Characterization of the Cellular Localization, Expression Level, and Function of SNP Variants of MRP2/ABCC2

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Purpose. The presence of single nucleotide polymorphisms (SNPs) has been reported for multidrug resistance–associated protein 2 (MRP2/ABCC2). The purpose of the current study was to characterize the localization, expression level, and function of MRP2 variants.
Methods. The expression and cellular localization of the wild-type and three kinds of reported SNP variants of MRP2 molecules were analyzed in LLC-PK1 cells after infection with the recombinant Tet-off adenoviruses. Their function was determined by using the isolated membrane vesicles from the infected LLC-PK1 cells.
Results. The transport activity for $E_2$17βG, LTC_4, and DNP-SG, normalized by the expression level of MRP2, was similar between the wild-type, V417I, and A1450T MRP2s. The transport activity of S789F MRP2 was slightly higher than that of wild-type MRP2. However, the expression level of S789F and A1450T MRP2 proteins was significantly lower compared with the wild-type and V417I MRP2. In addition, although the wild-type and V417I MRP2 were exclusively localized in the apical membrane, S789F and A1450T MRP2 were located in the apical membrane and also in the intracellular compartment.
Conclusions. These results suggest that the most frequently observed V417I substitution may not affect the *in vivo* function of MRP2, whereas the much less frequently observed S789F and A1450T may be associated with the reduced *in vivo* function.

KEY WORDS: biliary excretion; Dubin-Johnson syndrome; MRP2/ABCC2; SNPs.

INTRODUCTION

Attention has been focused on single nucleotide polymorphisms (SNPs) of drug metabolizing enzymes as one of the important factors influencing interindividual differences in drug disposition. For example, an important association has been reported between the genotype and phenotype of certain SNPs in cytochrome P450 (CYP) enzymes, such as CYP2C9, CYP2C19, and CYP2D6 (1–3). It is now possible to predict the *in vivo* drug disposition in subjects with SNP-type enzymes from *in vitro* data obtained using recombinant enzymes (1–3). In addition to these drug metabolizing enzymes, transporters also play important roles in determining drug disposition, although only a limited number of reports are available on the relationship between their genotype and phenotype (4–8).

In the current study, we have focused on the SNPs in MRP2/ABCC2, one of the ATP-binding cassette transporters expressed on the bile canalicular membrane, apical membrane of enterocytes, and renal tubules (8–14). It is established that MRP2 plays a primary role in the biliary excretion and in restricting the oral absorption of its substrates including anionic drugs and glutathione and glucuronide conjugates of xenobiotics (8–14). It has also been established that mutations in the MRP2 gene are responsible for the pathogenesis of the Dubin-Johnson syndrome (DJS), characterized by a defect in the biliary excretion of bilirubin glucuronides (8,15,16). Considering that some MRP2 mutations found in DJS patients are associated with the loss of transport activity and/or the ability to traffic to the apical membrane by the single amino acid alteration (17,18), it is possible that the MRP2 SNPs may also be associated with alterations in its function and, consequently, may be responsible for the interindividual differences in the disposition of substrate drugs (8).

Recently, the SNPs of MRP2 have been analyzed in 48 healthy Japanese subjects and also in 72 cell lines established from clinically dissected human tumors (8,19,20). In the Japanese volunteers, six kinds of SNPs have been identified for the MRP2 gene (19). The allele frequencies of frequently observed C-24T, G1249A, and C3972T SNPs are 18.8%, 12.5%, and 21.9%, respectively (19). Among them, only G1249A is associated with amino acid alterations, from Val to Ile at amino acid position 417 (19). Only one respective heterozygote subject was observed out of 48 volunteers for C2302T (exon 18, Arg768Trp), C2366T (exon 18, Ser789Phe), and G4348A (exon 31, Ala1450Thr) (19), and their allele frequency was calculated to be 1%. Among these three kinds of less frequently observed SNPs, C2302T (exon 18, Arg768Trp) has been identified as the mutation responsible for DJS (21). A detailed analysis has revealed that the maturation of this mutated protein is impaired, resulting in a low expression in cDNA-transfected LLC-PK1 cells (18).

