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Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1

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Abstract The human multidrug resistance protein MRP1 mediates transport of organic substrates conjugated to glutathione, glucuronide, or sulfate. The naturally occurring prostaglandins A_1 and A_2 can form two diastereomeric glutathione *S*-conjugates, and it has been speculated that these might be substrates for MRP1. Here we present evidence that polarized MDCKII cells expressing *MRP1* cDNA transport PGA_1-GS to the basolateral side of a cell monolayer, in accordance with the lateral localization of human MRP1 in these cells. Furthermore, we show that vesicles made from yeast cells expressing *MRP1* cDNA and from mouse erythrocytes (known to contain mrp1) actively accumulate both diastereomers of PGA_2-GS with a similar efficiency. Recently, we generated mice with a homozygous mutant *mrp1* allele. Uptake of PGA_2-GS in vesicles made from erythrocytes of these mice was 3.2 times lower than in wild-type vesicles, but was still significantly above background. This residual transport activity was partly inhibited by methotrexate and cAMP, whereas mrp1-mediated activity was unaffected by these compounds. We conclude that mouse erythrocytes contain at least two transport systems for PGA_2-GS. One of these is mrp1; the other one has not been identified yet, but can be inhibited by methotrexate and cAMP.

Key words: Multidrug resistance; Prostaglandin; Glutathione *S*-conjugate; GS-X pump

1. Introduction

Membrane proteins belonging to the ATP binding cassette (ABC) family of transport proteins play a central role in the defense of cells against toxic compounds. After exposure of mammalian tumor cells to one single cytotoxic drug, these cells can become resistant against a whole range of drugs by actively lowering the intracellular drug concentration. This phenomenon is known as multidrug resistance (MDR). Two human members of the ABC family have been identified that can render mammalian tumor cells MDR: the MDR1 P-glycoprotein (Pgp [1,2]) and the multidrug resistance protein (MRP1 [3,4]). Both transporters were identified because of their overexpression in MDR tumor cell lines.

Whereas Pgp preferentially transports hydrophobic amphipathic compounds, it has been demonstrated in experiments with inside-out membrane vesicles that MRP1 is able to transport a range of substrates that are conjugated to glutathione (GSH), glucuronide, or sulfate [5–11]. Transporters with these latter substrate characteristics are known as GS-X pumps [12], multispecific organic anion transporters [13], or leukotriene C_4 (LTC_4) transporters [14]. The exact mechanism by which MRP1 transports anti-cancer drugs has not been elucidated yet, as for most of these drugs negatively charged conjugates are not known to exist. However, both in vitro and in vivo experiments have demonstrated that glutathione is required for the transport of these compounds [10,15,16].

The excretion of conjugates of endogenous and xenobiotic compounds is of importance in detoxification and cellular homeostasis. One of the endogenous GS-X conjugates is LTC_4, which belongs to the cysteinyl leukotrienes that play an important role in the inflammatory response [17]. As LTC_4 is a high affinity substrate for MRP1 [5] it was postulated that it might be one of the physiologically relevant MRP1 substrates. Indeed, Wijnholds et al. [18] have shown that mice homozygous for a *mrp1* mutant allele, $mrp1^{-/-}$ mice, have an impaired response to an LTC_4-mediated inflammatory stimulus.

Another set of naturally occurring molecules that are involved in the regulation of the mammalian defense system are the prostaglandins which belong to a class of the cyclic 20-carbon fatty acids that are synthesized from poly-unsaturated fatty acid precursors in response to external stimuli such as cell injury and inflammation. The prostaglandins of the A and J class are characterized by the presence of a reactive α,β-unsaturated ketone moiety in the cyclopentenone ring. Treatment of cells with these molecules leads to numerous effects like activation of heat shock transcription factor [19], inhibition of viral replication [20], inhibition of the cell cycle [21,22], and induction of apoptosis [23]. The α,β-unsaturated carbonyl group in PGA and PGJ is susceptible to Michael addition reactions with the thiol groups of molecules like glutathione, cysteine and proteins. It has been shown that the reaction between GSH and PGA occurs efficiently both chemically and by the mediation of glutathione *S*-transferases (GSTs [24–26]) and that two diastereomers can be formed [25]. Conjugation of prostaglandins with GSH may result in inhibition of their mode of action [27,28], and it has been speculated that these conjugates are substrates for MRP1 [12,26], but this has not been demonstrated directly. Recently, we have established assay systems to study some of the transport characteristics of MRP1 [11,18,29,30]. Here we show that PGA-GS is transported by MRP1 by in vitro uptake experiments with inside-out vesicles and by transport experiments with polarized MDCKII cells transduced with a retrovirus containing *MRP1* cDNA.

