ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2

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Bilirubin is secreted from the liver into bile mainly as mono-glucuronosyl and bisglucuronosyl conjugates. We demonstrate for the first time that ATP-dependent transport of both bilirubin glucuronides is mediated by the multidrug resistance protein (MRP1) as well as by the distinct canalicular (apical) isoform MRP2, also termed cMRP or cMOAT (canalicular multispecific organic anion transporter). In membrane vesicles from *MRP1*-transfected HeLa cells mono[³H]glucuronosylbilirubin and bis-[³H]glucuronosylbilirubin (each at 0.5 μM) were transported with rates of 5.3 and 3.1 pmol/min per mg of protein respectively. Rat hepatocyte canalicular membrane vesicles, which contain Mrp2 (the rat equivalent of MRP2), transported mono-[³H]glucuronosylbilirubin and bis[³H]glucuronosylbilirubin at rates of 8.9 and 8.5 pmol/min per mg of protein, whereas membrane vesicles from mutant liver lacking Mrp2 showed no transport of the conjugates. In membrane vesicles from human hepatoma Hep G2 cells, which predominantly expressed MRP2, transport rates were 8.3 and 4.4 pmol/min per mg of protein for monoglucuronosylbilirubin and bisglucuronosylbilirubin respectively. ATP-dependent transport of the glutathione S-conjugate [³H]leukotriene C₄, an established high-affinity substrate for MRP1 and MRP2, was inhibited by both bilirubin glucuronides with IC$_{50}$ values between 0.10 and 0.75 μM. The ratios of leukotriene C₄ transport and bilirubin glucuronide transport, determined in the same membrane vesicle preparation, indicated substrate specificity differences between MRP1 and MRP2 with a preference of MRP2 for the glucuronides.

INTRODUCTION

Bilirubin, the main product of haem catabolism, is excreted from the hepatocytes into bile mainly as monoglucuronosylbilirubin and bisglucuronosyl bilirubin [1–5]. These glucuronides are formed in the endoplasmic reticulum by UDPglucuronosyl transferases of the UGT1 subfamily (reviewed in [6]).

The secretion of compounds against a concentration gradient is mediated by ATP-dependent export pumps. Among the members of the ATP-binding cassette transporter family present in the hepatocyte canalicular membrane is the ATP-dependent conjugate export pump [7–10], also termed canalicular multi-specific organic anion transporter (cMOAT) [11]. This transporter function is deficient in the hyperbilirubinaemic mutant Wistar rats GY/TR⁻ [12–14] and Eisai hyperbilirubinaemic rats (EHBR) [15], as well as in the human Dubin–Johnson syndrome [10,16,17]. Conjugated bilirubin has been considered a prominent substrate for this conjugate export pump. In the mutant rats bilirubin glucuronides accumulate in blood and liver and are eliminated largely by renal excretion [12,13]. The impairment of ATP-dependent transport of bilirubin glucuronides has been demonstrated with hepatocyte canalicular membrane vesicles from GY/TR⁻ mutants [18]. In addition to the transport of bilirubin glucuronides, the canalicular export of several other amphiphilic anions, including glutathione S-conjugates such as leukotriene C₄ (LTC₄) [19], and glucuronidated and sulphated bile salts [14], is deficient in these mutants. The multidrug resistance protein, MRP1, a cloned and sequenced member of

the ATP-binding cassette family of transport proteins [20], transports a similar spectrum of substrates. It has been demonstrated that MRP1 mediates the ATP-dependent transport of glutathione S-conjugates, such as LTC₄ and S-(2,4-dinitro-phenyl)glutathione [21–23], in addition to the transport of glucuronidated and sulphated compounds such as 6α-glu-curonosylhyodeoxycholate and 3α-sulphatolithocholyltaurine [24]. MRP1 has been detected in most cell types and tissues [20,25,26].

Recently, the cDNA for the canalicular (apical) conjugate export pump deficient in GY/TR⁻ and EHBR mutant rats has been cloned and sequenced [27,28]. This rat transport protein was termed cMoat [27] or cMrp [28]; the term Mrp2 is used here. The cDNA of the human homologue MRP2 (cMRP/cMOAT) has also been cloned and sequenced, and the protein has been localized to the hepatocyte canalicular membrane [10,28–30]. MRP2 is considered a functional isoform of MRP1. MRP2 is expressed in the apical membrane of polarized cells [10,29].

