MUTATIONAL ANALYSIS OF POLAR AMINO ACID RESIDUES WITHIN PREDICTED TRANSMEMBRANE HELICES 10 AND 16 OF MULTIDRUG RESISTANCE PROTEIN 1 (ABCC1): EFFECT ON SUBSTRATE SPECIFICITY

Da-Wei Zhang,¹ Kenichi Nunoya,² Monika Vasa, Hong-Mei Gu, Susan P. C. Cole, and Roger G. Deeley

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ABSTRACT:

Human multidrug resistance protein 1 (MRP1) has a total of 17 transmembrane (TM) helices arranged in three membrane-spanning domains, MSD0, MSD1, and MSD2, with a 5 + 6 + 6 TM configuration. Photolabeling studies indicate that TMs 10 and 11 in MSD1 and 16 and 17 in MSD2 contribute to the substrate binding pocket of the protein. Previous mutational analyses of charged and polar amino acids in predicted TM helices 11, 16, and 17 support this suggestion. Mutation of Trp⁶⁵⁶ in TM10 also affects substrate specificity. To extend this analysis, we mutated six additional polar residues within TM10 and the remaining uncharacterized polar residue in TM16, Asn¹²⁰⁸. Although mutation of Asn¹²⁰⁸ was without effect, two of six mutations in TM10, T550A and T556A, modulated the drug resistance profile of MRP1 without affecting transport of leukotriene C₄, 17β-estradiol 17-β-D-glucuronide (E₂₁₇βG), and glutathione. Mutation T550A increased vincristine resistance but decreased doxorubicin resistance, whereas mutation T556A decreased resistance to etoposide (VP-16) and doxorubicin. Although conservative mutation of Tyr⁶⁶⁸ in TM10 to Phe or Trp had no apparent effect on substrate specificity, substitution with Ala decreased the affinity of MRP1 for E₂₁₇βG without affecting drug resistance or the transport of other substrates tested. These analyses confirm that several amino acids in TM10 selectively alter the substrate specificity of MRP1, suggesting that they interact directly with certain substrates. The location of these and other functionally important residues in TM helices 11, 16, and 17 is discussed in the context of an energy-minimized model of the membrane-spanning domains of MRP1.

Human multidrug resistance protein 1 (MRP1) is a member of the “C” branch of the ATP-binding cassette transporter (ABC) superfamily and has been designated ABCC1. The predicted topology of MRP1 consists of a typical P-glycoprotein-like core region, composed of two membrane-spanning domains (MSDs 1 and 2), each with six transmembrane (TM) α-helices, and two nucleotide binding domains. In addition, MRP1 contains an NH₂-terminal MSD, MSD0, which consists of five TMs with an extracellular NH₂ terminus (Bakos et al., 1996; Hipfner et al., 1997; Kast and Gros, 1997). Thus, the protein contains three MSDs with a total of 17 predicted TM helices (5 + 6 + 6; Fig. 1).

MRP1 confers resistance to many commonly used natural product chemotherapeutic agents including anthracyclines, Vinca alkaloids, and epipodophyllotoxins, as well as methotrexate and certain heavy metal oxyanions (Cole et al., 1992, 1994). However, transport of unmodified drugs by MRP1 is both GSH- and ATP-dependent (Rappa et al., 1997; Loe et al., 1998; Renes et al., 1999). In some cases, GSH appears to be cotransported with these compounds (Rappa et al., 1997; Loe et al., 1998; Renes et al., 1999). Detailed in vitro transport measurements using MRP1-enriched inside-out membrane vesicles have demonstrated that MRP1 is capable of directly transporting many glutathione-, glucuronide-, and sulfate-conjugated organic anion conjugates, such as the glutathione conjugate cysteinyl leukotriene 4 (LTC₄), and glucuronate conjugate 17β-estradiol 17-β-D-glucuronide (E₂₁₇βG) in an ATP-dependent manner (Muller et al., 1994; Jedlitschky et al., 1996; Loe et al., 1996a,b). The mechanism by which MRP1 binds and transports such structurally unrelated cytotoxic drugs and conjugated organic anions remains an active area of study. In the absence of a crystal structure of the protein, identification of amino acid residues involved in determining substrate specificity and transport activity, coupled with structural predictions, has provided valuable insights into the mechanism by which MRP1 recognizes structurally diverse compounds.