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2. Materials and methods

2.1. Materials

[5,6(*n*)-^{3}H]Prostaglandin E_1 (PGE_1; 43 Ci/mmol), and [^{14}C]ethacrynic acid (EA; 15 mCi/mmol) were obtained from Amersham International, Little Chalfont, UK. [^{35}S]Glutathione (GSH; 57.5 Ci/mmol) was obtained from Dupont, Den Bosch, The Netherlands. The radioactive conjugate of EA was synthesized from [^{14}C]EA and glutathione as described [11]. [^{3}H]PGA_1 was freshly prepared from [^{3}H]PGE_1 by acid-catalyzed dehydration as previously described [31]. The glutathione *R*- and *S*-diastereomers of PGA_2 were synthesized by incubating [^{35}S]GSH (0.5 mM) and PGA_2 (5 mM) in a 50 mM K-phosphate buffer (pH 8.0) for 60 min at 25°C. Purification of the conjugates was performed by HPLC using a Zorbax C18 (250×4.6 mm) column. The solvent program started isocratically at 1 ml/min with 50 mM NH_4Ac (pH 3.4), acetonitrile (75:25, v/v) for 30 min, followed by a linear gradient to 50% acetonitrile in 30 min. Peak detection was performed at 200 nm and radioactivity was detected using on-line radiochemical detection. Peak fractions were collected on CO_2(s) and freeze-dried. After freeze-drying the fractions were redissolved in TS buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.4) and stored at −20°C. Rechromatography of the purified products showed that they were stable under these conditions. The purity of the *R*- and *S*-stereoisomers was 99% and 91%, respectively.

Methotrexate (Emethexate) was from Pharmachemie (Haarlem, The Netherlands), and other chemicals were from Sigma, unless stated otherwise.

2.2. Cell lines

The kidney-derived MDCK cell line strain II (MDCKII [32]) was cultured in Dulbecco's modified Eagle medium with 10% fetal calf serum. A *SalI-NotI* DNA fragment containing the complete predicted MRP1 open reading frame (pJ3Ω-MRP1 [33]) was inserted in the retroviral vector pCMV-neo [34]. Transfection of the retroviral packaging cell line Phoenix [35] and subsequent transduction of the MDCKII cells were exactly as described [31].

2.3. Transport assays in MDCKII cells

Export of [^{3}H]PGA_1-GS in MDCKII cells was determined by washing cells in Hanks' balanced salt solution (HBSS; 5.6 mM glucose, 10 mM HEPES, 5.4 mM KCl, 143 mM NaCl, 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, pH 7.4). 2 ml HBSS containing 2 μM [^{3}H]PGA_1 was added to both sides of the monolayer and cells were incubated at 37°C. Samples (200 μl) were taken at various time points and 100 μl of 4% (v/v) formic acid was added. Subsequently samples were extracted twice with 300 μl ethyl acetate and radioactivity in 200 μl of the water phase was determined by liquid scintillation counting.

2.4. Preparation of membrane vesicles and vesicle uptake studies

Preparation of microsomal vesicles from DTY168 yeast cells transformed with *MRP1* cDNA was exactly as described [11,30]. Transport of [^{14}C]EA-GS and PGA_2-[^{35}S]GS was measured by the rapid filtration technique using nitrocellulose filters as described before [11,30]. Glutathione conjugates of PGA_1 and PGA_2 used in inhibition experiments (Table 1) were made by incubating a mixture of 1 mM prostaglandin and 2 mM GSH in 100 mM K-phosphate buffer (pH 7.5) for 2 h at 30°C. PGE_1, although not giving any GSH conjugate, was treated similarly for comparison.

