Identification of Basic Residues Involved in Drug Export Function of Human Multidrug Resistance-associated Protein 2*

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Multidrug resistance-associated protein 2 (MRP2)/canalicular multispecific organic anion transporter (cMOAT) is involved in the ATP-dependent export of organic anions across the bile canalicular membrane. To identify functional amino acid residues that play essential roles in the substrate transport, each of 13 basic residues around transmembrane regions (TMs) 6–17 were replaced with alanine. Wild type and mutant proteins were expressed in COS-7 cells, and the transport activity was measured as the excretion of glutathione-methylfluorescein. Four mutants, K324A(TM6), K483A(TM9), R1210A(TM16), and R1257A(TM17), showed decreased transport activity, and another mutant, K578A(TM11), showed decreased protein expression. These five mutants were normally delivered to the cell surface similar to the other fully active mutants and wild type MRP2. The importance of TM6, TM16, and TM17 in the transport function of MRP2 is consistent with the previous observation indicating the importance of the corresponding TM1, TM11, and TM12 on P-glycoprotein (Loo, T. W., and Clarke, D. M. (1999) J. Biol. Chem. 274, 35388–35392). Another observation that MRP2 inhibitor, cyclosporine A, failed to inhibit R1230A specifically, indicated the existence of its binding site within TM16.

Transport across the hepatocellular canalicular membrane into the bile is a critical step in the elimination of endogenous and exogenous compounds in mammals (1, 2). These compounds are converted into amphiphilic anionic conjugates with glutathione, glucuronate, or sulfate by the catalysis of several hepatocellular enzymes (1, 2). The excretion of these conjugates into the bile is mediated by the 190-kDa multidrug resistance-associated protein 2 (MRP2), also termed canalicular multidrug resistance-associated protein (cMRP) (2–5) or canalicular multispecific organic anion transporter (cMOAT) (6, 7). The hepatobiliary secretion of such compounds is therefore strongly reduced in mutant rat strains lacking MRP2 expression such as the TR−rat and the EHBR rat (8–10). Similarly, a deficiency in human MRP2 expression because of a mutation in the gene causes decreased biliary excretion of bilirubin glucuronides, resulting in hereditary conjugated hyperbilirubinemia, known as Dubin-Johnson syndrome (8, 11, 12).

MRP2 belongs to the ATP-binding cassette transporter superfamily that includes other drug efflux pumps such as the 170 kDa P-glycoprotein (P-gp/MDR1) and the 190-kDa multidrug resistance-associated protein (MRP1) (11). MRP2 is closely related to MRP1 and categorized into CFTR/MRP subfamily, also called ABCC subfamily (13). MRP1, exhibiting a multidrug resistance phenotype in tumor cell lines, shares numerous substrates with MRP2, including glutathione conjugates (14, 15). MRP2 is predominantly expressed in canalicular membrane of hepatocyte (14), and also in the kidney, jejunum, and ileum. In contrast to MRP2, MRP1 is expressed in most tissues, but lower in liver, and localized at basolateral site in hepatocyte (6, 8, 9). This difference enhances the physiological significance of MRP2 on hepatobiliary excretion.

The mechanism of how MRP2 transports the substrates is beyond our sights. In the case of P-gp, predicted transmembrane (TM) regions appeared to be particularly important for its function because the sites for interaction with substrates are embedded in lipid bilayer (16, 17). Labeling of P-gp with photoactive analogs of drug substrates (18–21), cysteine-scanning mutagenesis studies using a thiol-reactive substrate (22), and mutational analysis (23–26) suggest that TM6, TM11, and TM12 are particularly important for drug-protein interaction. In MRP1-related transporters, the role of the N-terminal transmembrane regions of MRP1 was reported (27, 28), but the residues involved in substrate-binding have not been identified.

In this study, we tested whether the basic residues around TM6–TM17 of MRP2 participate in binding of substrates. We constructed 13 mutants by site-directed mutagenesis, and these mutants were expressed in COS-7 cells to test transport activity. The results suggested that Lys324, Lys483, Arg1210, and Arg1257 are involved in substrate-protein interaction, whereas Lys578 may be involved in stable expression of MRP2.

