Single amino acid substitution of rat MRP2 results in acquired transport activity for taurocholate

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Ito, Kousei, Hiroshi Suzuki, and Yuichi Sugiyama. Single amino acid substitution of rat MRP2 results in acquired transport activity for taurocholate. Am J Physiol Gastrointest Liver Physiol 281: G1034–G1043, 2001.—Multidrug resistance-associated protein 3 (MRP3), unlike other MRPs, transports taurocholate (TC). The difference in TC transport activity between rat MRP2 and MRP3 was studied, focusing on the cationic amino acids in the transmembrane domains. For analysis, transport into membrane vesicles from SF9 cells expressing wild-type and mutated MRP2 was examined. Substitution of Arg at position 586 with Leu and Ile and substitution of Arg at position 1096 with Lys, Leu, and Met resulted in the acquisition of TC transport activity, while retaining transport activity for glutathione and glucuronide conjugates. Substitution of Leu at position 1084 of rat MRP3 (which corresponds to Arg-1096 in rat MRP2) with Lys, but not with Val or Met, resulted in the loss of transport activity for TC, whereas the presence of neutral amino acids at the corresponding position of rat MRP3 is required for the transport of substrates.

Adenine 5′-triphosphate-binding cassette transporter superfamily; bile acid; site-directed mutagenesis; multidrug resistance-associated protein 2

Multidrug resistance-associated protein 2 (MRP2) belongs to the ATP-binding cassette (ABC) transporter family cloned from rat liver as a homologue of human MRP1 (4, 13, 27). MRP2 is highly expressed on the bile canicular membrane and is able to excrete structurally diverse organic anions into the bile as shown by comparing the behavior of normal and MRP2-deficient rats (16, 31). A hereditary defect in human MRP2 results in a form of hyperbilirubinemia referred to as Dubin-Johnson syndrome (15, 28, 32, 33). Substrates for MRP2 are very similar to those for MRP1 and include glutathione conjugates [e.g., 2,4-dinitrophenyl-S-glutathione (DNP-SG) and leukotriene C4 (LTC4)], glucuronide conjugates [e.g., 17β-estradiol 17-β-D-glucuronide (E217βG) and bilirubin glucuronide], non-conjugated organic anions (e.g., methotexate), and sulfate and glucuronide conjugates of certain bile acids (e.g., taurocholate-3-sulfate and cholate-3-O-glucuronide; Refs. 19, 31). Unlike the sulfate and glucuronide conjugates of certain bile acids, monovalent bile acids such as taurocholate (TC), cholate, and glycocholate (GC) are not transported by MRP2, but excreted into the bile via the bile salt export pump/sister of P-glycoprotein (Bsep/spgp; 6), which is also a member of the ABC transporter family. The primary structure of Bsep/spgp is more similar to that of rat multidrug-resistance protein 1b (mdr1b) and mdr2 with homology of 70% and 69%, respectively, than to that of the MRP family proteins (~50% homology with MRP1 and rat MRP2; 6).

MRP3, the third member of the MRP family, has been cloned as a homologue of MRP1 and MRP2 (9, 17, 20, 21, 34). Induction of MRP3 on the basolateral membrane of rat hepatocytes under cholestatic conditions (3, 20) led us to hypothesize that MRP3 compensates for the reduced ability of MRP2 to pump out common substrates from hepatocytes into blood. Indeed, MRP3 has been shown (10) to accept glucuronide conjugates as substrates although glutathione conjugates are poor substrates. In addition, it has been demonstrated (1, 11, 36) that MRP3 transports monovalent bile acids such as TC and GC. These findings clearly indicate that there is a difference in substrate specificity among MRP1, MRP2, and MRP3. Together with the localization of MRP3 on the basolateral membrane of cholangiocytes and the intestinal epithelium (22), possible involvement of MRP3 in the enterohepatic and/or cholehepatic circulation has been proposed, although further data are required.

