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

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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 Trp553 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 ABCB1. The predicted topology of MRP1 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., 1999a,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.
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; Haimerl et al., 2002, 2004; Koike et al., 2002). Two polar residues, Thr550 and Thr556, located in 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 Arg197, Arg202, and Glu1204, and the polar residue Trp1198, predicted to be within TM16, have been reported to be essential for function (Koike et al., 2004; Situ et al., 2004). We have now examined the role of additional residues with hydrogen-bonding capability within TM10 and -17. In TM10, Thr550, Thr552, Thr556, Thr570, and Thr5770 were individually mutated to Ala, and Tyr568 was mutated to Ala, Ser, Phe, and Trp. Asn208, within TM16 was replaced by Ala. These mutant proteins were then stably expressed in human embryonic kidney (HEK293) cells, and the transfectants were characterized with respect to their drug resistance profiles, as well as their ability to transport LTC4, E17βG, and GSH. Mutation N1208A in TM16 had no detectable effect on the function of MRP1. However, two polar residues, Thr550 and Thr556, located in TM10, play an important role in determining the drug resistance profile, and the aromatic side chain of the residue at position 568 of TM10 of MRP1 is important for E17βG transport.

Materials and Methods

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/mmol) 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 using a selection primer, 5’-GAGAGTGCACGATATCCGGT-GTG-3’, that mutates a unique NdeI site in the vector to an EcoRV restriction site. An oligonucleotide bearing the mismatched site at the residue to be mutated (underlined) was synthesized by ACGT Corp. (Toronto, ON, Canada) with the following sequence: 5’-GGTGCCCTTGTGCCATTTGCGCTCGTGTTCTA-3’. Mutations T550A, T552A, T556A, Y568A, Y568S, Y568F, Y568W, T570A, and N1208A were generated using the Quikchange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). 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. with the following sequences: T550A, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’; T552A, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’; T556A, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’; Y568A, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’; Y568S, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’; Y568F, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’; Y568W, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’; T570A, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’; and N1208A, 5’-GATCCCCGCGGGGCGGCGCTCCCTCTGCTGCTCGTCTACGGTAC-3’. After confirming all mutations by DNA sequencing (ACGT Corp.), DNA fragments containing the desired mutations were transferred into pCEBV7-MRP1. After reconstructing full-length expression vectors containing the mutations, the integrity of the mutated inserts and cloning sites was verified by DNA sequencing (ACGT Corp.).

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.

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 transfected cells 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.
Confocal Microscopy. Confocal microscopy was carried out as described previously (Zhang et al., 2001a,b). Briefly, 5 × 10^4 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 MRPm6. 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 Fluor 488). The subcellular localization of wild-type and mutant MRP1 as indicated in the figure. An ×25 optical section of the cells is shown to illustrate the distribution of the wild-type and mutant proteins between plasma and intracellular membranes.

Results

Expression of Mutant MRP1 in Stably Transfected HEK293 Cells. Thr^{550}, Thr^{552}, Thr^{556}, Thr^{564}, Tyr^{568}, and Thr^{570} within TM10, and Asn^{1208} 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 were determined using immunoblotting and densitometry (Fig. 2A). The expression levels of these mutant proteins in stably transfected HEK293 cells ranged from 50 to 90% of wild-type MRP1. Endogenous MRP1 in HEK293 cells transfected with the empty vector was undetectable under the conditions used (data not shown).

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]E₂17βG by Wild-Type and Mutant MRP1. To determine whether any of the mutations altered the efficiency with which the protein transported LTC₄ and E₂17βG, 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 each of the wild-type and 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]E₂17βG 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 E₂17βG.

