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Transport Function and Hepatocellular Localization of mrp6 in Rat Liver

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ABSTRACT

The multidrug resistance-associated proteins (Mrps) constitute a family of cellular export pumps of the ATP-binding cassette transporter superfamily and play an important role in hepatobiliary excretion. We investigated the transport function and subcellular localization of mrp6, a novel member of the mrp family, in rat liver. Transport studies in vesicles isolated from mrp6 expressing Sf9 cells identified the anionic cyclopentapeptide and endothelin receptor antagonist BQ-123 as a substrate of mrp6 ($K_m \sim 17$ μM). Besides BQ-123, which is also a substrate of mrp2 ($K_m \sim 124$ μM), no other common substrates were found for mrp2, mrp6, and the canalicular bile salt export pump Bsep. The cyclic peptides endothelin I and Arg^8-vasopressin were transported by mrp2 but not by mrp6. Using a polyclonal antiserum raised against a C-terminal peptide, mrp6 was found to be localized at the lateral and, to a lesser extent, at the canalicular plasma membrane of hepatocytes. The limited overlap of the substrate specificity with the canalicular export pumps mrp2 and Bsep indicates that mrp6 does not play a major role in canalicular organic anion excretion. However, its dual localization at the lateral and canalicular plasma membrane suggests that mrp6 might fulfill a "housekeeping" transport function involved in the regulation of paracellular and/or transcellular solute movement from blood into bile.

The liver excretes numerous drugs and other xenobiotics from blood into bile. The rate-limiting step in overall hepatic bile secretion is the transport of cholephilic compounds across the canalicular (apical) membrane of hepatocytes. These concentrative canalicular transport processes are mediated to a large extent by members of the ATP-binding cassette (ABC) transporter superfamily (Müller and Jansen, 1998; Stieger and Meier, 1998); they include 1) the multidrug resistance P-glycoprotein (rodents: mdr1 and mdr3; mdr1a, mdr1b, humans: MDR1) for canalicular excretion of cytotoxic organic compounds (mostly cations) (Kamimoto et al., 1989), 2) mdr2 (human: MDR3) for canalicular translocation of phosphatidylcholine (Oude Elferink et al., 1997), 3) the bile salt export pump (rodents: Bsep; human: BSEP) for intracanalicular concentration of bile salts such as taurocholate (Gerloff et al., 1998), and 4) the multidrug-resistance-associated protein mrp2 (human: MRP2) for the canalicular excretion of a wide variety of organic anions, including phase II biotransformation products such as bilirubindiglucuronide and glutathione conjugates, leukotriene C_4, and the cyclic pentapeptide BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]), an endothelin receptor antagonist (Oude Elferink et al., 1995; Keppler and König, 1997; Shin et al., 1997; Akhteruzzaman et al., 1999).

Although these canalicular polyspecific ABC transporters can account for ATP-dependent biliary excretion of a wide variety of cholephilic compounds, functional studies in mrp2-deficient rat strains have provided evidence for the presence of additional related transporters at the canalicular plasma membrane of hepatocytes (Jansen et al., 1993; Shin et al., 1997; Kusuhara et al., 1998; Akhteruzzaman et al., 1999). In fact, among the new members of the MRP family that have been identified in human tissues and cancer cell lines, MRP3 and MRP6 are expressed in the liver (Kool et al., 1997, 1999a). The same is also true for their rat homologs mrp3 (or MLP-2) and mrp6 (or MLP-1) (Hirohashi et al., 1998, 1999). Although its expression level in normal liver is low, mrp3 expression is induced in mrp2-deficient rat strains and during experimental cholestasis (Hirohashi et al., 1998, 1999; Ortiz et al., 1999). Although the exact physiological role of mrp3/MRP3 is not yet known, the preferred substrates of the rat mrp3 appear to be glucuronides rather than glutathione conjugates (Hirohashi et al., 1999). Because mrp3/MRP3 has been localized to the basolateral cell pole of hepatocytes by some (König et al., 1999; Kool et al., 1999b), but not all (Ortiz et al., 1999), investigators, it is unlikely that increased mrp3

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ABBREVIATIONS: ABC, ATP-binding cassette; GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); Bsep, canalicular bile salt export pump; mdr/MDR, multidrug resistance; mrp/MRP, multidrug resistance-associated protein.

expression can account for all residual canalicular organic anion and cyclic peptide excretion in mrp2-deficient rat strains (Jansen et al., 1993; Shin et al., 1997; Kusuhara et al., 1998; Akhteruzzaman et al., 1999).

In contrast to mrp3/MRP3, the human MRP6 (Kool et al., 1999a) and its rat homolog MLP-1 (Hirohashi et al., 1998) are highly expressed in normal liver. However, neither the transport function nor the exact hepatocellular localization of mrp6/MRP6 has been investigated. Therefore, we cloned mrp6 from rat liver, compared its transport activity with those of mrp2 and Bsep in transfected Sf9 cells, and investigated its hepatocellular localization by in situ immunofluorescence confocal microscopy. The results demonstrate that mrp6 can mediate ATP-dependent transport of the anionic cyclopentapeptide BQ-123 and that it is localized at the lateral and canalicular borders of normal rat hepatocytes.