Cumulative information on the primary structure and substrate specificity of MRP families has allowed us to take advantage of comparative studies on chimeric proteins and/or mutated proteins produced by site-directed mutagenesis to search for a region(s) of the protein involved in determining the particular substrate specificity of each of these cognate gene products. Stride et al. (30) clearly demonstrated that the anthracycline resistance and the ability to transport E217βG by MRP1 are conferred by the COOH-terminal third of the protein, using a chimeric approach to study human MRP1 and mouse MRP1 expressed in HEK 293 cells. Moreover, we (14) have recently demonstrated...
that the cationic amino acids in transmembrane domain (TM) 6 and TM11 of rat MRP2, the cationic charge of which is conserved among MRP1, MRP2, and MRP3, are involved in the recognition of glutathione conjugates. The role of cationic amino acids in TMs in transporting glutathione conjugates by human MRP2 has also been reported (29). In the present study, the cationic amino acids in the TMs of rat MRP2, the cationic charges of which are fully conserved among MRP1, MRP2, and MRP3 or conserved in MRP1 and MRP2 but not in MRP3, were substituted with neutral amino acids to examine whether these amino acids are involved in gaining access to monovalent bile acids. Substitution of the amino acid at position 586 (an amino acid with a cationic charge that is conserved among MRP1, MRP2, and MRP3) and position 1096 (an amino acid with a cationic charge that is conserved in MRP1 and MRP2 but not in MRP3) of rat MRP2 resulted in acquired transport activity for TC. The transport activity of mutant rat MRP3 with a mutation at position 1084, which corresponds to the amino acid at position 1096 of rat MRP2, was also investigated.

**EXPERIMENTAL PROCEDURES**

**Chemicals.** [3H]LTCA (165 Ci/mmol), [3H]E$_2$17βG (55 Ci/mmol), and [3H]TC were purchased from NEN Life Science Products (Boston, MA). [3H]DNP-SG (50 Ci/mmol) was synthesized enzymatically using [glycine-2-3H]glutathione (NEN Life Science Products), 1-chloro-2,4-dinitrobenzene, and glutathione S-transferase (Sigma Chemical, St. Louis, MO) as described previously (18), followed by purification by HPLC on LiChrosorb RP-18 column (Kanto Chemical, Tokyo, Japan). The solvent system used was H$_2$O-acetoni trile (22:78) containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The purity of [3H]DNP-SG was >99% throughout the experiments. Unlabeled DNP-SG was synthesized using the method of Hinchman et al. (7). Briefly, 2,4-dinitrofluorobenzene (0.01 mol; Sigma Chemical) dissolved in 1 ml MeOH was added slowly to GSH (0.01 mol) dissolved in 5 ml 1 N KHCO$_3$.

After incubation for 15 min, the solution was filtered and acidified to pH 2 with diluted HCl. The precipitate was collected by vacuum filtration. The recrystallization of DNP-SG was performed from boiling H$_2$O, E$_2$17βG, TC, ATP, GTP, CTP, UTP, AMP, creatine phosphate, and creatine phosphokinase were also obtained from Sigma Chemical. SF9 cells were maintained as a suspension culture at 27°C with serum-free Excel 420 (Nichirei, Tokyo, Japan) supplemented with an antibiotic-antimycotic mixture (LifeTechnologies, Tokyo, Japan).

**Plasmid construction.** Rat MRP2 cDNA inserted in the recombinant donor plasmid pFASTBAC1 was obtained as reported previously (14). A 5.2-kb ApaLI-SalI fragment of rat MRP3 cDNA containing the full-length coding region flanked by an untranslated sequence of 15 and 572 nt at the 5’ and 3’ ends, respectively, was excised from pBluescript SK(−) vector (10). This fragment was ligated with BamHI-SmaI linker and subsequently inserted into the BamHI and SalI site of the donor plasmid pFASTBAC1 (Life Technologies) downstream from the polyhedrin promoter.

