Charged Amino Acids in the Sixth Transmembrane Helix of Multidrug Resistance Protein 1 (MRP1/ABCC1) Are Critical Determinants of Transport Activity*

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The multidrug resistance protein, MRP1 (ABCC1), is an ATP-binding cassette transporter that confers resistance to chemotherapeutic agents. MRP1 also mediates transport of organic anions such as leukotriene C₄ (LTC₄), 17β-estradiol 17-β-(D-glucuronide) (E₂17G), estrone 3-sulfate, methotrexate (MTX), and GSH. We replaced three charged amino acids, Lys³³², His³³⁵, and Asp³³⁶, predicted to be in the sixth transmembrane (TM6) helix of MRP1 with neutral and oppositely charged amino acids and determined the effect on substrate specificity and transport activity. All mutants were expressed in transfected human embryonic kidney cells at levels comparable with wild-type MRP1, and confocal microscopy showed that they were correctly routed to the plasma membrane. Vesicular transport studies revealed that the MRP1-Lys³³² mutants had lost the ability to transport LTC₄, and GSH transport was reduced; whereas E₂17G, estrone 3-sulfate, and MTX transport were unaffected. E₂17G transport was not inhibited by LTC₄ and could not be photolabeled with [³H]LTC₄, indicating that the MRP1-Lys³³² mutants no longer bound this substrate. Substitutions of MRP1-His³³⁵ also selectively diminished LTC₄ transport and photolabeling but to a lesser extent. Kinetic analyses showed that Vₘₐₓ (LTC₄) of these mutants was decreased but Kₘ was unchanged. In contrast to the selective loss of LTC₄ transport in the Lys³³² and His³³⁵ mutants, the MRP1-Asp³³⁶ mutants no longer transported LTC₄, E₂17G, estrone 3-sulfate, or GSH, and transport of MTX was reduced by >50%. Lys³³², His³³⁵, and Asp³³⁶ of TM6 are predicted to be in the outer leaflet of the membrane and are all capable of forming intrahelical and interhelical ion pairs and hydrogen bonds. The importance of Lys³³² and His³³⁵ in determining substrate specificity and of Asp³³⁶ in overall transport activity suggests that such interactions are critical for the binding and transport of LTC₄ and other substrates of MRP1.

Multidrug resistance is responsible for significantly limiting the effectiveness of many anti-cancer drugs. In experimental model systems, multidrug resistance in vivo and in vitro is characterized by cross resistance to a broad range of cytotoxic drugs that may have little structural similarity to one another and may exert their cytotoxic effects through different cellular pathways. Multidrug resistance in human tumor cells is often associated with enhanced ATP-dependent drug efflux attributed to elevated expression of some members of the ATP-binding cassette (ABC) superfamily of transporter proteins. These include MRP1 (gene designation ABCC1), P-glycoprotein (gene ABCB1), and BCRP or MXR (gene ABCC2) (1–7).

The 190-kDa multidrug resistance protein, MRP1, is frequently overexpressed in drug-resistant tumor cell lines and is also expressed in a wide variety of human tumors. The xenobiotics transported by MRP1 range from complex heterocyclic natural products and chemotherapeutic agents such as vincristine, doxorubicin, and the folate antagonist methotrexate to arsenical and antimonal oxyanions (3, 8). MRP1 has also been shown to be an efficient ATP-dependent transporter of various conjugated organic anions, including a mediator of inflammation, the cysteinyl leukotriene LTC₄, the cholestatic glucuronide conjugated estrogen E₂17G, and the sulfate conjugate estrone 3-sulfate (8, 9). To transport unconjugated drugs such as vincristine, MRP1 requires the presence of reduced GSH, or analog, which appears to be co-transported with the drug (10–13).