EXPERIMENTAL PROCEDURES

Materials—LipofectAMINE and genetin were purchased from Life Technologies, Inc. 5-Chloromethylfluorescein diacetate (CMFDA) and monochlorobimane (mBCl) were obtained from Molecular Probes, Inc. (Eugene, OR). Cyclosporine A (CsA) and fluorescein isothiocyanate conjugated goat anti-rabbit IgG were purchased from Amersham Pharmacia Biotech.

Cells and Vectors—Human MRP2 expression vector, pCl-neo/MRP2, and CHO-K1 cells expressing human MRP2 (29), CHO/MRP2, were kindly provided by Dr. Kuwano (Kyusyu University, Kyusyu, Japan). In brief, NotI-tagged cDNA fragment encompassing the whole open reading frame of human MRP2 was cloned in the NotI site of mamma-

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Lian expression vector pCI-neo (Promega). CHO-K1 cells were transfected with pCI-neo/MRP2 and the stable clone CHO/10/1/1F was selected by 800 μg/ml G418. For mutagenesis, we constructed pCI-neo/ MRP2X, which contains a deletion of XhoI site in the mutagenizing site of pCI-neo, and pCI-neo/MPM2Y, which contains 1448V and L659V mutations, by the method of Kunkel (30). For this, the 1785-base pair Apol-484II fragment of pCI-neo/MPR2, which encodes the C-terminal half encompassing 2449–4207 amino acids of MRP2, was subcloned into pUC1816AE, which has Apol and Eco48II sites in the mutagenizing site, and mutations were introduced. Then the original Apol-484II fragment of pCI-neo/MPR2 vector was exchanged with the mutated one. Each mutation was confirmed by restriction enzyme analysis and DNA sequencing.

Cell Culture—CHO-K1 and CHO/10/1/1F cells were cultured in Eagle’s minimum essential medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 100 μg/ml kanamycin sulfate, and 100 U/ml penicillin in 5% CO2 at 37 °C. To keep MRP2 expression in CHO/10/1/1F, G418 was added at final concentration of 500 μg/ml in the medium. COS-7 cells were routinely maintained in Dulbecco’s modified Eagle’s medium containing 1 mM spermidine and 24 h later, cells were washed and overlaid with 1 ml of serum-free excretion assay medium and incubated at 37 °C for 12 min in excretion measurement (data not shown). Thereafter, the cells were washed twice with ice-cold excretion assay medium and incubated at 4 °C for 60 min with ice-cold excretion assay medium containing 5 μM CMFDA or 100 μM mBCl. Thereafter, the cells were washed twice with ice-cold excretion assay medium and incubated at 37 °C for 12 min in excretion assay medium containing 5 μM mBCl. In Ca2+-directed inhibition assay, cells were incubated with culture medium containing 10 μM Ca2+ at 37 °C for 30 min before preloading of CMFDA. 10 μM CsA was also included in preloading and excretion period. At designated time, 100-μl aliquots of the medium were collected, and the fluorescence of GS-MF and GS-B was determined by measuring the fluorescence at excitation of 490 nm and emission of 520 nm and at excitation of 380 nm and emission of 461 nm, respectively (SPECTRA MAX GEMINI; Molecular Devices). At the end of the experiment, the cells were solubilized by adding 1 ml of 0.1% Triton X-100 in PBS, and the fluorescence of cell lysate was also measured. The total amount of cell proteins was determined by the method of Bradford (31).

Study of Intracellular Accumulation of GS-MF—Cells seeded on coverslips were washed once with excretion assay medium and incubated at 37 °C for 15 min with excretion assay medium containing 2.5 μM CMFDA. Thereafter the cells were washed twice with excretion assay medium and incubated at 37 °C for 30 min. To visualize GS-MF accumulation, coverslips were mounted on slide glass and examined by a fluorescence microscope (model IX70; Olympus, Tokyo, Japan).