ABBREVIATIONS: MRP, multidrug resistance protein; ABC, ATP-binding cassette transporter; MSD, membrane-spanning domain; TM, transmembrane; mAb, monoclonal antibody; E₂₁₇βG, 17β-estradiol 17-β-D-glucuronide; GSH, glutathione; LTC₄, leukotriene C₄; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VP-16, etoposide; HEK, human embryonic kidney; PBS, phosphate-buffered saline.

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Photoaffinity labeling studies have implicated TMs 10, 11, 16, and 17 of MRP1 as components of the substrate binding pocket(s) of the protein (Daoud et al., 2001; Qian et al., 2001, 2002; Mao et al., 2002). Previously, we have demonstrated that certain amino acid residues with hydrogen-bonding side chains located in the putative TM11 and -17 of MRP1 are important, either for overall activity or substrate specificity of the protein (Ito et al., 2001; Zhang et al., 2001a,b, 2002, 2004; Haimeur et al., 2002, 2004; Koike et al., 2002). Thr550 and Thr556, located within TM10 of MRP1, have also been shown to be important for the transport activity of the protein (Koike et al., 2002, 2004). More recently, charged residues Arg1197, Arg1202, and Glu1204, and the polar residue Trp1198, predicted to be within TM16, have been described previously (Zhang et al., 2001a). Briefly, HEK293 cells were transiently transfected with the pCEBV7 vector containing the wild-type and mutant MRP1 cDNAs has been described previously (Zhang et al., 2001a). After determination of protein expression of exposed films.

Materials. Culture medium and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA). [3H]LTC4 (38 Ci/mmol) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and [3H]E17βG (44 Ci/μmol) and [3H]GSH from PerkinElmer Life Sciences (Boston, MA). Doxorubicin HCl, etoposide (VP-16), and vincristine sulfate were obtained from Sigma (St. Louis, MO).

Site-Directed Mutagenesis. Mutation T564A was generated using the Transformer Site-Directed Mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA). Templates were prepared as described previously (Zhang et al., 2004). Mutagenesis was then performed according to the manufacturer’s instructions. Oligonucleotides bearing mismatched bases at the residues to be mutated (underlined) were synthesized by ACGT Corp. (Toronto, ON, Canada) with the following sequence: 5'-GGTTGCGGTTGACATTCGATG-3'.

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FIG. 1. Topology of human MRP1. A, the predicted topology of human MRP1 with 17 TM helices. The putative TM10 and -16 are indicated by lighter shading. B, an expanded view of TM10 and -16. Mutated polar residues are indicated by shaded circles.

Determination of Protein Levels in Transfected Cells. Plasma membrane vesicles were prepared by centrifugation through sucrose, as described previously (Loo et al., 1996b; Zhang et al., 2001a). After determination of protein levels by the Bradford assay (Bio-Rad, Hercules, CA), total membrane protein (0.5 μg, 1.0 μg, and 1.25 μg) from transfectants expressing wild-type MRP1 and various mutant proteins were resolved on a 7.5% SDS-polyacrylamide gel electrophoresis and then transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). The proteins were detected with the mAb, MRPm6 (Alexis Biochemicals, San Diego, CA), and the signal was enhanced using Renaissance chemiluminescence reagent (PerkinElmer Life Sciences). Relative levels of MRP1 expression were determined by densitometry of exposed films.

Cell Lines and Tissue Culture. Stable transfection of HEK293 cells with the pCEBV7 vector containing the wild-type and mutant MRP1 cDNAs has been described previously (Zhang et al., 2001a). Briefly, HEK293 cells were transfected with pCEBV7 vectors containing mutant MRP1 using Fugene6 (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer’s instructions. After ~48 h, the transfected cells were supplemented with fresh medium containing 100 μg/ml hygromycin B. Approximately 3 weeks after transfection, the hygromycin B-resistant cells were cloned by limiting dilution and the resulting cell lines were tested for high level expression of the mutant proteins.
ATP or AMP (4 mM), 10 mM MgCl2, and [3H]LTC4 (50 nM, 200 Ci). At the (50 mM Tris-HCl, 250 mM sucrose, 0.02% sodium azide, pH 7.4) containing confocal microscope (Meridian Instrument Company, Inc., Kent, WA) (filter, MRP1 in the transfected cells was determined using a Meridian Insight MRPs, followed by linear transformation using a Hanes-Woolf plot. Kinetic parameters of ATP-dependent [3H]E2 uptake by membrane vesicles (2.5 µg of protein) were measured at various LTC4 concentrations (0.01–10 µM) for 1 min at 23°C in 25 µl of transport buffer containing 4 mM ATP and 10 mM MgCl2, followed by filtration using a Hanes-Woolf plot. Kinetic parameters of ATP-dependent [3H]E2 uptake were determined as described for [3H]LTC4, except that the temperature used was 37°C.

