Transport of Amphipathic Anions by Human Multidrug Resistance Protein 3

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ABSTRACT

The multidrug resistance-associated protein 1 (MRP1) and the canalicular multispecific organic anion transporter (cMOAT or MRP2) are ATP-binding cassette transporters that confer resistance to some anticancer drugs and efflux glutathione and glucuronate conjugates from the cell. The MRP subfamily of ABC transporters, however, contains at least four other members of which MRP3 (MOAT-D) bears the closest structural resemblance to MRP1. Although transfection studies have established that human MRP3 confers increased resistance to several anticancer agents, neither the substrate selectivity nor physiological functions of this transporter have been determined. Here we report the results of investigations of the in vitro transport properties of cloned human MRP3 using membrane vesicles prepared from MRP3-transfected HEK293 cells. It is shown that the expression of MRP3 is specifically associated with enhancement of the MgATP-dependent transport into membrane vesicles of the glucuronide estradiol 17β-D-glucuronide (E₂17βG), the glutathione conjugates 2,4-dinitrophenyl-S-glutathione (DNP-SG) and leukotriene C₄ (LTC₄), the antimitabolite methotrexate, and the bile acid glycocholate. DNP-SG, LTC₄, and E₂17βG are transported at moderate affinity and low capacity with K_m and V_max values of 5.7 ± 1.7 μM and 3.8 ± 0.1 pmol/mg/min, 5.3 ± 2.6 μM and 20.2 ± 5.9 pmol/mg/min, and 25.6 ± 5.4 μM and 75.6 ± 5.9 pmol/mg/min, respectively. Methotrexate and glycocholate are transported at low affinity and high capacity with K_m and V_max values of 776 ± 319 μM and 288 ± 54 pmol/mg/min and 248 ± 113 μM and 183 ± 34 pmol/mg/min, respectively. On the basis of these findings, the osmotic dependence of the transport measured and its inability to transport taurocholate, MRP3, like MRP1 and cMOAT, is concluded to be competent in the transport of glutathione S-conjugates, glucuronides, and methotrexate, albeit at low to moderate affinity. In contrast to MRP1, cMOAT, and all other characterized mammalian ABC transporters, however, MRP3 is active in the transport of the monoanionic human bile constituent glycocholate.

INTRODUCTION

Cellular resistance to anticancer agents is a significant problem in treating cancer patients. One well-established resistance mechanism involves expression of Pgp, an ATP-binding cassette transporter that functions to diminish intracellular drug concentrations (1, 2). Because the spectrum of drugs to which Pgp confers resistance is broad and includes several natural products such as anthracyclines, Vinca alkaloids, epipodophyllotoxins, and taxanes, the drug resistance phenotype associated with Pgp has been termed multidrug resistance. Multidrug resistance is not solely attributable to Pgp, however. Soon after the identification of Pgp, studies using peptide antisera directed at a conserved nucleotide binding fold epitope of Pgp disclosed a distinct M₉ 190,000 transporter in an anthracycline-resistant cell line that exhibited multidrug resistance and a drug accumulation defect but did not overexpress Pgp (3). MRP1, the cDNA that encodes this M₉ 190,000 protein, has been isolated (4) and shown in transfection studies to be capable of conferring a multidrug-resistant phenotype (5, 6). Unlike Pgp, which predominantly transports neutral or weakly cationic lipophilic compounds, MRP1 is a lipophilic anion transporter. Compounds known to be transported by MRP1 include glutathione S-conjugates, such as LTC₄ and DNP-SG, glucuronate conjugates such as E₂17βG, glutathione disulfide, and sulfated diatomic bile conjugates such as 3α-sulfatolithocholyltaurine (7–11).

