Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione

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The canalicular multispecific organic anion transporter (cMOAT), a member of the ATP-binding cassette transporter family, mediates the transport of a broad range of non-bile salt organic anions from liver into bile. cMOAT-deficient Wistar rats (TR⁻) are mutated in the gene encoding cMOAT, leading to defective hepatobiliary transport of a whole range of substrates, including bilirubin glucuronide. These mutants also have impaired hepatobiliary excretion of GSH and, as a result, the bile flow in these animals is reduced. In the present work we demonstrate a role for cMOAT in the excretion of GSH both *in vivo* and *in vitro*. Biliary GSH excretion in rats heterozygous for the cMOAT mutation (TR/tr) was decreased to 63 % of controls (TR/TR) (114 ± 24 versus 181 ± 20 nmol/min per kg body weight). Madin-Darby canine kidney (MDCK) II cells stably expressing the human cMOAT protein displayed > 10-fold increase in apical GSH excretion compared with wild-type MDCKII cells (141 ± 6.1 pmol/min per mg of protein versus 13.2 ± 1.3 pmol/min per mg of protein in wild-type MDCKII

cells). Similarly, MDCKII cells expressing the human multidrug resistance protein 1 showed a 4-fold increase in GSH excretion across the basolateral membrane. In several independent cMOAT-transfectants, the level of GSH excretion correlated with the expression level of the protein. Furthermore, we have shown, in cMOAT-transfected cells, that GSH is a low-affinity substrate for the transporter and that its excretion is reduced upon ATP depletion. In membrane vesicles isolated from cMOAT-expressing MDCKII cells, ATP-dependent S-(2,4-dinitrophenyl)glutathione uptake is competitively inhibited by high concentrations of GSH ($K_i \approx 20$ mM). We concluded that cMOAT mediates low-affinity transport of GSH. However, since hepatocellular GSH concentrations are high (5–10 mM), cMOAT might serve an important physiological function in maintenance of bile flow in addition to hepatic GSH turnover.

Key words: ABC transporters, bile, hepatocytes, liver, multidrug resistance.

INTRODUCTION

The liver plays a major role in the detoxification of a broad range of endogenous and xenobiotic compounds, a process in which bile formation is essential. Detoxification takes place in the hepatocyte and encompasses uptake across the basolateral membrane, intracellular metabolism and excretion into bile at the apical side of the cell. The canalicular multispecific organic anion transporter (cMOAT) is a transport protein which is expressed in the apical (canalicular) membrane of the hepatocyte, where it mediates the hepatobiliary transport of a wide range of organic compounds, including bilirubin glucuronides, as well as glutathione- and sulphate-conjugates [1]. The protein has been extensively characterized at the functional level using transport-deficient rats, the TR⁻ and the Eisai hyperbilirubinemic rats, two naturally occurring mutant Wistar and Sprague–Dawley strains respectively [1–3]. Both mutants have an autosomal recessive hepatobiliary transport defect characterized by a chronic conjugated hyperbilirubinemia, and are the animal model for the human Dubin-Johnson syndrome. The cDNAs encoding the cMOAT protein have recently been isolated from rat and human liver [4–8], and mutations were detected in the gene encoding this protein, both in the mutant rat strains and in patients with Dubin-Johnson syndrome [4,6,8,9], which resulted in a truncated and inactive transporter.

The cMOAT protein, also termed multidrug resistance protein (MRP)2, is a member of the ATP-binding cassette transporter family, and is closely related to the human MRP1, a plasma membrane protein which, when overexpressed, confers multidrug resistance to mammalian cells [10]. Recently, Evers et al. [11] showed increased transport of the anti-cancer drug vinblastine over the apical membrane of cMOAT-transfected cells. In addition, Kool et al. [12] reported that expression levels of cMOAT mRNA correlated with cisplatin resistance in several doxorubicin-selected cell lines. These data support a possible involvement of cMOAT in multidrug resistance. Transport via cMOAT and MRP1 has been functionally characterized using the organic anion S-(2,4-dinitrophenyl)glutathione (DNP-GS) as a model substrate. In addition to this mutual substrate, both proteins share a remarkable list of common substrates which are transported out of the cell at the cost of ATP hydrolysis [13]. The major differences between these two transporters are twofold: (i) cMOAT is highly expressed in liver, is low in kidney, colon, ileum and nerve, and is not detectable in other tissues [4,12], whereas MRP1 is ubiquitously expressed, with high expression in lung and testis and with very low expression in the liver [14]; (ii) cMOAT is localized in the apical membrane of epithelial cells [4,5,11], and MRP1 is localized in the lateral domain of the basolateral membrane [15].

Abbreviations used: cMOAT, canalicular multispecific organic anion transporter; TR⁻, cMOAT-deficient Wistar rat; MRP, multidrug resistance protein; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-GS, S-(2,4-dinitrophenyl)glutathione; MDCK, Madin-Darby canine kidney; NEM, N-ethylmaleimide; PCA, perchloric acid; HBSS, Hanks balanced salt solution; HBSS/a, HBSS without Phenol Red, supplemented with acivicin; DTT, dithiothreitol; ES, embryonic stem.

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Besides the impaired hepatobiliary excretion of amphiphilic organic anions, such as bilirubin glucuronides, *cMOAT*-deficient rats completely lack canalicular excretion of GSH [16]. The liver plays a crucial role in maintaining the interorgan homeostasis of cysteine by the excretion of GSH into the circulation ($\approx 30 \mu\text{M}$ in hepatic vein plasma [17]). Furthermore, a substantial amount of GSH is excreted into bile. Hepatocellular GSH concentrations range from 5–10 mM, whereas biliary concentrations range from 1–5 mM [18]. Lauterburg et al. [19] have demonstrated that biliary GSH is an important determinant in maintaining bile flow, suggesting that biliary GSH excretion is a relevant physiological event. The absence of biliary GSH in *cMOAT*-deficient rats suggests a role for this protein in biliary GSH excretion, a hypothesis which has been questioned by others [20], who have suggested that this is a secondary effect of the accumulation of organic anions in the liver of these animals. In the present report, evidence is presented that *cMOAT*, as well as MRP1, mediate low-affinity transport of GSH and that this transport is ATP-dependent. Furthermore, we show, in membrane vesicles isolated from MDCKII-*cMOAT* cells, that DNP-GS uptake is competitively inhibited by GSH.

