Mutation of a Single Conserved Tryptophan in Multidrug Resistance Protein 1 (MRP1/ABCC1) Results in Loss of Drug Resistance and Selective Loss of Organic Anion Transport*

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Multidrug resistance protein 1 (MRP1/ABCC1) belongs to the ATP-binding cassette transporter superfamily and is capable of conferring resistance to a broad range of chemotherapeutic agents and transporting structurally diverse conjugated organic anions. In this study, we found that substitution of a highly conserved tryptophan at position 1246 with cysteine (W1246C-MRP1) in the putative last transmembrane segment (TM17) of MRP1 eliminated 17β-estradiol 17-(β-D-glucuronide) (E217βG) transport by membrane vesicles prepared from transiently transfected human embryonic kidney cells while leaving the capacity for leukotriene C4 and verapamil-stimulated glutathione transport intact. In addition, in contrast to wild-type MRP1, leukotriene C4 transport by the W1246C-MRP1 protein was no longer inhibitable by E217βG, indicating that the mutant protein had lost the ability to bind the glucuronide. A similar phenotype was observed when Trp1246 was replaced with Ala, Phe, and Tyr. Confocal microscopy of cells expressing Trp1246 mutant MRP1 molecules fused at the C terminus with green fluorescent protein showed that they were correctly routed to the plasma membrane. In addition to the loss of E217βG transport, HeLa cells stably transfected with W1246C-MRP1 cDNA were not resistant to the Vinca alkaloid vincristine and accumulated levels of [3H]vincristine comparable to those in vector control-transfected cells. Cells expressing W1246C-MRP1 were also not resistant to cationic anthracyclines (doxorubicin, daunorubicin) or the electroneutral epipodophyllotoxin vinblastine. In contrast, resistance to sodium arsenite was only partially diminished, and resistance to potassium antimony tartrate remained comparable to that of cells expressing wild-type MRP1. This suggests that the structural determinants required for transport of heavy metal oxyanions differ from those for chemotherapeutic agents. Our results provide the first example of a tryptophan residue being so critically important for substrate specificity in a eukaryotic ATP-binding cassette transporter.

The 190-kDa multidrug resistance protein 1 (MRP1) is a member of a branch of the ATP-binding cassette (ABC) superfamily of transport proteins designated ABCB (1–3). When overexpressed in tumor cells, MRP1 (gene symbol ABCC1) confers resistance to anticancer drugs and other xenobiotics of remarkably diverse structures and charge. Thus, the resistance spectrum associated with MRP1 expression extends from cationic and neutral natural product drugs (e.g. vincristine, doxorubicin, and VP-16) to the antimitabolite methotrexate to arsenical and antimonials (4). MRP1 is also a primary active transporter of conjugated organic anions that include GSH-, glucuronide-, and sulfate-conjugated derivatives of both endo- and xenobiotics, suggesting a role for MRP1 in the disposition and elimination of these compounds (2).

The ability of MRP1 to confer drug resistance and transport conjugated organic anions is shared by at least two other proteins belonging to the ABC subfamily, MRP2 (ABCC2) and MRP3 (ABCC3) (5, 6). These three proteins share a common five-domain structure that distinguishes them from most other ABC transporters, which more typically have four structural domains. Thus, MRP1, -2, and -3 have a third membrane-spanning domain (MSP) with an extracytosolic N terminus that precedes a core structure consisting of two tandemly arranged units containing an MSD and a nucleotide-binding domain (see Fig. 1A) (7, 8). The drug resistance profiles and the specificity and relative affinities of the three proteins for organic anions are similar, but not identical (5, 8–10). For example, cisplatin resistance is associated with elevated expression of MRP2, but not MRP1 or MRP3 (8, 11). Moreover, MRP3 is a relatively poor transporter of GSH-conjugated organic anions compared with MRP1 and MRP2 (9). Such differences in substrate specificity are not unexpected given that the amino acid sequences of MRP2 and MRP3 are only 48 and 58% identical, respectively, to that of MRP1.

Two of the best characterized substrates of MRP1 are the GSH-conjugated arachidonic acid derivative leukotriene C4 (LTC4) and the glucuronidated estrogen 17β-estradiol 17-(β-D-glucuronide) (E217βG) (5, 12). MRP1 has also been shown in vitro to transport certain unconjugated xenobiotics, but only in the presence of GSH (13–16). Conversely, transport of GSH itself by MRP1 is markedly stimulated by a variety of structurally diverse heterocyclic molecules that may or may not be cotransported with this tripeptide (16, 17). The mechanism by which MRP1-mediated transport and/or cotransport of its conjugated and unconjugated substrates occurs is presently un-
clear. Nevertheless, it has been proposed that MRP1 contains a bipartite binding site(s) that can accommodate the structural and physical diversity of its substrates (3, 13, 18).