The molecular identification of these export pumps enabled us in the present study to characterize the transport of mono-glucuronosyl- and bisglucuronosyl-bilirubin mediated by these defined members of the MRP family. The ATP-dependent transport of bilirubin conjugates into inside-out oriented vesicles from *MRP1*-transfected HeLa cells was demonstrated and compared with Mrp2-mediated transport into rat hepatocyte cana-licular membrane vesicles by using the control vector-transfected HeLa cell membranes and canalicular membrane vesicles from GY/TR⁻ mutant liver as controls. Transport mediated by human

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MRP2 was characterized in membranes prepared from Hep G2 hepatoma cells, which have been shown to express large amounts of this protein under certain culture conditions.

MATERIALS AND METHODS

Materials

[^{3}H$_4$]LTC$_4$ (6.4 TBq/mmol) was obtained from Du Pont–New England Nuclear (Boston, MA, U.S.A.). UDP-[^{3}H]glucuronate (0.6 TBq/mmol) was from Biotrend (Köln, Germany). Unlabelled LTC$_4$ was obtained from Amersham Buchler (Braunschweig, Germany). Aprotinin, leupeptin, pepstatin, PMSF, bilirubin and unlabelled UDP-glucuronate were from Sigma Chemicals (Deisenhofen, Germany). Nick-spin columns filled with Sephadex G-50 fine were obtained from Pharmacia-LKB (Freiburg, Germany).

Antibodies

The polyclonal anti-MRP antibody B5 was raised in our laboratory in Heidelberg against the same C-terminal amino acid sequence (residues 1517–1531) of MRP1 as the 6KQ antibody described previously [31]. The polyclonal antibody EAG 15 was raised in rabbits against the C-terminal sequence (residues 1530–1541) of rat Mrp2 [28], and the polyclonal antibody EAG 5 was raised against the corresponding sequence of the human homologue MRP2 [10,28]. QCRL-1, an IgG$_1$ MRP1-specific monoclonal antibody produced from mice immunized with membranes from MRP1-overexpressing H69AR cells [32], was kindly provided by Dr. R. G. Deeley and Dr. S. P. C. Cole (Queen's University, Kingston, Ontario, Canada).

Cells

MRP1-transfected HeLa T5 and control HeLa C1 cells (transfected with the control vector) [33], kindly provided by Dr. R. G. Deeley and Dr. S. P. C. Cole (Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada), were grown in RPMI medium (Sigma) with 10 % (v/v) fetal calf serum under the selection pressure of G418 (Geneticin®). HepG2 cells, obtained from the Tumorbank of the Deutsches Krebsforschungszentrum, Heidelberg, were grown in RPMI medium supplemented with 10 % (v/v) fetal calf serum. All cell lines were kept in a humidified incubator [air/CO$_2$ (19:1); 37 °C].

Preparation of membrane vesicles

Plasma membrane vesicles from HeLa T5, control HeLa C1 and Hep G2 cells were prepared from hypotonically lysed cells as previously described for mastocytoma cells [34,35]. All membranes were prepared in the presence of proteinase inhibitors (0.3 μM aprotinin/1 μM leupeptin/0.1 mM PMSF/1 μM pepstatin). Membrane vesicles were frozen and stored in liquid nitrogen.

Membrane fractions enriched in bile canalicular membranes were prepared from livers of normal Wistar rats and from Mrp2-deficient GY/TR$^-$ mutant rats as described [36]. Mutant rats were obtained from Dr. F. Kuipers (University of Groningen, The Netherlands).

Immunoblotting

Membrane proteins (30 μg) were separated on a 7.5 % (w/v) polyacrylamide gel. Immunoblotting with the polyclonal antibodies B5, EAG 15 and EAG 5 was performed essentially by the method of Towbin [37] with a tank blotting system and an enhanced chemoluminescence horseradish peroxidase detection system (Amersham Buchler, Braunschweig, Germany). Quantification of the MRP1 band on the autoradiographic film was performed with a laser densitometer.

Synthesis and HPLC purification of bilirubin glucuronides

Bilirubin was incubated with ^{3}H-labelled or unlabelled UDP-glucuronate in the presence of microsomes (100 000 *g* pellet) prepared from *UGT1A1* (bilirubin glucuronosyltransferase)-transfected HeLa cells. These cells had been transfected with *UGT1A1* cDNA [38]. Transfection was performed with the eukaryotic expression vector pZeoSV (Invitrogen BV, Leek, The Netherlands).

