The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs

Glen Reid*, Peter Wielinga*, Noam Zelcer*, Ingrid van der Heijden, Annemieke Kuil, Marcel de Haas, Jan Wijnholds, and Piet Borst†

Division of Molecular Biology and Center of Biomedical Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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Prostaglandins are involved in a wide variety of physiological and pathophysiological processes, but the mechanism of prostaglandin release from cells is not completely understood. Although poorly membrane permeable, prostaglandins are believed to exit cells by passive diffusion. We have investigated the interaction between prostaglandins and members of the ATP-binding cassette (ABC) transporter ABCC [multidrug resistance protein (MRP)] family of membrane export pumps. In inside-out membrane vesicles derived from insect cells or HEK293 cells, MRP4 catalyzed the time- and ATP-dependent uptake of prostaglandin E_1 (PGE_1) and PGE_2. In contrast, MRP1, MRP2, MRP3, and MRP5 did not transport PGE_1 or PGE_2. The MRP4-mediated transport of PGE_1 and PGE_2 displayed saturation kinetics, with K_m values of 2.1 and 3.4 μM, respectively. Further studies showed that $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGA_1, and thromboxane B_2 were high-affinity inhibitors (and therefore presumably substrates) of MRP4. Furthermore, several nonsteroidal antiinflammatory drugs were potent inhibitors of MRP4 at concentrations that did not inhibit MRP1. In cells expressing the prostaglandin transporter PGT, the steady-state accumulation of PGE_1 and PGE_2 was reduced proportional to MRP4 expression. Inhibition of MRP4 by an MRP4-specific RNA interference construct or by indomethacin reversed this accumulation deficit. Together, these data suggest that MRP4 can release prostaglandins from cells, and that, in addition to inhibiting prostaglandin synthesis, some nonsteroidal antiinflammatory drugs might also act by inhibiting this release.

Prostaglandins are key mediators in the regulation of many physiological processes. They are involved in inflammatory responses and tumorigenesis, and their synthesis and metabolism are tightly regulated (1). The first step in prostaglandin synthesis is the production of arachidonic acid, which is released from membrane lipid primarily by cytosolic phospholipase A_2 (1). Arachidonic acid is then oxidized to the intermediate prostaglandin H_2 (PGH_2) by PGH synthases, also known as cyclooxygenase (COX)-1 and -2, and the recently identified COX-3 (2). These enzymes are known clinically as the targets of aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs) (3). Moreover, several recent studies have shown a link between COX-2 expression and carcinogenesis. Prostaglandins are overproduced by a variety of tumors, leading to the suggested prophylactic use of COX-2 inhibitors to decrease the incidence of colon cancer (4, 5). After COX-mediated synthesis, PGH_2 is further converted by tissue-specific prostaglandin synthases into PGE_2, $PGF_{2\alpha}$, PGD_2, prostacyclin, or thromboxane B_2, the biologically active molecules (1).

Prostaglandins are formed and secreted by most cells, and act as autocrine- or paracrine-signaling molecules. In many cases they exert their effects extracellularly via interaction with a family of G protein-coupled membrane receptors (reviewed in ref. 6), although some prostaglandins interact with the nuclear hormone receptor peroxisomal proliferator-activated receptor γ ($PPAR_\gamma$) (reviewed in ref. 7). The localized action of prostaglandins necessitates their efficient release, reuptake, and metabolism to initiate and terminate signaling. Many organic anion transporters (OAT) mediate the uptake of prostaglandins, and one of the best characterized is the prostaglandin transporter (PGT) (reviewed in ref. 8). PGT is thought to mediate prostaglandin uptake via an exchange mechanism, with lactate acting as the counter-ion (9). In contrast, the release of prostaglandins after their synthesis, a process essential for their activity, has received little attention. Despite being poorly membrane permeable, primary prostaglandins such as PGE_2 are widely assumed to diffuse passively from the cell. In contrast, another prostanoid, the proinflammatory leukotriene LTC_4, is transported by the multidrug resistance proteins MRP1 (10–12) and MRP2 (13), and $MRP1^{-/-}$ mice show an impaired response to inflammatory stimuli, associated with decreased LTC_4 secretion (14). Similarly, the metabolically inactive glutathione conjugate of PGA_1 (PGA_1-GS) is also transported by both MRP1 (15–17) and MRP2 (18), and MRP1 also transports the glutathione conjugate of the synthetic prostaglandin Δ^7-PGA_1 methyl ester (15). The primary prostaglandin PGE_2, however, was not found to be a substrate of MRP1 (17).

