The human multidrug-resistance-associated protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin

Igino RIGATO*†, Lorella PASCOLO*†, Cristina FERNETTI*†, J. Donald OSTROW‡ and Claudio TIRIBELLI*†¹

*Liver Research Center, Bldg. Q, AREA Science Park Basovizza, SS 14 Km 163.5, 34012 Trieste, Italy, †Department of Biochimica, Biofisica e Chimica delle Macromolecole, University of Trieste, 34012 Trieste, Italy, and ‡GI/Hepatology Division, Department of Medicine, University of Washington School of Medicine, Seattle, WA 98195, U.S.A.

Results of previous studies have suggested that UCB (unconjugated bilirubin) may be transported by MRP1/Mrp1 (multidrug-resistance-associated protein 1). To test this hypothesis directly, [³H]UCB transport was assessed in plasma-membrane vesicles from MDCKII cells (Madin–Darby canine kidney II cells) stably transfected with human MRP1 or MRP2; wild-type MDCKII cells served as controls. As revealed by Western blotting, transfection achieved abundant expression of MRP1 and MRP2. [³H]UCB uptake was measured in the presence of 60 μ M human serum albumin at a free (unbound) concentration of UCB (B_F) ranging from 5 to 72 nM and in the presence of 3 mM ATP or 3 mM AMP-PCP (adenosine 5'-[β , γ -methylene]triphosphate). MRP1-transfected vesicles showed transport activity three and five times higher respectively compared with MRP2 or wild-

type vesicles, whose transport did not differ significantly. [³H]UCB transport was stimulated 4-fold by 1.5 mM GSH, occurred into an osmotically sensitive space, was inhibited by 3 μ M MK571 and followed saturative kinetics with $K_m = 10 \pm 3$ nM (B_F) and $V_{max} = 100 \pm 13$ pmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹. UCB significantly inhibited the transport of LTC4 (leukotriene C4), a leukotriene substrate known to have high affinity for MRP1. Collectively, these results prove directly that MRP1 mediates ATP-dependent cellular export of UCB and supports its role in protecting cells from bilirubin toxicity.

Key words: ATP-binding-cassette protein (ABC) protein, cytotoxicity, glutathione, multidrug-resistance-associated protein 1 (MRP1), transport, unconjugated bilirubin.

INTRODUCTION

MRP1 (multidrug-resistance-associated protein 1) is the first identified member of the multidrug-resistance-associated protein subfamily of ABC transporters (ATP-binding-cassette transporters) [1]. Since its discovery in 1992, increased expression of MRP1 has been correlated with the development of multidrug resistance by cells previously sensitive to chemotherapy [2]. It was shown that MRP1 can transport many exogenous agents, such as chemotherapeutics (daunorubicin, etoposide and vincristine) [3–6], metalloid salts (antimony and arsenic) [7] and aflatoxin B1 [8]. MRP1 also transports endogenous compounds such as arachidonic acid derivatives [LTC4 (leukotriene C4) is the highest affinity substrate] [4,9–11], glutathione conjugates [3,4], glucuronide and sulphate conjugates of bile salts [12], GSSG [13,14], 17 β -oestradiol 17-(β -D-glucuronide) [5,12] and bilirubin glucuronides [15].

MRP1, and another multidrug resistance protein, MDR1, which shares many of the substrates transported by MRP1 [16], are both expressed in the blood–cerebrospinal fluid and blood–brain barriers, where they play important roles in preventing accumulation, in the cerebrospinal fluid and CNS (central nervous system) respectively, of a wide variety of xeno- and endo-biotics [17]. Of great importance among the latter is UCB (unconjugated bilirubin), which is toxic to CNS cells at high concentrations [18] and is responsible for the increasing prevalence of bilirubin-induced encephalopathy in severely jaundiced newborns [19,20].

Earlier studies conducted in our laboratory at clinically relevant UCB concentrations have suggested a role for one or more MRP proteins in the transport of UCB. Petrovic et al. [21] found that UCB is a substrate for both *YCF1* and *YLL015* gene products in *Saccharomyces cerevisiae*. Of the six MRP genes expressed,

YCF1 and *YLL015* show the highest homology for human *MRP1* and *MRP2* [22]. Pascolo et al. [23] observed that BeWo cells, grown in a polarized manner, concomitantly increase the export of UCB and the expression of MRP1; moreover, the efflux of UCB was almost abolished by MK571 [23], a general inhibitor of MRPs [4]. MK571 also increased apoptosis and impairment of mitochondrial function [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test] and decreased Trypan Blue exclusion in cultured astrocytes exposed to clinically relevant concentrations of UCB *in vitro* [24]. This indicated that one or more MRPs were functional in protecting these CNS cells from toxic effects of UCB. In the same paper, it was shown that UCB up-regulated the expression of MRP1 and engendered its translocation from the Golgi to the plasma membrane, suggesting a mechanism by which MRP1 limits UCB toxicity by exporting UCB out of the cell [24].