MATERIALS AND METHODS

Chemicals

1-Chloro-2,4-dinitro- ^{14}C benzene (^{14}C CDNB; specific activity 5.87 Ci/mol) was obtained from Amersham International (Houten, The Netherlands). The radiolabelled glutathione conjugate of CDNB, dinitrophenylglycine-2- ^3H glutathione (DNP- ^3H GS; 44.8 Ci/mmol) was synthesized from ^3H GS (New England Nuclear, Dreieich, Germany) and CDNB (Janssen Chimica, Beerse, Belgium) as described previously [21]. Acivicin, L-buthionine-(*S,R*)-sulphoximine, 2-deoxy-D-glucose and 5,5-dithio-bis-(2-nitrobenzoic acid) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). GSSG, GSH, NADPH, glutathione reductase (from yeast), dithiothreitol (DTT), ATP, NADP, glucose-6-phosphate dehydrogenase (from yeast), hexokinase, creatine kinase and creatine phosphate were from Boehringer (Mannheim, Germany). *N*-Ethylmaleimide (NEM) was from Serva (Heidelberg, Germany). Other chemicals were from E. Merck (Darmstadt, Germany). Nitrocellulose filters, 0.45- μm pore size were from Schleicher and Schuell (Dassel, Germany). Dulbecco's modified Eagle's medium was obtained from Bio Whittaker (Walkersville, MD, U.S.A.), and fetal-calf serum from Integro b.v. (Zaandam, The Netherlands).

Rats and bile cannulation

Normal Wistar rats (*TR/TR*) were obtained from Harlan (Zeist, The Netherlands). *cMOAT-MRP2*-deficient *TR*⁻ rats (*tr/tr*) (Wistar strain) were bred in our own colony. Cross-breeding of *TR/TR* and *tr/tr* rats yielded obligate heterozygous rats (*TR/tr*). Bile-duct cannulations were performed as described previously [16]. Briefly, homozygous (*TR/TR*, *tr/tr*) and heterozygous (*TR/tr*) male rats (300–350 g) were anaesthetized with pentobarbital (Nembutal, 50 mg/kg body weight, intraperitoneally) and the common bile duct was cannulated after distal ligation of the bile duct using a 0.9-mm tube. Bile samples were acidified immediately upon collection with 10% (v/v) perchloric acid (PCA) (final concentration 3.5%). From a different group of rats, livers were excised, frozen in liquid nitrogen and homogenized in PCA (final concentration 3.5%). Subsequently, acidified bile and liver samples were centrifuged (12000 *g* for 3 min), supernatants were neutralized with 3 M K_3PO_4 , and GSH was determined as described by Tietze [22]. Serum and bile

bilirubin levels were determined using a clinicochemical diazo assay with an automated Hitachi 747 analyser.

Cell lines

Polarized MDCKII cells (derived from proximal tubules) stably expressing the human *cMOAT* or human MRP1 proteins were generated by Evers et al. [11,23]. In the present study, three *cMOAT* clones, MDCKII-MOAT16, MDCKII-MOAT17 and MDCKII-MOAT27, and two MRP1 clones, MDCKII-MRP02 and MDCKII-MRP04, were all compared with the non-transfected MDCKII cells. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-bovine serum, 2% (v/v) glutamine and 250 units/ml penicillin/streptomycin.

DNP-GS efflux assay

Cells were grown to confluency on microporous polycarbonate membrane filters (0.4- μm pore size, 12-mm diam.) (Transwell[®] 3414; Costar Corporation, Cambridge, MA, U.S.A.) and the efflux of ^{14}C DNP-GS was determined after incubation of the cells with ^{14}C CDNB. Cells were washed and incubated at room temperature with 0.9 μM ^{14}C CDNB in Hanks balanced salt solution (HBBS; 143 mM NaCl/5.4 mM KCl/1.3 mM CaCl_2 /0.4 mM MgSO_4 /0.5 mM MgCl_2 /0.42 mM NaHCO_3 /0.44 mM KH_2PO_4 /0.34 mM Na_2HPO_4 /5.6 mM glucose/10 mM Hepes, pH 7.0), without Phenol Red, in both the apical and basal compartment. Samples of medium (200 μl) from both the apical and basal compartments were taken at various time points after the addition of ^{14}C CDNB. The hydrophilic ^{14}C DNP-GS was separated from the hydrophobic ^{14}C CDNB by extraction of the samples with ethyl acetate. The radioactivity in the ^{14}C DNP-GS-containing aqueous phase was estimated by liquid scintillation spectroscopy. To determine intracellular ^{14}C DNP-GS, 10% (v/v) PCA was added to the membrane filters, cells were scraped off and centrifuged at 12000 *g* for 10 min. The supernatant was extracted with ethyl acetate to remove unconjugated ^{14}C CDNB. The cell pellets were used for the determination of protein content using the Lowry method, with BSA as a standard.

GSH efflux assay

Cells, grown to confluency on microporous membrane filters, were washed in HBSS supplemented with 0.5 mM acivicin (HBSS/a), and subsequently incubated in HBSS/a. Samples of medium (200 μl) were taken from both the apical and basal compartments at 0–125 min, deproteinized by the addition of PCA (final concentration 10%) and centrifuged at 12000 *g* for 3 min. The supernatant was neutralized to pH 7.0 by the addition of 0.5 M Mops/5 M KOH. To determine intracellular glutathione levels, cells were scraped in 10% (v/v) PCA, centrifuged to remove precipitated proteins and neutralized with 0.5 M Mops/5 M KOH. Total glutathione (GSH plus GSSG) was determined according to the recycling method of Tietze [22]. Cell pellets were used for the estimation of protein content. The oxidized fraction of total glutathione was measured after trapping the reduced fraction with NEM, as described by Sacchetta et al. [24]. Briefly, after PCA precipitation, the samples were divided into two aliquots of 100 μl and either NEM (final concentration 1 mM) or sterile doubly distilled water was added. The samples were incubated, on ice, for 5 min and free NEM was degraded by increasing the pH to 12 by the addition of 3 M K_3PO_4 . After a 20 min incubation on ice, the samples were neutralized by the addition of PCA and subjected to the GSH-recycling reaction.

