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Hepatic Expression of Multidrug Resistance-Associated Protein-Like Proteins Maintained in Eisai Hyperbilirubinemic Rats

TOMOKO HIROHASHI, HIROSHI SUZUKI, KOUSEI ITO, KOTARO OGAWA, KAZUHIKO KUME, TAKAO SHIMIZU, and YUICHI SUGIYAMA

Graduate School of Pharmaceutical Sciences (T.H., H.S., K.I., K.O., Y.S.) and Graduate School of Medicine (K.K., T.S.) The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT

The biliary excretion of several organic anions is mediated by the canalicular multispecific organic anion transporter (cMOAT), which is hereditarily defective in mutant rats such as Eisai hyperbilirubinemic rats (EHBR). In addition, using a kinetic study with isolated canalicular membrane vesicles, we recently suggested the presence of ATP-dependent organic anion transporter(s) other than cMOAT in EHBR [*Pharm Res (NY)* **12:**1746–1755 (1995); *J Pharmacol Exp Ther* **282:**866–872 (1997)]. The aim of this study is to provide a molecular basis for the presence of multiplicity in the biliary excretion of organic anions in rats. Based on the homology with human multidrug resistance-associated protein (hMRP), two cDNA fragments encoding the carboxyl-terminal ATP-binding cassette region were amplified by reverse transcription-polymerase chain reaction from EHBR liver. These fragments exhibited approximately 70% amino acid identity with hMRP and rat cMOAT;, therefore, they were designated MRP-like proteins (MLP-1 and MLP-2). The cloned full length cDNA of MLP-1 and -2 from the Sprague-Dawley (SD) rat liver and colon cDNA library was composed of 1502 and 1523 amino acids, respectively, had the characteristics of ATP-binding cassette transporters, and exhibited homology with hMRP and rat cMOAT. Northern blot analysis indicated that MLP-1 is expressed predominantly in the liver in both SD rats and EHBR, whereas hepatic expression of MLP-2 was observed only in EHBR. In addition, MLP-2 was markedly induced by ligation of the bile duct in SD rat liver. In both SD rats and EHBR, MLP-2 was expressed predominantly in the duodenum, jejunum, and colon. These findings suggest that MLP-1 and MLP-2 might be novel members of the MRP family responsible for the excretion of organic anions from these epithelial cells, and that MLP-2 is an inducible one.

Biliary excretion is one of the major pathways for the elimination of xenobiotics. Many xenobiotics are converted to more hydrophilic metabolites by metabolizing enzymes responsible for oxidation (cytochrome P450) and/or conjugation (such as glutathione *S*-transferase or UDP-glucuronosyltransferase) and then are excreted into the bile. The biliary excretion of many organic anions and glutathione or glucuronide conjugates is mediated by a primary active transporter referred to as the cMOAT (Oude Elferink *et al.*, 1995; Yamazaki *et al.*, 1996; Keppler and König, 1997). Extensive studies in our own and other laboratories have suggested that substrates for cMOAT include nonbile acid organic anions [such as dibromosulfophthalein (Sathirakul *et al.*, 1993; Sathirakul *et al.*, 1994), cefodizime (a β-lactam antibiotic) (Sathirakul *et al.*, 1994), pravastatin (a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor) (Yamazaki *et al.*, 1997), temocaprilat (an angiotensin-converting enzyme inhibitor) (Ishizuka *et al.*, 1997) and the carboxylate forms of CPT-11 and its active metabolite (SN-38, a topoisomerase inhibitor) (Chu *et al.*, 1997), a cyclic anionic peptide (BQ-123, an endothelin antagonist) (Shin *et al.*, 1997)], glutathione conjugates [such as leukotriene C_4 (Ishikawa *et al.*, 1990) and DNP-SG (Kobayashi *et al.*, 1990)], and glucuronide conjugates [such as bilirubin glucuronide (Nishida *et al.*, 1992), E3040 glucuronide (Takenaka *et al.*, 1995a, 1995b) and SN-38 glucuronide (Chu *et al.*, 1997)] as reviewed previously

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[1] The sequences reported in this paper have been submitted to the GenBank with the accession numbers AB010466 (MLP-1) and AB010467 (MLP-2).

