MOLECULAR CLONING AND PHARMACOLOGICAL CHARACTERIZATION OF RAT MULTIDRUG RESISTANCE PROTEIN 1 (MRP1)

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ABSTRACT:
Multidrug resistance protein 1 (MRP1) transports a wide range of structurally diverse conjugated and nonconjugated organic anions and some peptides, including oxidized and reduced glutathione (GSH). The protein confers resistance to certain heavy metal oxynions and a variety of natural product-type chemotherapeutic agents. Elevated levels of MRP1 have been detected in many human tumors, and the protein is a candidate therapeutic target for drug resistance reversing agents. Previously, we have shown that human MRP1 (hMRP1) and murine MRP1 (mMRP1) differ in their substrate specificity despite a high degree of structural conservation. Since rat models are widely used in the drug discovery and development stage, we have cloned and functionally characterized rat MRP1 (rMRP1). Like mMRP1 and in contrast to hMRP1, rMRP1 confers no, or very low, resistance to anthracyclines and transports the two estrogen conjugates, 17β-estradiol-17-β-glucuronide (E217βG) and estrone 3-sulfate, relatively poorly. Mutational studies combined with vesicle transport assays identified several amino acids conserved between rat and mouse, but not hMRP1, that make major contributions to these differences in substrate specificity. Despite the fact that the rodent proteins transport E217βG poorly and the GSH-stimulated transport of estrone 3-sulfate is low compared with hMRP1, site-directed mutagenesis studies indicate that different nonconserved amino acids are involved in the low efficiency with which each of the two estrogen conjugates is transported. Our studies also suggest that although rMRP1 and mMRP1 are 95% identical in primary structure, their substrate specificities may be influenced by amino acids that are not conserved between the two rodent proteins.

Human multidrug resistance protein 1 (hMRP11) was originally cloned from a multidrug-resistant human small cell lung cancer cell line, H69AR, that was selected in the presence of doxorubicin (Cole et al., 1992). When overexpressed in tumor cells, hMRP1 confers resistance to anticancer drugs that include Vinca alkaloids, epipodophyllotoxins and anthracyclines, as well as some heavy metal oxynions (Cole et al., 1994; Grant et al., 1994; Zaman et al., 1994). MRP1 is now known to be a primary active transporter of conjugated organic anions (Leslie et al., 2001a; Borst and Oude Elferink, 2002). The list of substrates transported by MRP1 includes GSH conjugates such as the leukotriene LTC4, as well as a number of glucuronide and sulfate-conjugates (Leier et al., 1994; Muller et al., 1994; Jedlitschky et al., 1996; Loe et al., 1996a, 1997; Leslie et al., 2001b; Qian et al., 2001). Transport of certain anionic conjugates, as well as some unmodified chemotherapeutic drugs to which MRP1 confers resistance is augmented in the presence of GSH (Loe et al., 1996b, 1998; Ding et al., 1999; Renes et al., 1999; Leslie et al., 2001b; Qian et al., 2001). In some cases, cotransport of GSH can be demonstrated but not in others (Rappa et al., 1997; Loe et al., 1998, 2000; Leslie et al., 2001b; Qian et al., 2001). GSH alone is inefficiently transported by MRP1, although transport can be stimulated by compounds such as verapamil and a variety of different flavonoids (Loe et al., 2000; Qian et al., 2001; Leslie et al., 2001b).

Despite a high level of sequence conservation, the murine ortholog of MRP1, mMRP1, differs from the human protein with respect to its ability to confer resistance to anthracyclines and to transport the conjugated estrogen E217βG (Stride et al., 1996, 1997). Thus, the mouse protein fails to confer resistance to several commonly used anthracyclines and transports E217βG very poorly. These differences place some limits on the usefulness of the mouse as a preclinical model for the development of agents that might be used to selectively reverse drug resistance mediated by MRP1. To date, the substrate specificity of other mammalian MRP1 orthologs has not been characterized in any detail. However, bovine MRP1 (bMRP1) has been cloned recently, and the drug resistance phenotype of transfected cell...
lines was found to be similar to that of hMRP1 except that the bovine protein, like the murine protein, conferred no resistance to doxorubicin (Taguchi et al., 2002). In addition, VP-16 resistance conferred by the bovine protein was higher than that conferred by hMRP1. Since rats are widely used as experimental models at the preclinical drug development stage, we have cloned the MRP1 ortholog from skeletal muscle of Sprague-Dawley (SD) rats and directly compared its functional characteristics with hMRP1 and mMRP1. During preparation of this manuscript, the sequence of a rMRP1 cDNA, cloned from brain astrocytes derived from SD rats was reported (Yang et al., 2002).

