Mutations of Charged Amino Acids in or near the Transmembrane Helices of the Second Membrane Spanning Domain Differentially Affect the Substrate Specificity and Transport Activity of the Multidrug Resistance Protein MRP1 (ABCC1)

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ABSTRACT
Multidrug resistance protein 1 (MRP1) belongs to the ATP-binding cassette superfamily of transport proteins. In addition to drugs, MRP1 mediates the active transport of many conjugated and unconjugated organic anions. MRP1 consists of two membrane-spanning domains (MSD2 and MSD3) each followed by a nucleotide binding domain plus a third NH2-terminal MSD1. MSD2 contains transmembrane (TM) helices 6 through 11, and previously, we identified two charged residues in TM6 as having important but markedly different roles in MRP1 transport activity and substrate specificity by characterizing mutants containing nonconservative substitutions of Lys332 and Asp336. We have now extended these studies and found that the same-charge TM6 mutant K332R, like the nonconservatively substituted Lys332 mutants, exhibits a selective decrease in leukotriene C4 (LTC4) transport, associated with substantial changes in both \( K_m \) and \( V_{max} \) and LTC4 binding. The overall organic anion transport activity of the same-charge mutant of Asp336 (D336E) also remained very low, as observed for D336R. In addition, nonconservative substitutions of TM6-associated Lys319 and Lys347 resulted in a selective decrease in GSH transport. Of eight other charged residues in or proximal to TM6 to TM11 that were investigated, nonconservative substitutions of three of them [Lys396 (TM7), Asp436 (TM8), and Arg593 (TM11)] caused a substantial and global reduction in transport activity. However, unlike TM6 Asp336, wild-type transport activity could be re-established in these MRP1 mutants by conservative substitutions. We conclude that MSD2-charged residues in or proximal to TM6, TM7, TM8, and TM11 play critical but differential roles in MRP1 transport activity and substrate specificity.

MRP1 is a 190-kDa multidrug resistance protein that belongs to subfamily C of the ABC transporter superfamily and is often overexpressed in drug-resistant tumor cell lines (Haimeur et al., 2004). It is also expressed in a wide variety of human tumors and normal tissues. In vitro studies using membrane vesicles have demonstrated that MRP1 can actively transport a wide variety of structurally diverse compounds ranging from complex heterocyclic natural products and chemotherapeutic agents, such as vincristine, doxorubicin and the folate antagonist methotrexate, to arsenical and antimonial oxyanions (Borst et al., 2000; Deeley and Cole, 2003; Haimeur et al., 2004). MRP1 has also been shown to be an efficient ATP-dependent transporter of various conjugated organic anions, including a mediator of inflammation, the cysteiny1 leukotriene LTC4, and the glucuronide- and sulfate-conjugated estrogens \( E_2 \) and \( E_1 \). To transport unconjugated drugs such as vincristine, MRP1 requires the presence of GSH, which seems to be cotransported with the drug (Lee et al., 1998; Mao et al., 2000). MRP1 also transports small hydrophobic peptides (de Jong et al., 2001). Thus, the range of substrates that can be transported by MRP1, at least in vitro, is very broad.

The two basic structural elements of all known ABC transporters are a nucleotide-binding domain (NBD) and a hydrophobic region typically containing six transmembrane (TM)
α-helices, which together constitute a membrane-spanning domain (MSD) (Higgins, 1992). The most common structure of ABC transporters is one with four domains: two MSDs and two NBDs. However, MRP1 and several other members of the ABCC transport protein family have an additional NH₂-terminal MSD with five TM segments and an extracytosolic NH₂ terminus (Hipfner et al., 1997; Raab-Graham et al., 1999; Konig et al., 2003). Thus, most computer algorithms for predicting secondary structures of integral membrane proteins favor a structure in which human MRP1 possesses 17 TM α-helices configured 5 + 6 + 6 in its three MSDs, respectively (Fig. 1).

The role that the MSDs of MRP1 play in determining its transport activity and substrate specificity has recently been the subject of considerable investigation, primarily by structure-function studies using site-directed mutagenesis. These studies have shown that mutation of individual aromatic, polar, and charged amino acids in each of the three MSDs can affect the recognition and transport of one or several substrates in different ways (Ito et al., 2001a; Zhang et al., 2001, 2002, 2003; Koike et al., 2002; Haimeur et al., 2002; Yang et al., 2002; Leslie et al., 2003b). In one study, we showed that nonconservative substitutions of three charged amino acids predicted to cluster within an arc of approximately 100° on the same face of TM6 of MSD2 (Lys332, His335, and Asp336) had strikingly different effects on MRP1 transport activity and substrate specificity (Fig. 1) (Haimeur et al., 2002). Thus, Lys332 and, to a certain extent, His335 were found to be critical for the binding and transport of LTC₄ and GSH, whereas mutation of these residues had little or no impact on the transport of other organic anions. In contrast, nonconservative substitutions of Asp336 showed no such substrate selectivity because mutation of this acidic residue to either an oppositely charged or neutral amino acid essentially eliminated MRP1 transport activity altogether (Haimeur et al., 1999; Konig et al., 2003). Thus, most computer algorithms for predicting secondary structures of integral membrane proteins favor a structure in which human MRP1 possesses 17 TM α-helices configured 5 + 6 + 6 in its three MSDs, respectively (Fig. 1).

