Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues

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Multidrug resistance protein 4 (MRP4/ABCC4) is a member of the MRP subfamily, which in turn is a member of the superfamily of ATP-binding-cassette (ABC) transporters. Within the MRP subfamily, ABCC4, ABCC5 (MRP5), ABCC11 (MRP8) and ABCC12 (MRP9) have similar predicted membrane topologies. All lack the additional transmembrane domain, TMD_0, which is present in the other MRPs. Using cells stably overexpressing ABCC4, this study shows that ABCC4 exports GSH. ABCC4 also facilitates the efflux of cAMP. Depletion of intracellular GSH with DL-buthionine-(*S*,*R*)-sulphoximine led to decreased export of cAMP and a corresponding increase in intracellular cAMP was observed. ABCC4 also mediates resistance to purine analogues 9-(2-phosphonylmethoxyethyl)-adenine and 6-thioguanine. This resistance can be reversed by the presence of DL-buthionine-(*S*,*R*)-sulphoximine. We conclude that as well as nucleotide and nucleoside analogues, ABCC4 can mediate the export of GSH. In addition, GSH plays an important role in the function of ABCC4. Depletion of intracellular GSH adversely affects the export of cAMP by ABCC4. Resistance to nucleoside analogues is also adversely affected by depletion of cellular GSH.

Key words: ATP-binding cassette, cyclic nucleotides, glutathione *S*-conjugate export pump, multispecific organic anion transporter B.

INTRODUCTION

The multidrug resistance proteins (MRPs) belong to a large superfamily of transport proteins know as the ATP-binding cassette (ABC) proteins [1]. In eukaryotes, ABC proteins typically contain two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). Half molecules with one TMD and one NBD also exist [1]. The NBD contains two conserved motifs: the Walker A and B motifs separated by 90–120 amino acid residues. A third conserved sequence, known as the 'Signature' or 'C' motif is present just upstream of the Walker B motif [2,3]. Functionally, all ABC transporters utilize ATP hydrolysis to energize export of a large variety of molecules. The MRP–cystic fibrosis transmembrane conductance regulator subfamily is currently the largest in the human ABC transporter family. Members of this family (subfamily C) include nine MRP proteins (ABCC1–6 and ABCC10–12), the sulphonylurea receptors SUR1 (ABCC8) and SUR2 (ABCC9), and the cystic fibrosis transmembrane conductance regulator [1,4–6]. Several members of this subfamily also contain a third N-terminal TMD, which is known as TMD_0. This is linked to the remaining portion of the transporter by a hydrophilic linker, known as L_0. Among the nine MRP proteins, ABCC4, ABCC5, ABCC11 and ABCC12 do not contain the TMD_0 [1,4–6].

ABCC1 (MRP1) was first cloned in 1992 [7]. Following the cloning of ABCC1, eight new MRP proteins were cloned [6,8–12]. Much work on the functional characterization and the drug-resistance profiles has been carried out on ABCC1 and ABCC2. ABCC1 can confer resistance to a diverse variety of anticancer drugs including anthracyclines, vinca alkaloids and epipodophyllotoxins [5,13,14]. Subsequent work demonstrated that ABCC1 is capable of transporting diverse organic anions including leukotriene C_4 and other glutathione-derived S-conjugates, glucuronide- and sulphate-conjugates [15–18]. ABCC2 (MRP2) is also known as the canalicular multispecific organic anion transporter. Its physiological function is to facilitate bilirubin glucuronide secretion from hepatocytes into the bile [17]. ABCC2 has been shown to transport a similar range of organic anions as ABCC1, but with different affinity for ABCC1 [18]. Overexpression of ABCC2 also confers resistance to a variety of carcinostatic agents [19–22].

Three other members of the MRP subfamily, ABCC3 (MRP3), ABCC6 (MRP6) and ABCC10 (MRP7), have similar predicted membrane topologies as ABCC1 and ABCC2 [10–12]. ABCC3 also functions as an organic anion transporter. ABCC3 can transport glucuronide conjugates and glutathione-*S*-conjugates and has the additional ability of mediating the transport of monoanionic bile salts [23,24]. However, the drug-resistance profile for ABCC3 is not well characterized. In contrast, none of the typical organic anionic conjugates were transported by ABCC6. To date, ABCC6 has been shown to only transport the anionic cyclopentapeptide, cyclo-(D-Trp-D-Asp-L-Pro-D-Val-L-Leu) ('BQ-123') [25]. ABCC10 is a recent addition to the family and has not yet been characterized.