Characterization of the encoded rMRP1 was limited to a demonstration that Madin-Darby canine kidney cells transiently transfected with an rMRP1-expressing plasmid accumulated less calcein, a fluorescent MRP1 substrate, and that the accumulation deficit could be reversed by the MRP1 inhibitors indomethacin or MK571. Here, we have used the full-length rMRP1 cDNA cloned from skeletal muscle to generate stable transfectants of human embryonic kidney cells (HEK293). The pharmacological phenotypes of clonal populations of transfected cells were then characterized with respect to their resistance to a number of chemotherapeutic agents. In addition, we have examined the transport of potential physiological substrates, including LTC4, E217, doxorubicin, daunorubicin, and various HPLC chemicals, San Diego, CA). This mAb recognizes a linear epitope of 10 amino acids in hMRP1, 238GSDLWSLNKE, nine of which are conserved in both hMRP1 and mMRP1 (Wilson and Deeley, 1995; Stride et al., 1997). Consequently, we inserted the rat cDNA into this vector for transfection into PC7-hMRP1 into HindIII-Sfl digested pc7-mrP1. The vector encoding rMRP1/hMRP1 (1188–1531) was generated by ligation a HindIII-Sfl fragment encompassing nucleotides 2875–4823 of PC7-hMRP1 into HindIII-Sfl digested pc7-mrP1. The vector encoding rMRP1/hMRP1 (1188–1531) was generated by ligation a Sfl-Stul fragment encompassing nucleotides 3562–4823 of PC7-mrP1 into Sfl-Stul digested pc7-mrP1. The vector encoding rMRP1/hMRP1 (959–1187) was constructed by ligation a HindIII-Stul fragment encompassing nucleotides 2875–3562 of PC7-mrP1 into HindIII-Stul digested pc7-mrP1. Integrity of the hybrid constructs was confirmed by sequencing across cloning junctions or by restriction enzyme digests. A 1.45-kb HindIII-Sfl fragment was subcloned into the wild-type rMRP1 expression vector. The fragments in the full-length constructs were again confirmed by sequencing.

**Construction of Hybrid rMRP1/hMRP1 Expression Vectors.** The chimeric vector expression encoding rMRP1/hMRP1 (959–1531) was generated by ligation a HindIII-Sfl fragment encompassing nucleotides 2875–4823 of PC7-hMRP1 into HindIII-Sfl digested pc7-mrP1. The vector encoding rMRP1/hMRP1 (1188–1531) was generated by ligation a Sfl-Stul fragment encompassing nucleotides 3562–4823 of PC7-mrP1 into Sfl-Stul digested pc7-mrP1. The vector encoding rMRP1/hMRP1 (959–1187) was constructed by ligation a HindIII-Stul fragment encompassing nucleotides 2875–3562 of PC7-mrP1 into HindIII-Stul digested pc7-mrP1. Integrity of the hybrid constructs was confirmed by sequencing across cloning junctions or by restriction enzyme digests.

**Creation of Cell Lines Stably Expressing rMRP1.** HEK293 cells grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum were transfected with the rMRP1 expression vectors and cell lines expressing rMRP1 selected with 100 μg/ml hygromycin B (Roche Applied Science, Laval, PQ, Canada). Briefly, approximately 1.5 x 10^6 HEK293 cells were seeded in each well of 6-well plate and 24 h later, 1 μg of DNA and 3 μl of FuGENE 6 (Roche Applied Science) were mixed and added to the cells according to the manufacturer’s instructions. After 48 h, the transfected cells were subcultured 1:3 and supplemented with fresh medium. The medium was replaced with fresh medium containing 100 μg/ml hygromycin B 24 h later. Approximately 14 days post-transfection, the hygromycin B-resistant cells were seeded into 96-well plates at a density of 15 cells/plate. The cells were selected in hygromycin B for a further 3 to 4 weeks. After expanding the hygromycin-resistant clones, levels of MRP1 protein were determined by immunoblot analysis. In some cases, vectors were transiently transfected into HEK293 cells as follows. Approximately 1 x 10^5 cells were seeded in 175-cm^2 flasks, and 24 h later, 15 μg of DNA was mixed with 45 μl of FuGENE 6 according to the manufacturer’s instructions. After 48 to 72 h, HEK293 cells were harvested, and inside-out membrane vesicles were prepared as described previously (Loe et al., 1998).

**Measurement of Protein Levels in Transfected Cells.** The levels of MRP1 proteins were determined by immunoblot analysis of membrane protein fractions from transfected cells. After determination of protein levels by Bradford assay (Bio-Rad, Hercules, CA), proteins were resolved by SDS-polyacrylamide gel electrophoresis (7.5% gel) and subsequently transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA) by electroblotting. Rat MRP1, hMRP1, and mMRP1 proteins were determined by immunoblot analysis of membrane protein samples. Rat MRP1, hMRP1, and mMRP1 proteins were identified using the monoclonal antibody (mAb) MRP1 (Alexis Biochemicals, San Diego, CA). This mAb recognizes a linear epitope of 10 amino acids in hMRP1, 238GSDLWNLKE, nine of which are conserved in both hMRP1 and mMRP1.
mMRP1 and rMRP1 (Hipfner et al., 1998) (Fig. 1). Antibody binding was detected with horseradish peroxidase-conjugated goat anti-rat IgG (Pierce Chemical, Rockford, IL) followed by enhanced chemiluminescence detection (PerkinElmer Life Sciences, Boston, MA) and densitometry of the X-ray film.

