Substitution of Trp^{1242} of TM17 alters substrate specificity of human multidrug resistance protein 3


The ATP-binding cassette (ABC) proteins comprise a large superfamily of transmembrane proteins that use the energy of ATP hydrolysis to translocate their substrates across biological membranes (23). One branch of the superfamily, known as subfamily C, presently consists of 12 human proteins, 7 of which are designated as multidrug resistance proteins (MRPs) 1–7 (4, 9, 29). MRP1 (ABCC1) was the first of the MRP-related transporters to be cloned (7), and increased expression of this protein in tumor cells results in resistance to a remarkably diverse spectrum of anticancer drugs. MRP1 is also a primary active transporter of a variety of organic anions that include both endo- and xenobiotic molecules conjugated to GSH, glucuronide, and sulfate (9).

The ability of MRP1 to confer resistance in tumor cells and to transport conjugated organic anions is shared by the structurally related MRP2 (ABCC2) and MRP3 (ABCC3). All three proteins have an extracytoplasmic NH_{2} terminus and are predicted to have 17 transmembrane (TM) helices, which are organized as three membrane-spanning domains (MSD) (4, 28) (Fig. 1A). The first, MSD1, contains five TM segments, whereas MSD2 and MSD3 are each predicted to contain six TM segments in most topological models (12, 21, 24). MSD2 and MSD3 are each followed by a nucleotide-binding domain (NBD1 and NBD2), both of which are required for transport activity (9).

The partial cDNA sequence of human MRP3 (ABCC3) and its mapping to chromosome 17p21, as well as the tissue distribution of human MRP3 mRNA, were first described by Kool et al. (26). Shortly thereafter, the complete coding sequences of human MRP3 and rat Mrp3 were reported by several groups (3, 11, 22, 25, 27, 41). The 1527-amino acid MRP3 protein exhibits 58% identity with MRP1 and 48% identity with MRP2. Despite this primary sequence similarity, MRP3, like MRP1 and MRP2, has its own distinctive pattern of tissue distribution and substrate specificity.

The highest levels of MRP3 mRNA are found in the liver, colon, and small intestine, whereas lower levels have been detected in pancreas, kidney, prostate, placenta, adrenal gland, and different parts of the brain (3, 11, 22, 39). Immunohistochemical studies have for the most part corroborated the mRNA expression studies, and in all polarized epithelial cells examined to date MRP3, like MRP1, has been localized to basolateral membranes (22, 25, 27, 38). In hepatocytes and intrahepatic bile duct epithelial cells (cholangiocytes), expression of MRP3 is induced during cholestasis, when apical expression of Mrp2/MRP2 is disrupted, such as in TR rats and in humans with Dubin-Johnson syndrome. Under these conditions, MRP3 is thought to play a compensatory role in the basolateral efflux of toxic organic anions (32). Hepatic MRP3/Mrp3 can also be induced in response to a number of different xenobiotics (5, 22, 34).
of MRP3, as they are for MRP1 and MRP2. However, glucuronide conjugates appear to be transported by MRP3 with substantially greater efficiency than glutathione conjugates (14, 43, 46). Finally, in addition to cytotoxic drugs and conjugated organic anions, MRP3 transports primary bile acids, such as cholic acid, taurocholic acid, and glycocholic acid, as well as conjugated secondary bile acids such as taurolithocholate-3-sulfate and cholate 3-O-glucuronide (14, 46). In contrast, MRP1 and MRP2 transport only conjugated bile acids (15, 24, 32, 40). Thus the substrate specificity of MRP3 overlaps but is distinct from that of either MRP1 or MRP2.

Amino acid residues in MRP3 involved in the recognition and transport of its substrates remain largely unknown. In previous studies of the related MRP1 and MRP2, we demonstrated that a highly conserved Trp residue in the highly amphipathic TM17 of MSD3 plays a critical role in the substrate specificity of these transporters (17, 18) (Fig. 1B). In the present study, we have substituted the analogous residue in MRP3, Trp1242, with both conserved and nonconserved amino acids and examined the effects on the expression and substrate specificity of this bile salt transporter.