Bilirubin (0.5 mM) was added from a stock solution prepared in 100 % DMSO to the incubation mixture in 50 mM Tris/HCl, pH 7.4, containing 10 mM MgCl$_2$, 5 μM UDP-[^{3}H]glucuronate or 1 mM UDP-glucuronate, and 20 μg of microsomal protein. 5′-AMP (4 mM) was added to inhibit the degradation of UDP-glucuronate by nucleotide pyrophosphatase (EC 3.1.4.1). Incubations were terminated after 45 min at 37 °C by the addition of ethanol. Precipitated protein was removed; the supernatant was subjected to HPLC separation on a C$_{18}$ Hypersil column with a water/acetonitrile gradient running from 0 to 80 % (v/v) acetonitrile. The pH of the aqueous phase was adjusted to pH 4.8 with ammonium acetate. The identity of the separated metabolites with monoglucuronosyl- and bisglucuronosyl-bilirubin was established by electrospray ionization mass spectrometry [39]. Because the incubations with UDP-[^{3}H]glucuronate yielded mainly mono[^{3}H]glucuronosylbilirubin, this was incubated, after purification, for a second time with an excess of unlabelled UDP-glucuronate to yield bis[^{3}H]glucuronosylbilirubin with the same specific radioactivity.

Transport measurements with membrane vesicles

ATP-dependent transport of [^{3}H]LTC$_4$ (0.05 or 0.5 μM) into membrane vesicles and its inhibition by unlabelled mono-glucuronosyl- and bisglucuronosyl-bilirubin were measured by rapid filtration through nitrocellulose filters [35]. For the mono-[^{3}H]glucuronosyl- and bis[^{3}H]glucuronosyl-bilirubin as substrates, centrifugation of the vesicles through a gel matrix with Nick-spin columns [40] was performed. Membrane vesicles (30 μg of protein) were incubated in the presence of 4 mM ATP, 10 mM MgCl$_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase and labelled substrate, in an incubation buffer containing 250 mM sucrose and 10 mM Tris/HCl, pH 7.4. The final incubation volume was 55 μl. The substrate and inhibitor concentrations are given in the respective Figure legends. In the rapid filtration procedure, the aliquots (15 μl) were diluted in 1 ml of ice-cold incubation buffer and immediately filtered through nitrocellulose filters (0.2 μm pore size), which had been presoaked in incubation buffer, then rinsed twice with 5 ml of incubation buffer. Filters were dissolved in liquid scintillation fluid and counted for radioactivity. In the centrifugation procedure, Nick-spin columns (1 g of Sephadex G-50 per 2 ml) were prepared by being rinsed with 250 mM sucrose/10 mM Tris/HCl (pH 7.4) and then centrifuged at 400 *g* for 4 min at 4 °C before use. Aliquots (15 μl) of the incubations were diluted in 80 μl of ice-cold incubation buffer and immediately loaded on Sephadex G-50 columns. The columns were rinsed with 100 μl of incubation buffer and centrifuged at 400 *g* for 4 min at 4 °C. The effluents were collected and assayed for vesicle-associated radioactivity. In control experiments ATP was replaced by an equal concentration of 5′-AMP. Rates of net ATP-dependent transport were calcu-

lated by subtracting values obtained in the presence of 5′-AMP as a blank from those in the presence of ATP.
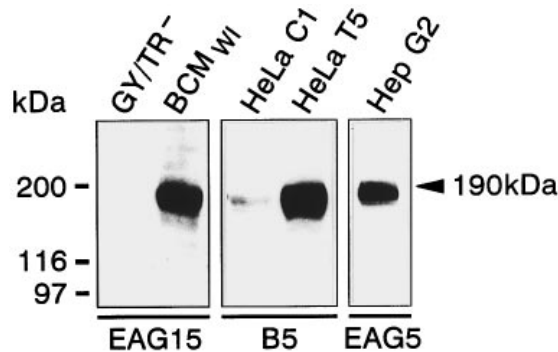
RESULTS

