Identification of a Nonconserved Amino Acid Residue in Multidrug Resistance Protein 1 Important for Determining Substrate Specificity

EVIDENCE FOR FUNCTIONAL INTERACTION BETWEEN TRANSMEMBRANE HELICES 14 AND 17*

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Murine multidrug resistance protein 1 (mrp1), differs from its human ortholog (MRP1) in that it fails to confer anthracycline resistance and transports the MRP1 substrate, 17β-estradiol 17-β-D-glucuronide (E217βG), very poorly. By mutating variant residues in mrp1 to those present in MRP1, we identified Glu1089 of MRP1 as being critical for anthracycline resistance. However, Glu1089 mutations had no effect on E217βG transport. We have now identified a nonconserved amino acid within the highly conserved COOH-proximal transmembrane helix of MRP1/mrp1 that is important for transport of the conjugated estrogen. Converting Ala1239 in mrp1 to Thr, as in the corresponding position (1242) in MRP1, increased E217βG transport 3-fold. Any mutation of mrp1 Ala1239, including substitution with Thr, decreased resistance to vincristine and VP-16 without altering anthracycline resistance. However, introduction of a second murine to human mutation, Q1086E, which alone selectively increases anthracycline resistance, into murp1A1239T restored resistance to both vincristine and VP-16. To confirm the importance of MRP1 Thr1242 for E217βG transport and drug resistance, we mutated this residue to Ala, Cys, Ser, Leu, and Lys. These mutations decreased E217βG transport 2-fold. Conversion to Asp eliminated transport of the estrogen conjugate and also decreased leukotriene C4 transport ~2-fold. The mutations also reduced the ability of MRP1 to confer resistance to all drugs tested. As with mrp1, introduction of a second mutation based on the murine sequence to create MRP1E1089Q/T1242A restored resistance to vincristine and VP-16, but not anthracyclines, without affecting transport of leukotriene C4 and E217βG. These results demonstrate the important role of Thr1242 for E217βG transport. They also reveal a highly specific functional relationship between nonconserved amino acids in TM helices 14 and 17 of both mrp1 and MRP1 that enables both proteins to confer similar levels of resistance to vincristine and VP-16.

Human multidrug resistance protein 1 (MRP1),1 a 190-kDa glycoprotein, is a member of the ATP-binding cassette (ABC) transporter superfamily (1–3). The predicted structure of MRP1 differs from that of a typical eukaryotic ABC transporter such as P-glycoprotein (P-gp). It contains a P-gp-like core region, which consists of two membrane-spanning domains (MSDs), each containing six transmembrane (TM) α-helices, and two nucleotide binding domains. However, MRP1 contains a third MSD predicted to consist of five TM α-helices with an extracellular NH2 terminus and a cytoplasmic linker connecting the additional MSD with the core region (3–7).

Like P-gp, MRP1 confers resistance to many commonly used, structurally diverse natural product chemotherapeutic agents including anthracyclines, Vinca alkaloids, and epipodophyllotoxins (7–10). However, several lines of evidence suggest that MRP1 and P-gp confer resistance to these drugs by different mechanisms. In vitro studies using P-gp-enriched membrane vesicles or purified reconstituted protein demonstrate that P-gp directly binds and transports its drug substrates in an ATP-dependent manner (11, 12). Under similar conditions, MRP1 will only transport unmodified amphipathic drugs, such as vincristine and daunorubicin, in the presence of GSH in addition to ATP (13–17). In some cases, GSH appears to be co-transported with these compounds (15).

In addition to conferring resistance to natural product drugs, MRP1 actively transports many glutathione-, glucuronide-, and sulfate-conjugated organic anions in a GSH-independent manner (16–20), although recent studies indicate that GSH may significantly enhance transport of some organic anion conjugates (21–23). Some of these conjugated compounds are potential physiological substrates, such as cysteinyl leukotriene C4 (LTC4) and 17β-estradiol 17-β-D-glucuronide (E217βG) (16–18). Knock-out mice lacking mrp1 have an impaired response to a leukotriene-mediated inflammatory stimulus (24). In addition, mrp1 has been shown to regulate dendritic cell migration to lymph nodes by effluxing LTC4 (25). Whether E217βG is a physiological substrate is not yet known, but plasma membrane vesicles enriched in MRP1 directly transport this cholestatic estrogen conjugate with a Km of 1–3 μM (18, 19, 26).

Despite the very broad substrate specificity of MRP1 and the relatively high level of primary structure conservation with mrp1 (87% identity), the murine and human proteins display some striking functional differences (26, 27). Although mrp1...
confers resistance to Vinca alkaloids and epipodophyllotoxins as efficiently as MRP1, cells overexpressing the murine protein do not show any detectable resistance to anthracyclines. In addition, despite the fact that MRP1 and mrp1 transport LTC4 with similar efficiency, the murine protein transports E217G very poorly (26). Studies using hybrid murine/human proteins revealed that the COOH-terminal third of MRP1 contains nonconserved amino acids that contribute to its ability to confer anthracycline resistance and to transport E217G efficiently (28).