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TTC-3; D430R, 5′-C AAC CTG CTG GTG GCC GTG CAC CCG AGC AGG TTC-3; D436R, 5′-C CCT GAG AGC TGC AGG GTG GCC CAC AGG TC-3’; D436E, 5′-C CCT GAG AGG TTC ATG GAA TTA GCC AGC AGG TAC-3’; D572R, 5′-C TAC GTG ACC ATG CAC GAG AAC ATC ATC GTG GAG-3’; E573R, 5′-C GGC TTC ATC AAC ATC CTG GAT G-3’; ATC CTG GAT G-3’; R593L, 5′-G GCC TTG TTC AAC ATC AAC ATC CTG AAG TTT CCC CTG AAC-3′.

The presence of the mutations was confirmed by sequencing and/or diagnostic restriction enzyme digests as appropriate. Mutant MRPI cDNAs were then digested with BamHI and Bsu36I to obtain a 1-kb fragment containing the altered sequence, which was used to replace the corresponding fragment in the wild-type pcDNA3.1(−) MRPIp1 plasmid. After cloning, the inserts were again sequenced to confirm the presence of the mutation. For selected mutants, MRPI-pcDNA3.1-GFP fusion constructs were generated by replacing the 1-kb BamHI/Bsu36I fragment in a pcDNA3.1(−)-GFP construct with the comparable fragment containing the desired mutation. Confocal microscopy was carried out as described previously (Haimeur et al., 2002).

Transfections with MRPI Expression Vectors. Wild-type and mutant pcDNA3.1(−)-MRPI expression vectors were transfected into SV40-transformed human embryonic kidney cells (HEK 293T). In brief, approximately 10 × 10⁶ cells were seeded in 150-mm plates, and 24 h later, DNA (16 μg) was added using FuGENE6 (Roche Diagnostics, Laval, PQ, Canada) according to the manufacturer’s instructions. After 72 h, the HEK 293T cells were harvested, and membrane vesicles prepared as described previously (Haimeur et al., 2002). Untransfected cells or cells transfected with empty vectors and vector containing wild-type MRPI cDNA were included as controls in all experiments. Levels of wild-type and mutant MRPI proteins were determined by immunoblotting as described below.

Measurement of MRPI Protein Levels in Transfected Cells. The expression levels of wild-type and mutant MRPI proteins were determined by immunoblot analysis of whole-cell lysates and membrane vesicles from transfected cells essentially as described using the human MRPI-specific murine MAb QCRL-1 (Hippner et al., 1996) and the Renaissance chemiluminescence detection system (PerkinElmer Life and Analytical Sciences). Relative levels of MRPI expression were estimated by densitometric analysis using a Chemi-Imager 4000 (Alpha Innotech, San Leandro, CA). Equal loading of protein was confirmed by amido black staining of the membrane.

MRPI1-Mediated Transport of [3H]-Labeled Substrates by Membrane Vesicles. Preparation of membrane vesicles from transfected HEK 293T cells has been described previously (Haimeur et al., 2002), and ATP-dependent vesicular uptake of [3H]-labeled substrates by the membrane vesicles was measured using a rapid filtration technique also as described previously (Haimeur et al., 2002). In brief, LTC₄ transport assays were performed at 23°C in a 50-μl reaction containing 50 nM LTC₄ (40 nCi), 4 mM AMP or ATP, 10 mM MgCl₂, creatine phosphate (10 mM), creatine kinase (100 μM · ml⁻¹), and 2 μg of vesicle protein. Uptake was stopped at selected times by rapid dilution in ice-cold buffer, and then the incubation mixture was filtered through glass fiber (type A/E) filters that had been presoaked with 3 mM glutamyltranspeptidase during transport, membranes were preincubated in 0.5 mM acivicin for 10 min at 37°C before measuring [3H]GSH uptake. MTX uptake was also measured as described previously (Haimeur et al., 2002). Assays were carried out at 37°C in a 60-μl reaction volume containing [3H]MTX (100 μM; 200 nCi), membrane vesicles (10 μg of protein), and other components as described above. Uptake was stopped after 20 min by rapid dilution in ice-cold buffer and processed as before.

Kinetic Analysis of ATP-Dependent [3H]LTC₄ and [3H]E₂₁₇G Uptake. Kₘ and Vₘₐₓ values of ATP-dependent LTC₄ transport by membrane vesicles (2.5 μg of protein) were determined by measuring initial rates of uptake at eight different [3H]LTC₄ concentrations (10–1000 nM) for 1 min at 23°C. For kinetics of [3H]E₂₁₇G uptake, substrate concentrations ranged from 0.25 to 25 μM, and vesicles were incubated for 1 min at 37°C in 50 μl of transport buffer containing components as described above. Data were analyzed using Prism software (GraphPad Software Inc., San Diego, CA), and kinetic parameters determined by linear regression analyses and Michaelis-Menten analysis.