Within the MRP subfamily, ABCC4 (MRP4), ABCC5 (MRP5), ABCC11 (MRP8) and ABCC12 (MRP9) are unique. All lack the TMD_0 domain but retain the L_0 linker [1,4–6,9,10]. ABCC4 and ABCC5 share less than 40% similarity with the other MRP proteins. Both proteins also share less than 40% similarity with each other. Nevertheless, ABCC4 and ABCC5 are much more similar to the other MRPs than to other members of the ABC superfamily [1,4–6,9,10].

Abbreviations used: ABC transporter, ATP-binding-cassette transporter; BSO, DL-buthionine-(*S,R*)-sulphoximine; Hep G2 cells, hepatocellular carcinoma cells; HBSS, Hanks balanced salt solution; MRP, multidrug resistance protein; MDCKII cells, Madin–Darby canine kidney II cells; MTS/PES, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H tetrazolium/phenazine ethosulphate; NBD, nucleotide-binding domain; PMEA, 9-(2-phosphonylmethoxyethyl)-adenine; RT-PCR, reverse transcriptase PCR; TMD, transmembrane domain.

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Unlike ABCC1 and ABCC2, ABCC4 and ABCC5 do not confer resistance against anthracyclines, vinca alkaloids or epipodophyllotoxins. However, ABCC4- and ABCC5-mediated resistance to purine analogues and other nucleoside-based antiviral drugs have been reported [26–28]. Subsequent studies by Jedlitschky et al. [29] showed that ABCC5 functions as an ATP-dependent export pump for cAMP and cGMP. Chen et al. [30] also recently showed that ABCC4 like ABCC5 mediate the efflux of cyclic nucleotides. Thus ABCC4 and ABCC5 function as nucleotide analogue pumps.

In the present study, we stably expressed the ABCC4 cDNA in Hep G2 cells and in Madin–Darby canine kidney II (MDCKII) cells. The results showed that ABCC4 is expressed in the basolateral membrane of MDCKII cells. Cells transfected with ABCC4 cDNA can secrete GSH. Depletion of intracellular GSH adversely affected the export of cAMP by ABCC4 and reversed ABCC4-mediated resistance to nucleoside analogues.

MATERIALS AND METHODS

Chemicals

All cell culture reagents were purchased from Life Technologies (Carlsbad, CA, U.S.A.), with the exception of glutamine and penicillin/streptomycin which were obtained from Sigma (St. Louis, MO, U.S.A.). LIPOFECTAMINE™ and OPTI-MEM I reduced serum medium were also from Life Technologies. All molecular biology reagents were obtained from New England Biolabs (Beverly, MA, U.S.A.). The pBluescript vector was from Strategene (La Jolla, CA, U.S.A.), whereas pcDNA6, the anti-V5 monoclonal antibody, and blasticidin were purchased from Invitrogen (Carlsbad, CA, U.S.A.). The FITC-conjugated anti-mouse antibody was purchased from DAKO (Copenhagen, Denmark). DL-Buthionine-(*S*,*R*)-sulphoximine (BSO) and 6-thioguanine were purchased from Sigma. 9-(2-phosphonylmethoxyethyl)-adenine (PMEA) was obtained from Moravek Biochemical (Brea, CA, U.S.A.), and forskolin was from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.).

Cell lines and cell culture

The Hep G2 and MDCKII cell lines used in this study were grown in complete medium consisting of Dulbecco's modified Eagle's medium, 1 mM sodium pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % fetal bovine serum. The cells were grown at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2.

Cloning of human ABCC4 cDNA

Total RNA was extracted from Hep G2 cells using the RNeasy Midi Kit (Qiagen, Hilden, Germany). The extraction was carried out according to the manufacturer's instructions. The full-length human ABCC4 cDNA was obtained with reverse transcriptase (RT)-PCR using total RNA isolated from Hep G2 and ABCC4 primers based on the published ABCC4 sequence (GenBank accession number AF071202, [9]). Three pairs of primers were used to generate three ABCC4 fragments: I, II and III. The sequence of the primers for fragment I were 5′-TGCGAATTCATGCTGCCCGTGTACCA-3′ (forward) and 5′-TGTGATATCTCATCAAGTA-3′ (reverse); for fragment II 5′-TGAGATATCACAGCGCAA-3′ (forward) and 5′-AGAGTTAACAAGGACGTAGA-3′ (reverse); and for fragment III 5′-CTTGTTAACTCTTCACAA-3′ (forward), and 5′-TGTCTCGAGCAGTGCTGTCTCGAA-3′ (reverse).