The more recently characterized MRP4 (ABCC4) has been shown to transport several physiological substrates such as cyclic nucleotides, steroid conjugates (19, 20), and folate (21). Like MRP1, MRP4 is also inhibited by the leukotriene antagonist MK571 (19, 22). Circumstantial evidence suggests that MRP4 might interact with prostaglandins as well. Early studies showed that the efflux of cAMP from erythrocytes is inhibited by several prostaglandins (23, 24), and we have recently found that the cellular efflux of cGMP by both MRP4 and MRP5 can also be inhibited by prostaglandins (25). Moreover, PGA_1 inhibits the ATP-dependent efflux of methotrexate, another MRP4 substrate (21), from Chinese hamster ovary cells (26). Based on these observations, we investigated the interaction between members of the MRP family and prostaglandins. We report here that MRP4 actively transports PGE_1 and PGE_2, whereas MRP1–3 and MRP5 do not.

Materials and Methods

Materials. [^{3}H]17β-Estradiol 17β-D-Glucuronide ($E_2$17βG; 40.5 Ci/mmol) was from NEN (Boston), [^{3}H]PGE_1 (49 Ci/mmol) and [^{3}H]PGE_2 (151 Ci/mmol) were from Amersham Biosciences, and [^{3}H]Alaninyl-d4TMP was synthesized from So324 as described (27). Rofecoxib, celecoxib, and diclofenac were from the pharmacy department of The Netherlands Cancer

Abbreviations: PG, prostaglandin; PGT, PG transporter; MRP, multidrug resistance protein; NSAIDs, nonsteroidal antiinflammatory drugs; RNAi, RNA interference; Sf9, *Spodoptera frugiperda*; COX, cyclooxygenase; ABC, ATP-binding cassette; OAT, organic anion transporter; pS, pSUPER; pRS, pRETROSUPER.

See commentary on page 9108.

*G.R., P.W., and N.Z. contributed equally to this work.

†To whom correspondence should be addressed. E-mail: p.borst@nki.nl.

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Institute (Amsterdam). All other chemicals and reagents were from Sigma.

Cell Lines and Culture Conditions. The generation and culture of HEK293/4.63 and HEK293/5I cells have been described (28, 29). The culture of *Spodoptera frugiperda* (Sf9) cells and the generation of baculovirus containing an MRP3 cDNA construct was reported by Zelcer *et al.* (30). Baculovirus containing MRP1 or MRP2 was from Balasz Sarkadi (Budapest) (31) and baculovirus containing MRP4 was from Remon van Aubel (Nijmegen, The Netherlands) (22).

Vesicular Transport Assays. Membrane vesicles from HEK293 and Sf9 cells were generated as described (27, 30), and the expression of transporters was evaluated by Western blot (29). The uptake of radiolabeled substrates by membrane vesicles was determined by the rapid filtration method as described (30). The ATP-dependent transport at 37°C was calculated by subtracting the uptake in the absence of ATP from that in the presence of ATP (4 mM), after correcting for nonspecific binding at 4°C. For inhibition studies, the uptake in the absence and presence of inhibitors was compared and expressed as the percentage of transport in the absence of inhibitor.

Cloning and Expression of PGT. PGT cDNA was cloned from human kidney total RNA by reverse transcription by using SuperScript reverse transcriptase (Invitrogen) and an oligo(dT) primer, and subsequent PCR amplification by using *Taq* polymerase with PGT-specific forward (5′-GGAATTCGCAGCCATGGGGC-TCC-3′) and reverse (5′-GCTCTAGAGTCAGATGAGGCCT-GCC-3′) primers containing *Eco*RI and *Xba*I restriction sites, respectively. An amplification product of ≈2 kb was subcloned into the pcDNA3 expression vector. The insert of the clone conferring the greatest PGE_2 uptake was sequenced and found to correspond to the previously cloned PGT (32) (NCBI accession no. NM005630), with the exception of three silent mutations.