The specific amino acids involved in the recognition, binding, and transport of MRP1 substrates are largely unknown. In previous studies, the differing abilities of human MRP1 and its murine ortholog to confer resistance to anthracycline drugs have allowed us to identify a nonconserved glutamic acid residue at position 1089 in putative transmembrane segment 14 (TM14) in the third C-proximal MSD (MSD3) of human MRP1 that is essential for its ability to confer resistance to this class of drugs (19). Anthracyclines such as doxorubicin and daunorubicin exist predominantly as cations at physiological pH, and consequently, it seems reasonable for a negatively charged membrane-embedded amino acid to be involved in conferring resistance to these drugs (20). In this study, we have identified a highly conserved tryptophan residue in predicted TM17, the last TM segment in MSD3, that is critical not only for enabling the transport of the conjugated organic anion 

**Experimental Procedures**

**Materials**—6,7-HgE17βG (55 Ci/mmol) and glycine-2-H1GSH (40–44.8 Ci/mmol) were purchased from PerkinElmer Life Sciences. [3H]Vin-cristine (44.8 Ci/mmol) and [14,15-3H]LTC4 (115.3 Ci/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). LTC4 was purchased from Calbiochem, and nucleotides, GSH, verapamil, 2-mercaptoethanol, acivicin, E17βG, and dithiothreitol were purchased from Sigma. Drugs and heavy metal oxyanions used in chemosensitivity assays were obtained as described previously (4).

**Vector Construction and Site-directed Mutagenesis**—The MRP1 expression vector pcDNA3.1(−)-MRP1K was cloned by constructing the EagI/BglII fragment from pMRP-fc-AT engineered with a perfect Kozak sequence into the NotI/KpnI site of the eukaryotic expression vector pcDNA3.1(−) (Invitrogen, Carlsbad, CA) (21).

**Construction of MRP1-GFP Fusion Proteins**—The 238-amino acid mouse MRP1 ortholog (as predicted by the SwissProt database) was identified by searching the NCBI database. The specific amino acids involved in the recognition, binding, and transport of MRP1 substrates are largely unknown. In previous studies, the differing abilities of human MRP1 and its murine ortholog to confer resistance to anthracycline drugs have allowed us to identify a nonconserved glutamic acid residue at position 1089 in putative transmembrane segment 14 (TM14) in the third C-proximal MSD (MSD3) of human MRP1 that is essential for its ability to confer resistance to this class of drugs (19). Anthracyclines such as doxorubicin and daunorubicin exist predominantly as cations at physiological pH, and consequently, it seems reasonable for a negatively charged membrane-embedded amino acid to be involved in conferring resistance to these drugs (20). In this study, we have identified a highly conserved tryptophan residue in predicted TM17, the last TM segment in MSD3, that is critical not only for enabling the transport of the conjugated organic anion E17βG, but also for the ability of MRP1 to confer resistance to both cationic and electroneutral natural product drugs.

First, the TGA stop codon of MRP1 was eliminated using polymerase (forward) and 5′-CCACGTACTTGAACTATCT-3′ (reverse) (substituted nucleotides are underlined). 5′-CCACGTACTTGAACTATCT-3′ was cloned into the pcDNA3.1(−)-MRP1K, and the fragments in the full-length constructs were sequenced once again. The specific amino acids involved in the recognition, binding, and transport of MRP1 substrates are largely unknown. In previous studies, the differing abilities of human MRP1 and its murine ortholog to confer resistance to anthracycline drugs have allowed us to identify a nonconserved glutamic acid residue at position 1089 in putative transmembrane segment 14 (TM14) in the third C-proximal MSD (MSD3) of human MRP1 that is essential for its ability to confer resistance to this class of drugs (19). Anthracyclines such as doxorubicin and daunorubicin exist predominantly as cations at physiological pH, and consequently, it seems reasonable for a negatively charged membrane-embedded amino acid to be involved in conferring resistance to these drugs (20). In this study, we have identified a highly conserved tryptophan residue in predicted TM17, the last TM segment in MSD3, that is critical not only for enabling the transport of the conjugated organic anion E17βG, but also for the ability of MRP1 to confer resistance to both cationic and electroneutral natural product drugs.

