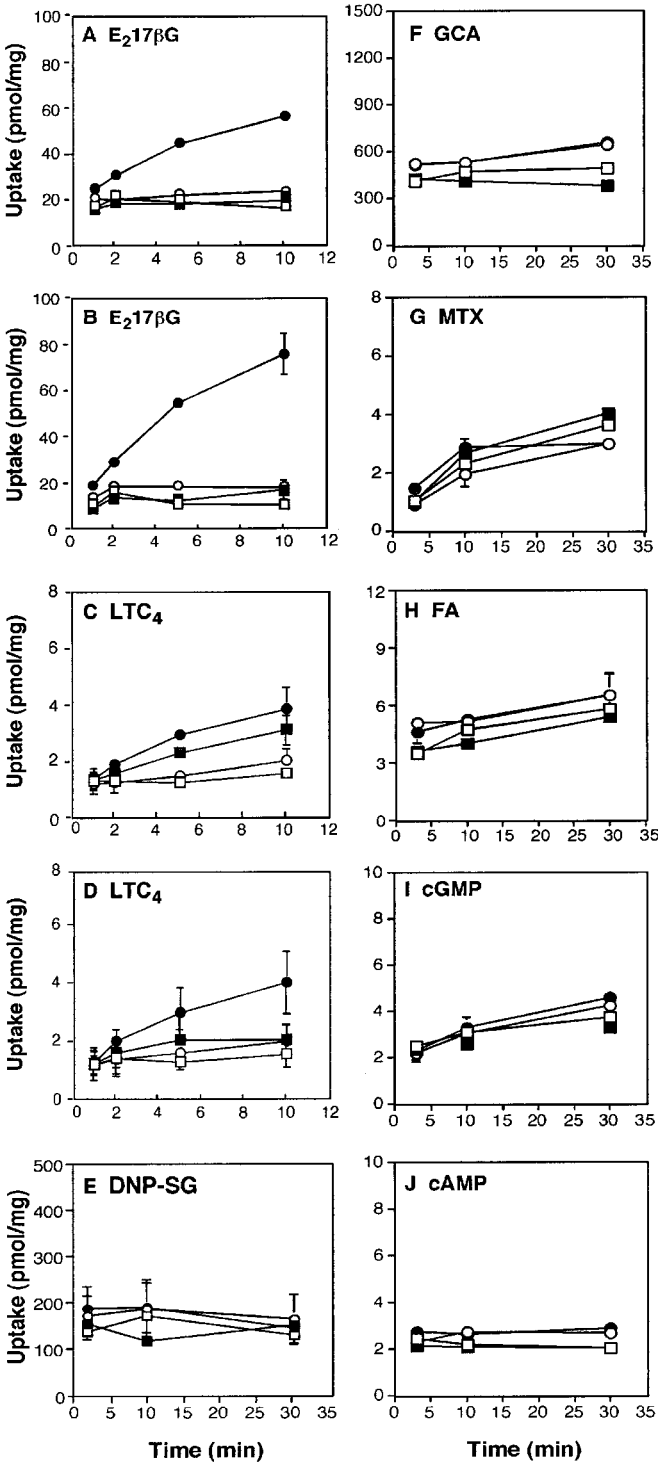

Fig. 2. Time course of MgATP-dependent uptake of [^{3}H]$E_2$17βG, [^{3}H]LTC_4, and other lipophilic anions into membrane vesicles. Membrane vesicles (10 μg) prepared from HEK-MRP7-C17 (A and C) and HEK-MRP7-C18 (B and D–J) (circles) or HEK293 cells transfected with parental plasmid (squares) were incubated at 37°C in uptake medium containing 5.0 μM [^{3}H]$E_2$17βG (A and B), 20 nM [^{3}H]LTC_4 (C and D), 10 μM [^{3}H]DNP-SG (E), 50 μM [^{14}C]glycocholic acid (F), 1.0 μM [^{3}H]methotrexate (G), 1.0 μM [^{3}H]folic acid (H), 1.0 μM [^{3}H]cGMP (I), and 1.0 μM [^{3}H]cAMP (J). Closed symbols, uptake from medium containing 4 mM MgATP; open symbols, uptake from medium containing 4 mM MgAMP. Representative experiments are shown, with the exception of C and D, which represent the data from five independent uptake experiments for [^{3}H]LTC_4. For other substrates, the values shown are means ± S.E. for duplicate determinations.