Chemosensitivity Testing. Drug resistance was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Stride et al., 1997). Mean values of quadruplicate determinations (± S.D.) were plotted using GraphPad software (GraphPad Software, Inc., San Diego, CA). IC₅₀ values were obtained from the best fit of the data to a sigmoidal curve. Relative resistance factors are expressed as the ratio of the IC₅₀ value of cells expressing rMRP1, hMRP1, or mMRP1 to the IC₅₀ value of cells stably transfected with the empty vector PC7. Resistance was determined in four separate concurrent experiments.

Transport by Membrane Vesicles. Plasma membrane vesicles were prepared as described previously (Loe et al., 1998) and ATP-dependent transport of [³H]LTC₄ into the inside-out membrane vesicles at 23°C was measured by a rapid filtration technique (Loe et al., 1996a,b; Qian et al., 2001). All data were corrected for the amount of [³H]LTC₄ that remained bound to the filter, which was usually <5% of the total radioactivity. ATP-dependent uptake of [³H]E₂₁₇G (400 nM, 32 nCi per time point) and [³H]estrone 3-sulfate (300 nM, 40 nCi per time point in the presence or absence of 1 or 3 mM GSH) were measured as described for [³H]LTC₄ except that the reactions were carried out at 37°C. Kinetic parameters of estrone 3-sulfate transport were determined by measuring the initial rate of [³H]estrone 3-sulfate uptake (in the presence or absence of 1 mM GSH) at eight different substrate concentrations between 0.15 and 16 μM. The amount of protein used in the assay varied from 2 μg/time point for vesicles containing human MRP1 to 4 μg/time point for vesicles containing the rat or murine protein. Transport in the presence of AMP was subtracted from transport in the presence of ATP and reported as ATP-dependent uptake. For each substrate concentration, ATP-dependent uptake was measured at a single time point of 1 min. Kₘ and Vₐₘₜ were determined using a Hanes-Woolf plot and Vₐₘₜ was then corrected for relative MRP1 protein expression levels.

Results

Comparison of Human, Mouse and Rat MRP1 Amino Acid Sequences. The rMRP1 sequence encoded by cDNAs isolated from skeletal muscle from SD rats is 99.8% identical to that recently described by Yang et al. (2002), which was derived from cDNAs isolated from brain astrocytes, also from SD rats. In addition, a third partial sequence for rMRP1 (amino acids 711-1532) isolated from astrocytes of Norway rats has been published (Hirrlinger et al., 2001). In the latter case, amino acid identity with our rMRP1 sequence is only 94.3%. In four of the five locations where differences occur between the partial sequence and our sequence, the residue present in our sequence is conserved between human and mouse MRP1 and the astrocyte-derived rMRP1 sequences. At the fifth location, Arg1003 of the partial sequence is Pro in both full-length rat sequences and the mouse sequence. In hMRP1, there is no corresponding residue.

The two SD rMRP1 cDNA sequences encode potential open reading frames of 1532 amino acids and differ at only three locations. The first at position 4 of the open reading frame of the astrocyte-derived
sequence is an Arg, as it is in both hMRP1 and mMRP1, whereas our rMRP1 sequence derived from skeletal muscle encodes a Ser at this location. In contrast, the Ser1382 of the skeletal muscle rMRP1 sequence is also a Ser in the partial rMRP1 sequence and in the mouse and human sequences but is a Pro residue in the sequence derived from rat astrocytes. This could be a significant change since it occurs in the second nucleotide binding domain (NBD2) between the conserved Walker A and ATP-binding cassette signature motifs. Similarly, Ile1473 of the skeletal muscle sequences is Ile in the partial rMRP1 sequence, hMRP1, and mMRP1 but is Val in the rMRP1 sequence from astrocytes. This conservative substitution is located just downstream of the Walker B motif in NBD2. At the moment, however, it is unclear whether these are genuine sequence polymorphisms or are the result of cloning artifacts.