ABBREVIATIONS: cMOAT, canalicular multispecific organic anion transporter; hMRP, human multidrug resistance-associated protein; MLP, multidrug resistance-associated protein-like protein; ABC, ATP-binding cassette; DNP-SG, *S*-(2,4-dinitrophenyl) glutathione; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole; CMVs, canalicular membrane vesicles; SD, Sprague-Dawley; EHBR, Eisai hyperbilirubinemic rats; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); kb; kilobase pair(s); SSC, standard saline citrate; SDS, sodium dodecyl sulfate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Oude Elferink *et al.*, 1995; Yamazaki *et al.*, 1996; Keppler and König, 1997). The substrate specificity of cMOAT described so far has been clarified from *in vivo*, *in situ*, and *in vitro* uptake experiments with isolated CMVs by comparing the transport properties in normal and mutant rats (such as TR^- and EHBR) whose cMOAT function is hereditarily defective (Oude Elferink *et al.*, 1995; Yamazaki *et al.*, 1996; Keppler and König, 1997). These mutant rats suffer from jaundice because of the impaired biliary excretion of bilirubin glucuronide via cMOAT;, therefore, they are good animal models for Dubin-Johnson syndrome in humans (Oude Elferink *et al.*, 1995; Yamazaki *et al.*, 1996; Keppler and König, 1997). Recently, we and others have focused on the fact that the substrate specificity of hMRP, also called MRP1, is similar to that of cMOAT (Keppler and Kartenbeck, 1996; Loe *et al.*, 1996), and have cloned the cDNA encoding rat cMOAT, also termed Mrp2 or canalicular Mrp, based on the homology with hMRP (Büchler *et al.*, 1996; Ito *et al.*, 1996; Paulusma *et al.*, 1996; Ito *et al.*, 1997). In addition, the mechanism for the mutation in TR^- (Paulusma *et al.*, 1996) and EHBR (Ito *et al.*, 1997) was determined. Recently, we found the ATP-dependent uptake of cMOAT substrates into the membrane vesicles prepared from cMOAT-cDNA transfected NIH/3T3 cells (Ito *et al.*, 1998). Madon *et al.* (1997) also succeeded in the functional analysis of cloned cMOAT cDNA by detecting the increased efflux of DNP-SG from cDNA-transfected COS-7 cells after preloading its precursor and from cRNA-injected *Xenopus laevis* oocytes after direct injection of this glutathione conjugate.

Although E3040 glucuronide is a good substrate for cMOAT, our recent kinetic study using CMVs from EHBR suggested that the ATP-dependent transport of E3040 glucuronide, but not that of DNP-SG, is maintained in EHBR to some extent (Takenaka *et al.*, 1995a). Moreover, the presence of another organic anion transporter(s) in SD rats was suggested by an inhibition study in CMVs; the ATP-dependent transport of E3040 glucuronide was not completely inhibited by excess DNP-SG sufficient to saturate DNP-SG uptake, whereas the uptake of DNP-SG was almost completely inhibited by E3040 glucuronide (Niinuma *et al.*, 1997). In addition, the ATP-dependent transport of E3040 glucuronide into CMVs from EHBR was not affected by DNP-SG (Niinuma *et al.*, 1997). These findings suggested the presence of multiple transport systems for the biliary excretion of organic anions and conjugated metabolites across the canalicular membrane (Niinuma *et al.*, 1997).

The object of the present study is to provide a molecular basis for the presence of novel members of the MRP family, particularly focusing on molecules that are also expressed in EHBR liver. In view of the fact that a series of primary active transporters possess highly conserved ABCs (Hyde *et al.*, 1990), RT-PCR was performed with degenerate primers for hMRP using poly(A)$^+$ RNA from EHBR liver as a template.

Materials and Methods

Animals. Male SD rats (250–300 g) and EHBR (250–350 g) were purchased from Charles River Japan (Kanagawa, Japan) and SLC (Shizuoka, Japan), respectively.

RNA isolation. Total RNA was prepared by a single-step guanidium thiocyanate procedure. Subsequently, poly(A)$^+$ RNA was purified using oligotex-dT30 (Takara Shuzo, Kyoto, Japan).

Amplification of cDNA fragments. cDNA fragments were amplified by RT-PCR with the total RNA of EHBR liver as a template by using a TaKaRa RNA LA PCR kit (Takara Shuzo). Degenerate primers were constructed on the basis of the conserved amino acid sequence in the carboxyl ABC region of hMRP (Ito *et al.*, 1996). The sequences of the forward and reverse primers were 5′-dGAGAAG-GTCGGCATCGTGGG(AGTC)CG(AGTC)AC(AGTC)GG-3′ and 5′-dGTCCACGGCTGC(AGTC)GT(AGTC)GC(TC)TC(AG)TC-3′, respectively (Ito *et al.*, 1996).[1] RT was performed using random primer at 30° for 10 min, 42° for 30 min, 99° for 5 min, and 5° for 5 min. Then PCR was carried out at 94° for 30 sec, 37° for 30 sec, and 72° for 1 min for 40 cycles using *Taq* polymerase. The amplified 421-bp PCR products were subcloned into the *Eco*RV site of pBluescript II SK(−) (Stratagene, La Jolla, CA) and the sequence was determined. PCR products were excised from the vector by digestion with *Eco*RI and *Hin*dIII and were used as probes for detection of their expression. Rat cMOAT cDNA probe (~1.0 kb) was prepared as described previously (Ito *et al.*, 1997).