Isolation of membrane vesicles and vesicle-uptake assay

Plasma membrane vesicles were isolated from MDCKII–MOAT27 as follows. Cells were washed with ice-cold PBS and scraped in ice-cold PBS containing 100 μ M PMSF and 2 mM EDTA (PBS/P/E). After collection, cells were centrifuged at 2500 rev./min for 5 min at 4 °C, and washed twice in PBS/P/E. Cells were swollen by incubation in hypotonic buffer (Tris/HCl, pH 7.4/250 mM mannitol/20 mM Hepes/100 μ M PMSF/2 mM EDTA) for 20 min, on ice, and subsequently homogenized with a Dounce homogenizer (tight pestle, 50 strokes) and centrifuged at 1000 *g* for 5 min at 4 °C. The supernatant was layered on top of a discontinuous sucrose gradient [19% (w/v) and 38% (w/v)] and centrifuged in a Beckman SW41 rotor for 2 h at 100000 *g* at 4 °C. The membrane fraction was collected from the 19%/38% sucrose interface, washed with 250 mM sucrose/20 mM Hepes/Tris/HCl, pH 7.0, and centrifuged (Beckman SW28 rotor) for 30 min at 100000 *g* at 4 °C. The membrane pellet was resuspended in 250 mM sucrose/20 mM Hepes/Tris/HCl, pH 7.0, and re-vesiculated by passing the suspension 30 times through a 27-gauge needle. Membrane vesicles were stored in liquid nitrogen. Uptake of DNP-[³H]GS was measured using the rapid filtration technique described previously [21]. Briefly, 20 μ l of membrane vesicles (30–100 μ g protein) were thawed at 37 °C and added to 20 μ l of pre-warmed (37 °C) reaction mixture (250 mM sucrose/20 mM Hepes/Tris, pH 7.0/20 mM MgCl₂/30 mM creatine phosphate/10 mM DTT/1 mM acivicin containing 1.2 mg creatine kinase/ml and 1 μ Ci DNP-[³H]GS/ml, with or without 4 mM ATP. In the competition experiments, different amounts of unlabelled GSH (5, 10 or 15 mM in 250 mM sucrose/Hepes/Tris, pH 7.0/5 mM DTT/0.5 mM acivicin), or unlabelled GSSG (10, 20, 50 or 100 μ M in Hepes/Tris, pH 7.0) were added to the reaction mixture, which contained 15 nM DNP-[³H]GS. In the competitive inhibition experiments, 15 mM unlabelled GSH, together with 12.5, 25, 50, 100 or 250 μ M DNP-GS (6 nM of which was DNP-[³H]GS) in 5 mM DTT/0.5 mM acivicin, were added to the reaction mixture. In control incubations GSH was replaced by an equimolar concentration of NaCl. Reactions were stopped by the addition of 3 ml of ice-cold stop buffer (250 mM sucrose/Hepes/Tris, pH 7.0). After rapid filtration, filters (presoaked in stop buffer containing 1 mg/ml BSA) were washed twice with 3 ml stop buffer. Filters were placed in liquid scintillation fluid and the radioactivity was estimated by liquid scintillation spectroscopy.

Western blotting

Crude membrane fractions were isolated as follows. Cells were scraped from the dishes with PBS/2 mM EDTA/200 μ M PMSF (500 μ l), centrifuged for 5 min at 1000 *g*, incubated for 20 min, on ice, in hypotonic buffer (250 mM mannitol/2 mM EDTA/200 μ M PMSF), and sonicated (5 pulses of 5 s at 10W). Homogenates were centrifuged for 1 h at 100000 *g* and the pellets were resuspended in PBS/2 mM EDTA/200 μ M PMSF. Proteins from membrane fractions (10 μ g of protein) were subjected to SDS/PAGE (7.5% gel) and electrophoretically transferred to nitrocellulose filters. The filters were blocked for at least 2 h in PBS containing 1% (w/v) BSA, 1% (w/v) dried milk powder and 0.05% (v/v) Tween 20 (PBS/M/T). Filters were incubated for 1 h with the monoclonal antibodies M₂III-6 and MRPr1 (both diluted to 1:1000 in PBS/M/T), which specifically recognize cMOAT [4,12] and MRP1 [25] respectively. Immune complexes were detected with horseradish-peroxidase-conjugated goat anti-(mouse) (for cMOAT) or rabbit anti-(rat) (for MRP1) immunoglobulins (1:3000 in PBS/M/T, 1 h), followed by

enhanced chemiluminescence (Amersham International, Little Chalfont, U.K.).

RESULTS

It has been suggested by others [20] that the GSH excretion defect in homozygous *TR*[−] rats is caused by hepatic accumulation of organic anions. To test this hypothesis we measured GSH excretion in rats heterozygous for the mutation in the *cMOAT* gene. Wild-type Wistar (*TR/TR*), heterozygous (*TR/tr*) and homozygous *TR*[−] (*tr/tr*) rats were characterized by determination of total serum bilirubin, biliary bilirubin and GSH excretion, after cannulation of the bile duct, and by liver GSH content (Table 1). Total serum bilirubin concentration was increased in *tr/tr* rats but the concentration in *TR/tr* was similar to that of control (*TR/TR*) rats. Biliary bilirubin excretion was decreased in *tr/tr* rats, but was similar in *TR/tr* and *TR/TR* rats. This indicates that the 50% expression of the *cMOAT* protein in *TR/tr* rats is sufficient to clear the endogenous organic anions, such as bilirubin glucuronides. In addition, the residual biliary bilirubin output in *tr/tr* rats suggests that the canalicular membrane contains (an)other, yet unidentified, transporter(s) which is/are involved in the excretion of bilirubin glucuronides, as has been suggested previously [26]. Biliary GSH excretion was hardly detectable in *tr/tr* rats, and it was significantly decreased to 63% of the *TR/TR* value in *TR/tr* rats (114 ± 24 versus 181 ± 20 nmol/min per kg body weight). In addition, the rate of biliary [¹⁴C]DNP-GS excretion in isolated perfused livers from *TR/tr* rats was 60% of the excretion rate in *TR/TR* (results not shown). In *TR/tr* rats the liver GSH content was increased (though not significantly) when compared with controls, whereas it was increased to 163% in *tr/tr* liver when compared with control values (Table 1). The data obtained with the *TR/tr* rats suggest, but do not prove, that the absence of biliary GSH output in *tr/tr* rats is a primary consequence of the absence of the *cMOAT* protein, and is not caused by hepatic accumulation of organic anions.

