Identification of an Amino Acid Residue in Multidrug Resistance Protein 1 Critical for Conferring Resistance to Anthracyclines

Received for publication, November 2, 2000, and in revised form, December 22, 2000
Published, JBC Papers in Press, January 23, 2001, DOI 10.1074/jbc.M010008200

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Murine multidrug resistance protein 1 (mrp1), unlike human MRP1, does not confer resistance to anthracyclines. Previously, we have shown that a human/murine hybrid protein containing amino acids 959–1187 of MRP1 can confer resistance to these drugs. We have now examined the functional characteristics of mutant proteins in which we have converted individual amino acids in the comparable region of mrp1 to those present at the respective locations in MRP1. These mutations had no effect on the drug resistance profile conferred by mrp1 with the exception of converting glutamine 1086 to glutamate, as it is in the corresponding position (1089) in MRP1. This mutation created a protein that conferred resistance to doxorubicin without affecting vincristine resistance, or the ability of mrp1 to transport leukotriene C4 (LTC4) and 17β-estradiol 17-(β-D-glucuronide) (E217βG). Furthermore, mutation Q1086D conferred the same phenotype as mutation Q1086E while the mutation Q1086N did not detectably alter the drug resistance profile of mrp1, suggesting that an anionic side chain was required for anthracycline resistance. To confirm the importance of MRP1 E1089 for conferring resistance to anthracyclines, we mutated this residue to Gln, Asp, Ala, Leu, and Lys in the human protein. The mutation E1089D showed the same phenotype as MRP1, while the E1089Q substitution markedly decreased resistance to anthracyclines without affecting LTC4 and E217βG transport. Conversion of Glu-1089 to Asn, Ala, or Leu had a similar effect on resistance to anthracyclines, while conversion to a positive amino acid, Lys, completely eliminated resistance to anthracyclines and vincristine without affecting transport of LTC4, E217βG, and the GSH-dependent substrate, estrone-3-sulfate. These results demonstrate that an acidic amino acid residue at position 1089 in predicted TM14 of MRP1 is critical for the ability of the protein to confer drug resistance particularly to the anthracyclines, but is not essential for its ability to transport conjugated organic anions such as LTC4 and E217βG.

Human multidrug resistance protein 1 (MRP1)1 is a member of the ATP-binding cassette transporter superfamily that confers resistance to a wide range of natural product drugs, including anthracyclines, Vinca alkaloids, and epipodophyllotoxins, as well as methotrexate and certain heavy metal oxoanions (1–4). The predicted structures of MRP1 and several of its related proteins differ from that of a typical eukaryotic ATP-binding cassette transporter such as P-glycoprotein (P-gp). MRPs 1, 2, 3, and 6 contain an additional NH2-terminal membrane-spanning domain with an extracellular NH2 terminus (5–9). Thus, MRP1 is predicted to contain three membrane-spanning domains with 5-6+6 transmembrane (TM) helices (6, 7) (Fig. 1). Based on comparisons of amino acid sequence and the intron/exon organization of their respective genes, the MRPs and the cystic fibrosis transmembrane conductance regulator appear to have evolved from a common four-domain ancestor, the progenitor of the C branch of the ATP-binding cassette superfamily (1, 10).

MRP1 and P-gp confer resistance to many of the same commonly used, structurally diverse natural product chemotherapeutic agents (2, 4, 11–13). However, unlike P-gp, MRP1 is capable of transporting a wide range of relatively hydrophilic organic glutathione, glucuronide and sulfate conjugates (14–17). Considerable evidence indicates that MRP1, in contrast to P-gp, requires GSH for the transport of some of its hydrophobic substrates, such as vincristine, daunorubicin and aflatoxin B1 and that in some cases, GSH may be co-transported with these compounds (18–22).

Presently, very little is known of the regions of MRP1 that are important for substrate bindage and transport. Studies in P-gp have demonstrated that drug-binding site(s) involve amino acids in TM helices 1, 4, 5, 6, 8, 11, and 12, as well as some amino acids in cytoplasmic regions of the protein (23–30). In an attempt to expedite identification of amino acid residues important for the transport of hydrophobic drugs and the more hydrophilic organic anion conjugates that are established MRP1 substrates, we have taken advantage of well-characterized functional differences between the human and murine orthologs of the protein (31–33). The murine ortholog, mrp1, and MRP1 are 87% identical, and both proteins confer resistance to Vinca alkaloids and epipodophyllotoxins with apparently similar efficiencies. However, mrp1 fails to confer resistance to any anthracycline that has been tested, including doxorubicin, daunorubicin, and epirubicin. In addition, mrp1 transports 17β-estradiol 17-(β-D-glucuronide) (E217βG) far less efficiently than MRP1, despite the fact that both proteins transport leukotriene C4 (LTC4) with similar kinetic parameters (31, 32).

P-gp, P-glycoprotein; TM, transmembrane; mAb, monoclonal antibody; E17βG, 17β-estradiol 17-(β-D-glucuronide); LTC4, leukotriene C4; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HEK, human embryonic kidney; CL, cytoplasmic loop.