Overall, the two deduced amino sequences for rMRP1 are 88 and 86.8% identical to hMRP1 (for skeletal muscle- and astrocyte-derived MRP1, respectively) and 95% identical to mMRP1 (Fig. 1) (Cole et al., 1992; Stride et al., 1996; Yang et al., 2002). When compared with hMRP1, rMRP1 and mMRP1 contain an additional Pro residue after amino acid 279 in the predicted third cytoplasmic loop (CL3) of MRP1. Additional Arg and Pro residues are also present after amino acid 1002 of the human sequence in rMRP1 and mMRP1, respectively. These residues are located in the first extracellular loop of membrane-spanning domain (MSD) 3. Lys 941 of hMRP1, present in the cytoplasmic linker region connecting NBD1 and MSD3, is absent in both the rat and mouse proteins. Mouse MRP1 lacks another single amino acid found at position 641 of hMRP1, which is located in NBD1. The residue is a Thr in hMRP1 and a Met in rMRP1. Mouse MRP1 is missing a further three amino acids that would correspond to hMRP1 amino acids 883–885 which are also located in the linker region connecting NBD1 to MSD3. These residues are Val-Thr-Gly in hMRP1 and Lys-Asn-Gly in rMRP1. In the human protein, this region can be deleted with no loss of LTC4 transport activity (Gao et al., 1992; Stride et al., 1996; Yang et al., 2002). When compared with previous reports of the size of mMRP1 mRNA, the probe hybridized with a mRNA of approximately 6.0 kb in RNA from the Sol 8 cell line. In the RNA from rat kidney, a single mRNA species was detected that was considerably larger than the mMRP1 mRNA and was estimated to be approximately 7.5 kb. Thus, the two rodent mRNAs differ considerably in the length of their untranslated sequence.

Stable Expression of rMRP1 in HEK Cells. An episcopal vector containing an expression cassette for rMRP1 was constructed as previously described for hMRP1 and mMRP1 and used to transfect HEK293 cells (Stride et al., 1997). HEK mMRP1 subpopulations were isolated by limiting cell dilution and screened for the level of rMRP1 expression. Those populations that expressed levels of rMRP1 approximately equivalent to the levels of mMRP1 and hMRP1 in previously characterized HEK transfectants were used for further studies. Immunoblot analysis of membrane-enriched fractions with mAb MRPr1 confirmed that the PC7-rMRP1-encoded protein comigrated with hMRP1 and mMRP1 and was present at a level equivalent to the levels of mMRP1 and hMRP1.

Comparison of Drug Resistance Profiles of HEKmMRP1, HEKhMRP1, and HEKhMRP1. Cell populations expressing human, mouse and rat proteins were tested concurrently for their resistance to a number of drugs using an MTT assay. Typical dose-response curves for cells exposed to vincristine, VP-16, doxorubicin, daunorubicin, and etoposide are shown in Fig. 4, and a summary of the relative resistance factors determined from four separate concurrent experiments is shown in Table 1. HEKhMRP1 conferred moderate resistance (7.0-fold) to vincristine as compared with hMRP1 (10.9-fold) and mMRP1 (11.7-fold). Resistance conferred by rMRP1 to VP-16 was also moderate (6.1-fold) when compared with that conferred by hMRP1 (9.0-fold) or mMRP1 (18.7-fold). As we have reported previously, mMRP1 did not confer resistance to the anthracyclines, doxorubicin, daunorubicin, and etoposide (1.1, 1.2 and 0.7-fold, respectively), whereas cells expressing hMRP1 conferred resistance to all three anthracyclines (7.0, 5.5, and 5.5-fold, respectively). HEKhMRP1 cells conferred no or very low resistance to doxorubicin, daunorubicin, and etoposide (1.9-, 1.6-, and 1.0-fold, respectively). Thus,
the anthracycline resistance profiles of rMRP1 were similar but not identical to those found for mMRP1.

**LTC₄ and E₁₇₈G Transport Function of rMRP1.** The transport of LTC₄ was examined using membrane vesicles isolated from HEK transfectants stably expressing rMRP1, hMRP1, or mMRP1. The data were then normalized for the relative expression levels of the orthologs in the membrane vesicles, as determined by immunoblotting and densitometry (shown in Fig. 3). The time course of LTC₄ uptake is shown in Fig. 5A. The rates of LTC₄ transport calculated at the 1 min time point were similar for all three proteins at 49.5, 48.2, and 42.0 pmol/mg/min for rMRP1, mMRP1, and hMRP1, respectively (Fig. 5C). The $K_m$ value for rMRP1 was 50 nM, which is similar to that previously obtained for the human and mouse proteins (data not shown) (Loe et al., 1996b; Stride et al., 1997, 1999).

![Graphs showing the LTC₄ and E₁₇₈G transport function of rMRP1.](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Relative Resistance Factor</th>
<th>HEK&lt;sub&gt;rMRP1&lt;/sub&gt;</th>
<th>HEK&lt;sub&gt;mMRP1&lt;/sub&gt;</th>
<th>HEK&lt;sub&gt;hMRP1&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td>Vincristine</td>
<td></td>
<td>7.0 ± 1.6</td>
<td>11.7 ± 0.3</td>
<td>10.9 ± 1.4</td>
</tr>
<tr>
<td>VP-16</td>
<td></td>
<td>6.1 ± 1.1</td>
<td>18.7 ± 1.1</td>
<td>9.0 ± 0.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td></td>
<td>1.9 ± 0.2</td>
<td>1.1 ± 0.0</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td></td>
<td>1.6 ± 0.4</td>
<td>1.2 ± 0.0</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>Epirubicin</td>
<td></td>
<td>1.0 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>5.5 ± 0.3</td>
</tr>
</tbody>
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**FIG. 4.** Relative resistance levels of stably transfected HEK293 cells.