To investigate the role of *cMOAT* in GSH transport in more detail, we generated MDCKII cells stably expressing *cMOAT* or *MRP1*. These cell lines were characterized for localizations and expression levels of the proteins, and the efflux rate of DNP-GS, a model substrate for both *cMOAT* and *MRP1*. *cMOAT* localized to the apical membrane, as shown by Evers et al. [11], whereas the *MRP1* localized to the lateral domain of the basolateral membrane (results not shown). The expression level of *cMOAT* was detected by Western blotting using monoclonal antibody M₂III-6, which was raised against rat *cMOAT* [4], but which specifically reacts also with human *cMOAT* [12]. *MRP1* was detected using monoclonal antibody MRPr1, which specifically reacts with the *MRP1* protein [25]. Analysis of crude membrane fractions of three independent MDCKII–MOAT and two MDCKII–MRP clones showed different amounts of the proteins; no signal was detected in untransfected MDCKII cells (Figure 1A). The initial transport rate of [¹⁴C]DNP-GS was determined by incubation of the cells with [¹⁴C]CDNB, a hydrophobic organic anion which rapidly diffuses into cells and subsequently is conjugated with GSH to [¹⁴C]DNP-GS. To distinguish between apical and basolateral transport, cells were grown to confluency on microporous membrane filters. The results of a representative experiment are shown in which the transport of [¹⁴C]DNP-GS over the apical (Figure 1B) and basolateral (Figure 1C) membrane was determined. Efflux of [¹⁴C]DNP-GS over the apical and basolateral membrane of MDCKII cells was comparable, and was most likely caused by endogenous, canine MRP-like transporters. In the transfectants,

Table 1 Characterization of wild-type Wistar (*TR/TR*), heterozygous (*TR/tr*) and homozygous (*tr/tr*) *cMOAT*-deficient *TR*⁻ rats

Parameters were determined in bile-duct cannulated rats as described in the Materials and methods section. Data are expressed as means $\pm$ S.D. (n = number of animals), as determined by the unpaired Student's *t*-test; * $P < 0.002$ for *tr/tr* and ** $P < 0.02$ for *TR/tr* when compared with *TR/TR* values.

Parameters	<i>TR/TR</i>	<i>TR/tr</i>	<i>tr/tr</i>
Serum bilirubin (μ M; n = 3)	1 $\pm$ 1	1 $\pm$ 1	50 $\pm$ 17*
Biliary bilirubin excretion (nmol/min; n = 2)	1.7/1.6	1.7/1.8	0.9/1.2
Biliary GSH secretion (nmol/min per kg body weight; n = 4)	181 $\pm$ 20	114 $\pm$ 24**	0.16 $\pm$ 0.09*
Liver GSH content (μ mol/gram of liver; n = 4)	6.4 $\pm$ 0.8	7.6 $\pm$ 0.5	10.4 $\pm$ 0.3*

Figure 1 Transport of [¹⁴C]DNP-GS over the apical and basolateral membrane of various *cMOAT*- and *MRP1*-expressing MDCKII cells

(A) Crude membrane fractions (10 μ g protein) were size-fractionated by SDS/PAGE (7.5% gel) and electroblotted. *cMOAT* was detected with monoclonal antibody *M₂/III-6* (lanes 1–4) and *MRP1* was detected with monoclonal antibody *MRP1* (lanes 5–7). Lanes 1 and 5: MDCKII; lanes 2, 3 and 4: MDCKII–MOAT27, MOAT16 and MOAT17; lanes 6 and 7: MDCKII–MRP02 and MRP04. Molecular-mass markers (kDa) are indicated on the left. Cells grown on microporous membrane filters were incubated at room temperature in both the apical (B) and basolateral (C) compartment with 0.9 μ M [¹⁴C]DNP and transport of [¹⁴C]DNP-GS across the apical and basolateral membrane of MDCKII (+), MDCKII–MOAT16 (■), MOAT17 (○), MOAT27 (●), MDCKII–MRP02 (▼) and MRP04 (▽) cells was measured at 3, 9, 15 and 21 min after loading. Data are expressed as means $\pm$ S.D. (n = 3).

Figure 2 Transport of GSH over the apical and basolateral membrane of various *cMOAT*- and *MRP1*-expressing MDCKII cells

Cells grown on microporous membrane filters were incubated at 37 °C in HBSS/a and allowed to excrete GSH across the apical (A) and basolateral (B) membrane for up to 2 h. GSH was determined at the time-points indicated as described in the Materials and methods section. MDCKII (+), MDCKII–MOAT16 (■), MOAT17 (○), MOAT27 (●), MDCKII–MRP02 (▼) and MRP04 (▽) cells. Data are expressed as means $\pm$ S.D. (n = 3).

apical efflux increased with increasing expression levels of *cMOAT* (MOAT27 > MOAT17 > MOAT16). In both MDCKII–MRP clones, basolateral efflux was increased to similar levels (despite the different *MRP1* expression levels), which suggests that, due to the high expression level, *MRP1*-mediated transport is not rate-limiting (in these cells the conjugation is probably rate-limiting). In *cMOAT* transfectants, intracellular [¹⁴C]DNP-GS content is inversely related to the excretion values of [¹⁴C]DNP-GS (MDCKII > MOAT16 > MOAT17 > MOAT27; re-

Table 2 GSH efflux rates and intracellular GSH levels in MDCKII cells and cells stably expressing the cMOAT (MDCKII–MOAT27, –MOAT17 or –MOAT16) and MRP1 (MDCKII–MRP02 or –MRP04) protein

GSH efflux rates were calculated from a representative experiment (performed in triplicate, at three different time points) as depicted in Figure 2, and are expressed as means $\pm$ S.D. Intracellular GSH was determined at the start ($t = 0$) and at the end of the experiment ($t = 2$ h). Data (nmol GSH/mg of protein) are expressed as means $\pm$ S.D.