A mixture of inside-out and inside-in vesicles was prepared from freshly isolated blood from wild-type and *mrp1*$^{-/-}$ mice as described [18,36]. Briefly, 4 mM EDTA was added to the blood samples and after centrifugation (1000×*g*, 10 min) erythrocytes were washed three times with buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl), and lysed in 10 mM Tris-HCl (pH 7.4), containing 1 mM PMSF, leupeptin (1 μg/ml, w/v), and aprotinin (1 μg/ml, w/v). Ghosts were centrifuged (20 000×*g*, 10 min), washed five times in 10 mM Tris-HCl (pH 7.4), and incubated in 1 mM Tris-HCl (pH 7.4) for 3 h. After centrifugation (100 000×*g*, 30 min, SW41 rotor), membranes were resuspended in 0.5 ml TS buffer. Vesicles were formed by passing the suspension five times through a 25 gauge needle. Vesicles were washed in TS buffer and resuspended in the same buffer at a protein concentration of approximately 4 μg/μl. After freezing in liquid nitrogen, membrane vesicles were stored at −80°C. Uptake of PGA_2-[^{35}S]GS diastereomers was measured by the rapid filtration method using nitrocellulose filters (0.45 μm pore size; Schleicher and Schuell). The reaction buffer was TS supplemented with 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg creatine kinase per ml, and the indicated amount of substrate. After equilibration at 37°C, 25–50 μg of vesicles were added. The final volume was 50 μl. Samples (20 μl) were taken at the indicated time points, diluted with 900 μl of ice-cold TS buffer, and applied to the presoaked membrane filters. Filters were rinsed with 7 ml of TS buffer. In control experiments ATP was omitted. Filters were placed in liquid scintillation fluid and radioactivity was determined by liquid scintillation counting.

3. Results

It has been shown before that PGA_1 is a potent inhibitor of MRP1 in intact S1 lung cancer cells transfected with *MRP1* cDNA, whereas PGE_1 is not [37]. We therefore examined whether prostaglandins or their GSH metabolites were able to inhibit the ATP-dependent transport mediated by MRP1 in vitro. As assay system we used microsomal vesicles isolated from *Saccharomyces cerevisiae* cells expressing human *MRP1* cDNA (strain DTY168(MRP1) [30]). This yeast strain has a disrupted *YCF1* gene [38] and therefore a very low endogenous level of GS-X pump activity [30,39]. As substrate we used the glutathione conjugate of ethacrynic acid ([^{14}C]EA-GS). EA-GS is a MRP1 substrate that gives low background binding to nitrocellulose filters in vesicle uptake studies and has a relatively low apparent K_m value (5 μM [11]). In uptake experiments with [^{14}C]EA-GS (0.2 μM) we did not observe any inhibition by either PGA_1, PGA_2 or PGE_1. In the presence of GSH, however, PGA_1 and PGA_2 were efficient inhibitors of [^{14}C]EA-GS transport, whereas PGE_1 was not (Table 1). This difference is most likely explained by the fact that the PGAs can form GSH conjugates, whereas PGE_1 cannot. Under the conditions employed, a hydrophilic conjugate between PGAs and GSH was formed as a reaction product partitioned in the aqueous layer after ethyl acetate extraction (data not shown). This product has been identified before as PGA-GS (see e.g. [24]).

To characterize the transport of PGA_2-GS in more detail

Table 1
Effect of prostaglandins and glutathione on ATP-dependent uptake of [^{14}C]EA-GS (0.2 μM) into microsomal vesicles from DTY168(MRP1) cells

Treatment			% of control
Control			100 ± 5
PGA1	+GSH	0.1 μM	99 ± 2
	+GSH	1 μM	65 ± 1
	+GSH	10 μM	15
	−GSH	10 μM	108 ± 3
PGA2	+GSH	0.1 μM	91 ± 3
	+GSH	1 μM	48 ± 2
	+GSH	10 μM	9
	−GSH	10 μM	104 ± 3
PGE1	+GSH	0.1 μM	98 ± 2
	+GSH	1 μM	100
	+GSH	10 μM	95 ± 3
	−GSH	10 μM	106 ± 1

DTY168(MRP1) vesicles were incubated at 25°C for 3 min. Prostaglandins and GSH were incubated as described in Section 2. Treatment '−GSH' indicates that prostaglandins were incubated in the absence of GSH. ATP-dependent uptake was calculated by subtracting values in the absence of ATP from values in the presence of ATP. In the control incubation uptake of [^{14}C]EA-GS was 249 ± 15 pmol/mg protein. Values are means of duplicate measurements ± the variation between measurements.