MRP1 belongs to subfamily C of the ABC transporter superfamily, which, in addition to MRP2–7, includes the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonylurea receptors, SUR1 and SUR2 (8, 14). Some members of the ABCC subfamily (MRP4, MRP5, CFTR, ABCC11, and ABCC12) have a typical four domain ABC transporter structure with two membrane-spanning domains (MSDs) and two nucleotide binding domains (8, 14, 15). However, the predicted topologies of the remaining members of this subfamily (MRP1, MRP2, MRP3, MRP6, and MRP7 as well as the K⁺ channel regulators, SUR1 and SUR2), together in some cases with biochemical studies, indicate that they contain an additional NH₂-terminal MSD with five TM segments and an extracytoplasmic NH₂ terminus (16–19). Thus, these ABCB transporters are predicted to have 17 TM segments, assumed to be α-helices, distributed among three MSDs: (MSD1, TM1–5; MSD2, TM6–11; and MSD3, TM12–17) (Fig. 1A). Overall, MRP2, MRP3, and MRP6 are the most closely related to MRP1 with respect to their structure and ability to transport conjugated organic anions. Like MRP1, MRP2 and MRP3 can also confer resistance to a variety of cytotoxic drugs (9, 19–25).

It has previously been noted that MRP1 contains a signifi-
cantly greater number of potentially charged amino acids in its predicted TM α-helices than P-glycoprotein (26). Since many of the molecules recognized and transported by MRP1 contain a relatively large hydrophobic domain as well as a hydrophilic domain with at least one anionic or cationic charge at physiological pH, it seems reasonable to suggest that charged amino acids in the TM segments of this protein might well play a role in determining its substrate specificity and transport activity (26–30). Hydropathy analyses predict that the first TM helix of M6, spans amino acids 320–340 and contains three potentially charged amino acids (Fig. 1). In the present study, we have mutated TM6 Lys332, His335, and Asp336, which are predicted to cluster in the exoplasmic leaflet of the membrane, and show that replacing them individually with a neutral or oppositely charged amino acid markedly affects the transport activity of MRP1. Importantly, we show that substitutions of Lys332 and to a lesser extent His335 selectively decrease the binding and transport of LTC₄ and GSH while leaving the transport of other organic anions unchanged. In contrast, substitutions of Asp336 show no such selectivity, since mutation of this amino acid markedly reduces or eliminates transport of all MRP1 substrates and thus appears critical for the overall activity of the protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—[14,15-3H]LTC₄ (115.3 Ci mmol⁻¹) was purchased from Amersham Biosciences. [6-3H]-[3H]Methotrexate (55 Ci mmol⁻¹), [6-3H]-Iodoacetate (30 Ci mmol⁻¹), [3H]- estrone-3-sulfate, and [3H]-glucuronide-2′-1′-HGH (40–44.8 Ci mmol⁻¹) were purchased from PerkinElmer Life Sciences. [3',5',7',4'-3H]Methotrexate sodium (17 Ci mmol⁻¹) was from Moravek Inc. (Brea, CA). LTC₄ was purchased from Calbiochem and nucleotides, GSH, acivicin, E₂17G, and dithiothreitol were purchased from Sigma.

**Vector Construction and Site-directed Mutagenesis**—The MRP1 expression vector pcDNA3.1−/MRP1K has been described previously (31). Site-directed mutagenesis was performed using the Transformer™site-directed mutagenesis kit (Clontech Laboratories Inc., Palo Alto, CA). The template for mutagenesis was prepared by cloning a 1.88-kb BamHI-SphI fragment from pCDNA3.1−/MRP1K into pGEM-3Z (Promega, Madison, WI). Mutations were generated according to the manufacturer’s instructions. The sequences of the individual sense strands, with the altered codons underlined and the corresponding changes in amino acids indicated in parentheses were as follows: (K332D) 5'-CTC ATG AGC TTC TTC TTC GAC GCC ATC CAG CAC GAG GAC CTG ATG-3'; (K332G) 5'-CTC ATG AGC TTC TTC TTC GAC GCC ATC CAG CAC GAG GAC CTG ATG-3'; (H335L) 5'-GGTTTC ATG AGC TTC TTC TTC GAC GCC ATC CAG CAC GAG GAC CTG ATG-3'; (H335Q) 5'-CCATTA ATG AGC TTC TTC TTC GAC GCC ATC CAG CAC GAG GAC CTG ATG-3'; (D336L) 5'-GGTTTC ATG AGC TTC TTC TTC GAC GCC ATC CAG CAC GAG GAC CTG ATG-3'; (D336R) 5'-CCATTA ATG AGC TTC TTC TTC GAC GCC ATC CAG CAC GAG GAC CTG ATG-3'; (D336N) 5'-GACAAT ATG AGC TTC TTC TTC GAC GCC ATC CAG CAC GAG GAC CTG ATG-3'; (D336K) 5'-G CAC GCC ATC CAG CAC GAG GAC CTG ATG-3'; (H335Q) 5'-GGTTTC ATG AGC TTC TTC TTC GAC GCC ATC CAG CAC GAG GAC CTG ATG-3'. The inserts were again sequenced to confirm the presence of the desired alteration. The altered sequence, which was used to replace the corresponding site, was cloned in frame with Ser65°FRET and Ser65°FRET/FRET(FRET) was used to construct MRP1-TM6 fusion proteins.

