Mutational Analysis of Ionizable Residues Proximal to the Cytoplasmic Interface of Membrane Spanning Domain 3 of the Multidrug Resistance Protein, MRP1 (ABCC1)

GLUTAMATE 1204 IS IMPORTANT FOR BOTH THE EXPRESSION AND CATALYTIC ACTIVITY OF THE TRANSPORTER*

The multidrug resistance protein MRP1 is an ATP-dependent transporter of organic anions and chemotherapeutic agents. A significant number of ionizable amino acids are found in or proximal to the 17 transmembrane (TM) helices of MRP1, and we have investigated 6 of these at the cytoplasmic interface of TM13–17 for their role in MRP1 expression and transport activity. Opposite charge substitutions of TM13 Arg1046 and TM15 Arg1132 did not alter MRP1 expression nor did they substantially affect activity. In contrast, opposite charge substitutions of TM16 Arg1202 and Glu1204 reduced protein expression by >80%; however, MRPI expression was not affected when Arg1202 and Glu1204 were replaced with neutral or same-charge residues. In addition, organic anion transport levels of the R1202L, R1202G, and R1202K mutants were comparable with wild-type MRP1. In contrast, organic anion transport by E1204L was substantially reduced, whereas transport by E1204D was comparable with wild-type MRP1, with the notable exception of GSH. Opposite charge substitutions of TM16 Arg1202 and TM17 Arg1249 did not affect MRP1 expression but substantially reduced transport. Mutants containing like-charge substitutions of Arg1202 or Arg1249 were also transport-inactive and no longer bound leukotriene C4. In contrast, substrate binding by the transport-compromised E1204L mutant remained intact. Furthermore, vanadate-induced trapping of azido-ADP by E1204L was dramatically increased, indicating that this mutation may cause a partial uncoupling of the catalytic and transport activities of MRP1. Thus, Glu1204 serves a dual role in membrane expression of MRP1 and a step in its catalytic cycle subsequent to initial substrate binding.

ATP-binding cassette (ABC)1 membrane proteins make up one of the largest protein superfamilies documented and are

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§§ The abbreviations used are: ABC, ATP-binding cassette; MSD, membrane spanning domain; TM, transmembrane; NBD, nucleotide binding domain; LTC4, leukotriene C4; E17βSO4, estrone sulfate; MTX, methotrexate; CL, cytoplasmic loop; mAb, monoclonal antibody.
cytosolic and membrane spanning regions of both halves of MRP1 that are required for the binding and/or transport of various substrates and are inhibitors of this transporter (3, 17–29). Of the individual residues identified, a significant number are ionizable ("charged") and located in M5D2 (TM6–11) and M5D3 (TM12–17) (19, 22–24, 27, 28). For example, we demonstrated previously (22, 24) that both conservative and nonconservative mutations of Lys332 in the inner leaflet of TM6 of M5D2 cause a selective loss of LTC4 and GSH transport, whereas mutation of the nearby Asp336 as well as Arg593 in TM11 essentially eliminates organic anion transport activity. In other studies, we showed that various substitutions of ionizable residues located in or proximal to the membrane-cytosol interface of TM14 in M5D3 could also differentially affect both MRP1 transport activity and substrate specificity (23, 24, 27, 28). For example, we demonstrated previously (22, 24) that both conservative and nonconservative mutations of Lys332 in the inner leaflet of TM6 of M5D2 cause a selective loss of LTC4 and GSH transport, whereas mutation of the nearby Asp336 as well as Arg593 in TM11 essentially eliminates organic anion transport activity. In other studies, we showed that various substitutions of ionizable residues located in or proximal to the membrane-cytosol interface of TM14 in M5D3 could also differentially affect both MRP1 transport activity and substrate specificity (23, 27). Because many organic anions transported by MRP1 are relatively hydrophilic and are formed inside the cell, it seems likely that they would first come into contact with MRP1 from the cytoplasm rather than from the membrane, as postulated for therophobic substrates of the P-glycoprotein transporter (29). Thus, it is reasonable to suppose that amino acids located at the hydrophobic surface of the MRP1 membrane model (Fig. 1).

In this paper, we have extended our studies of M5D3 by investigating the role of six additional ionizable residues predicted by hydropathy analyses to be in or proximal to the cytoplasmic interface of TM13 and -15 in the expression and transport activity of MRP1 (Fig. 1). Thus, Arg1046, Arg1131, Arg1197, Arg1202, Glu1204, and Arg1249 were targeted for substitution by site-directed mutagenesis, and the expression, or-ganionic anion transport properties, and, in some cases, the substrate and nucleotide binding properties of the resulting mutant proteins were examined.