Library construction and screening. For cDNA screening, the cDNA libraries were constructed from SD rat liver and colon using a kit (SuperScript Choice System; Life Technologies, Gaithersburg, MD) described previously (Ito *et al.*, 1997). Briefly, poly(A)$^+$ RNA was fractionated by sucrose density gradient and fractions containing RNA longer than ~5 kb were used as a template for cDNA construction using reverse transcriptase and an oligo(dT) primer. The conversion of RNA-cDNA hybrid into double-stranded cDNA was performed using RNase H in combination with DNA polymerase I and *Escherichia coli* DNA ligase. *Eco*RI-*Not*I adapters were then added to both ends of the double-stranded and blunt-ended cDNA. After phosphorylation with T_4 polynucleotide kinase, the resulting cDNA was subsequently ligated to *Eco*RI digested and calf intestinal alkaline phosphatase-treated λ ZAP II vector (Stratagene). Packaging of those recombinants into the λ-phage was performed using a kit (Gigapack III Gold Packaging Extract; Stratagene). Approximately 5×10^5 independent plaques on XL1-Blue strain were screened with the probe described. After two rounds of screening, single positive colonies were isolated. After coinfection with the M13 helper phage (ExAssist; Stratagene), the cDNA was excised in a pBluescript II SK(−) plasmid and rescued by SOLR strain.

DNA sequencing. DNA sequence analysis was performed in both directions using double-stranded cDNA as a template. A sequencer (Model 373 DNA Sequencer; Perkin Elmer, Foster City, CA) was used in combination with a kit (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase, FS; Perkin Elmer). The GenBank/EMBL database was searched with the cDNA sequence of the fragments using the BLAST program. Other sequence analysis and calculations were performed with the GENETYX-MAC program (Software Development Co., Tokyo, Japan).

Northern blot analysis. Poly(A)$^+$ RNA was separated on 0.7% agarose gel containing 3.7% formaldehyde and transferred to a nylon membrane (Biodyne; Pall Corporation, Glen Cove, NY), before fixation by baking for 2 hr at 80°. The membranes were prehybridized in hybridization buffer containing 4 × SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 5 × Denhardt's solution, 0.2% SDS, 0.1 mg/ml sonicated salmon sperm DNA and 50% formamide for 2 hr at 42° and hybridized for 10 hr at 42° in the same buffer with a ^{32}P-labeled cDNA probe that was prepared by a random primed labeling method (Rediprime; Amersham International, Buckinghamshire, UK). The hybridized membrane was washed in 2 × SSC and 0.1% SDS at room temperature for 20 min, followed by washing in 2 × SSC and 0.1% SDS at 55° for 20 min and then in 0.1 × SSC and 0.1% SDS at 55° for 20 min. Filters were exposed to a film (Hyperfilm-MP; Amersham International) at −100° using an intensifying screen. In some instances, the filters were exposed to an imaging plate followed by analysis using Fujix BAS 2000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Induction of transporters. The expression of transporters was examined in rats with cholestasis induced by bile-duct ligation. The proximal bile duct of SD rats was ligated under light ether anesthesia (Schrenk *et al.*, 1993). Three days after ligation, liver RNA was prepared to examine by Northern blot analysis the induction of transporters. The hybrid membranes were exposed to the imaging plates and quantified using a Fujix BAS 2000 image analyzer.

Results

Amplification of novel cDNA fragments from EHBR liver. To identify novel MRP family members that are maintained in EHBR liver, RT-PCR was performed using the degenerate primers based on the sequence of the hMRP carboxyl-terminal ABC region. Two novel 421-bp fragments were amplified from EHBR liver. The amino acid identity of one fragment was 62.6% with hMRP and 56.1% with rat cMOAT and that of the other fragment was 73.2% with hMRP and 71.5% with rat cMOAT. Based on the homology with hMRP, these fragments were designated as MLP-1 and -2, respectively. MLP-2 also showed high homology with MRP3 (84.1% amino acid identity), which has been identified by screening databases of human expressed sequence tags (Kool *et al.*, 1997), suggesting that MRP3 may be the human homologue of rat MLP-2. By screening the library, the sequences of the amino- and carboxyl-terminal ABC regions of MLP-1 and -2 were determined. The sequence alignment of rat MLP-1, MLP-2, cMOAT, hMRP and MRP3 (only carboxyl terminal) at the ABC regions indicates a high degree of similarity among these MRP family proteins (Fig. 1). Moreover, each ABC region had the Walker A and B motifs and the putative consensus pattern for the ABC transporter family signatures (Hyde *et al.*, 1990) (Fig. 1).

To examine the expression of MLP-1 and -2 in SD rat and EHBR liver, Northern blot analysis was performed. As shown in Fig. 2, the level of MLP-1 in the liver was almost the same for SD rats and EHBR, whereas the expression of MLP-2 was observed only in EHBR liver.

cDNA cloning of the MLP-1 and -2. Because it is possible that MLP-1 and -2 are organic anion transporters that are expressed in EHBR liver, cDNA cloning was performed. A full-length cDNA with a single open reading frame of 1502 and 1523 amino acids was cloned for MLP-1 and -2, respectively (Fig. 3). A BLAST search of the National Center for Biotechnology Information database showed that, at deduced amino acid levels, MLP-1 and 2 exhibit high overall identity with several ABC proteins such as hMRP, cMOAT, rabbit epithelial basolateral chloride conductance regulator, rat sulfonylurea receptor and yeast cadmium factor (Table 1). The identities with hMRP3–5, whose sequences are partially reported (Kool *et al.*, 1997), are also listed in Table 1. The high sequence identity, in particular within two ABC regions, was observed between MLPs and these ABC transporters (Table 1). Fig. 4 shows that the pattern of hydropathy plot of hMRP, rat cMOAT and rat MLP-1 and -2 resembles each other. A lesser degree of identity was observed with human cystic fibrosis transmembrane conductance regulator, rat Mdr1 and Mdr2.