Experimental Procedures

Materials. [γ-^{32}P]ATP (2500 Ci/mmol), [^{14}C]-1-chloro-2,4-dinitrobenzene ([^{14}C]CDNB) (10 mCi/mmol), and [propyl-3,4(n)-^{3}H]BQ-123 (42.0 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Buckinghamshire, England). [^{3}H]Taurocholic acid (2.1 Ci/mmol), [^{3}H]leukotriene C_4 (165 Ci/mmol), [*glycine*-2-^{3}H]glutathione (reduced form; GSH) (44.8 Ci/mmol), [^{3}H]estradiol-17β-D-glucuronide (49.0 Ci/mmol), [^{125}I]endothelin-I (2200 Ci/mmol), and [^{125}I]Arg^{8}-vasopressin (2200 Ci/mmol) were purchased from NEN Life Sciences Products Inc. (Boston, MA). [^{3}H]Sulfotaurolithocholic acid (0.79 Ci/mmol) was kindly provided by Dr. Alan F. Hofmann (Department of Medicine, University of California at San Diego). [^{14}C]2,4-Dinitrophenyl-*S*-glutathione was synthesized using [^{14}C]CDNB, GSH, and glutathione-*S*-transferase as described previously (Awasthi et al., 1981). Hybridomas producing monoclonal antibodies against aminopeptidase N were the generous gift of Dr. A. Quaroni (Cornell University, Ithaca, NY) (Quaroni and Isselbacher, 1985). The mouse monoclonal antibody CX 12H10 against connexin 26 was obtained from Zymed Laboratories (San Francisco, CA). Secondary antibodies conjugated to Cy2 or Cy3 were obtained from Amersham and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively.

Synthesis of [^{3}H]Glutathione (Oxidized Form; GSSG). [^{3}H]GSH was extracted with ethyl acetate to remove dithiothreitol (Butler et al., 1976) and diluted with water to obtain 1 μCi/μl. [^{3}H]GSSG was synthesized according to Flohé and Günzler (1984) with some modifications. Glutathione peroxidase from bovine erythrocytes (Sigma Chemical Co., St. Louis, MO) was resuspended in 0.05 M sodium phosphate buffer, pH 7.4, in the presence of 2 mg/ml BSA (protease free; Life Technologies), and after overnight dialysis against 0.05 M sodium phosphate buffer, pH 7.4, it was stored at −20°C in the presence of 50% glycerol. The incubation mixture of 150 μl containing 50 μCi of [^{3}H]GSH, 33 mM sodium phosphate buffer, pH 7.4, 1.33 mM unlabeled GSH, and 0.2 U of glutathione peroxidase was preincubated for 10 min at 37°C. After the addition of 50 μl of freshly prepared 5 mM H_2O_2, the incubation was continued for an additional 5 min. The mixture was then placed on ice and immediately diluted with the uptake solution. Directly after the [^{3}H]GSSG synthesis, a 50-μl sample was analyzed using HPLC (12.5-cm μBondak C18 column, elution with 50 mM formic acid, flow 0.8 ml/min, peak monitoring at 226 nm) (Winterbourn and Brennan, 1997). The purity of the synthesized GSSG was at least 99%.

Animals. Male Sprague-Dawley rats were obtained from RCC Ltd. (Füllinsdorf, Switzerland). Male GY/TR$^-$ rats were provided by Drs. Peter L. M. Jansen and F. Kuipers (University of Groningen, Groningen, the Netherlands) and bred in-house.

Cell Lines and Media. CHO (Chinese hamster ovary), COS-1 and COS-7 (African green monkey kidney, SV40 transformed), HeLa (human cervix epitheloid carcinoma), 3LL (mouse lung), MDCK (dog kidney), MH1C1 (rat Morris hepatoma), and V79 (Chinese hamster lung) were propagated in Iscove's modified Dulbecco's medium (Life Technologies, Rockville, MD) supplemented with 10% (v/v) FCS (Life Technologies) at 37°C and 5% CO_2 atmosphere in a humidified incubator. The Sf9 insect cells (PharMingen, San Diego, CA) were grown in Grace's Insect Medium Supplemented (Life Technologies) in the presence of 10% (v/v) FCS at 27°C in a humidified incubator.

Cloning of mrp6. A random and poly(dT) primed λgt-10 rat liver cDNA library (Clontech, Palo Alto, CA) was screened with two reverse transcription-polymerase chain reaction (RT-PCR) fragments of MRP1 as described previously (Madon et al., 1997). An isolated 1.80-kb mrp-like partial cDNA clone was used as a hybridization probe to screen a rat liver cDNA library prepared from size-fractionated poly(A)$^+$ RNA (Gerloff et al., 1998). This resulted in the isolation of a 5.7-kb clone that was sequenced on both strands and found to contain only four nucleotides upstream of the first initiation codon. To extend the 5′ end of this clone, 2 μg of total rat liver RNA was reverse transcribed using the 5′/3′ RACE kit (Boehringer-Mannheim Biochemica) and the specific mrp6 primer P-191 (5′-GGTAGCAG-CAGCCATGGCGATGGATGTAGAG-3′) (Microsynth, Balgach, Switzerland). Amplification was performed according to the kit instructions using P-193 (5′-GTAGATGGGGCCAAGGACCCAGAGG-TACAT-3′) as mrp6 specific primer. Gel electrophoresis of the final reaction product showed three main bands ranging between 240 and 295 bp. These fragments were gel purified (Qiagen DNA Extraction Kit; Qiagen, Hilden, Germany), subcloned into the pGEM-T vector (Promega, Madison, WI), and sequenced. This resulted in a final clone containing an additional 85 nucleotides at the 5′ end.