By contrast with [^{3}H]$E_2$17βG, for which uptake was consistently detected, uptake of the glutathione conjugate LTC_4 was observed in most but not all experiments, and the rate and extent of uptake were modest. The combined results of five independent uptake experiments for HEK-MRP7-C17 and HEK-MRP7-C18 membranes are shown in Fig. 2, C and D. Transport was not observed to any extent for the synthetic glutathione conjugate DNP-SG (Fig. 2E), nor for several other established substrates of MRP family members, including glycocholic acid, methotrexate, folic acid, cAMP, and cGMP (Fig. 2, F–J). Similarly, increased uptake of taurocholic acid was not observed (data not shown).

Osmotic Sensitivity of $E_2$17βG Transport by MRP7. The osmotic sensitivity of [^{3}H]$E_2$17βG uptake was examined to confirm that radiolabel retained by MRP7-enriched membrane vesicles represents transport of the substrate into the intravesicular compartment as opposed to nonspecific binding to the vesicles and/or filters. MgATP-dependent uptake of 5.0 μM [^{3}H]$E_2$17βG increased as a linear function of the reciprocal of the sucrose concentration of the uptake medium, indicating that transport was osmotically sensitive, as would be expected if the substrate were delivered into the intravesicular compartment (Fig. 3). By contrast, the sucrose concentration exerted only a moderate effect on substrate retention measured in medium containing MgAMP. The magnitude of the ordinate intercepts indicated that nonspecific substrate binding constituted roughly 27% of the radiolabel retained by MRP7-enriched membranes in media containing MgATP but as much as 57% of the radiolabel retained in media containing MgAMP.

Kinetics of $E_2$17βG Uptake by MRP7. The substrate concentration dependence of MgATP-energized [^{3}H]$E_2$17βG uptake by membrane vesicles prepared from HEK-MRP7-C18 exhibited saturation kinetics. When measured over a broad range of substrate concentrations, the initial rates of MgATP-dependent uptake of [^{3}H]$E_2$17βG approximated Michaelis-Menten kinetics (Fig. 4). Nonlinear least-squares fitting of the data to the Michaelis-Menten equation for three independent determinations yielded K_m and V_{max} values of 57.8 ± 15 μM and 53.1 ± 20 pmol/mg/min, respectively.

Inhibition of MRP7-Mediated Transport of $E_2$17βG. To gain further insight into the substrate selectivity of the pump, the capacity of a variety of compounds to inhibit MRP7-mediated transport of $E_2$17βG was examined. From these experiments, it was determined that both amphiphiles and uncharged lipophilic compounds were good inhibitors, as might be expected for a lipophilic anion transporter.

In the first instance, several amphipathic anions that are established substrates of MRP family members, including LTC_4, DNP-SG, methotrexate, and cAMP were analyzed (Table 1). The inhibitions exerted by these compounds were in accord with the uptake experiments (Fig. 2), in that LTC_4, for which some degree of transport, albeit modest, was detected, was the single most potent inhibitor (59.6% inhibition at 1 μM) of all of the compounds tested, whereas methotrexate and cAMP, compounds for which transport was not observed, were weak inhibitors (26 and 5.0% inhibition, respectively, at 100 μM). Although transport of DNP-SG was not observed in time-dependent uptake experiments (Fig. 2), this compound was a good inhibitor (53.6% inhibition at 10 μM), consistent with the notion that some glutathione conjugates may be transport substrates of the pump.

Next, several inhibitors of MRP family members and P-glycoprotein were tested. Of the MRP1 inhibitors examined, sulfinpyrazone, a general inhibitor of organic anion transporters, and MK571, which structurally resembles LTC_4,