siRNA Knock-Down Experiments. The MRP4 knock-down vectors pSUPER42 (pS42) and pRETROSUPER42 (pRS42) were constructed as described (33) by using the forward primer 5′-gatccccgatggtgcatgtgcaggatttcaagagaatcctgcacatgcaccatctttttgg-aaa-3′ and the reverse primer 5′-agcttttccaaaaagatggtgcatgtgc-aggattctcttgaaatcctgcacatgcaccatcggg-3′. The primers were annealed and cloned into pSUPER cut with *Hin*dIII and *Bgl*II to generate pS42. The pRS42 construct was constructed by cloning an *Xho*I and *Eco*RI fragment from pS42 into pRS. Transient transfections were done by calcium phosphate precipitation. To generate clones with a stable MRP4 knockdown, one cell per well was plated in 96-well plates and selected with puromycin, expanded (14–21 days), and screened by Western blot for MRP4 expression as before (29).

Cellular Prostaglandin Uptake. HEK293 (4×10^5 cells per well in a six-well plate) were transiently transfected with 2 μg of a PGT construct, with or without RNA interference (RNAi) constructs. On day 4 after transfection, the cells were washed with PBS and incubated at room temperature with 0.1 nM [^{3}H]PGE_1 or [^{3}H]PGE_2, in 500 μl of Hanks' balanced salt solution per well, in the presence or absence of inhibitors. At the indicated time points, uptake was stopped by washing the cells twice with ice-cold PBS. The cellular accumulation of [^{3}H]prostaglandin was determined by scintillation counting.

Results

Inhibition of MRP-Mediated Transport by Prostaglandins and NSAIDs. MRP1–4 have partially overlapping substrate specificities, despite their different tissue distribution, subcellular localization, and proposed physiological functions (34). Estradiol 17-β-D-glucuronide ($E_2$17βG) is transported by MRP1–4, and was therefore used to investigate the inhibitory effect of prostaglandins on these transporters. By using inside-out membrane vesicles from Sf9 cells (WT or those containing MRP1, MRP2, MRP3, or MRP4), we measured transport of $E_2$17βG in the absence and presence of the prostaglandins specified in Table 1. The uptake of $E_2$17βG was similar for each transporter, with initial uptake rates of 10.3 ± 0.3, 17.6 ± 0.1, 9.4 ± 0.4, and 11.2 ± 0.5 pmol/mg protein per 2 min for MRP1, MRP2, MRP3, and MRP4, respectively. Transport by MRP4 was strongly inhibited by all prostaglandins tested, whereas transport by MRP1 and MRP3 was not inhibited (Table 1). The transport of alaninyl-d4TMP, a specific MRP5 substrate (27), by vesicles derived from HEK293/MRP5 cells was slightly inhibited by prostaglandins at a concentration of 20 μM (Table 1). MRP2-mediated transport of $E_2$17βG was stimulated rather than inhibited by prostaglandins. However, transport of $E_2$17βG is stimulated by many compounds that are not necessarily transported by MRP2 (our unpublished results).

Table 1. Effect of prostaglandins on MRP-mediated transport

PG	μM	MRP4	MRP1	MRP2	MRP3	MRP5
PGE_1	1	67 ± 7				
	5	18 ± 4				
	20	4 ± 2	87 ± 4	162 ± 7	94 ± 5	69 ± 4
PGE_2	1	66 ± 1				
	5	35 ± 5				
	20	9 ± 2	98 ± 5	131 ± 1	86 ± 1	74 ± 3
$PGF_{1\alpha}$	1	79 ± 4				
	5	62 ± 1				
	20	29 ± 1	91 ± 4	138 ± 5	107 ± 3	87 ± 3
$PGF_{2\alpha}$	1	74 ± 1				
	5	42 ± 1				
	20	15 ± 2	107 ± 7	166 ± 10	101 ± 5	63 ± 3
PGA_1	1	53 ± 1				
	5	18 ± 5				
	20	2 ± 2	83 ± 3	182 ± 9	76 ± 1	51 ± 1
TXB_2	1	89 ± 1				
	5	56 ± 3				
	20	29 ± 6	66 ± 1	143 ± 10	76 ± 9	77 ± 10

Sf9 membrane vesicles containing MRP1, 2, 3, or 4 were incubated with 1 μM $E_2$17βG for 2 min; HEK293/MRP5 vesicles were incubated for 30 min with 1 μM alaninyl-*d4TMP*. Values are expressed as percentage of control and are the average ± SE of two independent experiments performed in triplicate.