**Transfections with MRP1 Expression Vectors**—Constructs containing wild-type and TM6 mutant pCDNA3.1−/MRP1 expression vectors were transfected into SV40-transformed human embryonic kidney cells (HEK293T). Briefly, 7 × 10⁵ cells were seeded in 150-mm plates, and 24 h later, DNA (18 μg) was added using FuGENE6 (Roche Diagnostics, Laval, Canada) according to the manufacturer’s instructions. After 72 h, the HEK293T cells were harvested, and inside-out membrane vesicles were prepared as described previously (32). Empty vector and vector containing the wild-type cDNAs were included as controls in all experiments. Levels of wild-type and TM6 mutant MRP1 proteins were determined by immunoblotting as described below.

**Measurement of MRP1 Protein Levels in Transfected Cells**—The expression levels of wild-type and TM6 mutant MRP1 proteins were determined by immunoblot analysis of membrane protein fractions from transfected cells essentially as described (33). Proteins were resolved on a 7%polyacrylamide gel and electrotransferred to a nylon membrane. Blots were blocked with 4% (w/v) skim milk powder for 1 h, followed by incubation with the human MRP1-specific murine monoclonal antibody QCR1-1 (diluted 1:10,000), which recognizes a linear epitope consisting of amino acids 918–924 (34). After washing, blots were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce) followed by application of Renaissance®chemiluminescence reagents. Relative levels of MRP1 expression were estimated by densitometric analysis using a ChemiImager™4000 (Alpha Innotech, San Leandro, CA).

**Confocal Microscopy**—HEK293T cells were seeded at 3.5 × 10⁴ cells/well in a six-well plate on coverslips coated with 0.1% gelatin in 2 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and transiently transfected with the MRP1-GFP cDNA constructs (1 μg of DNA/well) using FuGENE6 as before. Forty-eight hours later, the coverslips were washed once with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. After washing twice with PBS, the cells were permeabilized by adding 0.2% Triton X-100 in PBS. The coverslips were then incubated in RNase A (10 μg ml⁻¹) in 1% Triton X-100/1 μg bovine serum albumin in PBS) for 30 min at room temperature. The coverslips were washed again, and cell nuclei were stained in 1 ml of propidium iodide (2 μg ml⁻¹ in PBS) for 45 min in the dark. Finally, the coverslips were placed on slides containing one drop of Antifade Solution (Molecular Probes, Inc., Eugene, OR), and cells were examined using a Meridian InSight Plus confocal microscope equipped with an air-cooled argon laser. Images obtained with 488-nm excitation were pseudocolored and overlaid using Maxim DL software.

**MRP1-mediated Transport of H-Labeled Substrates by Inside-out Membrane Vesicles**—Preparation of inside-out membrane vesicles from transfected HEK293T cells has been described previously (32), and ATP-dependent uptake of H-labeled substrates by the membrane vesicles was measured using techniques described previously (10). Briefly, LTC₄ transport assays were performed at 23 °C in a 50-μl reaction containing 50 nM LTC₄ (50 nM; 40 nCi), 4 mM AMP or ATP, 10 mM MgCl₂, creatine phosphate (10 mM), creatine kinase (100 μg 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 in transport buffer. Radioactivity was quantitated by liquid scintillation counting. All data were corrected for the amount of [3H]LTC₄ that remained bound to the filter, which was usually <10% of the total radioactivity. Transport in the presence of AMP was subtracted from the transport in the presence of ATP to determine ATP-dependent LTC₄ uptake. ATP-dependent LTC₄ uptake was carried out in triplicate, and results were expressed as means ± S.D.