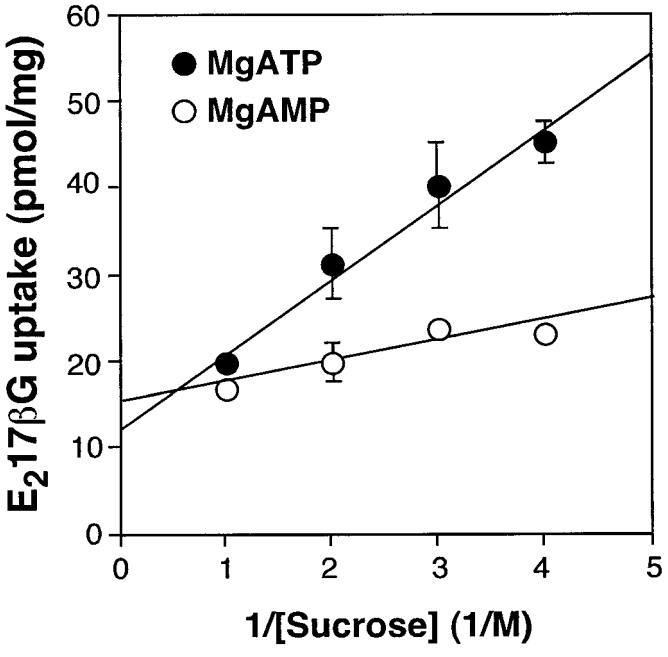

Fig. 3. Osmotic sensitivity of [^{3}H]$E_2$17βG uptake by MRP7. Membrane vesicles (10 μg) prepared from HEK-MRP7-C18 were preincubated in uptake medium containing 0.25 to 1.0 M sucrose for 5 min before measuring uptake of 5.0 μM [^{3}H]$E_2$17βG at 37°C in medium containing 4 mM MgATP (●) or 4 mM MgAMP (○). Uptake was measured at 5 min. Values shown are means ± S.E. for duplicate determinations.

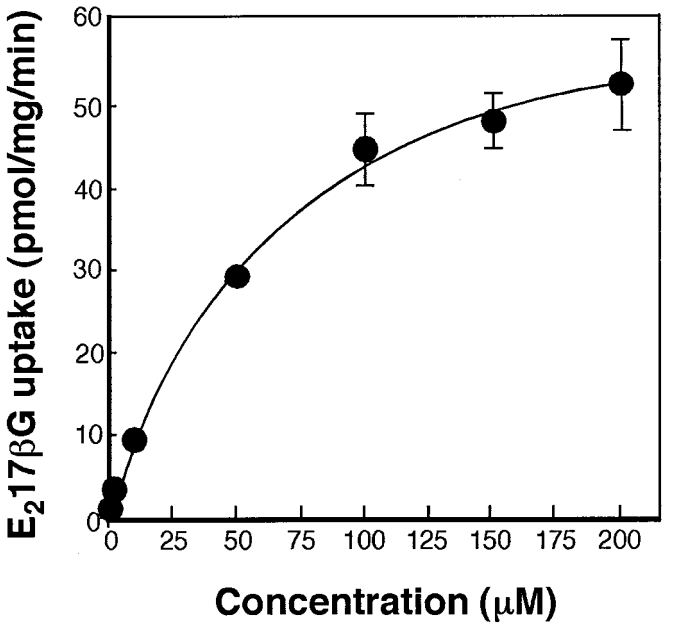

Fig. 4. Concentration dependence of [^{3}H]$E_2$17βG uptake by MRP7. The rates of MgATP-dependent uptake of [^{3}H]$E_2$17βG into membrane vesicles (10 μg) prepared from HEK-MRP7-C18 were measured at 37°C at various concentrations (0.4–200 μM). Values shown (means ± S.E.) are rates measured in the presence of MgATP minus rates measured in the presence of MgAMP for duplicate determinations. Uptake rates were measured at 5 min. The lines of best fit and kinetic parameters were computed by nonlinear least-squares analysis (Marquardt, 1963). A representative experiment is shown.

exhibited significant activities (60.5 and 57.8% inhibition at 30 μM). Probenecid however, was a weak inhibitor (11.2% inhibition at 100 μM). Phosphodiesterase inhibitors have recently been reported to be potent inhibitors of MRP5 (Jedlitschky et al., 2000). This class of compounds exerted a moderate degree of inhibition, in that 100 μM concentrations of sildenafil, trequinsin, and zaprinast exerted 59 to 51.7% inhibition. Of the three Pgp inhibitors tested, only cyclosporine A was a potent inhibitor (50.4% at 10 μM), whereas both verapamil (37.7% inhibition at 100 μM) and PSC833 (4.6% inhibition at 10 μM) were weak by comparison.