Immunodetection of conjugate export pumps in membrane vesicle preparations

For the characterization of the different membrane preparations, immunoblot analysis was performed by means of the antibodies EAG15, B5 and EAG5, directed against the C-terminal sequences of Mrp2, MRP1 and MRP2 respectively (Figure 1). In addition, the monoclonal antibody QCRL-1 directed against MRP1 was used. Rat Mrp2 was detected in the canalicular membranes from normal rats with antibody EAG15 [28], but not in the canalicular membrane preparation from transport-deficient GY/TR⁻ mutants. Rat Mrp1 was below detectability in purified canalicular membranes [28]. Detection with antibody B5 demonstrated the overexpression of MRP1 in the membranes from HeLa T5 cells. Antibody EAG5 showed a strong reaction with MRP2 in the Hep G2 hepatoma cell membranes (Figure 1). The latter membrane preparation was also probed with the monoclonal antibody QCRL-1 and revealed a low expression of MRP1 that was approx. 2 % of the amount of MRP1 detected in the HeLa T5 membranes as determined by analysis of the bands by laser densitometry.
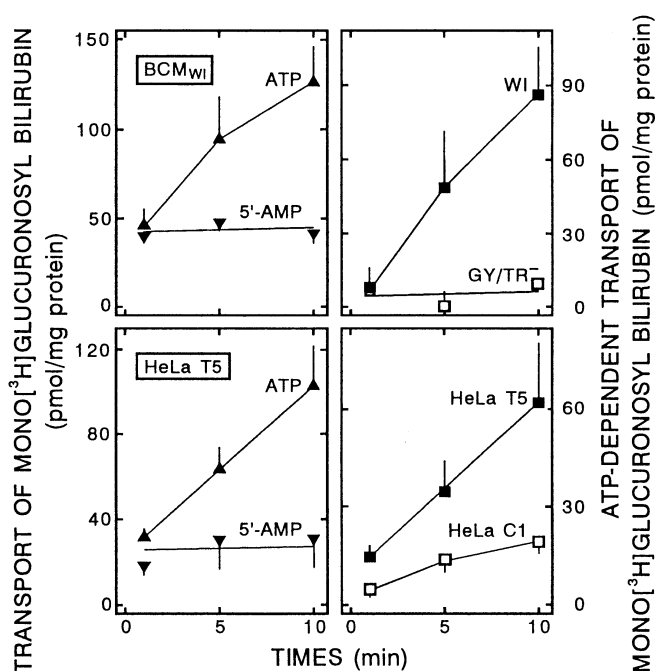
Transport of mono[³H]glucuronosyl bilirubin by Mrp2 and MRP1

Transport of mono[³H]glucuronosyl bilirubin (0.5 μM) into bile canalicular membrane vesicles (BCM) from normal Wistar rats, containing Mrp2, and from Mrp2-deficient GY/TR⁻ liver was compared with transport by membrane vesicles from HeLa T5 and control HeLa C1 cells. As shown in Figure 2, both the Mrp2-containing BCM of normal rats and the membranes from the HeLa T5 cells showed significant transport of mono[³H]-glucuronosylbilirubin. Transport rates determined with the MRP1-expressing HeLa T5 membranes were 60 % of those

Figure 2 Transport of mono[³H]glucuronosylbilirubin into BCM from normal or Mrp2-deficient rats and membrane vesicles from *MRP1*-transfected or control HeLa cells
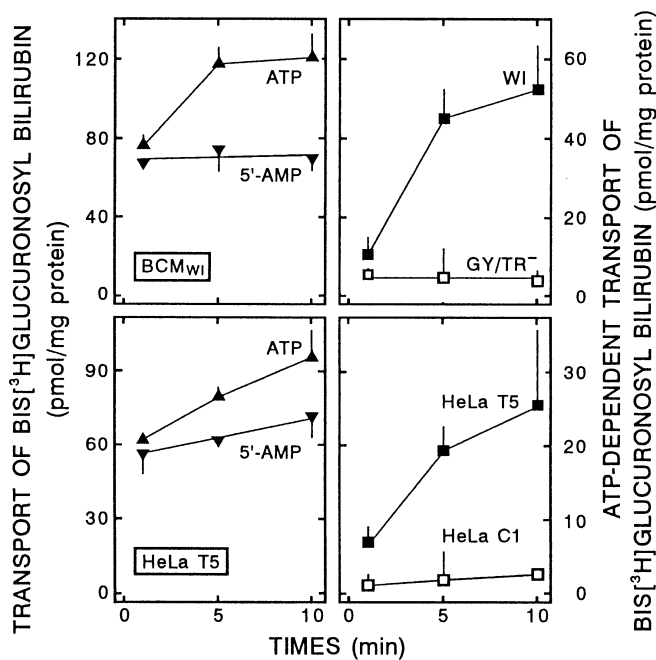
BCM from Wistar rats (BCM_WI, upper left panel) as well as membrane vesicles from HeLa T5 cells (lower left panel) were incubated with mono[³H]glucuronosylbilirubin (0.5 μM) in the presence of 4 mM ATP (▲) or 4 mM 5′-AMP (▼); the vesicle-associated radioactivity was determined by centrifugation of the vesicles through a gel matrix. The rates of net ATP-dependent transport into BCM from normal Wistar (WI) and from Mrp2-deficient GY/TR⁻ rats (upper right panel) as well as into membrane vesicles from HeLa T5 and from control HeLa C1 cells (lower right panel) were calculated by subtracting the blank values obtained in the presence of 5′-AMP from those obtained in the presence of ATP. Results are means ± S.D. for at least three experiments.