The purpose of the current study was to characterize the function of MRP2 SNPs. For the analysis, we used Tet-off recombinant adenovirus systems. The recombinant adenoviruses were used to infect LLC-PK1 cells in order to identify the cellular localization and function of MRP2 SNPs.

MATERIALS AND METHODS

Materials

[^{3}H]$E_2$17βG (51.0 μCi/nmol) and [^{3}H]LTC_4 (52.0 μCi/nmol) with purities of 98.5% and 99.0%, respectively, were purchased from New England Nuclear (Boston, MA, USA). Unlabeled and [^{3}H]-labeled 2,4-dinitrophenyl-*S*-glutathione ([^{3}H]DNP-SG, 44.8 μCi/nmol) were synthesized as previously

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ABBREVIATIONS: Ad, adenovirus; CYP, cytochrome P450; DNP-SG, 2,4-dinitrophenyl-S-glutathione; DJS, Dubin-Johnson syndrome; $E_2$17βG, 17β-estradiol 17β-D-glucuronide; ER, endoplasmic reticulum; K_m, Michaelis constant; LTC_4, leukotriene C_4; MOI, multiplicity of infection; MRP, multidrug resistance–associated protein; SNPs, single nucleotide polymorphisms; TRE, tet-responsive element; tTA, tetracycline-responsive transcriptional activator; V_{max}, maximum transport velocity.

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described (22). ATP, creatine phosphate, creatine phosphokinase, acivicin, and glutathione *S*-transferase were purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals used were commercially available and of reagent grade.

LLC-PK1 and HEK293 cells were cultured in Medium 199 (GIBCO BRL, Gaithersburg, MD, USA) and high glucose Dulbecco's Modified Eagle Medium (GIBCO BRL), respectively, after addition of 10% fetal bovine serum, peniciliin (100 U/ml), and streptomycin (100 mg/ml).

Construction of Recombinant Adenoviruses Containing MRP2

Human MRP2 cDNA located between the Not I and Nco I sites of pBluescript SK(-) (Stratagene, La Jolla, CA, USA) was provided by Dr. Kuwano (Kyushu University School of Medicine). According to the information about SNPs in the human MRP2 gene (19), point mutations were introduced into the wild-type MRP2 using the QuickChange site-directed mutagenesis kit (Stratagene). We have generated the following four kinds of missense mutations reported previously: Val417Ile, Arg768Trp, Ser789Phe, and Ala1450Thr. The introduction of the mutations was verified by full sequencing. The MRP2 cDNA was subcloned into the Not I and Nco I sites of the pTRE shuttle, resulting in the production of pTRE shuttle-MRP2. pTRE shuttle-MRP2 has I-CeuI and PI-SceI sites upstream and downstream of the MRP2 expression cassette, respectively. The I-CeuI/PI-SceI digested fragments of pTRE shuttle-MRP2 were ligated with I-CeuI/PI-SceI digested Adeno-X Viral DNA (BD Biosciences, Palo Alto, CA, USA), resulting in pAd-MRP2. pAd containing tetracycline-responsive transcriptional activator (tTA) (pAd-tTA) was purchased from Clontech (Palo Alto, CA, USA).

To generate the recombinant viruses, both pAd-MRP2 and pAd-tTA were digested with PacI. Linearized DNAs were transfected to HEK293 cells grown on a 12-well dish with Fugene 6 (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. Viruses (Ad-MRP2 and Ad-tTA) were prepared as previously described (23). Recombinant viruses were purified by CsCl gradient centrifugation, dialyzed with a solution containing 10 mM Tris (pH7.5), 1 mM $MgCl_2$, and 10% glycerol, and stored in aliquots at -80°C. Then, the resulting virus titer was determined as previously described (24).