Cell line	GSH efflux (pmol/min per mg of protein)		GSH _{intracellular} (nmol/mg of protein)	
	Apical	Basolateral	$t = 0$	$t = 2$
MDCKII	13.2 $\pm$ 1.3	68.3 $\pm$ 10	91.9 $\pm$ 1.0	92.8 $\pm$ 1.2
MDCKII–MOAT27	141 $\pm$ 6.1	26.5 $\pm$ 7.5	54.0 $\pm$ 2.2	41.2 $\pm$ 1.8
MDCKII–MOAT17	38.1 $\pm$ 2.3	25.5 $\pm$ 2.0	61.9 $\pm$ 0.6	55.6 $\pm$ 2.5
MDCKII–MOAT16	31.9 $\pm$ 6.1	27.9 $\pm$ 6.7	82.0 $\pm$ 2.0	70.5 $\pm$ 2.2
MDCKII–MRP02	4.1 $\pm$ 0.8	299 $\pm$ 63	39.0 $\pm$ 1.1	33.8 $\pm$ 0.8
MDCKII–MRP04	3.9 $\pm$ 1.0	266 $\pm$ 22	28.9 $\pm$ 2.8	28.7 $\pm$ 1.2

sults not shown). This demonstrates that, in these cells the glutathione-S-transferase-mediated conjugation rate is not a limiting factor, but that the DNP-GS excretion rates in the different clones are a direct consequence of the different cMOAT expression levels.

To study cMOAT- and MRP1-mediated GSH transport, cells were cultured to confluency on microporous membrane filters and incubated in HBSS/a. Acivicin was added to inhibit the γ -glutamyltranspeptidase, an enzyme present on the external surface of apical membranes which hydrolyses excreted GSH. Figure 2(A) shows apical and Figure 2(B) basolateral glutathione transport over a 125 min period in the MDCKII-cell lines. Less than 2 % of the glutathione was in the form of GSSG, both in the apical and basal medium, indicating that all glutathione is exported as GSH. The GSH export rate over the apical membrane was approx. ten times higher in MDCKII–MOAT27 (141 $\pm$ 6.1 pmol/min per mg of protein), three times higher in MDCKII–MOAT17 (38.1 $\pm$ 2.3 pmol/min per mg of protein) and 2.5 times higher in MDCKII–MOAT16 cells (31.9 $\pm$ 6.1 pmol/min per mg of protein), when compared with control MDCKII cells (13.2 $\pm$ 1.3 pmol/min per mg of protein) (Table 2). Similarly, in MDCKII–MRP02 and in MDCKII–MRP04 cells, GSH excretion rate over the basolateral membrane was increased approx. 4.4 times (299 $\pm$ 63 pmol/min per mg of protein) and 3.9 times (266 $\pm$ 22 pmol/min per mg of protein) respectively, when compared with parental MDCKII cells (68.3 $\pm$ 10 pmol/min per mg of protein). The extent of GSH

excretion relates to the expression levels of cMOAT and MRP1 (Figure 1A) and the [14 C]DNP-GS excretion pattern of these cell lines (Figures 1B and 1C). Under control conditions ($t = 0$), intracellular GSH levels were significantly decreased in cMOAT and MRP1 transfected cells as compared with the parental MDCKII cells (Table 2). After the efflux experiments ($t = 2$ h), intracellular GSH levels of transfected cells were decreased when compared with $t = 0$, but intracellular GSH levels in MDCKII cells were unchanged (Table 2). This decrease is most likely caused by lack of GSH synthesis during the experiment, due to the absence of cysteine and methionine in the incubation medium, combined with the high export rates in the transfectants. These data strongly suggest a direct role for the cMOAT and MRP1 protein in the excretion of GSH.

Further experiments were carried out with the high cMOAT-expressing cell line MDCKII–MOAT27. To assess whether the increased apical GSH efflux was dependent on ATP content of the cells, ATP synthesis was inhibited before incubation in HBSS/a. As a control for cMOAT-mediated transport, [14 C]DNP-GS transport was monitored after ATP depletion. It has been shown recently that this treatment results in a pronounced decrease in cMOAT-mediated [14 C]DNP-GS excretion from cMOAT-transfected cells, accompanied by accumulation of intracellular [14 C]DNP-GS [11]. The efflux of GSH into the apical compartment was decreased upon energy depletion (Table 3). The MDCKII–MRP02 cells showed a similarly reduced efflux rate, albeit at the basolateral membrane domain. These results suggest that transport of GSH is an ATP-dependent process. Surprisingly, energy depletion resulted in an increase in GSH efflux into the basal compartment in both parental MDCKII and MDCKII–MOAT27 cells compared with controls. This might be caused by activation of an endogenous GSH transporter upon ATP depletion. It is highly unlikely that this phenomenon is caused by cell death upon ATP depletion, as the intracellular content in MDCKII and MDCKII–MOAT27 cells is unchanged and increased respectively; in addition, apical efflux in parental MDCKII cells was not changed.