Photolabeling of MRPI with [3H]LTC₄. Wild-type and mutant MRPI membrane proteins were photolabeled with [3H]LTC₄ essentially as described previously (Loe et al., 1986). In brief, membrane vesicles enriched for wild-type and mutant MRPI cDNAs (50 μg of protein in 50 μl) were incubated with [3H]LTC₄ (0.5 μCi; 200 nM) at room temperature for 30 min and then frozen in liquid nitrogen. Samples were then alternately irradiated at 312 nm for 1 min using a CL-1000 Ultraviolet Crosslinker (Dialma, Mississauga, ON, Canada) and snap-frozen in liquid N₂ for 10 times. Radiolabeled proteins (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis. The gel was processed for autoradiography and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) at −70°C.

Results

MRPI Expression Levels of MSD2 Mutants in Transfected HEK Cells. To investigate whether charged amino acids predicted to be located in or near the TM α-helices of MSD2 (TM6–TM11) are important for MRPI function, we first mutated them, singly and in one case in combination, to an oppositely charged residue and, in several cases, to a nonpolar neutral and/or same-charge residue (Fig. 1). These MSD2 mutants were cloned into the pcDNA3.1(−) expression vector and expressed in HEK 293T cells as described previously (Haimeur et al., 2002). The expression levels of the mutant MRPI proteins were determined by immunoblot analysis of membrane vesicles prepared from the transfected cells, and in all cases but one, a single band of 190 kDa corresponding to MRPI was detected (Fig. 2). The exception was the Asp₄₄₀ mutant D430K which, like endogenous MRPI in vector control membrane vesicles, was undetectable under the conditions used. Densitometric analysis showed that the levels of the expressed MRPI mutants were comparable (80–120%) to wild-type MRPI, indicating that mutations of charged amino acids proximal to MSD2 (with the exception of Asp₄₄₀), did not significantly affect the biogenesis of MRPI. Comparable results were observed in immunoblots of whole-cell lysates of the transfected cells (data not shown).
Substitution of Lys^{332}, Asp^{336}, Lys^{319}, and Lys^{347} in or near TM6. We showed previously that nonconservative substitutions of Lys^{332} with either Asp (K332D) or Leu (K332L) led to a selective loss of transport of GSH and the GSH conjugate, LTC₄ (Haimeur et al., 2002). LTC₄ binding was also abrogated in these mutants as indicated by photo-labeling and competition experiments. When the Asp residue of the K332D mutant was mutated back to the wild-type Lys residue to create K(D)332K, LTC₄ and GSH transport was restored as expected (Fig. 3, A and B). To test whether the charge and/or steric properties (side-chain volume) of Lys^{332} were important for the substrate selective loss of transport activity, Lys^{332} was also replaced with Arg to create the same-charge mutant K332R. Unlike the K332D mutant, the conservatively substituted K332R had a significant level of LTC₄ transport activity (approximately 40% of wild-type MRP1 levels). These results indicate that the charge and, to some extent, the molecular volume of the Lys^{332} side chain are important for LTC₄ transport by MRP1. Analysis of kinetic parameters showed that the decrease in LTC₄ transport by the same-charge K332R mutant was largely caused by a decrease in its apparent uptake affinity for LTC₄ ($K_m = 552$ nM versus $115$ nM for wild-type MRP1), leading to an overall 6-fold decrease in LTC₄ transport efficiency ($V_{max}/K_m = 0.2$ versus 1.2 for wild-type MRP1) (Table 1).

To determine whether $[^3H]E217\beta G$ transport by the like-
charged K332R mutant that retained some LTC$_4$ transport activity could still be inhibited by this conjugated leukotriene, the effect of LTC$_4$ on [H]E$_{217}$G uptake by K332R was determined. The dose-response curves shown in Fig. 3C indicate that LTC$_4$ had a greater inhibitory effect on E$_{217}$G uptake by the K332R mutant than by the K332D mutant, but this effect was significantly less (50–70%) than the effect of LTC$_4$ on E$_{17}$G uptake by wild-type MRP1. These relative levels of inhibition by LTC$_4$ are consistent with the relative levels of LTC$_4$ transport by these three MRP1 proteins.

The same-charge mutant K332R, like the K332D and K332L mutants described previously (Haimer et al., 2002), exhibited transport levels of the conjugated estrogens E$_{217}$β and E$_{17}$SO$_4$ and the antifolate MTX that were comparable with wild-type MRP1 (Table 2); however, GSH transport by K332R was very low compared with wild-type MRP1 and similar to that which we reported previously for the K332D/L mutants (Fig. 3B). Overall, these observations indicate the Lys residue at position 332 is strictly required for full LTC$_4$ and GSH transport activity but not for transport of other organic anions (i.e., E$_{17}$βG, E$_{17}$SO$_4$, and MTX).