In a previous study, we demonstrated that mutating Glu1089 in TM14 of MRP1 to Gln, as present in mrp1, essentially eliminated the ability of the protein to confer anthracycline resistance without affecting transport of E217G (29). However, transport of the estrogen conjugate was increased in a mrp1/MPR1 hybrid containing a relatively highly conserved region of the human protein extending from amino acid 1188 to the COOH terminus (28). In the present study, we examined the consequences of replacing amino acid residues in this region that differ between the human and murine proteins. By doing so, we have identified a Thr residue at position 1242 in predicted TM17 of MRP1 that appears to account for the enhanced E217G transport observed with the hybrid protein. In mrp1, the corresponding amino acid is Ala1239. Reciprocal mutation of these residues in the human and murine proteins decreased or increased E217G transport, respectively. The nonconserved A1239/T1242 mutants fail to confer drug resistance (30). Unexpectedly, reciprocal exchange of the nonconserved Ala1239/Thr1242 residues in mrp1 and MRP1 also decreased the ability of both proteins to confer resistance to vincristine and VP-16. This alteration in specificity was not expected because wild-type mrp1 and MRP1 display no differences in their ability to confer resistance to these drugs (26). However, we also show that a second reciprocal exchange of nonconserved amino acids between the TM17 mutant human and murine proteins, at the previously identified residue in TM14 shown to be critical for anthracycline resistance (29), can in both cases restore the ability to confer resistance to vincristine and VP-16. This observation provides compelling evidence that the two pairs of nonconserved residues, Glu1089 and Thr1242 in MRP1 and Gln1089 and Ala1239 in mrp1, must act in concert to enable the proteins to confer resistance to these, and possibly other, chemotherapeutic drugs.

**Experimental Procedures**

**Materials**—Culture medium and fetal bovine serum were obtained from Life Technologies, Inc. [3H]LTC4 (38 Ci/mmol) was purchased from Amersham Pharmacia Biotech, and [3H]E17G (44 Ci/mmol) was from PerkinElmer Life Sciences. Doxorubicin HCl, etoposide (VP-16), and vincristine sulfate were obtained from Sigma, and epirubicin was from Amersham Pharmacia Biotech, and [3H]E217 was from Roche Molecular Biochemicals. Briefly, HEK293 cells were transfected with pCEBV7-mrp1 or pCEBV7-MRP1, after which the entire mutated inserts and the cloning sites were verified by DNA sequencing.

**Cell Lines and Tissue Culture**—Stable transfection of HEK293 cells with the pCEBV7 vector containing the wild type MRP1 cDNAs or wild type mrp1 cDNAs has been described previously (26, 29). All of the mutant constructs were analyzed as stable cell lines by Western blot analysis with a monoclonal antibody (mAb), MRP1, which cross-reacts with the murine and human proteins (26). The human proteins were detected with mAb QCR1-1 (32). Antibody binding was detected with horseradish peroxidase-conjugated goat anti-rat/anti-immunoglobulin G (Fierce), followed by enhanced chemiluminescence detection and X-Omat film (Kodak) (PerkinElmer Life Sciences). Densitometry on the film images was performed using a Calibrated Image Analyser v2.00 (Alpha Innotech Corporation, San Leandro, CA). The relative protein expression levels were calculated by dividing the integrated densitometry values obtained for 1.25, 2.5, and 5 μg of total membrane protein from transfectants expressing wild type MRP1/mrp1 were analyzed together with comparable amounts of membrane protein from cells expressing various mutant proteins by SDS-polyacrylamide gel electrophoresis (7.5% gel). Proteins were subsequently transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) by electroblotting.
Functional Analysis of Murine mrp1 and Human MRP1 Mutants

**RESULTS**

Transport of [3H]LTC4 and [3H]E17βG by Wild Type and Mutant Murine mrp1—Previous studies demonstrated that the murine/human hybrid protein in which amino acids 1185–1528 of mrp1 were replaced with the corresponding region of MRP1 (mrp1/MPRP1, 1185–1531) transported E17βG more efficiently than wild type murine protein with none detectable changes in the specificity of LTC4 transport (28). To further localize residues in this region responsible for enhancing E17βG transport activity, amino acids 1351–1528 of mrp1 were replaced with the corresponding part of MRP1. In addition, 9 residues between amino acids 1185 and 1350 of mrp1 that differ from the human protein were substituted with the corresponding amino acid(s) from MRP1 by single, double, or triple mutations (Figs. 1A and 2E). Episomal pCEBV7 expression vectors containing mutated forms of full-length mrp1 cDNAs were transfected into HEK293 cells, and populations of transfected cells were selected in hygromycin B. The resulting stably transfected cell populations were cloned by limiting dilution. Populations derived from clones that expressed high levels of mrp1 mutant proteins were used in all subsequent studies. The levels of mutant proteins relative to wild type mrp1 in previously characterized HEK transfectants were determined by immunoblotting and densitometry as described under “Experimental Procedures.”