However, MRP1-mediated efflux of UCB has not been directly demonstrated. To determine whether MRP1 could be involved in the transfer of UCB across cellular membranes, we studied the transport of [³H]UCB by plasma-membrane vesicles derived from MDCKII cells (Madin–Darby canine kidney II cells) transfected with human MRP1 or MRP2, using wild-type cells as controls. We also studied the effects of ATP, GSH and MK571 on this transport.

MATERIALS AND METHODS

Materials

[³H]UCB (29.25 mCi/mmol) was biosynthetically labelled *in vivo* and then highly purified from the bilirubin conjugates in bile, as described previously [25]. [³H]LTC4 (182.7 Ci/mmol) was

Abbreviations used: AMP-PCP, adenosine 5'-[β , γ -methylene]triphosphate ('pp[β , γ -CH₂]pA'); CNS, central nervous system; HSA, human serum albumin; LTC4, leukotriene C4; MDCKII cells, Madin–Darby canine kidney II cells; Mrp/MRP1, multidrug-resistance-associated protein 1; UCB, unconjugated bilirubin.

¹ To whom correspondence should be addressed, at Liver Research Center (email ctliver@csf.units.it).

obtained from PerkinElmer (Zaventem, Belgium). MK571 was obtained from Alexis Chemicals (VinciBiochem, Vinci, Florence, Italy). DMSO and chloroform were HPLC grade and obtained from Carlo Erba (Milan, Italy). HSA (human serum albumin; fatty acid-free) and all other chemicals and reagents, unless otherwise indicated, were reagent grade and obtained from Sigma-Aldrich (Milan, Italy).

Cell lines and culture conditions

MDCKII wild-type and clones 108 and 227, stably transfected with human *MRP1* and *MRP2* genes respectively [26], were gifts from Dr R. P. J. Oude Elferink (Academic Medical Center, Amsterdam, The Netherlands). The cells were cultured under standard conditions [26] in Dulbecco's modified Eagle's medium with 10 % (v/v) foetal calf serum and 1 % antibiotics (100 000 units/l penicillin and 100 mg/l streptomycin).

Anti-MRP antibodies

A specific anti-human MRP1 polyclonal antibody (A23) was used as described previously [27]. An anti-MRP2 polyclonal antibody, designated A22, was generated by immunizing a rabbit with a synthetic peptide (22 amino acid residues; GSPEELLQIPGPFYFM-AKEAGI). The antigen peptide was derived from the C-terminal region of human MRP2 sequence (GenBank® accession no. NM_000392), which has low homology with the corresponding regions of MRP1, MRP3, MRP4 and MRP5. Using the 'Antigenic' (European Molecular Biology Open Software Suite) program, the chosen region was found to contain antigenic sites. The IgG fraction was purified by affinity chromatography using SulfoLink coupling gel (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions.

Membrane vesicle preparations

Membrane vesicles were obtained from MDCKII 108 and 227 and wild-type cells as described previously [16,28], but with some modifications. Cells grown as indicated above were removed with trypsin (0.05 %)/EDTA (0.02 %, w/v) in PBS solution and washed three times with Dulbecco's PBS solution. Cell pellets were obtained by centrifugation (600 *g* for 5 min at 4 °C), incubated with lysis buffer (1 mM NaHCO₃, adjusted with HCl to pH 7.4) for at least 40 min at 4 °C and homogenized using a Potter Dounce (Wheaton, Millville, NJ, U.S.A.). The supernatant obtained after centrifugation at 1000 *g* for 8 min at 4 °C was further centrifuged at 100 000 *g* for 30 min at 4 °C. The pellet was resuspended in 250 mM sucrose and 10 mM Hepes/Tris (pH 7.4), and the plasma-membrane fraction was collected at the interface of a sucrose gradient (38 and 19 %, w/v) by centrifugation (200 000 *g* for 2 h at 4 °C). After washing once with 250 mM sucrose and 10 mM Hepes/Tris, pH 7.4 (100 000 *g* for 40 min at 4 °C), membrane vesicles were snap-frozen in liquid nitrogen after an additional homogenization. The protein concentration was measured by the bicinchoninic acid method [29] using BSA as a standard. The sidedness of the membrane vesicles was assessed by determining the activities of the plasma membrane-associated ectoenzyme 5'-nucleotidase [30] in the presence or absence of 0.2 % Nonidet P40 [5].