**Uptake of E₂17G** was measured in a similar fashion except that membrane vesicles (2 μg of protein) were incubated at 37 °C in a total reaction volume of 50 μl containing [3H]E₂17G (400 nm; 40 nCi) and components as described for [3H]LTC₄ transport. ATP-dependent transport of estrone 3-sulfate into membrane vesicles was measured as described above for [3H]E₂17G except that the initial substrate concentration was 300 nm (200 nCi) [3H]estrone-3-sulfate, and the reaction volume was 60 μl containing 3 mM GSH and 10 mM dithiothreitol. GSH uptake was also measured by rapid filtration with membrane vesicles (20 μg of protein) incubated at 37 °C for 20 min in a 65-μl reaction volume containing 100 μM [3H]GSH (1000 nCi/μmol) and 0.1 μCi [3H]g-γ-glutamyltranspeptidase during transport, membranes were preincubated in 0.5 mM acivicin for 10 min at 37 °C prior to measuring [3H]GSH uptake in the presence of apigenin (30 μM) (32). MTX uptake was also measured as described previously (35). Assays were carried out at 37 °C in a 60-μl reaction volume containing 25 μM MTX and other components as 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, Transport—Kₘ and Vₘₐₓ values of ATP-dependent LTC₄ transport by membrane vesicles (2.5 μg of protein) were determined by measuring uptake at eight different substrate concentrations. Data were analyzed using Graph Pad Prism™software and kinetic parameters determined by nonlinear regression analyses and Michaelis-Menten analysis.
Affected by Substitutions of Charged Amino Acids in TM6—

Expression of wild-type and TM6 mutant MRP1 proteins was confirmed by immunoblot analysis. In all cases, the MRP1-specific monoclonal antibody QCRL-1 detected a single band of 190 kDa in membrane vesicles prepared from transfected HEK293T cells (Fig. 3A). Densitometric analysis showed that all mutants were expressed at levels comparable with that of wild-type MRP1 (80–100%). The GFP fusion proteins of wild-type MRP1 and the TM6 MRP1 mutants were also expressed at similar levels (data not shown).

All TM6 Mutant MRP1 Proteins Are Correctly Routed to Plasma Membrane of Transfected HEK293T Cells—To determine whether the substitutions of Lys332, His335, and Asp336 impaired trafficking of MRP1 to the plasma membrane, the subcellular localization of the MRP1 TM6 mutants was compared with wild-type MRP1 by confocal laser-scanning fluorescence microscopy. For these experiments, GFP-tagged constructs encoding wild-type MRP1 and TM6 mutant MRP1 proteins were generated and transfected into HEK293 cells. When viewed under the confocal microscope, both wild-type and mutant MRP1 proteins exhibited an exclusively plasma membrane localization, confirming that the mutant proteins were correctly routed to the cell surface. Representative confocal micrographs of cells expressing GFP-tagged wild-type MRP1 and mutants K332D, H335E, and D336R are shown in Fig. 2.

Kinetic Analysis of [3H]LTC4 Uptake in His335 Mutant MRP1—To determine the effect of the TM6 mutations on MRP1 transport activity, a time course of ATP-dependent [3H]LTC4 uptake was performed with 69, 63, and 48 pmol [3H]LTC4 at levels that were 60% of wild-type MRP1 uptake 1 min compared to wild-type MRP1 by confocal laser-scanning fluorescence microscopy. For these experiments, GFP-tagged constructs encoding wild-type MRP1 and TM6 mutant MRP1 proteins were generated and transfected into HEK293 cells. When viewed under the confocal microscope, both wild-type and mutant MRP1 proteins exhibited an exclusively plasma membrane localization, confirming that the mutant proteins were correctly routed to the cell surface. Representative confocal micrographs of cells expressing GFP-tagged wild-type MRP1 and mutants K332D, H335E, and D336R are shown in Fig. 2.