To probe the structural requirements for transport of $E_2 17\beta G$, a series of conjugated and unconjugated estrogens were examined. These compounds (100 μM) exerted roughly comparable degrees of inhibition (39.3–54.9%) regardless of whether they were unsubstituted (17β-estradiol; 39.3%), D-ring glucuronides [16α,17β-estriol-16-(β-D-glucuronide) and $E_2 3SO_4 17\beta G$, 47.0 and 54.9%, respectively], A-ring glucuronides [16α,17β-estriol 3-(β-D-glucuronide) and 17β-estradiol 3-(β-D-glucuronide), 41.5 and 39.9%, respectively], or derivatives with nonglucuronide substituents (17α-ethynyl-17β-estradiol and 16α,17β-estriol 3-sulfate, 48.6 and 50.6%, respectively). Glucuronic acid itself at concentrations of up to 1 mM did not exert appreciable inhibition (1.3%). Three bile acids were next examined. The inhibitions exerted by the monoanionic bile acids taurocholate and glycocholate (55.5 and 41.6%, respectively, at 100 μM), were roughly comparable with the estrogens. However, the dianionic bile acid glycolithocholate-3-sulfate was a more potent inhibitor (58.2% at 30 μM, 18.9% at 100 μM; Table 1 and data not shown) than either the monoanionic bile acids or the series of estrogens.

Finally, to gain insight into the pump's potential for conferring resistance to natural product anticancer agents, four members of this class of compounds were examined. Several of these agents were surprisingly potent, in that 30 μM concentrations of vincristine, paclitaxel, and doxorubicin exerted inhibitions of 81.6, 77.9, and 81.8%. Etoposide was less potent by comparison (50.5% inhibition at 30 μM) and the inhibition exerted by cisplatin, an alkylating agent that is not thought to be a substrate of MRPs in its unmodified form, was barely discernible (1.3% at 30 μM).

TABLE 1

Inhibition of MRP7-mediated transport of $E_2 17\beta G$ Membrane vesicles prepared from HEK-MRP7-C18 were incubated at 37°C for 5 min in medium containing 1 μM [^{3}H]$E_2 17\beta G$ in the presence or absence of the indicated compounds

ATP-dependent uptake was calculated by subtracting values obtained in the presence of 4 mM MgATP from those in the presence of 4 mM MgAMP. Transport is expressed as percent of uptake in the absence of inhibitor. Values shown are means ± S.E. of at least three measurements performed in duplicate. The concentrations of cyclosporin A and PSC833 were limited by their solubilities.

Inhibitor	Concentration	Uptake
	μM	*% of control*
Control		100
LTC_4	1	40.4 ± 2.1
DNP-SG	10	46.4 ± 6.6
Methotrexate	100	74.0 ± 9.5
cAMP	100	95.0 ± 0.9
Sulfinpyrazone	30	39.5 ± 6.9
MK571	30	42.2 ± 7.9
Probenecid	100	88.8 ± 10
Cyclosporin A	10	49.6 ± 5.6
PSC833	10	95.4 ± 9.5
Verapamil	100	62.3 ± 11
Sildenafil	100	41.0 ± 3.7
Trequinsin	100	44.3 ± 8.6
Zaprinast	100	48.3 ± 7.5
17-βEstradiol	100	60.7 ± 5.9
16α,17β-Estriol 16-(β-d-glucuronide)	100	53.0 ± 6.4
$E_2 3SO_4 17\beta G$	100	45.1 ± 10.
16α,17β-Estriol 3-(β-d-glucuronide)	100	58.5 ± 13
17β-Estradiol 3-(β-d-glucuronide)	100	60.1 ± 9.7
17α-Ethynyl-17β-estradiol	100	51.4 ± 10
16α,17β-Estriol 3-sulfate	100	49.4 ± 6.4
Glucuronic acid	1000	98.7 ± 4.7
Taurocholate	100	44.5 ± 9.8
Glycocholate	100	58.4 ± 5.7
Glycolithocholate-3-sulfate	30	41.8 ± 5.9
Vincristine	30	18.4 ± 4.2
Paclitaxel	30	22.1 ± 5.4
Etoposide	30	49.5 ± 8.0
Doxorubicin	30	18.2 ± 6.5
Cisplatin	30	98.7 ± 8.0