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1 The abbreviations used are: MRP, multidrug resistance protein; P-gp, P-glycoprotein; TM, transmembrane; mAb, monoclonal antibody; E17βG, 17β-estradiol 17-(β-D-glucuronide); LTC4, leukotriene C4; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HEK, human embryonic kidney; CL, cytoplasmic loop.
Previously, we have demonstrated using hybrid human/murine proteins that the COOH-terminal third of MRP1 contains important determinants of the ability to confer anthracycline resistance and to transport \( \text{E}_{17} \text{G} \). These studies also identified the region from amino acid 959 to 1187 of MRP1 as being particularly important for anthracycline resistance (33). In the present study, we have used this information to guide the design of point mutations in mrp1 in which variant amino acids in the region between residues 955 and 1184 have been replaced with the corresponding amino acid from MRP1 and vice versa. These mutant human and murine proteins were then stably expressed in human embryonic kidney (HEK293) cells and the transfectants characterized with respect to their drug resistance profiles and their ability to transport LTC4, \( \text{E}_{17} \text{G} \), and, in the case of the human protein, estrone-3-sulfate. The results of these studies identify glutamate 1089 in MRp1 as being critical for the ability to confer resistance to anthracyclines. In contrast, mutations at this location had no detectable effect on the ability to transport either \( \text{E}_{17} \text{G} \) or \( \text{E}_{17} \text{G} \) and no effect on the GSH-stimulated transport of estrone-3-sulfate.

EXPERIMENTAL PROCEDURES

Materials—Culture medium and fetal bovine serum were obtained from Life Technologies, Inc. \( ^{3} \text{H} \text{LTC}_{4} (38 \text{ Ci/mmol}) \) was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, England) and [\(^{6,7-3} \text{H}\)estrone-3-sulfate (53 \text{ Ci/mmol}) from Perkin-Elmer Life Sciences. Doxorubicin HCl, etoposide (VP-16), and vincristine sulfate were obtained from Sigma, and epirubicin from IC Biomedicals (Irving, CA).

Site-directed Mutagenesis and Generation of Expression Vectors—All mutations were generated using the Transformer\textsuperscript{TM} site-directed mutagenesis kit (CLONTECH, Palo Alto, CA). Templates were prepared by cloning 1.5–1.7-kilobase pair restriction fragments of human MRP1 or murine mrp1 cDNAs into pCEBV7 (Promega). Mutagenesis was then performed according to the manufacturer’s instructions using a selection primer 5’-GAG AGT GCA CGA TAT CCG GTG TG-3’ that matches a unique \( NdeI \) site in the vector to an EcoRI restriction site. Oligonucleotides bearing mismatched bases at the residues to be mutated (underlined) were synthesized by Cortec DNA Service Laboratories (Kingston, Canada). They are as follows: mutation mrp1Q955K (5’-GGG GCC ATC TTG GCC TCC CGT C-3’), mutation MRP1E1089K (5’-CTT CCC ATG ATC CCG AAA CCA CTG-3’), mutation MRP1E1089L (5’-CTT CCC ATG ATC CCG GAG GTC-3’), mutation MRP1E1089Q (5’-CTT CCC ATG ATC CCG CAG GTC-3’), mutation Mrp1N961D (5’-GAC GTG TAC TGG GAC TAC ATG AAG-3’), mutation Mrp1R1044C (5’-GCG GGG GGC ATC TTG GCC TCC CGT C-3’), mutation Mrp1L1022S (5’-GAG AGT GCA CGA TAT CCG GTG TG-3’), mutation Mrp1L979M (5’-GGG GCC ATC TTG GCC TCC CGT C-3’). The double and triple mutants were also generated by this method. The Q955K/N961D double mutation used the N961D mutagenic primer with the cDNA containing Q955K mutation as a template. Similarly, the L979M/L1022S double mutation used the L979M mutagenic primer with cDNA containing F1040L mutation as a template. The R1944C/Y1051H/N1052S triple mutation used the R1044C mutagenic primer with the cDNA containing Y1051H/N1052S mutation as a template.

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 (32). All of the mutated MRP1 or Mrp1 constructs were analyzed as stably transected HEK293 cells grown in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine serum and 100 \( \mu \text{g/ml} \) of hygromycin B (Roche Molecular Biochemicals, Laval, Quebec). Briefly, HEK293 cells were transfected with pCEBV7 vectors containing mutant MRP1 or mrp1 cDNAs using Fugene6 (Roche) according to the manufacturer’s instructions. After ~48 h, the transfected cells were supplemented with fresh medium containing 100 \( \mu \text{g/ml} \) hygromycin B. Approximately 3 weeks after transfection, the hygromycin-resistant cells were screened by limiting dilution and the resulting cell lines tested for high level expression of the mutant proteins.

Determination of Protein Levels in Transfected Cells—Plasma membrane vesicles were prepared as described previously (15, 32). After determination of protein levels by Bradford assay (Bio-Rad), 2 \( \mu \)g of membrane protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% gel) and subsequently transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) by electroblotting. MRP1/mrp1 proteins were identified using the monoclonal antibody, MRPr1, which cross-reacts with murine and human Mrp1/MPR1 protein (34). Antibody binding was detected with horseradish peroxidase-conjugated goat anti-rat IgG (Pierce), followed by chemiluminescent peroxidase detection (Perkin-Elmer Life Sciences).