In our earlier study, we showed that substitution of the negatively charged TM6 Asp$^{336}$ residue with Arg or Leu essentially eliminated the transport of both conjugated and unconjugated MRP1 organic anion substrates (Haimer et al., 2002). In the present study, Asp$^{336}$ was replaced with Lys to create D336K, and a comparable global loss of transport activity was observed (Fig. 4A and Table 2). To test whether the charge of Asp$^{336}$ or the steric bulk of its side chain was responsible for the functional importance of this amino acid, the Lys in the D336K mutant was replaced with Glu to create the like-charge mutant D(K)336E. In contrast to the like-charge K332R mutant in which LTC$_4$ transport activity was partially restored, the like-charge D(K)336E mutant remained essentially inactive. Thus, LTC$_4$ and GSH uptake by D(K)336E was decreased 80–90%, respectively, compared with wild-type MRP1 (Fig. 4A), and transport of three other MRP1 substrates was decreased by 55 to 75% (Table 2). These data show that both the charge and size of the side chain of the Asp residue at position 336 are critical for MRP1 activity.

We speculated previously that ion pairing might be occurring between K332 and D336 in TM6 because they are within approximately one turn of an α-helix from one another (Haimer et al., 2002). To test this idea, the K332D/D336K double mutant in which these two residues were exchanged was created. If ion pairing was important (and the only interaction between these two residues), then this double mutant might be expected to have wild-type transport activity. However, as shown in Fig. 4B, LTC$_4$ transport by K332D/D336K was not detectable. Transport of other organic anions by this double mutant was also markedly reduced (Table 2). These findings indicate that even if Lys$^{332}$ and Asp$^{336}$ form an ion pair in the TM6 α-helix, additional bonding interactions of these two residues with other residues in MRP1 are required for full transport activity.

To further investigate the functional role of charged amino acids proximal to TM6, we also mutated Lys$^{339}$ and Lys$^{347}$. Lys$^{339}$ was replaced with Asp (K339D), and Lys$^{347}$ was replaced with Asp (K347D) as well as Leu (K347L). The effects of mutating either Lys$^{339}$ or Lys$^{347}$ were similar and substrate-specific. Thus the ability of the K319D, K347D, and K347L mutants to transport GSH was reduced by approximately 50% (Fig. 4C), whereas the transport of four other substrates (LTC$_4$, E$_{17}$G, E$_{17}$SO$_4$ and MTX) by these mutants remained comparable with wild-type MRP1 (Table 2). Thus, these two Lys residues seem particularly important for GSH transport by MRP1.

### Table 1

<table>
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<tr>
<th>Transfectants</th>
<th>K$_{m}$ (nM)</th>
<th>V$_{max}$ (pmol/mg/min)</th>
<th>V$<em>{max}$/K$</em>{m}$ × 10$^4$</th>
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<tr>
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### Table 2

Summary of organic anion transport activity of MRP1 mutants with substitutions of charged amino acids in and proximal to the TM helices (TM6–11) of MSD2.

<table>
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<tr>
<th>MRP1 Mutant</th>
<th>% Wild-Type MRP1 Transport Activity</th>
<th>LTC$_4$</th>
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<th>E$_{17}$SO$_4$</th>
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or substrate specificity of MRP1. In contrast, substitution of the nearby Lys396 residue with either an oppositely charged (K396E) or neutral (K396I) residue resulted in a substantial decrease in overall MRP1 transport activity (Fig. 5). Thus, LTC4, E217βG, and E23SO4 transport by the K396E and K396I mutants was reduced by 60 to 75% (Fig. 5, A-C) whereas GSH and MTX transport by these two mutants was reduced by approximately 50% (Fig. 5, D and E). Kinetic analyses indicated that both the apparent uptake affinity and the transport capacity of the K396E mutant for LTC4 and E217βG were altered. In the case of LTC4 transport, the $K_m$ was increased 4-fold, whereas the $V_{max}$ was reduced >2-fold, resulting in a 10-fold decrease in the transport efficiency of this substrate (Table 1). For E217βG transport, the $K_m$ was increased 2-fold, while the $V_{max}$ decreased 3-fold, resulting in 4.6-fold decrease in the overall transport efficiency of this substrate (Table 3).

To test whether the positive charge of the Lys396 side chain was the physical property critical for MRP1 transport function, the Glu in the K396E mutant was replaced with Arg to create the same-charge mutant K(E)396R. As shown in Fig. 5, uptake levels of all five organic anion substrates by the K(E)396R mutant were comparable with wild-type MRP1. The apparent $K_m$ and $V_{max}$ of K(E)396R for LTC4 and E217βG transport were also similar to wild-type MRP1 (Tables 1 and 3). Thus, unlike Lys332 and Asp336 in TM6, in which both charge and side-chain volume were essential for transport activity, the presence of a positive charge alone at position 396 seems sufficient for wild-type MRP1 transport function.