RT-PCR was carried out using the Access RT-PCR kit (Promega, Madison, WI, U.S.A.). Reverse transcription was carried out at 48 °C for 45 min followed by a denaturation step at 94 °C for 2 min. Following reverse transcription, the cDNA was subjected directly to PCR. Cycles (40) of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 68 °C for 2.5 min were carried out. A final extension at 68 °C was carried out for 9 min. The fragments were first cloned in pGEM-T vector and then subcloned into pBlueScript (pBS) vector to obtain the full-length MRP4 cDNA using the restriction sites *Eco*R1 and *Eco*RV (fragment I), *Eco*RV and *Hinc*II (fragment II), and *Hinc*II and *Xho*I (fragment III) sequentially. pBS–ABCC4 was then sequenced.

The translated protein sequence was identical with the published sequence (GenBank accession number AF071202), except for changes at position 18 (Ile to Leu), position 637 (Glu to Gly), position 664 (Ser to Phe), position 1103 (Ile to Val), position 1139 (Lys to Asn) and position 1289 (Lys to Arg). Of these, the mutation at position 1139 (Lys to Asn) has been reported (GenBank accession number XM_007206). All the amino acid changes were the result of single base changes within the codons. The changes at positions 18, 1103, 1139 and 1289 were all conservative substitutions.

The full-length ABCC4 cDNA was then cloned into pcDNA6/V5-His vector to generate the recombinant vector pcDNA6–ABCC4. The pcDNA6/V5-HisA vector was used to ensure the ABCC4 open reading frame was in frame with the V5 epitope and the polyhistidine sequence, which were located at the C-terminal end of the ABCC4 protein.

Transfection and selection of stable clones

The pcDNA6–ABCC4 construct was transfected into Hep G2 cell and MDCKII cells. Hep G2 or MDCKII cells were seeded into six-well plates 1 day before transfection. The number of cells seeded per well was 3×10^5. Transfection was done using LIPOFECTAMINE™ reagent. DNA (2 μg) was dissolved in 100 μl of OPTI-MEM I reduced serum medium. For each transfection, 6 μl of LIPOFECTAMINE™ reagent was diluted into 100 μl of OPTI-MEM medium. This diluted LIPOFECTAMINE™ reagent was then mixed with the DNA. The combined solutions were mixed gently and incubated at 24 °C for 45 min to allow DNA–liposome complexes to form. During the incubation, the cells in the six-well plates were washed twice with the OPTI-MEM medium. After washing, 0.8 ml of OPTI-MEM medium was added to each well. The DNA–liposome mixture was then added. The cells were then incubated for 5 h in a 95 % air/5 % CO_2 incubator at 37 °C. After 5 h, 1 ml of complete medium was added into each well and the cells were incubated overnight at 37 °C. The cells were then washed with PBS (containing 0.14 g/l KH_2PO_4, 9.0 g/l NaCl and 0.8 g/l Na_2HPO_4, pH 7.4) and fresh complete medium was added. Blasticidin (0.25 μg/ml) was added to the medium for selection 48 h after the start of transfection. When single colonies were observed, they were picked and subcultured. The colonies were expanded and two ABCC4 clones, ABCC4/Hep G2 and ABCC4/MDCKII were used for subsequent assays and analysis.

Immunoblotting

Immunoblotting was performed with cell lysates. The cells were trypsinized, and resuspended with 10 ml PSB. The samples were then centrifuged at 1000 ***g*** for 5 min, the supernatant was removed and the cells were resuspended in 200 μl PBS. Samples were separated by SDS/PAGE and transferred on to nitro-

cellulose membrane. An anti-V5 monoclonal antibody was used to detect the V5 epitope. The bound antibodies were visualized by enhanced chemiluminescence (ECL®; Amersham International).