MATERIALS AND METHODS

Materials. [6,7-3H]E217βG (55 Ci/mmol) and [3H(G)]taurocholic acid (2 Ci/mmol) and [3H(G)]taurocholate-3-sulfate and cholates 3-O-glucuronide salt (17 Ci/mmol) were from Amersham Pharmacia Biotech (Little Chalfont, UK), and [3',5',7',9-3H(n)]leucovorin di- 

...
ACA TTC GCT TTA AAC TCC ATG ATA CCA ATG ATG TCA G-3′, W1242Y (5′-G CAG GTG ACA TTC GCT TTA AAC TAC ATG ATA CCA ATG ATG TCA G-3′), and W1242P (5′-G CAG GTG ACA TTC GCT TTA AAC CCG ATG ATA CCA ATG ATG TCA G-3′). After the presence of all mutations was confirmed by a Dral diagnostic digestion, a 0.3-kb AgeI/PmaCI fragment containing the desired mutation was subcloned back into pcDNA3.1(+)MRP3 and the entire fragment in the full-length construct was sequenced.

**Transient transfections of MRP3 expression vectors.** Mutant pcDNA3.1(+)MRP3 expression vectors were transfected into SV40-transformed human embryonic kidney cells (HEK293T) as before (17). Briefly, 5 × 10^6 cells were seeded in 150-mm dishes, and 24 h later DNA (16 μg) was added using FuGENE 6 (Roche Diagnostics, Laval, PQ, Canada) according to the manufacturer’s instructions. After 48–72 h, the HEK293T cells were harvested and inside-out membrane vesicles were prepared as described previously (30). Empty vector pcDNA3.1(+) DNA and vector containing the wild-type MRP3 cDNA were included as controls in all transfection experiments.

**Measurement of MRP3 protein levels in transfected cells.** The relative levels of wild-type and Trp1242 mutant MRP3 proteins were determined by immunoblot analysis of membrane protein fractions from the transfected cells essentially as described (17). Proteins were resolved on a 6% polyacrylamide gel and electrophoresed to a nylon membrane. Membranes were blocked with 4% (wt/vol) skim milk powder in Tris-buffered saline with 0.1% Tween 20 (vol/vol) (TBST) for 1 h followed by incubation with the MRP3-specific murine MAb M Ab II-9 (Alexis, San Diego, CA) diluted 1:1,000 to 1:10,000 in 4% skim milk powder in TBST. After being washed, blots were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody diluted 1:10,000 (Pierce, Edmonton, AB, Canada) followed by application of Renaissance chemiluminescence blotting substrate (NEN Life Science). Relative levels of MRP3 protein expression were estimated by densitometric analysis using a Chemilager 4000 (Alpha Innotech, San Leandro, CA).

**MRP3-mediated transport of [3H]E217G and [3H]LTC4 by inside-out membrane vesicles.** ATP-dependent transport of [3H]labeled substrates by the membrane vesicles was measured by using a rapid filtration technique as described previously (18, 30). Briefly, time courses of E17G uptake were performed at 37°C in a 60-μl reaction volume containing 400 nM [3H]E217G (120 nCi), 4 mM AMP or ATP, 10 mM MgCl2, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and 6 μg of vesicle protein in transport buffer (50 mM Tris·HCl, 250 mM sucrose, pH 7.4). In some experiments, MTX, taurocholate, and glycocholate were added at the concentrations indicated. Uptake was stopped at the appropriate times by rapid dilution of an aliquot of the reaction mixture in ice-cold transport buffer and then filtered through glass fiber (type A/E) filters that had been presoaked in transport buffer. Radioactivity was quantitated by liquid scintillation counting. All data were corrected for the amount of [3H]E217G that remained bound to the filter, which was usually <10% of the total radioactivity. Uptake in the presence of AMP was subtracted from uptake in the presence of ATP to determine ATP-dependent [3H]E217G uptake. All assays were carried out in triplicate, and results were expressed as means ± SD. Uptake of [3H]LTC4 was measured in a similar fashion, except that membrane vesicles (6 μg protein) were incubated at 37°C for 5 min with [3H]LTC4 (500 nM; 80 nCi) and components as described for [3H]E217G transport.