GSH uptake was also measured by rapid filtration with membrane vesicles (20 µg of protein) incubated at 37°C for 20 min in a 60-µl reaction volume with [3H]GSH (100 µCi, 300 nCi) in the absence and presence of verapamil (100 µM). To minimize GSH catabolism by γ-glutamyltransferase during transport, membranes were preincubated in 0.5 mM acivicin for 10 min at 37°C before measuring [3H]GSH uptake in the presence of verapamil (100 µM).

Chemoresistance Testing. Drug resistance was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Cole et al., 1994; Zhang et al., 2001a). 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 is expressed as the ratio of the IC50 value of cells transfected with MRP1 expression vectors compared with cells transfected with empty vector. Resistance was determined in three or more independent experiments.

Results

Expression of Mutant MRP1 in Stably Transfected HEK293 Cells. Thr550, Thr552, Thr556, Thr564, Tyr569, and Thr570 within TM10, and Asn1308 within TM16 were replaced individually by Ala (Fig. 1). The episomal expression vector, pCEBV7, containing mutated forms of MRP1 cDNAs, was used to stably transfect HEK293 cells, and populations of transfected cells were selected in hygromycin B. The resultant stably transfected cell populations were cloned by limiting dilution, and subpopulations expressing high levels of MRP1 mutant proteins were used in subsequent studies. The levels of mutant proteins relative to wild-type MRP1 measured by immunoblotting (Fig. 2A). The expression levels of these mutant proteins in stably transfected HEK293 cells were determined by immunoblotting of membrane vesicle preparations and densitometry as described. Blots were probed with the MRP1-specific mAb MRP6. The numbers below the blot refer to the levels of the mutant MRP1 proteins relative to the levels of wild-type MRP1 proteins in membrane vesicles prepared from the stably transfected HEK293 cells. The relative levels of the mutant proteins in membrane vesicles were determined by densitometry using a Hanes-Woolf plot. The numbers below the blot refer to the levels of the mutant MRP1 proteins relative to the levels of wild-type MRP1 proteins in membrane vesicles prepared from the stably transfected HEK293 cells.

Fig. 2. Expression of mutant MRP1 in stably transfected HEK293 cells. A, expression levels of wild-type and mutant MRP1 proteins in membrane vesicles isolated from stably transfected HEK293 cells were determined by immunoblotting of membrane vesicle preparations and densitometry as described. Blots were probed with the MRP1-specific mAb MRP6. The numbers below the blot refer to the levels of the mutant MRP1 proteins relative to the levels of wild-type MRP1 proteins in membrane vesicles prepared from the stably transfected HEK293 cells. Values are the mean of three independent experiments. B, the subcellular localization of wild-type and mutant MRP1 was determined by confocal microscopy as described. MRP1 was detected using mAb MRP6. Location of MRP1 is indicated in green. Nuclei were stained with propidium iodide and are shown in red. Transfectants tested were expressing wild-type or mutant MRP1 as indicated in the figure. An x-y optical section of the cells is shown to illustrate the distribution of the wild-type and mutant proteins between plasma and intracellular membranes.

Confocal Microscopy. Confocal microscopy was carried out as described previously (Zhang et al., 2001a,b). Briefly, –5 × 106 stably transfected HEK293 cells were seeded in each well of a six-well tissue culture dish on coverslips. When the cells had grown to confluence, they were washed once in PBS and then fixed with 2% paraformaldehyde in PBS, followed by permeabilization using digitonin (0.25 mg/ml in PBS). MRP1 proteins were detected with the monoclonal antibody MRP6. Antibody binding was detected with Alexa Fluor 488 (Molecular Probes, Eugene, OR) anti-mouse IgG (H+L) (Fab′)2 fragment. Nuclei were stained with propidium iodide. Localization of MRP1 in the transfected cells was determined using a Meridian Insight confocal microscope (Meridian Instrument Company, Inc., Kent, WA) (filter, 620/40 nm for propidium iodide; 530/30 nm for Alexa Fluor 488).