**Chemosensitivity Testing**—Drug resistance was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (8, 26, 29). Briefly, cells diluted in culture medium were added to cells (100 μl/well) (Sigma) was added to 1 ml of transport buffer containing 4 mM ATP, 10 mM MgCl2, and [3H]LTC4 (50 nM, 25 nCi) for 1 min. ATP-dependent uptake of [3H]LTC4 (400 nM, 120 nCi) uptake was measured as described for [3H]LTC4 except that 20 μg of vesicle protein was used, and the reaction was carried out at 37°C. Kinetic parameters of ATP-dependent [3H]LTC4 uptake by membrane vesicles were prepared as described previously, and the reaction was carried out under the bound radioactivity was determined by scintillation counting. All data were corrected for the amount of [3H]LTC4 that remained bound to the filter in the absence of vesicle protein (usually <5% of the total radioactivity). [3H]LTC4 uptake was expressed relative to the total protein concentration in each reaction. ATP-dependent [3H]E17βG (400 nM, 120 nCi) uptake was measured as described for [3H]LTC4 except that 5 μg of vesicle protein was used, and the reaction was carried out at 37°C. Km and Vmax values of ATP-dependent [3H]LTC4 uptake by membrane vesicles (2.5 μg of protein) were measured at various LTC4 concentrations (0.01 to 2 μM) for 1 min at 23°C in 25 μl of transport buffer containing 4 mM ATP and 10 mM MgCl2 followed by nonlinear regression analyses. Kinetic parameters of ATP-dependent [3H]E17βG (0.1–16 μM) uptake were determined as described for [3H]LTC4, except that 5 μg of vesicle protein was used, and the reaction was carried out at 37°C. Confocal Microscopy—Confocal microscopy was carried out as described previously (29). Briefly, ~5 × 105 stably transfected HEK293 cells were seeded in each well of a six-well tissue culture dish on coverslips. When the cells had grown to confluence, they were washed once in phosphate-buffered saline and then fixed with 2% paraformaldehyde in phosphate-buffered saline, followed by permeabilization using digitonin (0.25 mg/ml in phosphate-buffered saline). MRP1 proteins were detected with the monoclonal antibody 2E6, which reacts with an epitope close to the COOH terminus of MRP1 (amino acids 1511–1528 of mrp1) by single, double, or triple mutations (Figs. 1A and 2E).
ATP-dependent transport of [3H]LTC4 and [3H]E217G was examined using plasma membrane vesicles from transfected HEK cells (Figs. 1 and 2). The levels of LTC4 uptake by vesicles prepared from HEK transfectants expressing wild type and mutant MRP1 were proportional to the relative expression levels of all mutant proteins tested and comparable with that of wild type MRP1 (Figs. 1B and 2A and B). Only two mutations increased the ability of the protein to transport E217G, a double mutation in which Ile1237 and Ala1239 were replaced with Val and Thr (I1237V/A1239T), and a single conversion of Ala1239 to Thr (A1239T) (Figs. 1C and 2C). Substitution of Ala1239 with Thr increased the ability of murine mrp1 to transport E217G ~3-fold, as did the double mutation I1237V/A1239T. However, when A1239 was mutated to Cys (A1239C) and Ser (A1239S), neither of the mutations had any effect on E217G transport activity (Fig. 2D). These results indicated a highly specific requirement for a Thr residue at position 1239 of mrp1 for efficient transport of the conjugated estrogen.

**Functional Analysis of Murine mrp1 and Human MRP1 Mutants**

Based on the findings with the mrp1A1239T mutation, we investigated how specific the requirement was for Thr at position 1239 of the human protein for its ability to transport E217G. Thus, Thr1242 was mutated to Ala, Cys, Ser, Leu, Lys, and Asp. Stably transfected cell populations expressing high levels of MRP1 mutant proteins were isolated as described for mrp1 mutations, and the levels of protein expression were determined by immunoblotting (Fig. 3A). The relative expression levels of wild type and mutant MRP1 proteins in the membrane vesicles were determined by immunoblotting with mAb QCRL-1 as described under “Experimental Procedures.”

**Closed symbols** represent uptake in the presence of 4 mM ATP; **open symbols** represent uptake in the presence of 4 mM AMP. Data shown have not been normalized to compensate for differences in protein expression levels. Transfectants tested were as follows: HEK_MRP1 ( ), HEK_MRP1A1239T (■), HEK_MRP1A1239C (○), HEK_MRP1A1239S (Δ), and HEK_MRP1A1239K (★).

**Mutational Analysis of Thr1242 in Predicted TM17 of Human MRP1**—Based on the findings with the mrp1A1239T mutation, we investigated how specific the requirement was for Thr at position 1242 of the human protein for its ability to transport E217G. Thus, Thr1242 was mutated to Ala, Cys, Ser, Leu, Lys, and Asp. Stably transfected cell populations expressing high levels of MRP1 mutant proteins were isolated as described for mrp1 mutations, and the levels of protein expression were determined by immunoblotting (Fig. 3A). The relative expression levels of wild type and mutant MRP1 proteins in the membrane vesicles were determined by immunoblotting with mAb QCRL-1 as described under “Experimental Procedures.”

**‘Closed symbols’** represent uptake in the presence of 4 mM ATP; **open symbols** represent uptake in the presence of 4 mM AMP. Data shown have not been normalized to compensate for differences in protein expression levels. Transfectants tested were as follows: HEK_MRP1 ( ), HEK_MRP1A1239T (■), HEK_MRP1A1239C (○), HEK_MRP1A1239S (Δ), and HEK_MRP1A1239K (★).
transport. However, conversion of Thr\textsuperscript{1242} to a negatively charged amino acid, Asp, essentially eliminated transport of \textit{E217G} and also decreased \textit{LTC}\textsubscript{4} transport ~2-fold (Figs. 3 and 4). These data support those obtained from studies of \textit{mrp1A1239} mutations and confirm the importance of Thr\textsuperscript{1242} in human MRP1 for efficient transport of \textit{E217G}.

Comparison of the Trafficking of Mutant Human Proteins with Wild Type MRP1 in Transfected HEK293 Cells—To determine whether the decrease in \textit{E217G} transport observed following mutation of Thr\textsuperscript{1242}, particularly the influence of substituting Thr with an acidic amino acid such as Asp, might be attributable to changes in trafficking of the protein, we compared the subcellular localization of wild type and mutant human proteins by confocal microscopy. As shown in Fig. 5, cells expressing mutations \textit{MRP1T1242D}, \textit{MRP1T1242C}, \textit{MRP1T1242A}, and \textit{HEKMRP1T1242A} showed a pattern of strong cell surface expression of wild type MRP1 and \textit{MRP1T1242A}, indicating that the trafficking was unaffected.

