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 Tyr566 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). 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; Jedlickscha 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.
of MRP1. However, two polar residues, Thr550 and Thr556, located in TM16 were replaced by Ala. These mutant proteins were then stably expressed in human embryonic kidney (HEK293) cells, and the transfectant cells were characterized with respect to their drug resistance profile, and the aromatic side chain of the residue at position 568 of TM10 of MRP1 is important for E217G 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]E217G (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 (Zhao et al., 2001). Mutagenesis was then performed according to the manufacturer’s instructions using a selection primer, 5'-GAGAGGACGATCATCGGTTG-3', that mutates a unique NdeI site in the vector to an EcoRV restriction site. An oligonucleotide bearing the mismatched site to be mutated (underlined) was synthesized by ACGT Corp. (Toronto, ON, Canada) with the following sequence: 5'-GGTGGCCTTGTGCCATTTGCCGTC-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'-GTCACATTTGCTGTCGCA-3'; T552A, 5'-GTCACATTTGCTGTCGCA-3'; T556A, 5'-GTCACATTTGCTGTCGCA-3'; Y568A, 5'-GTCACATTTGCTGTCGCA-3'; Y568S, 5'-GTCACATTTGCTGTCGCA-3'; Y568F, 5'-GTCACATTTGCTGTCGCA-3'; Y568W, 5'-GTCACATTTGCTGTCGCA-3'; Y570A, 5'-GTCACATTTGCTGTCGCA-3'; T570A, 5'-GTCACATTTGCTGTCGCA-3'; T570A, 5'-GTCACATTTGCTGTCGCA-3'; N1208A, 5'-GTCACATTTGCTGTCGCA-3'.
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
620/40 nm for propidium iodide; 530/30 nm for Fluor 488).

Confocal Microscopy. Confocal microscopy was carried out as described
previously (Zhang et al., 2001a,b). Briefly, −5 × 10⁵ 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 perme-
abilization 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).

Results
Expression of Mutant MRP1 in Stably Transfected HEK293 Cells. Thr⁵⁵⁵, Thr⁵⁵⁷, Thr⁵⁵⁶, Thr⁵⁶⁴, Tyr⁵⁶⁸, and Thr⁵⁷⁰ within TM10, and Asn¹²⁰⁸ within TM16 were replaced individually by Ala (Fig. 1).
The episomal expression vector, pCEBV7, containing mutated forms
of MRP1 cDNAs, was used to stably transfected 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 pro-
teins relative to wild-type MRP1 in previously characterized HEK
proteins were compared with cells transfected with empty vector.
Resistance was determined in three or more independent experiments.

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 μM, 300 Ci) in the absence and presence of verapamil
(100 μM). To minimize GSH catabolism by γ-glutamyltranspeptidase 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).

Chemosensitivity 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.

Determination 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 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]E217βG by MRPI. Because replacement of Tyr568 by Ala selectively decreased transport of E217βG, 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 MRPIY568F, MRPIY568S, and MRPIY568W were 90, 50, and 90% of wild-type MRPI, respectively (Fig. 4A). The effects of these mutations on the ability of MRPI to transport LTC4 and E217βG 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 E217βG 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]E217βG Transport by Wild-Type and Y568A Mutant MRPI. To more precisely determine the influence of mutation Y568A on the ability of MRPI to transport E217βG, we compared kinetic parameters for the wild-type and mutant proteins (Fig. 5). For wild-type MRPI 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 E217βG transport, a comparable analysis yielded $K_m$ values of 1.4 μM and 5.4 μM 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 MRPI for E217βG 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-
teins were determined using MTT assays. The results are presented as relative drug resistance factors in Table 1. Mutation of Asn1208 in TM16 had no effect on the ability of MRP1 to confer resistance to any of the three classes of drugs tested. However, mutation of two Thr residues in TM10 altered the drug resistance profile (Table 1). Substitution T550A reduced resistance to doxorubicin approximately 2-fold and increased resistance to vincristine approximately 4.5-fold, but had no effect on VP-16 resistance. In contrast, mutation T556A reduced the resistance to both VP-16 and doxorubicin approximately 2-fold without affecting vincristine resistance. Thus, elimination of the polarity of the side chains of residues at positions 550 and 556 differentially affects the drug resistance profile of MRP1, suggesting that these two Thr residues may interact directly with drug substrates.