Analysis of Inhibition of MRP7-Mediated Transport by LTC_4, MK571, Cyclosporine A, and Glycolithocholate-3-sulfate. The mechanism of inhibition was analyzed for three amphipathic anions—LTC_4, MK571, and glycolithocholate-3-sulfate—and for cyclosporine A. Lineweaver-Burk plots of $E_2 17\beta G$ uptake by HEK-MRP7-C18 membrane vesicles in the presence and absence of these compounds indicated that all four behaved as competitive inhibitors (Fig. 5). The $K_{i(app)}$ values yielded from double reciprocal plots indicated that LTC_4 was a potent inhibitor ($K_{i(app)}$ 1.5 μM). The $K_{i(app)}$ values for the other three compounds were ~10- to 23-fold higher than LTC_4 and fell in the rank order cyclosporine A ($K_{i(app)}$ = 14.4 μM) > MK571 (28.5 μM) > glycolithocholate-3-sulfate (34.2 μM).

Discussion

In a prior study, we assigned MRP7, on the basis of amino acid alignments, to the C family of ABC transporters (Hopper et al., 2001). This family is composed of both established lipophilic anion pumps (MRPs 1–6), the cystic fibrosis transmembrane conductance regulator (ABCC7) chloride channel, and proteins that regulate the activity of ion channels, such as SUR1 (ABCC8). Although we designated MRP7 as an MRP, analysis of the phylogenetic relationships among C family members indicates that MRP7 is about equally related to the members of an evolutionary branch in which both lipophilic anion pumps (MRPs 1–3 and 6) and ion channel regulators reside (SUR1, SUR2) (Hopper et al., 2001; Tammur et al., 2001). Hence, whether MRP7 functions as a lipophilic anion pump or is involved in the regulation of ion channels has been an open question. In the present study, the in vitro properties of human MRP7 were investigated to gain insight into its biochemical activity and potential physiological functions. The results showing that MRP7 is able to mediate the transport $E_2 17\beta G$, and to a lesser extent LTC_4, provide the first evidence that this protein indeed functions as a lipophilic anion transporter and is in accord with its inclusion in the MRP family.

With regard to the facility for the MgATP-energized transport of $E_2 17\beta G$, MRP7 is similar to MRP1, MRP2, MRP3, and MRP4, for which this compound is an established transport substrate (Table 2). However, there are considerable differences in the affinities of these pumps for $E_2 17\beta G$. MRP7 has the lowest affinity (K_m = 57.8 μM), MRP2, MRP3, and MRP4 have intermediate affinities (K_m = 7.2, 25.6, and 30.3 μM, respectively), and MRP1 has the highest affinity (K_m = 1.5/

2.5 μM) (Jedlitschky et al., 1996; Loe et al., 1996; Cui et al., 1999; Zeng et al., 2000; Chen et al., 2001). In addition to its lower affinity for $E_2 17\beta G$, MRP7 differs from other MRPs that have the facility for transport of conjugates with regard to its substrate range (Table 2). MRP1, MRP2, and MRP3 are able to transport glutathione conjugates such as LTC_4 and DNP-SG, methotrexate and folates, and, in the case of MRP3, monoanionic bile acids are also substrates (Leier et al., 1994; Hirohashi et al., 1999; Kawabe et al., 1999; Hirohashi et al., 2000; Zeng et al., 2000, 2001; Chen et al., 2002). Of these three classes of compounds, MRP7-mediated transport was detected only for glutathione conjugates (LTC_4); even in this case, transport was modest under the experimental conditions employed. MRP7 also differs from MRP4 and MRP5 in that the latter pumps are able to transport cyclic nucleotides (Jedlitschky et al., 2000; Chen et al., 2001). An additional difference between MRP7 and MRP4 is that the latter pump also has the facility for mediating the transport of methotrexate and folates (Chen et al., 2002). Finally, the substrate range of MRP7 is distinct from that of MRP6, for which transport of glutathione but not glucuronate conjugates was reported (Belinsky et al., 2002; Ilias et al., 2002).