NSAIDs are known to inhibit prostaglandin synthesis by inhibiting COX-1 or COX-2 activity. In these studies, inhibition of prostaglandin synthesis is determined by prostaglandin levels found in the medium, raising the possibility that these inhibitors also inhibit the release of prostaglandins. Therefore, we tested the effect of various NSAIDs on the transport mediated by MRP4 and MRP1. The common NSAIDs indomethacin, indoprofen, ketoprofen, and flurbiprofen inhibited both MRP4- and MRP1-mediated $E_2$17βG transport, but MRP4 proved more sensitive than MRP1 (Table 2). In contrast, diclofenac, rofecoxib, and celecoxib were equally poor inhibitors of both transporters (Table 2).

Transport of PGE_1 and PGE_2 by MRP4. The inhibition data from Table 1 suggest that prostaglandins might be high-affinity substrates of MRP4, but not of MRP1–3 or -5. To verify this hypothesis, we tested the ability of MRP1–4 in Sf9 cells to transport PGE_1 and PGE_2, and only MRP4 was able to do so (Fig. 1 *A* and *C*). We obtained similar results by using vesicles derived from human embryonic kidney cells (Fig. 1 *B* and *D*). However, in contrast to WT Sf9 vesicles, which did not accumulate PGE_1 and PGE_2, vesicles from HEK293 parental cells accumulated PGE_2, suggesting that these cells contain an ATP-

Table 2. Effect of NSAIDs on $E_2 17\beta G$ transport

NSAID	μM	MRP4	MRP1
Flurbiprofen	1	80 ± 3	
	5	60 ± 2	
	50	23 ± 1	92 ± 17
Ibuprofen	1	85 ± 6	
	20	50 ± 5	
	200	31 ± 2	79 ± 9
Indomethacin	1	68 ± 2	
	5	56 ± 8	102 ± 1
	50	11 ± 3	56 ± 11
Indoprofen	1	67 ± 1	
	5	45 ± 8	
	50	17 ± 5	105 ± 11
Ketoprofen	1	97 ± 2	
	5	81 ± 3	
	50	35 ± 3	98 ± 5
Diclofenac	100	61 ± 2	82 ± 2
Celecoxib	50	78 ± 4	105 ± 5
Rofecoxib	100	80 ± 3	103 ± 1

Sf9 membrane vesicles containing MRP4 or MRP1 were incubated with 1 μM [^{3}H]$E_2 17\beta G$ for 2 min. Values are expressed as percentage of control and are the average ± SE of two independent experiments performed in triplicate.

dependent transporter, possibly endogenous MRP4. Reduced glutathione, known to stimulate transport of some substrates by MRP1–3, did not support transport of PGE_1 or PGE_2 by MRP1–3 or MRP5, nor did it alter the transport of PGE_1 and