**Site-directed mutagenesis.** Site-directed mutagenesis was performed by the method of Kunkel et al. (23). Single-strand DNAs encoding rat MRP2 and MRP3 were rescued from CJ236 transfected with pBluescript SK(−) containing the expression cassette of rat MRP2-Aor51HI-SalI (13) and rat MRP3 cDNA (9) after they were infected with f1 helper phage. The resulting single-strand DNAs and respective mutagenic primers were heat denatured at 96°C for 1 min and then slowly cooled to 40°C to allow them anneal properly. An in vitro polymerase reaction was performed using Sequenase version 2.0 (Life Technologies) and T4 DNA ligase (Life Technologies) at 37°C for 90 min. The resulting double-stranded DNA was transfected to competent DH5α and spread onto LB agar plate containing 50 µg/ml of ampicillin. Mutant MRP2 and MRP3/pBluescript SK(−) were prepared from several colonies and verified by automated sequencing. The SmaI-SalI fragment of the wild-type MRP2 cDNA cassette in pFASTBAC1 was replaced with the mutant MRP2 cDNA cassette excised as an Aor51HI-SalI fragment from the mutant MRP2/pBluescript SK(−). Similarly, the NheI-SalI fragment of the wild-type MRP3 partial cDNA cassette (2,388 bp of coding region and 572 bp of 3’-noncoding region) was replaced with corresponding mutant MRP3 cDNA cassette excised as the NheI-SalI fragment from the mutant MRP3/pBluescript SK(−).

**Production and infection of recombinant baculovirus.** Recombinant baculovirus was prepared as described previously (14). SF9 cells were infected with an appropriate amount of the respective virus and cultured for 60–72 h in the presence of 5% fetal bovine serum. SF9 cells infected with the baculovirus carrying green fluorescent protein (GFP) cDNA were used as a control throughout the experiment (GFP control). Cells were harvested 60–72 h after infection, and subsequently, membrane vesicles were isolated from 1 to 2 × 10$^8$ SF9 cells using the standard method described previously with some modifications. Briefly, the harvested cells (1–2 × 10$^8$ cells) were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 7.4 at 4°C) and stirred gently for 1 h on ice in the presence of 2 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 1 µg/ml pepstatin, and 5 µg/ml aprotinin. The cell lysate was centrifuged at 100,000 g for 30 min at 4°C, and the resulting pellet was suspended in 10 ml of isotonic TS buffer (10 mM Tris-HCl, pH 7.4 at 4°C, and 250 mM sucrose) and homogenized with Dounce B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top of a 38% (wt/vol) sucrose cushion in 5 mM Tris-HEPES (pH 7.4 at 4°C) and centrifuged in a Beckman SW41 rotor at 280,000 × g for 45 min at 4°C. The turbid layer at the interface was collected, diluted to 23 ml with TS buffer, and centrifuged at 100,000 g for 30 min at 4°C. The resulting pellet was suspended in 400 µl of TS buffer. Vesicles were formed by passing the suspension 30 times through a 25-gauge needle with a syringe. The membrane vesicles were finally frozen in liquid nitrogen and stored at −80°C until use. Protein concentrations were determined by the Lowry method.

**Transport study.** The transport study was performed using a rapid filtration technique, as described previously (12). Briefly, 16 µl of transport medium (10 mM Tris, 250 mM sucrose, 10 mM MgCl$_2$, 5 mM ATP or AMP, and ATP-regenerating system (10 mM creatine phosphate and 100 µg/ml creatine phosphokinase), pH 7.4 at 37°C), containing radiolabeled compounds with or without unlabeled substrate, were preincubated at 37°C for 3 min and then rapidly mixed with 4 µl of membrane vesicle suspension (10 µg protein). The transport reaction was stopped by the addition of 1 ml ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.4 at 4°C). The stopped reaction mixture was filtered through a 0.45-µm HAWP filter (Millipore, Bedford, MA) and then washed twice with 5 ml of stop solution. The radioactivity retained on the filter was determined using a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan).
Western blot analysis. Expression of MRP2 and MRP3 protein on the Sf9 membrane was determined as described previously (12) with some modifications. Membrane vesicles from Sf9 cells were loaded onto a 8.5% polyacrylamide slab gel containing 0.1% SDS and then transferred onto a Pall Fluoro Trans W membrane filter (Ann Arbor, MI) by electroblotting. The filter was blocked with Tris-buffered saline containing 0.05% Tween 20 and 3% BSA for 10 h at 4°C and probed for 1 h at room temperature with polyclonal anti-MRP2 antibody raised against the upstream region of the COOH-terminal nucleotide-binding domain (amino acid residues 1272–1285; CP-2 antibody was supplied by Dr. J. Nakayama, Kumamoto University, Kumamoto, Japan) or anti-MRP3 antibody directed against 838–973 of the deduced rat MRP3 amino acid sequence (10) diluted with Tris-buffered saline containing 0.05% Tween 20 and 0.1% BSA (1:1,000). The primary antibody was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden) with a horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech) as the secondary antibody that was used after 2,000-fold dilution.

