Characterization of the Transport Properties of Cloned Rat Multidrug Resistance-associated Protein 3 (MRP3)*

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The membrane proteins belonging to the ATP binding cassette (ABC) transporter family play an important role in the extrusion of many substrates from cells. Among them, the GS-X pump plays an important role in cell survival (1). Many electrophiles entering cells are conjugated with glutathione and then extruded from the cells with the aid of GS-X pumps (1). Multidrug resistance-associated protein (MRP1), initially cloned from a multidrug resistant tumor cell line, was the first molecule to be associated with the GS-X pump activity (1–3). The substrate specificity of MRP1 was determined in a series of transport studies using MRP1-transfected or MRP1-overexpressing cells (1–3). MRP1 accepts many conjugates as substrates such as glutathione conjugates (e.g., 2,4-dinitrophenyl-S-glutathione (DNS-GS), glutathione disulfide, and leukotriene C4 (LTC4)), glucuronide conjugates (e.g., 17β estradiol-17β-D-glucuronide (E217G)), and sulfated conjugates of certain bile acids (e.g., 5α-sulfatolithocholyglycolaurine) (1–3).

Although MRP1 is widely expressed in many somatic cells, its hepatic expression is not marked. In the liver, canicular multispecific organic anion transporter (cMOAT/MDR2), another member of the GS-X pump family, is expressed on the bile canicular membrane, mediating the efficient biliary excretion of many organic anions (3–5). The similar substrate specificity of cMOAT/MDR2 and MRP1 has been established by comparing the transport properties across the bile canicular membrane between normal rats and mutant rats whose cMOAT/MDR2 activity is hereditarily defective (e.g., transport-deficient (TR−) rats and Eisai hyperbilirubinemic rats (EHBH)) (3–5). These mutant rats have been used as an animal model for Dubin-Johnson syndrome found in humans (6–14). cDNA cloning and functional analysis of its product, along with a mutation analysis (15, 16), have been performed in this and other laboratories.

It is possible that transporters other than cMOAT/MDR2 may be also involved in the hepatic transport of organic anions. Indeed, we were able to amplify two kinds of novel transporters, which were initially referred to as MRP-like protein (MLP) 1 and 2 from EHBR liver using RT-PCR with the degenerated primers designed for the highly conserved carboxyl-terminal ABC region of human MRP1 (17). The sequence alignment of the full-length of cDNA indicated that MLP-1 and 2 correspond to MRP6 and 3, respectively (17, 18). Northern blot analysis showed that the hepatic expression of MRP3 was significantly enhanced in EHBR compared with Sprague-Dawley (SD) rats, although the extent of MRP6 expression is comparable in the two rat strains (17). It is possible that MRP3 may compensate for the defective expression of cMOAT/MDR2 (17, 19).

In the present study, we examined the function of rat MRP3 using membrane vesicles from LLC-PK1 and HeLa cells transfected with an expression vector containing the cloned MRP3.

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1 The abbreviations used are: ABC, ATP binding cassette; CMVs, canalicular membrane vesicles; cMOAT, canalicular multispecific organic anion transporter; DNS-GS, 2,4-dinitrophenyl-S-glutathione; EHBR, Eisai hyperbilirubinemic rat; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole; LTC4, leukotriene C4; MIX, methyltriacryl glutathione human multidrug resistance-associated protein; Na-DNP-Cys, Na-acetyl-2,4-dinitrophenylcysteine; E17βG, 17β estradiol-17β-D-glucuronide; SD, Sprague-Dawley rats.
cDNA. MRP3 shows different substrate specificity to MRP1 and cMOAT/MPR2 in that glutathione conjugates are poor substrates for the former.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]LTC4 (111 μCi/nmol) and [3H]E17βG (55.0 μCi/nmol) were purchased from NEN Life Science Products. [3H]Methotrexate (30 μCi/nmol) was purchased from American Radiolabeled Chemicals, Inc. Unlabeled and [3H]-labeled DNP-SG (22.5 μCi/nmol) were synthesized enzymatically using [glycine-2-3H]glutathione (NEN Life Science Products) and 1-chloro-2,4-dinitrobenzene and glutathione S-transferase (Sigma) as described previously (21). [14C]E3040 glucuronide (84.5 μCi/nmol) and unlabeled E3040 glucuronide and sulfate were prepared from E3040 (supplied by Eisai Co., Ltd, Tsukuba, Japan) as described previously (22). N-acetyl-DNP-Cys was synthesized as described previously (23). 4-Methylumbelliferone glucuronide, 4-methylumbelliferone sulfate, LTC4, methotrexate, α-naphthyl β-D-glucuronide, estrone sulfate, and acivicin were purchased from Sigma.