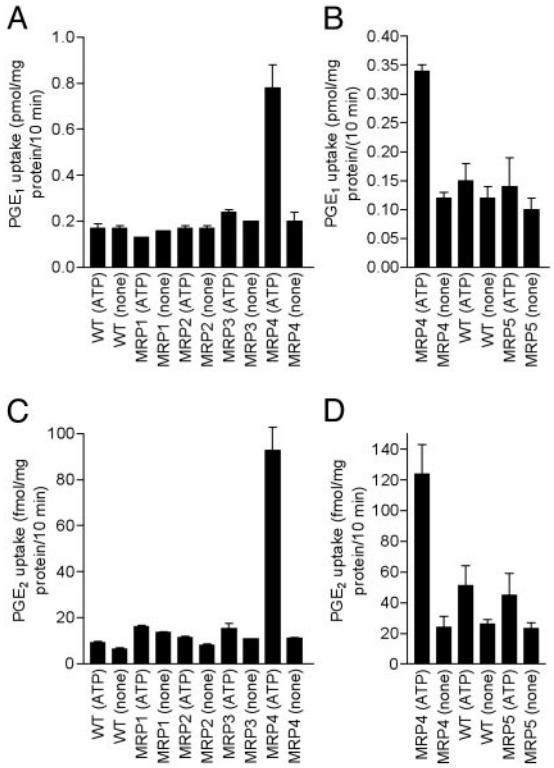

Fig. 1. Transport of PGE_1 and PGE_2 into membrane vesicles from Sf9 or HEK293 cells. Membrane vesicles (20 μg) were incubated with 41 nM [^{3}H]PGE_1 (*A* and *B*) or 6.6 nM [^{3}H]PGE_2 (*C* and *D*) for 10 min in the presence or absence of 4 mM ATP. The vesicles were derived from Sf9 cells infected with a WT or MRP1, 2, 3, or 4 cDNA-containing baculovirus (*A* and *C*), or MRP4- or MRP5-overexpressing and parental HEK293 cells (*B* and *D*). Values are the average ± SE of two independent determinations done in triplicate.

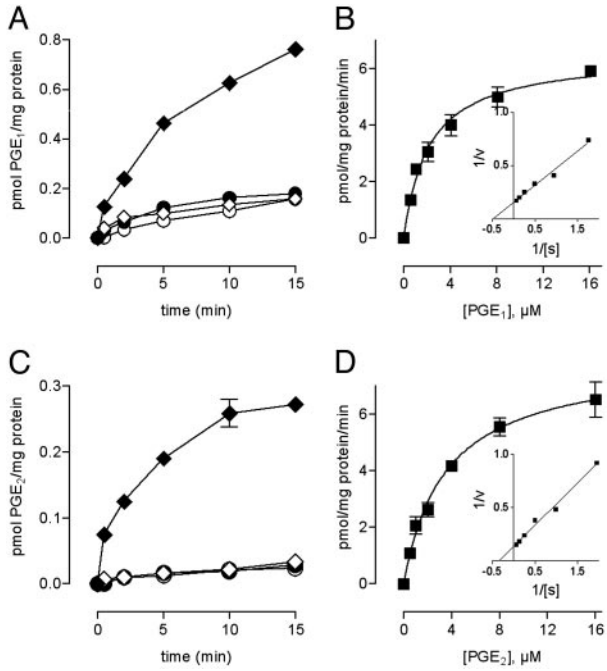

Fig. 2. Time and concentration dependence of PGE_1 and PGE_2 transport by MRP4. Membrane vesicles (100 μg of protein) from WT (circles) or MRP4-containing (diamonds) Sf9 cells were incubated at 37°C with 41 nM [^{3}H]PGE_1 (*A*) or 8.7 nM [^{3}H]PGE_2 (*C*) in the presence (filled symbols) or absence (open symbols) of ATP, and aliquots (20 μg of protein) were taken at the times indicated. To analyze the concentration dependence of uptake, MRP4 vesicles were incubated at 37°C for 3 min with 41 nM [^{3}H]PGE_1 (*B*) or 13 nM [^{3}H]PGE_2 (*D*) with unlabeled substrate added to the final concentration. The samples were incubated in the presence or absence of 4 mM ATP, and only the ATP-dependent uptake is plotted. Values are the average ± SE of representative experiments done in triplicate. (*Insets*) Lineweaver–Burk transformation.

PGE_2 mediated by MRP4 (data not shown). To further characterize the MRP4-mediated transport of PGE_1 and PGE_2, we used Sf9 vesicles. Transport of both compounds at 37°C was time- and concentration-dependent (Fig. 2), with apparent K_m and V_{max} values of 2.1 μM and 6.9 pmol/mg protein per minute for PGE_1 (Fig. 2*B*), and 3.4 μM and 6.4 pmol/mg protein per minute for PGE_2 (Fig. 2*D*).