Infection of Recombinant Adenovirus and Membrane Vesicle Preparation

For the preparation of the isolated membrane vesicles, LLC-PK1 cells cultured in a 15-cm dish were infected by recombinant adenoviruses containing the wild-type and SNP-type MRP2 (25 MOI) and tTA (25 MOI). As a negative control, cells were infected with Ad-tTA (50 MOI). Cells were harvested at 48 h after infection, and then the membrane vesicles were isolated from 1 to 2×10^8 cells using a standard method described previously in detail (25). Briefly, cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl, 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, 250 mM sucrose, pH 7.4 at 4°C) and homogenized with Dounce B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top of a 38% (w/v) sucrose solution in 5 mM Tris-HEPES, pH 7.4 at 4°C, and centrifuged in a Beckman SW41 rotor centrifuged 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 TS buffer. Vesicles were formed by passing the suspension 30 times through a 27-gauge needle using 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.

Immunoblots

For the Western blot analysis, the isolated membrane vesicles were dissolved in 3x SDS sample buffer (New England BioLabs, Beverly, MA, USA), and subjected to electrophoresis on a 7% SDS-polyacrylamide gel with a 4.4% stacking gel. The molecular weight was determined using a prestained protein marker (New England BioLabs). Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a blotter (Bio-Rad Laboratories, Richmond, CA, USA) at 15 V for 1 h. The membrane was blocked with 2.5% skimmed milk for 1 h at room temperature. Then, the membrane was incubated for 40 min at room temperature with anti-MRP2 rabbit serum, diluted with skimmed milk (1:500), which was raised against 12 amino acids at the carboxyl terminus of the deduced MRP2 sequence (EAGIENVNSTKF). For the detection of MRP2, the membrane was allowed to bind to Alexa Fluor 680 goat anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR, USA). The fluorescence was assessed in a densitometer (Odyssey, ALOKA, Tokyo, Japan).

Immunohistochemical Staining

For the immunohistochemical staining, LLC-PK1 cells were grown in 12-well dishes. Recombinant adenoviruses containing MRP2s (25 MOI) and tTA (25MOI) were infected 48 h prior to starting the experiments. After fixation with ice-cold methanol for 10 min and permeabilization in 1% Triton X-100 in PBS for 10 min, cells were incubated with the monoclonal antibody against MRP2 [M2-6 (Molecular Probes) diluted 40-fold in PBS] for 1 h at room temperature. Then, cells were washed three times with PBS and incubated with goat anti-mouse IgG Alexa 488 (Molecular Probes) diluted 250-fold in PBS for 1 h at room temperature and mounted in VECTASHIELD Mounitng Medium (Vector Laboratories, Burlingame, CA, USA). The localization of MRP2 protein was visualized by confocal lazer microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY, USA). *En face* views were used to determine the expression level of MRP2, and then areas were selected to collect localization of the signal.

Vesicle Transport Assays

The uptake study of [^{3}H]$E_2$17βG (400 nM), [^{3}H]LTC_4 (100 nM), and [^{3}H]DNP-SG (1 μM) was performed as previously reported (26). Briefly, the transport medium (10 mM Tris, 250 mM sucrose, and 10 mM $MgCl_2.6H_2O$, pH 7.4) con-

tained the ligands, 5 mM ATP, and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/μl of creatine phosphokinase). An aliquot of transport medium (17–18 μl) was mixed rapidly with the vesicle suspension (5 μg protein in 2–3 μl). The transport reaction was stopped by the addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.4). The stopped reaction mixture was passed through 0.45-μm HA filters (Millipore Corp.) and then washed twice with 5 ml of stop solution. The radioactivity retained on the filter was measured in a liquid scintillation counter (LS 6000SE, Beckman Instruments, Fullerton, CA, USA) after the addition of scintillation cocktail (Clear-sol I, Nacarai Tesque, Tokyo, Japan). Ligand uptake was normalized in terms of the amount of membrane protein.