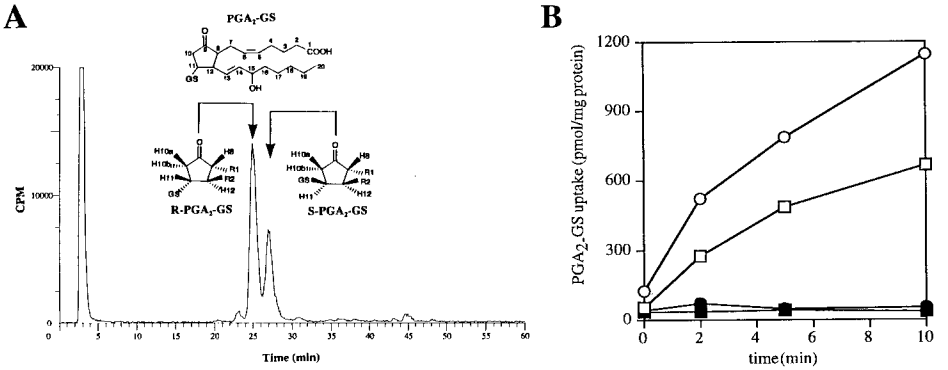

Fig. 1. A: Reversed phase HPLC radioactivity profile of the incubation of PGA_2 with glutathione. Incubation and elution were performed as described in Section 2. Structures of the PGA_2-GS diastereomers are indicated. B: Time-dependent PGA_2-[^{35}S]GS uptake in microsomal vesicles from DTY168(MRP1) cells. Vesicles were incubated in the presence of 3 μM of the *R*- (squares) or *S*- (circles) diastereomers. Samples were taken at $t = 0$, 2, 5, and 10 min. Open and solid symbols represent uptake in the presence and absence of ATP, respectively. Experiments were carried out in duplicate and variation was within the size of the symbols.

^{35}S-labeled conjugate was synthesized in vitro. During the reaction two diastereomers were formed, which could be separated from each other by HPLC (Fig. 1A). Vesicles of DTY168(MRP1) cells showed time- and ATP-dependent uptake of both diastereomers (Fig. 1B) with similar efficiency.

To further characterize the transport characteristics of both diastereomers of PGA_2-GS we analyzed transport in membrane vesicles prepared from mouse erythrocytes. Erythrocytes contain MRP1 [40] and by comparing erythrocyte inside-out plasma membrane vesicles prepared from wild-type and *mrp1*$^{-/-}$ mice we have shown recently that mrp1 is the high affinity EA-GS and dinitrophenyl glutathione (DNP-GS) transporter in the erythrocyte plasma membrane [18]. Comparison of time-dependent uptake of the PGA_2-GS diastereomers in vesicles made from wild-type and *mrp1*$^{-/-}$ mice showed that uptake was approximately 3.2 times higher in wild-type vesicles (Fig. 2A). Under the assay conditions employed uptake was linear with the protein concentration present in the assay (data not shown). The apparent K_m value for the ATP-dependent uptake in wild-type vesicles yielded a value of 0.8–1 μM for the *S*- and the *R*-diastereomers of PGA_2-GS (Fig. 2B). These results indicate that murine mrp1 transports PGA_2-GS in a non-stereoselective way.

Heasley and Brunton [24] have shown that the cAMP transport from pigeon erythrocytes is inhibited by PGA-GS and Saxena and Henderson [41] have described a transport activity in human erythrocyte membranes that might be involved in the transport of methotrexate (MTX). However, we have not been able to detect inhibition of MRP1-mediated transport by cAMP or MTX in in vitro uptake studies using membrane vesicles isolated from DTY168(MRP1) yeast cells (data not shown). Table 2 shows that MTX and cAMP partially inhibited uptake of a mixture of PGA_2-GS diastereomers in vesicles made from *mrp1*$^{-/-}$ mice, whereas the mrp1-mediated uptake was not significantly affected.

We and others have shown that MRP1 is present in the lateral plasma membrane in polarized cells [29,42]. Transport of substrates across the apical and basolateral plasma membrane can be distinguished by growing polarized cells as a monolayer on microporous membrane filters. On these filters, the physical barrier between the apical and basal compartment is formed by the tight junctions formed between the cells. In contrast to MRP1, one of the homologs of MRP1, MRP2 or cMOAT, is present in the apical membrane [31,43–45] and we have demonstrated that cMOAT is involved in transport of PGA_1-GS to the apical side of a monolayer of polarized MDCKII-cMOAT cells [31]. To investigate whether PGA_1-GS is a substrate for MRP1, we retrovirally transduced MDCKII cells with human *MRP1* cDNA. For detection of MRP1 we used the monoclonal antibody (mAb) MRPr1 [46]. Several clones were identified which contained substantial levels of MRP1 and subsequent immunolocalization experiments using confocal laser scanning microscopy demonstrated that MRP1 was present in the lateral plasma membrane in these cells, confirming the results with another kidney cell line of Evers et al. [29] (data not shown). To investigate whether PGA_1-GS was transported by MRP1, export was measured in MDCKII parental and MDCKII-MRP1 cells. Exported PGA_1-GS was separated from unconjugated PGA_1 by ethyl acetate extraction. By this method we formally cannot exclude that we also isolate other polar metabolites, e.g. PGA_1-*S*-Cys, which can be formed extracellularly by the mediation of γ-glutamyl transpeptidase. Fig. 3 shows that the parental cells transported comparable amounts of polar PGA_1 metabolites to both sides of the monolayer. In contrast, transport in the