5′ End Primer Extension. The oligonucleotides P-191 and P-193 were labeled using [γ-^{32}P]ATP (Amersham) and polynucleotide kinase (Boehringer-Mannheim Biochemica). Labeled oligonucleotide (200,000–500,000 cpm) was mixed with 75 μg of total rat liver RNA and precipitated in the presence of 0.5 M NaCl with one volume of isopropanol. After washing and drying, the pellet was resuspended in 50 μl of 50% formamid, 0.4 M NaCl, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)-HCl, pH 6.4, and 1 mM EDTA. The mixture was incubated for an initial 3 min at 85°C, and the hybridization was carried out for 18 h at 42°C. After a 10-fold dilution with water, the nucleic acids were precipitated with 1 volume of isopropanol. The washed and dried pellet was resuspended in 30 μl of water and used for primer extension with the Superscript II reverse transcriptase (Life Technologies). The primer extension product was precipitated with isopropanol, and after washing and drying, it resuspended in 10 μl of sequencing loading buffer. A 4-μl sample was separated on a urea sequencing gel.

In Vitro Transcription-Translation. For in vitro transcription-translation studies, the Promega TNT T7 Coupled Reticulocyte Lysate System was used in the presence of canine pancreatic microsomes (Promega) with 1 pmol of mrp6-PCR fragments containing the T7 promoter sequence at their 5′ end as described previously (Sarkar and Sommer, 1989; Lancaster et al., 1996). The PCR fragments covered nucleotides 63–656, 81–656, and 204–656 of the mrp6 cDNA sequence.

Expression in Sf9 Insect Cells. The mrp6 cDNA corresponding to the longest major transcript (starting at nucleotide 54; see Fig. 1) was cloned into the baculovirus transfer vector pFast-Bac1 (Life Technologies) by a combination of restriction digestion and PCR. The full-length mrp2 cDNA was subcloned into pFastBac1 using restriction enzymes cutting in the multiple cloning site of pKEH1 (Madon et al., 1997). The recombinant baculovirus strains were prepared using the Bac-to-Bac system (Life Technologies). mrp6 was expressed in Sf9 cells infected at a multiplicity of infection (m.o.i.) of 2.0 and cultured for 3 days; for mrp2 expression, Sf9 cells were infected at an m.o.i. of 7.5 and cultured for 2 days; and for Bsep expression, Sf9 cells were infected at a an m.o.i of 10, and the cells were harvested after 3 days (Gerloff et al., 1998). The isolation of the membrane vesicles from virus-infected Sf9 cells was performed as described in Gerloff et al. (1998). The vesicles were suspended in

SKMH [50 mM sucrose, 100 mM KNO_3, 12.5 mM $Mg(NO_3)_2$, 10 mM HEPES-Tris, pH 7.4) or SMH (250 mM sucrose, 10 mM $MgCl_2$, 10 mM HEPES-Tris, pH 7.4) buffer. The expression levels of mrp6 and mrp2 were controlled by Western blot analysis of the isolated membrane vesicles.

Uptake Studies. ATP-dependent substrate uptake was determined at 37°C with an ATP-regenerating system by a rapid filtration technique (Stieger et al., 1992) using vesicles isolated from control Sf9 cells (infected with the wild-type baculovirus) as well as from mrp6-, mrp2-, or Bsep-expressing Sf9 cells. The stop solution was ice-cold incubation buffer (SKMH or SMH), which was supplemented with 1% (w/v) BSA (Sigma Chemical Co.) in the case of BQ-123, endothelin-1, and Arg^8-vasopressin uptake experiments to reduce nonspecific filter binding.

Northern and Western Blotting. Northern blots were performed as described previously (Madon et al., 1997). Polyclonal antisera were raised in rabbits against oligopeptides as described previously (Stieger et al., 1994) using the C-terminal 16 amino acids of mrp6 and the C-terminal 11 amino acids of mrp2 coupled via an additional N-terminal cysteine residue to keyhole limpet hemocyanin (Neosystem, Strasbourg, France). To test the respective specificity of the antisera, isolated basolateral and canalicular rat liver plasma membrane vesicles (Meier et al., 1984), as well as vesicles isolated from Sf9 cells expressing mrp6, mrp2, or Bsep, were separated by SDS-polyacrylamide gel electrophoresis and processed by Western blotting as in Stieger et al. (1994).

Immunofluorescence Microscopy. Semithin (0.5–1 μm) sections from rat livers that had been perfusion fixed with 4% (w/v) paraformaldehyde were used as described previously (Stieger et al., 1994). For confocal through focus view methodology, 10-μm cryostat sections from native livers were fixed for 5 min in methanol at −20°C before further processing with antibodies, and data were acquired with a Leica (Glattbrugg, Switzerland) TCS 4D confocal laser scanning microscope.

Other Methods. Protein concentration was determined using the BCA protein assay kit from Pierce Chemical (Rockford, IL)

Results

Cloning of mrp6 from Rat Liver. Screening a size-fractionated rat liver cDNA library with MRP1 PCR fragments (Madon et al., 1997) eventually resulted in the isolation of a rat liver cDNA coding for a mrp-like protein of 1502 amino acids. Comparison of its amino acid sequence with already cloned rat and human mrps/MRPs revealed that the cloned mrp was 100% identical with rat MLP-1 (Hirohashi et al., 1998) and 79% identical with human MRP6 (Kool et al., 1999a) (Table 1). Considerable less amino acid identity was found with other members of the mrp/MRP family of ABC transporters such as the human MRP1 (Cole et al., 1992) and the rat mrp3 (Hirohashi et al., 1999) and mrp2 (Büchler et al., 1996; Paulusma et al., 1996) (Table 1). Less than 25% amino acid identity was found for the rat members of the mdr family of ABC transporters, including mdr1, mdr2, and the rat liver canalicular bile salt export pump Bsep (Gerloff et al., 1998) (Table 1). Because of its high degree of homology to MRP6 (Kool et al., 1999a), the cloned mrp appears to be the rat homolog of the human MRP6 and is therefore called mrp6 throughout this study.