Confocal Microscopy—Approximately 5–10\% stably-transfected HEK293 cells were seeded in each well of a six-well tissue culture dish on coverslips coated with 0.1% gelatin. When the cells had grown to confluence, they were washed once in PBS for 10 min and then fixed with 2% paraformaldehyde in PBS for 10 min. Cells were permeabilized using digitonin (0.25 mg/ml in PBS) for 10 min, treated with a blocking solution of 1% BSA in PBS for 10 min, and then followed by 1-h incubation in blocking solution containing 10 \( \mu \text{g/ml} \) RNase A and an antibody against MRP1. Antibodies used were a 1:5000 dilution of mAb QCRL-1, which recognizes amino acids 918–924 in the cytoplasmic connector region of MRP1, or a 1:5000 dilution of mAb MRp6, which reacts with an epitope close to the COOH terminus of MRP1 (amino acids 1510–1520) (34, 35). After washing with blocking solution for 10 min, cells were incubated with Alexa Fluor 488 anti-mouse IgG (H+L) (Fab\textsuperscript{\text{-}}) fragment for 1 h, washed with block solution for 10 min, and then incubated in propidium iodide (2% in H\textsubscript{2}O) for 5 min. Coverslips were mounted on the slides with one drop of Antifade reagent (Molecular Probes, Eugene, OR). Localization of MRP1 in the transfected cells was determined using a Meridian Insight confocal microscope (filter, 625 nm excitation). 550/50 nm for filter emission.

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 (4, 13, 32). Briefly, cells were seeded at 5 × 10\(^4\) cells/well in 100 \( \mu \text{L} \) of medium in culture dishes in 96-well tissue culture plate. The following day, various concentrations of drug diluted in culture medium were added to cells (100 \( \mu \text{L} \)well). After 24 h, 10 \( \mu \text{L} \) of an additional solution of 100 \( \mu \text{g/ml} \) of each drug from each well and the MTT reagent (25 \( \mu \text{Lw}ell, 2 \text{mg/ml} \) (Sigma) was added. After 3 h, the formazan was solubilized by mixing with HCl/ isopropanol (1:24) (100 \( \mu \text{L} \)well). Color density was determined using the ELX 800 UV spectrophotometer (Filter4, 570 nm). Mean values of quadruplicate determinations (± S.D.) were plotted using GraphPad software. IC\textsubscript{50} values were obtained from the best fit of the data to a sigmoidal curve. Relative resistance is expressed as the ratio of the IC\textsubscript{50} value of cells transfected with MRP1 and mrp1 expression vectors compared with cells transfected with empty vector. Resistance was determined in three or more independent experiments.

LTC\textsubscript{4}, E\textsubscript{17}G, and Estrone-3-sulfate Transport by Membrane Vesicles—Plasma membrane vesicles were prepared as described previously, and ATP-dependent transport of \( ^{3} \text{H} \text{LTC}_{4} \) into the inside-out membrane vesicles was measured by a rapid filtration technique (15, 16, 33). Briefly, vesicles (2.5 \( \mu \text{g} \) of protein) were incubated at 23 °C in 25 \( \mu \text{L} \) of transport buffer (50 mM Tris-HCl, 250 mM sucrose, 0.02% sodium azide, pH 7.4) containing 4 \( \mu \text{M} \) ATP, 10 mM MgCl\textsubscript{2}, and \( ^{3} \text{H} \text{LTC}_{4} \) (50 nm, 25 \( \mu \text{Ci} \)). At 1 min, 20 \( \mu \text{L} \) aliquots were removed and added to 1 ml of cold transport buffer followed by filtration through vacuum filtration through glass fiber filters (type AE, Gelman Sciences, Dorval, Quebec, Canada). Filters were immediately washed twice with 5 ml of cold transport buffer and then dried before the bound radioactivity was determined by scintillation counting. All data were corrected for the amount of \( ^{3} \text{H} \text{LTC}_{4} \) that remained bound in the filter in the absence of vesicle protein (usually <5% of the total radioactivity). \( ^{3} \text{H} \text{LTC}_{4} \) uptake was expressed relative to the protein concentration of the mem-
and with various concentrations of \[3H\]E217 the reaction was carried out at 37 °C for 2 min for preliminary analyses described for \[3H\]LTC4 except that 5

protein. In addition to the single substitutions, selected double mutations were made as indicated in Table I. Of the

seven, five are non-conservative and each of these was also

predicted in cytoplasmic residues in this region. Of the

seven, five are non-conservative. Each of the non-conserved amino acids in

MRP1 was replaced individually with the corresponding residue from MRP1. An additional seven sequence differences are pres-

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replaced with the corresponding amino acid from the human

protein. In addition to the single substitutions, selected double

and triple mutations were made as indicated in Table I.

The episomal expression vector, pCEBV7, containing mu-

tated forms of full-length mrp1 and MRP1 cDNAs was used to stably transfect HEK293 cells. From the initial populations of

transfectants, we isolated subpopulations by limiting cell dilution that, based on immunoblotting, expressed levels of each

mutant protein were approximately equivalent to the levels of wild type mrp1 and MRP1 in previously characterized HEK

transfectants. The only exceptions were HEK 293 subpopulations expressing mrp1Q1086N. This mutant protein reached levels that were slightly higher than those in the other sub-

populations used. The levels of mrp1 and MRP1 mutant proteins used in studies described below, relative to the respective

wild type protein, were determined by immunoblotting with mAb Mrp1, which reacts with both murine and human

MRP1(34), followed by densitometry (Fig. 2). Endogenous MRP1 in HEK293 cells transfected with the empty vector was undetectable under the conditions used (data not shown).