MATERIALS AND METHODS

Chemicals and Reagents— [3H]E217Glc, [3H]E1SO4, and glycine-2-3H(GSH (40–44.8 Ci mmol−1) were from PerkinElmer Life Sciences. [14,15,19,20]-32P]ATP (115 Ci mmol−1) was from New England Nuclear (Boston, MA). LTC4 was from Calbiochem, and E17G and E15SO4 were from Sigma. 8-Azido-[32P]ATP (15.7 Ci mmol−1) was from Affinity Labeling Technologies, Inc. (Lexington, KY).

Site-directed Mutagenesis—Amino acid substitutions were created using the QuikChangeTM site-directed mutagenesis kit (Stratagene). The template for mutagenesis was generated by cloning a 2-kb Xmal fragment (nucleotides 2337–4322) encoding amino acids 780–1440 of MRP1 M5D3 from pcDNA3.1–MRP1 DNA (16 µg) using FuGEnE 6 (Roche Applied Science) (48 µl) at a ratio of 1:3, as suggested by the manufacturer. Seventy two hours later, cells were harvested, and membrane vesicles were prepared as described (6). Protein concentrations were determined and then the vesicles were aliquoted and stored at -70 °C until needed.

To quantitate levels of MRP1 expression, membrane vesicle proteins (1 and 2 µg) were resolved on a 7% SDS-polyacrylamide gel and then transferred onto an Immobilon-P membrane (Millipore). mAb QCRL-1 was used to detect MRP1 (30), and the signal was enhanced using SuperSignal West Pico Chemiluminescent substrate (Pierce). Membrane vesicle proteins were quantitated using different batches of vesicles prepared from independent transfections.

Transfection of MRP1 Expression Vectors into HEK293T Cells, Preparation of Membrane Vesicles, and Quantitation of MRP1 Protein Levels—SV40-transformed human embryonic kidney cells (HEK293T) were seeded at 105 cells per 150-mm plate. Twenty four to 48 h later, cells were transfected with either mutant or wild-type MRP1 pcDNA3.1–MRP1 DNA (16 µg) using FuGENE 6 (Roche Applied Science) (48 µl) at a ratio of 1:3, as suggested by the manufacturer. Seventy two hours later, cells were harvested, and membrane vesicles were prepared as described (6). Protein concentrations were determined and then the vesicles were aliquoted and stored at -70 °C until needed.

To quantitate levels of MRP1 expression, membrane vesicle proteins (1 and 2 µg) were resolved on a 7% SDS-polyacrylamide gel and then transferred onto an Immobilon-P membrane (Millipore). mAb QCRL-1 was used to detect MRP1 (30), and the signal was enhanced using Renenese chemiluminescence reagent (PerkinElmer Life Sciences).

Relative levels of MRP1 expression were determined by densitometry of exposed films.

Membrane Vesicle Transport Studies—Uptake of [3H]LTC4, [3H]E1SO4, [3H]E17Glc, [3H]E15SO4, [3H]GSH, and [3H]MTX into membrane vesicles was measured following the rapid filtration method described (6, 22). Membrane vesicles prepared from HEK293T cells transfected with wild-type pcDNA3.1–MRP1 served as a positive control, whereas vesicles prepared from HEK293T cells transfected with empty vector pcDNA3.1– served as a negative control. ATP-dependent transport was determined by subtracting uptake values in the presence of AMP from uptake values measured in the presence of ATP. The results shown are mean values of duplicate or triplicate determinations in a single experiment and were confirmed in 1–3 additional experiments using different batches of vesicles prepared from independent transfections.

Photoactivation of MRP1 with [3H]LTC4, 8-Azido-[32P]ATP, and Orthovanaadate-induced Trapping of 8-Azido-[32P]ADP—Photoactivation of mutant and wild-type MRP1 proteins with [3H]LTC4 was carried out as described (6, 22). Briefly, membrane vesicles (50 µg of protein) were
Expression and Transport Properties of MRP1 Mutants Containing Opposite and Same Charge Substitutions of Arg<sup>1046</sup>, Asp<sup>1084</sup>, and Arg<sup>1131</sup> in or Near TM13–15—In the first series of experiments, the importance of ionizable amino acids located in or near predicted TM13–15 for MRP1 protein expression and function were investigated by replacing Arg<sup>1046</sup> and Arg<sup>1131</sup> with Asp and Glu, respectively. Levels of expression of the R1046D and R1131E mutant proteins were determined by the immunoblotting of membrane vesicles prepared from the transfected HEK293T cells with mAb QCRL-1. The TM14-associated D1084R mutant described previously (27) was included for comparison. A single 190-kDa band was detected in both the wild-type and mutant MRP1 preparations, whereas no immunoreactive bands were detected in membrane vesicles prepared from control transfected cells (Fig. 2A). Densitometry was performed on immunoblots of vesicle protein from three independent transfections, and the relative expression levels of the mutant MRP1 proteins were determined to be comparable (70–130%) to wild-type MRP1 levels, indicating that these mutations did not substantially affect the biogenesis of MRP1.