Transcription-Translation Initiation Sites of mrp6 cDNA/mRNA. Cloning of the full-length cDNA was tested by primer extension analysis. As demonstrated in Fig. 1A, three major transcripts containing 8, 16, and 18 nucleotides upstream of the first AUG were detected. Furthermore, two minor mRNA transcripts were found to contain 50 and 59 additional nucleotides (Fig. 1A). These results, together with the cDNA sequence obtained by 5′ rapid amplification of cDNA ends (Fig. 1B), demonstrate that the isolated mrp6 cDNA indeed represents a full-length clone. Because it was intended to functionally express mrp6 in Sf9 cells using the baculovirus system, which is known to use the first ATG encountered to initiate protein synthesis, we had to determine which of the two ATGs shown in Fig. 1B at positions 72 and 89 represents the initiation codon. Therefore, we next performed in vitro transcription-translation experiments. As illustrated in Fig. 1C, the highest amount of the expected 22-kDa translation product was obtained with a PCR fragment containing both ATGs (lane 1). If a PCR fragment starting after the first ATG was used, the translation efficiency decreased to ~50% (lane 2). However, if the first two ATGs were skipped and translation initiation was attempted at the third ATG (position 213 in Fig. 1B), no protein product was formed (lane 3). Similar results were obtained when the first or second ATG was mutated to other amino acids (data not shown). These results indicate that both ATGs at positions 72 and 89 (Fig. 1B) may be used as translation initia-

Fig. 1. Analysis of transcription-translation initiation sites of mrp6. A, 5′ end primer extension of mrp6 mRNA was performed using total rat liver RNA as described in *Materials and Methods*. The primer extension product (lane P) was run alongside the sequencing reactions (lanes A, C, G, and T) for accurate transcription start site determination. B, 5′ end sequence of the mrp6 cDNA. The shaded boxes mark the first, second, and third ATG of the mrp6 cDNA; asterisks indicate the positions of different transcription initiation sites as determined by primer extension. C, in vitro translation products were separated on a SDS-polyacrylamide gel and detected using autoradiography. As templates, PCR fragments starting 9 bp before the first ATG (nucleotides 63–656) (lane 1), 9 bp before the second ATG (nucleotides 81–656) (lane 2), or 9 bp before the third ATG (nucleotides 204–656) (lane 3) were used.

tion codons and hence contribute to the synthesis of the mrp6 protein. Therefore, both ATGs were included for subcloning into the vector for the expression of mrp6 in the baculovirus system.

Functional Expression and Characterization of mrp6 in Sf9 Cell Vesicles. To characterize its transport function, mrp6 was cloned into a baculovirus expression vector and expressed in insect Sf9 cells. For comparison, the canalicular bile salt export pump Bsep and the multispecific organic anion transporter mrp2 were also included. As summarized in Table 2, among the tested Bsep and mrp2 substrates only the anionic cyclic pentapeptide BQ-123 was also transported by mrp6. Thereby, both mrp2- and mrp6-mediated BQ-123 transport exhibited substrate saturability with apparent K_m values of ~ 24 and ~17 μM, respectively (Fig. 2). Because endogenous endothelin-1 is excreted into bile and has a similar ring structure as BQ-123 (Bluhm et al., 1993; Kraus et al., 1998), we wondered whether mrp2 and/or mrp6 might also be involved in the mediation of hepatobiliary transport of endothelin-1. As illustrated in Fig. 3, ATP-dependent transport of endothelin-1 was indeed found for mrp2, but not for mrp6, indicating that mrp2 is responsible for physiological excretion of endothelin-1 into bile (Bluhm et al., 1993; Kraus et al., 1998). In addition, cyclic Arg^8-vasopressin was also found to be a transport substrate of mrp2 (Fig. 3). Despite an extensive further search that included glycocholate, estrone-3-sulfate, dehydroepiandosteronesulfate, prostaglandins E_1 and E_2, thyroxine and triiodothyronine, vinblastine, doxorubicine, *para*-aminohippurate, the phosphatidylcholine derivative dibutyroyl-glycero-3-phosphatidylcholine, and the fluorescent aminophospholipid NBD-phosphatidylserine, no other substrates for mrp6 could be identified so far.

Organ Distribution and Cellular Localization of mrp6. To determine the expression of mrp6 in different tissues, Northern blot analysis was performed. As demonstrated in Fig. 4A, mrp6 mRNA is predominantly expressed in the liver and to a lesser extent in kidney, small intestine, and colon. No significant differences in hepatic expression of mrp6 were observed between the mutant GY/TR$^-$ and the wild-type (normal) rat strains. Although expressed in hepatocytes, mrp6 mRNA could be detected in neither the rat hepatoma cell line MH1C1 nor in any other cell lines derived from kidney, lung, cervix, or ovary (Fig. 4B).

To investigate the cellular and subcellular localization of mrp6 in rat liver, a polyclonal antiserum directed against a C-terminal synthetic peptide of mrp6 was raised in rabbits (Fig. 5). The antiserum reacted strongly with isolated rat liver basolateral and, to a lesser extent, with canalicular plasma membranes of rat liver (Fig. 5A). The recognized antigen exhibited an apparent molecular mass of 178.3 ± 5.7 kDa (n = 4). Because the exact immunolocalization of mrp6 depends on the specificity of the antiserum used, we compared the reactivity of the antiserum against the three ABC transporters mrp2, mrp6, and Bsep. As illustrated in Fig. 5B, each antiserum (α) reacted with its corresponding antigen expressed in Sf9 cell vesicles. Most importantly, the absence of any cross-reactivity of the mrp6 antiserum with mrp2 and/or Bsep strongly indicates that the raised antiserum is suitable for specific localization of mrp6 in rat liver.