Another anion transporter related to MRP1, cMOAT (or MRP2), has since been isolated (12–14) and demonstrated in experiments using the cloned human, rat, and rabbit cDNAs to share the biochemical substrate selectivity of MRP1 with regard to glutathione and glucuronate conjugates (15–20). cMOAT, however, differs from MRP1 in its tissue distribution. Whereas MRP1 is expressed in a broad range of tissues (21), cMOAT is primarily expressed in liver canalicular membranes, where it functions as an apical anion efflux pump (22). In mutant TR- and Eisai hyperbilirubinemic rats and in humans with Dubin-Johnson syndrome, hereditary defects in cMOAT are characterized by mild elevations in conjugated bilirubin and the inability to excrete certain exogenous organic anions (13, 14, 23–25). Transfection studies indicate that cMOAT is capable of conferring resistance to anticancer agents. However, its drug resistance profile is distinct from that of MRP1 (19, 20, 26).

As first suspected from the results of biochemical investigations (27) and confirmed by analyses of expressed sequence tags and other partial sequences, it is now clear that there are several human MRP subfamily members in addition to MRP1 and cMOAT (28–30). At the time of writing, the complete coding sequences and putative topologies have been determined for four MRP subfamily members from humans: MRP3 (MOAT-D, cMOAT2); MRP4 (MOAT-B); MRP5 (MOAT-C, SMRP); and MRP6 (MOAT-E, ARA; Refs. 31–37), and two from rat: MRP3 and MRP6 (38). Of these, MRP3 bears the closest resemblance to MRP1 in terms of overall sequence alignment (58% overall amino acid identity; 71 and 74% identity in its first and second nucleotide binding folds, respectively) and its possession of the third (NH₃-terminal) membrane-spanning domain also found in cMOAT and MRP6 but not in MRP4 and MRP5 (33).

The functional characteristics of human MRP3 have yet to be defined in any detail. The ability of MRP3 to confer resistance to certain natural product drugs and other anticancer agents has been assessed to some degree in transfected cell lines (39, 40), but nothing is known of its in vitro transport properties, substrate selectivity profile, or physiological functions. In the report that follows, we address these questions by the analysis of MRP3-mediated transport in membrane vesicles prepared from transfected HEK293 cells. In so doing, we demonstrate that MRP3 not only catalyzes the MgATP-energized transport of glutathione and glucuronate conjugates but also the high-capacity transport of the antimitabolite methotrexate and the bile acid glycocholate. In comparison to MRP1, MRP3 is a low-affinity glutathione and glucuronate conjugate transporter. However, unlike MRP1 and any other characterized mammalian ABC trans-
porter, MRP3 has the facility for transporting glycolate, a major constituent of human bile.

MATERIALS AND METHODS

Cell Lines and Materials. [3H]LTC₄ (110.5 Ci/mmol), [3H]E₂₁₇βG (44 Ci/mmol), and [3H]auricin acid (2.0 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). [3H]Methotrexate (26.8 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA), and [14C]glycine acid, sodium salt (56.0 Ci/mmol) was purchased from Amersham Life Science (Little Chatford, England). Unlabeled and [3H]DNP-SG (44.8 Ci/mmol) were synthesized enzymatically by conjugating unlabeled or glycine-2-[3H]glutathione (NEN Life Science Products) with 1-chloro-2,4-dinitrobenzene using human placental glutathione S-transferase (Sigma Chemical Co., St. Louis, MI) as described (41). E₂₁₇βG, LTC₄, glycine acid, methotrexate, ATP, and AMP were purchased from Sigma. Creatine kinase and creatine phosphate were purchased from Boehringer Mannheim (Indianapolis, IN). Polyclonal MRP3 antibody, the MRP3-transfected HEK293 cell line (HEK/MPR3–5), the control cell line transfected with parental vector (HEK/pcDNA3), and Si9 cells infected with an MRP3 baculovirus were described previously (39).

Immunoblot Analysis. Membrane proteins were separated by SDS-PAGE (6% acrylamide) as described (42). Proteins were transferred to nitrocellulose filters using a wet transfer system (43), and MRP3 was detected using polyclonal MRP3 (1:1000) antibody and alkaline phosphatase-conjugated secondary antibody.