Transport of [\( \text{H} \)]\( \text{LTC}_4 \) by Wild-Type and Mutant MRP1. We have shown that mutations T550A and T556A both affect the ability of MRP1 to confer drug resistance, whereas mutation Y568A only influenced the transport of \( \text{E}_2\text{17\betaG} \). One major distinction between MRP1-mediated transport of substrates such as \( \text{LTC}_4 \) and \( \text{E}_2\text{17\betaG} \), and drugs such as vincristine and daunorubicin, is a requirement for GSH, which may be cotransported with the unmodified drug (Rappa et al., 1997; Renes et al., 1999; Loe et al., 1998). Previously, we have reported that MRP1 exhibits low levels of ATP-dependent GSH 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 \( \text{LTC}_4 \) 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 Gin1237, 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 membrane 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. Thr525 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, Thr562 and Thr570 are predicted to be located in the outer leaflet region of TM10.

Similarly, conservative mutation of Tyr568, which is also predicted to
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 V0 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 Km and Vmax values for wild-type and mutant MRP1 are provided under Results.

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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>
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<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>HEKMRP1T570A</td>
<td>32.0 ± 2.4 (40.0)</td>
</tr>
<tr>
<td>HEKMRP1N1208A</td>
<td>25.6 ± 2.3 (32.0)</td>
</tr>
<tr>
<td>HEKMRP1Y568F</td>
<td>16.2 ± 0.9 (32.4)</td>
</tr>
<tr>
<td>HEKMRP1Y568W</td>
<td>28.9 ± 3.5 (32.1)</td>
</tr>
<tr>
<td>HEKMRP1Y568G</td>
<td>30.2 ± 2.7 (33.6)</td>
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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 (Fig. 7). Two of these residues, Trp1246 and Tyr1243, together with Trp553 and Phe594, 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 E217βG, only mutations of

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**FIG. 6.** ATP-dependent, verapamil-stimulated \[^3^H\]GSH uptake by membrane vesicles prepared from HEK293 cells stably transfected with wild-type or mutant MRP1. Membrane vesicles were incubated at 37°C with 100 μM GSH (300 nCi) in transport buffer in the presence of verapamil (100 μM) as described. Transfectants tested expressed wild-type or mutant MRP1 as indicated in the graphs. The normalized transport values were obtained by adjusting experimentally determined values (20-min time point) to compensate for differences in the relative levels of the wild-type and mutant proteins and are shown in B. Data shown in A have not been normalized to compensate for differences in expression levels. Values are the mean ± S.D. of three independent experiments.

**FIG. 7.** Energy-minimized model of MRP1 TMs 10, 11, 16, and 17. A, TMs 10, 11, 16, and 17, viewed in the plane of the membrane (top) or from the extracellular face (bottom). The predicted membrane interfaces are indicated by broken lines in the left panel. The energy-minimized model is based on the structure of the lipid A transporter from Vibrio cholerae, VC-Msba. The derivation of this model of MSD1 and MSD2 of MRP1 has been described in detail by Campbell et al. (2004). For clarity, other TMs in these two MSDs have been removed and the side chains of only selected residues have been shown using PyMol. Previously mutated residues in TMs 10, 16, and 17 that do or do not influence substrate specificity or overall activity are shown in red and blue, respectively. Residues in TM10 which, when mutated, alter substrate specificity or overall activity include: Thr550, Thr556, and Thr564 (green), Trp553 (yellow), Pro557 (violet), and Tyr568 (orange). The residue in TM17 indicated by the arrow is Arg1249, the side chain of which is approximately 7.5 Å from the side chain of Trp553. B, a schematic of TM10 and TM16 with mutated polar residues referred to in the text indicated by shaded circles.
Phe\textsuperscript{504} differentially affected transport of substrates including LTC\textsubscript{4} (Campbell et al., 2004). Despite the cross-linking of LTC\textsubscript{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 (Haimer et al., 2002, 2004). The predicted location of Tyr\textsuperscript{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 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\textsuperscript{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\textsubscript{217}\textbeta subsequent to initial binding, such as substrate translocation and release. However, kinetic analysis showed that the nonconservative mutation Y568A increased the apparent \(K_{m}\) for E\textsubscript{217}\textbeta 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\textsuperscript{568} is predicted to be within 2 Å of Pro\textsuperscript{543} in TM6, mutation of which results in a major decrease in transport of E\textsubscript{217}\textbeta 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 distance of E\textsubscript{217}\textbeta. 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\textsuperscript{543} and Tyr\textsuperscript{568} may occur subsequent to a conformational change triggered by initial docking of E\textsubscript{217}\textbeta with residues in the inner leaflet region of the protein.

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References


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