To obtain information on the affinity of GSH for the cMOAT protein in MDCKII–MOAT27 cells, the intracellular GSH concentration was modulated by preincubation of the cells with various concentrations of an inhibitor of GSH synthesis, L-buthionine-(S,R)-sulphoximine (0–100 μ M), for 24 h. This resulted in a decrease in intracellular GSH content, which was maximal (17 % of control) after incubation with 100 μ M L-buthionine-(S,R)-sulphoximine. The relationship between intracellular GSH levels and apical GSH efflux is depicted in Figure 3. The transport of GSH over the apical membrane is linearly related to the intracellular GSH concentration in MDCKII–MOAT27 cells. This indicates that, under all con-

Table 3 Effect of energy depletion on the efflux of GSH from MDCKII, MDCKII–MOAT27 and MDCKII–MRP02 cells

Cells grown on microporous membrane filters were preincubated for 15 min at 37 °C in either HBSS/a plus glucose (control) or HBSS/a minus glucose but supplemented with 2 mM deoxyglucose, 2 mM Na $_3$ N $_3$, 2 mM KCN and 20 mM KF (energy-depleted). Media were refreshed and cells were allowed to secrete GSH for 20 min at 37 °C. In a representative experiment (performed in triplicate) both apical- and basolateral-secreted GSH, intracellular GSH and ATP content were determined as described in the Materials and methods section. GSH and ATP are expressed as means $\pm$ S.D. in nmol/mg of protein ($n = 3$); n.d., not detectable (< 0.1 nmol/mg of protein).

Cell lines	Control				Energy-depleted			
	Apical	Basolateral	Intracellular	ATP	Apical	Basolateral	Intracellular	ATP
MDCKII	0.5 $\pm$ 0.3	n.d.	104.1 $\pm$ 4.7	36.0 $\pm$ 3.0	0.7 $\pm$ 0.5	3.4 $\pm$ 0.9	96.4 $\pm$ 1.7	8.3 $\pm$ 1.2
MDCKII–MOAT27	2.0 $\pm$ 0.2	n.d.	49.6 $\pm$ 3.3	30.1 $\pm$ 0.6	0.4 $\pm$ 0.1	1.7 $\pm$ 1.2	57.2 $\pm$ 3.7	4.6 $\pm$ 3.2
MDCKII–MRP02	0.1 $\pm$ 0.2	3.8 $\pm$ 1.2	25.3 $\pm$ 0.9	28.7 $\pm$ 3.0	0.1 $\pm$ 0.1	1.1 $\pm$ 0.9	29.6 $\pm$ 1.3	< 4

Figure 3 Relationship between intracellular GSH and apical GSH efflux in MDCKII-MOAT27 cells

Cells grown on microporous membrane filters were incubated with L-buthionine-(S,R)-sulphoximine (0, 10, 25, 50, 75 or 100 μ M) at 37 °C for 24 h. Subsequently, cells were incubated in HBSS/a and allowed to excrete GSH for 2 h. GSH excreted into the apical compartment and intracellular GSH was determined as described in the Materials and methods section. GSH excretion rate over the apical membrane (nmol/h per mg of protein) is plotted as a function of intracellular GSH. Data are from a representative experiment performed in duplicate.

Table 4 Effect of reducing agents on the oxidation of 15 mM GSH and on DNP-[3 H]GS-uptake rate into MDCKII-MOAT27 membrane vesicles

Membrane vesicles were incubated for 15 min at 37 °C in the presence of 15 mM GSH and different reducing agents. Reactions were stopped by addition of an equal volume of 20% (v/v) PCA, and the oxidized fraction of total glutathione (GSSG) was measured after trapping the reduced fraction with NEM as described in the Materials and methods section. Data are expressed as the means $\pm$ S. D. ($n = 3$). ATP-dependent DNP-[3 H]GS (22 nM) uptake into MDCKII-MOAT27 vesicles (30 μ g protein) was determined at 37 °C in the presence of reducing agents. Reactions were stopped after 15 min and radioactivity present in the vesicles was determined. Initial DNP-[3 H]GS uptake rates (fmol/min per mg of protein) are data from a duplicate experiment.

Reducing agent	GSSG (μ M)	DNP-[3 H]GS uptake (fmol/min per mg of protein)
Control (no reducing agent)	82.3 $\pm$ 14.4	30.1/27.9
1 mM DTT	22.5 $\pm$ 4.2	26.6/25.7
2.5 mM DTT	< 2	29.1/31.3
5 mM DTT	< 2	36.6/31.4
Glutathione reductase (0.125 mg/ml + 0.25 mM NADPH)	< 2	17.6/14.4

ditions, the intracellular GSH concentration is well below the K_m and that GSH is a low-affinity substrate for the cMOAT protein (see below).

Next we attempted to show uptake of GSH into membrane vesicles isolated from MDCKII-MOAT27 cells. As GSH is autoxidized to GSSG, a good cMOAT-substrate [1], we first determined the conditions needed to prevent GSH from becoming oxidized before studying the uptake of GSH or the inhibition of DNP-[3 H]GS uptake by GSH in membrane vesicles (see below). In a 15 min incubation in reaction mixture containing ATP, membrane vesicles (see the Materials and methods section) and 15 mM GSH, several reducing agents (including DTT and glutathione reductase) were tested for their ability to prevent oxidation of GSH during the experiment (Table 4). In addition,

Table 5 Effect of GSH and GSSG on the ATP-dependent uptake of DNP-[3 H]GS into MDCKII-MOAT27 membrane vesicles

Membrane vesicles (50 μ g protein) were incubated in the presence of 15 nM DNP-[3 H]GS, 5 mM DTT, 0.5 mM acivicin, 2 mM ATP, an ATP-regenerating system and various concentrations of GSH or GSSG for 15 min at 37 °C. Uptake of DNP-[3 H]GS was determined by rapid filtration, as described in the Materials and methods section. Data are expressed as percentages of controls (means $\pm$ S.D., $n = 3$).