**Substitution of Asp430 and Asp436 in or near TM8.** We previously showed that the naturally occurring Arg333→Ser single nucleotide polymorphism proximal to the cytoplasmic end of TM8 resulted in a decrease in LTC4 and E23SO4 transport and an increase in doxorubicin resistance (Conrad et al., 2002). In the present study, we investigated the effects of mutating the nearby acidic residues Asp430 and Asp436. When Asp430 was replaced with Lys, the resulting D430K mutant was not expressed (Fig. 2). In contrast, when Asp436 was replaced with Lys, the mutant was expressed but showed a significant decrease in overall organic anion transport activity (Fig. 6 and Table 2). Thus, vesicular uptake by the D436K mutant was decreased by approximately 60% for LTC4 (Fig. 6A), by 80% for E217βG (Fig. 6B), by 70% for E23SO4 (Fig. 6C), by 70% for GSH (Fig. 6D), and by 45% for MTX (Fig. 6E).

Kinetic analyses showed that the apparent $K_m$ (LTC4) of the D436K mutant was increased 2-fold and $V_{max}$ decreased >3-fold, resulting in an overall 7-fold decrease in LTC4 transport efficiency for this mutant (Table 1). Likewise, the apparent $K_m$ (E217βG) of D436K was increased 3-fold and $V_{max}$ decreased 3-fold, resulting in a 7.5-fold decrease in E217βG transport efficiency (Table 3). When the Lys residue in the D436K mutant was replaced with Glu to create the like-charged D(K)436E mutant, wild-type levels of transport activity were observed (Fig. 6, A-E). Kinetic analyses showed that the $K_m$ and $V_{max}$ values for LTC4 and E217βG were also similar to those for wild-type MRP1 (Tables 1 and 3). These data suggest that the charge rather than the volume of the Asp436 side chain is the key physical property of this residue required for MRP1 transport activity.

**Photolabeling of Wild-Type and Asp436 Mutant MRP1 Proteins with [3H]LTC4.** To determine whether the substantially reduced [3H]LTC4 transport activity of the Asp436 mutant D436K was associated with a decrease in substrate binding, photolabeling experiments were carried out with [3H]LTC4. Photolabeling was also carried out with DK436E and wild-type MRP1. As shown in Fig. 6F, [3H]LTC4 photolabeling of the D436K mutant was reduced by approximately 50% compared with wild-type MRP1. In contrast, photolabeling of the same-charged D(K)436E mutant was similar to wild-type MRP1 as was its LTC4 transport activity.
Substitution of Asp<sup>572</sup>, Glu<sup>573</sup>, Asp<sup>578</sup>, and Arg<sup>593</sup> in or near TM11. We next tested the functional importance of three negatively charged amino acids predicted to be in the short fifth extracellular loop between TM10 and TM11 (Asp<sup>572</sup>, Glu<sup>573</sup>, and Asp<sup>578</sup>) and a positively charged amino acid predicted to be in TM11 (Arg<sup>593</sup>). Asp<sup>572</sup>, Glu<sup>573</sup>, and Asp<sup>578</sup> were substituted with Arg, whereas Arg<sup>593</sup> was substituted with Glu as well as with the neutral, nonpolar Leu. The D572R, E573R, and D578R mutants exhibited transport activity profiles similar to wild-type MRP1, indicating that none of these negatively charged amino acids are critical for MRP1 transport activity (Table 2). In marked contrast, substitutions of TM11 Arg<sup>593</sup> with Glu or Leu substantially reduced MRP1 transport activity. As shown in Fig. 7, the LTC<sub>4</sub> (Fig. 7A), GSH (Fig. 7D), and MTX (Fig. 7E) transport activities of the R593E and the R593L mutants were reduced by 70 to 80% relative to wild-type MRP1. In addition, neither mutant transported the conjugated estrogens E<sub>2</sub>1βG (Fig. 7B) and E<sub>2</sub>3SO<sub>4</sub> (Fig. 7C). Kinetic analysis of LTC<sub>4</sub> transport by the R593E mutant showed that its decreased transport activity was caused by a 4-fold decrease in apparent uptake affinity (K<sub>m</sub> = 464 versus 115 nM for wild-type MRP1) and in transport capacity (2-fold decrease in V<sub>max</sub>), resulting in an overall 10-fold decrease in LTC<sub>4</sub> transport efficiency (Table 1).

To investigate the physical properties of Arg<sup>593</sup> important for MRP1 transport function, the Glu residue of the R593E mutant was replaced with Lys to create the same-charge mutant R(E)593K. As shown in Fig. 7, the transport activity of the R(E)593K mutant was comparable with wild-type MRP1 for all five organic anion substrates tested. Kinetic analyses of the R(E)593K mutant also showed that its apparent K<sub>m</sub> and V<sub>max</sub> values for LTC<sub>4</sub> were similar to those of wild-type MRP1 (Table 1). These findings indicate that the charge rather than the size of the Arg<sup>593</sup> side chain (as was observed for Lys<sup>396</sup>) is the most critical feature for MRP1 function.