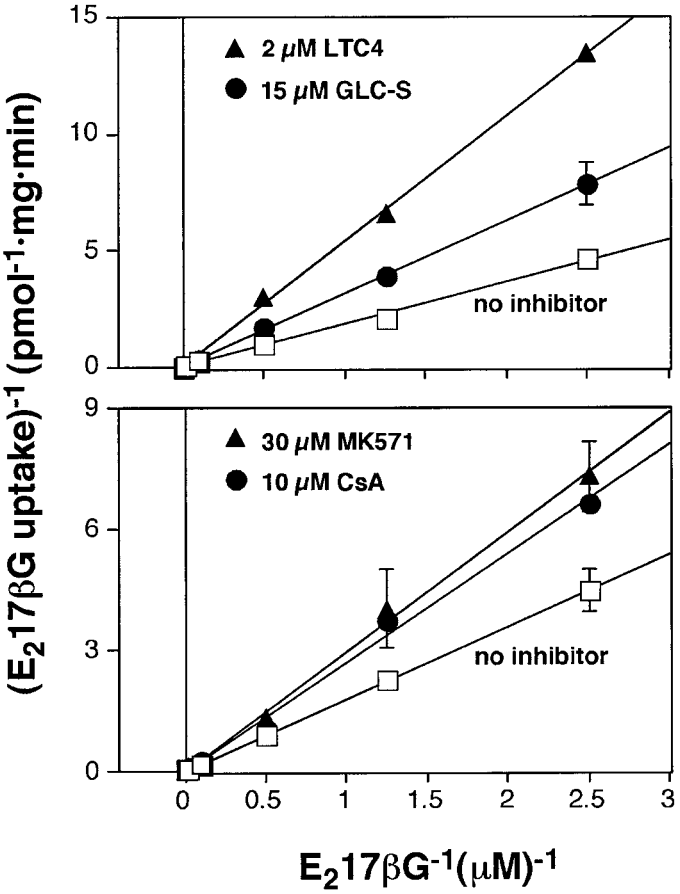

Fig. 5. Effect of LTC_4, glycolithocholate-3-sulfate, MK571, and cyclosporine A on [^{3}H]$E_2 17\beta G$ uptake by MRP7. The rates of MgATP-dependent uptake of [^{3}H]$E_2 17\beta G$ into membrane vesicles (10 μg) prepared from HEK-MRP7-C18 were measured at 37°C at various substrate concentrations (0.4–200 μM) in the absence (□, A) or presence of 2.0 μM LTC_4 (▲, A) and 15 μM glycolithocholate-3-sulfate (●, A), or the absence (□, B) or presence of 30 μM MK571 (▲, B) and 10 μM cyclosporine A (●, B). K_i values were determined from the double reciprocal plots. Values shown (means ± S.E.) are rates measured in the presence of MgATP minus rates measured in the presence of MgAMP for duplicate determinations. Representative experiments are shown.

Potential insights into the substrate binding pocket of MRP7 were provided by the analysis of inhibitors of $E_2 17\beta G$ transport. The results showing that either amphipathic anions or uncharged lipophilic molecules are good inhibitors of MRP7 are in accord with the notion, inferred from the finding that the pump transports glucuronate and glutathione conjugates, that the binding pocket of MRP7 has specific sites for both lipophilic and negatively charged ligands. Among a broad range of compounds examined, LTC_4 was by far the most potent inhibitor ($K_{i(app)}$ = 1.5 μM), consistent with the results of our uptake experiments, which suggested that it is a transport substrate of the pump. Two other amphiphiles, the leukotriene D_4 receptor antagonist MK571 and the dianionic bile acid glycolithocholate 3-sulfate, were also among the most potent inhibitors tested ($K_{i(app)}$ = 28.5 and 34.2 μM, respectively) but were ≥20-fold less potent than LTC_4. In addition, cyclosporine A, an uncharged lipophilic compound, exhibited significant inhibitory activity ($K_{i(app)}$ = 14.4 μM). These results suggest both similarities and differences between the MRP7 binding pocket and that of MRP1, in that LTC_4 and glycolithocholate 3-sulfate have been reported to be potent inhibitors of MRP1-mediated transport of $E_2 17\beta G$ (Loe et al., 1996). However, whereas the inhibition exerted by LTC_4 on MRP7-mediated transport was in the range of the effect reported for MRP1 ($K_{i(app)}$ = 0.53 μM), the latter transporter was ~24-fold more susceptible to inhibition by glycolithocholate 3-sulfate ($K_{i(app)}$ = 1.4 μM) compared with MRP7. Analysis of the effects of estrogens on transport by MRP7 revealed another striking difference compared with MRP1. We found that estrogen glucuronides such as $E_2 3SO_4 17\beta G$ were not par-

TABLE 2

Summary of amphipathic anion transport by human MRP family members

The data from several reports describing membrane vesicle transport assays are summarized (Leier et al., 1994; Jedlitschky et al., 1996, 2000; Loe et al., 1996; Chen et al., 1999, 2002; Cui et al., 1999; Kawabe et al., 1999; Zeng et al., 2000; Chen et al., 2001; Belinsky et al., 2002; Ilias et al., 2002). Membrane vesicle assays of methotrexate transport by MRP5 have not been reported. However, it is inferred from the results of the drug sensitivity analysis of MRP5-transfected cells that this compound is not a transport substrate (Wijnholds et al., 2000). Empty spaces denote compounds for which transport by the indicated pumps has not been analyzed in the literature.