Induction of MLP-2 by cholestasis in SD rat liver. The effect of cholestasis induced by bile duct ligation on the expression of cMOAT, MLP-1 and -2 in SD rat liver was examined by Northern hybridization (Fig. 5). The induction ratio was calculated as a percentage of controls in five samples after correcting the mRNA loading amount by rehybridization of GAPDH. MLP-2 was markedly induced by bile-duct ligation. Although we can not calculate the induction ratio of MLP-2 precisely because the expression was not detected in

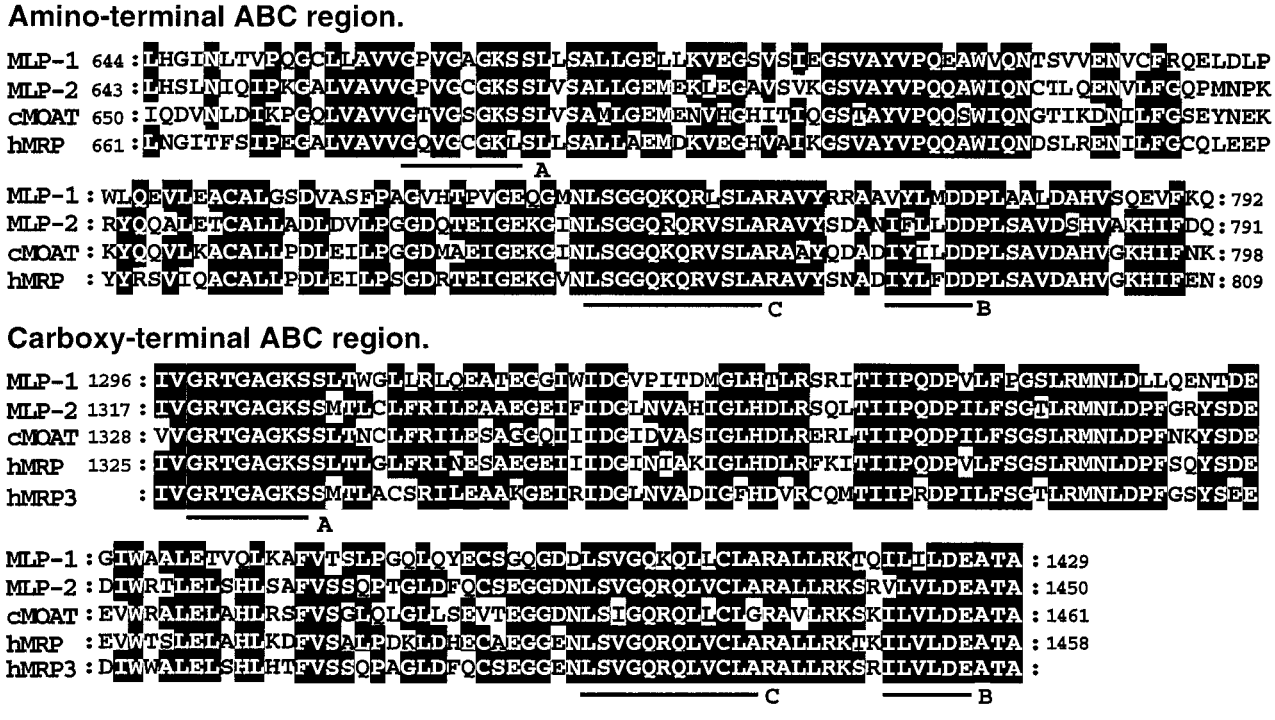

Fig. 1. Alignment of the deduced amino acid sequence in the amino- and carboxyl-terminal ABC regions of rat MLP-1, MLP-2, cMOAT, hMRP and hMRP3 (only carboxyl terminal). *White type on black background*, at least three amino acids are identical at that position; *numbers*, amino acid position of MLP-1, MLP-2, cMOAT, and hMRP. *Single lines* and *A*, *B*, and *C*, Walker A and B motifs and active transport family signature (Hyde *et al.*, 1990), respectively.

untreated SD rats (Fig. 2), MLP-2 was induced by at least 607 ± 189% (mean ± standard error) compared with controls, assuming that the detection limit was 20% above membrane background ($p < 0.01$). In contrast, MLP-1 mRNA was reduced to 65 ± 8% compared with untreated control rats ($p < 0.05$). In agreement with previous findings (Trauner *et al.*, 1997), cMOAT mRNA levels also slightly decreased (to 80 ± 15%), although this did not reach statistical significance.