Western blotting

Plasma-membrane vesicles (10 µg of protein) were fractionated by SDS/PAGE (7 % acrylamide gel) and blotted on to a nitrocellulose membrane (0.2 µm Protran BA 83; Schleicher and Schuell, Dassel, Germany) with a semi-dry blotting system

(Sigma, St. Louis, MO, U.S.A.). After blocking for 60 min with 4 % (w/v) milk in TTBS solution [500 mM NaCl, 20 mM Tris/HCl (pH 7.5) and 0.2 % Tween 20], the membrane was incubated overnight with a 1:1000 dilution of the specific A23 antibody [27] or a 1:500 dilution of the A22 antibody to reveal respectively MRP1 or MRP2. After washing three times (for 10 min) with 4 % milk in TTBS solution, the membrane was incubated for 60 min with a 1:5000 dilution of goat anti-rabbit IgG antibody conjugated with peroxidase (Sigma-Aldrich). The peroxidase reaction was performed with the ECL®-Plus Western blotting detection system (Amersham Biosciences, Milan, Italy). After transfer to a Kodak film, the bands were visualized by Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Transport of [³H]UCB by vesicles

Frozen vesicles were rapidly thawed at 37 °C, passed six times through a 25-gauge needle and then placed on ice. [³H]UCB from a freshly opened vial was dissolved in chloroform at a concentration of 0.85 mM and aliquots were dried under nitrogen. Immediately before each incubation, an aliquot was dissolved in DMSO (1.4 µl of DMSO per µg of [³H]UCB) [31] and then diluted with 100 vol. of a medium containing 60 µM HSA in 250 mM sucrose, and 10 mM Hepes, adjusted with Tris to pH 7.0. As reported previously [31,32], 20 µl of membrane vesicles (1.8–2.2 mg of protein/ml) were added to 80 µl of the medium containing 60 µM HSA and different concentrations of [³H]UCB. GSH (1.5 mM) and 3 mM ATP-Mg²⁺ or 3 mM AMP-PCP (adenosine 5'-[β,γ-methylene]triphosphate) were then added just before starting incubations at 37 °C. To avoid displacement of bilirubin from its binding site on albumin, an ATP-regenerating system was omitted.

For most of the experiments, the final UCB concentrations were 14–20 µM, yielding free (unbound) UCB concentrations (*B_F*) calculated to be 17–28 nM [33]. To determine *K_m* and *V_{max}*, we tested UCB concentrations from 5 to 34 µM, yielding *B_F* values between 5 and 72 nM. To minimize the photodegradation of UCB, all the experimental procedures were performed under a dim red light.

Transport into vesicles was measured [30], after incubation at 37 °C for different time intervals, by adding 1.0 ml of ice-cold stop solution, consisting of the transport medium without HSA or UCB. Each sample was rapidly vacuum-filtered through a nitrocellulose membrane (HAWP 0.24; Millipore, Bedford, MA, U.S.A.) and the filter was washed three times with 3.5 ml of ice-cold stop solution and dried at room temperature. Radioactivity retained on the filter was determined by a liquid-scintillation spectrometer (Beta V; Kontron, Milan, Italy) after the addition of scintillation medium (Packard Bioscience, Groningen, The Netherlands) with automatic quench corrections using external reference standards. The difference between the transport measured in the presence of 3 mM ATP versus the non-hydrolysable ATP analogue (AMP-PCP) was considered to be ATP-dependent transport of [³H]UCB. With or without ATP, the net contribution of MRP1 to UCB uptake was expressed as the difference between the transport measured in vesicles from MRP1-transfected cells versus wild-type cells.

