Transport of bile acids in multidrug-resistance-protein 3-overexpressing cells co-transfected with the ileal Na^+-dependent bile-acid transporter

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Many of the transporters involved in the transport of bile acids in the enterohepatic circulation have been characterized. The basolateral bile-acid transporter of ileocytes and cholangiocytes remains an exception. It has been suggested that rat multidrug resistance protein 3 (Mrp3) fulfills this function. Here we analyse bile-salt transport by human MRP3. Membrane vesicles from insect (*Spodoptera frugiperda*) cells expressing MRP3 show time-dependent uptake of glycocholate and taurocholate. Furthermore, sulphated bile salts were high-affinity competitive inhibitors of etoposide glucuronide transport by MRP3 ($IC_{50} \approx 10\ \mu M$). Taurochenodeoxycholate, taurocholate and glycocholate inhibited transport at higher concentrations ($IC_{50} \approx 100$, 250 and 500 μM respectively). We used mouse fibroblast-like cell lines derived from mice with disrupted *Mdr1a*, *Mdr1b* and *Mrp1* genes to generate transfectants that express the murine apical Na^+-dependent bile-salt transporter (Asbt) and MRP3. Uptake of glycocholate by these cells is Na^+-dependent, with a K_m and V_{max} of $29 \pm 7\ \mu M$ and 660 ± 63 pmol/min per mg of protein respectively and is inhibited by several organic-anion transport inhibitors. Expression of MRP3 in these cells limits the accumulation of glycocholate and increases the efflux from cells preloaded with taurocholate or glycocholate. In conclusion, we find that MRP3 transports both taurocholate and glycocholate, albeit with low affinity, in contrast with the high-affinity transport by rat Mrp3. Our results suggest that MRP3 is unlikely to be the principal basolateral bile-acid transporter of ileocytes and cholangiocytes, but that it may have a role in the removal of bile acids from the liver in cholestasis.

Key words: ASBT (ileal apical Na^+-dependent bile-acid transporter), cholestasis, MRP3 (multidrug-resistant protein 3), transport.

INTRODUCTION

The enterohepatic circulation of bile acids is the result of the co-ordinated action of membrane-transport and bile-acid-binding proteins [1–4]. Bile acids are synthesized in the liver from cholesterol and are secreted into bile. In the intestine bile acids assist in absorbing cholesterol, fat and fat-soluble vitamins. Extensive intestinal absorption of bile salts results in less than 5% faecal loss of the daily bile-acid pool. Absorbed bile acids are returned to the liver, where they are taken up into hepatocytes. In recent years many of the transport systems involved in the enterohepatic circulation of bile acids have been characterized at the molecular level. At the sinusoidal membrane of hepatocytes, bile acids are mainly taken up from blood by the Na^+/taurocholate co-transporting polypeptide (Ntcp) [5,6]. Secretion of bile acids into bile at the canalicular membrane of hepatocytes is mainly mediated by the canalicular bile salt export pump (BSEP), also known as 'Sister of P-glycoprotein', a membrane protein belonging to the ATP-binding-cassette ('ABC') family of transporters [7,8]. Absorption of bile salts into enterocytes in the intestine occurs mainly in the distal ileum through the action of the apically located ileal Na^+-dependent bile-acid transporter (ASBT) [9–12]. In the enterocytes, bile acids are transported across the basolateral membrane into the portal vein. The transporter for this last step has not been identified yet, and remains one of the 'missing links' in the transport of bile acids in the enterohepatic circulation.

Recently, two distinct transport proteins have been implicated as possible candidates for the basolateral transport system of enterocytes. The first, described by Lazaridis et al. [13] is a truncated form of Asbt (t-Asbt) which is the result of differential splicing. The t-Asbt localizes to the basolateral membrane of ileocytes and cholangiocytes. When expressed in *Xenopus* oocytes it mediates Na^+-independent taurocholate efflux. A second candidate is the multidrug resistant protein 3 (MRP3) [14–16]. MRP3 localizes to the basolateral membranes of polarized cells, is expressed in human [17] and in mouse intestine (N. Zelcer, A. Kuil and P. Borst, unpublished work) and is highly up-regulated in the livers of rats made cholestatic by bile-duct ligation, or in cholestatic human liver [15,18–22]. These findings suggest that MRP3 plays a role in the reabsorbtion of bile salts from the intestine and in the removal of toxic organic anions from the liver under cholestatic conditions. Initial studies with rat Mrp3 verified this and demonstrated that rat Mrp3 transports not only organic anions with a preference towards the sulphated and glucuronidated ones [23], but also the bile acids taurocholate, glycocholate, taurochenodeoxycholate 3-sulphate and taurolithocholate 3-sulphate, laying a possible link between Mrp3 and the enterohepatic circulation of bile acids [24]. Recently, human MRP3 has been demonstrated to transport glycocholate, but not taurocholate [25]. Additionally, whereas rat Mrp3 has a high affinity for taurocholate and glycocholate, human MRP3 seems to have a low affinity for the latter.

Abbreviations used: ASBT and Asbt, human and murine ileal apical Na^+-dependent bile-acid transporter respectively; BSEP and Bsep, human and murine bile-salt export pump respectively; $E_2 17\beta G$, 17β-oestradiol 17β-D-glucuronide; His_6, hexahistidine tag; MDCK, Madin–Darby canine kidney; MRP3 and Mrp3, multidrug resistance protein 3 of human and rat or mouse respectively; Ntcp, Na^+/taurocholate co-transporting polypeptide; OATP, organic anion transport protein; Sf9, *Spodoptera frugiperda* (fall armyworm); t-ASBT, truncated ASBT; TR^-/EHBR, Transport mutant/Eisai hyperbilirubinaemic rat; 4-MUS, 4-methylumbelliferone sulphate.