As illustrated in Fig. 6, hepatocellular expression of mrp6 is predominantly confined to the lateral border of hepato-

TABLE 1

Sequence comparison of mrp6 with other ABC transporters

Sequence comparison was performed using the Bestfit program of the Wisconsin Sequence Analysis Package.

Transporter	Amino Acids	Identity	Similarity	Accession Number
mrp6 (rat)	1502	100.0	100.0	U73038
MLP-1 (rat)	1502	100.0	100.0	AB010466
MRP6 (human)	1503	79.4	86.7	AF076622
MRP1 (human)	1531	44.6	65.7	P33527
mrp3 (rat)	1523	40.2	62.3	AF072816
ebcr (rabbit)	1564	37.1	60.7	Z49144
mrp2 (rat)	1541	36.8	59.4	L49379
ycf1 (yeast)	1515	35.2	57.3	P39109
cftr (mouse)	1476	28.6	53.2	P26361
mdr2 (rat)	1278	24.6	50.5	Q08201
mdr1 (rat)	1277	24.1	49.4	P43245
Bsep (rat)	1321	23.0	47.9	U69487

TABLE 2

Comparison of the substrate specificities among Bsep, mrp2, and mrp6

Vesicles were isolated from Sf9 cells infected either with wild-type baculovirus (control) or baculovirus containing Bsep, mrp2, or mrp6. Uptake experiments were performed as described in *Materials and Methods*. In the case of BQ-123 uptake, the incubation solution was supplemented with 1 mg/ml BSA (protease free; Life Technologies). The following substrates at concentrations given in parentheses were used: TCA, taurocholate; STLC, sulfotaurolithocholic acid; DNP-SG, *S*-(2,4-dinitrophenyl)-glutathione; LTC_4, leukotriene C_4; and E-17β-G, estradiol 17β-D-glucuronide. ATP-dependent uptake rates are given as pmol substrate/mg protein × min as mean ± S.D. of triplicate determinations.

Substrate	Control Vesicles	Bsep Vesicles	Mrp2 Vesicles	Mrp6 Vesicles
TCA (2 μM)	1.1 ± 0.8	18.3 ± 2.9*	1.1 ± 0.7	1.0 ± 0.4
STLC (2 μM)	3.2 ± 1.7	3.7 ± 1.4	41.1 ± 3.7*	2.5 ± 0.3
GS-DNP (25 μM)	6.5 ± 2.4	6.3 ± 3.4	81.4 ± 7.2*	7.4 ± 0.5
LTC_4 (0.05 μM)	0.7 ± 0.2	0.9 ± 0.3	3.6 ± 0.3*	0.7 ± 0.04
E-17β-G (10 μM)	8.5 ± 1.2	7.9 ± 0.2	56.8 ± 6.5*	7.2 ± 2.6
GSSG (100 μM)	1.4 ± 1.5	1.0 ± 1.3	51.7 ± 2.7*	0.3 ± 0.02
BQ-123 (5 μM)	0.2 ± 0.1	0.3 ± 0.1	11.6 ± 0.3*	5.9 ± 0.3*

* Values significantly different from control values ($P < .02$).

cytes (green fluorescence) with the sinusoidal plasma membrane domain being virtually devoid of mrp6 (Fig. 6A). Furthermore, bile canaliculi as visualized with a monoclonal antibody against aminopeptidase N (red fluorescence) showed partial overlapping fluorescence (yellow), indicating expression of mrp6 at the canalicular plasma membrane as well (Fig. 6, A and B). Because its lateral localization could indicate a possible role of mrp6 in intercellular communication, we also investigated the expression of mrp6 in relation to the gap junction protein connexin 26. Although some overlapping fluorescence (yellow) was seen at the periphery of some but not all gap junctions, most mrp6 (green fluorescence) and connexin 26 (red fluorescence) did not overlap in a confocal through a focus view of the cryostat sections (Fig. 6C). These results indicate predominant expression of mrp6 outside the gap junctional area and therefore do not support a major functional role of mrp6 in gap junction-mediated intercellular communication.

Discussion

In the present study, the rat mrp6 has been identified as an ATP-dependent transporter of the anionic cyclopentapeptide BQ-123 and localized to the lateral and to the canalicular plasma membrane domains of rat hepatocytes. The cloned mrp6 is similar in sequence to the previously reported MLP-1 (Hirohashi et al., 1998) and exhibits a 79% amino acid identity with the human MRP6 (Kool et al., 1999a). The latter does not contribute to the multidrug-resistant phenotype, and its overexpression has been found to be invariably associated with amplification of the *MRP1* gene, which is localized adjacent to the *MRP6* gene on human chromosome 16p13 (Kool et al., 1999a). Both mrp6 and MRP6 exhibit a similar tissue distribution, with the highest expression in liver, followed by kidney, intestine, and colon (Fig. 4) (Kool et al., 1999a). Thus, in contrast to mrp1 (Roelofsen et al., 1997) and mrp3 (Hirohashi et al., 1998), mrp6 is significantly expressed in normal rat liver and its expression is not induced in mrp2-deficient rat strains (Fig. 4) or during experimental cholestasis (Hirohashi et al., 1998).