Transport of [³H]LTC4 by vesicles and its inhibition by added UCB

LTC4 transport was determined by rapid ultrafiltration, as described for [³H]UCB, except that HSA was omitted from the medium to avoid competitive displacement between LTC4 and UCB at the binding domain on HSA and allow the determination of the true free concentration of the two substrates. A mixture of

87 % [^3H]LTC4 and 13 % unlabelled LTC4 was dissolved in the uptake medium (sucrose 250 mM, Hepes 10 mM and Tris, pH 7.0) to a final concentration of 150 nM. Purified unlabelled UCB was dissolved in DMSO ($0.71 \mu\text{g}/\mu\text{l} = 1.2 \text{ mM}$), and $2 \mu\text{l}$ of this solution, or DMSO alone, was diluted with 24 ml of the uptake medium. The labelled LTC4 mixture was added to the UCB/DMSO or DMSO solutions to obtain final concentrations of 150 nM LTC4 and 100 or 0 nM UCB respectively.

Statistical analysis

Results are expressed as means $\pm$ S.D. Each data point represents the average of four measurements in at least three different vesicle preparations. A paired t test was used to calculate the statistical significance between the different experimental conditions.

RESULTS

Experimental considerations

After the preparation of the incubation medium, very little degradation of highly pure [^3H]UCB is expected to occur during the subsequent 1–3 min of incubation with vesicles. In most experiments, the radioactivity retained by the vesicles ranged from 400 to 1200 d.p.m. (disintegrations per min) (approx. 10 % of the initial amount in the medium), with blank values (without vesicles) usually lower than 20 % of the whole signal. Even if all radiolabelled impurities present in the medium were taken up by the vesicles, this would represent less than 30 d.p.m. or 10 % of the total uptake. Therefore the radioactivity assayed in the vesicles was considered equivalent to [^3H]UCB.

The orientation of membrane vesicles obtained from the three different cell lines (MRP1-transfected, MRP2-transfected and wild-type), assessed by the accessibility of 5'-nucleotidase activity, was comparable, with the percentage of inside-out vesicles ranging from 20 to 27 %.

The comparable percentage of inside-out orientation in all the three vesicle preparations suggests that the markedly higher UCB transport found in the MRP1-transfected vesicles is accounted for by the presence of this transporter.

Effects of GSH and ATP on [^3H]UCB transport

As shown in Figure 1, in the absence of GSH, ATP-dependent [^3H]UCB transport was low, but was 40 % higher ($P < 0.01$) in vesicles obtained from MRP1-transfected MDCKII cells compared with wild-type cells. When 1.5 mM GSH was added, transport of [^3H]UCB increased significantly in vesicles from both cell types, but much more strikingly in those expressing MRP1 (4 times that found in the absence of GSH; $P = 0.006$). Consequently, all subsequent experiments were performed in the presence of 1.5 mM GSH. Substitution of ATP with non-hydrolysable AMP-PCP (3 mM each) decreased [^3H]UCB transport by MRP1-transfected vesicles from 100 ± 20 to $6 \pm 2 \text{ pmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$, indicating that this UCB transport was almost entirely ATP-dependent.

Time course of [^3H]UCB transport

Figure 2 shows the time course of the MRP1-dependent [^3H]UCB transport during 180 s, measured as the difference between the ATP-dependent transport by vesicles from MRP1-transfected cells versus wild-type cells. In vesicles from wild-type cells, the transport was much lower and linear throughout ($y = 0.30x + 4.43$, $r^2 = 0.88$) (results not shown). The 60 s time point yielded

Figure 1 Effect of ATP and GSH on [^3H]UCB transport

Transport of [^3H]UCB was measured in plasma-membrane vesicles obtained from MRP1-transfected and wild-type MDCKII cells after 60 s of incubation with [^3H]UCB ($B_F = 23 \text{ nM}$) in the presence of $60 \mu\text{M}$ HSA. ATP-dependent [^3H]UCB transport, calculated as the difference between the transport measured in the presence of 3 mM Mg^{2+} ATP versus AMP-PCP, was measured in plasma-membrane vesicles from wild-type MDCKII cells (white bars) and MRP1-transfected MDCKII cells (black bars). $\$P < 0.01$; $*P = 0.006$.

Figure 2 Time course of [^3H]UCB transport and dependence on MRP1 activity

Transport of [^3H]UCB, in the presence of 3 mM ATP and 1.5 mM GSH was measured serially in vesicles from MRP1-transfected and wild-type MDCKII cells. The thick solid line shows the MRP1-dependent transport, expressed as the difference between the two cell types. Transport was rather slow in the first 30 s, then increased linearly up to 60 s and reached a plateau from 90 to 180 s.

adequate counts for an accurate radioassay, but safely preceded the plateau; it was used, therefore, in all subsequent experiments.