**MRP3-mediated transport of [3H]MTX, [3H]leucovorin, and [3H]taurocholate by inside-out membrane vesicles.** [3H]MTX uptake was also measured by rapid filtration essentially as described (17). Assays were performed at 37°C in 60 μl transport buffer containing 1 μM [3H]MTX (250 nCi per reaction), 4 mM AMP or ATP, an ATP-regenerating system, and membrane vesicles (10 μg protein). Uptake was stopped at 0 min, diluted, and filtered as above for [3H]E217G transport. Experiments were also carried out at an initial substrate concentration of 100 μM MTX.

**RESULTS**

**MRP3-Trp1242 mutants are expressed in human embryonic kidney cells.** Based on our observation that substitution of MRP1-Trp1246 and MRP2-Trp1254 at the predicted COOH-proximal cytosol-membrane interface of TM17 can dramatically affect the substrate specificity of MRP1 and MRP2, respectively, cDNA constructs were generated in which the analogous residue, Trp1242, in MRP3 was replaced with several different amino acids. These included nonconservative substitutions with a nonaromatic nonpolar amino acid (Ala; W1242A-MRP3) and a nonaromatic polar amino acid (Cys; W1242C-MRP3), as well as conservative substitutions with aromatic polar (Tyr; W1242Y-MRP3) and nonpolar (Phe; W1242F-MRP3) amino acids. Trp1242 was also replaced with a α-helix-disrupting residue (Pro; W1242P-MRP3). The cDNA constructs were transfected into HEK293T cells, and after 48–72 h the cells were harvested, the membrane vesicles were prepared, and relative MRP3 protein levels were determined by immunoblotting. As shown in Fig. 2, wild-type MRP3 and the four mutants (W1242A-, W1242C-, W1242F-, and W1242Y-MRP3) were expressed at similar levels. In contrast, the W1242P-MRP3 mutant was expressed at significantly lower levels, suggesting that this mutation affects the biogenesis or stability of the protein. Mean expression levels of the mutant MRP3 proteins relative to wild-type MRP3 were as follows: W1242A, 1.1 ± 0.3; W1242C, 1.0 ± 0.2; W1242Y, 1.0 ± 0.1; W1242F, 1.0 ± 0.3; and W1242P, 0.6 ± 0.2 (4–6 independent transfections).

**Substitution of Trp1242 enhances the ability of MRP3 to transport [3H]E217G.** Time courses of ATP-dependent [3H]E217G uptake were determined for the W1242A-, W1242C-, W1242F-, and W1242P-MRP3 mutants by using inside-out membrane vesicles prepared from transfected HEK293T cells (Fig. 3A).

Unexpectedly, four of the five mutants (W1242A-, W1242C-, W1242F-, and W1242Y-MRP3) transported this glucuronide substrate at levels that were substantially higher than those of wild-type MRP3. In contrast, transport by W1242P-MRP3 was almost undetectable. At 3 min, [3H]E217G uptake in membrane
vesicles enriched for W1242A-, W1242C-, and W1242F-MRP3 was 2.5- to 3-fold higher than for wild-type MRP3, whereas $[^3\text{H}]\text{E}_{217}\beta\text{G}$ uptake by the most conservatively substituted mutant, W1242Y-MRP3, was ~7-fold higher (Fig. 3B).

**Effect of Trp1242 substitutions on MRP3-mediated transport of $[^3\text{H}]\text{MTX}$.** Membrane vesicles prepared from HEK293T cells transfected with wild-type MRP3 transported $[^3\text{H}]\text{MTX}$ at a rate of ~15 pmol·mg protein$^{-1}·\text{min}^{-1}$ (Fig. 5A). This rate is more than two-fold higher than those reported previously for human MRP3 expressed in stably transfected HEK293T cells, which may simply reflect higher levels of MRP3 expression in our HEK transfectants (45). However, when Trp1242 was replaced with either nonconserved or conserved amino acids, a significant reduction in ATP-dependent $[^3\text{H}]\text{MTX}$ uptake was observed in all cases (Fig. 5A). At 10 min, ATP-dependent $[^3\text{H}]\text{MTX}$ uptake levels by the W1242A, W1242C, W1242F, and W1242Y
mutants were ~12–26% those of wild-type MRP3 [after the transport activity of the pcDNA3.1(+) vector control was subtracted and values normalized for relative MRP3 protein expression] (Fig. 5B). A similar reduction in MTX transport activity was observed when assays were carried out at a 100-fold higher initial substrate concentration (100 μM [3H]MTX; data not shown).