Table 2
Effect of methotrexate and cAMP on ATP-dependent uptake of PGA2-[^{35}S]GS uptake into membrane vesicles from erythrocytes

Treatment		Vesicles	
		wild-type	mrp1$^{-/-}$
Control		100 ± 8	100 ± 5
Methotrexate	50 μM	nd	61 ± 15
	100 μM	107 ± 8	53 ± 7
	250 μM	nd	51 ± 6
	1 mM	90 ± 6	33 ± 20
cAMP	100 μM	114 ± 8	54 ± 5
	250 μM	nd	50 ± 11
	500 μM	118	50 ± 11

Plasma membrane vesicles from wild-type or mrp1$^{-/-}$ erythrocytes were incubated with a mixture of PGA_2-[^{35}S]GS diastereomers (8.5 μM) in the presence or absence of the indicated compounds. Vesicles (8 and 16 μg protein for wild-type and mrp1$^{-/-}$, respectively) were incubated at 37°C for 10 min. Relative transport rates (% of control) were calculated by subtracting the values in the absence of ATP from those in the presence of ATP. Means ± variation between duplicate measurements are shown. Experiments were repeated with two independent batches of vesicles and substrate. nd, not determined.

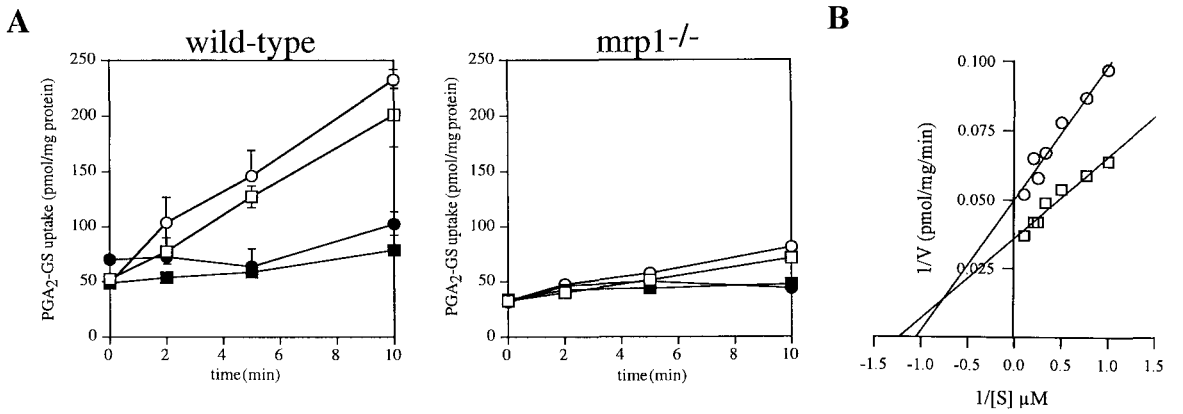

Fig. 2. A: Time course of uptake of PGA_2-[^{35}S]GS diastereomers into erythrocyte plasma membrane vesicles from wild type and *mrp1*$^{-/-}$ mice. Membrane vesicles from wild-type and mrp1$^{-/-}$ erythrocytes were incubated in the presence of 3.5 μM or 3.9 μM PGA_2-GS *R*- (squares) or *S*- (circles) form, respectively. Samples were taken at $t = 0$, 2, 5, and 10 min. Open and solid symbols represent uptake in the presence and absence of ATP, respectively. Experiments were carried out in duplicate and bars indicate variation between measurements. B: Lineweaver-Burk plots of the concentration dependence of uptake of PGA_2-[^{35}S]GS *R*- (squares) and *S*-form (circles). The concentration of substrate was in the range of 1–10 μM and the incubation time was 5 min. ATP-dependent uptake was calculated by subtracting values in the absence of ATP from those in the presence of ATP.

MDCKII-MRP1 cells was preferentially to the basolateral side of the monolayer, whereas transport to the apical side was strongly reduced. This basolateral transport activity is in accord with the lateral localization of MRP1 in these cells. Similar results were obtained with another MDCKII-MRP1 clone (data not shown).