Tissue expression of MLP-1 and -2. To examine the tissue distribution of MLP-1 and -2, Northern blot analysis was performed in SD rats and EHBR and the expression was compared between rats after correcting the mRNA loading amount by rehybridization of GAPDH. As shown in Fig. 2, MLP-1 was expressed predominantly in the liver of both rat strains. Although low expression of MLP-1 was observed in duodenum and kidney at the same level for both strains, the jejunum expression was enhanced somewhat in EHBR compared with SD rats (Fig. 2). High expression of MLP-2 was observed in intestinal tissues such as duodenum, jejunum and colon and, to a lesser extent, in kidney and lung (Fig. 2). In these tissues, the extent of MLP-2 expression was almost the same for SD rats and EHBR (Fig. 2).

The tissue distribution patterns of four MRP family proteins (rat MLP-1, MLP-2, cMOAT and hMRP) are summarized in Table 2. The expression of MRP in human tissues was detectable in lung, testis, kidney and spleen by Northern blot and RNase protection assay as reviewed by Loe *et al.* (1996) (Table 2). The relative expression level of rat cMOAT was estimated based on our previous Northern blot data (Ito *et al.*, 1997) after normalizing the mRNA loading amount by the expression of GAPDH. The relative expression levels of MLP-1 and -2 were determined based on Fig. 2 in the same manner. cMOAT in normal rats is extensively expressed in liver and, to a lesser extent, in duodenum, kidney and jejunum (Ito *et al.*, 1997) (Table 2). The expression pattern of cMOAT is similar to that of MLP-1 in SD rats (Table 2). In addition, the expression of both cMOAT (Ito *et al.*, 1997) and MLP-1 in jejunum was enhanced somewhat in EHBR (Fig. 2). On the other hand, high expression of MLP-2 was observed in intestinal tissues, which was different from both hMRP (lung-type) and cMOAT (liver-type) (Table 2).

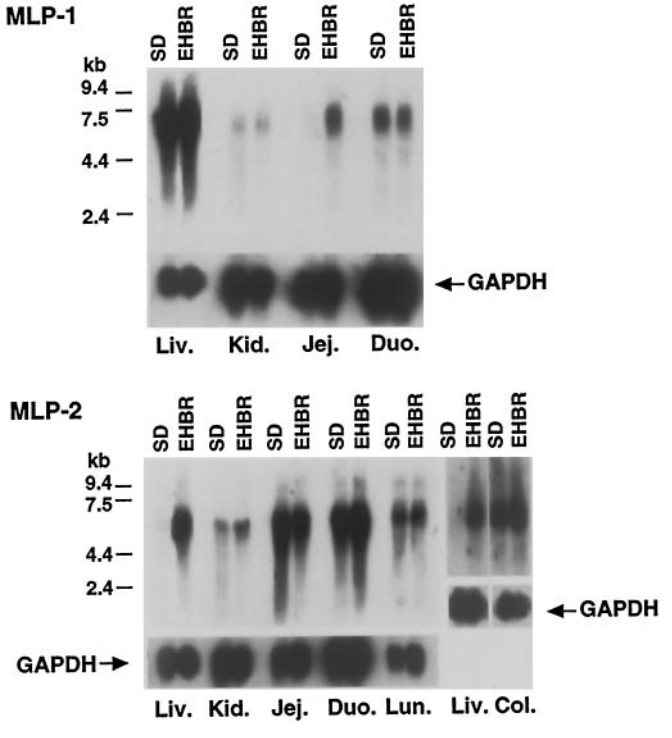

Fig. 2. Northern blot analysis of MLP-1 and MLP-2. Five micrograms of poly(A)$^+$ RNA from tissues of SD rats and EHBR was loaded in each lane. The autoradiograph of the blot, hybridized with ^{32}P-labeled 421-bp cDNA fragments of MLP-1 and -2, had an 80-hr exposure at −100° with an intensifying screen. Rehybridization was performed with ^{32}P-labeled GAPDH cDNA as a loading control and an autoradiograph of the blots had a 24 hr exposure in the same way. *Liv.*, liver; *Kid.*, kidney; *Duo.*, duodenum; *Jej.*, jejunum; *Lun.*, lung; *Col.*, colon.

Discussion

It has been established that the biliary excretion of many organic anions is mediated by cMOAT which is hereditarily defective in EHBR (Oude Elferink *et al.*, 1995; Yamazaki *et al.*, 1996; Keppler and König, 1997). Previous studies from this laboratory indicated that approximately one third of the ATP-dependent transport activity of E3040 glucuronide in CMVs from SD rats, however, was maintained in EHBR (Takenaka *et al.*, 1995a). This was in marked contrast to the fact that the ATP-dependent transport of DNP-SG in CMVs from EHBR was almost completely abolished (Takenaka *et al.*, 1995a). To characterize this ATP-dependent transporter maintained even in EHBR, we recently performed a kinetic study using CMVs (Niinuma *et al.*, 1997). In SD rat CMVs, the transport of DNP-SG was almost completely inhibited by E3040 glucuronide in a competitive manner, whereas the transport of E3040 glucuronide was partially inhibited by excess DNP-SG (Niinuma *et al.*, 1997). Furthermore, low affinity ATP-dependent transport of E3040 glucuronide was observed in EHBR CMVs, which is not affected by DNP-SG, suggesting the presence of a primary active transporter distinct from cMOAT, in both SD rats and EHBR (Niinuma *et al.*, 1997). In the present study, RT-PCR was performed to establish the molecular features of this novel transporter.