The classic substrates of the mrp family of membrane transporters are phase 2 biotransformation products such as glucuronide, sulfate, and glutathione conjugates. However, none of these typical anionic mrp2 substrates were transported by mrp6 as demonstrated in vesicles isolated from transfected Sf9 cells (Table 2). These results suggest that the main function of mrp6 is somewhat different from that of the other mrps and does not involve typical detoxification and drug-resistance mechanisms. This view is supported by the absence of mrp6 induction in mrp2-deficient rat strains. On the other hand, the anionic cyclopentapeptide BQ-123 was identified as a common substrate of mrp2 and mrp6 (Table 2, Fig. 2). This potent and selective endothelin A receptor antagonist is rapidly eliminated in bile without any biotransformation. Our data confirm that mrp2 plays a decisive role in the biliary excretion of BQ-123 (Shin et al., 1997). Furthermore, the physiological peptides endothelin-1 and Arg^8-vasopressin have been identified as new substrates of mrp2 (Fig. 3). Although these results provide an explanation for the biliary excretion of endothelin-1 in the isolated perfused rat liver (Bluhm et al., 1993), the physiological significance of mrp2-mediated Arg^8-vasopressin transport remains unknown. However, based on transport studies in canalicular vesicles isolated from mrp2-deficient rat liver, it has been

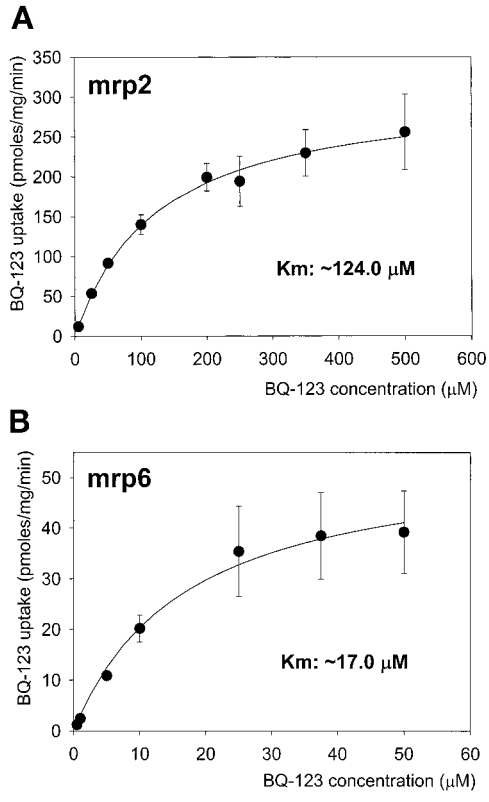

Fig. 2. Uptake kinetics of the endothelin receptor antagonist BQ-123 by membrane vesicles isolated from mrp2 and mrp6 expressing Sf9 cells. mrp2 and mrp6 vesicles were resuspended in SMH and SKMH buffer (see *Materials and Methods*), respectively. Incubations were carried out in the same buffers supplemented with 1 mg/ml BSA (protease free; Life Technologies) in the presence of increasing concentrations of BQ-123. Initial (linear) uptake rates were determined at 3.5 min for mrp2 and at 2.0 min for mrp6 in the absence or presence of 4 mM ATP. ATP-dependent uptake rates were calculated by subtracting uptake values in the absence of ATP from total uptake values in the presence of ATP. The values are the mean ± S.D. of triplicate determinations in two separate experiments.

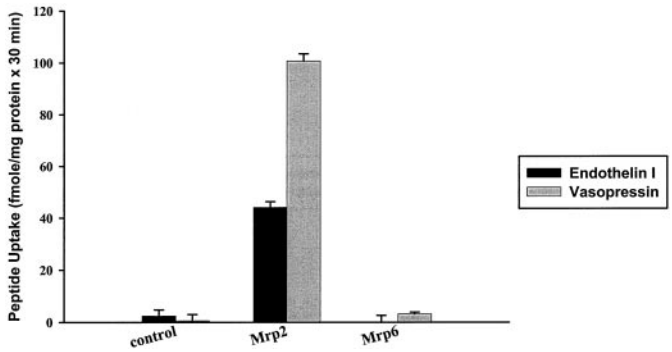

Fig. 3. Transport of endothelin-1 and Arg^8-vasopressin by membrane vesicles isolated from mrp2 and mrp6 expressing Sf9 cell vesicles. Vesicles from Sf9 cells infected with either wild-type baculovirus (control) or baculovirus containing mrp2 or mrp6, were resuspended in SKMH buffer. Uptake of endothelin-1 or Arg^8-vasopressin (each at a concentration of 0.22 nM) was measured in the same buffer supplemented with 1 mg/ml BSA (protease free; Life Technologies) during 30 min in the presence and absence of 4 mM ATP as described in *Materials and Methods*. ATP-dependent uptake of a representative experiment is given as the differences between uptake values in the presence or absence of ATP. The values are mean ± S.D. of triplicate experiments.

repeatedly suggested that additional ATP-dependent carriers must be involved in overall biliary elimination of cyclic and linear hydrophobic peptides (Ziegler et al., 1994; Yamada et al., 1996; Takahashi et al., 1997). In this regard, our finding that mrp6 exhibits a ~7-fold higher affinity for BQ-123 compared with mrp2 (Fig. 2) indicates that mrp6 is potentially also involved in the hepatocellular handling of cyclic oligopeptides. A major role of mrp6 in biliary elimination of peptidic compounds, however, would require a polar expression of mrp6 at the canalicular membrane of rat hepatocytes. Therefore, we developed specific mrp6 antibodies (Fig. 5) and investigated the subcellular expression of mrp6 in rat liver.