Cellular localization of ABCC4

The ABCC4/Hep G2 cells were grown on glass cover slips, whereas the ABCC4/MDCKII cells were plated on membrane inserts (0.4 μm pore size, 24 mm diameter; Corning Costar) and grown for 3 days to allow the cells to polarize. Cells were fixed with 4% paraformaldehyde for 30 min. This was followed by treatment with a solution containing 0.5% (v/v) Triton X-100, 1% serum, 2.5% skimmed milk and PBS for 15 min to permeablize the fixed cells. The cells were then stained with an anti-V5 antibody. After 16 h, the permeabilized cells were incubated with the secondary antibody for 2 h. The secondary antibody used was a FITC-conjugated anti-mouse antibody. After each step, cells were washed thoroughly with PBS before application of the next reagent. The cells were then counterstained with 20 μg/ml propidium iodide solution for 5 min and then mounted onto glass slides. The cells were viewed with a confocal microscope. The FITC-conjugated secondary antibody has an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

Cytotoxicity assay

All assays were performed in 96-well tissue culture plates. In each well, 2.5×10^3 cells were seeded in a volume of 100 μl of complete medium. After 24 h, PMEA or 6-thioguanine was added at various concentrations. Controls consisted of cells incubated with the respective solvents for each of the drugs used. After 48 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H tetrazolium/phenazine ethosulphate reagent (MTS/PES; supplied as the CellTiter 96 Aqueous One Solution Cell Proliferation Assay from Promega) was added to each well. After incubation at 37 °C for 30 min, the absorbance at 490 nm was measured. All assays were performed in triplicate. The results were used to calculate the IC_{50} of the various compounds. At least five drug concentrations were used to determine IC_{50} values.

In order to determine the effect of BSO on cytotoxic resistance, 2.5×10^3 cells were seeded in each well and allowed to recover for 24 h. The medium was then changed to medium containing 0.5 mM BSO and PMEA or 6-thioguanine, and the cells were incubated at 37 °C for 24 h. The medium was then changed to medium containing PMEA or 6-thioguanine, and the cells were allowed to grow for another 24 h. CellTiter 96 Aqueous One Solution Reagent (20 μl) was then added to each well, and the cells were incubated at 37 °C for another 30 min. Absorbance at 490 nm was then measured.

GSH-efflux assay

Cells were harvested from 75 cm² flasks using trypsin and centrifuged briefly at 1000 ***g*** for 5 min. The cells were washed twice with 10 ml of Hanks balanced salt solution (HBSS, containing 5.8 mM K^+, 143 mM Na^+, 1.3 mM Ca^{++}, 0.8 mM Mg^{2+}, 146 mM Cl^-, 0.8 mM phosphate, 4.2 mM HCO_3^- and 10 mM Hepes, pH 7.4)/0.5 mM acivicin, and resuspended in 1 ml of HBSS/5.6 mM glucose containing 0.5 mM acivicin. Samples of the suspensions (300 μl) were taken out and used for GSH-efflux assay. The cells were incubated in a 37 °C water bath with shaking for 100 min. After 100 min, cell viability was determined by Trypan Blue staining. The cells were then centrifuged at 2000 ***g*** for 2 min. The supernatant was collected and the pellet was washed twice and resuspended in 150 μl of PBS containing 1% SDS.

GSH measurement was carried out as described by Fernandez-Checa and Kaplowitz [31] with modifications. This assay was chosen as it measured specifically GSH and has been shown to be comparable with the recycling assay. In essence, GSH measurement was carried out using a modification of the reaction between MCB and GSH catalysed by GST that was partially purified from rat liver (B. G. Lim and K. P. Wong, Department of Biochemistry, National University of Singapore, unpublished work).

Efflux of cyclic AMP

Cells (1×10^4) were seeded into each well of the 96-well plate, and incubated overnight at 37 °C. The cells were then incubated in complete medium containing 5 mM forskolin at 37 °C for 60 min. The cellular and extracellular cAMP levels were determined using the cAMP enzyme immunoassay system (Amersham Pharmacia Biotech).

In order to determine the effects of BSO on efflux of cAMP, 5×10^3 cells were seeded per well and allowed to recover for 24 h. The medium was then removed and fresh medium containing 0.5 mM BSO was added. After 24 h, forskolin was added and the cAMP levels were determined as described above. Cell viability (by Trypan Blue staining) and cell number were determined at the end of the 60 min incubation period.