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H335E (dark brown) were determined by immunoblotting with monoclonal antibody QCRL-1. Proteins in membrane vesicles used for transport assays were prepared from HEK293T cells expressing wild-type and TM6 mutant MRP1 cDNA constructs. HEK293T cells were transfected with the pcDNA3.1(−)MRP1-GFP, pcDNA3.1(−)MRP1K332D-GFP, pcDNA3.1(−)MRP1H335E-GFP, and pcDNA3.1(−)MRP1D336R-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 the signal was collected with a 600/40-nm bandpass filter.

**FIG. 2.** Confocal laser-scanning fluorescence micrographs of transfected HEK293T cells expressing GFP-tagged wild-type and TM6 mutant MRP1 cDNA constructs. HEK293T cells were transfected with wild-type MRP1 (WT-MRP1), TM6 mutant MRP1 proteins. Membrane vesicles and Inhibition by LTC4 and H335Q mutants, respectively.

**FIG. 3.** Time course of [3H]LTC4 uptake by membrane vesicles prepared from HEK293T cells expressing wild-type and TM6 mutant MRP1 proteins. Membrane vesicles were incubated at 23 °C with 50 nM [3H]LTC4 in transport buffer for the times indicated. A, MRP1 expression levels of wild-type (WT-MRP1) and TM6 mutant proteins in membrane vesicles used for transport assays were determined by immunoblotting with monoclonal antibody QCRL-1. B, wild-type MRP1 (■), MRP1 mutants K332D (▲) and K332L (●), and control empty pcDNA3.1(−) vector (○). C, wild-type MRP1 (■), MRP1 mutants H335E (◇), H335L (□), and H335Q (●), and control pcDNA3.1(−) vector (○). D, wild-type MRP1 (■), MRP1 mutants D336L (▲) and D336R (●), and control empty pcDNA3.1(−) vector (○). Results shown are means ± S.D. of triplicate determinations in a single experiment. Similar results were obtained in at least three additional independent experiments.

and H335Q mutants, respectively.

[3H]E217G Uptake by TM6 Mutant MRP1-enriched Membrane Vesicles and Inhibition by LTC4—We further explored the importance of Lys332, His335, and Asp336 for the transport activity of MRP1 by determining time courses of [3H]E217G uptake by these mutants (Fig. 5). The Lys332 mutants (Fig. 5A) and His335 mutants (Fig. 5B) exhibited the same levels of E217G uptake as wild-type MRP1. In contrast, substitution of Asp336 with either Leu or Arg led to a complete loss of E217G uptake. Thus, as shown in Fig. 5C, MRP1-Asp336 mutants D336L and D336R exhibited the same E217G uptake activity as the empty vector control. These observations indicate that substitutions of Lys332 and His335 selectively reduce LTC4 transport but leave E217G uptake intact, whereas replacing Asp336 eliminates MRP1-mediated transport of both conjugated organic anions altogether.

Reciprocal and competitive inhibition of LTC4 and E217G transport has been reported previously (10, 36). Consequently, to determine whether E217G transport by the TM6 mutant MRP1 proteins that either no longer transport LTC4 or exhibit reduced LTC4 transport activity could still be inhibited by this cyssteinyl leukotriene, [3H]E217G uptake by the Lys332 and His335 mutants in the presence of LTC4 was examined. As shown in Fig. 6A, LTC4 at a concentration of 2 μM completely inhibited [3H]E217G uptake by wild-type MRP1 as expected. In contrast, LTC4 had very little effect (<15%) on E217G uptake by MRP1 mutants K332D and K332L, indicating that loss of LTC4 transport in these mutants is associated with a loss of binding of this substrate. On the other hand, LTC4 was still able to inhibit E217G uptake by MRP1 mutants H335E, H335L, and H335Q, which is consistent with only a partial reduction in LTC4 transport activity observed with these mutants (Fig. 6B).
Mutations abrogate photolabeling and hence binding of this compound. These results are consistent with the inability of these mutants to transport LTC₄. For the H335D, H335L, and H335Q MRP1 mutants, photolabeling was decreased by 40–45%.