Kinetic Parameters of [\textit{3H}]\textit{LTC}\textsubscript{4} and [\textit{3H}]\textit{E217G} Transport—We have shown that interconversion of Ala\textsuperscript{1239} in \textit{mrp1} and Thr\textsuperscript{1242} in MRP1 affected the ability of the proteins to transport \textit{E217G} with no apparent effect on \textit{LTC}\textsubscript{4} transport. To determine the influence of these mutations on transport more precisely, we compared \textit{Km} and \textit{Vmax} values for the wild type murine and human proteins with those of mutant \textit{mrp1A1239T} and \textit{MRP1T1242A} (Fig. 6). For wild type \textit{mrp1} and \textit{mrp1A1239T}, the \textit{Km} values and normalized \textit{Vmax} values for \textit{LTC}\textsubscript{4} were essentially identical (Fig. 6A and Table 1). For \textit{E217G} transport, \textit{Km} values were 2.0 and 1.3 \textmu M for wild type \textit{mrp1} and \textit{mrp1A1239T}, respectively, and the normalized \textit{Vmax} value for \textit{mrp1A1239T} was ~2-fold higher than that for wild type \textit{mrp1} (Table 1).

The kinetic parameters of ATP-dependent \textit{LTC}\textsubscript{4} and \textit{E217G} transport were also examined for the wild type and mutant human proteins (Fig. 6, C and D). For \textit{E217G}, \textit{Km} and normalized \textit{Vmax} values were essentially identical (Table 1). Consistent with the results obtained with the \textit{mrp1} mutant, substitution of Thr\textsuperscript{1242} with Ala in MRP1 decreased the normalized \textit{Vmax} value for the mutant ~2-fold relative to wild type MRP1. Apparent \textit{Km} values for wild type protein and \textit{MRP1T1242A} were 0.5 and 0.7 \textmu M, respectively (Table 1). Thus, the \textit{mrp1A1239T} and \textit{MRP1T1242A} mutations increased or decreased the \textit{Vmax/\textit{Km}} ratio for \textit{E217G} 3-fold, respectively (Table 1).

Effect of Mutation \textit{mrp1A1239T} and \textit{MRP1T1242A} on the Inhibition of \textit{mrp1}/MRP1-mediated \textit{LTC}\textsubscript{4} Transport by \textit{E217G}—As an alternative means of assessing the effects of the TM17 mutations on the interaction between \textit{E217G} and the human and murine proteins, we examined the ability of the conjugated estrogen to inhibit transport of \textit{LTC}\textsubscript{4}. IC\textsubscript{50} values for wild type and mutant murine and human proteins were obtained from the best fit of the inhibition data to a sigmoidal curve (Fig. 7). For the wild type murine protein, the IC\textsubscript{50} value for \textit{E217G} was 127 \textmu M compared with 27 \textmu M for mutation \textit{mrp1A1239T}. In \textit{MRP1}, substitution of Thr\textsuperscript{1242} with Ala increased the IC\textsubscript{50} value from 15 to 194 \textmu M. These results are independent of protein expression levels and provide strong evidence that the increase or decrease in \textit{E217G} transport by \textit{mrp1A1239T} and \textit{MRP1T1242A}, respectively, is at least partially attributable to changes in the affinity of the proteins for this substrate.

Resistance Profiles of Wild Type and TM17 Mutant Murine/ Human Proteins—In addition to the differences in \textit{E217G} transport activity, the hybrid protein \textit{mrp1}/MRP1 (1188–1531) has been shown to increase the resistance to anthracyclines ~2-fold relative to wild type \textit{mrp1} (28). Consequently, we ex-
MRP1 on Transport Activity and Drug Resistance Profiles

The kinetic parameters of LTC₄ and E₁7βG uptake by vesicles from HEK cells transfected with vectors encoding wild type and mutant proteins were determined as described in the legend to Fig. 6. The normalized Vₘₐₓ values were obtained by adjusting determined Vₘₐₓ values to compensate for differences in the relative levels of the wild type and mutant proteins.

| Transfectant | Kₑₘ | Vₘₐₓ | Normalized Vₘₐₓ | Vₘₐₓ/Kₑₘ (E₁7βG) |
|--------------|-----|-------|----------------|------------------|
| HEK₉₁⁰⁺     | 132 | 2.0   | 187            | 157              |
| HEK₉₁⁰⁺₁₂₃⁹⁸ | 149 | 1.3   | 153            | 158              |
| HEKMRP₁      | 116 | 0.5   | 213            | 213              |
| HEKMRP₁₂₄₂₂ | 114 | 0.7   | 255            | 190              |

**TABLE I** Kinetic parameters of LTC₄ and E₁7βG uptake by vesicles from HEK cells transfected with vectors encoding wild type and mutant proteins

**FIG. 7. Effect of E₁7βG on ATP-dependent LTC₄ transport by wild type or mutant mrp1/MRP1.** The rates of [³H]LTC₄ uptake by vesicles were determined as described in the legend to Fig. 1 except that the reaction was carried out at a fixed LTC₄ concentration (50 nM) in the presence of various concentrations of E₁7βG (0.5–160 μM). Values shown are means ± S.D. of triplicate determinations in a single experiment. The transfectants tested were HEK₁₁⁰⁺ (○) and HEK₉₁⁰⁺₁₂₃⁹⁸ (●) (A) and HEKMRP₁ (▼) and HEKMRP₁₂₄₂₂ (●) (B). IC₅₀ values for the inhibition of [³H]LTC₄ uptake by E₁7βG were obtained from the best fit of the data to a sigmoidal curve. Details of IC₅₀ values are provided under “Results.”