Interestingly, mrp6 was found to be localized at the lateral and canalicular border of rat hepatocytes (Fig. 6). This dual localization of mrp6 is difficult to reconcile with an exclusive role of mrp6 in biliary excretion. In general, active pumping of organic solutes into the lateral intercellular cleft is expected to result in sinusoidal solute outflow rather than in paracellular solute movement across tight junctions into bile canaliculi. This is especially true for anionic organic compounds such as BQ-123, because the hepatic tight junctions exhibit high permselectivity for negatively charged organic solutes (Bradley and Herz, 1978). In addition, mrp6-mediated intercellular accumulation of osmotically active compounds would reduce the physiological osmotic bile to plasma gradient, thereby reducing the paracellular water flow from blood into bile. On the other hand, mrp6 appears to be present at the canalicular membrane as well (Fig. 6), where it could be involved in the active biliary excretion of BQ-123 and other cholephilic organic compounds. Alternatively, the localization of mrp6 on both sides of the tight junctions may suggest a role of mrp6-mediated transport in the regulation of tight junctional permeability. Such a function of mrp6 in the regulation of the paracellular permeability, however, should be independent of vasopressin transport, because this cyclic polypeptide was found to be transported by mrp2 but not by mrp6 (Fig. 3). We also could not find any evidence for the involvement of mrp6 in phospholipid translocation using the phosphatidylcholine derivative dibutyroyl-glycero-3-phosphatidylcholine and the fluorescent aminophospholipid NBD-phosphatidylserine (data not shown). Hence, the physiological function and endogenous substrate specificity of mrp6 remain to be elucidated.

Besides mrp6, mrp1/MRP1 has been previously shown to be mainly localized at the lateral membrane of rat and human hepatocytes (Roelofsen et al., 1997), and MRP3 was

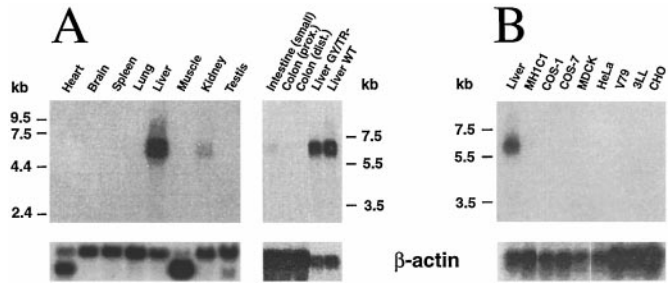

Fig. 4. Northern blot analysis of mrp6 in different rat tissues and cell lines. A, 1 μg of poly(A)$^+$ RNA isolated from different rat tissues was separated on a 1.2% agarose-formaldehyde gel and after transfer analyzed by hybridization under high stringency conditions using a PCR fragment of mrp6 (nucleotides 122–647). GY/TR$^-$ rats are deficient in functional mrp2 (Kusuhara et al., 1998) WT, wild-type (normal) rats. B, 5 μg of total RNA obtained from rat liver and the indicated cell lines (see *Materials and Methods*) was analyzed as described above. For both blots, a human β-actin probe (Clontech) was used as a positive control.

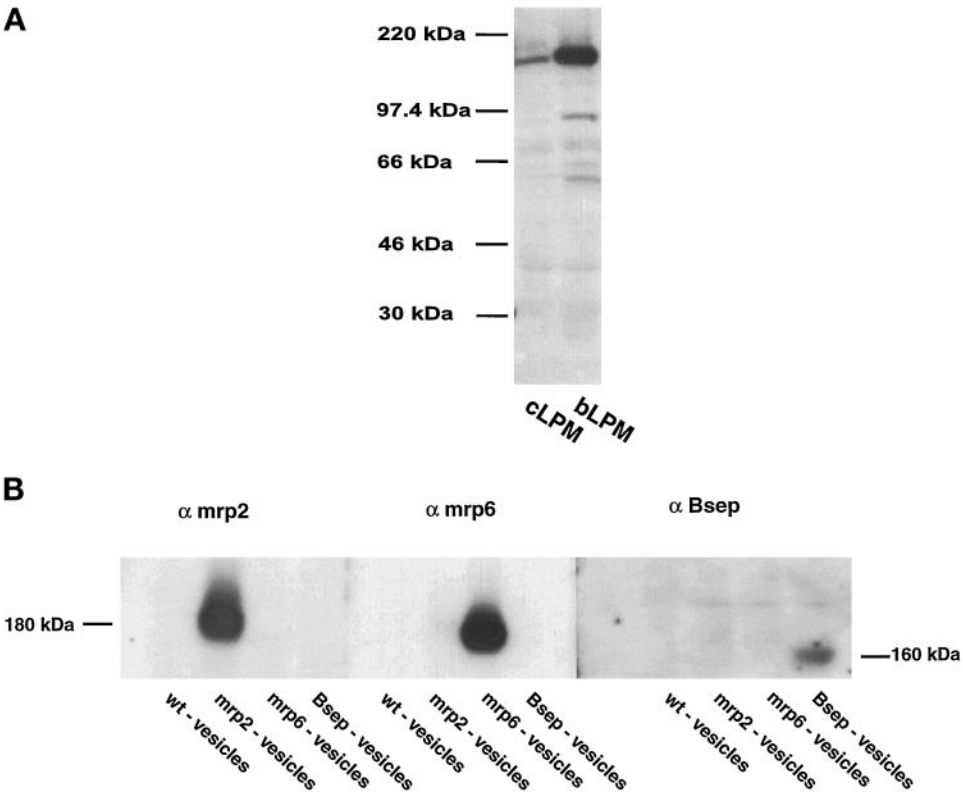

Fig. 5. Characterization of the polyclonal antiserum against mrp6. A, lanes were loaded with 125 μg of protein of basolateral (bLPM) and canalicular (cLPM) plasma membrane vesicles, respectively. Western blotting was performed as described in *Materials and Methods*, and the blot incubated was with the polyclonal antiserum against the C-terminus of mrp6. B, lanes were loaded with 75 μg of protein of wild-type (wt) vesicles and vesicles expressing mrp2, mrp6 or Bsep (see *Materials and Methods*). Western blotting with antibodies (α) against mrp2, mrp6, or Bsep was performed as described in *Materials and Methods*.