To determine the functional consequences of replacing Arg<sup>1046</sup>, Asp<sup>1084</sup>, and Arg<sup>1131</sup> with an oppositely charged amino acid, the ability of the R1046D, D1084R, and R1131E mutants to transport different organic anions was tested. Fig. 2, B and C, shows that uptake levels of E<sub>17βG</sub> and LTC<sub>4</sub>, respectively, by the R1046D and R1131E mutants were moderately reduced (by 30–50%) compared with wild-type MRP1, whereas uptake by the D1084R mutant was substantially reduced by >90%. Similarly, levels of E<sub>17βG</sub> and MTX uptake by the R1046D and R1131E mutants were comparable with or moderately different from wild-type MRP1, whereas uptake of these organic anions by D1084R was substantially diminished (<25% of wild-type MRP1) (Fig. 2, D and E). These data are summarized in Table I, and overall, they indicate that mutation of Arg<sup>1046</sup> and Arg<sup>1131</sup> caused only a moderate effect or no effect on MRP1 transport activities. In contrast, replacement of Asp<sup>1084</sup> with an oppo-

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Fig. 2. Expression levels and transport activities of TM13–15 MRP1 mutants containing substitutions of Arg<sup>1046</sup>, Asp<sup>1084</sup>, and Arg<sup>1131</sup>. A, MRP1 expression in membrane vesicles prepared from HEK293T cells transfected with empty vector [pcDNA3.1] and vector containing wild-type (WT-MRP1) and mutant (R1046D, D1084R, D1084E, and R1131E) cDNAs was determined by immunoblotting with mAb QCRL-1. Shown is a representative immunoblot of membrane vesicles (1 and 2 μg of protein) from a single transfection. The relative expression levels of the mutants were estimated by densitometry and are indicated directly below the blot. Similar values were obtained in 2–3 additional independent transfections. B–F, levels of <sup>3</sup>H-labeled organic anion uptake by the membrane vesicles shown in A were determined and corrected to take into account any differences in MRP1 protein expression (empty pcDNA3.1 vector control [open bars], wild-type MRP1 [black bars], and mutants R1046D, D1084R, D1084E, and R1131E [gray bars])

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To measure 8-azido-ATP labeling of MRP1, membrane vesicles (20 μg of protein) were incubated for 10 min at 37°C in a transport buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose) containing 5 mM MgCl<sub>2</sub> and 1 μM 8-azido-[α-<sup>32</sup>P]ATP (31, 32). After incubation on ice for 5 min, the samples were exposed to UV light at 302 nm on ice for 5 min. The reactions were stopped by the addition of ice-cold Tris-EGTA buffer, and the membrane proteins were collected by centrifugation and then subjected to SDS-PAGE and exposed to film as before. To measure orthovanadate-induced trapping of 8-azido-[α-<sup>32</sup>P]ADP by MRP1, membrane proteins (20 μg) were incubated in transport buffer (20 μl) containing 5 mM MgCl<sub>2</sub>, 1 mM sodium orthovanadate, and 1 μM 8-azido-[α-<sup>32</sup>P]ATP at 37°C for 15 min (31, 32). Membrane proteins were then collected by centrifugation, UV cross-linked, resolved by SDS-PAGE, and exposed to film as before. To determine whether orthovanadate-induced trapping of ADP influences photolabeling of MRP1 by LTC<sub>4</sub>, membrane proteins (50 μg) were incubated with 5 mM MgCl<sub>2</sub> in the presence or absence of 5 μM ATP and 1 μM orthovanadate for 20 min at room temperature followed by addition of PHILTC<sub>4</sub> (200 nM, 110 nCi) and PHE<sub>2</sub> for a further incubation for 30 min (20). Samples were UV cross-linked, subjected to SDS-PAGE, and exposed to film as before.