Confocal Microscopy. To determine whether the mutations that caused substantial changes in the transport properties of MRP1 also affected the proper routing of MRP1 to the plasma membrane, the subcellular localization of these mutants was compared with wild-type MRP1 by confocal laser scanning fluorescence microscopy. For these experiments, GFP-tagged constructs encoding wild-type MRP1 and the mutant MRP1 proteins (D336E, K332D/D336K, K396E, D436K, and R593E) were generated and transfected into HEK 293T cells (Koike et al., 2002). When viewed under the confocal microscope, both wild-type and the mutant GFP-tagged MRP1 proteins exhibited an exclusively plasma membrane localization, confirming that they were all correctly routed to the cell surface.

![Fig. 5. ATP-dependent 3H-labeled organic anion uptake into membrane vesicles enriched for MRP1 mutants containing substitutions of Lys<sup>396</sup>. Time courses of [3H]LTC<sub>4</sub> uptake (A), [3H]E<sub>2</sub>1βG uptake (B), and GSH-stimulated [3H]E<sub>2</sub>3SO<sub>4</sub> uptake (C) by wild-type (WT) MRP1 (■), mutants K396E (○), K396I (△), and K(E)396R (□), and empty pcDNA3.1(−) vector control (○). D, apigenin-stimulated [3H]GSH uptake and [3H]MTX uptake (E) at 20 min by wild-type MRP1 (■), mutants K396E, K396I, and K(E)396R (□), and empty pcDNA3.1(−) vector control (○). Results shown are means (± S.D.) of triplicate determinations in a single experiment. Relative MRP1 protein expression levels in the membrane vesicles were as shown in Fig. 2. Similar results were obtained in at least two additional independent experiments.](molpharm.aspetjournals.org)
Discussion

The aims of the present study were 2-fold. The first was to better define the physical properties of TM6 Lys332 and Asp336 that contribute to the transport activity and substrate specificity of MRP1 (Haimeur et al., 2002). The second was to determine whether any of 11 additional charged amino acids in or proximal to TM6 and the other five TM helices of MSD2 (TM7–TM11) also play a role in organic anion transport by MRP1.

The critical importance of TM6 Lys332 as a highly substrate-selective determinant of MRP1 LTC4 and GSH transport activities was first confirmed by demonstrating that, despite maintaining a positive charge at position 332, transport of LTC4 and GSH by the K332R mutant remained significantly lower than wild-type MRP1. The reduced LTC4 transport activity of K332R was associated with a substantial reduction (5-fold) in the apparent LTC4 uptake affinity (Km), whereas Vmax was unchanged. Thus the smaller side chain of an Arg residue cannot fully compensate for the loss of Lys at position 332, indicating that both the volume and charge of the Lys332 side chain are critical for LTC4 binding and transport. This is not the case for other substrates, because the transport of E217βG, E13SO4 and MTX by the Lys332 mutants remained comparable with wild-type MRP1. In a similar way, a like-charge substitution of Glu for Asp at position 336 in the same TM helix of MRP1 resulted in a loss of overall transport activity comparable with that observed with Arg, Lys, or Leu substitutions (Haimeur et al., 2002). Thus, both the charge and volume of the Asp336 side chain are also critical for MRP1 transport activity.

We postulated previously that because they are separated by approximately one helical turn from each other, a salt bridge or ion pair critical for maintaining TM6 in a transport-competent configuration might exist between Lys332 and Asp336. However, the inactivity of the double-exchange mutant K332D/D336K suggests that this is unlikely to be the case (Fig. 2). It is interesting that both Lys332 and Asp336 are strictly conserved in MRP1/Mrp1 mammalian orthologs but are only moderately conserved in homologs of the human MRP/ABCC subfamily. Nevertheless, in a study of rat Mrp2, transport activity of K332R was associated with a substantial reduction (5-fold) in the apparent LTC4 uptake affinity (Km), whereas Vmax was unchanged. Thus the smaller side chain of an Arg residue cannot fully compensate for the loss of Lys at position 332, indicating that both the volume and charge of the Lys332 side chain are critical for LTC4 binding and transport. This is not the case for other substrates, because the transport of E217βG, E13SO4 and MTX by the Lys332 mutants remained comparable with wild-type MRP1. In a similar way, a like-charge substitution of Glu for Asp at position 336 in the same TM helix of MRP1 resulted in a loss of overall transport activity comparable with that observed with Arg, Lys, or Leu substitutions (Haimeur et al., 2002). Thus, both the charge and volume of the Asp336 side chain are also critical for MRP1 transport activity.

We postulated previously that because they are separated by approximately one helical turn from each other, a salt bridge or ion pair critical for maintaining TM6 in a transport-competent configuration might exist between Lys332 and Asp336. However, the inactivity of the double-exchange mutant K332D/D336K suggests that this is unlikely to be the case (Fig. 2). It is interesting that both Lys332 and Asp336 are strictly conserved in MRP1/Mrp1 mammalian orthologs but are only moderately conserved in homologs of the human MRP/ABCC subfamily. Nevertheless, in a study of rat Mrp2.
mutational analyses of the analogous Lys$^{325}$ and Asp$^{329}$ residues showed that they too could not be functionally replaced with like-charge amino acids (Ito et al., 2001b).