HEK cells stably transfected with vectors expressing rMRP1 (■), hMRP1 (○), and mMRP1 (●) and with vector alone (□) were tested using a standard MTT assay for resistance to vincristine, VP-16, doxorubicin, daunorubicin, and epirubicin. The results shown are those of a typical experiment. Values are mean ± S.D. of quadruplicate determinations.
Previously, we have shown that hMRP1 transports E217/H9252G relatively efficiently while transport of this substrate by mMRP1 is very poor (Stride et al., 1997). Here, we show that transport of E217/H9252Gb by HEKrMRP1 membrane vesicles was less than 10% that of HEKhMRP1 vesicles but was similar to that of vesicles containing the mouse protein (Fig. 5B). The ATP-dependent transport calculated at the 1 min time point by HEKrMRP1, HEKhMRP1, and HEKhMRP1 was 9.7/1.6, 8.7/3.3, and 139.9/22.7 pmol/mg/min, respectively.

Transport of [3H]LTC4 and [3H]E217G by Hybrid and Mutant Proteins. Previous studies demonstrated that murine/human hybrid proteins in which amino acids 955-1184 or 1185–1528 of mMRP1 membrane vesicles was less than 10% of that of HEKhMRP1 vesicles but was similar to that of vesicles containing the mouse protein (Fig. 5B). The ATP-dependent transport calculated at the 1 min time point by HEKhMRP1, HEKhMRP1, and HEKhMRP1 was 9.7 ± 1.6, 8.7 ± 3.3, and 139.9 ± 22.7 pmol/mg/min, respectively.

Fig. 5. Transport of [3H]LTC4 and [3H]E217G.

[3H]LTC4 (A) and [3H]E217G (B) uptake was measured in membrane vesicles prepared from HEKhMRP1(■), HEKhMRP1(○), HEKhMRP1(●), and HEKhMRP1(□). For the panel inserted in B, the scale of [3H]E217G was expanded to show transport by membrane vesicles containing rMRP1 and mMRP1. Histograms show [3H]LTC4 (C) and [3H]E217G (D) uptake by the membrane vesicles at the 1 min time point. In all cases, experiments were repeated at least twice with similar results. Shown are typical experiments in which the data have been normalized to the rate of uptake of hMRP1 vesicles taking into account differences in the levels of the rat and murine protein. Shown are the mean ± S.D. of triplicate determinations.

To further localize residues important for transport, the region encoding TMs 12–17 of human, murine, and rat MRP1 were compared. Residues that are identical between murine and rat MRP1, but nonconservatively substituted in hMRP1 were selected for analysis.
Such amino acids in rMRP1 were substituted with the corresponding residue from hMRP1, and membrane vesicles were isolated from transiently transfected HEK293 cells. Expression levels of the mutant proteins, which varied from 1.0 to 1.4 relative to rMRP1, are shown in Fig. 6D. LTC4 uptake for the mutant proteins was proportional to their expression levels. Therefore, LTC4 transport was unaffected by these mutations (Fig. 6E). Similarly, mutations L983M, L1029S, and V1106C had no effect on E217G transport whereas S1101N increased the transport by approximately 50% (Fig. 6F). However, the A1243T mutation increased E217G uptake 3.7-fold relative to wild-type rMRP1. This is similar to the increase found in the equivalent mMRP1 mutation, A1239T (Zhang et al., 2001a). A 2-fold increase in transport activity was also noted for the Q1090E mutation. The same mutation in mMRP1, Q1086E, had no significant effect on E217G transport. In addition, the double mutation Q1090E/A1243T increased E217G transport approximately 9-fold to 57.6 pmol/mg/3 min (Fig. 6F).

Transport of [3H]Estrone 3-Sulfate. MRP1 is expressed at high levels in the testis in both humans and mice where it may play a role in the efflux of steroid conjugates (Flens et al., 1996; Wijnholds et al., 1998). We have shown previously that hMRP1 is capable of transporting estrone 3-sulfate, which is one of the more abundant estrogen conjugates formed in the testis (Qian et al., 2001). However, transport of estrone 3-sulfate by hMRP1, unlike E217G, is stimulated approximately 10-fold by GSH and its other nonreducing derivatives, including S-methyl GSH. The ability of murine and rat MRP1 to transport this estrogen conjugate and the dependence of the transport on the presence of GSH has not been determined. Consequently, we compared the ability of the three orthologs to transport [3H]estrongonate sulfate in the absence and presence of GSH. In the absence of GSH, transport activities obtained with HEKnMRP1, HEKnMRP1, and HEKhMRP1 membrane vesicles were 5.7 ± 3.0, 5.0 ± 2.6, and 6.5 ± 1.9 pmol/mg/3 min, respectively (Fig. 7). In the presence of 1 mM GSH, the transport activity of HEKnMRP1 and HEKnMRP1 increased 3- to 4-fold to 24.3 ± 3.0 and 14.0 ± 2.9 pmol/mg/3 min, respectively, while the transport activity of the HEKhMRP1 vesicles increased approximately 9-fold to 57.6 ± 10.4 pmol/mg/3 min (Fig. 7). Thus the basal rate of transport of this substrate by the rodent proteins appears to be similar to that of the human protein. However, transport is 2- to 3-fold less responsive to the presence of GSH than transport observed with the human protein.