Effect of Trp1242 substitutions on MTX inhibition of MRP3-mediated transport of [3H]E217βG. MTX has previously been reported to inhibit E217βG transport by MRP3 (14, 43). Consequently, the effect of MTX on [3H]E217βG uptake by the MRP3-Trp1242 mutants with reduced MTX transport activity was examined. The results in Fig. 5C show that [3H]E217βG uptake by wild-type MRP3 was inhibited >90% by 1 mM MTX as expected. [3H]E217βG uptake by the four Trp1242 mutant MRP3 proteins was also inhibited by MTX, although to a much lesser degree (~35%).

Only the most conservatively substituted W1242Y-MRP3 mutant transports [3H]leucovorin. In addition to MTX, MRP3 has been reported to transport folic acid and another folic acid analog, leucovorin (N5-formyltetrahydrofolic acid) (45). To determine if Trp1242 substitutions also affected MRP3-mediated transport of the latter substrate, [3H]leucovorin uptake into membrane vesicles prepared from transfected cells expressing wild-type MRP3 and W1242A-, W1242C-, W1242F-, and W1242Y-MRP3 mutants was examined. For wild-type MRP3 membrane vesicles, the levels of [3H]leucovorin uptake were 1.32 ± 0.12 nmol/mg compared with 0.18 ± 0.04 nmol/mg for the empty vector control (Fig. 6A). Of the four Trp1242 mutants tested, only the most conservatively substituted mutant, W1242Y-MRP3, transported [3H]leucovorin at levels comparable to those of wild-type MRP3. In contrast, [3H]leucovorin transport by the less conservatively substituted Trp1242 mutants was reduced by >75% compared with wild-type MRP3 after subtraction of uptake by vector control membrane vesicles and normalization of mu-
tant MRP3 protein levels to wild-type MRP3 protein levels (Fig. 6B).

Substitution of Trp1242 has no significant effect on [3H]taurocholate transport by MRP3. Rat Mrp3 has been shown to transport monovalent bile acids such as taurocholate and glycocholate, whereas human MRP3 has been reported to transport only glycocholate (1, 14, 46). However, we found that ATP-dependent [3H]taurocholate uptake by wild-type MRP3 membrane vesicles was readily detectable at a level of 331 ± 90 pmol/mg protein compared with 70 ± 18 pmol/mg protein for the vector control vesicles (Fig. 7A). Taurocholate uptake by the W1242A-, W1242C-, W1242F-, and W1242Y-MRP3 mutants was then examined and, in all cases, was not significantly different from uptake by wild-type MRP3 (Fig. 7B). Consistent with this observation, [3H]E217G uptake by W1242A-, W1242C-, W1242F-, and W1242Y-MRP3 could still be inhibited by taurocholic acid (40–60%) at concentrations of 50 and 100 μM (Fig. 7C). Glycocholic acid also inhibited [3H]E217G uptake by the four MRP3-Trp1242 mutant proteins, and this inhibition was similar to that observed with wild-type MRP3. At concentrations of 50 and 100 μM, inhibition by glycocholic acid ranged from 40 to 80% (Fig. 8).

The transport activities of the MRP3-Trp1242 mutants for the five substrates tested are summarized in Table 1. For comparison, the effects of nonconservative (Ala) and conservative (Tyr) substitutions of MRP1-Trp1246 (18), MRP2-Trp1254 (17), and MRP3-Trp1242 on transport activity are summarized in Table 2.

DISCUSSION

In addition to its potential role as a cause of drug resistance in tumor cells in which it is overexpressed, MRP3 is a primary active transporter of both primary
and secondary bile salts (13, 20, 46). The majority of bile salt transport in the liver takes place across the apical canalicular membrane and is mediated by bile salt export protein (BSEP, ABCC11) (32). However, because of the relatively high level of MRP3 expression in the liver, its inducibility in this tissue, and its location on basolateral membranes, it has been postulated that MRP3 may contribute to the enterohepatic circulation of bile salts that is essential for the maintenance of bile acid and cholesterol homeostasis (16, 32, 34). In this respect, the substrate specificity of MRP3 differs from the related MRP1 and MRP2, which confer resistance to a much broader range of anticancer drugs than MRP3 but are limited to transporting only conjugated bile salts. Thus all three of these ABCC proteins transport conjugated organic anions, but there are marked differences in the affinity and efficiency with which they do so (2, 24, 29, 40, 46).