Data analysis. Uptake rates were fitted to the Michaelis-Menten equation using a nonlinear least-squares program (MULTI; 35) to calculate the kinetic parameters.

RESULTS

Preparation of membrane vesicles from Sf9 cells infected with baculoviruses carrying mutated MRP2 cDNA. Comparison of the amino acid sequence among MRP1, MRP2, and MRP3 indicated that six cationic charges (Lys-308 and -325 and Arg-586, -1019, -1201, and -1226 in rat MRP2) are conserved among MRP1, MRP2, and MRP3 and two cationic charges (Arg-1096 and -1206 in rat MRP2) are conserved in MRP1 and MRP2, and MRP3 and two cationic charges (Arg-1096 and -1206 in rat MRP2) are conserved in MRP1 and MRP2, respectively.

Fig. 1. Membrane topology of rat multidrug resistance-associated protein 2 (MRP2) and alignment of transmembrane domains of MRP families. A: membrane topology of rat MRP2 was predicted by the Kite and Doolittle algorithm, taking into account the reported model for human MRP1 (2, 8). Location of substituted cationic amino acid residues is indicated by ● (amino acids with a cationic charge that is conserved among MRP1, MRP2, and MRP3) and ▲ (amino acids with a cationic charge that is conserved in MRP1 and MRP2 but not in MRP3). B: detailed alignment of transmembrane domain (TM) 6, 11, 13, 14, 16, and 17 of rat MRP2 is shown compared with that of human and mouse MRP1 (hMRP1 and mMRP1, respectively), hMRP2, rat MRP2 (rMRP2), rabbit MRP2, hMRP3, and rMRP3. Predicted TM are underlined. The location of cationic amino acids is indicated by ● (amino acids with a cationic charge that is conserved among MRP1, MRP2, and MRP3) and ▲ (amino acids with a cationic charge that is conserved in MRP1 and MRP2 but not in MRP3). MSD, membrane-spanning domain regions; NBD, nucleotide-binding domain.
MRP2 but not in MRP3 (Fig. 1B; 14). We have focused on the role of these eight cationic amino acids in determining the TC transport activity and prepared mutated rat MRP2 by substituting neutral amino acids (Leu or Met). Membrane vesicles isolated from Sf9 cells infected with baculoviruses carrying the respective mutant MRP2 cDNA were analyzed. As shown in Fig. 2, Western blot analysis revealed detectable levels of expression of wild-type MRP2 and mutant MRP2 on the Sf9 cell membrane except for mutants with Met substituted for Arg-1201 and -1226 (R1201M and R1226M, respectively), which were expressed below the detection limit (data not shown) as reported previously (14). The molecular mass of wild-type and mutant MRP2 was also comparable (~175 kDa; Fig. 2).