Resistance Profiles of Wild Type Murine mrp1 and Proteins Mutated in the Region between Amino Acids 955 and 1184—

The relative resistance of transfectants expressing each of the

mrp1 mutant proteins was determined by MTT assays following

exposure to various concentrations of vincristine and doxorubinc. The results are summarized as IC_{50} values and as relative resistance factors (Table I). None of the mutations significantly affected the level of resistance conferred to vincristine. The only mutation that affected doxorubicin resistance was the conversion of glutamate 1086 to glutamate (Q1086E), as it is at the corresponding position, 1089, in MRP1. The introduction of glutamate resulted in a relative resistance to doxorubicin of 4–5-fold, as did mutation of this residue to aspartate (Q1086D). In contrast, a conservative mutation to asparagine (Q1086N) had no effect (Table I). Typical survival curves for transfectants expressing wild type mrp1 and the mutant proteins Q1086E, Q1086D, and Q1086N are shown in

Fig. 3. These results clearly indicate the importance of the

residue at this position in influencing substrate specificity and suggest that a negatively charged side chain may be an impor-

tant prerequisite for conferring resistance to doxorubicin.

Mutational Analysis of Glutamate 1089 (Glu-1089) in Pre-

dicted TM14 of Human MRP1—Based on the findings with mrp1 Q1086E and Q1086D mutations, we investigated how critical glutamate 1089 was for the ability of the human MRP1 to confer resistance to anthracyclines, and whether or not muta-

tions of this residue also affected resistance to Vinca alka-

loids and epipodophyllotoxins. Thus, glutamate 1089 was muta-

ted to aspartate, glutamine, alanine, leucine, and lysine and chemosensitivity assays were carried out.

Cells expressing MRP1E1089D had a resistance profile indistinguishable from transfectants expressing the wild type

protein (Table II). In contrast, conversion of glutamate 1089 to glutamine, as it is in the murine protein, essentially eliminated the ability of MRP1 to confer resistance to doxorubicin, dauno-

rubinc, and epirubicin, as did mutations MRP1E1089A, MRP1E1089L, and MRP1E1089K (Table II). In addition, muta-

tion of glutamate 1089 to glutamine, alanine, and leucine decreased the relative resistance to vincristine by 55–65%, while mutation to lysine essentially eliminated resistance to this drug. The three mutations that introduced amino acids with uncharged side chains had less effect on VP-16 resistance, decreasing the resistance to this drug by 30–40% (Table II). The HEK293mrp1E1089K transfec-

tants also retained some resistance to VP-16, with a relative resistance factor of 6.5 compared with 15.6 for transfectants expressing wild type protein.

Typical survival curves for transfectants expressing wild type

MRP1 and the mutant proteins E1089Q, E1089D, and E1089K are shown in Fig. 4. These data confirm the essential role played by glutamate 1089 in MRP1 with respect to the ability...
Functional Analysis of Murine mrp1 and Human MRP1 Mutants

The resistance of HEK293 cells transfected with expression vectors encoding wild type and mutant murine 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/mutant mrp1-transfected cells by the IC50 value for control transfectants. The values shown represent the mean ± S.D. of IC50 values determined from three to six independent experiments.

Table I

| Transfectant | Vincristine IC50 nM | Doxorubicin IC50 nM | Relative resistance factor |
|--------------|---------------------|---------------------|---------------------------|
| HEKAC7       | 4.57 ± 0.85         | 0.059 ± 0.004       | 1                         |
| HEKmrp1      | 84.45 ± 11.70       | 0.047 ± 0.005       | 18.4 ± 2.6                |
| HEKmrp1Q1086D| 79.22 ± 9.20        | 0.041 ± 0.005       | 19.4 ± 3.5                |
| HEKmrp1Q1086E| 81.45 ± 10.20       | 0.045 ± 0.007       | 17.3 ± 2.0                |
| HEKmrp1Q1086N| 77.08 ± 6.60        | 0.058 ± 0.006       | 17.8 ± 2.2                |
| HEKmrp1N1052S| 89.32 ± 10.20       | 0.051 ± 0.006       | 16.8 ± 1.5                |
| HEKmrp1Q1086L| 75.14 ± 9.50        | 0.066 ± 0.007       | 19.5 ± 2.2                |
| HEKmrp1L1022S| 84.7 ± 9.38         | 0.060 ± 0.007       | 16.4 ± 2.1                |
| HEKmrp1E1089K| 75.68 ± 10.95       | 0.043 ± 0.007       | 19.1 ± 2.1                |
| HEKmrp1E1089Q| 72.96 ± 9.69        | 0.059 ± 0.007       | 16.5 ± 2.4                |
| HEKmrp1R1044C| 82.56 ± 10.50       | 0.060 ± 0.008       | 15.9 ± 2.1                |
| HEKmrp1L1022S| 82.39 ± 11.35       | 0.059 ± 0.006       | 18.0 ± 2.2                |
| HEKmrp1E1089C| 82.58 ± 9.87        | 0.067 ± 0.005       | 18.2 ± 2.5                |
| HEKmrp1Q1086E| 92.23 ± 8.46        | 0.037 ± 0.004       | 18.0 ± 2.2                |
| HEKmrp1Q1086E| 73.32 ± 12.28       | 0.271 ± 0.044       | 20.0 ± 1.9                |
| HEKmrp1Q1086N| 81.62 ± 14.65       | 0.249 ± 0.036       | 16.0 ± 2.7                |

![Image](http://www.jbc.org/)

**Fig. 2.** Immunoblot showing the expression levels of the mutant murine and human MRP1 proteins. Membrane proteins (2 μg) prepared from each cell line were separated by SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane as described under “Experimental Procedures.” Blots were probed with mAb MRPr1, which reacts with both the mouse and human proteins (34). For comparison, membrane proteins from cells expressing the wild type mrp1/MPR1 are also shown. Under the experimental conditions used, no endogenous MRP1 was detectable in control HEK293AC7 transfectants (data not shown).

of the protein to confer resistance to anthracyclines and also indicate that elimination of a negatively charged side chain at this location reduces the efficiency with which the protein confers resistance to other classes of natural product drugs.