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In the present study we further characterize bile-acid transport by human MRP3 in membrane vesicles from *Spodoptera frugiperda* (Sf9; fall armyworm) insect cells that were infected with a recombinant MRP3 cDNA containing baculovirus [26]. To study the possibility that Asbt and MRP3 co-operate under physiological conditions in vectorial transport of bile acids across enterocytes and cholangiocytes, we also generated a fibroblast cell line that stably expresses either one or both transporters.

EXPERIMENTAL

Materials

[^{3}H]Taurocholate (2 or 3 Ci/mmol) and [^{3}H]17β-oestradiol 17β-D-glucuronide ($E_2$17βG) (44 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA, U.S.A.). [^{14}C]Glycocholate (56 mCi/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.). The synthesis of [^{3}H]etoposide glucuronide was as previously described [26]. MK571 [a leukotriene C_4 analogue and a known high-affinity inhibitor of MRP1 ($K_i \approx 0.1$ μM)] was from Biomol (Plymouth Meeting, PA, U.S.A.). All other chemicals and reagents were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell lines

The fibroblast cell line used in the present study, the generation of clones that stably overproduce MRP3 and growth conditions were described previously [26]. Cell lines were regularly checked for the absence of *Mycoplasma* infection.

Construction of a cell line expressing MRP3 and Asbt

A cDNA insert encoding mouse Asbt-His_6 (His_6 is a hexahistidine tag) [27] was excised from pZmISBT using *Eco*RI, blunted, and cloned into the *Eco*RV site of the retroviral pBabeBLEO vector [28]. The correctness of the resulting pBabeBLEO-Asbt-His_6 vector was verified by digestion analysis. The pBabeBLEO-Asbt-His_6 vector was transfected into the amphotropic retroviral packaging cell line Phoenix [28]. The virus particles produced were used to transduce cells that overproduce MRP3 (M3 cells) and the control clone V1, which does not. As controls, the same lines were transduced with virus particles that contained no Asbt-His_6 cDNA insert. After 48 h, the cells were split and stable clones were selected with bleomycin (25 μg/ml). Resistant clones were expanded and analysed for Asbt-His_6 expression using a mouse monoclonal antibody directed against the His_6 tag (Qiagen, Leusden, The Netherlands) according to the manufacturer's protocol. MRP3 was detected using the monoclonal antibody M_3II-9 (1:250) as previously described [15]. Both primary antibodies were followed by a rabbit anti-mouse IgG–horseradish peroxidase conjugate (1:1000) and visualized by enhanced chemiluminescence (ECL®, Amersham, Little Chalfont, U.K.). For detection of murine Bsep and Mrp3 we used the monoclonal antibody C219 and a rabbit serum directed against Mrp3 respectively (N. Zelcer, I. Bot and P. Borst, unpublished work). The clones selected for study did not differ in morphology, size or volume (results not shown).

Bile-acid accumulation assays

Short-term bile-acid-accumulation assays were performed as described by Saeki et al. [27]. Briefly, 1×10^6 cells/well were plated 1 day prior to assay. On the following day, cells were washed twice with 37 °C pre-warmed wash buffer (250 mM mannitol/20 mM Tris/HCl, pH 7.4). Subsequently, cells were incubated with 37 °C pre-warmed uptake buffer [100 mM NaCl (or choline chloride)/3 mM KH_2PO_4/10 mM Tris/HCl (pH 7.4)/glucose (4.5 g/litre)] containing [^{14}C]glycocholate or [^{3}H]taurocholate at the indicated concentrations. Similar results were obtained when the buffer system reported by Sun et al. [11] was used (results not shown). Unless otherwise indicated, uptake into the cells was measured after 5 min. Assays were terminated by washing the cells twice with cold wash buffer containing 500 μM taurocholate or glycocholate. The cell monolayer was lysed with 1 ml of 0.2 M NaOH at 37 °C for at least 1 h, and aliquots were taken to determine cellular protein and cell-associated radioactivity using liquid-scintillation counting in a Packard 1900CA counter (Packard, Meriden, CT, U.S.A.). To study the inhibition of Asbt-mediated bile-acid uptake, inhibitors at the indicated concentrations were added to the uptake buffer.

Bile-acid efflux assays

Efflux assays were performed as described above for uptake assays, with the exception that, following the uptake phase, cells were washed twice with cold wash buffer containing 500 μM non-radioactive taurocholate or glycocholate and incubated with 1 ml of efflux buffer [100 mM choline chloride/3 mM KH_2PO_4/10 mM Tris/HCl (pH 7.4)/glucose (4.5 g/litre)]. At the indicated times, samples (100 μl) were taken and their radioactive content determined by liquid-scintillation counting. At the end of the assay, cells were processed as described above.

Preparation of membrane vesicles

Sf9 cells were infected with MRP3-expressing baculovirus at a multiplicity of infection of 1. After incubation at 27 °C for 3 days, cells were harvested by centrifugation at 500 ***g*** for 5 min. The pellet was resuspended in ice-cold hypo-osmotic buffer (0.5 mM Na^+ phosphate/0.1 mM EDTA, pH 7.4) supplemented with protease inhibitors (2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 10 μM pepstatin) and incubated at 4 °C for 90 min. The suspension was centrifuged at 4 °C at 100000 ***g*** for 40 min and the pellet was homogenized in ice-cold TS buffer (50 mM Tris/HCl/250 mM sucrose, pH 7.4) using a tight-fitting Dounce homogenizer. After centrifugation at 500 ***g*** at 4 °C for 10 min, the supernatant was centrifuged at 4 °C at 100000 ***g*** for 40 min. The pellet was resuspended in TS buffer and passed 20 times through a 27-gauge needle. The vesicles were dispensed in aliquots, frozen in liquid nitrogen, and stored at −80 °C until use.