We had also found previously that substitution of Glu¹⁰⁸⁹ in MRP1 with Gln essentially eliminated resistance to anthracyclines without affecting either LTC₄ or E₁7βG transport. However, unlike the reciprocal mutation in mrp1, which had no detectable effect on vincristine and VP-16 resistance, replacement of Glu¹⁰⁸⁹ with uncharged amino acids decreased the capacity of the protein to mediate vincristine and VP-16 resistance by ~60 and ~40%, respectively (29). Consequently, a double mutation of MRP1 was also made, in which Glu¹⁰⁸⁹ was replaced with Gin and Thr¹²⁴² was substituted with Ala (E1089Q/T1242A). Stably transfected cell populations expressing high levels of the mutant protein were isolated, and the levels of protein expression were determined by immunoblotting (Fig. 8B). As in the case of mrp1, the second mutation in TM14 of MRP1 had no additional effect on transport of either LTC₄ or E₁7βG over and above that observed with the single TM17 mutation (Fig. 8, E and F). Trafficking of the double mutation was also comparable with that of wild type MRP1 (Fig. 5).

In contrast to the lack of effect of the second reciprocal mutation in TM14 on transport of E₁7βG by either mrp1A₁₂₃⁹⁸ or MRP1T₁₂₄₂₂, introduction of the TM14 mutations into both proteins markedly affected their ability to confer drug resistance. Mutation mrpQ₁₀₈₉E/A₁₂₃⁹⁸ increased the resistance of the mutant protein mrp1A₁₂₃⁹⁸ to confer resistance to both vincristine and VP-16 (Table II). After normalizing for differences in expression levels, the double mutation increased resistance to doxorubicin and epirubicin 5-fold (Table II), as observed previously with the single mutation mrpQ₁₀₈₉E (29). Similarly, introduction of the E1089Q mutation into MRP1T₁₂₄₂₂ increased resistance to vincristine and VP-16 despite the fact that the single TM14 mutation in the human protein has been shown to decrease resistance to both of these drugs (29). However, the E1089Q mutation eliminated the ability of MRP1T₁₂₄₂₂ to confer resistance to anthracyclines (Table III), as observed previously when this amino acid substitution was introduced into wild type MRP1 as a single mutation (29). These results indicate a highly specific requirement for the combination of Gln¹⁰⁸⁹ and Ala¹₂₃⁹ in mrp1 and Glu¹⁰⁸⁹ and Thr¹²⁴² in MRP1 for the ability of both proteins to confer resistance to vincristine and VP-16.

amined the drug resistance profiles of transfected expressing mrap1 mutations A₁₂₃⁹⁸, A₁₂₃⁹₈, and A₁₂₃⁹. The results are summarized as relative resistance factors in Table II. None of the mutations increased resistance to either doxorubicin or epirubicin. However, they all decreased the ability of the murine protein to confer vincristine resistance by 6–7-fold and reduced the resistance to VP-16 by 40–45% (Table II).

When comparable studies were carried out with the human mutant proteins, MRP1T₁₂₄₂₂, MRP1T₁₂₄₂₃, MRP1T₁₂₄₂₅, MRP1T₁₂₄₁₂₄₂₂, and MRP1T₁₂₄₂₂, we found that these mutations also decreased the ability of the protein to confer vincristine and VP-16 resistance by ~40% and reduced the ability of MRP1 to confer resistance to doxorubicin or epirubicin by 2–3-fold (Table III). Thus, in both the human and murine proteins, Thr¹²⁴² and Ala¹₂₃⁹, respectively, are involved in conferring resistance to vincristine and VP-16 and, in MRP1, also resistance to anthracyclines.

**Effect of Double Mutations in TM14 and TM17 of mrap1 and MRP1 on Transport Activity and Drug Resistance Profiles**—We demonstrated previously that converting Gln¹⁰⁸⁹ in mrap1 to Glu, as it is in mRP1, increased the relative resistance to doxorubicin 4–5-fold. However, this mutation had no influence on the ability of the protein to transport LTC₄ and E₁7βG (29).

Given the observed effect of the mrap1A₁₂₃⁹⁸ and MRP1T₁₂₄₂₂ mutations on drug resistance, including, in the case of MRP1T₁₂₄₂₂, resistance to anthracyclines, we examined whether the mutations in TM helices 14 and 17 had any combined effect on transport or drug specificity. To do so, we made a double mutation in mrap1, in which Gln¹⁰⁸⁹ was replaced with Glu and Ala¹₂₃⁹ was substituted with Thr (Q1086E/A1239T). The double mutant was stably expressed in HEK293 cells, and the transport of both LTC₄ and E₁7βG by membrane vesicles was examined (Fig. 8, A, C, and D). The levels of uptake of the two substrates by vesicles containing either mrap1A₁₂₃⁹⁸ or mrapQ₁₀₈₉E/A₁₂₃⁹T were proportional to the relative expression levels of the proteins (Fig. 8, A, C, and D). Thus, in the murine protein, mutation Q1086E has no significant effect on the ability of mutant mrap1A₁₂₃⁹⁸ to transport either LTC₄ or E₁7βG.