localized to the basolateral domain of hepatocytes (Kool et al., 1999b). In contrast to mrp6, however, the level of mrp1/MRP1 in normal hepatocytes is very low. The lateral expression of mrp1/MRP1 was markedly induced in proliferating hepatocyte-derived cells and found to be dependent on the establishment of cell-cell contacts (Roelofsen et al., 1997). Similarly, the canalicular expression of mrp2 was decreased and the lateral expression of mrp1 was increased in cultured rat hepatocytes (Roelofsen et al., 1999). Because mrp1 and mrp2 exhibit closely related transport functions, the proliferation-dependent switch from apical mrp2 to lateral mrp1 expression preserves glutathione conjugate excretion and protects proliferating hepatocytes against certain cytotoxic substances. Although mrp6 expression remains to be investigated in quiescent and proliferating cultured hepatocytes, preliminary studies in regenerating rat liver after partial hepatectomy indicated no significant changes in the expression of mrp6 (data not shown). Hence, in contrast to the induction of basolateral mrp3 in mrp2-deficient rat strains and during cholestasis (see above) and unlike the up-regulation of lateral mrp1 expression in proliferating hepatocytes, constitutive mrp6 expression is preserved in hepatocytes independent of apical mrp2 levels, cholestasis, and/or the cell cycle. This stable mrp6 expression under various physiological and pathophysiological situations suggests that mrp6 functions as an important constitutive "housekeeping" transporter in normal and abnormal hepatocytes. In this regard, the peptidic nature of the xenobiotic substrate BQ-123 might point to physiological small peptides as possible endogenous mrp6 substrates that might be involved in cellular signaling and/or autocrine or paracrine regulation of hepatocellular functions.

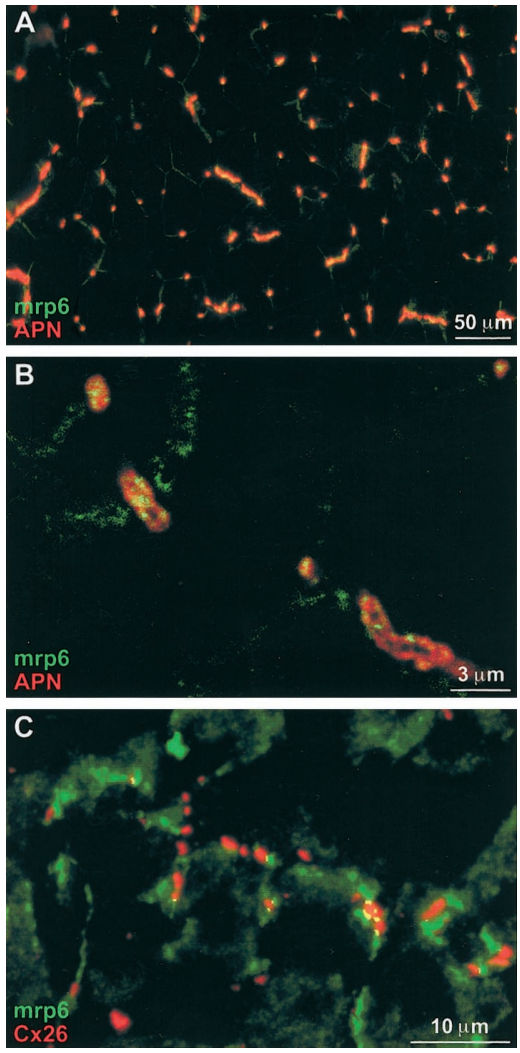

Fig. 6. Cellular localization of mrp6 in rat liver. A, semithin cryosections of rat liver were used for indirect immunofluorescence and probed with antiserum against mrp6 (green) and with a monoclonal antibody recognizing the canalicular enzyme aminopeptidase N (red). mrp6 was expressed in the lateral as well as in the canalicular plasma membrane domain of hepatocytes as suggested by yellow signal originating from overlap of green mrp6 and red aminopeptidase N signal in the canalicular plasma membrane. B, a singular 0.3-μm optical section from the same specimen was obtained by confocal laser scan microscopy. Canaliculi (red) show a patchy mrp6 signal (yellow), whereas lateral membrane domains are green. The two mrp6-positive membranes in the top left corner are cut obliquely. C, cryostat sections (10 μm) were immunoreacted with antiserum against mrp6 (green) and with a monoclonal antibody against the gap junction protein connexin 26 (red). A stack of 22 confocal images was visualized as maximum intensity projection and pixels showing overlap of green and red channels were highlighted in yellow. Colocalization of mrp6 and connexin 26 is restricted to the periphery of some gap junctions only, indicating no direct participation of mrp6 in intercellular communication.

In conclusion, mrp6 has been characterized as a lateral and canalicular ABC transporter in rat hepatocytes. This dual localization of mrp6 is unique, because the other hepatic ABC transporters are localized selectively at either the canalicular (i.e., mdr1/MDR1, mdr2/MDR3, Bsep/BSEP, and mrp2/MRP2) or the basolateral (i.e., mrp1/MRP1 and mrp3/MRP3) plasma membrane domains of hepatocytes. Although mrp6 can mediate ATP-dependent transport of the anionic cyclopentapeptide BQ-123, its physiological transport function and regulation appear to be different from the other hepatic mrps, such as mrp1, mrp2, and mrp3. Elucidation of the constitutive cellular function of mrp6 can be most probably be best achieved by the generation of mrp6 knock-out mice.

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