LTC4, E217βG, and GSH Transport by Membrane Vesicles. Plasma membrane vesicles were prepared as described previously, and ATP-dependent transport of [3H]LTC4 into the inside-out membrane vesicles was measured by a rapid filtration technique (Loe et al., 1996b; Zhang et al., 2001a). Briefly, vesicles (10 µg of protein) were incubated at 23°C in 100 µl of transport buffer (50 mM Tris-HCl, 250 mM sucrose, 0.02% sodium azide, pH 7.4) containing ATP or AMP (4 mM), 10 mM MgCl2, and [3H]LTC4 (50 nM, 200 Ci). At the indicated times, 20-µl aliquots were removed and added to 1 ml of ice-cold transport buffer, followed by filtration through a glass fiber filter (type A/E; Gelman Sciences, Dorval, QC, Canada). Filters were immediately washed twice with 4 ml of cold transport buffer. The bound radioactivity was determined by scintillation counting. All data were corrected for the amount of [3H]LTC4 that remained bound to the filter in the absence of vesicle protein (usually <5% of the total radioactivity). [3H]LTC4 uptake was expressed relative to the total protein concentration in each reaction. ATP-dependent uptake of [3H]E217βG (400 nM, 120 Ci) was measured as described for [3H]LTC4, except that the temperature used was 37°C.

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 is expressed as the ratio of the IC50 value of cells transfected with MRP1 expression vectors compared with cells transfected with empty vector. Resistance was determined in three or more independent experiments.

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To determine whether these mutations influenced trafficking of the protein, we compared the subcellular localization of wild-type and mutant MRP1 by confocal microscopy. The subcellular distribution of the mutated proteins assessed by immunoreactivity with the MRP1-
specific mAb MRPm6 was indistinguishable from that of cells expressing wild-type protein (Fig. 2B). In all cases, strong plasma membrane staining was observed, indicating that trafficking was unaffected.

**Transport of [3H]LTC4 and [3H]E217/H9252G by Wild-Type and Mutant MRP1.** To determine whether any of the mutations altered the efficiency with which the protein transported LTC4 and E217/H9252G, we examined ATP-dependent uptake of these compounds by membrane vesicles prepared from HEK transfectants expressing each of the mutant proteins (Fig. 3). The levels of LTC4 uptake by vesicles prepared from HEK transfectants expressing either wild-type or mutant MRP1 were proportional to the relative expression levels of the wild-type and mutant proteins (Fig. 3, A to C). Thus, these polar residues may not be involved in the binding and transport of LTC4. ATP-dependent transport of [3H]E217/H9252G was also examined (Fig. 3, D to F), only replacement of Tyr568 with Ala decreased the transport efficiency by approximately 60%, indicating that the polar and/or the bulky aromatic side chain of the residue at position 568 is important for the ability of MRP1 to transport E217/H9252G.

**Effect of Mutations Y568S, Y568F, and Y568W on the Transport of [3H]LTC4 and [3H]E217/H9252G by MRP1.** Because replacement of Tyr568 by Ala selectively decreased transport of E217/H9252G, this residue was also mutated to Ser, Phe, and Trp. These three mutations were then stably expressed in HEK293 cells. Immunoblotting indicated that the expression levels of mutant MRP1Y568F, MRP1Y568S, and MRP1Y568W were 90, 50, and 90% of wild-type MRP1, respectively (Fig. 4A). The effects of these mutations on the ability of MRP1 to transport LTC4 and E217/H9252G were then examined (Fig. 4, B and C). Like mutation Y568A, substitution of Tyr568 with Ser did not affect the transport of LTC4 but decreased E217/H9252G transport. However, replacement of Tyr568 with the more conservative residues, Phe and Trp, had no effect on transport of either compound (Fig. 4, B and C). Thus, the aromaticity or steric bulk of the residue at position 568, but not the side chain polarity, plays a role in determining the efficiency of transporting the conjugated estrogen.