Figure 1 Immunodetection of the conjugate export pumps in BCM from normal and transport-deficient rats and in membrane vesicles prepared from *MRP1*-transfected and control HeLa cells as well as from Hep G2 cells

BCM prepared from the liver of normal and Mrp2-deficient Wistar rats, plasma membranes from HeLa T5 and control HeLa C1 cells as well as membrane vesicles from MRP2-expressing Hep G2 cells (30 μg of protein) were subjected to SDS/PAGE [7.5% (w/v) gel] and subsequently transferred to nitrocellulose membranes. The polyclonal antibodies EAG 15 (left panel), B5 (middle panel) and EAG 5 (right panel), directed against the C-terminal sequence of Mrp2, MRP1 and MRP2 respectively, were used for detection as described in the Materials and methods section. The amount of MRP1 in Hep G2 membranes was estimated from immunoblots with the monoclonal anti-MRP1 antibody QCRL-1 and was quantified by laser densitometry to be approx. 2% of the amount expressed in HeLa T5 membranes.

Table 1 Transport rates determined for the bilirubin glucuronides and LTC₄ in membrane vesicles containing MRP1, MRP2 or Mrp2

Membrane vesicles were incubated with mono[³H]glucuronosylbilirubin (MGB), bis[³H]glucuronosyl bilirubin (BGB) or [³H]LTC₄, each at a concentration of 0.5 μM, and the rates of ATP-dependent transport were calculated as described in the legend to Figure 2 (mean values from triplicate determinations; S.D. less than 15% of the mean). For each membrane vesicle preparation the ratio of ATP-dependent LTC₄ transport to the ATP-dependent transport of the bilirubin glucuronides in the same membrane vesicle preparation was calculated. Abbreviation: BCM_Wi, BCM from Wistar rats.

| | Transport rate (pmol/min per mg of protein) | | | | |
Membrane	LTC₄	MGB	BGB	LTC₄/MGB	LTC₄/BGB
HeLa T5 (MRP1)	119	5.3	3.1	23	38
BCM_Wi (Mrp2)	62	8.9	8.5	7	7
Hep G2 (MRP2)	28	8.3	4.5	3	6

determined with the normal Mrp2-expressing BCM (Table 1). Under the conditions and at the glucuronide concentrations used, no significant transport was detected with the BCM from the Mrp2-deficient GY/TR⁻ rats, whereas the control HeLa C1 membranes showed a transport rate of 1.7 pmol/min per mg of protein, which is in agreement with the protein detection shown in Figure 1. ATP-dependent transport of 5 μM [³H]cholyltaurine

Figure 4   Transport of mono[³H]glucuronosyl and bis[³H]glucuronosyl-bilirubin into membrane vesicles from HepG2 cells

Transport of 0.5 μM mono[³H]glucuronosylbilirubin (left panel) and bis[³H]glucuronosyl bilirubin (right panel) was measured in membrane vesicles from MRP2-expressing Hep G2 cells in the presence of ATP (▲) or 5′-AMP (▼) and the rates of net ATP-dependent transport (■) were calculated. Results are means ± S.D. for three experiments.