RESULTS

Expression of Human MRP2 in LLC-PK1 Cells Using Recombinant Tet-off Adenoviruses

The expression level of MRP2 was examined by Western blot analysis of the membrane fraction isolated from the MRP2-expressing LLC-PK1 cells. It was found that the human MRP2 antibody recognized the band with an approximate molecular weight of 190 kDa (Fig. 1), which is consistent with the previous report (27). The expression level of V417I, S789F, and A1450T MRP2 was 82% and 80%, 23% and 25%, and 18% and 8%, respectively, in duplicate experiments (Fig. 1). R768W MRP2 showed no staining, presumably due to rapid degradation as previously reported (18).

Localization of Human MRP2 in LLC-PK1 Cells

The localization of human MRP2 was determined by immunohistochemical staining. Using an antibody against human MRP2, the fluorescence signal was detected exclusively at the apical membrane of LLC-PK1 cells after simultaneous infection of Ad-tTA and Ad-wild-type MRP2 (Fig. 2). No specific fluorescence signal was observed at the apical membrane or in the cytoplasm after infection of either Ad-tTA or Ad-MRP2 (Fig. 2). We also analyzed the cellular localization of MRP2 variants. As a positive control to determine the localization of MRP2, we also determined the localization of R768W MRP2, which has been reported to be responsible for the sideration of the DJS (18). In our experimental system, R768W MRP2 was located in the intracellular compartment of LLC-PK1 cells, which is consistent with the previous report (18). V417I MRP2 showed the same localization as wild-type MRP2 (Fig. 2), whereas two variants (S789F and A1450T MRP2s) were expressed not only at the apical surface, but also intracellularly (Fig. 2). The fraction of A1450T MRP2 located intracellularly was higher than that of S789F MRP2 (Fig. 2).

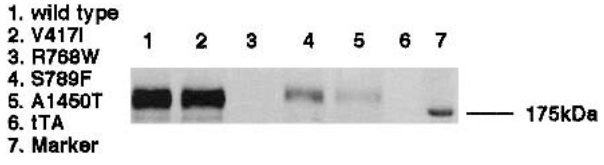

Fig. 1. Expression level of MRP2 in LLC-PK1 cells. Expression level of the wild-type and SNP variants of MRP2 proteins was determined in the isolated membrane vesicles (5 μg) from LLC-PK1 cells by Western blot analysis.

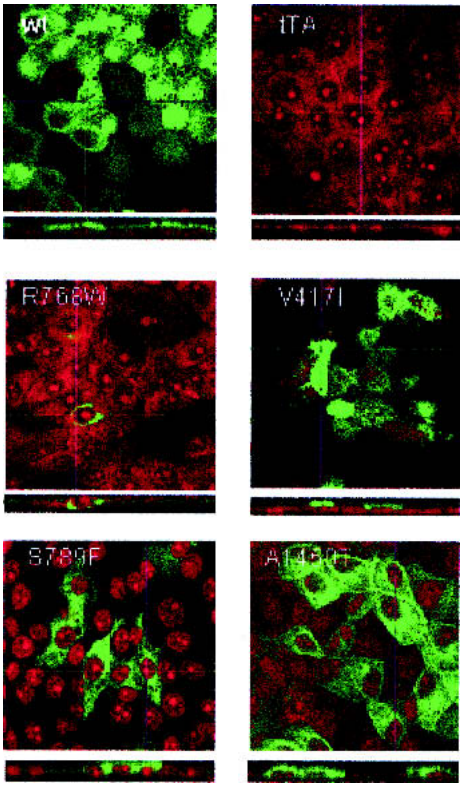

Fig. 2. Localization of MRP2 in LLC-PK1 cells. The immunolocalization of MRP2 molecules in the infected LLC-PK1 cells was determined using polyclonal antibody for human MRP2 (green). Nuclei were stained with PI (red). The top and bottom parts show the en face and the Z-sectioning images, respectively.