Effect of MRP expression on [^3H]UCB transport by plasma-membrane vesicles and the effect of MK571

Figures 3(A,1) and 3(A,2) show the Western blots used to check the expression of MRP1 and MRP2 proteins respectively in the membrane vesicle preparations from transfected cells. HepG2 cells were used as a positive control for both proteins, whereas MDCKII wild-type cells served as a negative control. As expected, using the specific anti-MRP1 antibody A23, MRP1 was clearly visible in vesicles from MDCKII 108 cells, but could not be detected in vesicles from MRP2-transfected MDCKII 227 and wild-type MDCKII cells (Figure 3A, 1); this confirms the absence

Figure 3 Expression of MRP1 and MRP2 proteins in transfected cells and effect of MK571 on ATP-dependent $[^3\text{H}]\text{UCB}$ transport by membrane vesicles from those cells

(A) The expression of MRP1 and MRP2 was assessed by Western-blot analysis of membrane vesicles obtained from transfected MDCKII cells. (A, 1) MRP1 revealed by A23 antibody after transfection with MRP1 (lane 108). No expression was detected in vesicles obtained from wild-type MDCKII cells (lane W) or MRP2-transfected cells (lane 227). Lane H shows the lower expression of MRP1 in vesicles obtained from untransfected HepG2 cells, used as positive control. (A, 2) MRP2 detection by A22 antibody. No expression was detected in vesicles from wild-type MDCKII (lane W) or MRP1-transfected MDCKII (lane 108) cells, whereas expression was observed in vesicles from MRP2-transfected cells (lane 227) and in HepG2 cells (positive control). (B) ATP-dependent transport of $[^3\text{H}]\text{UCB}$, determined as in Figure 1, in plasma-membrane vesicles obtained from the three strains of the MDCKII cell line (MRP1-transfected, MRP2-transfected and wild-type) in the absence (black, mid-grey and white bars) or in the presence (light-grey bars) of the MRP inhibitor, MK571 (3 μM). In the absence of the inhibitor, transport by vesicles from MRP1-transfected cells was three times that of MRP2-transfected cells and five times that of wild-type cells (for each, $P < 0.001$), whereas MRP2-transfected cells did not differ significantly from wild-type. MK571 inhibited UCB transport by 50% in vesicles from MRP1-transfected cells ($P < 0.001$), whereas there was no significant inhibitory effect on vesicles from MRP2-transfected or wild-type cells.

of cross-reactivity of antibody A23 with human MRP2. Similarly, the antibody against human MRP2 specifically detected this protein only in the MRP2-transfected MDCKII clone. This shows also that the two antibodies do not cross-react with endogenous Mrps of canine MDCKII cells.

As shown in Figure 3(B), in the presence of GSH, the expression of MRP1 enhanced ATP-dependent $[^3\text{H}]\text{UCB}$ transport almost 3-fold compared with MRP2-transfected vesicles and almost 5-fold compared with wild-type vesicles (for each, $P < 0.001$), with no significant difference between MRP2 and wild-type vesicles. In contrast (results not shown), the transport of $[^3\text{H}]\text{LTC}_4$, a substrate also for MRP2 [34], was increased by 2.5 times in vesicles obtained from MRP2-transfected cells, confirming the functionality of the transfected MRP2. These results establish the involvement of MRP1, but not MRP2, in the ATP-dependent transport of UCB, and also excludes the possibility that transfection *per se* might have stimulated transport activity.

To assess further the involvement of MRP1 in $[^3\text{H}]\text{UCB}$ transport, we tested the effect of MK571, a general inhibitor of MRPs

Figure 4 Effects of changes in vesicular volume on MRP1-dependent transport of $[^3\text{H}]\text{UCB}$

ATP-dependent transport of $[^3\text{H}]\text{UCB}$ by plasma-membrane vesicles from MRP1-transfected MDCKII cells was determined at $B_F = 23$ nM, in the presence of 60 μM HSA and 1.5 mM GSH, and was expressed as the difference between the transport at 60 s in the presence of 3.0 mM ATP versus 3.0 mM AMP-PCP. The intravesicular volume was changed by varying the sucrose concentration from 250 to 1000 mM. ATP-dependent UCB uptake was linearly correlated with the reciprocal of the sucrose concentration ($y = 10631x + 16.1$, $r^2 = 0.90$, $P < 0.001$), indicating that the ATP-dependent UCB transport by the MRP1-transfected vesicles occurred into an osmotically sensitive space.