RESULTS

Expression and Transport Properties of MRP1 Mutants Containing Opposite and Same Charge Substitutions of Arg<sup>1046</sup>, Asp<sup>1084</sup>, and Arg<sup>1131</sup> in or Near TM13–15—In the first series of experiments, the importance of ionizable amino acids located in or near predicted TM13–15 for MRP1 protein expression and function were investigated by replacing Arg<sup>1046</sup> and Arg<sup>1131</sup> with Asp and Glu, respectively. Levels of expression of the R1046D and R1131E mutant proteins were determined by the immunoblotting of membrane vesicles prepared from the transfected HEK293T cells with mAb QCRL-1. The TM14-associated D1084R mutant described previously (27) was included for comparison. A single 190-kDa band was detected in both the wild-type and mutant MRP1 preparations, whereas no immunoreactive bands were detected in membrane vesicles prepared from control transfected cells (Fig. 2A). Densitometry was performed on immunoblots of vesicle protein from three independent transfections, and the relative expression levels of the mutant MRP1 proteins were determined to be comparable (70–130%) to wild-type MRP1 levels, indicating that these mutations did not substantially affect the biogenesis of MRP1.

To determine the functional consequences of replacing Arg<sup>1046</sup>, Asp<sup>1084</sup>, and Arg<sup>1131</sup> with an oppositely charged amino acid, the ability of the R1046D, D1084R, and R1131E mutants to transport different organic anions was tested. Fig. 2, B and C, shows that uptake levels of E<sub>17βG</sub> and LTC<sub>4</sub>, respectively, by the R1046D and R1131E mutants were moderately reduced (by 30–50%) compared with wild-type MRP1, whereas uptake by the D1084R mutant was substantially reduced by >90%. Similarly, levels of E<sub>17βG</sub> and MTX uptake by the R1046D and R1131E mutants were comparable with or moderately different from wild-type MRP1, whereas uptake of these organic anions by D1084R was substantially diminished (<25% of wild-type MRP1) (Fig. 2, D and E). These data are summarized in Table I, and overall, they indicate that mutation of Arg<sup>1046</sup> and Arg<sup>1131</sup> caused only a moderate effect or no effect on MRP1 transport activities. In contrast, replacement of Asp<sup>1084</sup> with an oppo-
gmentation with Asp1084 mutants provided there is an acidic side chain are critically important for MRP1-mediated LTC4 and E217G transport by MRP1. In the present study, we found that these nonconservative substitutions also resulted in a substantial loss (＞70%) of E1SO4 and MTX transport as well (results not shown).

Because the overall transport activity of the oppositely charged Asp1084 mutant was greatly diminished (Fig. 2, B–E), a like-charge substituted D1084E mutant was created to clarify whether it was the loss of the acidic character or a change in size of the amino acid side chain that was responsible for the diminished activity. Like the D1084R mutant, the D1084E mutant showed substantially reduced LTC4 uptake levels (＜20% of wild-type MRP1) (27) (Fig. 2C). In contrast to D1084R, however, the D1084E mutant exhibited E17βG (Fig. 2B), E1SO4 (Fig. 2D), and MTX (Fig. 2E) uptake levels that were similar or only moderately reduced compared with those of wild-type MRP1. Because of the apparent selectively greater loss of LTC4 transport activity by the D1084E mutant, GSH uptake by this mutant was also examined and compared with that of D1084R. As shown in Fig. 2F, apigenin-stimulated GSH uptake by the D1084R and D1084E mutants was reduced by ＞90 and 80%, respectively. These data are summarized in Table I, and overall, our results indicate that both the acidic character and the size of the Asp1084 side chain are critically important for MRP1-mediated LTC4 and GSH transport activities. On the other hand, moderate to wild-type E17βG, E1SO4, and MTX transport activity can be supported by Asp1084 mutants provided there is an acidic side chain at position 1084.

Selective Loss of MRP1 Expression in Mutants Containing Opposite Charge Substitutions of Arg1197, Arg1202, Glu1204, and Arg1249 in or Near TM13–17—In the next series of experiments, the importance of ionizable amino acids located in or near predicted TM16 and TM17 for MRP1 protein expression was investigated by initially replacing Arg1197, Arg1202, Glu1204, and Arg1249 with oppositely charged residues, and levels of expression were determined by immunoblotting of membrane vesicle proteins as before (Fig. 3A). The relative mean expression levels of the R1197E and R1249D mutants from 2 to 3 independent transfections were comparable with wild-type MRP1 (110 and 70%, respectively). In contrast, expression levels of the R1202D and E1204K mutants were substantially reduced (by ＞75%). A Northern blot analysis performed on total RNA isolated from the transfected cells indicated that the R1202D and E1204K mRNA levels were comparable with wild-type MRP1 mRNA levels, thus excluding the possibility that the low R1202D and E1204K protein levels could be caused by differences in transfection efficiency (results not shown).