Confocal Microscopy of MRP1, MRP1E1089Q, and MRP1E1089K-transfected HEK293 Cells—To determine whether the effects of mutations in TM14 on drug resistance profiles might be attributable in part to changes in trafficking of MRP1, we examined the subcellular localization of MRP1E1089Q and MRP1E1089K, as well as wild type MRP1 by confocal microscopy. Mutant and wild type MRP1 was detected using mAbs against two different regions of the protein: mAb QCRL-1 and mAb MRPM6, which recognize defined linear epitopes in the linker region and near the COOH terminus of the protein, respectively (35). As shown in Fig. 5, cells expressing the mutant proteins showed a pattern of strong plasma membrane staining comparable with that of cells expressing wild type MRP1, indicating that the trafficking of both mutant proteins was unaffected.

Transport of [3H]LTC4 and [3H]E217 G by Wild Type and Mutant Proteins—In addition to differences in the drug resistance profiles conferred by mrp1 and MRP1, we have shown previously that the two proteins differ with respect to their ability to transport some potential physiological substrates. For example, mrp1 and MRP1 transport LTC4 with similar efficiency, but mrp1 is a much less efficient transporter of E217G when compared with the human protein (32). As in the case of resistance to anthracyclines, studies with hybrid proteins implicated sequence differences in the COOH-terminal one-third of MRP1 and mrp1 as being primarily responsible for differences in the efficiency with which the two proteins transport this substrate (33). To determine whether any of the mutations of mrp1 that affected the ability to confer resistance
to anthracyclines altered the efficiency with which the protein transported either LTC₄ or E₂₁₇βG, we examined ATP-dependent uptake of these compounds by membrane vesicles prepared from HEK transfectants expressing mrp1Q₁₀₈₆E, mrp₁Q₁₀₈₆D, and mrp₁Q₁₀₈₆N. Despite the effect of the mrp₁Q₁₀₈₆E and mrp₁Q₁₀₈₆D mutations on doxorubicin resistance, none of the mutations appeared to affect transport of either LTC₄ or E₂₁₇βG (Fig. 6, A and C).

In view of the effect of mutations of glutamate 1089 in the human protein on resistance to vincristine and VP-16 in addition to the anthracyclines, we also examined LTC₄ and E₂₁₇βG transport by wild type and mutant human MRP1, including MRP1, E₁₀₈₉Q, E₁₀₈₉D, and E₁₀₈₉K. However, none of these mutations had any detectable influence on transport including the E₁₀₈₉K mutation that significantly decreased resistance to all drugs tested (Fig. 6, B and D).

Kinetic Parameters of [³H]LTC₄ and [³H]E₂₁₇βG Transport—Initial transport studies revealed no effect of mutations of glutamine 1086 in murine mrp1 or glutamate 1089 in human MRP1 on transport of either LTC₄ or E₂₁₇βG. However, to determine more thoroughly whether these mutations may have altered the affinity of mrp1 or MRP1 for these substrates, we also measured the Km and Vmax for both LTC₄ and E₂₁₇βG. The rate of uptake by membrane vesicles was determined at a number of substrate concentrations and a non-linear regression analysis of the combined data was used to determine a Km and Vmax for each protein (Fig. 7). For mrp1 and mutant mrp1Q₁₀₈₆E, which were expressed at comparable levels, the Km and Vmax for LTC₄ transport were virtually identical (Km = 148 nM, Vmax = 206

### Table II

| Transfectant         | Vincristine (IC₅₀) | VP-16 (IC₅₀) | Doxorubicin (IC₅₀) | Epirubicin (IC₅₀) | Daunorubicin (IC₅₀) |
|----------------------|-------------------|--------------|-------------------|------------------|---------------------|
| HEKMRP1              | 15.3 ± 3.1        | 15.6 ± 1.5   | 7.8 ± 0.9         | 8.0 ± 1.1        | 4.0 ± 0.5           |
| HEKMRP1E₁₀₈₆D       | 14.8 ± 0.9        | 13.1 ± 2.7   | 7.7 ± 0.4         | 7.5 ± 0.2        | Not tested          |
| HEKMRP1E₁₀₈₉Q       | 6.1 ± 0.3         | 9.5 ± 1.2    | 1.5 ± 0.1         | 1.3 ± 0.1        | 1.1 ± 0.4           |
| HEKMRP1E₁₀₈₆D       | 5.5 ± 0.5         | 6.7 ± 0.7    | 1.2 ± 0.3         | 1.1 ± 0.0         | Not tested          |
| HEKMRP1E₁₀₈₉N       | 7.2 ± 1.3         | 9.8 ± 0.9    | 1.4 ± 0.9         | 1.4 ± 0.6        | Not tested          |
| HEKMRP1E₁₀₈₉K       | 1.1 ± 0.1         | 6.5 ± 1.1    | <1                | <1               | <1                  |

**FIG. 4.** Resistance of stably transfected HEK293 cells to vincristine (A and B), doxorubicin (C and D), and VP-16 (E and F). The relative resistance of cells expressing wild type human MRP1 (HEK293 MRP1, ▲), mutant proteins (HEK293 MRP1E₁₀₈₆Q, ▼; HEK293 MRP1E₁₀₈₆D, ●), and cells transfected with the empty expression vector (HEK293 pC7, □) was determined as described under “Experimental Procedures.”