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side-out membrane vesicles were prepared from transiently transfected HEK293T cells or stably transfected HeLa cells, and ATP-dependent transport of \(^{3}H\)-labeled substrates by the membrane vesicles was measured using a rapid filtration technique as described previously (13). Briefly, LTC\(_{50}\) transport assays were performed at 23 °C in a 50-μl reaction containing 50 mM LTC\(_{50}\), (40 nCi), 4 mM AMP or ATP, 10 mM MgCl\(_{2}\), 10 mM creatine phosphate, 100 μg/ml creatine kinase, and 2–4 μg of vesicle protein in transport buffer (50 mM Tris-HCl, 250 mM sucrose, pH 7.4). Uptake was stopped at selected times by rapid dilution in ice-cold buffer, and then the reaction was filtered through glass-fiber filters (type A/E) that had been presoaked in transport buffer. Radioactivity was quantitated by liquid scintillation counting. All data were corrected for the amount of \(^{3}H\)HLTC\(_{50}\) that remained bound to the filter, which was usually <10% of the total radioactivity. Transport in the presence of AMP was subtracted from transport in the presence of ATP to determine ATP-dependent LTC\(_{50}\) uptake. All transport assays were carried out in triplicate, and results are expressed as means ± S.D.

Uptake of \(^{3}H\)E\(_{17}\)G was measured in a similar fashion, except that membrane vesicles (2–4 μg of protein) were incubated at 37 °C in a total reaction volume of 50 μl containing E\(_{17}\)G (400 nM, 40 nCi) and the components as described for LTC\(_{50}\) transport. \(^{3}H\)GSH uptake was also measured by rapid filtration with membrane vesicles (20 μg of protein) incubated at 37 °C in a 60-μl reaction volume with 100 μM \(^{3}H\)GSH (300 nCi/reaction) (17). To minimize GSH catabolism by γ-glutamyltransferase during transport, membranes were preincubated in 0.5 mM acivicin for 10 min at 37 °C prior to measuring \(^{3}H\)GSH uptake in the presence of verapamil (100 μM) (16).

Chemosensitivity Testing and \(^{3}H\)-Labeled Drug Accumulation in Stably Transfected HeLa Cell Lines—The relative drug resistance of the HeLa cell lines stably transfected with wild-type and mutant MRP1 cDNAs was determined using a tetrazolium-based microtiter plate assay (25). Briefly, HeLa cells (1.25 × 10\(^6\) cells/well), incubated at 37 °C for 24 h before the addition of drug, and then incubated for a further 72 h before the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2 mg/ml of PBS). After 3 h at 37 °C, isopropyl alcohol and 1 N HCl (24:1) were added to solubilize the formazan crystals, and the absorbance at 570 nm was determined using an ELX800 microplate reader (Bio-Tek Instruments, Inc.). IC\(_{50}\) values were obtained from the dose-response curves using GraphPAD Prism\textsuperscript{®} software. All assays were carried out in quadruplicate and reported as means ± S.D. Relative resistance was calculated as the IC\(_{50}\) of the wild-type or mutant MRP1-transfected HeLa cell line divided by the IC\(_{50}\) of the HeLa cell line transfected with empty pcDNA3.1(−) vector alone.

\(^{3}H\)Vincentine accumulation in intact cells was measured as described previously (25). Briefly, HeLa cells (1.25 × 10\(^6\) cells/ml) were incubated at 37 °C in the presence of 1 μM \(^{3}H\)Vincentine (1 μCi/ml) in RPMI 1640 medium supplemented with 5 mM HEPS (pH 7.0), 10 mM glucose, and 5% fetal bovine serum. Aliquots of suspended cells were removed at 0 and 60 min, and accumulation of \(^{3}H\)Vincentine was stopped by rapid dilution into ice-cold PBS. Cells were washed twice with PBS, and the cell pellets were solubilized in 1% SDS. Cell-associated radioactivity was determined by liquid scintillation counting.