Preparation of Membrane Vesicles and Transport Experiments. Membrane vesicles were prepared as described (8) and stored in liquid nitrogen. Transport experiments were performed using the rapid filtration method, as described (8), with modifications. Briefly, 100 μl of reaction buffer [10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 4 mM ATP or AMP, 10 mM MgCl₂, 10 mM creatine phosphate, and 100 μg/ml creatine kinase] containing radiolabeled compounds, with or without unlabeled carrier, was preincubated at 37°C for 1 min and then rapidly mixed with 5–10 μl of membrane vesicle suspension (30 μg protein). At the indicated times, 20-μl aliquots were removed and added to 1 ml of ice-cold washing buffer [10 mM Tris-HCl (pH 7.4), 250 mM sucrose] and filtered through 0.22-μm pore size GVWP polyvinylidene fluoride filters (Millipore, Bedford, MA). The filters were washed once with 5 ml of ice-cold washing buffer, and radioactivity was measured by liquid scintillation counting. Membrane protein was estimated by a modification of the method of Lowry et al. (see Ref. 44).

Data Analysis. Kinetic parameters were computed by nonlinear least squares analysis (45) using the Ultrafit computer software package (BioSoft, Ferguson, MO).

RESULTS

Transport of Glutathione and Glucuronate Conjugates into Membrane Vesicles. MRP3-dependent transport was assayed on density-fractionated membrane vesicles prepared from HEK293 cells transfected with MRP3 expression vector (HEK/MPR3–5 cells; Ref. 39). To assess the contribution made by MRP3-independent transport to overall uptake, parallel measurements were also performed on membrane vesicles prepared from HEK293 cells transfected with parental plasmid (HEK/pcDNA3 cells; Ref. 39).

Immunoblot analysis of membranes prepared from HEK/MPR3–5 cells revealed a broad, intensely immunoreactive M₁ band after reaction with polyclonal MRP3 antibody, which was absent from cells transfected with empty vector (Fig. 1). The higher M₂ species in HEK/MPR3–5 membranes was inferred to be a more heavily glycosylated form of the lower M₁ species because the latter comigrated with recombinant MRP3 expressed in insect (Si9) cells (Fig. 1), which are known to be only partially competent in protein glycosylation.

Glutathione and glucuronate conjugates are established transport substrates for MRP1 and cMOAT, the closest relatives of MRP3. Therefore, to explore the transport capabilities of MRP3, the glutathione S-conjugates, LTC₄, and DNP-SG, and the glucuronide E₂₁₇βG were selected as model compounds for the initial investigations. Although all three of these conjugates were subject to MgATP-stimulated, time-dependent transport by MRP3, E₂₁₇βG was transported at the highest rate under the conditions used. Whereas little or no E₂₁₇βG uptake into HEK/MPR3–5 vesicles in media containing MgAMP instead of MgATP or into HEK/pcDNA3 vesicles in media containing either MgATP or MgAMP was detected, initial rates of MgATP-dependent uptake of ~3 pmol/mg/min were obtained with HEK/MPR3–5 vesicles incubated in 1380 nm [³H]E₂₁₇βG (Fig. 2A). By comparison, the rates and extents of MRP3-specific, ATP-dependent [³H]DNP-SG and [³H]LTC₄ uptake were moderate. Although an uptake increment consequent on MRP3 was evident for both [³H]DNP-SG and [³H]LTC₄, the background rates of uptake of these compounds were appreciable (Fig. 2, B and C). At an initial concentration of 1.6 μM, DNP-SG was transported at 2.1 and 1.2 pmol/mg/min by HEK/MPR3–5 membranes and at 1.5 and 1.4 pmol/mg/min by HEKpcDNA3 membranes in media containing MgATP and MgAMP, respectively (Fig. 2B). The corresponding values for the uptake of 20 nm LTC₄ were 0.20, 0.08, 0.12 and 0.08 pmol/mg/min (Fig. 2C).