Factors governing the substrate specificity of the MRP-related transporters are complex, as can be expected for any relatively large polytopic membrane protein that recognizes such a variety of different substrates. To date, a number of conserved and nonconserved amino acids in different regions of MRP1 have been shown to affect the substrate specificity and transport efficiency of this protein (8, 9, 18, 47, 48). Similarly, sequence analyses of MRP2/Mrp2 in humans with Dubin-Johnson syndrome and in hyperbilirubinemic TR/HB rats, respectively, as well as site-directed mutagenesis studies, have identified amino acids important for the function of this transporter (17, 19, 20, 33, 37, 42). In contrast, comparatively little is known about the amino acids that determine the substrate specificity of MRP3. However, by taking advantage of amino acid sequence differences between rat Mrp3 and Mrp2, Ito et al. (20) recently identified a nonconserved Leu residue at position 1084 in predicted TM14 of rat Mrp3 as being important for the ability of this protein to transport taurocholic acid. Thus substitution of Leu1084 in Mrp3 with Lys (as it is in MRP1) eliminated both taurocholic acid and E217G uptake by these mutants could still increase rather than decreased. Also in contrast to the rat Mrp3-Leu1084 mutant, the MRP3-Trp1242 mutant, transport activity was markedly increased rather than decreased. In the present study, we have shown that mutation of the highly conserved Trp1242 in TM17 of human MRP3, like the nonconserved Leu1084 in rat Mrp3, significantly alters the ability of this protein to transport E217G, but in the case of MRP3-Trp1242, transport activity was markedly increased rather than decreased. Also in contrast to the rat Mrp3-Leu1084 mutant, the MRP3-Trp1242 mutants retained their ability to transport taurocholic acid and E217G transport by these mutants could still be inhibited by bile acid. Thus it appears that E217G and taurocholic acid share an overlapping but not identical set of binding determinants in a substrate-binding pocket of this transporter. In the present study, we have shown that mutation of the highly conserved Trp1242 in TM17 of human MRP3, like the nonconserved Leu1084 in rat Mrp3, significantly alters the ability of this protein to transport E217G, but in the case of MRP3-Trp1242, transport activity was markedly increased rather than decreased. Also in contrast to the rat Mrp3-Leu1084 mutant, the MRP3-Trp1242 mutants retained their ability to transport taurocholic acid and E217G transport by these mutants could still be inhibited by bile acid. Thus it appears that E217G and taurocholic acid share an overlapping but not identical set of binding determinants in a substrate-binding pocket of MRP3/Mrp3.

Our observation that all substitutions of MRP3-Trp1242 markedly stimulated E217G uptake was unexpected, because both conservative and nonconservative substitutions of the analogous Trp1246 in MRP1 had the opposite effect and essentially eliminated transport of this conjugated estrogen, as did nonconservative substitutions of MRP2-Trp1254 (17, 18) (Table 2). Trp residues with their large indole (benzopyrrole) side chains have the greatest steric bulk of all of the amino acids (36). Thus all Trp substitutions examined in the present study resulted in mutant MRP3 proteins

### Table 1. Transport activities of variously substituted MRP3-Trp1242 mutants

<table>
<thead>
<tr>
<th>Substrate</th>
<th>W1242A</th>
<th>W1242C</th>
<th>W1242F</th>
<th>W1242Y</th>
<th>W1242P</th>
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<tbody>
<tr>
<td>E217G</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>LTC4</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
<td>n.d.</td>
</tr>
<tr>
<td>Leucovorin</td>
<td>↔️</td>
<td>↔️</td>
<td>↔️</td>
<td>↔️</td>
<td>n.d.</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>↔️</td>
<td>↔️</td>
<td>↔️</td>
<td>↔️</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

MRP, multidrug resistance protein; E217G, 17β-estradiol 17β-(glucuronide); LTC4, leukotriene C4; MTX, methotrexate; ↔️, no effect; ↑↑, increased 2.5- to 3-fold compared with wild-type MRP3 activity; ↓↓, decreased 7-fold; ↓, decreased 25–50% compared with wild-type MRP3 activity; ↓↓↓, decreased 50–75%; ↓↓↓↓, decreased ≥ 75%; n.d., not determined.