RESULTS

Identification of Trp\(^{1246}\) as a Functionally Important Amino Acid in MRP1—During the course of a mutational analysis of proline residues in MRP1, we generated an MRP1 mutant in which proline at position 196 was replaced with alanine (P196A). When stably expressed in HeLa cells, the ability of the structural and functional importance of this amino acid. A W1246C Substitution Causes Complete Loss of \(^{3}H\)GSH Transport Activity in Membrane Vesicles from Transiently Transfected HEK293T Cells—As a first step to confirming the importance of Trp\(^{1246}\), this amino acid was substituted with cysteine in the transmembrane helices are those predicted by the MEMSAT algorithm. B, alignment of putative transmembrane segment TM17 of MRP1 and related ABC proteins showing conservation of Trp\(^{1246}\) in this subfamily of the ABC superfamily of transporter proteins. The numbers to the right indicate the number of amino acids in the sequences that are identical to the 23 amino acids (positions 1228–1250) of TM17 of MRP1. C, helical wheel projection of the amino acid sequence of putative TM17 of MRP1. Shaded circles indicate amino acids that can participate in hydrogen-bonding interactions. NBD, nucleotide-binding domain; Hum, human; mus, Mus musculus; Ysc, yeast S. cerevisiae; Lt, L. tarentolae; At, A. thaliana; CFTR, cystic fibrosis transmembrane conductance regulator.
observed to be essentially proportional to their relative levels of expression (Fig. 2B). Subsequent kinetic analyses of LTC₄ transport by the W1246C-MRP1 mutant indicated that its affinity for this substrate was reduced compared with wild-type MRP1 (Kₘ = 189 nM versus 79 nM), but the Vₘₐₓ values for the two proteins were similar (40.6 versus 35.1 pmol/mg/min). These results indicate that substitution of Trp1246 with a nonpolar non-aromatic amino acid (M) inhibits LTC₄ transport by W1246C-MRP1. In contrast, the W1246A mutant did not transport E₂₁⁷G (Fig. 3). As shown in Fig. 3A, the levels of expression of the mutant proteins were comparable or slightly higher than those of wild-type MRP1. The LTC₄ transport levels of the W1246A-MRP1 mutant (Fig. 3B) and the W1246F-MRP1 and W1246F-MRP1 mutants (Fig. 3C) were similar to those of wild-type MRP1 and the W1246C-MRP1 mutant. In contrast, like the W1246C mutant, the W1246A mutant did not transport E₂₁⁷G (Fig. 3D). E₂₁⁷G transport by the W1246Y and W1246F mutants was also extremely low (≈10% of wild-type MRP1) (Fig. 3E).

**Verapamil-stimulated Transport of GSH Remains Intact after Substitution of Trp₁²⁴⁶**—To determine whether the Trp₁²⁴⁶ substitution might affect the transport of other MRP1 substrates, GSH uptake was measured in membrane vesicles prepared from HEK293T cells transiently transfected with wild-type (WT-MRP1) and mutant MRP1 proteins in transiently transfected HEK293T cells, A, relative levels of wild-type (WT-MRP1) and mutant MRP1 proteins in the membrane vesicles were determined by immunoblotting with the MRP1-specific murine mAb QCRL-1 as described under “Experimental Procedures.” The numbers below the blot refer to the relative levels of MRP1 proteins. B, shown is the time course of LTC₄ uptake in membrane vesicles prepared from HEK293T cells transiently transfected with wild-type MRP1 (■), mutant W1246C-MRP1 (■), and empty control (○) cDNA expression vectors. Membrane vesicles were incubated at 23 °C with 50 nM [³H]LTC₄ in transport buffer for the times indicated. Results shown are means ± S.D. of triplicate determinations in a single experiment. Similar results were found in three additional independent experiments. C, shown is the time course of E₂₁⁷G uptake in membrane vesicles prepared from HEK293T cells transiently transfected with wild-type MRP1 (■), mutant W1246C-MRP1 (■), and control empty (○) cDNA expression vectors. Membrane vesicles were incubated at 37 °C with 400 nM [³H]E₂₁⁷G in transport buffer for the times indicated. Results shown are means ± S.D. of triplicate determinations in a typical experiment. Similar results were found in three additional independent experiments. D, shown is the time course of LTC₄ uptake in membrane vesicles prepared from HEK293T cells transiently transfected with wild-type MRP1 (■), mutant W1246C-MRP1 (■), and control empty (○) cDNA expression vectors. Membrane vesicles were incubated at 37 °C with 400 nM [³H]E₂₁⁷G in transport buffer for the times indicated. Results shown are means ± S.D. of triplicate determinations in a single experiment. Similar results were obtained in a second experiment.

Conservative and Nonconservative Substitutions of MRP1 Trp₁²⁴⁶ Cause Loss of E₂₁⁷G Transport Activity—To determine whether the loss of E₂₁⁷G transport activity was related specifically to a Cys substitution at position 1246, several different amino acids were introduced in place of Trp₁²⁴⁶. These included substitution with a nonpolar non-aromatic amino acid (A); W1246A-MRP1) as well as conservative substitutions with polar (Tyr; W1246Y-MRP1) and nonpolar (Phe; W1246F-MRP1) aromatic amino acids. Membrane vesicles were prepared from HEK293T cells transiently transfected with these constructs, and the ATP-dependent transport of LTC₄ and E₂₁⁷G was determined (Fig. 3). As shown in Fig. 3A, the levels of expression of the mutant proteins were comparable or slightly higher than those of wild-type MRP1. The LTC₄ transport levels of the W1246A-MRP1 mutant (Fig. 3B) and the W1246F-MRP1 and W1246F-MRP1 mutants (Fig. 3C) were similar to those of wild-type MRP1 and the W1246C-MRP1 mutant. In contrast, like the W1246C mutant, the W1246A mutant did not transport E₂₁⁷G (Fig. 3D). E₂₁⁷G transport by the W1246Y and W1246F mutants was also extremely low (≈10% of wild-type MRP1) (Fig. 3E).