Figure 3   Mrp2-mediated and MRP1-mediated transport of bis[³H]-glucuronosylbilirubin

Transport of bis[³H]glucuronosylbilirubin (0.5 μM) was studied in BCM from normal Wistar (WI) or Mrp2-deficient GY/TR⁻ rats (upper panels) as well as in membrane vesicles from HeLa T5 and control HeLa C1 cells (lower panels) in the presence of ATP (▲) or 5′-AMP (▼) (left panels). The rates of net ATP-dependent transport are shown in the right panels. Results are means ± S.D. for at least three experiments.
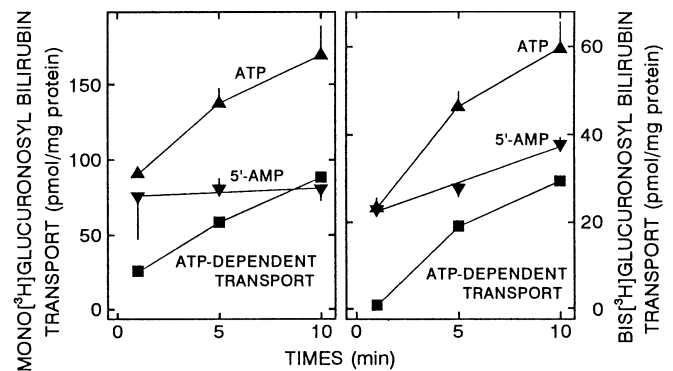
was measured as a control to indicate that the BCM of the GY/TR⁻ rats were functionally intact for the transport of another substrate by another ATP-dependent transport system [41–43]. The transport rate determined with [³H]cholyltaurine was 206 ± 30 (mean ± S.D.) pmol/min per mg of protein. For comparison, in normal rat BCM [³H]cholyltaurine was transported in the presence of ATP at a rate of 273 ± 27 pmol/min per mg of protein [40].

Transport of bis[³H]glucuronosylbilirubin by Mrp2 and MRP1

The membrane preparations described above were also used to study the transport of bis[³H]glucuronosylbilirubin by Mrp2 and MRP1. ATP-dependent transport was significant with bisglucuronosylbilirubin at a concentration of 0.5 μM (Figure 3). As with monoglucuronidated bilirubin, the BCM from normal Mrp2-expressing rats showed a higher transport activity for the glucuronide than the MRP1-expressing HeLa T5 membranes (Table 1). There was no significant ATP-dependent transport of bisglucuronosylbilirubin in BCM from GY/TR⁻ liver. The transport rates in the control HeLa C1 membranes were less than 0.5 pmol/min per mg of protein.

ATP-dependent transport of mono[³H]glucuronosyl bilirubin and bis[³H]glucuronosylbilirubin into membrane vesicles from human hepatoma Hep G2 cells expressing MRP2

Transport of mono[³H]glucuronosyl- and bis[³H]glucuronosyl-bilirubin was measured at a concentration of 0.5 μM in membranes from human Hep G2 cells (Figure 4), which express, under the conditions described here, mainly MRP2 and a very

small amount of MRP1 (Figure 1). Similarly to the results obtained for MRP1 (Figures 2 and 3), monoglucuronosylbili-rubin was a better transport substrate than bisglucuronosyl bilirubin for human MRP2 in these membranes (Figure 4).

Relative transport rates for [³H]leukotriene C₄, mono[³H]glucuronosylbilirubin and bis[³H]glucuronosylbilirubin in membrane vesicles containing different MRP isoforms

The relative rates of ATP-dependent transport of [³H]LTC₄, mono[³H]glucuronosylbilirubin and bis[³H]glucuronosylbili-rubin were determined in human Hep G2 membranes rich in MRP2, in Mrp2-containing rat BCM and in membranes from HeLa T5 cells (Table 1). LTC₄, at a concentration of 0.5 μM, was transported at a higher rate by MRP1 in HeLa T5 membranes than by Mrp2 in the BCM from normal rats or by MRP2 in Hep G2 membranes (Table 1). Conversely, transport rates of both bilirubin glucuronides by the MRP2-containing HepG2 and the Mrp2-containing BCM membrane preparations were higher than with MRP1-rich membrane vesicles from HeLa T5 cells. The ratios of the transport rates of these different substrates in a defined membrane vesicle preparation indicate a preference of MRP2 (and Mrp2) for the bilirubin glucuronides as substrates in comparison with the glutathione conjugate LTC₄ (Table 1).