Transporter	Substrate				
	Glutathione Conjugates	Glucuronate Conjugates	Monoanionic Bile Acids	Cyclic Nucleotides	Methotrexate
MRP1	+	+			+
MRP2	+	+			+
MRP3	+	+	+		+
MRP4	±	+		+	+
MRP5	−	−		+	−
MRP6	+	−		−	−
MRP7	+	+	−	−	−

−, transport not detected; +, transport detected.

ticularly good inhibitors, and were comparable in activity to a variety of other substituted and unsubstituted estrogens. By contrast, D-ring glucuronides such as $E_2 3SO_4 17\beta G$ were reported to be very potent inhibitors of $E_2 17\beta G$ transport by MRP1, with a $K_{i(app)}$ value (1.4 μM) roughly comparable with the magnitude of the inhibition exerted by LTC_4 on either MRP7 (Table 1) or MRP1-mediated transport (Loe et al., 1996). This difference in susceptibility to inhibition by certain estrogen glucuronides may reflect the ~30-fold higher affinity of MRP1 for $E_2 17\beta G$ (K_m = 1.5–2.5 μM) compared with MRP7 (K_m = 57.8 μM).

Analysis of the effects of P-glycoprotein inhibitors on MRP7-mediated transport of $E_2 17\beta G$ revealed an interesting similarity between this pump and MRP2 and MRP3. Our measurements indicate that whereas cyclosporine A is a good inhibitor of MRP7, two other P-glycoprotein inhibitors, PSC833 and verapamil, were quite weak by comparison. A similar pattern of inhibition has been described for MRP2 and MRP3; we found that cyclosporine A, but not PSC833 or verapamil, was a good inhibitor of MRP3-mediated transport of methotrexate and that cyclosporine A, but not PSC833, was reported to be a good inhibitor of LTC_4 transport by MRP2 (Chen et al., 1999; Zeng et al., 2001). If MRP7 is capable of functioning as a drug efflux pump, which is a distinct possibility in view of its susceptibility to inhibition by a variety of natural product anticancer agents (Table 1), it will be of interest to determine whether cyclosporine A and other inhibitors we tested, such as MK571 and sulfinpyrazone, can also function as resistance modulators.

The determination that $E_2 17\beta G$ is a substrate of MRP7 indicates that this pump represents one of at least five MRP family members that function in phase III (cellular extrusion) of detoxification of compounds that have been metabolized by the covalent addition of glucuronic acid. In view of the panoply of UDP glucuronosyl transferases that have been identified and the broad range of endogenous compounds and xenobiotics that are metabolized by these enzymes (King et al., 2000), it is perhaps not surprising that cells can deploy several different pumps for effluxing these conjugates. It is also important to bear in mind that relatively few glucuronate conjugates have been analyzed with regard to their susceptibility to transport by MRPs and that as more conjugates are characterized, distinguishing features among these pumps may emerge. Additional insights into the physiological functions of MRP7 will require a better understanding of its substrates, tissue-specific expression pattern, and subcellular distribution. At present, human MRP7 expression has been analyzed mainly at the transcript level. Previously, using an reverse transcription-polymerase chain reaction assay, we detected MRP7 expression in a range of human tissues (Hopper et al., 2001). However MRP7 transcript was not detected by Northern blot analysis in this initial analysis, suggesting the possibility that its expression is modest in many of the tissues analyzed. In the case of murine MRP7, the highest levels of transcript were detected in heart, liver, skeletal muscle, and kidney (Kao et al., 2002). The availability of MRP7 immunological reagents and transfected cell lines should facilitate the analysis of the tissue specific expression of the protein as well as the determination of whether the pump has the facility for conferring resistance to anticancer agents.

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