RESULTS

Expression and localization of ABCC4 in Hep G2 and MDCKII cells

The full-length ABCC4 cDNA was obtained as described in the Materials and methods section. Two stable clonal cell lines

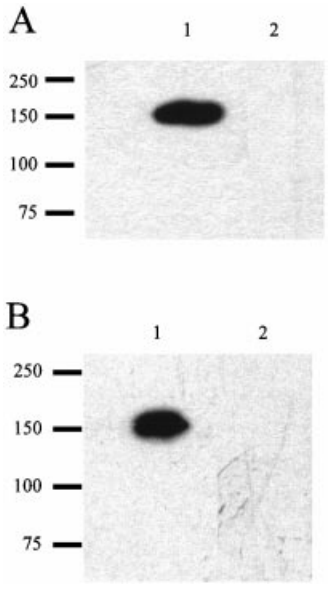

Figure 1 Expression of ABCC4

Western-blot analysis of ABCC4 expression in (**A**) Hep G2 cells and (**B**) MDCKII cells. Lane 1 shows the ABCC4-expressing clone and lane 2 shows the control clone. The molecular mass (kDa) is indicated.

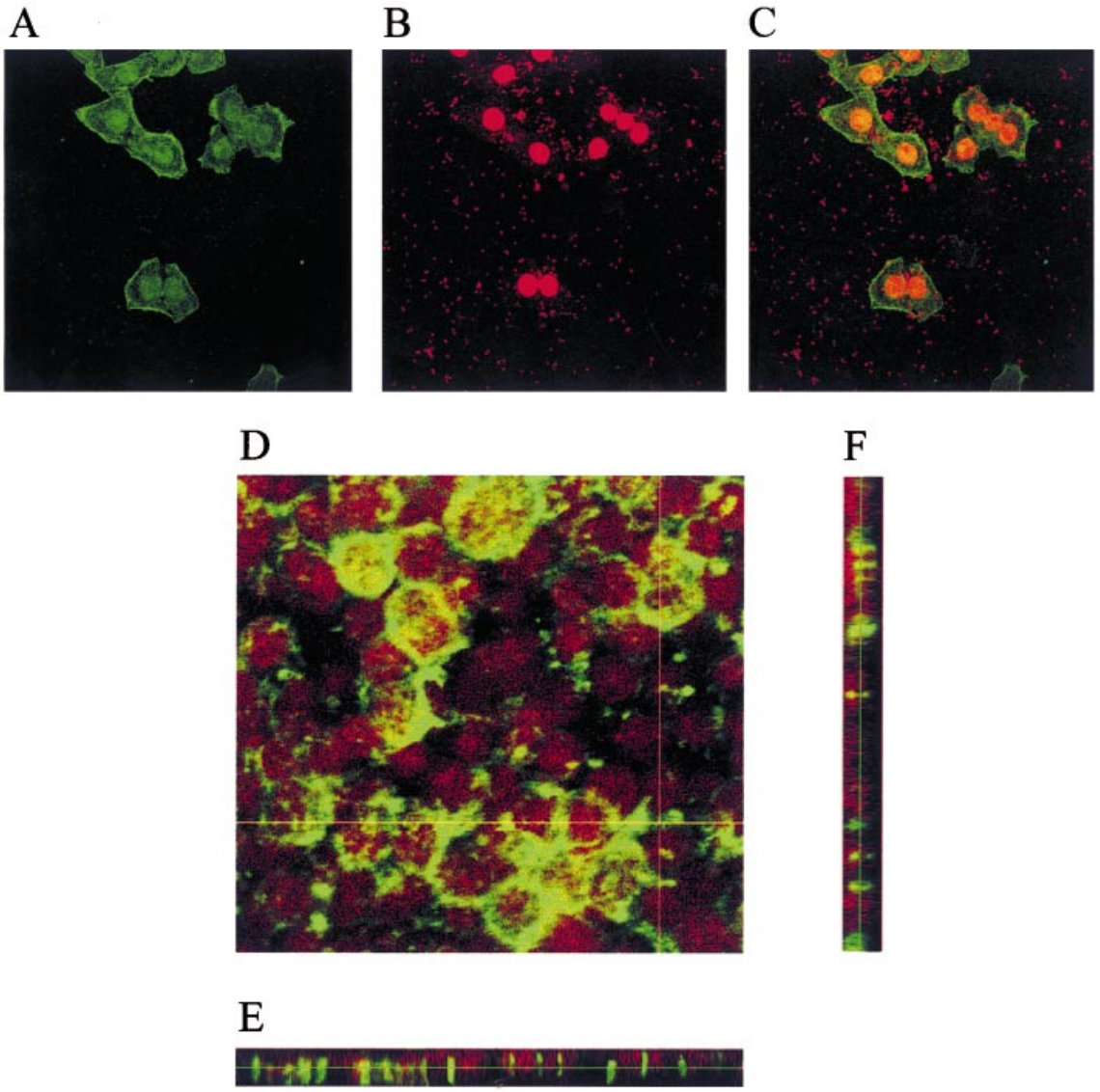

Figure 2 Immunostaining of Hep G2 cells (A–C) and MDCKII cells (D–F) overexpressing ABCC4