Inhibition of [³H]leukotriene C₄ transport by monoglucuronosyl-bilirubin and bisglucuronosylbilirubin

Both bilirubin conjugates were potent inhibitors of the ATP-dependent transport of LTC₄ (0.05 μM) mediated by MRP1, Mrp2 and MRP2 (Figure 5). In rat BCM, LTC₄ transport was inhibited by monoglucuronosylbilirubin and bisglucuronosyl-bilirubin with IC_{50} values of 0.12 and 0.10 μM respectively. In HeLa T5 membranes the IC_{50} values of 0.42 and 0.75 μM for monoglucuronosylbilirubin and bisglucuronosylbilirubin re-spectively were higher than in the BCM. In Hep G2 membranes rich in MRP2, IC_{50} values of 0.28 and 0.40 μM were obtained for monoglucuronosylbilirubin and bisglucuronosylbilirubin re-spectively (Figure 5).
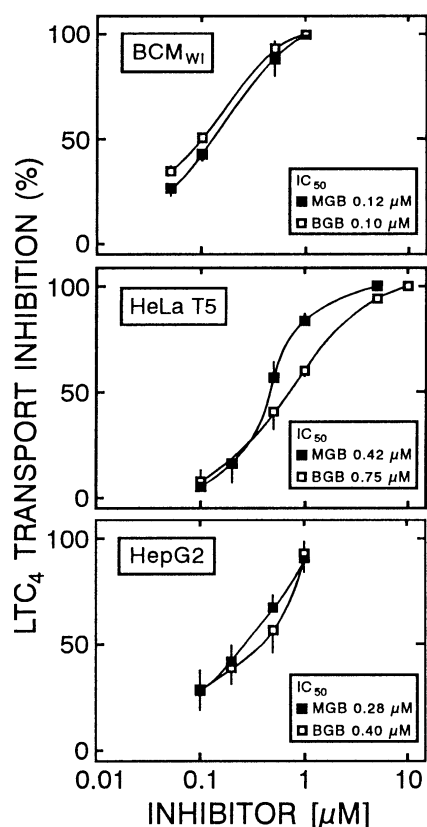
Figure 5   Inhibition of ATP-dependent [³H]LTC₄ transport by mono-glucuronosyl- and bisglucuronosyl-bilirubin

ATP-dependent transport of [³H]LTC₄ (0.05 μM) was measured in rat BCM (top panel), in membrane vesicles from HeLa T5 cells (middle panel) and in predominantly MRP2-expressing HepG2 membranes (bottom panel) in the presence of various concentrations of unlabelled monoglucuronosylbilirubin (MGB) and bisglucuronosylbilirubin (BGB). The IC₅₀ values were calculated. Results are means ± S.D. for three experiments.

DISCUSSION

The identification of MRP1 as a primary active ATP-dependent export pump for glutathione S-conjugates suggested that it might have a substrate specificity similar to that of the *MRP2* gene-encoded conjugate export pump in the hepatocyte canalicular membrane [21–23]. It has been suggested on the basis of studies in normal and transport-deficient mutant rats [8–15], and has been demonstrated more recently for recombinant MRP1, that, in addition to glutathione S-conjugates, certain glucuronidated and sulphated compounds can be transport substrates for both conjugate export pumps [24,44]. MRP1-mediated ATP-dependent transport was shown for the steroid conjugates 17β-glucuronosyloestradiol, 6α-glucuronosylhyodeoxycholate, and 3α-sulphatolithocholyltaurine, as well as for the epipodophyllo-toxin conjugate glucuronosyletoposide [24]. Despite the fact that amphiphilic anions of considerably different structures are transported by the same protein, a remarkable selectivity could be observed in some cases. This is exemplified by some steroid conjugates, among them 3α-sulphatooestradiol and cholyl-taurine, which are not MRP1 substrates [24]. *MRP1* was originally cloned and sequenced from multidrug-resistant lung cancer cells [20] and encodes an integral membrane glycoprotein of approx. 190 kDa [33]. The recently cloned rat *mrp2* cDNA, as well as its human homologue *MRP2*, also encodes membrane glyco-

proteins of approx. 190 kDa with a similar predicted structure [10,27–30]. The amino acid sequences of these canalicular (apical) isoforms, however, are markedly different from that of MRP1. The amino acid sequence of Mrp2 displays 48 % identity with, and 68 % similarity to, MRP1 [28]. Therefore it was also of interest to study possible differences in substrate specificity or function. Because of the demonstration of the selective loss of Mrp2 in GY/TR⁻ and EHBR mutant canalicular membranes [27,28], the canalicular membrane vesicles from normal compared with mutant rat liver provide a well-defined tool for the characterization of Mrp2-mediated transport. Bilirubin glucuronides have been considered physiologically the most important substrates for Mrp2.