### Table 2. Effects of nonconservative (Ala) and conservative (Tyr) substitutions of MRP1-Trp1246, MRP2-Trp1254, and MRP3-Trp1242 on transport activity of common substrates

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substitution</th>
<th>LTC4</th>
<th>E117G</th>
<th>MTX</th>
</tr>
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<tbody>
<tr>
<td>MRP1-Trp1246</td>
<td>Ala</td>
<td>100</td>
<td>&lt;10</td>
<td>&lt;10*</td>
</tr>
<tr>
<td></td>
<td>Tyr</td>
<td>100</td>
<td>&lt;10</td>
<td>10*</td>
</tr>
<tr>
<td>MRP2-Trp1254</td>
<td>Ala</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Tyr</td>
<td>30</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>MRP3-Trp1242</td>
<td>Ala</td>
<td>70</td>
<td>250</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Tyr</td>
<td>65</td>
<td>700</td>
<td>20</td>
</tr>
</tbody>
</table>

Data are from Ito et al. (17, 18) and the present study, with exceptions (*) noted (I. Letouneau, C. J. Oleschuk, R. G. Deeley, and S. P. C. Cole, unpublished observations).

Fig. 8. Effect of glycocholic acid on [3H]E217G uptake by wild-type and Trp1242 mutant MRP3 proteins. Membrane vesicles from cells expressing wild-type MRP3 (WT-MRP3) and W1242A, W1242C, W1242F, and W1242Y mutant MRP3 were incubated at 37°C with 400 nM [3H]E217G in transport buffer and other components for 3 min in the absence (open bars) or presence of glycocholate (50 μM, grey bar; 100 μM, solid bar). Each bar represents the mean ± SD of triplicate determinations.
with less steric bulk at position 1242 than is found in the wild-type protein. This could allow for greater freedom of motion in the E$_{217}$G-binding pocket of MRP3 or possibly enhance the flexibility of TM17, which might increase the transport efficiency of this substrate. However, the highest level of E$_{217}$G uptake was observed with W1242Y-MRP3, which is the most conservatively substituted mutant with respect to steric bulk, aromaticity, and H-bonding capability of the lateral side chain. Thus only a relatively small reduction in steric bulk seems required to enhance E$_{217}$G transport efficiency by MRP3.

In contrast to E$_{217}$G uptake, the variously substituted MRP3-Trp$^{1242}$ mutants all showed reduced LTC$_4$ transport, but the decrease was moderate (25–50%). We showed previously that the MRP1-Trp$^{1246}$ was critical for E$_{217}$G transport as well as being essential for conferring drug resistance (18). However, LTC$_4$ transport by the MRP1-Trp$^{1246}$ mutants was similar to wild-type MRP1 with just a small decrease in $K_m$. This contrasts with the findings with MRP2-Trp$^{1254}$ mutants, where nonconservative substitutions completely eliminated the ability of MRP2 to transport this cysteinyll leukotriene (17) (Table 2).

MRP1 and MRP2 are low-affinity transporters of GSH, and, at least in the case of MRP1, GSH transport can be markedly enhanced by a variety of compounds, including vincristine, phenylalkylamines such as verapamil, and bioflavonoids such as apigenin (2, 28, 29, 31, 35). In contrast, despite the higher degree of amino acid similarity between MRP1 and MRP3 than between MRP1 and MRP2, there is no evidence that MRP3 transports GSH alone (27, 43) nor can it be stimulated by verapamil or bioflavonoids (C. J. Oleschuk, I. Letourneau, R. G. Deeley, and S. P. C. Cole, unpublished observations). Efflux of drugs such as the Vinca alkaloids vincristine and vinblastine and the anthracyclines doxorubicin and daunorubicin by MRP1 and MRP2 seems to occur in a cotransport manner with GSH. Whether the inability of MRP3 to confer resistance to these two classes of drugs is related to its inability to transport GSH is not yet known. Also uncertain is whether the apparent inability of MRP3 to transport GSH is related to the relatively poor affinity of this transporter for GSH conjugates compared with glucuronide conjugates. However, it is clear that factors other than binding of a GSH moiety contribute to GS-X and GSH transport, because LTC$_4$ transport by MRP3 is readily detectable.