Vesicular-transport assays

The time- and concentration-dependent uptake of various substrates into MRP3-containing vesicles was studied following the rapid-filtration method previously described [26]. Briefly, membrane vesicles containing 20 μg of protein were incubated with the indicated concentration of substrate in 50 μl of TS buffer in the presence of 4 mM ATP or AMP, 10 mM $MgCl_2$, 10 mM creatine phosphate and creatine kinase (100 μg/ml). At the indicated time, the reaction mixture was diluted in 1 ml of ice-cold TS buffer and immediately filtered through a pure-cellulose filter (0.45 μm pore size). The filter was washed twice with 3 ml of ice-cold TS buffer and the radioactivity retained on the filter measured by liquid scintillation.

Statistics

Differences were tested with a two-tailed, non-paired Student's *t* test. A P value of < 0.05 was considered significant.

RESULTS

Bile-acid vesicular-uptake studies

To study transport of bile acids by MRP3 we used vesicular-transport assays with membrane vesicles generated from Sf9 cells infected with a recombinant baculovirus encoding MRP3. These vesicle membranes contain higher levels of MRP3 protein than

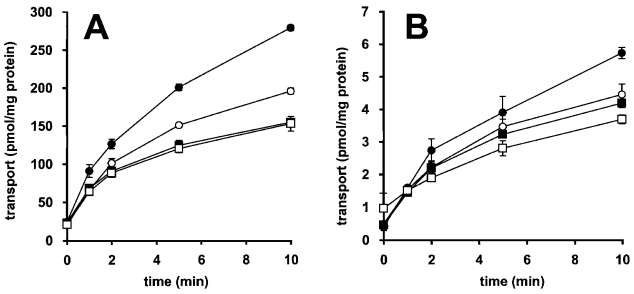

Figure 1 Transport of glycocholate and taurocholate by MRP3

Membrane vesicles from Sf9 cells infected with MRP3 (circles) or wild-type (squares) baculovirus were incubated at 37 °C with 10.5 μM [^{14}C]glycocholate (**A**) or 0.27 μM [^{3}H]taurocholate (**B**) in the presence of 4 mM ATP (closed symbols) or AMP (open symbols). Each point and bar is the mean value ± S.D. for experiments performed in triplicate.

Table 1 Effect of bile acids on MRP3-mediated etoposide glucuronide transport

Membrane vesicles prepared from Sf9 insect cells infected with a baculovirus coding for MRP3 were incubated at 37 °C for 5 min with 94 nM [^{3}H]etoposide glucuronide with or without inhibitors. Inhibition is expressed as percentage of ATP-dependent uptake measured in the absence of inhibitors. Values are means ± S.E.M. for experiments ($n = 3$) done in duplicate.

Inhibitor	Concn. (μM)	Uptake (% of control)
Glycocholate	500	30 ± 3
	250	46 ± 1
	83	68 ± 1
	27	95 ± 2
Taurocholate	500	55 ± 2
	250	68 ± 3
	83	90 ± 1
	27	99 ± 6
Taurodeoxycholate	500	9 ± 0
	250	26 ± 2
	100	50 ± 2
	25	85 ± 4
Taurochenodeoxycholate	500	6 ± 1
	250	21 ± 2
	100	51 ± 3
	25	77 ± 8
Taurolithocholic acid sulphate	50	8 ± 0
	20	25 ± 4
	5	70 ± 1
Glycolithocholic acid sulphate	20	29 ± 0
	5	74 ± 1
	1	95 ± 4
Lithocholic acid sulphate	20	24 ± 3
	5	61 ± 1
	1	100 ± 4
MK571	20	40 ± 4
	5	77 ± 3
	1	98 ± 0

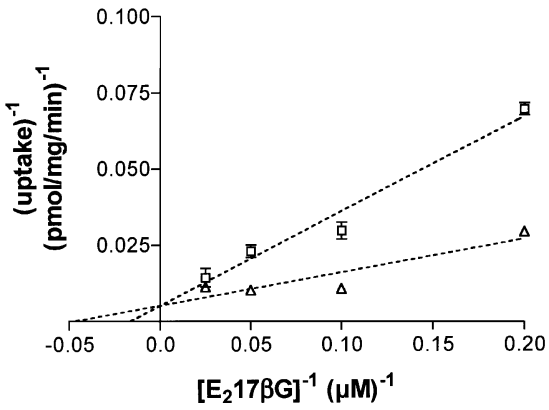

Figure 2 Lineweaver–Burk plot of MRP3-mediated $E_2 17\beta$G transport and its inhibition by taurolithocholic acid sulphate

Rates of ATP-dependent transport of [^{3}H]$E_2 17\beta$G by MRP3 were determined in membrane vesicles from Sf9 cells infected with a MRP3 baculovirus at four different concentrations in the absence (triangles) or presence of 20 μM taurolithocholic acid sulphate (squares) at 37 °C for 2 min. The ATP-dependent transport of $E_2 17\beta$G in membrane vesicles from Sf9 cells infected with a wild-type baculovirus were subtracted from those obtained in membrane vesicles with MRP3, and each point represents the mean ± S.D. from triplicate determinations.

we have been able to obtain in transfected mammalian cells and can be used to study organic-anion transport by MRP3 [26]. With these vesicles, we found a time- and ATP-dependent uptake of both glycocholate and taurocholate into MRP3 vesicles that is higher than with vesicles generated from Sf9 cells infected with wild-type baculovirus (Figure 1). However, as is evident from Figure 1, Sf9 cells contain a background transporter for both of these bile acids which does not require ATP. Our attempts to saturate this endogenous transporter by increasing the concentration of bile salt used in the assay did not decrease the background. The endogenous bile-salt transporter precluded the determination of reliable kinetic parameters for bile-salt transport by MRP3 and we therefore took an indirect approach. We previously showed that, in the same vesicular system, etoposide glucuronide, the major metabolite of the anticancer drug etoposide formed *in vivo*, is a good substrate of MRP3 [26]. We therefore used the inhibition of MRP3-mediated etoposide glucuronide transport to study the interaction of MRP3 with bile acids and other organic anions (Table 1). MRP3-mediated transport of etoposide glucuronide was inhibited by several bile acids in a dose-dependent manner. Sulphated bile acids showed strong inhibition of MRP3-mediated transport at low concentrations ($IC_{50} \approx 10\ \mu$M), as has been found previously for rat Mrp3 [24]. Taurolithocholic acid sulphate inhibited the transport of $E_2 17\beta$G in a competitive fashion with a K_i of 11 μM (Figure 2). MK571 also inhibited MRP3, although at much higher concentrations than those required for MRP1 (Table 1).