Anti-MRP2 Antiserum—Anti-MRP2 antiserum was established by the immunization of rabbit (New Zealand White) with FYFMKEAGI-ENVNSTRF peptide of MRP2 corresponding to C-terminal amino acids 1528–1545.

Immunoblot Analysis of MRP2 Expression—COS-7 cells transfected with MRP2 expression vector were disrupted in RIPA buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin) and centrifuged at 15,000 rpm for 20 min. The supernatant was recovered and the protein concentration was determined by the method of Bradford (31).

RESULTS

Visualization of Intracellular Accumulation of GS-MF with Fluorescence Microscope—We have examined MRP2-mediated excretion of a fluorescent substrate, GS-MF. GS-MF is produced by sequential modification of CMFDA within the cell cytosol. At first CMFDA, which can pass through the plasma membrane, is hydrolyzed by cytoplasmic esterase, and then the metabolite was conjugated with glutathione by glutathione S-transferase (32). The resulting GS-MF is now dependent on specific transporter like MRP2 to go across the membrane. This was shown in Fig. 1. COS-7 cells transfected either with pCI-neo/1/1/1/1 for or pCI-neo, CHO-K1, and CHO/1/1/1/1 were seeded on coverslips and incubated with 2.5 μM CMFDA. After washing, the cells were further incubated with CMFDA-free medium. In CHO/1/1/1/1/1 and COS-7 cells transfected with pCI-neo/1/1/1/1/1, intracellular fluorescence was apparently lower than that of control CHO-K1 and vector-transfected COS-7 cells (Fig. 1). This result reveals that GS-MF is actively excreted by pCI-neo/1/1/1/1/1.2 transfection in these cell systems.

Quantitative Assay of MRP2-mediated Transport Activity—The excretion of GS-MF and GS-B from intact cells or MRP2-transfected cells was examined by using fluorescent substrate, CMFDA, and mBCl. Like GS-MF, GS-B is also a fluorescent glutathione conjugate synthesized within the cells from mBCl. Cells preloaded with 5 μM CMFDA or 100 μM mBCl were incubated in medium without these fluorescent substrate, and the excreted GS-MF or GS-B in the medium was quantitated every 3 min. In CHO/1/1/1/1 and COS-7 cells transfected with pCI-neo/1/1/1/1, the excretion rate of GS-MF into the medium was 3.7- and 3.2-fold higher than that of the control CHO-K1 and COS-7 cells with vector alone (t = 12 min), respectively (Fig. 2). The excretion of GS-B into the medium from CHO/1/1/1/1/1 and COS-7 cells transfected with pCI-neo/1/1/1/1/1 was 3.5- and 2.6-fold higher than that of the control cells (t = 12 min), respectively (Fig. 3). Because of the excretion of GS-MF and GS-B during preloading period with their precursors at 4 °C, the amount of accumulated GS-MF and GS-B in the cells expressing MRP2 was about 30% less than that of control cells at the starting point (t = 0 min) or after incubation period (t = 9 or 12 min) of excretion measurement (data not shown). Therefore, the excreted amount of GS-MF and GS-B was normalized by the amount of GS-MF and GS-B remaining in the cells after excretion measurement (t = 9 or 12 min).

Mutagenesis and Expression in COS-7 Cells—It was previously characterized that P-gp transports wide variety of cationic substrates (33). The mechanism of how those substrates could be recognized by P-gp was speculated to be due to an interaction of negatively charged amino acid residues in transmembrane region of P-gp with cationic substrates (34). Conversely, it is supposed that positively charged amino acid residues are important in anionic substrate transporting MRP2.
To explore this possibility, we constructed MRP2 mutants that are carrying mutations at basic amino acid residues around transmembrane regions by site-directed mutagenesis. We replaced each of 13 basic residues (Lys316, Lys324, Lys329, His439, Lys483, Lys578, Arg590, Arg1023, His1042, Arg1100, Arg1210, Arg1230, and Arg1257) around transmembrane regions from TM6 to TM17 with alanine (Fig. 4). The wild type or mutant MRP2 was transiently expressed in COS-7 cells, and the expression amount was compared by immunoblot analysis (Fig. 5) and immunocytochemical analyses (Fig. 6) with rabbit anti-MRP2 C terminus antiserum. The expression amount of each mutants was consistent in both immunoblot and immunocytochemical analyses. Except for K578A, all of the mutants were expressed at nearly the same level when compared with wild type MRP2.
Only the expression of K578A was faint in both immunoblot and immunocytochemistry.