Inhibiting compound	DNP-GS uptake (% of control)
GSH (mM)	
0	100 $\pm$ 8
5	82 $\pm$ 2
10	60 $\pm$ 2
15	51 $\pm$ 5
GSSG (μ M)	
0	100 $\pm$ 8
10	89 $\pm$ 15
20	85 $\pm$ 9
50	76 $\pm$ 11
100	73 $\pm$ 3

the effect of these reducing agents on the uptake rate of DNP-[3 H]GS were studied. In the presence of 5 mM DTT, negligible amounts of GSSG ($< 2 \mu$ M) were formed and no inhibitory effect on DNP-[3 H]GS uptake was observed. These data demonstrate that 5 mM DTT was sufficient to prevent 15 mM GSH from oxidizing to GSSG during the time course of the experiment. These conditions were therefore used in subsequent experiments. Next we attempted to measure directly ATP-dependent uptake of [3 H]GSH into membrane vesicles obtained from MDCKII-MOAT27 cells. The presence of cMOAT in the vesicle preparation was assessed by Western-blot analysis (results not shown) and functionality of the protein was assessed by the uptake of the model organic anion DNP-[3 H]GS in the absence and presence of ATP. Uptake of DNP-[3 H]GS was increased five-fold in the presence of ATP, when compared with uptake into MDCKII vesicles, and was linear up to 20 min; the γ -glutamyltranspeptidase inhibitor acivicin (final concentration 0.5 mM) had no effect on uptake (results not shown). Using membrane vesicles, we studied the uptake of 0.25 μ M [3 H]GSH in the absence and presence of ATP. No significant uptake rates could be measured, nor was there a difference between membrane vesicles isolated from transfected and parental cells or between incubations in the presence and absence of ATP. This was most likely because of the low affinity of GSH for the transporter, which led to extremely low transport rates at the relatively low concentration used.

Since several attempts failed to demonstrate cMOAT-mediated [3 H]GSH transport directly, we studied inhibition of ATP-dependent DNP-[3 H]GS uptake by GSH in MDCKII-MOAT27 vesicles. Table 5 shows that, in the presence of 5, 10, and 15 mM GSH, DNP-[3 H]GS (15 nM) uptake was inhibited. In the same experiment, the GSSG concentration was determined and found to be less than 2 μ M. In parallel, the effect of GSSG on DNP-[3 H]GS transport was determined and increasing the GSSG concentration up to 20 μ M had no significant effect on DNP-[3 H]GS uptake. These results suggest that DNP-GS transport is inhibited by high concentrations of GSH and that this is not due to the low amounts of GSSG formed during the incubation period.

To assess whether inhibition of DNP-GS uptake by GSH was competitive, experiments were performed with and without a fixed concentration of GSH and with various concentrations of

Figure 4 Double-reciprocal plot of initial DNP-GS uptake into MDCKII-MOAT27 membrane vesicles in the absence of and in the presence of 15 mM GSH, as a function of DNP-GS concentration

Membrane vesicles (100 μ g protein) were incubated as described in Table 5 in the absence (●) or presence (○) of 15 mM GSH and various concentrations of DNP-GS (12.5, 25, 50, 100 or 250 μ M, of which 6 nM was DNP-[3 H]GS). Initial DNP-GS uptake was determined by rapid filtration. Results are from a representative experiment performed in triplicate.

DNP-[3 H]GS. The results of a representative experiment are shown in Figure 4. DNP-GS uptake (in the absence and presence of 15 mM GSH) into MDCKII-MOAT27 membrane vesicles was a saturable process which obeyed Michaelis-Menten kinetics. Analysis of the results, depicted as a Lineweaver-Burk plot, reveals a K_m of 70 ± 12 μ M for DNP-GS. The intercept of the two curves on the y -axis demonstrates that GSH competitively inhibits the cMOAT-mediated uptake of DNP-GS with a K_i of $\approx 20 \pm 0.6$ mM, calculated from the change in the slope of the line. This again suggests that GSH is a low-affinity substrate for the cMOAT protein.

DISCUSSION

The transport processes involved in hepatobiliary excretion of GSH have been a matter of controversy for a number of years. Inoue et al. [27] demonstrated electrogenic uptake of GSH in canalicular membrane vesicles of rat liver, which was inhibited by GSSG, suggesting an identical biliary excretion pathway for both substrates. GSH excretion was also shown to be inhibited by bromo- and dibromo-sulphophthalein, and by glucuronide/sulphate conjugates of lithocholic acid, whereas no inhibition was observed for sulphobromophthalein-glutathione [28,29]. The identification and characterization of the transporter responsible for the excretion of these organic anions, cMOAT or MRP2, helped to clarify this field considerably. TR $^-$ and Eisai hyperbilirubinemic rats, which lack this ABC-transporter, are impaired in the hepatobiliary transport of a whole range of anionic substrates and, in addition, have very much lower biliary GSH levels [1-3,16]. The simplest interpretation of this observation is that the absence of biliary GSH is a direct effect of the absence of the cMOAT protein, as hypothesized by Oude Elferink et al. [16]. Alternatively, the existence of an additional, multispecific GSH carrier was proposed by Fernandez-Checa et al. [20]. According to their hypothesis, the absence of biliary GSH could be caused by *cis*-inhibition of this alternative GSH carrier by accumulated intrahepatic cMOAT substrates and/or by loss of *trans*-stimulation by cMOAT substrates normally present in bile. They demonstrated electrogenic, ATP-independent and low-

affinity ($K_m \approx 15$ mM) transport of GSH into canalicular membrane vesicles of both wild-type and cMOAT-mutated rats. GSH transport in membrane vesicles was *cis*-inhibited and *trans*-stimulated by DNP-GS. Subsequently, this group isolated the cDNA encoding the putative canalicular glutathione transporter, RcGshT, from rat liver (GenBank U06845) [30], but later this was found to be a cloning artefact. GenBank searches indicated that large parts of the RcGshT cDNA sequence are highly similar to an *Escherichia coli* genomic sequence (AE000369) and a part of the *E. coli* K-12 genome (U28375). The conclusion must be that there is, at present, no evidence for a separate GSH carrier in the canalicular membrane of the hepatocyte. In the present study, we have described a role for the cMOAT protein in the transport of GSH. Although we cannot exclude the possibility that activation of the cMOAT protein regulates the activity of endogenous GSH channels in the apical membrane, we consider this, based on our experimental data, a rather unlikely possibility.