Transport Activity of MRP2

After confirming the expression level, the uptake of MRP2 substrates was examined using membrane vesicles. Typical substrates of MRP2, such as [^{3}H]$E_2$17βG, [^{3}H]LTC_4, and [^{3}H]DNP-SG were clearly taken up into membrane vesicles expressing the wild-type MRP2 in an ATP-dependent manner (Figs. 3a, 3e and 3i). Figure 3 also shows the time profiles for the uptake of MRP2 substrates by MRP2 variants. Then, the uptake given as the amount of ligand taken up per milligram membrane protein (Fig. 3) was normalized by the expression level of MRP2s by considering the results of the Western blot analysis (Fig. 1), in order to identify the intrinsic transport activity of each MRP2 (Fig. 4). As shown in Fig. 4, no significant difference was observed between the wild-type and V417I MRP2s, whereas S789F MRP2 showed approximately a 1.4- to 2.0-fold higher uptake of three substrates compared with the wild-type MRP2 (Fig. 4). For A1450T, the expression level was too low to detect the transport function of $E_2$17βG (Fig. 3d), whereas the normalized uptake of DNP-SG and LTC_4 was almost identical to that of the wild-type (Figs. 4b and 4c).

Moreover, the kinetic parameters for the transport of [^{3}H]DNP-SG by wild-type and S789F MRP2s were determined (Fig. 5). The K_m values were 24.5 ± 1.5 and 10.7 ± 1.8 μM, and V_{max} values were 3.02 ± 0.14 and 0.430 ± 0.049