Degenerate PCR primers were prepared for the ABC region of hMRP, an ATP-dependent transporter for organic anions (such as glutathione- and glucuronide-conjugates). RT-PCR with degenerate primers resulted in amplification of MLP-1 and -2 from EHBR liver (Fig. 1). Of these two novel cDNAs, MLP-1 was expressed in both SD rat and EHBR liver to the same extent (Fig. 2). The cloned MLP-1, which had two ABC regions with Walker A and B motifs and the active transporter family signatures (Hyde *et al.*, 1990) (Fig. 3), exhibited high homology with hMRP and rat cMOAT (Table 1). In addition, the pattern of hydropathy plot of MLP-1 is similar to that of these transporters (Fig. 4). These findings suggested that MLP-1 may encode a primary active transporter which is responsible for the ATP-dependent transport of glucuronide in CMVs from EHBR (Takenaka *et al.*, 1995a; Niinuma *et al.*, 1997), although the functional analysis and determination of intracellular localization remain to be clarified.

The hepatic expression of MLP-2, which also exhibits similar structure with MRP and cMOAT (Fig. 3 and 4), was significantly enhanced in EHBR compared with SD rats (Fig. 2), suggesting that MLP-2 is an inducible transporter. The fact that the cholestasis induced by common bile duct ligation

Fig. 3. Deduced amino acid sequence of rat MLP-1 (A) and MLP-2 (B). *Single lines* and *A*, *B*, and *C*, Walker A and B motifs and active transport family signature (Hyde *et al.*, 1990), respectively.

resulted in an increase in the expression of MLP-2 in SD rat liver (Fig. 5) is consistent with this hypothesis. Although the hepatic expression of mdr 1a and 1b is induced in rats by cholestasis induced by either bile duct ligation or α-naphthylisothiocyanate-induced cholestasis in rats (Schrenk *et al.*, 1993), the mechanism for the induction may differ between MLP-2 and mdr 1, because the expression of mdr 1 was not induced in EHBR (Suzuki H, Ogawa K, Hirohashi T and

TABLE 1
Amino acid identities of MLP-1 and MLP-2 with related ABC transporters

Domain		Entire length	Upstream of ABC-1	ABC-1	Between ABC-1 and 2	ABC-2	Downstream of ABC-2
Amino acids		1–1502	1–643	644–792	793–1280	1281–1437	1438–1502
MLP-1							
hMRP	1531 aa	42.8	35.5	58.4	41.6	62.2	47.6
Rat cMOAT	1541 aa	38.3	25.2	51.0	34.2	57.1	42.9
Rat MLP-2	1523 aa	40.2	35.7	57.7	35.0	59.0	50.0
Rabbit EBCR	1565 aa	38.6	26.7	51.0	33.7	59.0	41.3
Rat SUR	1582 aa	33.0	21.9	39.1	26.3	51.3	36.2
YCF1	1515 aa	32.7	26.3	48.3	28.5	51.6	46.9
Human MRP3[a]	485 aa				41.2	56.4	53.1
Human MRP4[a]	126 aa					58.6	39.7
Human MRP5[a]	222 aa					54.7	39.7
Amino acids		1–1523	1–642	643–791	792–1301	1302–1458	1459–1523
MLP-2							
hMRP	1531 aa	55.7	52.4	67.8	48.7	73.2	73.4
Rat cMOAT	1541 aa	45.7	35.7	66.4	43.3	71.8	56.2
Rabbit EBCR	1565 aa	45.2	36.7	63.1	42.6	68.8	53.1
Rat SUR	1582 aa	36.7	24.0	44.8	30.3	51.6	38.6
YCF1	1515 aa	38.0	29.4	51.0	35.3	73.1	58.5
Human MRP3[a]	485 aa				79.1	84.1	89.2
Human MRP4[a]	126 aa					51.6	52.5
Human MRP5[a]	222 aa					55.7	54.2

EBCR, epithelial basolateral chloride conductance regulator; SUR, sulfonylurea receptor; YCF1, yeast cadmium factor; ABC-1, amino-terminal ABC; ABC-2, carboxyl-terminal ABC.
[a] Partial sequences were reported by Kool *et al.*, 1997.