Osmotic Sensitivity of [³H]E₂₁₇βG Transport. To confirm that retention of radiolabel by the membrane vesicles was largely attributable to the transport of substrate into the intravesicular compartment rather than nonspecific binding to the membrane vesicles and/or membrane filters, the osmotic sensitivity of E₂₁₇βG uptake was examined. As shown in Fig. 3, the rate of MgATP-dependent uptake into HEK/MPR3–5 membrane vesicles increased as a linear function of the reciprocal of the osmoticum (sucrose) concentration of the uptake medium, suggesting that the vesicles were osmotically responsive and that the transported species was delivered into an osmotically active compartment. By contrast, osmotically exerted only a modest effect on substrate retention measured in media containing MgAMP, suggesting that an appreciable fraction of the apparent uptake measured under non-energized conditions represented binding to the membrane vesicles and/or membrane filters, not genuine uptake into the vesicles (Fig. 3). The magnitude of the ordinate intercepts indicated that substrate binding constituted no more than 10–15% of the radioactivity retained by HEK/MPR3–5 membranes in media containing MgATP but as much as 50% of the radioactivity retained by the same membranes in media containing MgAMP (Fig. 3).

Kinetcs of [³H]E₂₁₇βG, [³H]DNP-SG, and [³H]LTC₄ Uptake. The substrate concentration dependence of MgATP-energized E₂₁₇βG, DNP-SG, and LTC₄ uptake by membrane vesicles prepared from HEK/MPR3–5 cells approximated Michaelis-Menten kinetics. When measured over a broad range of concentrations, the initial rate of MgATP-dependent uptake of all three conjugates, enumerated as...
the difference between uptake in media containing MgATP and media containing MgAMP, exhibited saturation kinetics (Fig. 4). Nonlinear least squares fitting of the uptake data to the Michaelis-Menten equation (45) yielded $K_m$ and $V_{max}$ values of $25.6 \pm 5.4$ μM and $75.6 \pm 5.9$ pmol/mg/min for $[^{3}H]E_{2}17\beta G$, $5.7 \pm 1.7$ μM and $3.8 \pm 0.1$ pmol/mg/min for $[^{3}H]DNP-SG$, and $5.3 \pm 2.6$ μM and $20.2 \pm 5.9$ pmol/mg/min for $[^{3}H]LTC_4$ (Table 1). The efficiencies ($V_{max}:K_m$ ratios) of MgATP-dependent uptake therefore fell in the rank order LTC_4 (3.8) > E_217βG (3.0) >> DNP-SG (0.7; Table 1).

The transport measured was largely attributable to MRP3 in that membrane vesicles prepared from HEK/pcDNA3 cells catalyzed negligible MgATP-dependent uptake of $E_217\beta G$, DNP-SG, or LTC_4 at all of the concentrations examined (Fig. 4).

Transport of $[^{3}H]$Methotrexate and $[^{14}C]$Glycocholate by MRP3. Having established that human MRP3 is able to transport glutathione and glucuronate conjugates and knowing from previous studies performed in ours and other laboratories that its expression confers resistance to the antimetabolite methotrexate (39, 40), the capacity of membranes from HEK/MRP3–5 to catalyze the uptake of this compound and other amphipathic anions, such as the bile acids glycocholate and taurocholate, was tested.

From these experiments, it was determined that MRP3 is not only competent in the transport of $E_217\beta G$, DNP-SG, and LTC_4 but also competent in the transport of methotrexate and glycocholate. $[^{3}H]$Methotrexate (0.5 μM) and $[^{14}C]$glycocholate (50 μM) were transported by HEK/MRP3–5 membrane vesicles at 0.26 pmol/mg/min and 110 pmol/mg/min, respectively, over the first 5 min of the assays, whereas the corresponding rates for HEK/pcDNA3 membranes were 0.12 and 46 pmol/mg/min (Fig. 5, A and B). In strict contrast to
Table 1  Summary of kinetic parameters for MgATP-dependent amphipathic anion transport by membrane vesicles prepared from HEK/MRP3–5 cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/mg/min)</th>
<th>Replicates</th>
<th>$V_{max} \cdot K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E$_2$17βG</td>
<td>25.6 ± 5.4</td>
<td>75.6 ± 5.9</td>
<td>2</td>
<td>3.0</td>
</tr>
<tr>
<td>DNP-SG</td>
<td>5.7 ± 1.7</td>
<td>3.8 ± 0.1</td>
<td>4</td>
<td>0.7</td>
</tr>
<tr>
<td>LTC$_4$</td>
<td>5.3 ± 2.6</td>
<td>20.2 ± 5.9</td>
<td>3</td>
<td>3.8</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>776 ± 319</td>
<td>288 ± 54</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Glycocholate</td>
<td>248 ± 113</td>
<td>183 ± 34</td>
<td>3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Kinetic parameters were computed as described in the legend to Fig. 4 and in “Materials and Methods.” Values shown are means ± SE.