**Preparation of Transfected Cells**—Rat MRP3 cDNA, excised with EcoRI from pBluescript II SK− (vector) (17), was then inserted into the EcoRI site in a mammalian expression vector (pcNX2; supplied by Dr. J. Miyazaki, Osaka University, School of Medicine) (24). After transfection with lipofectamine (Life Technologies, Inc.), the LLC-PK1 and HeLa cells were maintained in the presence of 800 μg/ml creatine phosphokinase. In some instances, the membrane vesicles were treated with acivicin (final concentration of 6 mM) at 2 °C for 50 min. The length of the transcript in MRP3-transfected LLC-PK1 and HeLa cells was confirmed by Northern blot analysis (Fig. 1). The expression of rat MRP3 in the transfected cells was quantified by Northern blot analysis using radiolabeled probes encoding the carboxyl-terminal ABC region of rat MRP3 according to the method described previously (9, 17). Filters were exposed to Fuji imaging plates (Fuji Photo Film Co., Ltd., Kanagawa, Japan) for 120 min. The expression level of MRP3 was determined in several independently transfected cell populations as described previously (25). The expression of rat MRP3 in the transfection vector-transfected LLC-PK1 cells revealed that the saturation uptake of [3H]E217βG in MRP3-transfected LLC-PK1 and HeLa cells was comparable with the band observed in Sprague-Dawley rats (Fig. 1).

**RESULTS**

**Uptake of Glucuronides and Glutathione Conjugates into Membrane Vesicles**—The expression of rat MRP3 in the transfected cells was confirmed by Northern blot analysis (Fig. 1). The length of the transcript in MRP3-transfected LLC-PK1 and HeLa cells was comparable with the band observed in Sprague-Dawley rats (Fig. 1).

The time profiles for the uptake of [3H]E17βG, [3H]E3040 glucuronide, [3H]DNP-SG, and [3H]LTC4 by the membrane vesicles from LLC-PK1 cells are shown in Fig. 2. The ATP-dependent uptake of [3H]E17βG and [3H]E3040 glucuronide at 10 min was 4.1-fold and 6.9-fold higher in MRP3-transfected LLC-PK1, respectively, compared with the control vector-transfected LLC-PK1 (Fig. 2, a and b). In contrast, the ATP-dependent uptake of [3H]DNP-SG and [3H]LTC4 was not stimulated by MRP3 transfection (Fig. 2, c and d). The same results were obtained in membrane vesicles from MRP3-transfected HeLa cells (Fig. 3). These results indicate that MRP3 preferentially accepts these two glucuronides as substrates, whereas these two glutathione conjugates are poor substrates of MRP3.

**Transport Kinetics of [3H]E217βG**—The ATP-dependent uptake of [3H]E217βG into membrane vesicles was saturable (Fig. 4). Nonlinear regression analysis of the uptake by MRP3- and control vector-transfected LLC-PK1 cells revealed that the saturation uptake can be described by a Michaelis-Menten equation: $V_{\text{max}} = \frac{V_{\text{max}}}{K_m} + \frac{V_{\text{max}}}{K_m}$.

The time profiles for the uptake of [3H]E17βG, [3H]E3040 glucuronide (b), [3H]DNP-SG (c), and [3H]LTC4 (a) into membrane vesicles. Membrane vesicles (10 μg of protein) from MRP3- (circles) or vector- (squares)-transfected LLC-PK1 cells were incubated at 37 °C in medium containing 100 mM [3H]E17βG (panel a), 10 μM [3H]E3040 glucuronide (panel b), 100 nM [3H]DNP-SG (panel c), and 5 mM [HILTC], (panel d) in the presence (closed symbols) and absence (open symbols) of ATP. Each point and vertical bar represents the mean ± S.E. of triplicate determinations (closed symbols) or the mean value from two determinations (open symbols).
Membrane vesicles from MRP3-transfected LLC-PK1 or HeLa cells were incubated at 37 °C for 5 min LLC-PK1 cells or 10 min HeLa cells in medium containing 100 nM [3H]E217G with or without (control) the inhibitors. ATP-dependent uptake was calculated by subtracting values in the presence of 5 mM AMP from those in the presence of 5 mM ATP. Transport was expressed as percent of the control uptake. Data represent mean ± S.E. of triplicate determinations.