To determine the kinetic parameters of estrone 3-sulfate transport and the effect of GSH, ATP-dependent uptake in the presence and absence of 1 mM GSH was determined for a number of substrate concentrations ranging from 0.15 to 16 μM. A nonlinear regression analysis (Fig. 7, B and C) and Hanes-Woolf transformations (not shown) of the data yielded similar Km and Vmax values. The Km for hMRP1 was determined to be 0.83 μM in the presence of GSH and 4.2 μM in the absence of GSH, consistent with the Km values reported previously (Qian et al., 2001; Zhang et al., 2001b). The Km values for mMRP1 and rMRP1 were either identical to each other, 1.1 μM in the
presence of GSH, or very similar to each other, 3.2 and 3.4 μM, respectively, in the absence of GSH. Thus, GSH increases the apparent affinity of MRP1 for estrone sulfate approximately 5-fold in the case of hMRP1 and 3-fold in the case of the rodent proteins. The $V_{\text{max}}$ values obtained in the absence of GSH (corrected for relative MRP1 protein expression) were 64, 85, and 109 pmol mg$^{-1}$ protein min$^{-1}$ for hMRP1, mMRP1, and rMRP1, respectively. In the presence of GSH, the $V_{\text{max}}$ values were 144, 117, and 110 pmol mg$^{-1}$ protein min$^{-1}$ for hMRP1, mMRP1, and rMRP1, respectively. Thus, GSH increased the $V_{\text{max}}$ for hMRP1 and mMRP1 by 2.25 and 1.4 fold, respectively. No effect on the $V_{\text{max}}$ of rMRP1 was detected. As an indicator of the overall effect of GSH on the efficiency of estrone sulfate transport, the $V_{\text{max}}/K_m$ values for the human, mouse, and rat protein increased 11.6-, 3.8-, and 3.2-fold, respectively.

To identify regions of the protein responsible for the difference in GSH dependence, we examined estrone 3-sulfate transport by the rat/human hybrid proteins. Two of them, rMRP1/hMRP1 (959–1531) and rMRP1/hMRP1 (959–1187), displayed a GSH dependence that was approximately equivalent to that of hMRP1 (Fig. 8A). The GSH dependence of the hybrid mMRP1/hMRP1 (1187–1531) was comparable with that of wild-type rMRP1. Thus, the region of the human protein that appears primarily responsible for its greater response to GSH is located between amino acids 959 and 1187. However, the two mutations that affected $E_{17}BG$ transport, Q1090E and A1243T, and the double mutation, Q1090/A1243T, did not alter the GSH dependence or the basal rate of estrone 3-sulfate transport (Fig. 8A). Thus, different determinants appear to be important for transport of the two estrogen conjugates.

The Effect of Doxorubicin on Transport of Estrone 3-Sulfate.

We have shown previously that doxorubicin inhibits hMRP1-mediated transport of LTC4 and $E_{17}BG$ (Loe et al., 1996b, 1998). To determine whether doxorubicin also inhibits GSH-stimulated estrone 3-sulfate transport, membrane vesicles from HEK293 cells transiently transfected with wild-type and hybrid hMRP1 and MRP1 expression vectors were tested in the standard vesicle transport assay, with and without addition of 50 μM doxorubicin. Doxorubicin inhibited estrone 3-sulfate transport in vesicles containing the human protein by 70% while having no effect on transport by the rat protein, consistent with the inability of rMRP1 to confer anthracycline resistance (Fig. 8B). Significantly, doxorubicin had a negative effect on transport of estrone 3-sulfate by all three hybrid proteins although the most significant reduction in transport occurred with the hybrid containing the corresponding residue in rMRP1 from glutamine to glutamate, with the ability of doxorubicin to inhibit estrone 3-sulfate transport but to a lesser extent than found for the Q1090E mutation. With the exception of the mouse, little is known of functional differences between hMRP1 and its orthologs in species commonly used as models for the preclinical study of drug efficacy and disposition. Since the rat is a frequently used experimental animal model for such studies, we have cloned rMRP1 and examined its function using whole cells or membrane vesicles prepared from stably trans-
Membrane vesicle preparation in the absence of 50 μM doxorubicin was expressed as a percentage of the uptake measured for each mean and range of triplet determinations from two experiments. In the presence of 3 mM GSH. The data have been normalized to compensate for wild-type rMRP1. In B, uptake of [3H]estrone 3-sulfate in the presence of 50 μM doxorubicin was expressed as a percentage of the uptake measured for each membrane vesicle preparation in the absence of 50 μM doxorubicin. Shown are the mean and range of triplet determinations from two experiments.