(**A**) ABCC4 expression in Hep G2 cells was detected by indirect immunofluorescence with an anti-V5 antibody (green). (**B**) Nucleic acids were counterstained with propidium iodide (red). (**C**) Both signals (from **A** and **B**) were superimposed, showing the membrane localization of ABCC4. (**D**–**F**) ABCC4 expression in MDCKII monolayer was detected by indirect immunofluorescence and confocal laser scanning microscopy using an anti-V5 antibody (green). (**D**) shows the top view of a monolayer, (**E**) the vertical *x*/*z* section and (**F**) the *y*/*z* section. The lines indicate the positions where the sections were made. Nucleic acids were counterstained with propidium iodide (red).

ABCC4/Hep G2 and ABCC4/MDCKII were established after transfection of Hep G2 cells and MDCKII cells with the ABCC4-vector construct, followed by selection with blasticidin. Two control blasticidin clones, v/Hep G2 and v/MDCKII were also obtained from transfection with the parental vector, pcDNA6.

Expression of ABCC4 was first analysed using immunoblotting. ABCC4 was cloned in frame with a V5 epitope and a polyhistidine sequence at the C-terminal. The predicted mass of ABCC4 is approx. 149 kDa, whereas that with the V5 and polyhistidine sequence is approx. 153 kDa. Using an anti-V5 antibody, a protein of approx. 150 kDa was detected in both ABCC4/Hep G2 and ABCC4/MDCKII cell (Figure 1), but not in v/Hep G2 nor in v/MDCKII cells. Immunostaining of both ABCC4/Hep G2 and ABCC4/MDCKII cells showed that ABCC4 was localized to the plasma membrane (Figures 2A–2C). Further analysis using immunostaining of ABCC4/MDCKII cells by confocal microscopy showed that ABCC4 was localized to the basolateral membrane (Figures 2D–2F). This is similar to the observations made in the basal cells of the prostatic glandular epithelium. In these cells, ABCC4 was localized in the basal and lateral portions of the plasma membrane [27].

Excretion of GSH from ABCC4-overexpressing cells

Cells transfected with the ABCC1, ABCC2 or ABCC5 gene constructs secrete GSH and cannot maintain intracellular GSH levels in simple salt media. This was also observed for the ABCC4-transfected Hep G2 cells. The amount of GSH excreted by ABCC4/Hep G2 cells incubated in HBSS/glucose containing 0.5 mM acivicin was 2.67 ± 0.18 pmol/10^4 cells per min compared

Table 1 GSH-efflux rates and intracellular GSH levels in Hep G2 cells, in Hep G2 cells stably expressing ABCC4 and in Hep G2 blasticidin clone (v/Hep G2)

Intracellular and extracellular GSH was determined at the end of the experiment (100 min) and the results are expressed as means ± S.D. *Compared with Hep G2 and v/Hep G2 cells, $P < 0.05$, ANOVA analysis.

Cell line	GSH efflux (pmol/min per 10^4 cells)	Intracellular GSH (nmol/10^4 cells)
Hep G2	0.96 ± 0.16	1.22 ± 0.14
v/Hep G2	0.53 ± 0.18	1.35 ± 0.12
ABCC4/Hep G2	2.67 ± 0.18*	0.38 ± 0.06*

Table 2 Drug sensitivity of ABCC4-transfected cells to purine analogues

IC_{50} is the concentration at which cell growth is inhibited by 50%. At least five drug concentrations were used to determine IC_{50} values. Experiments at each concentration were carried out in triplicate.

Cell line	IC_{50} for PMEA (μM)	IC_{50} for 6-thioguanine (μM)
Hep G2	173.5	12.8
v/Hep G2	215.2	12.2
ABCC4/Hep G2	424.5	28.1
MDCKII	217.4	28.9
v/MDCKII	194.0	26.6
ABCC4/MDCKII	398.0	62.8

Table 3 Effect of BSO on drug sensitivity

v/Hep G2 and ABCC4/Hep G2 cells were incubated with and without BSO, as well as in the absence or presence of 200 μM PMEA, 400 μM PMEA or 20 μM 6-thioguanine. The viability of the cells was determined using the MTS/PES assay. *Compared with similarly treated v/Hep G2 cells, $P < 0.01$, ANOVA analysis. †Compared with ABCC4/Hep G2 cells treated with drug only, $P < 0.05$, ANOVA analysis.