MRP1 Expression Is Re-established in Mutants Containing Neutral and Same-Charge Substitutions of TM16 Arg1202 and Glu1204—The role of Arg1202 and Glu1204 in the stable expres-

### Table I

| Mutation | E17βG | LTC4 | E1SO4 | MTX | GSH |
|----------|-------|------|-------|-----|-----|
| TM13     | R1046D| 115  | 70    | 80  | 120 |
| TM14     | D1084R| ＜10  | ＜10   | ＜15 | ＜10 |
| TM15     | R1131E| 70   | 50    | 80  | 60  |
| TM16     | R1197E| ＜10  | ＜10   | ＜15 | ＜10 |
| TM17     | R1202G| 115  | 115   | 75  | 70  |

*The values shown are means of duplicate or triplicate determinations and are derived from Fig. 2, 4, and 5 (see figure legends for details). For clarity, numbers have been rounded to the nearest 5%, and S.D. values (which were typically ＜10%) were omitted.

*ND, not done.

![Fig. 3](http://example.com/figure3.png)
sion of MRP1 in HEK cell membranes was further explored by replacing Arg^{1202} with the hydrophobic Leu, and Glu^{1204} with Leu and Asp. Arg^{1202} is highly conserved in MRP1 orthologs and homologs except MRP3 (Table II). Consequently, Arg^{1202} was also mutated to Gly because this latter amino acid is present in the corresponding position in MRP3. Immunoblots showed that expression levels of the R1202G and R1202L mutants (Fig. 3B) and the E1204L and E1204D mutants (Fig. 3C) ranged from 80 to 225% of wild-type MRP1. These results indicate that unlike substitutions with oppositely charged residues, neutral and same-charge substitutions of Arg^{1202} and Glu^{1204} did not adversely affect MRP1 biogenesis.

**Transport Activities of the Expressed Arg^{1202} and Glu^{1204} Mutants**—In the next series of experiments, the organic anion transport activities of the neutrally substituted, expressed mutants of Arg^{1202} (Fig. 4, A–D) and Glu^{1204} (Fig. 4, E–I) were assessed. At 1 min, E_{17}βG and LTC_{4} uptake by the R1202G and R1202L mutants was comparable with wild-type levels (Fig. 4, A and B). Levels of E_{17}SO_{4} uptake by the R1202G mutant were reduced by ~25%, whereas uptake by the R1202L mutant was reduced by ~50% (Fig. 4C). Uptake levels of MTX by the R1202G mutant was moderately reduced (by ~30%), whereas uptake by R1202L was comparable with wild-type MRP1 (Fig. 4D). In contrast to the neutrally substituted Arg^{1202} mutants, E_{17}βG uptake by the neutrally substituted Glu^{1204} mutant E1204L was <10% of wild-type MRP1 levels (Fig. 4E). In addition, LTC_{4} uptake by E1204L was reduced by 50% (Fig. 4F), and E_{17}SO_{4} uptake was reduced by 90% (Fig. 4G). On the other hand, MTX uptake was comparable with wild-type MRP1 (Fig. 4H). These results are summarized in Table I.

To determine whether the substrate-selective loss of transport function observed in the E1204L mutant was because of the loss of the acidic character or the change in the size of the Arg1197 and Arg1249 side chains is important for overall, our data indicate that neither the basic character nor the size of the Arg^{1197} and Arg^{1249} side chains is important for MRP1 expression, but both are critical for organic anion transport activity.

**Effect of Glu^{1204}, Arg^{1197}, and Arg^{1249} Mutations on Photolabeling with [^{3}H]LTC_{4} and 8-Azido-[α-^{32}P]ATP**—In the next series of experiments, those same-charge or neutrally substituted mutants that showed substantially reduced transport activities (R1197K, E1204L, and R1249K) were further examined to determine whether their loss of transport activity was accompanied by a decrease in substrate binding. As shown in Fig. 6A, [^{3}H]LTC_{4} photolabeling of the R1197K and R1249K mutants was 20 and ~10% of wild-type MRP1, respectively (Fig. 5A). Similarly, uptake levels of LTC_{4} (Fig. 5B), E_{17}SO_{4} (Fig. 5C), and MTX (Fig. 5D) by the R1197K and R1249K mutants were reduced to ~10% of wild-type MRP1 uptake levels. These results are summarized in Table I, and overall, our data indicate that neither the basic character nor the size of the Arg^{1197} and Arg^{1249} side chains is important for MRP1 expression, but both are critical for organic anion transport activity.

**Transport Activities of TM16/17 Arg^{1197} and Arg^{1249} Mutants**—Unlike the R1202D and E1204K mutants, oppositely charged substitutions of Arg^{1197} (R1197E) and Arg^{1249} (R1249D) did not adversely affect expression of MRP1 (Fig. 3A). Nevertheless, these mutants displayed a global and substantial loss of transport activity (Fig. 5, Table I). Thus, levels of E_{17}SO_{4} uptake by both mutants were <10% of wild-type MRP1 levels (Fig. 5A), whereas LTC_{4} uptake by the R1197E and R1249D mutants was <15% of wild-type MRP1 (Fig. 5B). Uptake of E_{17}SO_{4} by the R1197E mutant was <15% of wild-type MRP1, whereas uptake of this sulfated estrogen by the R1249D mutant was reduced by >90% (Fig. 5C). Finally, the R1197E and R1249D mutants showed MTX transport activity that was not significantly different from the empty vector control (Fig. 5D).