We had also found previously that substitution of Glu¹⁰⁸⁹ in MRP1 with Gln essentially eliminated resistance to anthracyclines without affecting either LTC₄ or E₁7βG transport. However, unlike the reciprocal mutation in mrap1, which had no detectable effect on vincristine and VP-16 resistance, replacement of Glu¹⁰⁸⁹ with uncharged amino acids decreased the capacity of the protein to mediate vincristine and VP-16 resistance by ~60 and ~40%, respectively (29). Consequently, a double mutation of MRP1 was also made, in which Glu¹⁰⁸⁹ was replaced with Gin and Thr¹²⁴² was substituted with Ala (E1089Q/T1242A). Stably transfected cell populations expressing high levels of the mutant protein were isolated, and the levels of protein expression were determined by immunoblotting (Fig. 8B). As in the case of mrap1, the second mutation in TM14 of MRP1 had no additional effect on transport of either LTC₄ or E₁7βG over and above that observed with the single TM17 mutation (Fig. 8, E and F). Trafficking of the double mutation was also comparable with that of wild type MRP1 (Fig. 5).

In contrast to the lack of effect of the second reciprocal mutation in TM14 on transport of E₁7βG by either mrap1A₁₂₃⁹⁸ or MRP1T₁₂₄₂₂, introduction of the TM14 mutations into both proteins markedly affected their ability to confer drug resistance. Mutation mrapQ₁₀₈₉E/A₁₂₃⁹T increased the capacity of the mutant protein mrap1A₁₂₃⁹⁸ to confer resistance to both vincristine and VP-16 (Table II). After normalizing for differences in expression levels, the double mutation increased resistance to doxorubicin and epirubicin 5-fold (Table II), as observed previously with the single mutation mrapQ₁₀₈₉E (29). Similarly, introduction of the E1089Q mutation into MRP1T₁₂₄₂₂ increased resistance to vincristine and VP-16 despite the fact that the single TM14 mutation in the human protein has been shown to decrease resistance to both of these drugs (29). However, the E1089Q mutation eliminated the ability of MRP1T₁₂₄₂₂ to confer resistance to anthracyclines (Table III), as observed previously when this amino acid substitution was introduced into wild type MRP1 as a single mutation (29). These results indicate a highly specific requirement for the combination of Gln¹⁰⁸⁹ and Ala¹₂₃⁹ in mrap1 and Glu¹⁰⁸⁹ and Thr¹²⁴² in MRP1 for the ability of both proteins to confer resistance to vincristine and VP-16.
The resistance of HEK293 cells transfected with expression vectors encoding wild type and mutant mrp1 relative to that of cells transfected with empty vector was determined using a tetrazolium salt-based microtiter plate assay. Data were analyzed as described under "Experimental Procedures." The relative resistance factor was obtained by dividing the IC50 values for wild type/mrp1-transfected cells by the IC50 value for control transfecants. The values shown represent the mean ± S.D. of relative resistance factors determined from 3–6 independent experiments. Resistance factors normalized for differences in the levels of mutant proteins expressed in the transfected populations used are shown in parentheses.

| Transflectant | Vincristine | VP-16 | Doxorubicin | Epirubicin |
|---------------|-------------|-------|-------------|------------|
| HEKmrp1       | 26.2 ± 3.1 (26.2) | 25.4 ± 4.0 (25.4) | <1 | <1 |
| n = 6         | n = 6       | n = 3 | n = 3       | n = 3     |
| HEKmrp1T1242K| 4.6 ± 0.1 (5.1)  | 13.1 ± 1.4 (14.5) | <1 | <1 |
| n = 6         | n = 6       | n = 3 | n = 3       | n = 3     |
| HEKmrp1T1242C| 3.1 ± 0.4 (4.5)  | 9.8 ± 0.9 (14.2) | <1 | <1 |
| n = 5         | n = 5       | n = 3 | n = 3       | n = 3     |
| HEKmrp1T1242S| 4.2 ± 0.2 (6.0)  | 11.7 ± 1.3 (16.7) | <1 | <1 |
| n = 6         | n = 3       | n = 3 | n = 3       | n = 3     |
| HEKmrp1Q1086E| 18.6 ± 7.0 (34.6) | 18.7 ± 4.0 (35.7) | 2.6 ± 0.2 (5.0) | 2.7 ± 0.3 (5.2) |
| n = 6         | n = 6       | n = 3 | n = 3       | n = 3     |

**Table II**

Relative drug resistance of HEK293 cells expressing wild type and mutant mrp1

The resistance of HEK293 cells transfected with expression vectors encoding wild type and mutant MRP1 relative to that of cells transfected with empty vector was determined as described in Table II. The values shown represent the mean ± S.D. of relative resistance factors determined from 3–6 independent experiments. Resistance factors normalized for differences in the levels of mutant proteins expressed in the transfected populations used are shown in parentheses.