**Kinetic Parameters of [3H]LTC4 and [3H]E217/H9252G Transport by Wild-Type and Y568A Mutant MRP1.** To more precisely determine the influence of mutation Y568A on the ability of MRP1 to transport E217/H9252G, we compared kinetic parameters for the wild-type and mutant proteins (Fig. 5). For wild-type MRP1 and Y568A, the $K_m$ and normalized $V_{max}$ values for LTC4 uptake were essentially identical. Linear regression using a Hanes-Woolf transformation yielded values of 115 nM and 143 nM, and 76.8 pmol/mg/min and 64 pmol/mg/min, for the $K_m$ and $V_{max}$ values of wild-type and Y568A proteins, respectively (Fig. 5, B and C). For E217/H9252G transport, a comparable analysis yielded $K_m$ values of 1.4 $\mu$M and 5.4 $\mu$M and normalized $V_{max}$ values of 170 pmol/mg/min and 190 pmol/mg/min for wild-type and mutant proteins, respectively (Fig. 5, D and E). Thus, mutation of Tyr568 seems to decrease the affinity of MRP1 for E217/H9252G approximately 4-fold without affecting its transport capacity.

**Resistance Profiles of Wild-Type and Mutant Human Proteins.** The drug resistance profiles of transfectants expressing mutant pro-
transport that can be dramatically stimulated by verapamil (Loe et al., 2000). Thus, we examined the effects of mutations made in TM10 and -16 on verapamil-stimulated GSH transport by MRP1 to determine whether any mutations that affected drug resistance also influenced the GSH transport (Fig. 6). As observed with the effects of the mutations on LTC4 transport, none of these mutations had any significant effect on verapamil-stimulated GSH transport, consistent with the suggestion that they modify interactions between MRP1 and the drug, rather than altering the ability to bind and transport GSH.

**Discussion**

Photolabeling studies indicate that amino acids in predicted TM helices 10, 11, 16, and 17 are probable components of the substrate binding pocket of MRP1 (Daoud et al., 2001; Qian et al., 2001; Mao et al., 2002). Mutational studies have also shown that a number of polar amino acids in TM helices 11, 16, and 17 are major determinants of substrate specificity and overall transport activity of the protein (Ito et al., 2001; Zhang et al., 2001a,b, 2002, 2004; Haimeur et al., 2002, 2004; Koike et al., 2002). The majority of the functionally important polar residues in TM11 and TM17 are in the predicted inner leaflet region of the membrane (Ito et al., 2001; Zhang et al., 2001b, 2002, 2004; Haimeur et al., 2004; Situ et al., 2004). Mapping of these residues onto an energy-minimized model of the tertiary structure of MSD1 and MSD2 of MRP1 suggests that most of their side chains project toward, or line, a central cavity presumed to be the translocation pathway of the protein (Campbell et al., 2004). Thus, they are available to interact with substrate or the side chains of amino acids in neighboring TM helices (Fig. 7). To provide additional experimental evidence for or against the proposed structure, we have extended the mutational analysis to polar residues in TM10 and mutated the remaining uncharacterized polar residue in TM16.

The predicted outer leaflet region of TM16 is devoid of polar amino acids (Fig. 1B), and mutation of the vicinal cysteine residues, Cys1205 and Cys1209, was found previously to have no effect on substrate specificity or overall activity (Olsen et al., 1998). Similarly, mutation of Arg1202 had no effect on transport activity of MRP1. Conversely, replacement of Glu1204 with Leu or Arg1197 with Glu or Lys affected either substrate specificity or overall transport activity of MRP1 (Situ et al., 2004). Mutation of Trp1198 to Ala also dramatically decreased overall transport activity (Koike et al., 2002). Based on the model shown (Fig. 7), these amino acids, and several other functionally important residues in TM17, cluster in the predicted inner leaflet region of the two TMs with their side chains projecting toward TMs 10 and 11. The remaining polar residue in TM16, Asn1208, is also predicted to project into the translocation pore (Fig. 7). However, mutation of Asn1208 had no effect on transport or drug resistance. Similarly, mutation of two polar residues in TM17, Ser1235 and Glu1237, with side chains predicted to project into the pore, also had no effect (Fig. 7) (Zhang et al., 2002). In all cases, these residues are located toward the outer leaflet of the protein relative to the cluster of amino acids that affect substrate specificity or overall activity.