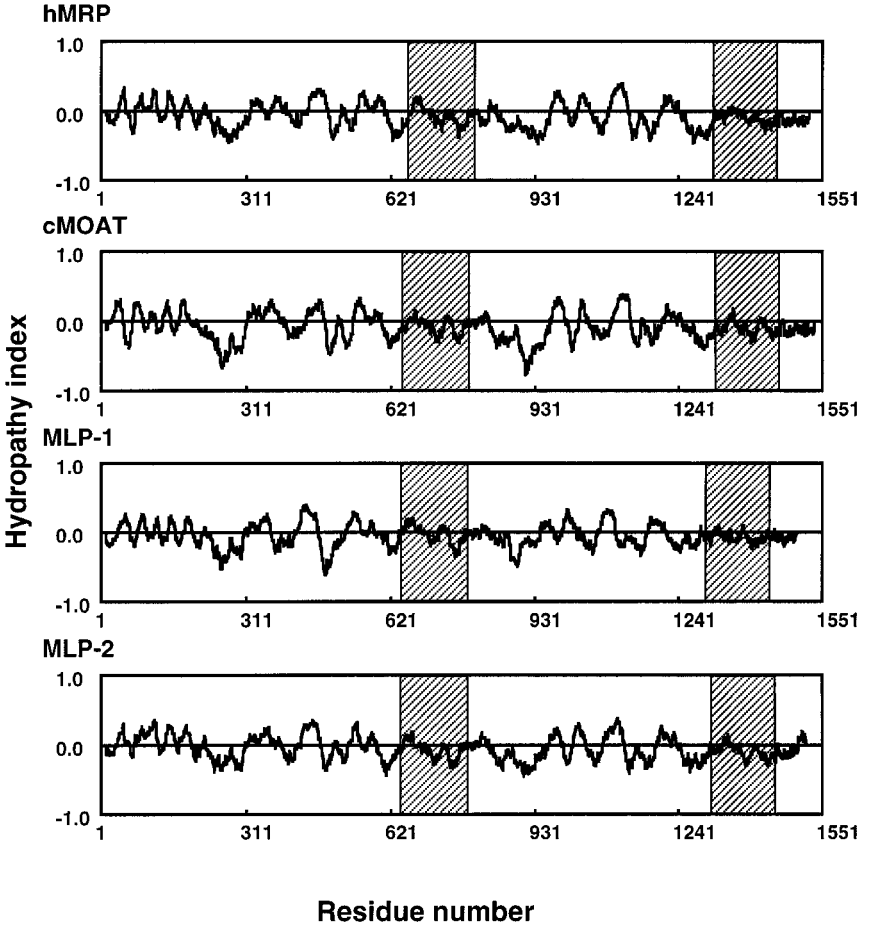

Fig. 4. Hydropathy plot of hMRP, rat cMOAT, MLP-1 and MLP-2. Hydropathy plotting was performed by the method of Eisenberg *et al.* (1984) with a window of 21 amino acids. *Ordinate: positive numbers*, hydrophobicity; *negative numbers*, hydrophilicity. *Shadows*, position of ABC regions.

Sugiyama Y, unpublished observations). It is possible that the gene expression of MLP-2 was induced by endogenous substrate(s) for cMOAT. One of the most plausible candidates for the induction may be conjugated and unconjugated bilirubin, because plasma concentration of total bilirubin in the bile-duct-ligated rats (3.62 mg/100 ml) (Schrenk *et al.*, 1993) and in EHBR (4.02 mg/100 ml) (Sathirakul *et al.*, 1993) was much higher than that of untreated normal rats (0.154 mg/100 ml) (Sathirakul *et al.*, 1993). It may be plausible that MLP-2 compensates for the function of cMOAT in EHBR, because it has been reported that, in mdr 1a knock-out mice, the increased expression of mdr 1b, whose substrate specificity resembles that of mdr 1a, compensates for the hepatic function of mdr 1a (Schinkel *et al.*, 1994). The induced expression of MLP-2 was in marked contrast to the somewhat reduced expression of cMOAT and MLP-1 after bile duct ligation (Fig. 5). Collectively, as with the metabolic enzymes, the transporters responsible for the excretion of xenobiotics may be classified as house-keeping (cMOAT and MLP-1) or inducible (MLP-2).

In normal rats, MLP-2 exhibited high expression in intestinal tissues (Fig. 2). Because the intestinal epithelium is directly exposed to a number of xenobiotics, it is equipped with a number of metabolizing enzymes (Peters *et al.*, 1989) (such as cytochrome P-450, UDP-glucuronosyltransferase and glutathione *S*-transferase) and a transporter (P-glycoprotein) responsible for the excretion of hydrophobic and amphipathic compounds (Gatmaitan and Arias, 1993). In addition to P-glycoprotein, cumulative evidence suggests the presence of another efflux transporter for organic anions in intestinal tissues; in Caco-2 cells, active excretion of DNP-SG (Oude Elferink *et al.*, 1993) and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (Collington *et al.*, 1992) has been reported. Furthermore, intestinal excretion of ethinylestradiol glucuronide (Schwenk *et al.*, 1982) and 1-naphthol glucuronide (de Vries *et al.*, 1989a) was demonstrated in rats in *in situ* experiments. Because kinetic analysis indicated that the intestinal excretion of 1-naphthol glucuronide was not significantly different between Wistar rats and TR$^-$ rats, it was suggested that a primary active transporter other than cMOAT may be responsible for the excretion of organic anions from intestinal cells (de Vries *et al.*, 1989b). It may be possible that MLP-2, an ABC transporter superfamily member, is responsible for the intestinal excretion of organic anions.