Effect of Mutations Y₅₆₈S, Y₅₆₈F, and Y₅₆₈W on the Transport of [³H]LTC₄ and [³H]E₂₁βG by MRP1. Because replacement of Tyr⁵₆₈ by Ala selectively decreased transport of E₂₁βG, 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 E₂₁βG 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 E₂₁βG 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]E₂₁βG Transport by Wild-Type and Y₅₆₈A Mutant MRP1. To more precisely determine the influence of mutation Y₅₆₈A on the ability of MRP1 to transport E₂₁βG, we compared kinetic parameters for the wild-type and mutant proteins (Fig. 5). For wild-type MRP1 and Y₅₆₈A, the Kₘ and normalized Vₘ₅ 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 Kₘ and Vₘ₅ values of wild-type and Y₅₆₈A proteins, respectively (Fig. 5, B and C). For E₂₁βG transport, a comparable analysis yielded Kₘ values of 1.4 μM and 5.4 μM and normalized Vₘ₅ 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 E₂₁βG 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 (Løe 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 sig-
ificant 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 transloca-
tion 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 re-
maining 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 Arg₁ₕ₀₂ 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 Trp¹₁₉₀ 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, Ser₁₃₅ and
Glu₁₃₅, 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
positioned toward the outer leaflet of the membrane relative to the
center 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 Tyr⁶₅₈. 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 Tyr⁶₅₈, which is also predicted to

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
membrane vesicle preparations and densitometry as described in the legend to
Fig. 2A. [³H]LTC₄ (B) and [³H]E₂₁₇G (C) uptake by wild-type and mutant proteins
was determined as described in the legend to Fig. 3. The normalized
transport values were obtained by adjusting experimentally determined values
(1-min time point) to compensate for differences in the relative levels of the
wild-type and mutant proteins. 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 (0.01–10 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 \([S] / V\) 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

Relative drug resistance of HEK293 cells transfected with wild-type and mutant MRP1

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.

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Vincristine</th>
<th>VP-16</th>
<th>Doxorubicin</th>
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<tbody>
<tr>
<td>HEKMRP1</td>
<td>36.0 ± 7.5 (36.0)</td>
<td>12.5 ± 2.7 (12.5)</td>
<td>9.6 ± 0.9 (9.6)</td>
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<tr>
<td>HEKMRP1T₅₅₀A</td>
<td>144.9 ± 27.3 (161.0)</td>
<td>12.4 ± 2.3 (13.8)</td>
<td>3.9 ± 0.3 (4.3)</td>
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<td>HEKMRP1T₅₅₂A</td>
<td>21.6 ± 3.5 (36.0)</td>
<td>7.5 ± 1.6 (12.5)</td>
<td>5.5 ± 2.1 (9.2)</td>
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<tr>
<td>HEKMRP1T₅₆₆A</td>
<td>25.0 ± 6.8 (41.6)</td>
<td>3.4 ± 1.4 (5.6)</td>
<td>2.5 ± 1.3 (4.1)</td>
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<tr>
<td>HEKMRP1T₅₆₄A</td>
<td>12.1 ± 2.6 (24.2)</td>
<td>5.7 ± 2.8 (11.4)</td>
<td>4.3 ± 0.8 (8.6)</td>
</tr>
<tr>
<td>HEKMRP1Y₅₆₈A</td>
<td>33.4 ± 4.9 (41.7)</td>
<td>11.3 ± 2.4 (14.1)</td>
<td>7.9 ± 1.9 (9.9)</td>
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<tr>
<td>HEKMRP1Y₅₆₈A</td>
<td>32.0 ± 2.4 (40.0)</td>
<td>10.4 ± 1.8 (13.0)</td>
<td>7.2 ± 0.9 (9.0)</td>
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<td>HEKMRP1N₁₂₀₈A</td>
<td>25.6 ± 2.3 (32.0)</td>
<td>14.7 ± 1.8 (18.3)</td>
<td>8.8 ± 2.4 (11.0)</td>
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<td>HEKMRP1Y₅₆₈S</td>
<td>16.2 ± 0.9 (32.4)</td>
<td>6.8 ± 0.9 (13.6)</td>
<td>5.1 ± 0.4 (10.2)</td>
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<tr>
<td>HEKMRP1Y₅₆₈F</td>
<td>28.9 ± 3.5 (32.1)</td>
<td>9.8 ± 1.7 (10.9)</td>
<td>8.3 ± 0.4 (9.2)</td>
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<tr>
<td>HEKMRP1Y₅₆₈W</td>
<td>30.2 ± 2.7 (33.6)</td>
<td>9.6 ± 2.3 (10.7)</td>
<td>9.2 ± 2.5 (10.2)</td>
</tr>
</tbody>
</table>
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 E217\textsuperscript{H9252}G without affecting the transport of LTC\textsubscript{4} or GSH, or resistance to any drug tested. Mutation of two of the five Thr residues, Thr\textsuperscript{550} and Thr\textsuperscript{556}, differentially affected drug resistance without altering transport of the organic anion conjugates tested. Thr\textsuperscript{550} and Thr\textsuperscript{556} are predicted to be in the inner leaflet region of TM10, and the side chains of both residues align with that of Trp\textsuperscript{553}. 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 Thr\textsuperscript{550} and Thr\textsuperscript{556} 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 Thr\textsuperscript{550} and Thr\textsuperscript{556} mutations had no effect on basal or verapamil-stimulated GSH transport (Loe et al., 2000). Overall, the effects of these two mutations suggest that Thr\textsuperscript{550} and Thr\textsuperscript{556} 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 Asn\textsuperscript{597} and Asn\textsuperscript{1245} 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.