**FIG. 5.** Confocal microscopy of cells expressing wild type (HEK293 MRP1) and mutant (HEK293 MRP1E₁₀₈₆Q; HEK293 MRP1E₁₀₈₆D; HEK293 MRP1E₁₀₈₉K) human MRP1 proteins. Cells were grown and stained for immunofluorescence detection of MRP1 as described under “Experimental Procedures.” MRP1 was detected using mAb QCRL-1 (left), which recognizes amino acids 918–924 in the cytoplasmic connector region of MRP1, or mAb MRPm6 (right), which reacts with a cytoplasmic COOH-terminal fragment of MRP1 (amino acids 1510–1520) (34, 35). Location of MRP1 is indicated in green. Nuclei were stained with propidium iodide and are shown in red.
which when normalized to the level of wild type protein yielded MRP1 proteins as indicated in the figure. Transport either LTC4 or E217 tamate had no significant effect on the ability of the protein to contribute to its ability to transport E217G (32). Previously, we have taken advantage of transport of E217G, membrane vesicles (5 μg of membrane protein) were incubated at 37 °C with 400 nm [3H]E217G for 2 min. Transfectedants were expressing wild type or mutant murine human MRP1/MPR1 proteins as indicated in the figure.

The kinetics of ATP-dependent LTC4 and E217G transport were also examined for the wild type and mutant proteins (Fig. 7, C and D). In these experiments, the levels of wild type MRP1 determined by immunoblotting and densitometry were approximately twice as high as that of the mutant MRP1E1089Q (Fig. 7G). The Km values for LTC4 transport obtained with vesicles containing wild type MRP1 or mutant MRP1E1089Q were similar, 116 and 108 nM, respectively. The values determined for wild type and mutant MRP1 were also virtually identical (337 and 352 pmol mg⁻¹ min⁻¹, respectively) (Fig. 7B). Thus, in murine mrp1, converting glutamine 1086 to glutamate had no significant effect on the ability of the protein to transport either LTC4 or E217G.

The kinetics of ATP-dependent LTC4 and E217G transport were also examined for the wild type and mutant human proteins (Fig. 7, C and D). In these experiments, the levels of wild type MRP1 determined by immunoblotting and densitometry were approximately twice as high as that of the mutant MRP1E1089Q (Fig. 7G). The Km values for LTC4 transport obtained with vesicles containing wild type MRP1 or mutant MRP1E1089Q were similar, 116 and 108 nM, respectively. The values determined for wild type and mutant MRP1 were also virtually identical (337 and 352 pmol mg⁻¹ min⁻¹, respectively) (Fig. 7B). Thus, as obtained with mrp1, mutation of glutamate 1086 in the human protein did not affect transport of GSH dependent substrates such as LTC4, and E217G, and drugs such as vincristine and daunorubicin, is a requirement for GSH, which may be co-transported with the unmodified drug (19–21). Recently, we have shown that GSH can also enhance the transport of some anionic conjugates such as estrogen-3-sulfates (36). Since transport of these compounds is more amenable to kinetic analysis than hydrophobic drugs such as vincristine and the anthracyclines, we used [3H]estrone-3-sulfate to investigate whether the MRP1E1089K mutation had affected the transport of GSH dependent substrates. In the membrane vesicles used, immunoblotting and densitometry indicated that the levels of mutant protein were ~70% that of wild type MRP1. The kinetic parameters of estrone-3-sulfate transport for membrane vesicles prepared from cells transfected with the wild type and mutant MRP1 were then determined in the presence and absence of GSH (Fig. 7, E and F). In the presence of GSH, a Km value of 1.1 μM was obtained for MRP1, which was essentially identical to that determined for mutant MRP1E1089Q or MRP1E1089K (1.0 and 1.1 μM, respectively). The Km values for MRP1, MRP1E1089Q, and MRP1E1089K were also similar in the absence of GSH (3.5, 3.5, and 3.9 μM, respectively). The Vmax values for transport by membrane vesicles from wild type MRP1 or mutations MRP1E1089Q and MRP1E1089K were 403, 191, and 289 pmol mg⁻¹ min⁻¹, respectively, in the presence of GSH and 185, 110, and 150 pmol mg⁻¹ min⁻¹, respectively, in its absence. When normalized for differences in expression levels, the Vmax values for wild type MRP1 or mutations MRP1E1089Q and MRP1E1089K were similar (403, 382, and 413 pmol mg⁻¹ min⁻¹, respectively, in the presence of GSH; 185, 222, and 214 pmol mg⁻¹ min⁻¹, respectively, in the absence of GSH). These findings demonstrated that, in the presence or absence of GSH, mutations MRP1E1089Q and MRP1E1089K behaved in a manner very similar to that of the wild type protein.