Transport of [3H]TC into mutant MRP2-expressing membrane vesicles. The uptake of [3H]E217G was stimulated by ATP in mutants with Met substituted for Lys-308, -325, and Arg-1019 (K308M, K325M, and R1019M, respectively), Leu substituted for Arg-586 and -1096 (R586L and R1096L, respectively), as well as wild-type MRP2, whereas the mutant with Met substituted for Arg-1206 (R1206M) lost the transport activity for both [3H]E217G and [3H]LTC4 (data not shown). The ATP-dependent uptake of [3H]TC by R586L and R1096L was significantly higher than the GFP control (Fig. 3). The uptake of [3H]TC by R586L and R1096L was linear at least for the first 5 min in the presence of ATP (Fig. 4). Moreover, to confirm that the TC molecules associated with the membrane vesicles reflect transport into the intravesicular space, rather than binding to the vesicle surface, the uptake of [3H]TC was examined with media of different osmolarities. As shown in Fig. 4B, the uptake of [3H]TC by both R586L and R1096L was osmotically sensitive. To confirm the nucleotide dependence of TC transport by these mutant MRP2, the uptake of [3H]TC by R586L and R1096L was measured in the presence of several kinds of nucleotides at a concentration of 5 mM. Of the four nucleotide triphosphates tested, ATP was shown to stimulate TC uptake most efficiently (Fig. 4C) for both mutant MRP2, although other nucleotides can also stimulate the uptake by up to 20–40% of that in the presence of ATP (Fig. 4C). In contrast, AMP was unable to support TC uptake (data not shown).

Concentration dependence of TC, E217G, and DNP-SG uptake by R586L and R1096L. The initial velocity for the uptake of [3H]TC by R586L and R1096L was determined in the presence of unlabeled TC (0.7–500 μM; Fig. 5). Kinetic analysis yielded Michaelis constant (K_m) values of 28.8 ± 6.9 and 41.6 ± 5.6 μM for R586L and R1096L, respectively (Table 1). Transport kinetics was also determined for E217G and DNP-SG in wild-type MRP2, R586L, and R1096L (Fig. 5 and Table 1). As shown in Table 1, K_m for E217G were 7.46 ± 3.39, 1.46 ± 0.30, and 5.00 ± 0.16 μM for wild-type MRP2, R586L, and R1096L, respectively. K_m values for DNP-SG were 62.6 ± 10.6, 134 ± 29, and 101 ± 13 μM for wild-type MRP2, R586L, and R1096L, respectively (Table 1).

Mutual effect of TC and other MRP2 substrates. To gain insight into the nature of the acquired TC transport activity by R586L and R1096L, the effect of unlabeled TC on the transport of authentic substrates for MRP2, including [3H]DNP-SG and [3H]E217G, was investigated in R586L and R1096L compared with wild-type MRP2. The uptake of [3H]DNP-SG and [3H]E217G by wild-type MRP2 was increased in the presence of unlabeled TC (Fig. 6, A and B), whereas that by R586L and R1096L was reduced with an approximate IC_50 of 200 μM (Fig. 6, A and B). Moreover, transport of [3H]TC by R1096L was increased ~2.4- and 1.9-fold in the presence of unlabeled DNP-SG (200 μM) and E217G (37.5 μM), respectively (Fig. 6, C and D), whereas that by R586L was not affected by up to 600 μM of DNP-SG or 75 μM of E217G (Fig. 6, C and D).

Transport of [3H]E217G and [3H]TC by R586L and R1096-mutant MRP2 and L1084-mutant MRP3. The role of Arg at positions 586 and 1096 in rat MRP2 in
the transport of TC was further studied by substituting
these amino acids with Lys (R586K and R1096K) or
other neutral amino acids [Ile for R586 (R586I) and
Met for R1096 (R1096M)]. Mutant MRP2 proteins were
detected as bands of 175 kDa (Fig. 7A) at the same
position as wild-type MRP2. ATP-dependent uptake of
\[^{3}H\]E_{2}17\beta G was observed in all R586 and R1096 mutant
MRP2 (Fig. 8A). In addition, significant ATP-dependent
uptake of \[^{3}H\]TC was also observed for R586 and R1096
MRP2 mutants, except for R586K (Fig. 8A).