Apigenin-stimulated [³H]GSH and GSH-stimulated [³H]Estrone 3-Sulfate Uptake Is Variously Affected by Substitutions of Charged Amino Acids in TM6 of MRP1—In addition to LTC₄ and E₁₇βG, membrane vesicles enriched for the TM6 MRP1 mutants were examined for their ability to transport [³H]GSH and [³H]estrone 3-sulfate. In previous studies, we have shown that MRP1 exhibits low levels of ATP-dependent GSH transport that can be markedly stimulated by a variety of compounds including the bioflavone apigenin (37, 38). Similarly, estrone 3-sulfate by itself is a poor substrate for MRP1, but uptake of this conjugated estrogen is increased 5-fold in the presence of physiological concentrations of GSH (30, 39). Thus, apigenin and GSH were included in membrane vesicle transport assays to allow more accurate measurements of [³H]GSH uptake and [³H]estrone 3-sulfate uptake, respectively.

When membrane vesicles from cells expressing the Lys³³², His³³⁵, and Asp³³⁶ MRP1 mutants were examined, it was found that apigenin-stimulated GSH uptake activity was reduced in all cases. The relative levels of [³H]GSH uptake by the MRP1 K332D and K332L mutants were less than 15% of wild-type MRP1 (Fig. 7A) after subtracting basal [³H]GSH transport by membrane vesicles from the empty vector-transfected control cells. GSH uptake by the His³³⁵ substituted MRP1 mutants
Membrane vesicles were incubated at 37 °C and K332D and K332L; B. 

ent [3H]estrone 3-sulfate uptake by membrane vesicles prepared D336R. 

shaded bars MRP1-transfected (open bars) from wild-type transfected (WT-MRP1) (pcDNA3.1( 

3-sulfate in transport buffer containing 3 mM GSH and 10 mM dithio-

control (WT-MRP1), TM6 mutant MRP1, and the empty vector control 

from HEK293T cells transfected with cDNAs encoding wild-type 

/H9262 was also reduced, but only by ~60% (Fig. 7B). Substitution of Asp336 with Leu (D336L) reduced GSH uptake to ~25% of wild-type MRP1 levels, whereas substitution of this negatively charged residue with a positively charged residue (D336R) further reduced uptake of this tripeptide to just above basal levels observed with vesicles from the empty vector control (Fig. 7C).

In contrast to [3H]GSH uptake, both neutral and negatively charged substitutions of Lys332 and His335 had essentially no effect on GSH-stimulated uptake of [3H]estrone 3-sulfate (Fig. 7, D and E). This suggests that these mutants are still capable of binding GSH although GSH transport is not detectable. On the other hand, as observed for LTC4, E217βG, and GSH uptake, both Leu and Arg substitutions of Asp336 eliminated estrone 3-sulfate transport by MRP1 (Fig. 7F).

[3H]MTX Uptake in TM6 Mutant MRP1-enriched Membrane Vesicles—Several groups have reported previously that MRP1 mediates ATP-dependent uptake of MTX (22, 40, 41). In the membrane vesicles prepared from the transfected HEK cells used in this study, we found that MTX uptake by wild-type MRP1 was ~6.6 ± 0.6 nmol mg of protein ~20 min⁻¹ compared with 1.1 nmol ± 0.4 mg of protein⁻¹ 20 min⁻¹ observed with control vesicles prepared from cells transfected with the pcDNA3.1( vector alone. Substitution of Lys332 with a neutral (K332L) or negatively charged (K332D) amino acid had no effect on MTX uptake by MRP1. Similarly, MTX uptake by the H335E, H335L, and H335Q MRP1 mutants was comparable with wild-type MRP1. In contrast, MTX uptake was reduced by ~50% when Asp336 was substituted with Leu (D336L) and by ~65% when substituted with Arg (D336R) (not shown).