[4,7]. As shown in Figure 3(B), the addition of 3 μM MK571 inhibited, by almost 50%, the ATP-dependent $[^3\text{H}]\text{UCB}$ transport by MRP1-transfected vesicles ($P = 0.001$), whereas no significant inhibition was observed in MRP2-transfected or wild-type samples. These results confirm that the ATP-dependent transport of UCB is mediated by an MRP protein and that this transport is almost completely accounted for by MRP1.

Effect of osmotically induced changes in vesicular volume

Transport of $[^3\text{H}]\text{UCB}$ was linearly correlated with the reciprocal of the osmolarity (1/osM) of the outer medium ($y = 10631x + 16.1$, $r^2 = 0.90$, $P < 0.001$; Figure 4), indicating that UCB uptake occurred into an osmotically sensitive space. By extrapolating to infinite sucrose concentration (1/osM = 0), theoretically representing zero intravesicular space, it could be calculated that, at physiological iso-osmolarity (250 mM sucrose), less than 25% of UCB is adsorbed on the vesicles and the remaining 75% is transported into the vesicle interior.

Kinetics of $[^3\text{H}]\text{UCB}$ transport

ATP-dependent transport (60 s) of $[^3\text{H}]\text{UCB}$ by vesicles from MRP1-transfected MDCKII 108 cells showed saturative kinetics over the range of B_F values below aqueous saturation (not shown). In contrast, vesicles from wild-type cells showed a linear relationship ($y = 0.10B_F + 0.01$, $r^2 = 0.99$) between transport and B_F (not shown). Figure 5 shows that the difference between these values, representing MRP1-dependent transport, also followed saturative kinetics. From the double-reciprocal Lineweaver–Burk plot (Figure 5, inset), $K_m = 10 \pm 3$ nM (B_F) and $V_{\max} = 100 \pm 13$ pmol $\cdot$ min $^{-1}$ $\cdot$ (mg of protein) $^{-1}$ were derived for the MRP1-dependent transport of $[^3\text{H}]\text{UCB}$.

Transport of leukotriene and its inhibition by UCB

To study further whether UCB is a substrate for MRP1, we measured the effect of UCB on the ATP-dependent transport of $[^3\text{H}]\text{LTC}_4$, the substrate with the highest known affinity for MRP1

Figure 5 Kinetics of MRP1-dependent transport of [^3H]UCB

MRP1-dependent transport was calculated as the difference between the ATP-dependent transport of [^3H]UCB at 60 s measured in plasma-membrane vesicles from MRP1-transfected cells versus wild-type MDCKII cells. At B_F varying from 0 to 72 nM in the presence of 60 μM HSA, 3 mM ATP and 1.5 mM GSH, MRP1-dependent [^3H]UCB transport was saturative. Inset: Lineweaver-Burk plot of the MRP1-dependent transport, from which $K_m = 10$ nM and $V_{\max} = 100$ pmol of UCB $\cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ were determined (the point at 72 nM, which is above the aqueous saturation of UCB, was excluded).

[11]. Transport was assessed in the presence of GSH (1.5 mM) and ATP (3 mM) in vesicles from both MRP1-transfected MDCKII cells and wild-type MDCKII cells, and the difference was considered to be the MRP1-dependent transport. The addition of 100 nM unlabelled UCB reduced the transport of 150 nM LTC4 from 8.1 ± 0.7 to 2.7 ± 0.25 pmol $\cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ ($P = 0.0002$), which is consistent with the hypothesis that the two substrates share a common MRP1-mediated transport mechanism.

DISCUSSION

The marked enhancement of UCB transport by vesicles from MDCKII cells, transfected to overexpress MRP1, proves directly, for the first time, that UCB is a substrate for MRP1. It was also shown that this transport is strictly dependent on the presence of ATP and GSH. The marked inhibition by UCB of the MRP1-dependent transport of LTC4, a known high-affinity substrate of MRP1, further supports a role for this ABC protein in the transport of UCB. These results extend previous indirect evidence obtained in yeast [21], BeWo cells [23] and cultured glial cells [24] for a role of MRP1 in UCB transport.