Nonconservative substitutions of two other positively charged Lys residues at positions 319 and 347 NH$_2$- and COOH-proximal to TM6, respectively, had little effect except to cause a moderate and selective reduction in GSH transport by MRP1. On the other hand, GSH-stimulated E$_3$SO$_4$ transport by the Lys$^{319}$ and Lys$^{347}$ mutants was comparable with wild-type MRP1. These observations suggest that the mutations affected the transport rather than the binding of GSH because cotransport of GSH is not required to stimulate E$_3$SO$_4$ transport by MRP1. Taken together, our observations are consistent with the conclusion that the TM6 region of MSD2 has a particularly important role in GSH transport by MRP1.

In addition to the four charged residues in or proximal to TM6 discussed above, MSD2 contains nine charged amino acids that, according to several topological models developed using common computer algorithms, are predicted to be located in or proximal to TM7 to TM11 (Hipfner et al., 1997). When these residues were replaced with oppositely charged amino acids, all but one of the resulting mutants could be expressed. The exception was the Asp$^{430}$ mutant D430K. Northern blot analyses showed that the D430K-expressing cells contained levels of the mutant MRP1 mRNA that were comparable with those of wild-type MRP1. Northern blot analyses showed that the D430K-expressing cells contained levels of the mutant MRP1 mRNA that were comparable with those of wild-type MRP1. These two basic residues are both relatively well-conserved in other ABC family members but are predicted to be on opposite faces of an α-helix that extends through the membrane as TM8 well into the cytoplasm (Campbell et al., 2004). Consequently, they would differ in their accessibility for interactions with other regions of MRP1 that are believed to occur during substrate binding and translocation. Whether or not differences in the relative accessibility of Arg$^{394}$ and Lys$^{396}$ are related to the differences in their functional importance for MRP1 transport activity is not yet known.

Unlike the oppositely charged mutant of Asp$^{430}$ discussed earlier, the TM8 D436K mutant was expressed at levels comparable with those of wild-type MRP1. However, the transport activity of this mutant was substantially reduced. Two other Asp residues shown to be important for overall MRP1 transport activity include the previously discussed...

![Fig. 7. ATP-dependent uptake of $^3$H-labeled organic anions into membrane vesicles enriched for MRP1 mutants containing substitutions of TM11 Arg$^{593}$. Time courses of $^3$HILTC$_4$ uptake (A), $^3$HIE$_{17/26}$G uptake (B), and GSH-stimulated $^3$HI$_2$SO$_4$ uptake (C) by wild-type MRP1 (●), mutants R593E (○), R593L (□), and empty pcDNA3.1(-) vector control (□). Results shown are means (± S.D.) of triplicate determinations in a single experiment. Relative MRP1 protein expression levels in the membrane vesicles were as shown in Fig. 2. Similar results were obtained in at least two additional independent experiments.](molpharm.aspetjournals.org)
Asp<sup>336</sup> in TM6 (Haimeur et al., 2002) and Asp<sup>1084</sup> proximal to the cytoplasmic end of TM14 in MSD3 (Zhang et al., 2003) (D. Situ, A. Haimeur, G. Conseil, K. E. Sparks, D. Zhang, R. G. Deeley, S. P. C. Cole, manuscript in preparation). Despite a comparable loss of transport activity, nonconservative mutations of Asp<sup>336</sup> completely abrogated [<sup>3</sup>H]LTC<sub>4</sub> photolabeling, whereas similar mutations of Asp<sup>1084</sup> had no effect. In the present study, the reduced transport activity of the D436K mutant was associated with reduced LTC<sub>4</sub> binding, and in this way, the D436K mutant is more similar to the Asp<sup>336</sup>, Lys<sup>396</sup>, and Asp<sup>436</sup> mutants. Thus, the nonconservative substitutions in both the apparent \( K_m \) and \( V_{max} \) for these substrates had occurred. It is interesting that reintroducing conservative substitutions of Lys<sup>396</sup> and Asp<sup>436</sup> [as in \( K(E)396R \) and \( D(K)436E \)], re-established wild-type transport activity, indicating that the charges of the side chains, rather than their molecular volumes, are the most important property of these amino acids for MRP1 transport function. These findings contrast with those for TM6 Asp<sup>336</sup>; in which the transport activity of the same-charge D(K)336E mutant remained very low.

Substitutions of Arg<sup>593</sup> (TM11) resulted in a somewhat different phenotype from those observed for the Lys<sup>396</sup>, Asp<sup>336</sup>, Lys<sup>396</sup>, and Asp<sup>436</sup> mutants. Thus, the nonconservatively substituted R593E and R593L mutants displayed a complete loss of E<sub>2</sub>17βG and E<sub>1</sub>3SO<sub>4</sub> transport and a substantial loss of LTC<sub>4</sub>, GSH, and MTX transport. On the other hand, the like-charge substitution of Arg<sup>593</sup> with Lys resulted in a fully functional MRP1 R(E)593K. This indicates that a positive charge at position 593 in TM11 is sufficient for MRP1 function, and changes in the side chain volume seem less important. It is interesting that transport activity was also retained when the analogous Arg<sup>336</sup> residue of rat Mrp2 was replaced with Lys but not when replaced with Leu, although it should be noted that only a limited number of substrates were tested in this latter study (Ito et al., 2001b).