**DISCUSSION**

Human MRP1 and murine mrp1 confer resistance to Vinca alkaloids and epipodophyllotoxins with approximately the same efficiency. However, despite the fact that the orthologs are relatively highly conserved (87% identity overall), only the human protein confers resistance to anthracyclines (31, 32). In addition, although mrp1 and MRP1 transport LTC4 with similar kinetic parameters, they differ markedly in their ability to transport E217G (32). Previously, we have taken advantage of these differences to map regions of the human protein that contribute to its ability to transport E217G much more efficiently than mrp1 and to confer resistance to anthracyclines. Analysis of the drug resistance profiles conferred by hybrid proteins and their in vitro transport characteristics localized residues important for both anthracycline resistance and transport of E217G to the COOH-terminal third of MRP1. More detailed analyses revealed that regions between amino acids 959–1187 and 1188–1531 both contributed approximately equally to the ability of the human protein to transport E217G (33). However, the hybrid containing MRP1-(959–1187) was approximately twice as effective as the hybrid containing the region MRP1-(1188–1531) at conferring anthracycline resistance (33). We have now examined the consequences of converting all non-conservative differences in mrp1 in the region between amino acids 955 and 1184 to the corresponding amino acid present in human MRP1 to begin identification of specific residues involved in conferring resistance to anthracyclines and to determine whether the same residues also enhance the transport of E217G.

Site-directed mutagenesis studies of the P-gps have identified single amino acid residues at various locations in the protein that influence substrate specificity but similar infor-
FIG. 7. Kinetics of ATP-dependent [3H]LTC₄ (A and C), [3H]E₂17βG (B and D), and [3H]estrone-3-sulfate (E and F) uptake by wild type and mutant proteins. The relative expression levels of wild type and mutant MRP1 proteins in the membrane vesicles used for examining kinetic parameters are shown in panel G. The proteins were determined by immunoblotting with mAb MRP1 as described in the legend to Fig. 2. The *numbers below the blot* refer to the relative levels of MRP1 proteins. The initial rate of ATP-dependent [3H]LTC₄ (A and C) and [3H]E₂17βG (B and D) uptake by membrane vesicles prepared from HEK293 cells transfected with wild type or mutant murine/human proteins was measured at various LTC₄ concentrations (0.01–2 μM) for 1 min at 23 °C and at various E₂17βG concentrations (0.2–16 μM) for 1 min at 37 °C in transport buffer, as described under “Experimental Procedures.” The initial rate of ATP-dependent [3H]estrone-3-sulfate uptake was also examined at various estrone-3-sulfate concentrations (0.075–16 μM) for 1 min at 37 °C in transport buffer in the presence or absence of 1 mM GSH (E and F). Data were plotted as $V_0$ versus $[S]$ to confirm that concentration range selected was appropriate to observe both zero-order and first-order rate kinetics. For LTC₄ and E₂17βG transport, the transfectants tested were HEK*-mrp1 (■), HEK*-mrp1Q1086E (△) (panels A and B), HEKMRP1 (○), and HEKMRP1E1089Q (●) (panels C and D). For estrone-3-sulfate transport, the transfectants tested were HEK*mrp1 (●), HEK*mrp1Q1086E (△), HEKMRP1 (○), HEKMRP1E1089Q (●), and HEKMRP1E1089K (○) (panels E and F). Closed symbols represent uptake in the presence of 1 mM GSH; open symbols represent uptake in the absence of GSH. Kinetic parameters were determined from non-linear regression analysis of the combined data. Details of $K_m$ and $V_{max}$ values for all three substrates are provided under “Results.”
mation about the MRP family is only just beginning to emerge (37). Most of the residues identified in P-gp are located in predicted TM helices, predominantly TMs 1, 6, 11, and 12, although single proline residues in TMs 4 and 10 have also been shown to influence substrate specificity (23). The region of MRP1/mrp1 we have analyzed includes TM helices 12–15, which because of the five additional NH2-proximal TM helices corresponds topologically to TM helices 7–10 of P-gp, as well as predicted CL7 of MRP1/mrp1 corresponding to CL4 of P-gp.

Although most residues implicated to date in determining the substrate specificity of P-gp are located in TM helices, CL4 is a cytoplasmic region where mutation of specific glycine residues to valine has been shown to increase resistance to colchicine and doxorubicin (26).

Four non-conservative differences between MRP1 and mrp1 are present in CL7. However, in contrast to the results obtained from mutagenesis studies of CL4 of P-gp, replacement of the variant residues in mrp1 with those present in MRP1 had no detectable effect on its drug resistance profile. Furthermore, single mutations in TM helices 12–15 of the mouse protein that converted non-conservative differences between mrp1 and MRP1 to the human sequence also had no effect, with the exception of glutamine 1086. Conversion of this residue to glutamate, as it is in MRP1, increased the ability of mrp1 to confer resistance to anthracyclines to a level approximately equivalent to that conferred by the original hybrid containing amino acids 959–1187 of the human protein. This result combined with the lack of effect of mutating other variant amino acids strongly suggests that, within this region, glutamate 1089 is critical for the ability of the hybrid protein to confer anthracycline resistance. Consistent with previous results obtained using mrp1/MRP1 hybrids, the mutation was selective with respect to anthracycline resistance and had no effect on the ability of mrp1 to confer resistance to vincristine and VP-16, or to transport LTC4 and E217G.