Fig. 4. Transport of \[^{3}H\]TC by R586L
and R1096L. A: time profiles for the
uptake of \[^{3}H\]TC (100 nM) by R586L (Δ)
and ( ), and R1096L (●) and ( □) in
the presence of ATP (Δ and ●) or AMP ( ■
and □) at 37°C. Values are means of
duplicate determinations. B: osmotic
sensitivity of \[^{3}H\]TC (100 nM) uptake
into membrane vesicles expressing
R586L and R1096L. Membrane vesicles
were incubated at 37°C for 5 min
in medium containing several different
concentrations of sucrose (0.25–1 M) in
the presence of ATP or AMP. Symbols
are the same as given in A. Values are
means ± SE of triplicate determina-
tions. Where bars are not shown, SE is
contained within the limits of the sym-
bol. C: the uptake of \[^{3}H\]TC (100 nM)
by R586L (filled bars) and R1096L
(crosshatched bars) was measured in
the presence of the indicated nucleo-
tides (5 mM) at 37°C for 5 min. No
ATP-regenerating system was in-
cluded except ATP-regenerating sys-
tem (ATP reg). Values represent %con-
trol values obtained in the presence of
ATP-regenerating system and are
means ± SE of triplicate determina-
tions.

Fig. 5. Eadie-Hofstee plot for the transport of TC (A), 17β-estradiol 17-β-d-glucuronide (E_{2}17\beta G; B),
and 2,4-dinitrophenyl-S-glutathione (DNP-SG; C) by mutant MRP2. The initial velocity of uptake (v) of \[^{3}H\]TC (5 min),
[^{3}H]E_{2}17\beta G (2 min), and \[^{3}H]DNP-SG (2 min) by wild-type MRP2 ( ●), R586L (Δ), and R1096L (■) was examined at
37°C in the presence of unlabeled compounds (concentration (c)). Values represent the MRP2-dependent uptake
determined by subtracting the uptake into MRP2-expressing vesicles from that into the GFP control vesicles. The
solid line represents the fitted line. Values are means ± SE of triplicate determinations. Where bars are not shown,
SE is contained within the limits of the symbol.
Moreover, Leu at position 1084 in rat MRP3, which corresponds to Arg at position 1096 in rat MRP2 (Fig. 1B), was substituted by other cationic and neutral amino acids including Arg (L1084R MRP3), Lys (L1084K MRP3), Met (L1084M MRP3), and Val (L1084V MRP3). Bands of 175 kDa were also detected in wild-type and mutant MRP3, except for L1084R MRP3, in which only an 80-kDa band was detectable (Fig. 7B). ATP-dependent transport of E217βG and TC was detectable in wild-type MRP3, L1084M MRP3, and L1084V MRP3, but not in L1084K MRP3 (Fig. 8B).

Concentration dependence of TC uptake by wild-type MRP3, L1084M MRP3, and L1084V MRP3. Transport kinetics was determined for [3H]TC uptake by mutant MRP3 and compared with that of wild-type MRP3 to examine whether the substitution of Leu at 1084 of rat MRP3 affects the transport properties. The initial velocity of uptake of [3H]TC by these MRP3 was determined in the presence of unlabeled TC (0.2–150 μM; Fig. 9). Kinetic analysis yielded $K_m$ of 52.2 ± 5.8, 37.3 ± 4.5, and 73.1 ± 11.7 μM for wild-type MRP3, L1084M MRP3, and L1084V MRP3, respectively (Table 2). No statistically sig-

Table 1. Kinetic parameters for transport of TC, E217βG, and DNP-SG by wild-type MRP2, R586L, and R1096L

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<th>TC</th>
<th>E217βG</th>
<th>DNP-SG</th>
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<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
<td>$K_m$</td>
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<tr>
<td>WT MRP2</td>
<td>ND</td>
<td>7.46 ± 3.39</td>
<td>ND</td>
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<tr>
<td>R586L</td>
<td>28.8 ± 6.9</td>
<td>0.189 ± 0.022</td>
<td>5.00 ± 0.16</td>
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<tr>
<td>R1096L</td>
<td>41.6 ± 5.6</td>
<td>0.277 ± 0.029</td>
<td>5.00 ± 0.16</td>
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Values are means ± computer-calculated SD. Values for Michaelis constant ($K_m$; μM) and maximum velocity ($V_{max}$; nmol min$^{-1}$ mg$^{-1}$) were calculated on the basis of the data shown in Fig. 5. TC, taurocholate; E217βG, 17β-estradiol 17-(β-D-glucuronide); DNP-SG, 2,4-dinitrophenyl-S-glutathione; ND, not determined. *$P < 0.05$, significantly different from $K_m$ of wild-type (WT) multidrug resistance-associated protein 2 (MRP2) by ANOVA followed by Dunnett’s test.