Since sulphated bile salts are high-affinity competitive inhibitors of MRP3, we tested the effect of 4-methylumbelliferone sulphate (4-MUS), another sulphated compound on MRP3-mediated transport. Previously, Hirohashi et al. [23] demonstrated that 4-MUS stimulated the ATP-dependent transport of $E_2 17\beta$G by rat Mrp3. We find a similar effect with human MRP3. Transport of $E_2 17\beta$G by MRP3 was stimulated in a dose-dependent manner by 4-MUS (Figure 3). This stimulation appears to be specific for the substrate $E_2 17\beta$G, as we did not observe it with etoposide glucuronide or leukotriene C_4 as substrate (results not shown). It is also specific for the sulphate conjugate of 4-methylumbelliferone, as the glucuronide only

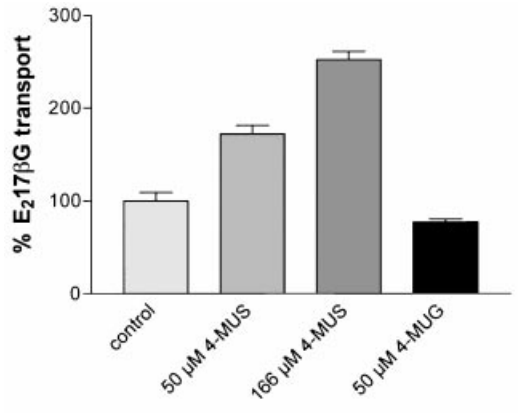

Figure 3 Stimulation of MRP3-mediated $E_2 17\beta G$ transport by 4-MUS

Membrane vesicles from Sf9 cells infected with MRP3 were incubated at 37 °C with 1 μM [^{3}H]$E_2 17\beta G$ for 2 min in the absence or presence of 4-methylumbelliferone conjugates. The ATP-dependent transport of [^{3}H]$E_2 17\beta G$ by MRP3 was calculated by subtracting the transport in the presence of 4 mM AMP from that of transport in the presence of 4 mM ATP. Each bar represents the mean value $\pm$ S.D. for three experiments done in triplicate. Abbreviation: 4-MUG, 4-methylumbelliferone glucuronide.

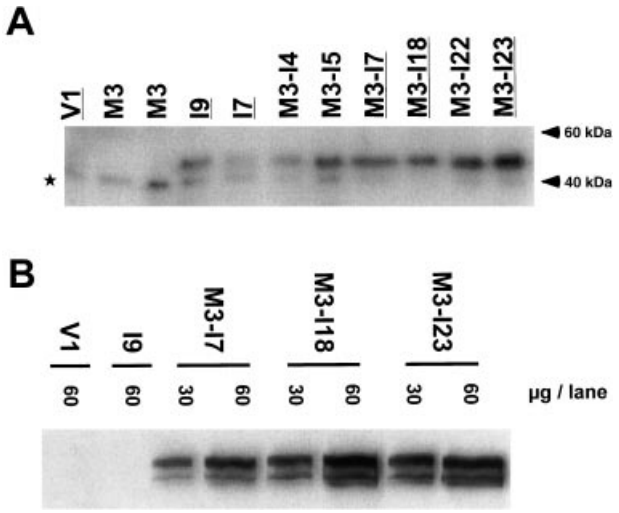

Figure 4 Immunoblot analysis of Asbt and MRP3 in selected clones

A 40 μg portion of total cell protein was size-fractionated on an 1% (w/v)-SDS/7.5% (w/v)-polyacrylamide gel and Asbt-His_6 expression in selected clones was studied by Western-blot analysis using a monoclonal antibody against the His_6 tag (**A**). An Asbt-His_6 band is present in cells transduced with the Asbt cDNA construct (I clones). A non-specific band is seen in all the clones [marked by a star (★)]. Underlined clones were used in subsequent studies. MRP3 was detected (**B**) by using monoclonal antibody M_3II-9 [15].

inhibited MRP3-mediated transport (Figure 3). The stimulation is specific for MRP3, as we did not observe it with MRP1, tested under identical conditions in Sf9 vesicles (results not shown). We did see a stimulation of background leukotriene C_4 transport by 4-MUS in Sf9 vesicles not containing MRP3. This was not seen with $E_2 17\beta G$ as substrate (results not shown). Finally, despite the capacity of 4-MUS to stimulate $E_2 17\beta G$ transport by MRP3, it did not stimulate MRP3-mediated glycocholate transport at a concentration of up to 166 μM (results not shown).

Table 2 Summary of [^{14}C]glycocholate and [^{3}H]taurocholate accumulation in Asbt/MRP3 clones

Cells were incubated either with 7 μM [^{14}C]glycocholate or [^{3}H]taurocholate or 0.25 μM [^{3}H]taurocholate in uptake buffer for 5 min at 37 °C and the retained radioactivity was determined. Each value represents the mean $\pm$ S.D. for experiments done in duplicate.