It is not known at present whether TM11 Arg<sup>593</sup>, which is in the inner leaflet of the membrane bilayer, plays a structural or dynamic role, or both. However, the lack of LTC<sub>4</sub> binding by R593E and the global reduction in its transport activity indicates that Arg<sup>593</sup> is important for high-affinity binding of LTC<sub>4</sub> and presumably other substrates as well. It is also clear that this region of TM11 is particularly critical for MRP1 transport activity because we have shown recently that nonconservative substitutions of either of the nearby Phe<sup>594</sup> or Pro<sup>595</sup> also result in a global loss of organic anion transport and LTC<sub>4</sub> binding (Campbell et al., 2004; Koike et al., 2004). Major photoaffinity drug binding sites have also been identified in a proteolytic

**Fig. 8.** Confocal laser scanning fluorescence micrographs of transfected HEK 293T cells expressing GFP-tagged wild-type and MSD2 mutant MRP1 cDNA constructs. The subcellular localization of wild-type and selected MSD2 mutant MRP1 cDNA constructs was determined by confocal microscopy (Haimeur et al., 2002). HEK 293T cells were transfected with the wild-type pCDNA3.1(−)-MRP1-GFP and mutant pCDNA3.1(−)-MRP1-D336E-GFP, pCDNA3.1(−)-MRP1-K332D/D336K-GFP, pCDNA3.1(−)-MRP1-K396E-GFP, pCDNA3.1(−)-MRP1-D436K-GFP, and pCDNA3.1(−)-MRP1-R593E-GFP expression vectors as indicated, and cells were viewed 48 h later under the confocal microscope. GFP signals were collected with a 530/30-nm bandpass filter. Nuclei were stained with propidium iodide, and signals were collected with a 600/40-nm bandpass filter.

**Fig. 9.** Topological model of MRP1 MSD2 based on crystal structures of bacterial lipid transporter MsbA. A schematic diagram of MSD2 of MRP1 shows the approximate boundaries of TM6 to TM11 derived from the crystal structure of the *Vibrio cholerae* lipid transporter MsbA and an energy-minimized P-glycoprotein simulation (Campbell et al., 2004). The locations of the charged amino acids in and proximal to MSD2 that were predicted by the *V. cholerae* MsbA-based model of MRP1 MSD2 to be proximal to the TM9-cytosol interface, whereas hydrophathy-based topology algorithms predict them to be located well into cytoplasmic loop 5. ECL, extracellular loop; CL, cytoplasmic loop.
fragment of MRP1 that encompasses TM10 and TM11 (Ser542 to Arg676) (Daoud et al., 2001).

There is presently little direct experimental evidence defining the boundaries and interhelical packing interactions of the 17 TM helices of MRP1 or how they are modified during a cycle of substrate binding and transport that is coupled to nucleotide binding and hydrolysis. We recently developed models of MSD2 and MSD3 of MRP1 that are derived from the crystal structures of two bacterial MsbA lipid transporters and a P-glycoprotein simulation (Campbell et al., 2003, 2004; Stenham et al., 2003). According to our models, TM6 Lys532 and Asp536 are in the inner leaflet of the membrane facing in toward a pore or chamber through which substrates are presumed to be translocated (Fig. 9). Likewise, Asp486 at the TM8-cytosol interface and Arg593 in the inner leaflet of TM11 (Fig. 9) line the pore or substrate translocation pathway of MRP1 (Campbell et al., 2004). Lys596 at the TM7-cytosol interface could also face inward, although the predicted position of this residue is more variable in other models of MSD2. Overall, however, the locations of these five charged residues predicted by our model are consistent with their demonstrated importance as determinants of MRP1 transport activity and substrate specificity.

Unlike the hydropathy-based TM-predicting algorithm of Eisenberg et al. (1984), our crystal-based models of MSD2 place two additional charged residues, Lys546 and Lys548, at the membrane-cytosol interface of TM9 (Fig. 9). However, the side chains of these residues face outward from the putative translocation pathway of MRP1 (Campbell et al., 2004). As might be predicted with such a location, nonconservative mutations of Lys546 and Lys548 had no significant effect on MRP1 transport activity (results not shown).

Finally, it is interesting to note that the amino acid analogous to Arg676 in CFTR is Arg547, and there is evidence for the existence of a salt bridge between this residue and Asp592 in the COOH-proximal MSD that is important for CFTR function (Cotten and Welsh, 1999). CFTR-Asp592 is not conserved in MRP1; instead, a polar residue, Gln1026, is found at the analogous position in TM13 of MSD3. Whether or not MRP1-Arg593 interacts with Gln1026 via hydrogen bonding or interacts with an acidic or polar residue elsewhere in MSD3, neither is currently under investigation. Characterization of the interhelical bonding interactions of other functionally important charged residues we have identified with either charged or polar residues is also the subject of ongoing studies.

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References

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