Mutation of Trp553 and Pro557 in TM10 alters the overall transport activity of MRP1 rather than substrate specificity (Koike et al., 2002, 2004). To further examine the role of amino acids in TM10, we mutated five Thr residues and Tyr568. Mutations of Thr552, Thr564, and Thr570 had no effect on either the drug resistance profile or organic anion transport activity. Thr552 is predicted to be in the inner leaflet region of the membrane, but its side chain projects away from the predicted pore. As found with polar residues in TMs 11, 12, 16, and 17 of MRP1 that do not affect substrate specificity, Thr564 and Thr570 are predicted to be located in the outer leaflet region of TM10. Similarly, conservative mutation of Tyr568, which is also predicted to

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**Fig. 4.** ATP-dependent [3H]LTC4 and [3H]E217G uptake by membrane vesicles prepared from HEK293 cells transfected with wild-type or mutant MRP1. A, expression levels of wild-type and mutant MRP1 proteins in membrane vesicles isolated from stably transfected HEK293 cells were determined by immunoblotting. Values are the mean ± S.D. of 3 independent experiments.
FIG. 5. Kinetics of ATP-dependent [3H]LTC4 and [3H]E217βG uptake. A, expression levels of wild-type and mutant MRP1 proteins in membrane vesicles isolated from transiently transfected HEK293 cells were determined by immunoblotting of membrane vesicle preparations and densitometry as described in the legend to Fig. 2A. The numbers below the blot refer to the levels of mutant MRP1 proteins relative to the levels of wild-type MRP1 protein in membrane vesicles prepared from the stably transfected HEK293 cells. B and C, the initial rate of ATP-dependent [3H]LTC4 uptake by membrane vesicles prepared from HEK293 cells transfected with wild-type or mutant proteins was measured at various LTC4 concentrations (0.01–1 μM) for 1 min at 23°C as described. D and E, [3H]E217βG uptake was determined as described for [3H]LTC4 except that the reactions were carried out at 37°C with various concentrations of E217βG (0.1–16 μM). Values are the mean ± S.D. of triplicate determinations in a single experiment. Similar results were obtained from one more experiment. B and D, data were plotted as \( V_0 \) versus \([S]\) to confirm that the concentration range selected was appropriate to observe both zero-order and first-order rate kinetics. C and E, data were plotted as \([S]/V_0 \) versus \([S]\). The transfectants tested were HEKMRP1 (■), and HEKMRP1Y568A (▲). Kinetics parameters for LTC4 and E217βG transport were determined from nonlinear and linear regression analysis of the combined data. Details of \( K_m \) and \( V_{max} \) values for wild-type and mutant MRP1 are provided under Results.

### TABLE 1

<table>
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<tr>
<th>Transfectant</th>
<th>Drug (Relative Resistance Factor)</th>
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<tr>
<td></td>
<td>Vincristine</td>
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<tr>
<td>HEKMRP1</td>
<td>36.0 ± 7.5 (36.0)</td>
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<tr>
<td>HEKMRP1T550A</td>
<td>144.9 ± 27.3 (161.0)</td>
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<tr>
<td>HEKMRP1T552A</td>
<td>21.6 ± 3.5 (36.0)</td>
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<tr>
<td>HEKMRP1T556A</td>
<td>25.0 ± 6.8 (41.6)</td>
</tr>
<tr>
<td>HEKMRP1T564A</td>
<td>12.1 ± 2.6 (24.2)</td>
</tr>
<tr>
<td>HEKMRP1Y568A</td>
<td>33.4 ± 4.9 (41.7)</td>
</tr>
<tr>
<td>HEKMRP1Y568F</td>
<td>32.0 ± 2.4 (40.0)</td>
</tr>
<tr>
<td>HEKMRP1Y568W</td>
<td>25.6 ± 2.3 (32.0)</td>
</tr>
<tr>
<td>HEKMRP1N1208A</td>
<td>16.2 ± 0.9 (32.4)</td>
</tr>
<tr>
<td>HEKMRP1Y568S</td>
<td>28.9 ± 3.5 (32.1)</td>
</tr>
<tr>
<td>HEKMRP1Y568W</td>
<td>30.2 ± 2.7 (33.6)</td>
</tr>
</tbody>
</table>