DISCUSSION

Two of the best characterized substrates of MRP1 are the physiological metabolites LTC4 and E217βG, which show reciprocal competitive inhibition of each other’s transport. Consequently, it has been suggested that these two conjugated organic anions bind to the same or mutually exclusive binding sites on the protein (10, 19, 36, 42). However, it is clear that the binding sites for these two substrates are not identical, since substitutions of single amino acids in TM17 can eliminate or reduce E217βG transport while leaving LTC4 transport essentially intact (31, 43). In the present study, we have now identified two basic amino acids, Lys332 and His335, in the highly amphipathic TM6 of MRP1 that play a critical and selective role in the binding and transport of LTC4. Thus, replacing Lys332 with either Leu or Asp eliminated the ability of MRP1 to transport LTC4 (and markedly reduced GSH transport) without affecting the transport of other organic anions including E217βG, estrone 3-sulfate, and MTX. The MRP1-Lys332 mutants also could not be photolabeled with LTC4, and E217βG transport by these mutants could no longer be inhibited by LTC4. Moreover, since the loss of LTC4 transport and binding was the same whether Lys332 was replaced with a neutral or negatively charged amino acid, it may be concluded that the loss of the positive charge at position 332 rather than the introduction of a neutral or negative charge is responsible for the phenotype observed.

Despite the fact that the MRP1-Lys332 mutant proteins no longer transported LTC4, they still retained the ability to transport GSH, albeit at a much reduced level. In addition, GSH-stimulated estrone 3-sulfate transport by these mutants remained comparable with that observed with wild-type MRP1. One possible explanation for these findings is that MRP1 may contain two binding sites for GSH, one of which is involved in the stimulation of estrone 3-sulfate transport and the other of which is involved in the transport of GSH itself. If indeed more than one site exists, our results indicate that only the site for GSH transport is affected by the Lys332 mutations (44). MRP1-Lys332 is quite well conserved among ABCC subfamily members, including MRP2 and MRP3, and the murine, canine, Saccharomyces cerevisiae, and Leishmania tarentolae orthologs of MRP1, which are known to transport organic anions. However, mutation of Lys332 in human MRP2 (which is analogous to MRP1-Lys332) had no effect on transport of glutathione-conjugated methylfluorescein, suggesting that this amino acid is not critical for the transport of all GSH-conjugated substrates (29). On the other hand, Ito et al. (28) showed that the analogous Lys325 in rat Mrp2 was important for LTC4 transport as well as for transport of GSH-conjugated 2,4-dinitrophenyl.

Substitution of MRP1-His335 also diminished GSH and LTC4 transport by MRP1, but the effect on LTC4 transport was considerably less than observed with the MRP1-Lys332 mutants. The retention of some activity allowed us to determine
the kinetic parameters of LTC₄ transport by the His₃³₅ mutant, which revealed that their apparent affinity for this substrate was similar to that of wild-type MRP1. However, their Vₘₐₓ values were significantly decreased, as was photolabeling by [³H]LTC₄. This suggests that these mutations caused a reduction in both the efficiency of binding and translocation of this substrate, although why the reduction in photolabeling was not associated with a change in Kₘ is not clear. MRP1-His₃³⁵ is relatively poorly conserved among ABCC family members and is present only in the canine, mouse, and yeast orthologs of MRP1 (Fig. 1B). MRP2, MRP3, and MRP6 have a much lower LTC₄ transport efficiency than MRP1, but whether this might be related to the absence of a His residue in the analogous position of MRP1-His₃³⁵ in these other transporters is not known.

Substitutions of MRP1-Asp₃³⁶ had a more dramatic effect on MRP1 transport activity than substitutions of either Lys₃³² or His₃³⁵ in that the D336L and D336R mutants did not transport four of the five organic anion substrates tested, and transport of the fifth, MTX, was reduced by at least 50%. Thus, mutations of MRP1-Asp₃³⁶ caused a nonselective overall loss of MRP1 transport activity, suggesting that this residue plays a more global and essential structural role in assembling or maintaining MRP1 in a transport-competent state. Among the ABCC proteins, MRP1-Asp₃³⁶ is the most conserved of the three charged residues examined that are all clustered on the same face of TM6 in the outer leaflet of the membrane. The functional importance of this acidic residue may also be conserved, since the analogous Asp₃²⁹ in rat Mrp2 has been demonstrated to be important for transport of the GSH-conjugated substrates, 2,4-dinitrophenyl-S-glutathione and LTC₄, and, to a lesser extent, E₁₇βG and other conjugated organic anions (28). Interestingly, a missense mutation of the analogous acidic residue in CFTR, E₃₉₂K, has been associated with a benign cystic fibrosis phenotype (45), and in vitro studies indicate that it is not critical for the chloride conducting-activity of CFTR (46).