To determine whether the charge or size of the Arg^{1197} and Arg^{1249} side chains were important for MRP1 transport function, the like-charge substituted mutants R1197K and R1249K were created. Expression levels of these mutants were comparable or somewhat greater than wild-type MRP1 (results not shown). Nevertheless, ATP-dependent E_{17}βG uptake by the R1197K and R1249K mutants was 20 and ~10% of wild-type MRP1, respectively (Fig. 5A). Similarly, uptake levels of LTC_{4} (Fig. 5B), E_{17}SO_{4} (Fig. 5C), and MTX (Fig. 5D) by the R1197K and R1249K mutants were reduced to <10% of wild-type MRP1 uptake levels. These results are summarized in Table I, and overall, our data indicate that neither the basic character nor the size of the Arg^{1197} and Arg^{1249} side chains is important for MRP1 expression, but both are critical for organic anion transport activity.
In the present study, we have extended our previous investigations on the importance of ionizable residues for the expression and activity of MRP1 by examining the consequences of mutating Arg1046, Arg1131, Arg1197, Arg1202, Glu1204, and Arg1249. These residues are predicted by hydropathy analyses (33) to be located in or proximal to the cytoplasmic interface of the TM helices of MSD3 (Fig. 1), and all six of them (or at least their charge) are quite highly conserved among mammalian ABC C family members with the exception of Arg1131 (Table II). Substitutions of two of the residues (TM16 Arg1197 and TM17 Arg1249) with oppositely charged residues had no substantial reduction or only a moderate reduction in transport activity.

Furthermore, orthovanadate-induced trapping of 8-azido-[α-32P]ADP of the R1197K and R1249K mutants was also comparable with wild-type MRP1 (Fig. 6B). However, vanadate-induced trapping of 8-azido-[α-32P]ADP was substantially increased (~4-fold), specifically in the COOH-proximal NBD2 (Fig. 6C) (27). E1204L could still be photolabeled with LTC4 despite substantially reduced transport of this organic anion, [3H]LTC4 labeling of the E1204L mutant was also comparable with wild-type MRP1 (Fig. 6C). However, vanadate-induced trapping of 8-azido-ADP by E1204L altered the substrate binding properties of MRP1. As shown in Fig. 6D, [3H]LTC4 labeling of wild-type MRP1 was abolished in the presence of ATP alone and in the presence of ATP and vanadate together, as expected (27). A similar decrease in [3H]LTC4 labeling of the E1204L mutant was observed under the same conditions.

DISCUSSION

In the present study, we have extended our previous investigations on the importance of ionizable residues for the expression and activity of MRP1 by examining the consequences of mutating Arg1046, Arg1131, Arg1197, Arg1202, Glu1204, and Arg1249. These residues are predicted by hydropathy analyses (33) to be located in or proximal to the cytoplasmic interface of the TM helices of MSD3 (Fig. 1), and all six of them (or at least their charge) are quite highly conserved among mammalian ABC C family members with the exception of Arg1131 (Table II). Substitutions of two of the residues (TM13 Arg1046 and TM15 Arg1131) with oppositely charged residues had no substantial effect on MRP1 protein expression levels and caused either no reduction or only a moderate reduction in transport activity.
were incubated with [3H]LTC4 (200 nM; 250 nCi) followed by UV cross-linking, SDS-PAGE, and fluorography. The relative levels of [3H]LTC4 in the text.

We reported previously (27) that nonconservative substitutions of the four MSD3 residues, viz. Arg1197, Arg1202, Glu1204, and Arg1249, like the Asp1084 described previously (27), revealed them to be critically important for MRP1 expression and/or function (Fig. 7A).

We reported previously (27) that nonconservative substitutions of Asp1084 proximal to TM14 caused a substantial reduction in E217βG, LTC4, and GSH transport and drug resistance, and we have now shown that these mutations also reduce MTX and E1204 transport activity, demonstrating a global disruption of MRP1 activity. We have also found that the same-charge mutant, D1084E, has significant transport activity with respect to E217βG, MTX, and E1204 whereas transport of GSH and the glutathione conjugate LTC4 remains quite low. Thus both the acidic character and smaller volume of the Asp1084 side chain (compared with Glu) appear to be particularly crucial for GSH and LTC4 transport, whereas only a negative charge at this position is important for transport of other organic anions. However, neither physical property of Asp1084 appears necessary for substrate binding, because GSH is still able to stimulate E217βG transport indicating that GSH binding to D1084E is intact. Similarly, D1084E and D1084R can still be photolabeled with LTC4 as well as wild-type MRP1. The altered transport properties of the Asp1084 mutants have been attributed to their inability to convert from a high to low affinity binding state (27).