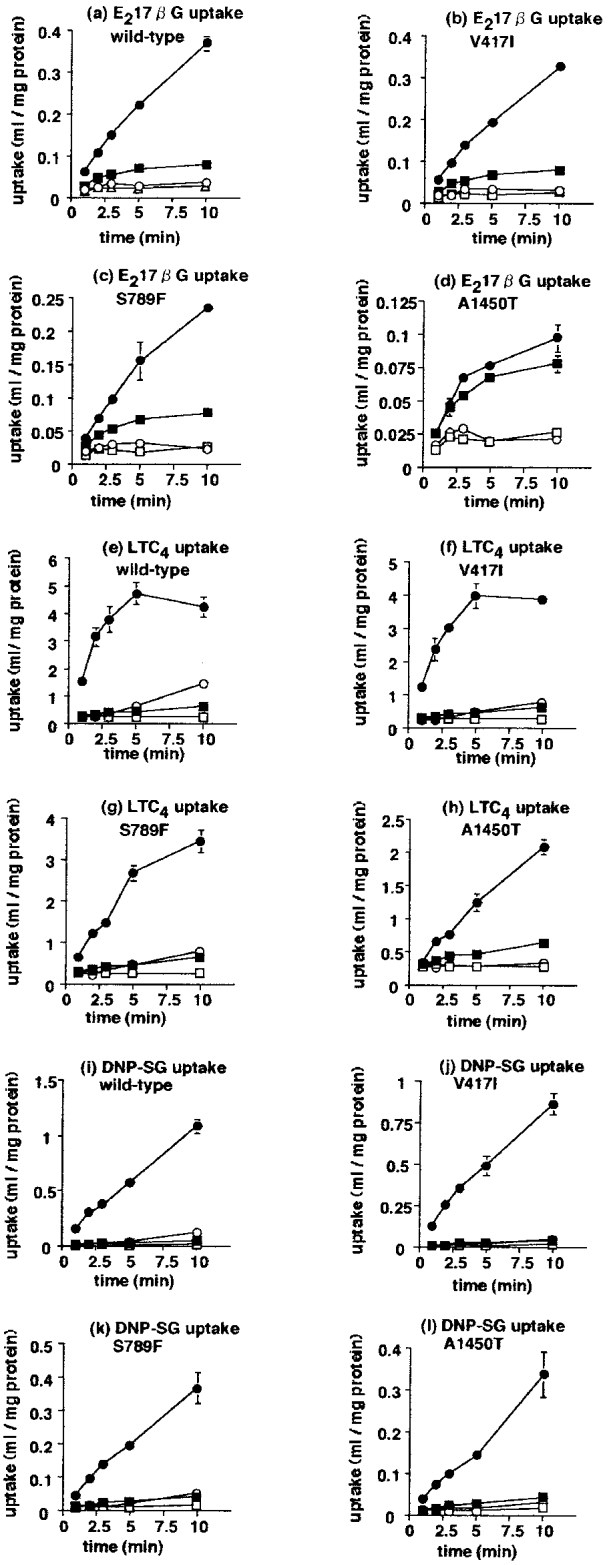

Fig. 3. Time profiles for the transport of ligands mediated by MRP2 expressed in LLC-PK1 cells. Time profiles for the uptake of (a–d) $E_2$17βG, (e–h) LTC_4, and (i–l) DNP-SG were measured in isolated membrane vesicles prepared from LLC-PK1 cells expressing the wild-type and SNP variants of MRP2 proteins. Membrane vesicles were incubated at 37°C with [^{3}H]$E_2$17βG (400 nM), [^{3}H]LTC_4 (100 nM), and [^{3}H]DNP-SG (500 nM), in the medium with or without ATP. Note that the absolute values of the ordinate are different among MRP2 variants. Key. ●, wt-MRP2 or SNPs variants with ATP; ○, wt-MRP2 or SNPs variants without ATP; ■, tTA with ATP; □, tTA without ATP.

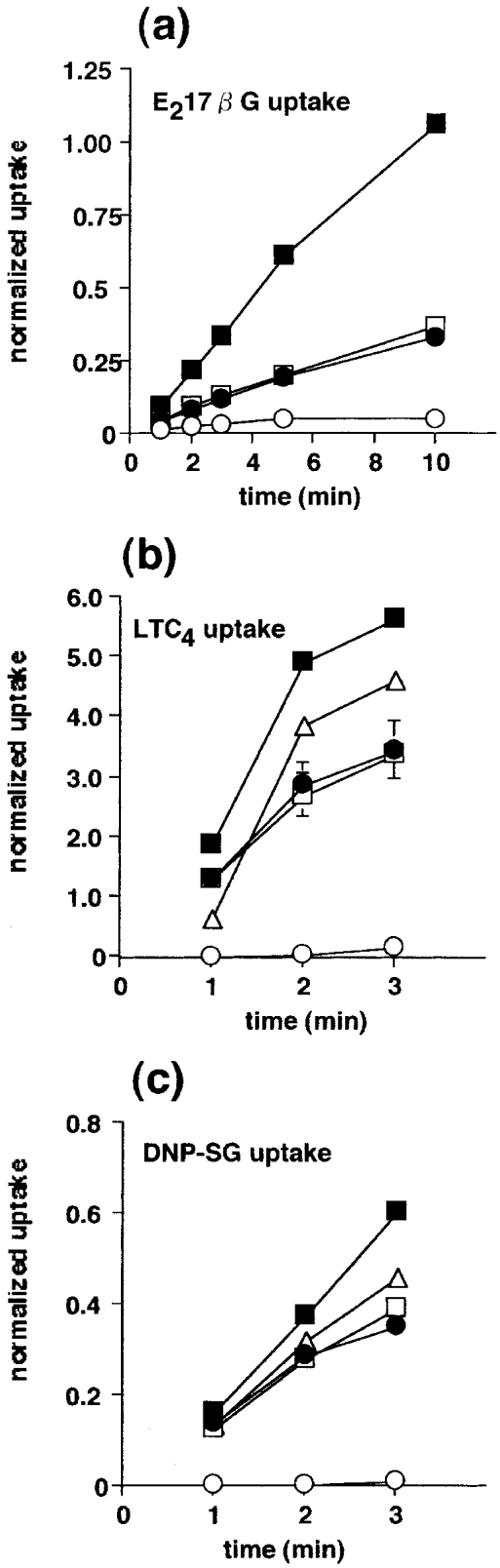

Fig. 4. Transport activity of MRP2 variants after normalization of their expression levels. Uptake of (a) $E_2$17βG, (b) LTC_4, and (c) DNP-SG into isolated membrane vesicles from LLC-PK1 cells expressing MRP2 variants was compared after normalization of their exprssion levels. Transport activity (Fig. 3) was normalized by the protein expression level determined by Western blot analysis (Fig. 1). The uptake of $E_2$17βG by A1450T was too low to be normalized by the expression level (Fig. 3d). Key: ○, tTA; ●, wt-MRP2; □, V417I; ■, S789F; △, A1450T.