**Table I**

| Inhibitor            | Concentration | % Control |
|----------------------|---------------|-----------|
| LLC-PK1 cells        |               |           |
| Control              | 100           |           |
| E3040 glucuronide    | 5             | 34.9 ± 2.6|
|                     | 10            | 19.2 ± 0.5|
|                     | 50            | 8.5 ± 1.2 |
|                     | 200           | 2.1 ± 0.4 |
| E3040 sulfate        | 5             | 169.3 ± 2.5|
|                     | 50            | 145.8 ± 5.0|
|                     | 100           | 98.8 ± 5.5|
|                     | 500           | 25.5 ± 0.7|
| 4-Methylumbelliferone| 10           | 74.7 ± 2.2|
| glucuronide          |               |           |
|                     | 50            | 44.7 ± 0.9|
|                     | 100           | 32.6 ± 0.7|
|                     | 500           | 14.1 ± 0.5|
| 4-Methylumbelliferone| 10           | 156.5 ± 10.6|
| sulfate              |               |           |
|                     | 50            | 321.8 ± 13.1|
|                     | 100           | 423.9 ± 9.6|
|                     | 500           | 576.7 ± 96.4|
| MTX                  | 10            | 59.0 ± 1.4 |
|                     | 50            | 40.0 ± 1.3 |
|                     | 300           | 22.4 ± 1.1 |
|                     | 1000          | 10.7 ± 0.9 |
| α-Naphthyl-β-N-glucuronide | 5         | 91.5 ± 2.2 |
|                     | 20            | 61.4 ± 2.1 |
|                     | 50            | 44.8 ± 2.0 |
|                     | 200           | 23.6 ± 0.9 |
| Estrone sulfate      | 10            | 100.8 ± 4.8|
|                     | 50            | 90.8 ± 4.6 |
|                     | 100           | 77.8 ± 4.0 |
|                     | 500           | 33.3 ± 0.4 |
| NAc-DNP-Cys          | 5             | 102.2 ± 5.7|
|                     | 20            | 125.2 ± 3.4|
|                     | 100           | 115.6 ± 1.8|
|                     | 500           | 97.7 ± 1.9 |
| DNP-SG               | 10            | 80.9 ± 1.4 |
|                     | 50            | 61.4 ± 1.8 |
|                     | 100           | 46.5 ± 2.2 |
|                     | 500           | 14.4 ± 0.6 |
| LTC₄                 | 0.1           | 97.4 ± 4.1 |
|                     | 0.25          | 91.9 ± 3.3 |
|                     | 1             | 75.7 ± 1.5 |
|                     | 2.5           | 57.0 ± 0.5 |
| HeLa cells           |               |           |
| E3040 glucuronide    | 2             | 62.2 ± 0.6|
|                     | 10            | 30.2 ± 1.4 |
|                     | 50            | 11.1 ± 1.1 |
| DNP-SG               | 5             | 79.2 ± 0.3 |
|                     | 20            | 67.8 ± 3.8 |
|                     | 100           | 40.2 ± 2.8 |
| LTC₄                 | 0.1           | 104.4 ± 0.5|
|                     | 0.25          | 87.2 ± 4.1 |
|                     | 1             | 76.9 ± 1.0 |
| MTX                  | 100           | 57.1 ± 2.1 |
|                     | 300           | 35.2 ± 1.5 |
|                     | 1000          | 18.5 ± 1.2 |