	Accumulation (pmol/mg of protein)		
Clone	7 μM Glycocholate	0.25 μM Taurocholate	7 μM Taurocholate
V1	3 $\pm$ 0 (3)*	0.1 $\pm$ 0 (3)	n.d.†
I9	402 $\pm$ 35 (14)	8 $\pm$ 1 (3)	239 $\pm$ 31 (3)
M3-I18	444 $\pm$ 34 (11)	12 $\pm$ 2 (3)	303 $\pm$ 58 (3)
M3-I23	454 $\pm$ 44 (9)	10 $\pm$ 2 (3)	282 $\pm$ 41 (3)

* Number of experiments.
† n.d. not determined.

Generation of stable cell lines co-expressing Asbt and MRP3

The problems encountered with MRP3-mediated vesicular bile-acid transport led us to develop a complementary cellular-based approach. To recapitulate the physiological situation where Asbt and MRP3 localize to opposing membranes of polarized cells, we attempted to generate Madin–Darby canine kidney-II (MDCK-II) cells that express both transporters. Although we managed to obtain clones that express both transporters in the correct membrane, we could not study the contribution of MRP3 to the basolateral transport of bile acids owing to the high activity of an uncharacterized endogenous basolateral bile-acid transporter. As an alternative, we transduced fibroblast cell lines that over-produce MRP3 with virus generated from the pBabeBLEO/Asbt-His_6 retroviral construct. These fibroblasts were generated from the kidneys of mice that have a homozygous disruption of *Mdr1a*, *Mdr1b* and *Mrp1*, and MRP3 expression in these cells results in resistance to the anticancer drug etoposide [26]. We screened clones that were resistant to 25 μM bleomycin by protein blot analysis (Figures 4A and 4B) and by a functional assay in which we determined the Na^+-dependency of [^{3}H]taurocholate and [^{14}C]glycocholate uptake (results not shown). In all the clones transduced with the Asbt-His_6 cDNA construct (I clones), a specific band was detected with an approximate size of $\approx$ 45 kDa, as has been reported by others [29]. In these clones, uptake of bile acids was completely dependent on the presence of Na^+, since replacement of Na^+ by choline resulted in bile-acid uptake levels similar to those found in control clones transduced with the empty pBabeBLEO virus (results not shown). MRP3 in all the M3 clones is detected as a double band (Figure 4B), presumably resulting from differential glycosylation. We have previously shown in other cells that treatment with tunicamycin, which blocks *N*-glycosylation, results in the two MRP3 bands shifting to one band which runs with higher mobility [15]. A subset of the clones having similar bile-acid uptake was selected for further study.

Bile-acid uptake and efflux assays with Asbt/MRP3 cells

We loaded cells for 5 min with 0.25 μM [^{3}H]taurocholate or either 7 μM [^{3}H]taurocholate or 7 μM [^{14}C]glycocholate and monitored the subsequent efflux. Cellular uptake in the clones studied was similar (Table 2) and, once cells were washed and Na^+ was replaced by choline, a higher fraction of the accumulated radioactivity was recovered in the efflux media of MRP3-containing clones. This indicates that MRP3 transports both

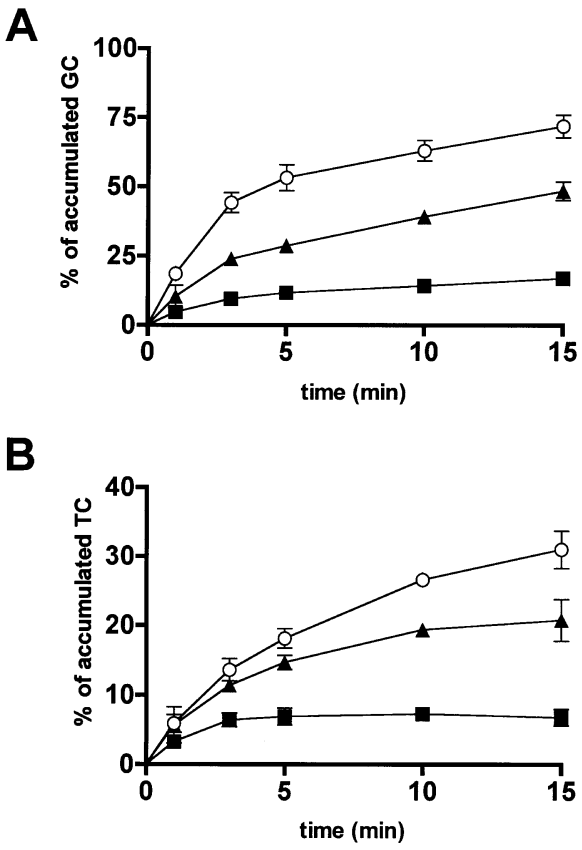

Figure 5 Increased efflux of glycocholate and taurocholate from MRP3-expressing cells

Selected clones were incubated for 5 min at 37 °C with 7 μM [^{14}C]glycocholate (**A**) or 0.25 μM [^{3}H]taurocholate (**B**) in uptake buffer, washed, and allowed to efflux into Na^{+}-free buffer. The fraction of the intracellular radioactivity that was retrieved in the efflux medium is plotted. The 100% values are presented in Table 2. Values for clones I9 (■), M3-I23 (▲) and M3-I18 (○) represent the mean value ± S.D. for experiments done in triplicate ($n = 4$).

Table 3 Inhibition of [^{14}C]glycocholate uptake into Asbt cells by common organic-anion-transport inhibitors

I9 cells were incubated at 37 °C for 5 min with 7 μM [^{14}C]glycocholate in the absence or presence of various concentrations of organic-anion-transport inhibitors. Inhibition is expressed as the percentage of [^{14}C]glycocholate uptake in the absence of inhibitor. Each value represents the mean ± SE.M. for experiments done in duplicate ($n = 3$).