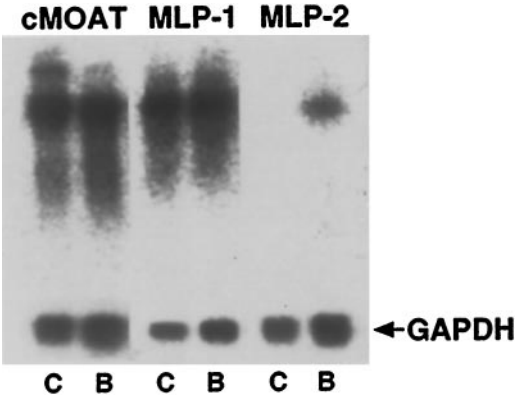

Fig. 5. Northern blot analysis of hepatic expression of cMOAT, MLP-1 and MLP-2 in SD rats 3 days after common bile duct ligation. Two micrograms of poly(A)$^+$ RNA was used for analysis. The membranes hybridized with ^{32}P-labeled cDNA probes were exposed to the imaging plates for 4 hr (cMOAT), 24 hr (MLP-1 and -2) and 8 hr (GAPDH) followed by analysis using a Fujix BAS 2000 image analyzer. *C*, control (untreated); *B*, bile duct ligation.

TABLE 2

Tissue distribution of rat MLP-1, MLP-2, cMOAT, and hMRP

Northern blot was performed as described in the legend to Fig. 2. The expression of cMOAT in SD rats was determined by Northern blot analysis (Ito *et al.*, 1997). The expression of MRP in human tissues was determined by Northern blot and RNase protection assay as reviewed by Loe *et al.* (1996).

Probe tissue	MLP-1 rat	MLP-2 rat	cMOAT rat	hMRP
Liver	+++[a]	[b]	++++[a]	+[a]
Kidney	+[a]	+[a]	+[a]	++[a]
Duodenum	++[a]	+++[a]	++[a]	+[a]
Jejunum	[b]	+++[a]	+[a]	+[a]
Colon	[b]	+++[a]	N.D.	+[a]
Lung	[b]	++[a]	[b]	++++[a]
Spleen	[b]	[b]	(+)[c]	++[a]
Brain	[b]	[b]	(+)[c]	+[a]
Heart	[b]	[b]	(+)[c]	+[a]
Testis	N.D.	N.D.	[b]	++++[a]

[a] The relative expression level is indicated by + symbols in the following order: ++++ > +++ > ++ > +.
[b] The expression was not detectable by Northern blot analysis (data not shown).
[c] In these tissues, a shorter band (~4 kb) was observed compared with other tissues (5.0, 6.3, and 8.3 kb) (Ito *et al.*, 1997). N.D., not determined.

The results of the present study provide a molecular basis for the presence of MRP family proteins. An ATP-dependent efflux system(s) for organic anions is expressed in many somatic cells, such as heart sarcolemmal (Ishikawa, 1989) and red blood cells (Kondo *et al.*, 1980), as well as in the epithelium of liver and intestine. Such efflux pumps are also observed in many kind of cultured human cell lines (Olive and Board, 1994). The physiological role of these transporters may be to exclude anionic xenobiotics entering from the circulating blood/intestinal lumen and anionic waste materials produced within cells. Because of the localization of efflux transporters on the canalicular membrane, the liver is endowed with the ability to eliminate endogenous and xenobiotic organic anions from the circulating blood by excreting them into bile (Oude Elferink *et al.*, 1995; Yamazaki *et al.*, 1996; Keppler and König, 1997). In the same manner, localization of such transporters on central endothelial cells may allow the blood-brain barrier to restrict entry of organic anions (Suzuki *et al.*, 1997). In addition, some tumor cells acquire multidrug resistance by overexpression of MRP and its related protein(s) (Ishikawa *et al.*, 1996; Loe *et al.*, 1996; Kool *et al.*, 1997). Recently, by screening the database of human expressed sequence tags, Kool *et al.* (1997) cloned three hMRP homologues (MRP3, 4 and 5). MRP3 shows particularly high homology with MLP-2 (Fig. 1; Table 1), which indicates that MRP3 is the human homologue of rat MLP-2. They reported that the MRP3 is expressed in the human liver, duodenum, colon and adrenal gland (Kool *et al.*, 1997). If we consider that MLP-2 is an inducible transporter, it is possible that the expression of MRP3 in the liver was induced in the human subject(s) used in their study (Kool *et al.*, 1997).

In conclusion, we identified MLP-1 and -2 as novel members of the MRP family that may be responsible for the biliary and intestinal excretion of organic anions. The results of the present study also provided a molecular basis for the presence of multiple systems for the extrusion of organic anions from many kinds of somatic cells. Drug disposition in

the body and/or tumor cells can be altered by modifying the activity of these efflux transporters.

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Send reprint requests to: Yuichi Sugiyama, Ph.D., Professor, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan. E-mail: bxg05433@niftyserve.or.jp