GS-MF Excretion of Mutant MRP2 Proteins—First of all, we examined the excretion of GS-MF from COS-7 cells transfected with pCI-neo/MRP2V, which contains conservative amino acid changes as I448V and L653V, or pCI-neo/MRP2X, which contains a small deletion at the multi-cloning site of the vector. MRP2X-transfected cells retained almost the same GS-MF excretion activity with wild type MRP2, and MRP2V-transfected cells also retained the comparable activity (70% of wild type MRP2), indicating that the mutation did not severely affect the MRP2 function (Fig. 7). Next, we studied transport activity of MRP2 mutant proteins. Fig. 8 shows the excretion of GS-MF from COS-7 cells transfected with MRP2 mutants of TM6–TM11. In K324A and K483A mutants, the excretion of GS-MF decreased about 40% compared with MRP2V. As to K578A mutant, GS-MF excretion decreased to the level of control (Fig. 8); however, expression level of this mutant also decreased (Figs. 5 and 6). Fig. 9 also presents the excretion of GS-MF from COS-7 cells transfected with MRP2 mutants of TM12–TM17. In R1210A and R1257A mutants, the excretion of GS-MF decreased approximately to the level of control, but expression level was comparable with MRP2X (Fig. 7). As shown in Fig. 10, we observed with confocal fluorescence microscopy that these mutant MRP2s including K578A were expressed at the cell surface. Therefore, the defect in the transport function of these mutants was clearly due to the functional defect except for K578A. The decrease in transport activity of K578A was mainly due to the decrease in the expression level.

Inhibition of GS-MF Excretion by CsA in MRP2 Mutants—We further examined whether basic residues of transmembrane regions are involved in CsA-directed inhibition of the substrate transporting activity of MRP2. Cells expressing active MRP2 mutants were incubated with 10 μM CsA, and extrusion of GS-MF was measured in the existence of 10 μM CsA. In R1230A mutant, the MRP2-mediated transport activity was not inhibited by CsA, whereas the other active mutants were suppressed on their transport activity up to 50% (Fig. 11).

DISCUSSION

According to the fact that MRP2 is involved in excretion of amphiphilic anionic conjugates, it has been predicted that positively charged amino acid residues in transmembrane helices are involved in substrate binding of MRP2. MRP1 and its related transporters have 17 transmembrane helices; however, the N-terminal five helices (TM1–TM5) have been shown to be dispensable for substrate translocating activity on MRP1 (28). In conjunction with other drug-extruding transporters, the core unit essential for transport activity seems limited on the tandem repeated structure of six-transmembrane helices and a nucleotide-binding domain. Hence, we tested whether the basic
residues around transmembrane regions of TM6–TM17 of MRP2 participate in binding of substrates by site-directed mutagenesis and transport activity assay.

First, we established a method for assaying transport activity of MRP2 by using fluorescent substrates, GS-MF and GS-B, which are glutathione-conjugated metabolites derived from CMFDA and mbCl, respectively. In CHO-K1 and COS-7 cells transfected with a MRP2 expression vector, the excretion of GS-MF and GS-B into the medium was 3–4-fold higher than that of the cells transfected with vector alone (Figs. 2 and 3). Although Evers et al. (35) reported that in nonpolarized cells exogenous MRP2 was not efficiently expressed at cell surface, there was no difference observed between wild type and the mutant MRP2s in their cell surface expression. The results indicated that the method developed here is useful to measure the transport activity of MRP2 and its mutants in these living cells.