	Percentage A_{490} compared with control	
Treatment	v/Hep G2	ABCC4/Hep G2
BSO only	94.49 ± 2.48	97.53 ± 3.73
200 μM PMEA	63.68 ± 3.78	88.08 ± 5.22*
200 μM PMEA/BSO	57.38 ± 3.15	62.27 ± 3.83†
400 μM PMEA	23.86 ± 2.26	66.21 ± 5.59*
400 μM PMEA/BSO	24.39 ± 9.48	39.51 ± 1.72†
20 μM 6-thioguanine	37.11 ± 3.53	65.10 ± 7.54*
20 μM 6-thioguanine/BSO	41.04 ± 3.98	47.11 ± 7.07†

with 0.53 ± 0.18 pmol/10^4cells per min in v/Hep G2 cells. Intracellular GSH was also significantly reduced in ABCC4 overproducing cells (Table 1). Cell viability as determined by Trypan Blue staining was not affected in these experiments.

Cytotoxic assays and the effect of BSO

ABCC4 and ABCC5 have been shown to act as plasma membrane drug efflux pumps and can confer resistance to PMEA and 6-thioguanine. In the present study, resistance to PMEA and 6-thioguanine was observed in both ABCC4 clones (Table 2), but not against $CdCl_2$ (results not shown).

To examine the role of cellular GSH in ABCC4-mediated resistance, cellular GSH was depleted by treatment with 0.5 mM BSO, an inhibitor of γ-glutamylcysteine synthetase. It has been shown previously [32] that treatment of Hep G2 cells with 0.5 mM BSO led to a significant decrease in intracellular GSH. Cellular GSH levels were comparable with untreated v/Hep G2 and untreated ABCC4/Hep G2 cells. Following treatment with BSO, intracellular GSH levels decreased by 56%, from 1.00 ± 0.04 nmol/10^4 cells to 0.44 ± 0.09 nmol/10^4 cells in ABCC4/Hep G2 cells. Exposure of the cells to BSO did not affect the viability of the cells. However, the presence of BSO did lead to increased sensitivity of ABCC4/Hep G2 cells to PMEA and 6-thioguanine (Table 3). A corresponding decrease in cellular GSH was observed in BSO-treated v/Hep G2 cells (from 1.14 ± 0.22 nmol/10^4 cells to 0.57 ± 0.02 nmol/10^4 cells), but there was little change in the sensitivity of these cells to PMEA and 6-thioguanine (Table 3).

Export of cAMP by MRP4/Hep G2 cells

We next examined if cells that overproduce ABCC4 can effectively export cAMP. Forskolin, an activator of adenylate cyclase [33], was added to stimulate the synthesis of intracellular cAMP. Treatment with forskolin led to a significant increase in intracellular cAMP in both v/Hep G2 and ABCC4/Hep G2 cells (Table 4). However, increased extracellular cAMP was only observed in ABCC4/Hep G2 cells (Table 4). Pre-treatment of ABCC4/Hep G2 cells with BSO led to decreased extracelluar cAMP following stimulation by forskolin (Table 4). A corresponding increase in the intracellular cAMP of ABCC4/Hep G2 cells was also observed. As determined by Trypan Blue staining, cell viability was not affected by treatment with BSO and forskolin (results not shown).

DISCUSSION

The present study was carried out primarily to further characterize the function of ABCC4. Increased GSH secretion was observed in our ABCC4-transfected cells. This is in line with

Table 4 Effect of BSO on the export of cAMP

v/Hep G2 and ABCC4/Hep G2 cells were left untreated, incubated with the solvent (DMSO), incubated with forsoklin, or with forsoklin and BSO. The intracellular and extracelluar cAMP concentrations were determined. *Compared with the corresponding untreated cells, $P < 0.01$, ANOVA analysis. †Compared with ABCC4/Hep G2 cells treated with forskolin only, $P < 0.05$, ANOVA analysis.