The results obtained with mrp1 mutants implicated glutamate 1089 of the human protein in the ability to confer anthracycline resistance. However, the levels of resistance conferred by both the mrp1/MRP1 959–1187 and the mrp1Q1086E mutant protein were ~60% that of the wild-type protein (33). The fact that the hybrid and mutant mrp1 were less effective than the wild type human protein left open the possibility that glutamate 1089 may contribute to, but not be essential for, resistance to this class of drugs. However, the results obtained with MRP1 mutants confirmed that glutamate 1089 was essential for anthracycline resistance. They also revealed that mutation of this residue, in contrast to the comparable mrp1 mutations, also affected resistance to other drugs. Mutation of glutamate 1089 to glutamine essentially eliminated anthracycline resistance, confirming the crucial role of this residue. However, unlike the reciprocal mutation in the murine protein, which had no effect on resistance to other types of drugs, the MRP1E1089Q mutation also decreased resistance to vincristine and VP-16, although to a lesser extent than the anthracyclines. This was observed to a similar degree with all of the mutations that eliminated a negative charge in the amino acid side chain. The difference in the effect of the mrp1Q1086E and MRP1E1089Q mutations raises the interesting possibility that the introduction of glutamate 1089 in MRP1 may have been a critical event in acquisition of the ability to confer resistance to anthracyclines, and that the protein has evolved so that this residue now has a more general role in drug binding and/or transport. Whether or not this is the case should become apparent as the drug resistance profiles and sequences of MRP1 orthologs in other species become available.

Studies of P-gp mutants of histidine 61 in predicted TM1 suggested that the size of the side chain of the residue influenced substrate specificity. It was observed that substitution by an amino acid with a small side chain increased relative resistance to vinblastine while introduction of a large side chain increased resistance to smaller substrates including colchicine, VP-16 and doxorubicin (28). We observed no differences between the mutant proteins, in which various neutral amino acids had been substituted for glutamate 1089, suggesting that a negative charge in the side chain was the predominant feature influencing the drug resistance profile of the wild type protein. This was supported by the results obtained by substitution of glutamate 1089 with aspartate, which resulted in no detectable change in resistance profile, and by conversion to lysine, which essentially eliminated resistance to both anthracyclines and vincristine, and substantially decreased resistance to VP-16. Thus, the drug resistance profile of MRP1 appears primarily dependent on the charge rather than size or hydrophobicity of the residue at position 1089. The relatively pronounced effect that the introduction of a positive charge at this location has on resistance to anthracyclines and vincristine may be attributable to the fact that the former are strongly and the latter is weakly cationic at physiological pH while VP-16 is uncharged.

The effect of the MRP1Q1089K mutation on the ability of the protein to confer resistance to all of the drugs tested suggested either that the overall transport activity of the protein was diminished or that the mutation may have adversely affected trafficking causing retention of the protein in intracellular membranes. This mutation results not only in a net gain of two positive charges in predicted TM helix 14, but also in the introduction of adjacent lysine residues (Fig. 1), creating the possibility that charge repulsion might contribute to an alteration in the conformation of this region of the protein. However, confocal microscopy revealed no evidence of a trafficking defect that might be indicative of misfolding of the protein. In addition, the protein retained full activity with respect to the transport of LTC4 and E217G, confirming that the effect of the mutation was specific for the chemotherapeutic drugs tested and did not alter the transport efficiency of these two conjugated organic anions. The mechanistic explanation for this specificity is presently not known. One major distinction between MRP1 mediated transport of LTC4 and E217G, and drugs such as vincristine and daunorubicin, is a requirement for GSH, which may be co-transported with the unmodified drug (19–21). We have recently shown that GSH can also markedly enhance the efficiency with which some conjugated organic anions such as estrone-3-sulfate are transported. Using this substrate, it has been possible to demonstrate that GSH both decreases the $K_m$ and increases the $V_{max}$ for transport of the conjugated estrogen (36). This provided the opportunity to determine whether mutations that specifically affected drug resistance were influencing the GSH dependence of the transport process. However, comparison of the GSH dependence of estrone-3-sulfate transport by the both the MRP1E1089Q, which eliminated anthracycline resistance and reduced vincristine resistance, and the MRP1E1089K mutation, which eliminated resistance to both anthracyclines and vincristine, revealed no kinetic differences from the wild type protein. Thus, the effects of the mutations at position 1089 in human MRP1 appear to be due primarily to the structure of the substrate.

Several previous studies have demonstrated that, under appropriate conditions, drugs such as vincristine and possibly doxorubicin act as competitive inhibitors of LTC4 transport, suggesting that they bind to common or mutually exclusive sites on the protein. In some cases, the presence of GSH has been shown to markedly increase the inhibitory potency of
these drugs (15, 16, 20). The results of experiments described here together with earlier competition studies, indicate that at least some of the residues within these sites that are critical for the binding of unmodified drugs differ from those required for binding and transport of conjugates such as LTC$_4$ and E$_2$17βG. Very recently we have also identified a single amino acid, mutation of which eliminates the ability of MRP1 to confer drug resistance and to transport E$_2$17βG but leaves LTC$_4$ transport intact. Knowledge of these sites may assist in the design of MRP1 reversing agents that spare at least some of the physiological functions of the protein while abrogating its ability to confer drug resistance.

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