DISCUSSION

In the present study the in vitro transport properties of human MRP3 were investigated to gain insight into its substrate selectivity and potential physiological functions. In the first instance, glutathione and glucuronate conjugates and the antimetabolite methotrexate were selected as target compounds, because the former two classes of compounds are well-established substrates of MRP1 and cMOAT, the closest known relatives of MRP3 (33), and transport of methotrexate by cMOAT has been inferred previously from pharmacokinetic studies in cMOAT-deficient rats (46). In agreement with the close structural similarity and high degree of amino acid identity of MRP3 with MRP1 (58%) and cMOAT (47%) and the results of previous cellular and kinetic studies, we found that MRP3 can indeed transport the glucuronide E$_2$17βG, the glutathione S-conjugates LTC$_4$, and DNP-SG, and methotrexate in an MgATP-dependent manner. In vitro transport of DNP-SG is consistent with the observation that MRP3-transfected MDCKII cells exhibit enhanced apical to basolateral transport of this conjugate (40). In vitro transport of methotrexate is consistent with the observation that MRP3-transfected cells are resistant to this organic anion (39, 40).

Although these results indicate that the substrate range of MRP3 is similar to that of MRP1 and cMOAT, there are significant differences in the kinetic properties of these transporters (Table 2). The cysteinyl leukotriene LTC$_4$ is a high-affinity substrate of human MRP1 ($K_m = 0.097 \mu M$; Ref. 8) and an intermediate affinity substrate of human cMOAT ($K_m = 0.24–1.0 \mu M$; Refs. 19, 20) but a markedly lower affinity substrate of MRP3 ($K_m = 5.3 \mu M$). Likewise, the $K_m$ for E$_2$17βG transport by MRP3, 25.6 μM, is considerably higher than the values of 1.5 and 2.5 μM reported for MRP1 (10, 11) and 7.2 μM reported for cMOAT (19). The smallest difference determined was for DNP-SG, the $K_m$ of which for MRP3-mediated transport, 5.7 μM, is only slightly higher than the value of 3.6 μM reported for MRP1 (Ref.
10; the kinetic parameters of DNP-SG transport have not been reported for cMOAT).

We speculate that the lower affinity of MRP3 for glutathione conjugates may be related to the more restricted resistance profile this transporter is capable of conferring by comparison with MRP1 and cMOAT. MRP1 has been demonstrated to confer resistance to a broad range of natural products, including anthracyclines, etoposide, vincristine, and actinomycin D (47–49). cMOAT has been implicated in resistance to anthracyclines and vincristine in three reports and to etoposide in one report (19, 20, 26). MRP3, by contrast, has been observed to confer resistance to etoposide and very low levels of resistance to vincristine in transfected HEK293 cells (39) and resistance to etoposide in transduced ovarian carcinoma cells (40), but resistance to anthracyclines and actinomycin D has not been detected.

The facility with which MRP1 transports amphipathic anions is probably relevant to resistance to natural product drugs, although these compounds are not known to form covalent conjugates within the cell. The results of cellular transport studies using MRP1-deficient ES cells and in vitro transport measurements indicate that the natural products etoposide and vincristine are cotransported with free glutathione (50, 51). Analogous experiments on cMOAT imply a similar mechanism for the transport of vinblastine by this transporter (52). It has therefore been proposed that MRP1, and by implication cMOAT, has a bipartite substrate-binding pocket consisting of a binding site for glutathione or other bulky organic anions and another binding site for hydrophobic molecules. There are currently no data concerning the biochemical mechanism whereby MRP3 confers resistance to natural product agents. However, if by analogy with MRP1 and cMOAT, MRP3 cotransports natural products with glutathione, its lower affinity for glutathione conjugates might signify a diminished capacity for binding the free glutathione required for transport of hydrophobic drugs.