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A Conserved Tryptophan and MRP1 Substrate Specificity

Verapamil-stimulated ATP-dependent \[^{3}H\]GSH uptake in transfected cells expressing Trp\[^{1246}\] MRP1 mutants. A, shown are immunoblots of membrane vesicles prepared from transiently transfected HEK293T cells as described in the legend to Fig. 3A. B, membrane vesicles were preincubated with avicin and then incubated with 300 nm \[^{3}H\]GSH in the presence of 100 \(\mu\)M verapamil in transport buffer for 30 min at 37 °C as described under "Experimental Procedures." Results shown are means \(\pm\) S.D. of triplicate determinants in a single experiment. Similar results were obtained in two additional experiments. WT-MRP1, wild-type MRP1.

Trim1246 Mutant MRP1 Molecules Are Correctly Routed to the Plasma Membrane—To ensure that the loss of transport activity in the Trim1246 MRP1 mutants was not caused by impaired trafficking of the mutant molecules to the plasma membrane, GFP-tagged constructs of wild-type MRP1 and mutants W1246C and W1246A were generated and transiently transfected into HEK293T cells. When viewed under the confocal microscope, both the wild-type and mutant MRP1 proteins were observed to localize strongly to the plasma membrane, indicating that in the transiently transfected cells, the mutants were correctly routed to the cell surface (Fig. 5). Studies with HEK293T and HeLa cells transfected with wild-type MRP1-GFP cDNA showed that fusion of GFP to the C terminus of wild-type MRP1 did not affect the Ltc\(_4\) and E\(_2\)17βG transport activities or the drug resistance-conferring properties of the protein.4

Drug Resistance Is Lost and Accumulation Is Restored in Stably Transfected Trim1246 Mutant MRP1 HeLa Cells—In addition to its ability to transport organic anions such as GSH, E\(_2\)17βG, and Ltc\(_4\), MRP1 confers resistance to natural product anticancer drugs by reducing drug accumulation in cells in which it is overexpressed. To examine whether Trim1246 plays a role in conferring drug resistance, stably transfected cell lines were established by transfection of HeLa cells with pcDNA3.1(−)−MRP1K and pcDNA3.1(−)−W1246C-MRP1. After G418 selection and cloning by limiting dilution, stable cell lines were obtained, and uniformity of protein expression levels was established by flow cytometric and immunoblot analyses. The transfected HeLa cell lines were then tested for vincristine resistance using a tetrazolium salt-based chemosensitivity assay. As expected, HeLa cells expressing wild-type MRP1 displayed \(\sim\)8-fold resistance to this drug (Fig. 6A). In contrast, the IC\(_{50}\) of the W1246C-MRP1 mutant for vincristine was similar to that of the vector control-transfected cell line. When accumulation of \[^{3}H\]vincristine was measured, steady-state concentrations of the drug in the W1246C-MRP1-transfected cells were comparable to those in vector control-transfected cells (33.83 \(\pm\) 1.94 versus 34.25 \(\pm\) 2.24 pmol/10\(^6\) cells/h) (Fig. 6B). However, in wild-type MRP1-transfected cells, vincristine accumulation was reduced to 19.44 \(\pm\) 1.11 pmol/10\(^6\) cells/h (\(\sim\)5% of vector control-transfected cells), consistent with results obtained in previous studies (4).

The drug resistance phenotype of the W1246C-MRP1-transfected cells was further characterized by determining the sensitivity of these cells to the cationic anthracyclines doxorubicin and daunorubicin as well as the electroneutral epipodophyllotoxin VP-16 (etoposide). As shown in Fig. 7, the IC\(_{50}\) values of the W1246C-MRP1-transfected cells were comparable to those in vector control-transfected cells (33.83 \(\pm\) 1.94 versus 34.25 \(\pm\) 2.24 pmol/10\(^6\) cells/h) (Fig. 6B). However, in wild-type MRP1-transfected cells, vincristine accumulation was reduced to 19.44 \(\pm\) 1.11 pmol/10\(^6\) cells/h (\(\sim\)5% of vector control-transfected cells), consistent with results obtained in previous studies (4).