Treatment	Concn. (μM)	Accumulation of glycocholate (%)
Benzbromarone	250	10 ± 4
	100	19 ± 7
	25	69 ± 4
Sulphinpyrazone	500	52 ± 2
	100	85 ± 2
	10	99 ± 0
Indomethacin	250	50 ± 3
Taurocholate	40	51 ± 2

glycocholate and taurocholate (Figure 5), a finding that supports our results with the vesicular transport system. The efflux rates of glycocholate and taurocholate correlate well with the level of MRP3 expression in the clones studied (Figure 4). Clone M3-I18, which has the highest expression of MRP3, has also the highest rate of bile-acid efflux, followed by M3-I23. A third MRP3 clone, M3-I7, in which MRP3 expression is lower than that of M3-I23 (Figure 4) had taurocholate-efflux rates which were intermediate between those of clone M3-I23 and the control clone I9, despite having a lower accumulation of taurocholate (results not shown). Loading the cells with 7 μM taurocholate instead of 0.25 μM leads to a proportional increase in the intracellular taurocholate concentration (Table 2). Under these conditions, the efflux mediated by MRP3 is also proportionally increased, indicating that the capacity of taurocholate transport by MRP3 in these cells is high (results not shown). In these assays we consistently recovered 95–100 % of the radioactivity. Interestingly, the control clone I9, which has no MRP3, nevertheless showed a low level of bile-acid efflux (Figure 5). A similar result was obtained with an additional control clone, namely I7 (results not shown). By protein blot analysis we could not detect the presence of mouse Mrp3 or Bsep. When 25 μM taurocholate was included in the efflux buffer, the basal bile-salt efflux from the control clone was further stimulated by 20 % at 10 min. This suggests that our cells contain an organic-anion-transport-protein (OATP)-like transporter that is able to exchange the intracellular [^{3}H]taurocholate for extracellular taurocholate [30]. Presumably, the MRP3 clones contain this system as well.

To further verify that MRP3 is responsible for the transport of bile acids in our clones, we attempted to inhibit it. Since no specific high-affinity inhibitors are known for MRP3, we tested a panel of inhibitors of organic-anion transport that have been shown to inhibit MRP3 in intact cells and membrane vesicles (Table 3). The tested inhibitors are commonly used drugs and are often used to inhibit MRPs. Unexpectedly, however, all the inhibitors tested inhibited the Na^{+}-dependent uptake of bile acids by Asbt at concentrations lower than those required to inhibit MRP3. Because of this complication, we could only add the inhibitor during the efflux phase of our experiments. Under these conditions 2 mM sulphinpyrazone substantially inhibited MRP3-mediated glycocholate transport. Transport of glycocholate at 5 min by clones M3-I18 and M3-I23 was reduced to 46 ± 16 (mean ± S.D.) and 56 ± 16 % of transport in the presence of inhibitor, whereas transport by the control clone V1 was only slightly reduced to 81 ± 10 % ($n = 3$).

Time- and concentration-dependent uptake of glycocholate by cells expressing Asbt with and without MRP3

Glycocholate was chosen instead of taurocholate for these studies since MRP3 has a higher affinity for this bile salt (Figure 1 and Table 1) and its efflux by MRP3 is higher (Figure 5). The accumulation of [^{14}C]glycocholate with time is initially similar in clones V1-I9 and M3-I18. However, after 10 min, the accumulation in clone M3-I18, which contains MRP3 along with Asbt, is reduced compared with the control V1-I9 clone (Figure 6A). Presumably, at that time, MRP3-mediated glycocholate efflux can partially counter the influx that is mediated by Asbt. However, the rate of influx remains higher than that of the efflux process, since the cells continue to accumulate [^{14}C]glycocholate with time. Therefore, to get a rough estimate for the rate of MRP3 transport of [^{14}C]glycocholate in the M3-I18 clone, we determined the kinetic parameters of uptake of [^{14}C]glycocholate in the control line. The transport of [^{14}C]glycocholate by Asbt in clone I9 showed saturable kinetics, with K_m and V_{max} values of 29 ± 7 μM and 660 ± 63 pmol/min per mg of protein ($n = 3$) respectively (Figure 6B). MRP3 would have to transport [^{14}C]glycocholate at a rate similar to this in order to partially counteract the accumulation mediated by Asbt. The estimated

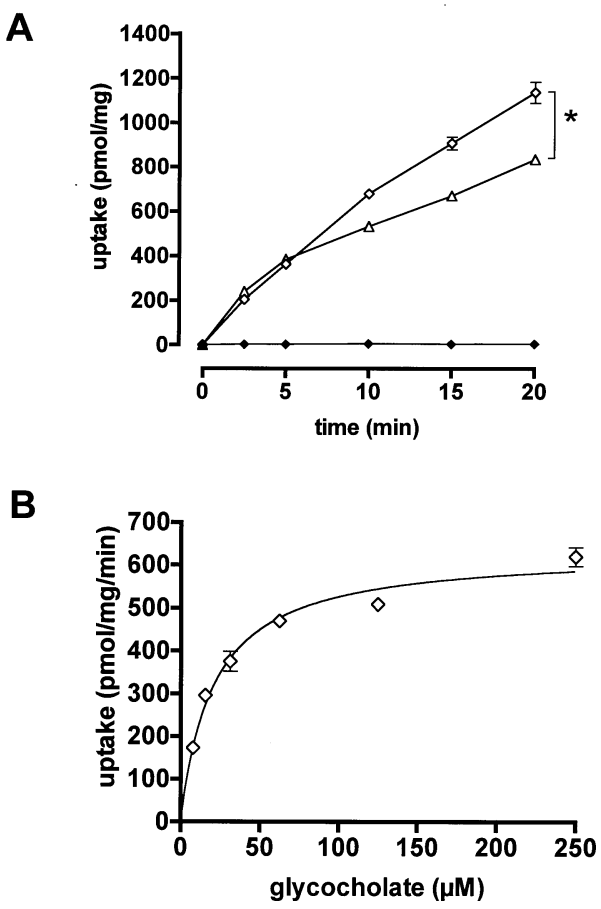

Figure 6 Characterization of glycocholate uptake in Asbt/MRP3 cells

(**A**) Selected clones were incubated at 37 °C with 7 μM [^{14}C]glycocholate for the indicated time at which cellular accumulation of radioactivity and protein content were determined. Values for clones V1 (◆), I9 (◇) and M3-I18 (△) are means ± S.D. for three experiments done in duplicate. *$P < 0.01$. (**B**) Concentration-dependent uptake of glycocholate into I9 cells which have Asbt, but no MRP3. I9 cells were incubated for 5 min at 37 °C with various concentrations of [^{14}C]glycocholate. Values are means ± S.D. for a representative experiment done in triplicate.