| Transflectant | Drug (relative resistance factor) |
|---------------|----------------------------------|
|               | Vincristine | VP-16 | Doxorubicin | Epirubicin |
| HEKmrp1       | 18.6 ± 3.1 (18.6) | 18.9 ± 2.0 (18.9) | 6.7 ± 0.9 (6.7) | 9.3 ± 0.4 (9.3) |
| n = 6         | n = 6       | n = 6 | n = 6       | n = 6     |
| HEKmrp1T1242A| 14.5 ± 0.9 (10.8) | 13.6 ± 1.2 (10.1) | 2.6 ± 0.2 (2.0) | 3.2 ± 0.3 (2.4) |
| n = 5         | n = 5       | n = 5 | n = 5       | n = 5     |
| HEKmrp1T1242C| 9.7 ± 0.3 (9.7)  | 10.7 ± 0.3 (10.7) | 3.1 ± 0.2 (3.1) | 3.3 ± 0.3 (3.3) |
| n = 3         | n = 3       | n = 3 | n = 3       | n = 3     |
| HEKmrp1T1242S| 9.1 ± 0.5 (11.4) | 9.6 ± 0.7 (11.9) | 2.5 ± 0.5 (3.1) | 2.6 ± 0.4 (3.2) |
| n = 3         | n = 3       | n = 3 | n = 3       | n = 3     |
| HEKmrp1T1242L| 10.7 ± 3.3 (11.9) | 10.4 ± 1.2 (10.7) | 2.8 ± 0.9 (2.9) | 2.9 ± 3.9 (3.0) |
| n = 3         | n = 3       | n = 3 | n = 3       | n = 3     |
| HEKmrp1T1242D| 6.5 ± 1.2 (9.3)  | 7.0 ± 0.5 (10.0) | 2.3 ± 0.2 (2.5) | 2.4 ± 0.1 (2.6) |
| n = 3         | n = 3       | n = 3 | n = 3       | n = 3     |
| HEKmrp1T1242K| 5.4 ± 0.3 (11.5) | 4.8 ± 0.3 (12.0) | 1.5 ± 0.2 (3.7) | 2.1 ± 0.5 (4.1) |
| n = 3         | n = 3       | n = 3 | n = 3       | n = 3     |
| HEKmrp1T1242G| 18.3 ± 5.3 (26.9) | 17.8 ± 5.1 (26.2) | <1 | <1 |
| n = 5         | n = 5       | n = 3 | n = 3       | n = 3     |

**Table III**

Relative drug resistance of HEK293 cells expressing wild type and mutant MRP1

The resistance of HEK293 cells transfected with expression vectors encoding wild type and mutant MRP1 relative to that of cells transfected with empty vector was determined as described in Table II. The values shown represent the mean ± S.D. of relative resistance factors determined from 3–6 independent experiments. Resistance factors normalized for differences in the levels of mutant proteins expressed in the transfected populations used are shown in parentheses.

**Discussion**

Human MRP1, like P-gp, confers resistance to many hydrophobic natural product chemotherapeutic agents including Vinca alkaloids, epipodophyllotoxins, and anthracyclines (7–10). However, unlike P-gp, MRP1 is able to transport many relatively hydrophilic organic anion conjugates including the two well characterized possible physiological substrates, LTC4 and E2_17 G (26). Previous studies with mrp1/MRP1 hybrids suggested that one of the most highly conserved regions of the two proteins including TM16 and TM17 extending to the COOH terminus contains variant residues important for the transport of this substrate (28). We have now examined the consequences of converting all nonconserved amino acid residues in mrp1 in the region between amino acids 1185 and 1528 to the corresponding amino acid present in MRP1 to identify specific residue(s) involved in the efficient transport of E2_17 G. We have also determined whether the same residue(s) influence the drug resistance profile of the protein.

Replacement of the variant residues in the cytoplasmic COOH-terminal region of mrp1 with those present in the human protein had no significant effect on its ability to transport either LTC4 or E2_17 G (29). Amino acid(s) that selectively affect transport of organic anion conjugates without altering the drug resistance profile conferred by Mrp1 or MRP1 have not been identified. However, we have shown recently that mutation of a highly conserved tryptophan residue at position 1246 in TM17 of MRP1 that eliminates the ability of the protein to confer drug resistance also abolishes E2_17 G transport but leaves LTC4 transport and verapamil-stimulated GSH transport relatively intact (30).

Earlier comparisons of the substrate specificity of mrp1 and MRP1 indicated that the efficiency of LTC4 transport by the two proteins was similar but that mrp1 was a very poor transporter of E2_17 G (26). Previous studies with mrp1/MRP1 hybrids suggested that one of the most highly conserved regions of the two proteins including TM16 and TM17 extending to the COOH terminus contains variant residues important for the transport of this substrate (28). We have now examined the consequences of converting all nonconserved amino acid residues in mrp1 in the region between amino acids 1185 and 1528 to the corresponding amino acid present in MRP1 to identify specific residue(s) involved in the efficient transport of E2_17 G. We have also determined whether the same residue(s) influence the drug resistance profile of the protein.
transport. In contrast, mutation of Ala1239 to Cys, Ser, and Thr decreased the ability of mrp1 to confer vincristine and VP-16 resistance. Since wild type mrp1 does not confer resistance to anthracyclines, the effect of these mutations on resistance to this class of drugs could not be assessed. Replacement of the comparable amino acid residue in MRP1, Thr1242, with Ala, Cys, Ser, Leu, Lys, or Asp also decreased the ability of the protein to confer resistance to vincristine and VP-16 and, in this case, the anthracyclines tested.