Fig. 6. Mutual effects of TC and authentic MRP2 substrates in wild-type MRP2, R586L, and R1096L. The effect of TC on the uptake of [3H]DNP-SG (50 nM; A) and [3H]E217βG (55 nM; B) by wild-type MRP2 (●), R586L (▲), and R1096L (▼) was examined at 37°C. The effect of DNP-SG (C) and E217βG (D) on the uptake of [3H]TC (100 nM) by R586L and R1096L was examined at 37°C. The MRP2-dependent uptake was obtained by subtracting the uptake into GFP control vesicles from that into respective MRP2-expressing vesicles in the presence of ATP. Results are given as a ratio to the control values determined in the absence of unlabeled compounds. Values are means ± SE of triplicate determinations. Where bars are not shown, SE is contained within the limits of the symbol.
significant differences were observed in the $K_m$ values for TC among wild-type and L1084-mutated MRP3.

**DISCUSSION**

In the present study, we substituted eight cationic amino acids in TMs of rat MRP2 with neutral amino acids (Fig. 1) to examine the transport activity of these mutated MRP2. The ability to transport $[^3H]$TC was acquired by substituting Arg at position 586 or 1096 with Leu (Fig. 3). The osmotic sensitivity of TC accumulation by membrane vesicles expressing R586L and R1096L (Fig. 4B) suggests that TC molecules are indeed transported in an ATP-dependent manner by these mutated transporters. Along with other MRP-family proteins (10, 24), ATP was the most effective nucleotide triphosphate in stimulating the transport of TC concerned (Fig. 4C). The transport was saturable with calculated $K_m$ of 28.8 ± 6.9 and 41.6 ± 5.6 mM (Table 1) for R586L and R1096L, respectively. These values are slightly higher than that reported in rat Bsep/spgp (5.3 μM) (6) or MRP3 (15.9 μM) (11). Although R1096L acquired the ability to transport TC, the transport characteristics of this mutant with respect to conjugated metabolites are similar to those of wild-type MRP2 (Table 1). In contrast, the $K_m$ values of R586L for the transport of E217βG have been reported to be significantly reduced (by a factor of 5) compared with wild-type MRP2 (14).

To further characterize the acquired transport site for TC in R586L and R1096L, the mutual effects of TC and conjugated metabolites were examined. TC exhibited an inhibitory effect on the uptake of $[^3H]$DNP-SG and $[^3H]$E217βG in both R586L and R1096L, with IC$_{50}$ of ~200 μM (Fig. 6, A and B), which were approximately seven and five times higher than the respective $K_m$ values of R586L and R1096L for the transport of TC (Table 1). In contrast, transport of $[^3H]$TC was not affected by DNP-SG or E217βG in R586L and was

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**Fig. 7.** Western blot analysis of mutant MRP2 and mutant MRP3. Membrane vesicles (50 μg protein) were separated on an 8.5% polyacrylamide slab gel containing 0.1% SDS. The fractionated proteins were transferred onto a membrane filter by electroblotting and analyzed using polyclonal anti-MRP2 antiserum (A) and anti-MRP3 antiserum (B).