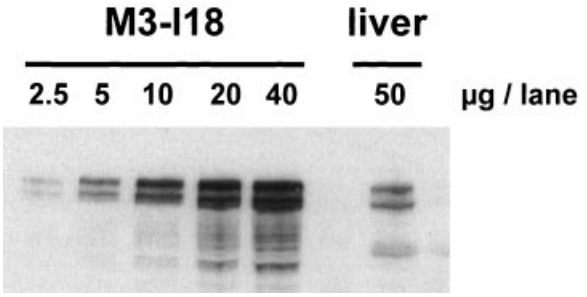

Figure 7 Immunoblot comparison of MRP3 levels in normal human liver and clone M3-I18

A 50 μg portion of total cell protein from normal human liver or the indicated amounts from clone M3-I18 were size-fractionated on an 1%-SDS/7.5%-polyacrylamide gel and MRP3 expression in the samples was studied by Western-blot analysis using the monoclonal antibody M_3II-9 [15].

high rate of transport we obtain in the transfected clones is the result of the high, and probably non-physiological, levels of MRP3 in these cells. However, as Figure 7 clearly demonstrates, the level of MRP3 in clone M3-I18 is, when assessed by densitometry, only 10-fold higher than that found in normal human liver.

DISCUSSION

In recent years many of the major transporters involved in the enterohepatic circulation of bile acids have been cloned, biochemically characterized and their altered expression in the cholestatic state described [4,31]. Moreover, their importance for the enterohepatic circulation has been further illustrated in knockout mice [32,33] and in the TR$^-$/EHBR (Transport mutant/Eisai hyperbilirubinaemic rat) naturally occurring Mrp2-deficient rats [34]. Despite these advances, the molecular identity of the basolateral bile-acid transporter of enterocytes and cholangiocytes remains elusive. Two transporters have been recently suggested to fulfill this task, namely t-Asbt and MRP3. Differential splicing of *Asbt* results in t-Asbt, a transporter that localizes to the basolateral membranes of enterocytes and cholangiocytes. When expressed in *Xenopus* oocytes, t-Asbt mediates Na^+-independent efflux of taurocholate [13]. Alternatively, Mrp3 might play a role in bile-acid transport. MRP3 is highly induced in cholestatic livers of humans and rats, suggesting that this transporter is important for the removal of toxic anions from the liver [15,18,19,21] and direct transport of bile acids by rat Mrp3 [24], and human MRP3 was observed in vesicular transport assays [25]. However, a large discrepancy was found between the two MRP3 orthologues. Whereas rat Mrp3 transports taurocholate with relatively high affinity ($K_m \approx 16$ μM), human MRP3 does not [24,25]. Additionally, transport of sulphated bile acids, increased in cholestasis, has not been studied for MRP3.

We initially studied MRP3-mediated bile-acid transport in vesicular transport assays. Using this system we could demonstrate a clear time- and ATP-dependent transport of both glycocholate and taurocholate, as has been found for rat Mrp3 (Figure 1). However, despite the high content of MRP3 in these membranes, the determination of the kinetic parameters for this transport was hampered by the presence of a substantial non-ATP-dependent bile-acid-transport activity in the control membranes. This background transport activity posed no problem when Bsep-mediated taurocholate transport was studied in Sf9-derived membranes. Presumably this is due to the high affinity of Bsep for taurocholate ($K_m \approx 5$–7 μM) [7,35]. Accordingly, this would suggest that the affinity of MRP3 for taurocholate and glycocholate is low. Support for this notion comes from our inhibition data (Table 1), where relatively high concentrations of these bile acids are required to inhibit the MRP3-mediated etoposide glucuronide transport. The apparent IC_{50} for glycocholate is ≈ 250 μM, a value that is in good agreement with the published K_m of MRP3 for this compound [25]. Taurocholate at a concentration of up to 500 μM could not inhibit 50% of the MRP3-mediated transport, indicating that the affinity of MRP3 for this bile acid is even lower.

MRP3 expression is highly induced under conditions of impaired bile flow or under conditions where MRP2 expression is diminished, for example as in Dubin–Johnson-syndrome patients or in their animal counterparts, TR$^-$/EHBR rats [15,18–21]. Additionally, *in vitro* exposure of enterocytes to 100 μM chenodeoxycholic acid increases MRP3 RNA transcript levels 3-fold, indicating that its transcription is sensitive to bile-acid levels [36]. Taken together, these observations suggest that MRP3 may compensate for the lack of MRP2 transport of

organic anions and/or function as a bile-acid transporter when their levels are elevated. With this in mind, it is noteworthy that MRP3-mediated transport is competitively inhibited by low concentrations of sulphated bile acids (Table 1 and Figure 2), which are made in increased amounts during cholestasis [37]. This indicates that sulphated bile acids are high-affinity substrates of human MRP3, as has been demonstrated for the rat isoform [24]. Since sulphated bile acids are also high-affinity substrates of rat Mrp2 [35], the high up-regulation of Mrp3 in the absence of functional Mrp2, as is seen in EHBR rats, might serve to remove these metabolites from the liver. We expect the induction of MRP3 in Dubin–Johnson patients to serve a similar purpose. MRP3-mediated transport is also inhibited by taurochenodeoxycholate (Table 1, $IC_{50} \approx 100\ \mu M$), the preferred substrate of Bsep [7]. The up-regulation of MRP3 may provide an escape route for this bile acid in some progressive-familial-intrahepatic-cholestasis patients or in bile-duct-ligated rats in which bile efflux by Bsep is not diminished [38].