Resistance to Potassium Antimony Tartrate Is Retained, but Resistance to Sodium Arsenite Is Reduced, in W1246C-MRP1-expressing Cells—We have previously shown that in addition to conferring resistance to anticancer drugs, both human and murine MRP1 can confer low level resistance to arsenical and antimonal oxoanions (4, 28). When tested for sensitivity to potassium antimony tartrate, the IC\(_{50}\) of the W1246C mutant HeLa cells was comparable to that of cells expressing wild-type MRP1 (40 versus 30 \(\mu\)g/ml) (Fig. 8A). Thus, both cell lines showed a similar level of resistance (6–8-fold) to this heavy metal oxoanion. In contrast, W1246C mutant cells displayed reduced resistance to sodium arsenite (Fig. 8B). Thus, the IC\(_{50}\) of W1246C mutant HeLa cells for sodium arsenite was 1 \(\mu\)g/ml.

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4 W. Qiu, A. Haimeur, R. G. Deeley, and S. P. C. Cole, unpublished observations.
compared with 3.5 μg/ml for cells expressing wild-type MRP1 and 0.6 μg/ml for vector control-transfected cells.

**DISCUSSION**

MRP1 was first cloned and identified as a protein capable of effluxing cationic and electroneutral chemotherapeutic agents and subsequently demonstrated to be a transporter of GSH-, glucuronide-, and sulfate-conjugated organic anions (1, 2, 28–31). Earlier studies from our group and others demonstrating competitive inhibition between structurally diverse substrates indicated that MRP1 contains mutually exclusive substrate-binding sites and TM structures that allow the recognition and passage of cationic, anionic, and electroneutral molecules. Thus, among ABC transporters, MRP1 and its related proteins have a remarkably broad capacity to transport substrates of striking physical and chemical diversity. However, the molecular basis for the breadth of this transport capacity is largely unknown.

Previous studies have shown that portions of the first 280 amino acids of MRP1 encompassing the N-terminal MSD (MSD1) and the cytoplasmic loop connecting it to the second MSD (MSD2) are important for its expression in mammalian cell membranes as well as its ability to transport at least some of its substrates (32, 33). In the course of a mutational analysis of this region of MRP1, we derived a mutant in which Pro196 was replaced with Ala by site-directed mutagenesis (34). When stably expressed in HeLa cells, the ability of this MRP1 mutant to transport organic anions was diminished, and in particular, E217bG transport was not detectable. Moreover, this mutant

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**FIG. 6. Vincristine resistance and accumulation in stably transfected HeLa cells.** A, vincristine sensitivity of HeLa cell lines stably transfected with the pcDNA3.1(−) vector (○), pcDNA3.1(−)-MRP1K (■), and pcDNA3.1(−)-W1246C-MRP1 (▵) was determined using a tetrazolium salt-based chemosensitivity assay as described under “Experimental Procedures.” Each point represents the mean ± S.D. of quadruplicate determinations in a single experiment. Similar results were obtained in two additional experiments. B, [3H]vincristine (VCR) accumulation in HeLa cell lines stably transfected with the pcDNA3.1(−) vector (white bar), pcDNA3.1(−)-MRP1K (wild-type MRP1 (WT-MRP1); black bar), and pcDNA3.1(−)-W1246C-MRP1 (gray bar). Cells (1 × 10⁶) were incubated with [3H]vincristine for 1 h at 37 °C, and accumulation was stopped by the addition of ice-cold PBS. Cell-associated radioactivity was quantitated by liquid scintillation counting. Each bar represents the mean ± S.D. of triplicate determinations in a single experiment. Similar results were obtained in one additional experiment.

**FIG. 7. Resistance of MRP1-transfected cells to chemotherapeutic drugs.** HeLa cell lines stably transfected with the pcDNA3.1(−) vector (○), pcDNA3.1(−)-MRP1K (■), and pcDNA3.1(−)-W1246C-MRP1 (▵) were exposed to doxorubicin (A), daunorubicin (B), and VP-16 (C) for 72 h at 37 °C, and then cell viability was measured as described under “Experimental Procedures.” The results shown are those of a typical experiment in which each data point represents the mean ± S.D. of quadruplicate determinations in a single experiment. Similar results were obtained in a second experiment.
protein failed to confer drug resistance. In contrast, a second independently derived transfected cell line also expressing an MRP1 protein with the same P196A mutation displayed a phenotype similar to that of cells expressing wild-type MRP1. It was subsequently discovered that the transfected MRP1 in the first P196A mutant cell line had acquired a second non-engineered mutation causing the substitution of Cys for Trp at position 1246. Consequently, we generated a single W1246C mutant of MRP1 so that its phenotype could be compared with that of the double P196A/W1246C mutant.