The observation that the apical GSH-efflux rate is proportional to the expression level of the cMOAT protein suggests, but does not prove, that cMOAT is directly involved in GSH transport. Obligate heterozygote rats (TR/tr), having 50% of normal cMOAT protein expression levels, have intermediate biliary excretion rates of both DNP-GS and GSH when compared with control rats (TR/TR), whereas, in homozygous tr/tr rats, this transport is completely abolished (Table 1). The same tendency was observed in the cMOAT-expressing MDCKII cells with regard to DNP-GS and GSH excretion (Figure 1). MDCKII cells transfected with MRP1 cDNA showed similar characteristics of GSH transport, but, in this case, over the basolateral membrane domain. The background DNP-GS and GSH transport in parental MDCKII cells is most likely due to the expression of endogenous, canine MRP-like transporters, as proposed by Evers et al. [11]. Previously, we reported an increase in GSH excretion from non-small-cell lung carcinoma cells transfected with MRP1 cDNA [31]. In addition, it was recently reported that GSH excretion is modulated by the expression level of the MRP1 protein in embryonic stem (ES) cells, where there is a homozygous disruption of the *MRP1* gene (ES *Mrp* -/-); the rate of GSH efflux was approx. twice as low as that of the wild-type cells (ES *Mrp* +/+) [32]. Taken together, these results suggest that both cMOAT and MRP1 mediate the transport of GSH.

Both *in vivo* and in the transfected cells the intracellular GSH levels inversely correlated with the expression level of the cMOAT protein. Absence of the cMOAT protein in tr/tr rats resulted in a significant increase in hepatocellular GSH levels, whereas in TR/tr rats the intracellular GSH levels were slightly but not significantly increased (Table 1). In addition, high cMOAT expression levels in the MDCKII-MOAT clones resulted in low intracellular GSH levels (Table 2). These data indicate that the intracellular GSH levels are determined by the cMOAT expression level, i.e. the rate of GSH efflux from the cell. Similar data were obtained for the MDCKII-MRP1 clones. In addition to our data, Koike et al. [33] have also observed an inverse correlation between cMOAT expression and intracellular GSH levels; transfection of HepG2 cells with a cMOAT anti-sense cDNA resulted in decreased cMOAT expression and a concomitant increase in intracellular GSH levels. Similarly, intracellular GSH levels in ES *Mrp* -/- cells were increased compared with wild-type ES cells [32].

Three mechanisms can be proposed by which the cMOAT protein transports GSH across the membrane. (i) cMOAT could mediate the transport of reversible, short-lived complexes of GSH with unknown intracellular compounds. Such a mechanism probably applies for arsenicals, antimony, and bismuth. When

these compounds were infused into rats they were excreted into bile with a concomitant increase in hepatobiliary efflux of GSH [34,35]; both arsenite excretion as well as (arsenite-induced) GSH excretion was completely abolished in TR^- rats, suggesting that the arsenite-glutathione complex is a cMOAT substrate (R. P. J. Oude Elferink, unpublished work). Indeed, it has been proposed that arsenite is excreted as a short-lived complex with GSH, $As(SG)_3$ [36]. In addition, arsenite-induced GSH excretion has been reported previously for the MRP1 protein [31,32]. (ii) cMOAT could mediate the co-transport of an endogenous substrate with GSH. Such a mechanism has been proposed for GSH-induced transport of vincristine by MRP1. Loe et al. [37] demonstrated that uptake of vincristine into MRP1-enriched membrane vesicles was increased in the presence of high GSH concentrations. It is, however, not known whether vincristine is transported preferentially as a short-lived complex, or that binding of GSH to MRP1 induces a conformational change and subsequently facilitates binding of vincristine, which results in excretion of this substrate together with GSH. (iii) cMOAT could mediate the transport of free GSH without the need for other substrates. The peculiar transport characteristics of GSH observed in the present study (Figure 3), are most likely because of the very low affinity of GSH for the transporter. If mechanisms (i) and/or (ii) exist, equimolar amounts (i.e. millimolar) of GSH and the complexing compound or the co-substrate should be present in bile. Since these have not been found, as yet, we favour the hypothesis that cMOAT itself mediates the transport of GSH (hypothesis iii).

Although the proportional correlation between cMOAT expression level and GSH excretion rates (Figures 1 and 2, Table 2) strongly suggests cMOAT-mediated GSH transport, no direct ATP-dependent uptake of GSH by cMOAT-enriched membrane vesicles could be demonstrated. We presume that this is caused by the low affinity of GSH for cMOAT. This implies that considerable rates of GSH transport can only be reached at high GSH concentrations, as occurs in the cytosol. Large amounts of labelled GSH would be required in the incubation medium in order for substantial amounts of the label to be taken up by the vesicles. Another drawback to our experimental setup is that, in the membrane vesicles isolated from MDCK cells, only a small fraction of the vesicle preparation will be present in the correct orientation (i.e. inside-out); the remainder of the vesicles will be in the right-side-out orientation and will be functionally silent. In this respect it is interesting to note that Rebbor et al. [38] very recently demonstrated ATP-dependent (low-affinity) GSH transport in secretory vesicles isolated from the *sec6-4* mutant strain of *Saccharomyces cerevisiae*. The K_m of this transport system for GSH (19 ± 5 mM) was almost identical to the K_m for cMOAT observed in the present study. Indeed, Rebbor et al. [39] directly demonstrated ATP-dependent, low-affinity GSH transport by the yeast cadmium factor (YCF1), a yeast MRP-like transporter with high amino-acid sequence identity to cMOAT (42%) and very similar transport characteristics [40,41]. The membrane vesicles used in the study of Rebbor et al. [39] are, by virtue of their isolation as intracellular vesicles, predominantly in the correct functional orientation, and thus, in this system, the functional volume of the vesicle preparation will be much larger. In the present study, the competitive inhibition experiment (Figure 4) indicates that DNP-GS and GSH compete for the same substrate binding site, suggesting direct cMOAT-mediated GSH transport.

We conclude that the cMOAT protein mediates the transport of GSH. It remains speculative whether this represents a physiologically important mechanism. GSH may contribute to the bile flow. The driving force for bile formation is partly bile-acid-

dependent and partly bile-acid-independent [1]. In *tr/tr* rats, bile-acid-independent flow is reduced by 50%, whereas the bile-acid-dependent flow is unaffected. Lauterburg et al. [19], by modulating intrahepatic GSH levels and determining GSH excretion and bile-acid-independent flow, reported a linear relation between bile-acid-independent flow and biliary GSH excretion, indicating that GSH, indeed, might be an important determinant of bile-acid-independent flow in the rat.

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