Introduction of a Pro residue at position 1242 was the only substitution that completely eliminated the conjugated organic anion transport activity of MRP3. This substitution would not only change the spatial volume occupied by the side chain and disrupt the potential H-bonding and aromatic stacking interactions of the amino acid at this position, but it would also introduce a kink into TM17 at the predicted membrane-cytosol interface (36). The loss of H-bonding and aromatic stacking interactions alone cannot fully account for the inactivity of the W1242P-MRP3 mutant, because the Ala, Cys, Phe, and Tyr mutants all retained some transport activity. Rather, it is more likely that the unique helix-modifying properties of Pro caused a more global disruption of the substrate-binding pocket of MRP3 that was sufficient to abrogate the overall transport activity of the protein. In this regard, it is also of interest that none of the substitutions of MRP3-Trp$^{1242}$ significantly affected protein expression levels, with the exception of the Pro substitution. Again, it may be that the helix-modifying properties of this amino acid adversely affected the proper packing of the TM segments of MRP3 so as to reduce the stability of the protein, which could also contribute to the elimination of transport.

Previously, we reported that MTX transport was essentially eliminated by all MRP2-Trp$^{1254}$ substitutions (17), and the same is true of the MRP1-Trp$^{1246}$ mutants (I. Letourneau, C. J. Oleschuk, K. Ito, R. G. Deeley, and S. P. C. Cole, unpublished observations; see Table 2). However, the MRP3-Trp$^{1242}$ mutants retained a low level of MTX transport activity, which is consistent with the observation that MTX was still able to partially inhibit E$_{217}$G uptake by the MRP3-Trp$^{1242}$ mutants. These findings suggest that the conserved Trp residue may be more important for maintaining the architecture of the MTX substrate-binding pocket of MRP1 and MRP2 than MRP3. We also found that, with the exception of the Tyr substitution, all MRP3-Trp$^{1242}$ mutations caused a marked decrease in the ability of MRP3 to transport leucovorin. This agent is used clinically as a rescue agent to reduce bone marrow toxicity associated with antifolate treatment (6), and it has been previously reported that the affinity of MRP3 is about threefold lower for leucovorin than it is for MTX (45). The basis for this difference in affinity is unknown but is likely to be related to differences in the interactions of these drugs with a folate-binding pocket in MRP3. These drugs, like the Trp residue itself, are capable of both H-bonding and aromatic stacking interactions. It is not presently possible to know whether or not such direct interactions occur between MRP3-Trp$^{1242}$ and these drugs. However, if they do, they must do so in a somewhat different way for leucovorin than for MTX, which may account for the difference in affinities of these compounds for MRP3 as well as for the ability of the W1242Y-MRP3 mutant to transport the former but not the latter drug. As a polar aromatic amino acid, Tyr is considered a conservative substitution of Trp. Nevertheless, the two amino acids differ in certain significant ways that could affect interactions with different MRP3 substrates. These differences include the spatial volumes occupied by the side chains of Trp and Tyr (174 Å$^3$ and 143 Å$^3$, respectively) as well as the potential H-bonding interactions of the indole ring of the Trp side chain vs. the hydroxyl group of the Tyr side chain.

In summary, our studies show that the highly conserved Trp residue in MRP3 at the membrane-cytosol interface of TM17, as shown previously for MRP1 and MRP2, plays a unique and selective role in substrate recognition by this protein. Importantly, they show that, although the transport of a variety of organic
anion substrates are affected by changes in this amino acid, binding and transport of bile salts appear to remain intact. Furthermore, our findings, together with those of Ito et al. (20), who identified a critical Leu residue in TM14 of rat Mrp3, suggest that, as we have shown previously for MRP1 (47), interactions between TM14 and TM17 may be required for MRP3/Mrp3 to transport at least some of its substrates. Finally, they also reaffirm the important notion that prediction of the substrate specificity of an ABC transporter solely on the basis of its amino acid conservation, either with homologs in the same species or orthologs in different species, is not possible.

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