MRP3 and Asbt localize to opposite membranes in polarized cells [11,15,29]. Accordingly, a simple model would suggest that bile acids taken up at the apical membrane by Asbt are transported across the basolateral membrane by MRP3. Optimally, polarized cells expressing both transporters would serve to test this hypothesis, as recently demonstrated for OATP8 (SLC21A8) and MRP2 [39]. However, it turned out that MDCK-II cells that stably express mouse or rat Asbt have a high capacity basolateral efflux system for taurocholate and glycocholate (N. Zelcer and P. Borst, unpublished work). The nature of the basolateral transporter of bile acids in the MDCK-II cells has not been further studied. As an alternative to the MDCK-II cells, we used a fibroblast cell line that expresses both MRP3 and Asbt (Figure 4). Although these cells are not polarized, they have previously proved to be suitable for studying transport of MRP3 substrates [26]. Clones that express MRP3 along with Asbt show a substantial increase in glycocholate and taurocholate efflux compared with the control clone (Figure 5), in agreement with our inhibition results (Table 1). The rates of efflux from these cells correlate well with the levels of MRP3 (Figure 4). Furthermore, cells that express Asbt accumulate glycocholate in a time-dependent manner (Figure 6). If the cells additionally express MRP3, the accumulation of glycocholate slows down at a higher intracellular concentration. Assuming a cell volume of $1 \times 10^{-15}\ m^3$, the effect of MRP3 becomes apparent when intracellular concentrations of glycocholate reach about $400\ \mu M$, again indicating that the affinity of MRP3 for this bile acid is low. Yet, despite the low affinity, the capacity of transport is high, as it can partially counteract the influx of glycocholate by Asbt, which has a high V_{max} (Figure 6). Low affinity coupled to a high transport rate of glycocholate by MRP3 was also found by Zeng et al. in membrane vesicles from HEK293 cells overexpressing MRP3 [25]. In accordance with this, when we increased the taurocholate concentration used for loading the cells from $0.25\ \mu M$ to $7\ \mu M$, the initial efflux rate increased as well. We realize that the transfected cell system used by us to study bile-salt transport is rather unphysiological, not only because it is not polarized, but also because it lacks the bile-acid-binding protein present in the ileal mucosa which facilitates intracellular transport and protects against the detergent effects of bile salts [40]. Moreover, the level of MRP3 in our transfected cells is substantially higher than in normal human liver (Figure 7). However, it has been shown that MRP3 levels can go up at least 10-fold in rats or human liver under pathological conditions [19–21], bringing the MRP3 concentration into the same range as in our transfected cells. Taken together, our results suggest that MRP3 can contribute to bile-salt efflux, at least in liver cells under some pathological conditions, and are compatible with the notion that MRP3 transports bile salts with low affinity and high capacity *in vivo*.

Interestingly, control clones, which only express Asbt, also had low levels of bile-acid efflux (Figure 5), as has been seen in many cell types. In fact, even cells which are not normally exposed to bile acids, such as *Xenopus* oocytes, contain an unidentified bile-acid transporter [41]. Low-level expression of endogenous t-Asbt might explain the basal efflux we see in the control cells, but expression of t-Asbt seems to be restricted to specific cells in the ileum, liver and kidney [9,10,29], and we do not expect our fibroblast cell line to express it. More likely, members of the rodent Oatp family of transporters might be expressed in our fibroblast cell line, and this is supported by the *trans*-stimulation of efflux by taurocholate that we find in the control clones, a characteristic of Oatps observed by others [30,42] and studied in the basolateral membrane of isolated cholangiocytes [43] and ileocytes [44].

The question can be raised as to whether the low affinity for bile acids that we find for MRP3 could be due to the requirement of an additional anion for efficient co-transport, as has been shown for several MRP1 substrates. MRP1 transport of oestrone 3-sulphate is GSH-dependent [45], and etoposide glucuronide transport by MRP1 is stimulated by the presence of GSH [46]. Similarly, we find that $E_2 17\beta G$ transport by MRP3 is strongly stimulated by the presence of 4-MUS (Figure 3), raising the possibility that optimal transport of bile acids by MRP3 requires a co-substrate. No stimulation of glycocholate transport by 4-MUS was seen, however. We also consider a requirement of a co-substrate for optimal bile-acid transport by MRP3 unlikely, as we obtain similar results for bile-acid transport by MRP3 in cellular-transport and vesicular-uptake assays. Nevertheless, we cannot rule out the possibility that our cellular system lacks an organic anion present in the cells in which MRP3 is normally active that can stimulate the transport of bile acids.

Our results indicate that MRP3 transports glycocholate and taurocholate with a low affinity and high capacity. This indicates that MRP3 does not substantially contribute to bile-acid transport under physiological conditions and is therefore not the main basolateral transporter of bile acids in ileocytes and cholangiocytes. During cholestasis, however, plasma concentrations of bile acids may reach levels of up to $500\ \mu M$ [37]. Under these conditions MRP3 is highly induced and, as shown here, could play a substantial role in protecting cells against the toxic build-up of intracellular bile salts. Sulphation of bile acids and their urinary excretion is increased in the cholestatic state as well [37]. MRP3, like its rat orthologue, has a high affinity for sulphated bile acids and it is therefore conceivable that, in cholestasis, MRP3 plays a major role in transporting sulphated bile acids into the circulation from which they can be excreted into urine.

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