	v/HepG2 cells		ABCC4/HepG2 cells	
Treatment	Intracellular cAMP (fmol/10^4 cells)	Extracellular cAMP (fmol/10^4 cells)	Intracellular cAMP (fmol/10^4 cells)	Extracellular cAMP (fmol/10^4 cells)
Untreated	14.91 ± 77.25	115.83 ± 30.88	46.49 ± 29.02	96.96 ± 51.79
DMSO	29.02 ± 120.21	111.61 ± 47.49	63.33 ± 41.48	194.99 ± 56.09
Forsoklin	3980.91 ± 677.57*	233.39 ± 16.08	1919.03 ± 362.26*	1683.52 ± 356.91*
Forsoklin/BSO	5669.84 ± 836.21*	149.52 ± 42.73	3102.81 ± 397.24*†	682.48 ± 148.47*†

observations that MRP proteins can facilitate the excretion of GSH from cells. Studies using membrane vesicles from ABCC1-overexpressing cells have shown that GSH is not a substrate for ABCC1 [34]. However, the export of GSH has been observed using intact cells. This has led to the suggestion that ABCC1-mediated export of GSH occurs only in association with unidentified endogenous factors present in intracellular compartments [35]. Cells expressing ABCC2 and ABCC5 have also been shown to excrete GSH [28,36].

ABCC1 is a glutathione-*S*-conjugate export pump and has been shown to transport glutathione-*S*-conjugates such as leukotriene C_4 and dinitrophenyl glutathione. However, ABCC1-overexpressing cells also exhibit decreased sensitivity to drugs that do not form glutathione-*S*-conjugates. It is now well established that ABCC1 transports vincristine and daunorubicin, not only in an ATP-dependent manner, but also in a GSH-dependent fashion [37,38]. Depletion of intracellular GSH resulted in a complete reversal of resistance to doxorubicin, daunorubicin, vincristine and VP-16 [39]. In addition, GSH can also enhance the ABCC1-mediated transport of glucuronosyl conjugates [40,41]. In the light of these observations, we examined the role of glutathione in ABCC4-mediated resistance to nucleoside analogues.

The two ABCC4 clones used in the present study also showed an increase in the resistance to PMEA and 6-thioguanine. This concurs with the observations that ABCC4 and ABCC5 were able to confer resistance to nucleoside analogues such as PMEA, azidothymidine, 6-thioguanine and 6-mercaptopurine [26–28,30]. Efflux of PMEA, AZT, 6-mercaptopurine or their metabolites from cells overexpressing ABCC4 and ABCC5 has been reported [28,30]. Analysis of the supernatant of HEK 293 cells over-producing ABCC5 loaded with 6-mercaptopurine showed that about 50 % of the excreted molecules is 6-mercaptopurine itself [28], indicating that ABCC5 also facilitates the export of 6-mercaptopurine. It has been reported that there was no uptake of 6-mercaptopurine using ABCC4-membrane vesicles [30]. The lack of uptake has been attributed to the need for cellular conversion of 6-mercaptopurine to di- and/or tri-phosphorylated metabolites. However, it may also be possible that this may not be the sole reason. Our results showed that depletion of intracellular GSH led to increased sensitivity to PMEA and 6-thioguanine. Thus the lack of uptake in membrane vesicles may be due to the absence of GSH and/or intracellular molecules.

To further examine if GSH has a role in the export of nucleotides, we examined the efflux of cAMP in BSO-treated and untreated cells. Stimulation of ABCC4-overexpressing cells with forskolin, an activator of adenylate cyclase, led to increased intracellular and extracellular levels of cAMP. Depletion of cellular GSH led to a decrease in extracellular cAMP with a corresponding increase in intracellular cAMP level. In control v/Hep G2 cells, only an increase in intracellular cAMP was observed and little extracellular cAMP was present. Compared with ABCC5, ABCC4 has a higher affinity for cAMP [30]. ABCC4 is not ubiquitously expressed in human tissues, but is highly expressed in the prostate. ABCC4 is also expressed in the testis and ovary [9]. In these organs, cAMP and cAMP-protein-dependent kinase are important molecules in mediating intracellular events [42], and ABCC4 may have a role in regulating the signalling events by regulating cAMP efflux. In addition, cAMP may also be involved in intercellular signalling. Such a role has been described and is particularly well characterized in the mold *Dictyostelium discoideum* [43,44]. However, little is know about the role of cAMP in intercellular signalling in mammalian systems.

The present study and those of others have shown that ABCC4 and ABCC5 are transporters for nucleotides and nucleoside analogues. The physiological contribution of these transporters to the regulation of cyclic nucleotide signalling will need to be further examined. In addition, nucleoside analogues are an important class of therapeutic agents. To date, the potential contribution of ABCC4 and ABCC5 to clinical resistance to nucleoside analogues remains uncharacterized.

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