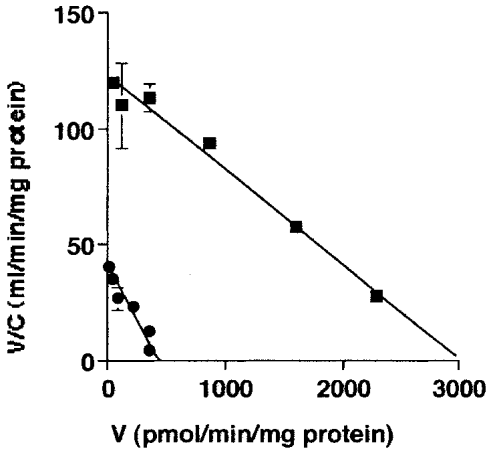

Fig. 5. Eadie-Hofstee plot of the ATP-dependent uptake of [^{3}H]DNP-SG. Saturation of the ATP-dependent uptake of [^{3}H]DNP-SG was determined using the membrane vesicles from LLC-PK1 cells expressing wild-type (squares) and S789F MRP2s (circles). Membrane vesicles were incubated at 37°C for 2 min in medium containing [^{3}H]DNP-SG (0.1 μM) with or without ATP and ATP-regenerating system. The ATP-dependent uptake was obtained by subtracting the value in the absence of ATP from that in its presence. Each point and bar represent the mean ± SE of three different experiments. The solid line represents the fitted line.

nmol·min^{-1}·mg^{-1} protein, for the membrane vesicles expressing the wild-type and S789F MRP2s, respectively. The transport activity of S789F MRP2 under linear conditions, calculated by correcting the V_{max} value by the protein expression level prior to the division by the K_m value, was approximately 1.3-fold higher than that of the wild type.

DISCUSSION

In the current study, we have characterized the effect of three kinds of SNPs on the expression level, localization, and function of MRP2 proteins. To examine the function of MRP2, it is necessary to establish experimental systems in which the MRP2 proteins are highly expressed. We focused on recombinant adenovirus system, as high expression of exogenous genes can be expected. Initially, we constructed wild-type MRP2 expressing adenovirus vector. However, the viruses were not generated after the introduction of the linearized adenoviral DNA, due to the death of the transfected HEK293 cells. Because it is possible that high expression of MRP2 is lethal to transfected HEK 293 cells, we constructed the Tet-off adenovirus vector containing wild-type MRP2 (pAd-MRP2). The Tet-off system was used because proteins cannot be expressed unless simultaneous infection of Ad-tTA is performed.

After confirming that the wild-type MRP2 is expressed on the apical membrane with a molecular weight of 190 kDa as established previously (Figs. 1 and 2) (27), the transport activity was examined using membrane vesicles. The rank order for the transport activity, defined as the initial velocity for the uptake of ligands divided by the ligand concentration in the medium, was in the order LTC_4 >> DNP-SG > $E_2$17βG (Fig. 3), which is consistent with the previous report involving isolated membrane vesicles from the HEK293 cells stably expressing wild-type human MRP2 (28), although the order of DNP-SG and $E_2$17βG was opposite in some reports, depending on the different experimental conditions (26).

To compare the transport activity of MRP2 variants per molecule, the uptake was normalized by the band density determined in the Western blot analysis (Fig. 4). V417I and S789F MRP2s showed similar substrate specificity for glucuronide- and glutathione-conjugates to that of wild-type MRP2 (Figs. 3 and 4). In contrast, the transport activity of glutathione-conjugates (LTC_4 and DNP-SG) in membrane vesicles expressing A1450T MRTP2 was 1/4 to 1/5 of that in the membrane vesicles expressing wild-type MRP2, whereas A1450T MRP2-mediated transport of $E_2$17βG was much less than wild-type MRP2-mediated transport of this glucuronide-conjugate (Figs. 3 and 4). These results suggest that the alteration in amino acid residue at 1450 may affect the mode of substrate discrimination between glutathione- and glucuronide-conjugates. The presence of amino acid residues in MRP2 molecules which are important in discriminating glutathione- and glucuronide-conjugates has also been suggested by a series of site-directed mutagenesis studies (29).