**Fig. 8.** Effect of doxorubicin on GSH-stimulated [3H]estrone 3-sulfate transport.

[44x222]LTC4, E217βG. Transport of the physiological substrates LTC4 and E217βG has been shown to be important for the ability of the protein to confer anthracycline resistance and/or to transport E217βG, which is markedly stimulated by GSH (Qian et al., 2001). The ability of the rodent MRP1 proteins to transport this substrate had not been determined. We found that the basal rates of transport of estrone 3-sulfate are comparable for all three proteins. However, at saturating concentrations (300 nM), GSH stimulated rMRP1- and mMRP1-mediated transport 3- to 4-fold compared with 9-fold stimulation for hMRP1. Kinetic analysis of the three orthologs revealed that in the presence of 3 mM GSH. The data have been normalized to compensate for differences in MRP1 protein levels as shown in Fig. 6. In A, uptake of [3H]estrone 3-sulfate was expressed as a percentage of uptake measured for vesicles containing wild-type rMRP1. In B, uptake of [3H]estrone 3-sulfate in the presence of 50 μM doxorubicin was expressed as a percentage of the uptake measured for each membrane vesicle preparation in the absence of 50 μM doxorubicin. Shown are the mean and range of triplet determinations from two experiments.

**Fig. 8.** Effect of doxorubicin on GSH-stimulated [3H]estrone 3-sulfate transport.

[44x222]LTC4, E217βG, and the GSH-dependent estrogen conjugate, estrone 3-sulfate, in the same cell background. The ability of hMRP1 to confer resistance to anthracyclines, epipodophyllotoxins, and the Vinca alkaloids has been well characterized (Cole et al., 1994; Grant et al., 1994; Zaman et al., 1994; Stride et al., 1997). In addition, we have shown that although the ability of mMRP1 to confer resistance to VP-16 and vincristine is similar to that of mMRP1, the mouse protein confers essentially no resistance to the anthracyclines (Stride et al., 1997). Cell lines expressing bMRP1 also showed no resistance to doxorubicin (Taguchi et al., 2002). Here we find that rMRP1 conferred moderate resistance to both VP-16 and vincristine but no, or very low, resistance to doxorubicin, daunorubicin, and epirubicin. Thus, the resistance profile of rMRP1 is similar to that of mMRP1. We previously showed by site-directed mutagenesis that the nonconserved Gln1089 in hMRP1, which corresponds to Gln1086 in mMRP1, is critical for resistance to doxorubicin (Zhang et al., 2001b). In both the bovine and rat proteins, the equivalent residue is Gln (Gln1088 in bMRP1 and Gln1086 in rMRP1) as it is in mMRP1 (Taguchi et al., 2002). Thus, there is a strong correlation between the presence of Gln at this location in the protein and the inability of MRP1 to confer anthracycline resistance.

The efficiency with which the mouse, rat, and human MRP1s transport the physiological substrates LTC4 and E217βG was also compared. Human and mouse MRP1s transport LTC4 with equal efficiency, and no difference in the ability of rMRP1 to transport this substrate was observed (Stride et al., 1997). In the case of E217βG however, the level of transport in the rMRP1 membranes was similar to that of mMRP1 and less than 10% that obtained for membranes containing hMRP1. We have recently identified another conjugated estrogen, estrone 3-sulfate, that can be efficiently transported by hMRP1, but in contrast to E217βG, transport of this compound is markedly stimulated by GSH (Qian et al., 2001). The ability of the rodent MRP1 proteins to transport this substrate had not been determined. We found that the basal rates of transport of estrone 3-sulfate are comparable for all three proteins. However, at saturating concentrations (300 nM), GSH stimulated rMRP1- and mMRP1-mediated transport 3- to 4-fold compared with 9-fold stimulation for hMRP1. Kinetic analysis of the three orthologs revealed that this difference in response is attributable to differential effects of GSH on both $K_m$ and $V_{max}$. GSH decreased the $K_m$ for estrone sulfate approximately 3-fold for both rodent proteins and 5-fold for hMRP1. In addition, the $V_{max}$ of hMRP1 was increased more than 2-fold compared with only 1.4-fold for mMRP1 and the $V_{max}$ of the rat protein was unaffected. The reasons for the differences in GSH stimulation are presently unknown. However, since they appear to be saturating GSH concentrations, it appears likely that a step after initial GSH binding is involved. This is supported by our recent demonstration that a photoactivatable derivative of GSH, azidophenacyl GSH, which can substitute functionally for GSH, labels hMRP1 and mMRP1 comparably (Qian et al., 2002).