The exact roles of TM6 Lys₃³², His₃³⁵, and Asp₃³⁶ in the mechanism of substrate binding and translocation by MRP1 are unknown, although it is interesting to note that residues in the analogous TM1 of CFTR form part of the chloride channel lining of this protein (46, 47). It is possible that the selective loss or reduction of LTC₄ and GSH transport by mutations of MRP1-Lys₃³² and His₃³⁵ occurs because the side chains of these amino acids are required to form ionic or hydrogen bonding interactions directly with the glutathione moiety of these substrates. Additionally, side chain interactions between Lys₃³² and His₃³⁵ and other amino acids, either in the same or a different TM helix of MRP1, may be required to maintain the architecture of the LTC₄ and GSH binding pocket(s). Thus mutations in these basic residues may cause a shift in TM helix packing that makes the LTC₄ and GSH binding site and/or translocation pathway less accessible to substrate. On the other hand, substitutions of Asp₃³⁶ may perturb normal packing of the TM helices of MRP1 or impede their movement in response to substrate translocation to such a degree that binding and transport of all substrates is markedly reduced or eliminated altogether. Further investigations of MRP1 helix-helix interactions such as those described for several TM segments of CFTR (48, 49) may help to distinguish among these possibilities.

There are numerous examples of proteins where side chains of charged amino acids are known to form ion pairs or salt bridges with oppositely charged amino acids and thus provide protein stability and/or promote interactions with ligands. In addition, the introduction of salt bridges at the x and x + 4 positions in short peptides and on protein surfaces can facilitate α-helix formation (50). Lysₓ and Aspₓ are located just four amino acids apart and thus could potentially form an intrahelical stabilizing salt bridge with one another. However, if this were their sole interaction, then it might be expected that mutation of either amino acid would result in the same phenotype, and clearly this was not the case. It is thus highly likely that these charged residues form additional ion pairs and/or hydrogen bonds with neighboring polar or charged amino acids in other TM helices of MRP1.

Ion pair interactions between TM helices have been reported for a variety of transporters and ion channels. For example, it has been shown that a salt bridge can occur between oppositely charged amino acids in TM2 and TM11 of the rat vesicular monoamine transporter, and when disrupted, high affinity recognition of its substrate serotonin is lost (51). Similarly, mutation of Lys₁₃¹ or His₁₃⁸₈ that make up a salt bridge between TM2 and TM8 in the rat vesicular acetylcholine transporter results in loss of substrate binding and transport (52). Whether or not the three MRPI TM6 amino acids studied here form ion pairs with oppositely charged residues or form hydrogen bonds with polar amino acids in other TM segments that are essential for MRPI transport activity remains to be determined.

In summary, we have identified three potentially charged residues in the region of TM6 predicted to be in the outer leaflet of the plasma membrane that are critical for the transport activity of MRPI. Mutations of two of them, Lys₃³² and His₃³⁵, selectively affect the recognition and transport of GSH and the GSH conjugate LTC₄. We previously showed that the naturally occurring mutation of MRPI-Arg₄₃₃ to Ser predicted to be located in the fourth cytoplasmic loop in close proximity to the membrane interface of TM8 results in a 2-fold decrease in LTC₄ and estrone 3-sulfate transport efficiency. Like the present findings with the Lys₃³² and His₃³⁵ mutants, E₁₇βG transport was unaffected by the Arg₄₃₃ → Ser mutation (30). Thus, potentially charged amino acids both in the TM segments of MRP2 and in at least one of the cytoplasmic loops connecting them appear particularly important for MRPI-mediated transport of LTC₄. Additional charged residues in other regions of MRP1 are currently being investigated to determine their participation, if any, in the substrate specificity and transport activity of MRPI.

Acknowledgments—We are grateful to Dr. Ken-ichi Ito and Curtis Oleshchuk for helpful discussions and advice. Maureen Rogers is thanked for expert word processing and assistance in the preparation of figures.
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