The highly conserved Arg1202 and Glu1204 residues in TM16 are predicted to be well embedded in the membrane bilayer, which is usually considered to be an energetically unfavorable environment for ionizable amino acids (34). Mutants containing opposite charge substitutions of either of these residues were very poorly expressed, which suggests that the introduction of an opposite charge at either position 1202 or 1204 impairs insertion of MRP1 into the membrane bilayer or causes aberrant TM helix packing and misfolding, which presumably then targets the mutant protein for degradation. This idea is supported by the observation that membrane expression of the R1202D and E1204K mutants in mammalian cells can be substantially increased when transfected cells are grown at lower temperatures (30 °C) where the stringency of the proofreading machinery for monitoring protein folding is diminished.2 Because neutral (Leu, Gly) substitutions of TM16 Arg1202 and Glu1204 did not affect MRP1 expression in any significant way, it may be concluded that it is the opposite charge of the side chain of the substituting amino acids on the nonexpress-
ing R1202D and E1204K mutants that is key to MRP1 protein destabilization. In this regard, it is worth noting that replacing Arg1202 with an Asp (or Glu1204 with a Lys) results in a potential net gain of two charges in the cytoplasmic half of TM16 as well as placing two same-charge ionizable residues in relatively close proximity to one another which could well perturb the helical geometry of TM16 and contribute to misfolding or aberrant TM helix-packing.

In addition to being readily expressed, the neutrally substituted mutants of TM16 Arg1202 (R1202G and R1202L) exhibited transport activities that were, in the case of most substrates, similar to those of wild-type MRP1. Our findings are consistent with a recent report that a neutrally substituted Arg1202 mutant (R1202G) could still transport LTC4 (28). Most interestingly, when the analogous residue in human MRP2 (Arg1210) was mutated to Ala, the ability of MRP2 to mediate cellular efflux of a fluorescent GSH conjugate was substantially reduced (35). Whether or not this reflects a different role for this TM16 Arg residue in GSH conjugate binding and transport by MRP1 and MRP2 remains to be determined.

Unlike the neutrally substituted Arg1202 mutants, transport of organic anions by the neutrally substituted Glu1204 mutant E1204L was substantially reduced or eliminated with the exception of MTX. Nevertheless, the substrate (LTC4)-binding site of E1204L remained intact. Furthermore, GSH transport remained very low, although other MRP1 transport activities of the same-charge E1204D mutant were comparable with wild-type MRP1. Thus, on the one hand, MRP1 can accommodate significant changes (except an opposite charge) at position 1202 and still retain some of its transport activities. On the other hand, both the acidic character and the volume of Glu1204 need to be preserved for full MRP1 function. These findings suggest that the TM16 Glu1204 side chain is critical for establishing interhelical hydrogen bonding or ion pair interactions that are important for substrate transport as well as stable membrane expression of the MRP1 protein.

Previous studies have indicated that TM16 may form part of a drug-binding site on MRP1 (18, 20). Our present data indicate that Glu1204 is not an essential component of this site but instead is more likely to be involved in a step of the transport process subsequent to initial substrate binding. This was shown to be the case when the nucleotide interactions of the transport-compromised E1204L mutant were examined. Previous studies have shown that the NBDs of MRP1, unlike P-glycoprotein, do not contribute equally to the activity of the

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**Fig. 7. Predicted secondary structure and an atomic three-dimensional surface model of the core membrane spanning regions of MRP1 (MSD2 and MSD3) based on molecular dynamics simulations.** A, the schematic diagram of MSD3 shows the approximate boundaries of TM12 to TM17 based on an atomic model derived using the crystal structure of the *V. cholerae* lipid transporter MsbA as template (39). The location of the ionizable residues in or proximal to the cytoplasmic face of MSD3 shown in this study and shown previously (27) to be important for MRP1 expression and/or function is indicated. B, three-dimensional surface representations of the atomic model of the core membrane spanning regions of MRP1 (MSD2-TM6–11 (magenta flat ribbon); MSD3-TM12–17 (deep purple flat ribbon)) (39) showing a side view (left) and a view onto the cytoplasmic face (right). The surface (translucent grey) was generated using a probe radius of 1.40 Å (ViewerPro 4.2 software, www.accelrys.com) rolling over the TM residue side chains. Left, the distance between the α-carbons of the amino acids predicted to be at the ends of the TMs is ~35 Å, consistent with the thickness of the hydrophobic core (30–40 Å) of the lipid bilayer of a mammalian cell plasma membrane. Right, the cytoplasmic view shows how the mutation-sensitive TM16 Arg1202 (orange), TM16 Glu1204 (yellow), and TM17 Arg1249 (green) residues could all face into the substrate translocation pathway of MRP1.