The MRP1-mediated transport of several unconjugated amphipathic substrates is linked to the presence of GSH, which modulates the affinity of the transporter for the substrate [5,9,16]. In GSH-depleted cells, cellular efflux of a number of antimitotic drugs is almost undetectable, increasing their intracellular concentration [4,7]. Our studies show that GSH is also critically involved in the transport of UCB by MRP1 (Figure 1). Thus a reduction in intracellular concentration of GSH due to oxidative stress [35] might decrease UCB efflux. Owing to the antioxidant properties of UCB at low concentrations [36], the resultant retention of UCB could help to counteract the cellular oxidative stress. In cells that are more severely damaged, a greater accumulation of UCB could promote apoptotic removal of these cells [37].

MK571 is considered as the most effective and specific inhibitor of MRPs generally, but is not specific for MRP1 [4,7,16,23,38,39]. Thus the inhibition of ATP-dependent UCB transport by MK571 is not, in itself, proof that MRP1 is involved. However, MK571 inhibited ATP-dependent UCB transport in vesicles from cells transfected with MRP1 but not MRP2 (Figure 3B). In addition, UCB transport was similar in plasma-membrane vesicles obtained from both MRP2-transfected and wild-type cells (Figure 3B), even though the MRP2 in transfected vesicles was functionally active in ATP-dependent transport of LTC4. This is in agreement with our previous evidence that ATP-dependent transport of UCB is not impaired in canalicular membrane vesicles from TR $^-$ /GY mutant rats that lack Mrp2 [30]. Collectively, these results indicate that MRP1/Mrp1, but not MRP2/Mrp2, is involved in the ATP-dependent transport of UCB.

It has been reported that Mdr1a, one of the two rodent homologues of MDR1, transports UCB [40], thus protecting the brain against accumulation of UCB [40–42]. However, the physiological relevance of these studies must be questioned, since they were conducted with incubation media or infusates containing UCB at concentrations significantly above its aqueous solubility [43].

UCB has been shown to be a potent antioxidant [36], but is toxic when its free concentration exceeds its aqueous solubility limit (70 nM) [18]. *In vitro* studies conducted at relevant B_F levels revealed that cultured neurons and astrocytes suffer both plasma-membrane and mitochondrial damage at B_F levels slightly to moderately above the solubility limit [18]. In contrast, exposure to UCB at B_F levels below 70 nM protects cultured neurons and their mitochondria against oxidative stress induced by hydrogen peroxide; this protection is diminished at higher B_F values, where the toxic effects of UCB became dominant [44]. Therefore it is expected that the physiological intracellular concentration of UCB should be kept low enough for its antioxidant effect to predominate. These findings suggest that, to prevent cytotoxicity, there should be a transport system that mediates efflux of excess of UCB and thus maintains a low physiological intracellular concentration of the pigment.

The ubiquitous presence of MRP1 in many cells [1], coupled with the demonstration that this ABC protein may export UCB from the cell, suggests that this transporter may function throughout the organism to protect cells against accumulation of toxic levels of UCB. The high affinity of MRP1 for UCB ($K_m \approx 10$ nM) is in line with this conclusion, since the intracellular B_F is probably no higher than that in plasma [33,45], although this has not been measured directly. Evidence for a role of MRP1 in the regulation of intracellular levels of UCB has been reported previously from our laboratory. A high correlation between UCB export and MRP1 expression in BeWo cells favoured the hypothesis of involvement of MRP1 in cellular export of UCB [23]. Additionally, in rats with increased bilirubin levels due to phenylhydrazine-induced haemolysis, gene and protein expressions of Mrp1 were increased in both the liver and spleen [46]. In cultured mouse astrocytes, expression of Mrp1 was up-regulated by exposure to UCB *in vitro*, accompanied by translocation of Mrp1 from the Golgi to the plasma membrane [24]. Thus, besides Mrp1 probably limiting intracellular accumulation of UCB, UCB also amplifies the protective effect of this transporter by enhancing its expression. Enhancing the activity of MRP1, therefore, represents a potential therapeutic approach to protect against bilirubin neurotoxicity in severely jaundiced newborns [47]. Transport of UCB by other MRPs, such as the newly discovered MRP4 [48], or other ABC proteins, such as MDR1 [40], needs to be further explored using clinically relevant concentrations of UCB [49]. To date, however, the present study with MRP1 constitutes the only direct

evidence for the transport of UCB by a human ABC protein, and unequivocally demonstrates the role of human MRP1 in exporting UCB from cells.

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