In addition, kinetic parameters for the transport of [^{3}H]DNP-SG were compared between wild-type and S789F MRP2s. The K_m value for the wild-type MRP2-mediated transport of [^{3}H]DNP-SG was 24.5 μM, which is consistent with the value reported using isolated membrane vesicles from MDCK cells stably expressing human MRP2 (27). The analysis revealed that the K_m and the corrected V_{max} values of [^{3}H]DNP-SG for S789F MRP2-mediated transport are approximately 43% and 57% that of wild-type MRP2, respectively. Consequently, the transport activity of S789F MRP2, defined as the corrected V_{max}/K_m, is approximately 1.3-fold higher than that of wild-type MRP2, which is consistent with its transport activity defined as the initial velocity for the uptake of ligands divided by the ligand concentration in the medium (Fig. 4).

The cellular localization and the expression level of MRP2 variants were also determined in LLC-PK1 cells. Wild-type and V417I MRP2s exhibited apical localization, which is consistent with the localization under physiological conditions. Moreover, the expression level of V417I MRP2 in isolated membrane vesicles was the same as that of wild-type MRP2. Together with the finding that the transport activity of V417I MRP2 is the same as that of wild-type MRP2, this suggests that it is possible that these frequently found SNPs may not affect the disposition of substrate drugs among individuals. In contrast, S789F and A1450T MRP2s were expressed not only at the apical surface, but also in the intracellular compartment. Furthermore, the expression level of these two MRP2 variants in membrane vesicles was lower than in wild type, although the molecular weight was similar in the Western blot analysis. It is possible that the low expression of S789F and A1450T MRP2s may be due to the degradation of the protein synthesized in the infected cells. Indeed, both R768W MRP2 mutant and MRP2 mutant, which lacks R1392 and M1393 found in DJS patients, were core glycosylated and localized predominantly within the endoplasmic reticulum (17,18). It was also suggested that these mutations lead to the impaired maturation and trafficking of the protein from the ER to the Golgi complex (17,18). It is possible that the two variants examined in the current study (S789F and A1450T) are also degraded in the same manner. In addition, it is also plausible that the stability of mRNA for

S789F and A1450T MRP2s is low compared with that of the wild type.

Due to this low expression level on the apical surface, it is possible that the function of S789F and A1450T MRP2s under *in vivo* conditions is much lower compared with that of wild-type MRP2. However, although the MOI of the adenovirus for each MRP2 variant was adjusted in the current study, the result in LLC-PK1 cells may not reflect the physiological expression and/or the localization pattern. It may be necessary to determine the expression level and/or the localization of each MRP2 variant in the intact liver/intestinal tissue.

Of the 48 Japanese subjects studied, both S789F and A1450T SNPs were only found in one heterozygous subject and, consequently, their allele frequency was calculated to be only 1%. If we consider the fact that the incidence of DJS in Japan is approximately one case per 300,000 subjects (30), the allele frequency of the mutation associated with the abolished MRP2 function is expected to be 0.3–0.4%. Because S789F and A1450T MRP2s are located within the first and second nucleotide binding domains, respectively, where the DJS mutations are frequently located, it is possible that S789F and A1450T SNPs are associated with the pathogenesis of DJS.

In conclusion, we have compared the localization, expression level, and transport activity of SNP variants of MRP2. It is suggested that the most frequently observed amino acid substitution (V417I) may not affect the drug disposition mediated by MRP2. Although the transport activity per MRP2 molecule was not significantly decreased in much less frequently observed S789F and A1450T MRP2s, their expression level was significantly lower than wild-type MRP2, which may be associated with their intracellular localization. It is possible that the *in vivo* function of these two kinds of variants may be lower than wild-type MRP2.

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