In the present study we show for the first time that both monoglucuronosylbilirubin and bisglucuronosylbilirubin are transported ATP-dependently by human recombinant MRP1 (Figures 2 and 3). The conjugation of bilirubin with ³H-labelled glucuronate of high specific radioactivity yielded labelled substrates well suited for transport assays in isolated membrane vesicles. The relative transport rates of both bilirubin glucuronides and of LTC₄ (Table 1), as well as inhibition studies (Figure 5), indicated differences in the substrate specificities of both transporters. Although we do not know the absolute transporter protein content in the membrane preparations and therefore cannot compare absolute transport rates, the ratios of LTC₄ and bilirubin glucuronide transport determined in a single membrane preparation were significantly different between MRP1-expressing and Mrp2-expressing membranes. For LTC₄, the transport rates obtained with normal rat liver BCM were markedly lower than with HeLa T5 membranes, whereas for monoglucuronosyl and bisglucuronosyl bilirubin the transport rates were significantly higher in the canalicular membranes (Table 1). In HeLa T5 membranes monoglucuronosyl bilirubin was transported at a significantly higher rate than the bis-glucuronide; however, both bilirubin conjugates were transported at similar rates in BCM (Figures 2 and 3 and Table 1). Under the conditions used, we could not detect a significant ATP-dependent transport of bilirubin glucuronides in BCM from the Mrp2-deficient GY/TR⁻ rat liver (Figures 2 and 3). This finding is in line with previous studies by Nishida et al. [18]. *In vivo*, however, the biliary output of bilirubin and its conjugates in GY/TR⁻ rats is decreased to only approx. 40 % of normal [12]. In bile from the mutant rat, bisglucuronosylbilirubin is the major bilirubin conjugate, whereas in normal rat bile mono-glucuronosyl- and bisglucuronosyl-bilirubin are present in almost equal proportions [12]. The presence of bilirubin glucuronides in the bile of GY/TR⁻ rats indicates that a low-affinity secretion of these conjugates is still active in the canalicular membrane of the mutants. It has been suggested that this residual biliary excretion is caused by a non-ATP-dependent transport process driven by the membrane potential and possibly mediated by the same protein [11,18]. This proposed bifunctional activity of the transporter can now be excluded after the molecular identification of the canalicular conjugate export pump and the demonstration of the absence of Mrp2 in GY/TR⁻ membranes [27,28]. These observations indicate that, at least at higher bilirubin glucuronide concentrations, an additional transport system might become involved. This system would not mediate the transport of other known Mrp2 substrates, such as LTC₄, whose biliary excretion in the GY/TR⁻ rats is impaired by more than 95 % [19,45].

The Hep G2 cell line has been chosen for a study of bilirubin glucuronide transport mediated by human MRP2 because of the high expression of the MRP2 protein and the very low MRP1 expression in these cells under our culture conditions (Figure 1). As has been observed for Mrp2 in rat canalicular membranes,

the ratios of the transport rates of LTC_4 and bilirubin glucu-ronides were markedly lower than in HeLa T5 membranes (Table 1). This indicates that MRP2 is better suited for bilirubin glucuronide transport and MRP1 is more active in LTC_4 transport. In contrast with rat Mrp2, human MRP2 seems to transport the more hydrophobic monoglucuronosylbilirubin significantly better than the more hydrophilic bisglucuronosyl bilirubin. The effective inhibition of MRP1-mediated and MRP2-mediated transport of the high-affinity substrate LTC_4 by both bilirubin glucuronides suggests a high-affinity binding of both bilirubin glucuronides to MRP1, Mrp2 and MRP2 (Figure 5).

Our studies establish that monoglucuronosyl bilirubin and bisglucuronosylbilirubin are substrates for ATP-dependent transport by human MRP1, MRP2 and the rat homologue Mrp2. Moreover, measurements of relative transport rates sug-gest that substrate specificity differences exist between both conjugate export pumps. These kinetic differences should be characterized in further detail after the reconstitution of purified MRP1 and MRP2 into proteoliposome vesicles.

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