Nac-DNP-Cys had no inhibitory effect in LLC-PK1 at a concentration of 500 μM (Table I). In contrast, E3040 sulfate and 4-methylumbelliferone sulfate enhanced the uptake of glutathione conjugates into membrane vesicles.
MRP1 and cMOAT/MRP2 in that LTC4 is a much better substrate than the glutathione conjugates. In addition, the transport characteristics of MRP3 are the same if not that of glutathione conjugates (DNP-SG and LTC4), was markedly stimulated by MRP3 transfection (Figs. 2 and 3), consistent with the finding that the $V_{max}$ of MRP3-mediated transport of E217G with an IC$_{50}$ of 20–50 $\mu$m (Table I), consistent with the hypothesis that this compound is also recognized by MRP3. The inhibitory effect of E3040 glucuronide (IC$_{50}$ < 5 $\mu$m) and 4-methylumbelliferone glucuronide (IC$_{50}$ ~ 50 $\mu$m) was in marked contrast to the stimulatory effect of the corresponding sulfates (Table I). Although the mechanism for stimulation still remains unclear, such a stimulatory effect by E3040 sulfate and 4MU sulfate has been demonstrated in uptake of DNP-SG into CMVs from SD rats (32). Moreover, the low affinity of MRP3 toward NAc-DNP-Cys suggests that MRP3 gene may not encode MOAT4, whose transport properties had previously been characterized in mouse L1210 cells (33). It has been shown that MOAT4 mediates the low affinity transport of DNP-SG ($K_m = 450$ $\mu$m) and exhibits high sensitivity toward NAc-DNP-Cys ($K_0 = 5.0$ $\mu$m) and $\alpha$-naphthyl $\beta$-D-glucuronide ($K_0 = 8.5$ $\mu$m) (33). Because NAcDNP-Cys did not affect MRP3-mediated transport, even at a concentration of 500 $\mu$m, irrespective of the fact that DNP-SG can act as an inhibitor with an IC$_{50}$ of 50–100 $\mu$m (Table I), suggests that MOAT4 differs from MRP3.

Previously, we found that the expression of MRP3 is induced in EHBR liver (17). It is also induced in SD rat liver by phenobarbital treatment and by treatment which increases plasma bilirubin and/or its glucuronide (e.g. the cholestasis induced by common bile duct ligation and by $\alpha$-naphthylisothiocyanate treatment) (17, 19). It is plausible that, in EHBR, MRP3 is induced to compensate for the physiological function of cMOAT/ MRP2 to excrete bilirubin glucuronides from hepatocytes (17, 19). This hypothesis has been proposed from the previous finding that, in mdr 1a knock-out mice, the hepatic function of mdr 1a is compensated for by the increased expression of mdr 1b, whose substrate specificity resembles that of mdr 1a (34). Although we reported that E3040 glucuronide is taken up by CMVs from EHBR in an ATP-dependent manner (32), the comparison of the transport properties between CMVs and MRP3-expressing membrane vesicles suggested that the uptake into EHBR CMVs may not be mediated by MRP3. This suggestion was proposed based on the finding that neither the uptake of E217G nor MTX (35) was stimulated by the addition of ATP if CMVs were isolated from EHBR. Together with the finding that all of the E$_{217}$G, E3040 glucuronide, and MTX are transported via MRP3 (Figs. 2, 3, and 5) suggests that the previously described ATP-dependent transport of E3040 glucuronide in CMVs from EHBR should be attributed to another transporter.

The extensive expression of MRP3 in rat and human intestinal tissues may be related to the intestinal excretion of glucuronides. Indeed, intestinal excretion of ethinylestradiol glucuronide (36) and 1-naphthol glucuronide (37) has been demonstrated in rats in $in situ$ experiments. In addition, the extent of excretion of 1-naphthol glucuronide was comparable in normal and cMOAT/MRP2-deficient rats, suggesting the presence of another transporter responsible for the excretion of this glucuronide (38). It is possible that MRP3 is responsible for the intestinal excretion of this conjugated metabolite.

In conclusion, although MRP3 mediates the transport of several kinds of organic anions, the substrate specificity of

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$^2$ K. Niinuma, Y. Kato, H. Suzuki, C. A. Tyson, V. Weizer, J. E. Dabbs, R. Froelich, C. E. Green, and Y. Sugiyama, Y., submitted for publication.

$^3$ A. Morikawa, H. Suzuki, T. Hirohashi, and Y. Sugiyama, unpublished observation.
MRP3 is in marked contrast to that of MRP1 and cMOAT/MRP2 in that glutathione conjugates are poor substrates for MRP3. It is possible that MRP3 acts as an inducible transporter compensating for the cMOAT/MRP2 function. In addition, MRP3 may be responsible for the cellular extrusion of glucuronide conjugates in the small intestine.

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