When transiently transfected into HEK293T cells, W1246C-MRP1 could be expressed at levels comparable to those of wild-type MRP1 and also transported LTC4 with a similar efficiency. Moreover, verapamil-stimulated GSH transport was measured as described under “Experimental Procedures.” The results shown are those of a typical experiment in which each data point represents the mean ± S.D. of quadruplicate determinations in a single experiment. Similar results were obtained in two additional experiments.

To examine the drug resistance phenotype of the W1246C mutation, stably transfected HeLa cells were generated. In addition to the selective loss of E17G transport, we found that W1246C-MRP1-expressing cells were no longer resistant to natural product chemotherapeutic agents, including the electroneutral VP-16 and the cationic vincristine and anthracyclines. Consistent with this loss of drug resistance, vincristine accumulation in intact cells expressing W1246C-MRP1 was comparable to that in vector control-transfected cells, which was ~2-fold higher than vincristine accumulation in HeLa cells expressing wild-type MRP1. On the other hand, the W1246C-MRP1-expressing cells were still resistant to antimony tartrate and partially resistant to sodium arsenite. Taken together, these observations suggest that the structural determinants in MRP1 necessary for recognition and transport of these heavy metal oxyanions differ from those for the natural product chemotherapeutic agents.

Current topological models of MRP1 are based on predictions from computer-based algorithms for which there are limited supporting biochemical data. Although similar in many respects, some models differ in their placement of several TM segments in the MSDs (7, 35). However, with the exception of the PredictProtein model, which predicts only four TM helices in MSD3, all of the algorithms place Trp1246 in the last TM segment close to the cytoplasmic face of the membrane (Fig. 1A). This tryptophan residue is extremely well conserved among MRP (ABCC) subfamily members, including MRPs -2, -3, -4, and -6 and murine MRP1. It is also found in the cystic fibrosis transmembrane conductance regulator, the sulfonylurea receptor SUR1, the \textit{Saccharomyces cerevisiae} cadmium resistance protein Ycf1, \textit{Leishmania tarentolae} PGPA, and the \textit{Arabidopsis thaliana} conjugate transporter AtMRP3 (Fig. 1B). However, it is not conserved in human P-glycoprotein (MDR1) or other members of the ABCB subfamily to which this multidrug resistance protein belongs. The last TM helix in MSD3 of MRP1 has a highly amphipathic character, with Trp1246 and other amino acids with hydrogen-bonding side chains densely clustered on one side of the α-helix (Fig. 1C) and predominantly in the region predicted to be in the inner leaflet of the plasma membrane (Fig. 1A). The last predicted TM helix of other ABCB subfamily members in which Trp1246 is conserved such as MRP2 and MRP3 has a similar amphipathic character, lending support to the idea that the extensive hydrogen-bonding capacity of putative TM17 may play an important role in the substrate-binding and transport properties of these proteins. Interestingly, the topologically comparable TM segment in P-glycoprotein (TM12) has also been demonstrated to contain key determinants of substrate specificity, but TM12 of this ABC transporter is significantly less amphipathic than TM17 of MRP1, -2, and -3 (36–38). This difference may be relevant to the apparent differences in the substrate specificity and transport mechanisms of MRP1 and P-glycoprotein (2, 3). MRP1 Trp1246 may function to either maintain or form part of a substrate-binding pocket, and/or it may interact directly with the natural product drugs and E17G. The facts that the Trp1246 mutants were expressed at comparable levels and correctly routed to the plasma membrane in the transiently transfected cells and that their LTC4 - verapamil-stimulated GSH transport activities remained intact indicate that the mutations did not perturb the global structural integrity of the

\[ \text{FIG. 8. Resistance of MRP1-transfected HeLa cells to heavy metal oxyanions.} \]

\[ \text{HeLa cell lines stably transfected with the pdDNA3.1(−) vector (○), pdDNA3.1(−)-MRP1K (△), and pdDNA3.1(−)-W1246C-MRP1 (■) were exposed to potassium antimony tartrate (A) or sodium arsenite (B) for 72 h at 37 °C, and then cell viability was measured as described under “Experimental Procedures.” The results shown are those of a typical experiment in which each data point represents the mean ± S.D. of quadruplicate determinations in a single experiment. Similar results were obtained in two additional experiments.} \]
protein. The inability of the variously substituted Trp\textsuperscript{1246} MRP1 mutants to transport E\textsubscript{217}βG suggests that tryptophan possesses specific physical and/or chemical properties that are essential for recognition and transport of some MRP1 substrates. Thus, the absence of E\textsubscript{217}βG transport activity and drug resistance in the W1246C-MRP1 mutant can be explained by the fact that although cysteine can participate in hydrogen bonding, it is considerably smaller than tryptophan and lacks aromaticity. However, the introduction of a phenylalanine or tyrosine residue at position 1246 also resulted in the loss of aromaticity. However, the introduction of a phenylalanine or tyrosine residue at position 1246 also resulted in the loss of aromaticity. However, the introduction of a phenylalanine or tyrosine residue at position 1246 also resulted in the loss of aromaticity.