The resistance of HEK293 cells transfected with expression vectors encoding wild-type and mutant MRP1 relative to that of cells transfected with empty vector was determined using a tetrazolium salt-based microtiter plate assay. The relative resistance factor was obtained by dividing the \( IC_{50} \) values for wild-type/mutant MRP1-transfected cells by the \( IC_{50} \) value for control transfectants. The values shown represent the mean (±S.D.) of relative resistance values determined from three independent experiments. Resistance factors normalized for differences in the levels of mutant proteins expressed in the transfectant populations used are shown in parentheses.
be in the outer leaflet, to either Phe or Trp had no effect on substrate specificity. However, nonconservative mutation to Ala or Ser selectively decreased transport of E217G/H9252 without affecting the transport of LTC4 or GSH, or resistance to any drug tested. Mutation of two of the five Thr residues, Thr550 and Thr556, differentially affected drug resistance without altering transport of the organic anion conjugates tested. Thr550 and Thr556 are predicted to be in the inner leaflet region of TM10, and the side chains of both residues align with that of Trp553. Taken together, these findings confirm the role of TM10 in determining substrate specificity and overall transport activity of MRP1.

We have previously proposed that hydrogen bonding may be a common form of interaction between MRP1 and its substrates (Ito et al., 2001; Zhang et al., 2001a,b, 2002, 2003a,b). The differential effect of eliminating the hydrogen-bonding potential of Thr550 and Thr556 on drug resistance supports this suggestion. However, since the transport of vincristine and doxorubicin by MRP1 is GSH-dependent, we also examined the possibility that these mutations might influence interaction of MRP1 with GSH rather than drug (Loe et al., 1998; Renes et al., 1999). Nonetheless, the Thr550 and Thr556 mutations had no effect on basal or verapamil-stimulated GSH transport (Loe et al., 2000). Overall, the effects of these two mutations suggest that Thr550 and Thr556 may form hydrogen bonds with some drug substrates, such as doxorubicin and VP-16. The increase in resistance to vincristine observed with the T550A mutation may be attributable to the smaller size of the Ala side chain that favors transport of the larger drug. Similar behavior was observed after Ala mutations of Asn597 and Asn1245 in TM11 and TM17, respectively (Zhang et al., 2002, 2004). Like the T550A mutation, these mutations decreased resistance to VP-16 and increased resistance to vincristine.

Thr550, Thr556, and the previously identified Trp553 in TM10, together with Phe594 in TM11, are predicted to be in the inner leaflet region and to project toward functionally important residues in TM17.
Phe^{504} differentially affected transport of substrates including LTC_4 (Campbell et al., 2004). Despite the cross-linking of LTC_4 to regions spanning these helices, amino acids important for specific interaction with this substrate seem to be located in other TM helices, including TMs 6, 9, and 15 (Haimeur et al., 2002, 2004).

The predicted location of Tyr^{568} close to the extracellular/membrane interface distinguishes it from other polar residues in TM10 and the polar residues in TMs 11, 16, and 17 that influence substrate specificity. Many of the conjugated substrates of MRPI are relatively hydrophilic, and it seems likely that they interact with the protein from the cytosol, rather than by diffusion through the membrane. Consequently, the predicted location of Tyr^{568} at the distal end of the translocation pathway raised the possibility that it might be involved in a step in the transport of E_{217}B subsequent to initial binding, such as substrate translocation and release. However, kinetic analysis showed that the nonconservative mutation Y568A increased the apparent \( k_{\text{m}} \) for E_{217}B approximately 5-fold, without altering \( V_{\text{max}} \). Thus, the mutation seems to affect a step that we are presently unable to distinguish kinetically from substrate binding. The side chain of Tyr^{568} is predicted to be within 2 Å of Pro^{543} in TM6, which results in reduced transport in E_{217}B and, to a lesser extent, the transport of other substrates (Koike et al., 2004).

Based on our current model of MRPI, the longest distance between side chains of residues in TM17 close to the cytosol/membrane interface that influence transport and those in the outer leaftlet of TM6 and TM10 is approximately 22 Å. This is approximately equivalent to the longest dimension of E_{217}B. Thus, it is feasible that binding of at least some substrates may involve contacts that span both lipid bilayers. However, since binding of some substrates by MRPI seems to cause conformational changes in the membrane-spanning domains (Manciu et al., 2003), it is also possible that the interaction with residues such as Pro^{543} and Tyr^{568} may occur subsequent to a conformational change triggered by initial docking of E_{217}B with residues in the inner leaftlet region of the protein.

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References