Functional differences between the mouse and human proteins have allowed us to define domains and/or amino acid residues of MRP1 important for the ability of the protein to confer anthracycline resistance and/or to transport E217βG. Using a strategy based on the functional characterization of various hybrid proteins, we have identified individual, nonconserved amino acids that are major determinants of substrate specificity (Stride et al., 1999; Zhang et al., 2001a,b, 2002). Rat/human hybrids, like the comparable mouse/human hybrids, also showed that E217βG transport is partially restored by substitution of the COOH-proximal third of the rat protein with the human sequence (Stride et al., 1999). Division of this region to create hybrids containing amino acids 959 to 1187 or 1188 to 1531 of the human sequence (Stride et al., 1999). Division of this region to create hybrids containing amino acids 959 to 1187 or 1188 to 1531 of the human protein decreased transport without eliminating it, suggesting that determinants for E217βG transport are present in both regions. A number of nonconservative differences in amino acid sequence between mouse/rat and human MRP1 were tested for their effect on LTC4 and E217βG transport, including the rMRP1 TM14 and TM17 mutations Q1090E and A1243T. None of the mutations had any effect on LTC4 transport. The rMRP1 TM17 A1239T mutation increased E217βG transport 4-fold. A similar effect on E217βG transport was noted for the corresponding mutation, T1239A, in mMRP1. A 2-fold increase in transport was also found with the rMRP1 TM14 mutation,
The conjugate must involve interaction with a set of amino acid residues.

These mutations, the results indicate that the binding of this estrogen stimulated transport of estrone 3-sulfate was not affected by any of the other amino acids, that are not conserved between rat and mouse proteins, may influence the interaction of the TM14 Glu with the estrogen glucuronide. The only other mutation that affected E17βG transport by rMRP1 was also in TM14, this mutation, L1101N, increased transport by approximately 50%.

Previously, we showed that the hMRP1 TM 14 mutations E1089Q and E1089K, which either reduce or completely eliminate resistance to doxorubicin and vincristine, had no effect on estrone 3-sulfate transport (Zhang et al., 2001b). Here we show that the TM14 and TM17 mutations, as well as the TM14/TM17 double mutation, which affect E17βG transport, had no effect on either the basal or GSH-stimulated transport of estrone 3-sulfate. Thus despite the shared steroid nucleus, these residues are unlikely to form direct interactions with the latter compound. However, the results of studies with the rat/human hybrids clearly show that the GSH stimulation of estrone 3-sulfate transport can be rescued by substituting amino acids 959 to 1187 of the rat protein with the corresponding region of the hMRP1, indicating that different, nonconserved amino acids within this region contribute to the greater efficiency with which hMRP1 transports the two estrogen conjugates.

Previously, we have shown that chemotherapeutic agents, such as vincristine and doxorubicin, can act as inhibitors of both LTC4 and E17βG transport by hMRP1, suggesting that they interact with common or mutually exclusive portions of the substrate binding pocket(s) (Ito et al., 1996a,b, 1998). When the ability of doxorubicin to inhibit GSH-stimulated estrone 3-sulfate transport by the rat and human proteins was directly compared, we found that a concentration of drug, which inhibited transport by hMRP1 by approximately 70%, resulted in only 20% inhibition of transport by rMRP1. This strongly suggests that the decreased ability of the rat protein to confer resistance to the anthracycline is attributable to a decreased ability to bind the drug. We found that all three rat/human hybrid proteins, comparable with mouse/human hybrids that restore doxorubicin resistance (Stride et al., 1999), also restore the ability of the drug to inhibit transport, as did the single mutation in TM14, Q1086E, that is critical for resistance to this class of drugs (Zhang et al., 2001b). In addition, the TM17 mutation A1239T that in the mouse protein had no detectable effect on doxorubicin resistance (Zhang et al., 2001a), also enhanced the ability of doxorubicin to inhibit transport by rMRP1. This suggests, as observed with the different effect of the TM14 mutations on E17βG transport, that other amino acids, which are not conserved between the rat and mouse proteins, may contribute to the binding of anthracyclines and the estrogen glucuronide. Since GSH-stimulated transport of estrone 3-sulfate was not affected by any of these mutations, the results indicate that the binding of this estrogen conjugate must involve interaction with a set of amino acid residues that is at least partially distinct from those involved in binding the anthracyclines and E17βG.

Overall, these studies serve to emphasize that the use of rodent models for preclinical testing of drugs targeted to hMRP1, or for the investigation of drug disposition mediated by this transporter and possibly its homologues, should be accompanied by in vitro assays that permit comparison of the transport and/or binding characteristics of the test compound by both rodent and human proteins.

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References