The effects of the reciprocal Ala/Thr substitutions on drug resistance of both mrp1 and MRP1 were unexpected, because exchanging MSD3 and the COOH-terminal regions of the two proteins had no effect on resistance to vincristine and VP-16 (28). The consequences of mutating mrp1A1239 and MRP1T1242, with the exception of the T1242D mutation, are essentially the same as those observed following mutation of the highly conserved Trp1242, which abolished drug resistance and E217G transport (30). Together, our results are also consistent with a direct involvement of residues in TM17 in substrate binding, as suggested by recent photoaffinity labeling studies in which cross-linking to a proteolytic fragment containing TM16 and TM17 of MRP1 was observed (34). The topologically comparable transmembrane helix in P-gp, due to the five additional NH2-proximal TMs in MRP1/mrp1, is TM12, which has been demonstrated to play an important role in substrate specificity and drug binding. Triple substitution of the nonconserved residues, Leu975, Val982, and Phe983, with Ala collectively abrogates drug transport, drug-stimulated ATP hydrolysis, and photoaffinity labeling with the drug analogues, [125I]iodoaryladizidoprazosin, while having minimal effect on [α-32P]8-azo-ATP labeling and basal ATPase activity of the protein (35). Use of the thiol-reactive substrate, dibromomane, together with cysteine-scanning mutagenesis has confirmed that hydrophobic residues Leu975, Val982, and Ala985 in TM12 are important for the interaction of substrates with P-gp (36, 37). In addition, mutation F978A in TM12 of Pgp decreases resistance to colchicine and doxorubicin with little effect on resistance to vinblastine or actinomycin D (38).

The mechanism by which various substitutions of the threonine residue at position 1242 in MRP1 and the alanine residue at position 1239 in mrp1 affect E217G transport and drug resistance is presently unclear. It has been proposed that MRP1 binds its substrates via hydrogen bond formation with the electron donor (or hydrogen bond acceptor) groups of the substrate (39). The helical wheel projection for MRP1 TM17 reveals a highly amphipathic character with amino acid residues possessing hydrogen bond donor and acceptor side chains arrayed preferentially on one side of the helix and amino acid residues with non-hydrogen-bonding side chains on the other side (30, 39). However, substitution of Thr1242 with either Cys or Ser, which retain hydrogen bonding capability, decreased the ability of the protein to transport E217G and to confer drug resistance. Their effect was similar to that of the MRP1T1242A mutation, suggesting that the size of the side chain, in addition to hydrogen bonding capability, is also critical for retaining substrate specificity and transport capacity. That the exchange of Ala and Thr affects the affinity of the mouse and human proteins for E217G is supported by the results of experiments in which we examined the ability of the estrogen conjugate to inhibit LTC4 transport. We have shown previously that E217G competitively inhibits LTC4 transport by MRP1 with a K_i of 22 μM (18), which is in reasonable agreement with the IC50 value of 15 μM determined in this study. In contrast, the IC50 for LTC4 transport by MRP1 T1242A was ~130 μM, a value similar to that obtained for wild type mrp1, while the IC50 of mrp1A1239T decreased to ~30 μM.

It has been proposed that the transport of anionic/cationic substrates by MRP1 is facilitated by cationic/anionic acid residues present in the transmembrane helices (39). We have shown previously that an acidic amino acid at position 1089 in TM14 of MRP1 is critical for the ability of the protein to confer resistance to antheracyclines, which are cationic at physiological pH, and that substitution with a positively charged amino acid essentially eliminates drug resistance (29). Similarly, the replacement of basic residues (Lys, Arg) in the predicted transmembrane domains in the related human protein, MR2, with Ala has been reported recently to decrease the ability of the protein to transport an organic anion substrate, glutathione-methylfluorescein (40). It has also been reported recently that the charged amino acids in transmembrane helices of rat mrp2 may play an important role in the recognition and/or transport of its conjugated substrates. Substitution of Lys825 with Met or Arg596 with Leu markedly decreases the ability of rat mrp2 to transport the glutathione conjugates, 2,4-dinitrophenyl-S-glutathione and LTC4 (41). However, we found in this study that mutation of Thr1242 to Lys did not enhance LTC4 transport and actually decreased transport of E217G. In addition, mutation of Thr1242 to either a negatively or positively charged amino acid similarly decreased the ability of the protein to confer resistance to all of the drugs tested including the cationic anthracyclines. Taken together, these findings indicate both a strong restriction on the size of the residue side chain at position 1242 and a re-
requirement for hydrogen bonding capacity for the protein to function as an efficient transporter of relatively bulky amphipathic, sterically rigid drugs and E_{17}{\beta}G, compared with the smaller more structurally flexible substrate, LTC_{4}. Only substitution of Thr^{1242} with Asp affected transport of both E_{17}{\beta}G transport and, albeit to a lesser extent, LTC_{4}. One possible explanation for this observation is that the interaction of LTC_{4} with other residues in a common substrate-binding pocket of the protein, such as Trp^{1246} (30), may be affected by charge repulsion from the aspartate side chain.

Having found previously that mutation of Glu^{1088} in TM14 of MRP1 to Gln not only markedly reduced that ability of the protein to confer resistance to anthracyclines but also, to a lesser extent, to vincristine and VP-16 (29), we investigated the effect of combining this mutation, and the reciprocal mutation in TM17 of MR1 introduced as a second mutation completely rescued the effect of the TM17 mutations on vincristine and VP-16 resistance. Previously, we have assumed that the similar levels of resistance that wild type mrp1 and MRP1 confer to these substrates interact with different, but partially shared sets of determinants in a common binding pocket on the protein (15, 16, 18, 21, 22, 28–30).

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Identification of a Nonconserved Amino Acid Residue in Multidrug Resistance Protein 1 Important for Determining Substrate Specificity: EVIDENCE FOR FUNCTIONAL INTERACTION BETWEEN TRANSMEMBRANE HELICES 14 AND 17

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