4. Discussion

By using polarized cells expressing *MRP1* cDNA and in vitro uptake studies we show in this communication that MRP1 transports the glutathione *S*-conjugates of PGAs with a relatively high affinity (K_m 1 μM). Our data are in agreement with the previous hypothesis that MRP1 might transport these compounds [12,47]. Bogaards et al. [25] demonstrated that PGA-GS conjugates are formed both chemically and by the mediation of GSTs. Whereas it was observed by these authors that GSTs show a striking stereoselectivity for the formation of either the *S*- or the *R*-form of the conjugate we show here that MRP1 transports both diastereomers with equal efficiency. Furthermore, we present evidence that the mouse erythrocyte plasma membrane contains at least two transport systems for PGA_2-GS, mrp1 and a second unidentified one. In contrast to mrp1, the second transport activity is susceptible to inhibition by methotrexate and cAMP.

PGA is the dehydration product of PGE and several reports indicate that PGAs inhibit the proliferation of cells by inducing cell cycle arrest (e.g. [22]) and apoptosis [23]. Intracellularly PGAs are conjugated to GSH which has been suggested to result in the inhibition of its antiproliferative mode of action (see [27,28]). Elevated levels of GSH are frequently found in tumor cells and these will shift the equilibrium reaction between PGA and GSH to the formation of conjugates that will be subsequently removed from the cell by MRP1. This suggests that MRP1 protects tumor cells not only by transporting cytotoxic drugs, but also by exporting endogenously formed toxic compounds. However, it remains to be tested whether in vivo MRP1 or other GS-X pumps play a critical role in this process.

We have shown recently that the high affinity DNP-GS and EA-GS transporter in the erythrocyte is mrp1 by comparing erythrocyte membrane vesicles prepared from wild-type and *mrp1*$^{-/-}$ mice [18]. However, some residual uptake activity was detectable in the mrp1$^{-/-}$ vesicles. This transport activity is probably identical to the low affinity erythrocyte GS-X pump that has been described before [36,41]. Based on detailed kinetic studies with several organic anion transport in-

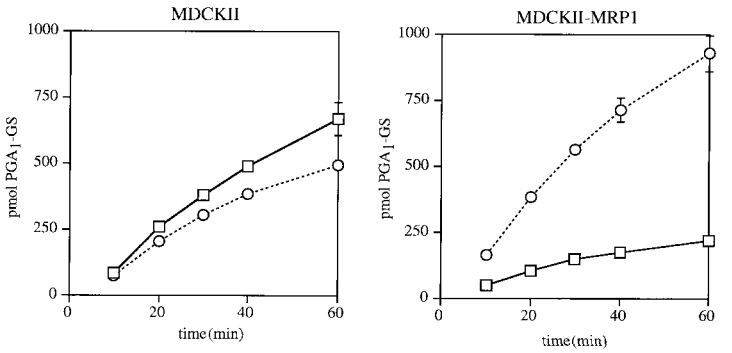

Fig. 3. Export of [^{3}H]PGA_1-GS from MDCKII and MDCKII-MRP1 cells. Cells were incubated at 37°C with [^{3}H]PGA_1 (2 μM) in both the apical and basolateral compartment and samples were taken at $t = 10$, 20, 30, 40, and 60 min. Samples were acidified with formic acid and extracted twice with an equal volume of ethyl acetate. Squares and continuous line: transport to the apical side. Circles and broken lines: transport to the basolateral side. The experiment was performed in duplicate and repeated three times.

hibitors Saxena and Henderson [41] concluded that this transporter is most likely homologous to the methotrexate transporter that they identified in L1210 cells [48]. Our results also support the presence of a methotrexate transporter in erythrocyte membranes. However, as we could only inhibit 50% of the transport activity in mrp1$^{-/-}$ vesicles we cannot exclude that there is a third PGA_2-GS transporter present in the erythrocyte plasma membrane. Based on its high affinity for bilirubin ditaurate evidence for the existence of such an additional transporter was found in human erythrocytes [41].

Analysis of data bases with expressed sequence tags has shown that besides MRP1 and cMOAT four other MRP homologs exist and one of these might be responsible for methotrexate or cAMP transport in the erythrocyte [49,50]. It has recently been shown that cMOAT can transport methotrexate [51], but cMOAT is not present in erythrocytes [36]. MRP5 is a possible candidate for one of the unidentified transport activities, as it is ubiquitously expressed in human tissues [50]. Experiments are in progress to investigate this possibility.

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