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**Fig. 8.** Transport of $[^3H]$E217βG and $[^3H]$TC by mutant MRP2 and MRP3. A: uptake of $[^3H]$E217βG (55 nM; 2 min; left) and $[^3H]$TC (100 nM; 5 min; right) by GFP control and wild-type MRP2 and its mutants was examined at 37°C. B: uptake of $[^3H]$E217βG (55 nM; 5 min; left) and $[^3H]$TC (100 nM; 5 min; right) by GFP control and wild-type MRP3 and its mutants was examined at 37°C. Results are given as the ATP-dependent uptake determined by subtracting the uptake in the presence of AMP from that in the presence of ATP. Values are means ± SE of triplicate determinations. Where error bars are not shown, SE is minimal. **P < 0.01, the uptake into MRP2-expressing membrane vesicles was significantly different from GFP control by ANOVA followed by Dunnett’s test. #P < 0.05, ##P < 0.01, the uptake into MRP3-expressing membrane vesicles was significantly different from GFP control by ANOVA followed by Dunnett’s test.
enhanced in R1096L by these conjugates (Fig. 6, C and D), suggesting that the acquired interaction sites for TC in R586L and R1096L may not be exactly the same as those for DNP-SG or E217βG. Because mutual inhibition has been observed between DNP-SG, E217βG, and TC in wild-type MRP3 (11), the molecular mechanism for substrate recognition differs between MRP3 and these mutant MRP2 (R586L and R1096L). Moreover, TC exhibited a stimulatory effect on the wild-type and these mutant MRP2 (R586L and R1096L). More.

The role of Arg at positions 586 and 1096 in rat MRP2 was additionally explored by introducing fur.

Table 2. Kinetic parameters for transport of TC by WT MRP3, L1084MRP3, and M1084MRP3

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<th>$K_m$ (μM)</th>
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<tr>
<td>WT MRP3</td>
<td>52.2 ± 5.8</td>
<td>0.605 ± 0.047</td>
</tr>
<tr>
<td>L1084MRP3</td>
<td>37.3 ± 4.5</td>
<td>0.522 ± 0.049</td>
</tr>
<tr>
<td>M1084MRP3</td>
<td>73.1 ± 11.7</td>
<td>0.451 ± 0.056</td>
</tr>
</tbody>
</table>

Values are means ± computer-calculated SD. $K_m$ (in μM) and $V_{max}$ (in nmol·min$^{-1}$·mg$^{-1}$) were calculated on the basis of the data shown in Fig. 9.
duced (Fig. 7B). Although we do not have a satisfactory explanation to account for the production of a shorter band of ~80 kDa in L1084RMRP3, it is possible this mutant protein may have easy access to some type of protease(s) that may be produced due to the Arg substitution at position 1084. Loo and Clarke (25) demonstrated that the substitution of Gly with Cys at position 341, located in the middle of the TM6 of P-glycoprotein, resulted in cleavage of the extracellular loop located between TM1 and TM2 to produce a truncated protein product of 130 kDa. To examine the role of the amino acid at position 1084 in rat MRP3, we also substituted L1084 with Lys (L1084KMRP3), Met (L1084MMRP3), and Val (L1084VMRP3). Because the molecular weight of these three mutant MRP3 is the same as that of wild-type MRP3 (Fig. 7B), we assumed that the extent of glycosylation of the mutant proteins is not altered by introduction of the mutation. The transport activity for both TC and E217G was completely abolished in L1084KMRP3 (Fig. 8B), irrespective of exhibiting the same protein expression level as wild-type MRP3 (Fig. 7B). Disruption of the conformation required for transport activity, produced by introduc-
ing a cationic amino acid inside the potential α-helix region, may be one possible reason for the disappearance of TC and E217G transport activity in L1084KMRP3. As far as L1084MMRP3 and L1084VMRP3 are concerned, the K values for TC were similar to that of wild-type MRP3 (Fig. 9 and Table 2). In addition, although some reduction in transport activity for TC was found for L1084MMRP3 and L1084VMRP3, compared with wild-type MRP3, the reduction in transport activity for E217G was comparable with that for TC (Fig. 8B), suggesting that the recognition/transport of TC and E217G cannot be distinguished by these mutants.

In conclusion, we have demonstrated that substitution of a single cationic amino acid in the predicted TM11 and TM14 provides rat MRP2 with a transport site for monovalent bile acids, which is distinguishable from the native transport sites for glutathione and glucuronide conjugates. These findings provide new insights into the multispecific and potential transport activity of MRP families.

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