The substrate selectivity we describe for human MRP3 is different from that described for rat MRP3 (79.6% identity). In transport studies using membrane vesicles prepared from two different rat MRP3-transfected cell lines, uptake of E$_2$17βG and methotrexate was detected, but transport of the glutathione $S$-conjugates LTC$_4$ and DNP-SG was not (53). This may simply reflect the fact that there was appreciable uptake of LTC$_4$ and DNP-SG in the control vesicles used for the studies of rat MRP3 that obscured the contribution made by MRP3. Alternatively, these results may denote a genuine difference in substrate selectivity and physiological functions between the rat and human transporters. Consistent with the latter possibility, the reported localization of rat MRP3 in liver differs from that of the human transporter. Rat MRP3 was reported to be localized to canalicular membranes in the liver (54), whereas human MRP3 has been reported to be localized in basolateral hepatocyte membranes in one study and in basolateral membranes of cholangiocytes with lesser levels in basolateral membranes of hepatocytes surrounding portal tracts in another (40, 55). Although the unexpected difference in the localizations of the human and rat proteins warrants further investigation, particularly in view of the potential for immunological cross-reactivity between closely related members of the MRP subfamily, the data currently available suggest that the functions of human and rodent MRP3 are not exactly equivalent.

The observation that human MRP3 transports glycocholate at high rates, albeit at low affinity, is an intriguing property of this transporter. Sister of Pgp (also known as the bile salt export pump), which localizes to canalicular membranes of hepatocytes, is the only other mammalian ABC transporter that has been shown to transport mono- and di-valent bile acids (56, 57). Contrary to human MRP3, which transports glycocholate but not taurocholate, experiments using cloned rat sister of Pgp expressed in S9F cells indicate that it transports taurocholate and several unconjugated bile acids but not glycocholate (57). Moreover, although our experiments show that MRP3 transports glycocholate, the basolateral localization of human MRP3 suggests that it cannot function to transport this compound into the bile. Instead, human MRP3 likely assumes other functions in the liver.

In cholestatic conditions, elevated levels of bile acids appear in the blood. However, the mechanism by which bile acids are transported from hepatocytes into sinusoidal blood is unknown. We therefore suggest that MRP3 may function to transport glycocholate and possibly other bile acids (but not taurocholate in the case of human MRP3) from hepatocytes into the blood under these conditions. Although the localization of rat MRP3 protein requires further investigation, the observation that elevations in its levels occur as a consequence of cholestasis induced by bile duct ligation (38, 54) adds support to this possibility. That elevated levels of MRP3 expression have been detected in patients that have Dubin-Johnson syndrome and are therefore deficient in cMOAT (55), as well as in rat strains deficient in cMOAT (38, 54), is similarly consistent with the notion that MRP3 may function as a basolateral compensatory mechanism to eliminate from hepatocytes potentially toxic compounds that are ordinarily excreted into bile. We speculate that in Dubin-Johnson syndrome and other cMOAT-deficient conditions, MRP3 functions to transport into sinusoidal blood the glucuronate and glutathione conjugates that are normally exported across bile canaliculi by cMOAT (cMOAT does not transport monovalent bile acids). In the context of the enterohepatic circulation of bile acids, it is also pertinent to note that in addition to liver, high levels of MRP3 transcript are found in small intestine (and several other tissues; Refs. 30, 32, 36, and 38). Bile acids taken up at the apical surface of enterocytes of the small intestine via the ileal bile salt transporter (58) must then be transported across the basolateral membrane into portal blood. However, a transporter capable of performing this function has yet to be identified. The possibility that MRP3 subserves this function is particularly attractive. These and other questions concerning the physiological roles of MRP3 and its significance for clinical drug resistance are important issues that remain to be resolved.

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