For mutagenesis, we constructed human MRP2 expression vectors, pCI-neo/MRP2V and pCI-neo/MRP2X. pCI-neo/MRP2X-transfected COS-7 cells retained almost the same GS-MF excretion activity with the wild type, and pCI-neo/MRP2V-transfected COS-7 cells also retained the comparable activity, 70% of the wild type (Fig. 7). pCI-neo/MRP2V contains mutations of I448V and L653V, which were located in TM8 and NBD1, respectively. In patients with Dubin-Johnson syndrome, loss of function mutations was occasionally found in NBD1 (12). Likewise, the mutation of NBD1 in pCI-neo/MRP2V might cause slightly decreased activity of MRP2V-mediated transport.

To gain insight into the substrate-binding site of MRP2, we constructed 13 mutants, each of which was replacing basic amino acid residue by alanine. Except for a mutant K578A, the protein expression level of these mutants in COS-7 cell was comparable with that of wild type (Figs. 5 and 6). Among these mutants, K578A, R1210A, and R1257A were the most influenced by mutation concerning substrate translocating activity.
(Figs. 8 and 9). The pattern of cytochemical staining of these mutants was not changed with wild type MRP2 (Fig. 10). These results suggested that Arg1210 and Arg1257 are important residues for substrate transport activity of MRP2. As to K578A, the excretion of GS-MF and protein expression was coincidentally lowered (Figs. 5, 6, and 8). Therefore, it was thought that Lys578 has a role in protein expression rather than substrate translocation. In K324A and K483A mutants, GS-MF excretion also moderately decreased (Fig. 8). Protein expression level (Figs. 5 and 6) and membrane localization (Fig. 10) were normal in these mutants, so that it is indicated that Lys324 and Lys483 are also involved in substrate transport function of MRP2. Lys324, Lys483, Arg1210, and Arg1257 are located in TM6, TM9, TM16, and TM17, respectively. In the case of P-gp, TM1, TM6, TM11, and TM12 are important in recognition and binding of substrate (22, 36, 37). TM6, TM16, and TM17 of MRP2 correspond to TM1, TM11, and TM12 of P-gp. Together with these findings, it is suggested that Lys324, Lys483, Arg1210, and Arg1257 of MRP2 are constituent of substrate binding site. Although there is a difference in the chemistry of residues
involved in substrate transport, it is intriguing to speculate that the functional transmembrane helices of MRP2 and P-gp for substrate binding are almost common.

Next, we examined whether basic residues of TMs are also involved in CsA inhibition. CsA is known to inhibit MRP2 transport activity through binding to but not to be transported by MRP2 (38). The previous study has shown that 10 μM CsA causes drug accumulation in MRP2 expressing LLC-PK1 cells as much as the level of control LLC-PK1 (29). Hence, we tried to see the effect of 10 μM CsA on mutant MRP2 expressors. In most active mutants excretion of GS-MF was inhibited by CsA as well as wild type MRP2, whereas in R1230A mutant excretion of GS-MF was not influenced (Fig. 11). This result clearly indicated that Arg1230 is an essential residue for the inhibition of CsA, either through direct binding of CsA or coordinating to block substrate translocation with CsA bound on another site. Interestingly, in contrast to the fact that both Arg1210 and Arg1257 are located at intracellular side of TM16 and TM17, respectively, Arg1230 is located at extracellular side of TM16. So far determined in P-gp, important residues are located at intracellular side (39). The unique location of Arg1230 may indicate a novel functional relationship on CsA inhibition site and substrate translocation pathway of MRP2.

In summary, the results of this study showed that the basic residues in TM6, TM9, TM16, and TM17 are involved in substrate binding and a residue in TM11 is involved in stable expression of MRP2. This is the first indication that transmembrane basic residues are truly important for substrate translocation on MRP2. Like P-gp, these functional residues of MRP2 are located in intracellular side of the corresponding transmembrane helices. In this study, we used glutathione conjugate as a substrate for MRP2. Further studies are required to determine the role of basic residues by using other substrates, glucronate, and sulfate conjugates.

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