The view shown, the cytoplasmic domains and parts of the TM helices in the outer leaflet of the lipid bilayer have been “hidden” to enhance the visibility of the mutation-sensitive ionizable residues.
transporter (36, 37). Thus, inactivation of NBD2 abolishes transport by MRP1, but inactivation of NBD1 results in only a partial loss of activity. Our demonstration that vanadate-induced trapping of azido-ADP by the mutant E1204L protein (and specifically by NBD2) was substantially increased suggests that the mutation may impair the ability of NBD2 to release ADP after hydrolysis of ATP, which could in turn impair substrate translocation and/or release. Alternatively, the E1204L mutant may hydrolyze ATP and release ADP very rapidly in the absence of vanadate but be unable to proceed through a second catalytic cycle (36). In effect, the mutation may diminish the coupling of the catalytic activity of MRP1 to transport in a way that affects some substrates more than others. Further studies are underway to distinguish among these possibilities. As mentioned previously, Glu\textsuperscript{1204} is located well within the membrane bilayer in the putative substrate translocation pathway of MRP1. Thus, the altered catalytic activity and impaired transport of the E1204L mutant suggests that Glu\textsuperscript{1204} (or at least the region in which it resides) could play a role in the signaling between the substrate translocation pathway and NBD2.

Unlike Arg\textsuperscript{1202} and Glu\textsuperscript{1204}, substitution of Arg\textsuperscript{1197} and Arg\textsuperscript{1249} with oppositely charged residues did not adversely affect expression of MRP1 but instead caused a substantial reduction in transport activity. Our observations with respect to the R1249D mutant are consistent with those of Ren et al. (19) who reported that Ala substitution of Arg\textsuperscript{1249} impaired MRP1-mediated LTC\textsubscript{4} transport and reduced vincristine resistance. However, we also found that the same-charge mutants of Arg\textsuperscript{1197} and Arg\textsuperscript{1249} were essentially transport-inactive and furthermore could no longer be photolabeled with LTC\textsubscript{4}. These findings were unexpected because same-charge mutations of other basic and acidic residues in MSD2 and MSD3 have generally been reported to retain substrate binding and transport activities of the protein (24, 27). The lack of LTC\textsubscript{4} labeling of the R1197K and R1249K mutants indicates that their binding site for LTC\textsubscript{4} (and likely other organic anions) has been disrupted. Thus, Arg\textsuperscript{1197} and Arg\textsuperscript{1249}, which are predicted to be at the membrane-cytosol interface of TM16 and TM17, respectively, act as more than just topological determinants and may well be critical for maintaining the architecture of the substrate binding site(s) of MRP1 (38). The bulkier, less ionizable Lys side chains in the R1197K and R1249K mutants presumably either cannot form or are prevented from forming the interhelical and/or intrahelical interactions that are established by Arg in wild-type MRP1 for proper folding into a functional transporter. However, our data show that disruption of these interactions does not destabilize MRP1 to the point where its expression in the membrane is impaired, nor does it appear to affect the catalytic activity of the transporter.

The precise arrangement and boundaries of the 17 TM α-helices of MRP1 are not yet known, although several models have been proposed. We have recently derived an atomic model of MSD2 and MSD3 places three additional basic residues (Arg\textsuperscript{1138}, Lys\textsuperscript{1141} and Arg\textsuperscript{1142}) close to the NH\textsubscript{2}-proximal end of the cytoplasmic loop (CL7) near the cytosol-membrane interface of TM15 (39) (Fig. 7a). In addition, we have noted that CL7, which is extensively α-helical, contains several clusters of ionizable amino acids. Initial characterization of MRP1 mutants containing substitutions of these residues shows that at least some of them are critical for expression and/or organic anion transport. Ongoing studies are aimed at a better understanding of the role that ionizable residues in CL7 and other cytoplasmic loops play in the transporter mechanism and substrate specificity of MRP1.

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Mutational Analysis of Ionizable Residues Proximal to the Cytoplasmic Interface of Membrane Spanning Domain 3 of the Multidrug Resistance Protein, MRP1 (ABCC1): GLUTAMATE 1204 IS IMPORTANT FOR BOTH THE EXPRESSION AND CATALYTIC ACTIVITY OF THE TRANSPORTER
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