Our data clearly demonstrate that Trp\textsuperscript{1246} is critical for the recognition and transport of E\textsubscript{217}βG by MRPI. However, although the human and murine orthologs of MRPI differ markedly in their ability to transport this conjugated estrogen, this amino acid is conserved in the two proteins, suggesting that other, nonconserved residues are important as well (28). Thus, it seems probable that several conserved and nonconserved determinants within the MSDs and possibly cytosolic regions of MRPI come together to form a multiparticle binding pocket for this organic anion and, furthermore, that substitution of just one amino acid can be sufficient to abrogate E\textsubscript{217}βG recognition and transport activity as well as the ability to confer drug resistance. Our recent finding that wild-type MRPI, but not W1246A-MRP1, transports the O-glucuronide of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol indicates that Trp\textsuperscript{1246} is important for the transport of other glucuronide conjugates as well.

It has been proposed (and there is considerable evidence to support the notion) that substrates for P-glycoprotein are taken up from the inner leaflet of the plasma membrane (39). Whether or not this is also true for every MRPI substrate is not known, but may depend on the physical characteristics of the substrate, such as its hydrophobicity, aromaticity, and charge, as well as certain aspects of its chemical structure. The fact that a hydrophobic-aromatic amino acid close to the cytosolic face of the protein is of such critical importance for the binding and transport of E\textsubscript{217}βG suggests that this organic anion may be taken up by MRPI from the cytoplasm. Why LT\textsubscript{C}β transport is not affected by the Trp\textsuperscript{1246} substitutions is unclear. It is possible that steric complementarity is more important for interaction of MRPI with E\textsubscript{217}βG than with LT\textsubscript{C}β. In support of this idea are our earlier findings that a change in the site of glucuronidation on the D-ring of 16α,17β-estradiol from the 17β-position to the 16α-position increased the K\textsubscript{s} for E\textsubscript{217}βG transport 30-fold (26). The LT\textsubscript{C}β molecule is significantly less rigid and more lipophilic than E\textsubscript{217}βG. These physical properties of LT\textsubscript{C}β may make its recognition and binding less affected by the changes in the architecture of a multiparticle substrate-binding pocket caused by replacement of the Trp\textsuperscript{1246} residue as well as possibly favoring its uptake from the membrane leaflet.

As mentioned previously, we have recently shown that the ability of MRPI to confer resistance to catonic anthracycines is lost when Glu\textsuperscript{1089} is substituted with a glutamine residue (19). This finding, together with our present data, indicates that recognition and transport of these drugs depend not only on the presence of a membrane-embedded negatively charged amino acid in putative TM14, but also on the presence of the bulky indole Trp\textsuperscript{1246} in TM17. Thus, these data raise the possibility that interactions between TM14 and TM17 are required for MRPI to confer drug resistance. The comparable interaction in P-glycoprotein would be between TM9 and TM12. However, such an interaction has not been reported, providing further evidence of significant differences between these two drug resistance proteins with respect to the mechanisms by which they recognize and transport xenobiotics.

As has been proposed for other multidrug transporters (20), MRP1 substrate molecules may penetrate the hydrophobic core of the protein, where they form a number of van der Waals and stacking interactions with the surrounding hydrophobic and aromatic residues. Our data suggest that for a broad range of MRPI substrates, aromatic stacking interactions, hydrogen-bonding interactions, and possibly \(\pi\)-bonding interactions with Trp\textsuperscript{1246} (and possibly other amino acids in TM17) are also important, as are electrostatic interactions with negatively charged amino acids such as Glu\textsuperscript{1089} in the case of cationic drugs (19). Whether or not anionic substrates have electrostatic interactions with positively charged residues in MRP1 is not yet known. However, the predicted MSDs of MRPI and related proteins contain a significant number of membrane-embedded arginine and lysine residues. Indeed, it has recently been reported that mutants of the related protein MRPI, in which several basic residues in MSD2 and MSD3 have been replaced by other amino acids show decreased transport activity (40, 41). Thus, additional molecular and pharmacological studies are anticipated to continue to provide important information on the structural features that determine how MRPI binds and transports both their charged and electroneutral substrates.

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Mutation of a Single Conserved Tryptophan in Multidrug Resistance Protein 1 (MRP1/ABCC1) Results in Loss of Drug Resistance and Selective Loss of Organic Anion Transport

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