Thr\textsuperscript{550}, Thr\textsuperscript{556}, and the previously identified Trp\textsuperscript{553} in TM10, together with Phe\textsuperscript{594} in TM11, are predicted to be in the inner leaflet region and to project toward functionally important residues in TM17 (Fig. 7). Two of these residues, Trp\textsuperscript{1246} and Tyr\textsuperscript{1243}, together with Trp\textsuperscript{553} and Phe\textsuperscript{594}, have been postulated to form part of an aromatic basket at the cytoplasmic entrance to the translocation pathway (Ito et al., 2001; Koike et al., 2002; Zhang et al., 2002; Campbell et al., 2004). Similar clusters of functionally important aromatic and polar amino acids are present in the inner leaflet regions of TM11 and TM16 (Fig. 7). Although mutation of residues in these clusters has been shown to selectively affect the ability of MRP1 to confer resistance to various drugs and to transport E\textsubscript{2}17\betaG, only mutations of
Phe<sup>506</sup> differentially affected transport of substrates including LTC<sub>4</sub> (Campbell et al., 2004). Despite the cross-linking of LTC<sub>4</sub> 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 (Haimer et al., 2002, 2004).

The predicted location of Tyr<sup>568</sup> 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 MRP1 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<sup>568</sup> at the distal end of the translocation pathway raised the possibility that it might be involved in a step in the transport of E<sub>2</sub>17βG subsequent to initial binding, such as substrate translocation and release. However, kinetic analysis showed that the nonconservative mutation Y568A increased the apparent <i>K<sub>m</sub></i> for E<sub>2</sub>17βG approximately 5-fold, without altering <i>V<sub>max</sub></i>. Thus, the mutation seems to affect a step that we are presently unable to distinguish kinetically from substrate binding. The side chain of Tyr<sup>568</sup> is predicted to be within 2 Å of Pro<sup>545</sup> in TM6, mutation of which results in a major decrease in transport of E<sub>2</sub>17βG and, to a lesser extent, the transport of other substrates (Koike et al., 2004).

Based on our current model of MRP1, the longest distance between side chains of residues in TM17 close to the cytosol/membrane interface that influence transport and those in the outer leaflet of TM6 and TM10 is approximately 22 Å. This is approximately equivalent to the longest dimension of E<sub>2</sub>17βG. 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 MRP1 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<sup>545</sup> and Tyr<sup>568</sup> may occur subsequent to a conformational change triggered by initial docking of E<sub>2</sub>17βG with residues in the inner leaflet region of the protein.

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References

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