It has been suggested that, after synthesis, prostaglandins are released passively from the producing cells via diffusion across the plasma membrane (8). To investigate the permeability of our membrane vesicles for prostaglandins, we carried out vesicular uptake experiments in the presence of orthovanadate (V_i) and EDTA, two inhibitors of the ATPase function of ABC transporters (Fig. 3). The addition of these compounds to the reaction before starting the assay completely abrogated the time- and ATP-dependent uptake of both $E_2 17\beta G$ (Fig. 3*A*) and PGE_1 (Fig. 3*B*). When the compounds were added during the reaction (after either 5 or 10 min), however, there was a striking difference in the effect of V_i/EDTA on the uptake of $E_2 17\beta G$ and PGE_1. In the case of $E_2 17\beta G$, uptake stopped after the addition of V_i/EDTA, and the amount of radioactivity associated with the vesicles decreased gradually over time to ≈50% of the initial value (Fig. 3*A*). Similar results were obtained when methotrexate was used as the substrate (data not shown). In contrast, all of the PGE_1 associated with the vesicles was immediately lost after the addition of V_i/EDTA (Fig. 3*B*), showing that the ability of PGE_1 to cross the plasma membrane of HEK293 cells is much greater than that of $E_2 17\beta G$. Addition of hexokinase and glucose to deplete ATP levels yielded similar

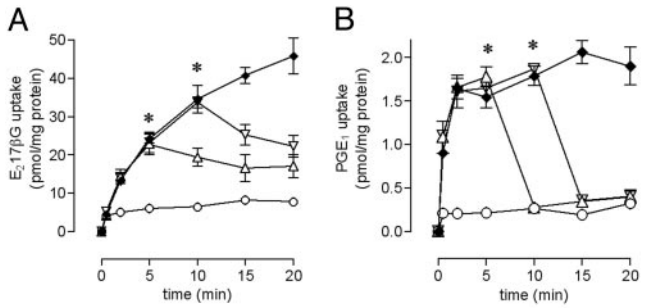

Fig. 3. Leakage of $E_2$17βG and PGE_1 from inside-out membrane vesicles. Membrane vesicles derived from HEK293/4.63 cells were incubated with 1 μM $E_2$17βG (*A*) or 51 nM PGE_1 (*B*) at 37°C (as in Fig. 2, except that the $MgCl_2$ concentration was reduced to 5 mM), and aliquots corresponding to 15 μg of protein were filtered at the indicated times (filled symbols). To determine the permeability of the vesicles, a combination of 5 mM orthovanadate and 5 mM EDTA was added to stop ATP-dependent uptake (open symbols), either at the beginning of the reaction or after 5 or 10 min (asterisks), and the reaction was followed until 20 min. Values are the average ± SE of two experiments done in triplicate.

results (data not shown). Given the high efflux rate of PGE_1, it is probable that the apparent initial prostaglandin uptake rates measured in Fig. 2 are underestimated and that the real maximal MRP4-mediated rates of prostaglandin transport are higher than the V_{max} values calculated here.

MRP4-Mediated Efflux of PGE_1 and PGE_2 from HEK293 Cells. To further characterize prostaglandin transport by MRP4, we investigated the effect of MRP4 expression on the accumulation of PGE_1 and PGE_2 in HEK293 parental cells and transfectants expressing PGT (Fig. 4). The uptake of prostaglandins into parental HEK293 cells was low but increased substantially after transfection with PGT. Experiments were carried out at room temperature because the rate of influx at 37°C was too rapid to allow measurement of initial rates (data not shown). The accumulation of prostaglandins in MRP4-overexpressing HEK293/4.63 cells was also rapid after transfection with PGT; in contrast, the steady-state accumulation was consistently 2-fold lower than that found in the parental cells expressing PGT (Fig. 4 *A* and *B*). HEK293/4.3 cells, which have a lower level of MRP4 overexpression, accumulated intermediate levels of PGE_2 (Fig. 4*B*). Moreover, the accumulation deficit could be reversed by incubating the cells with indomethacin during the uptake period. In fact, indomethacin, an inhibitor of MRP4 previously shown not to interact with PGT, raised the steady-state intracellular prostaglandin levels in both parental and MRP4-overexpressing HEK293 cells (Fig. 4*C*). These results indicate that the steady-state prostaglandin levels in the cells result from the equilibrium between uptake mediated by PGT and active prostaglandin efflux mediated by an indomethacin-sensitive transporter.

Having found that MRP4 expression led to a decrease in the steady-state accumulation of prostaglandins, we tested the ability of MRP4 to efflux either PGE_1 or PGE_2. Cells were first loaded with prostaglandins as detailed above, and, after washing, the efflux into medium was followed. Initial rates of efflux from the cells were extremely rapid, with most of the accumulated prostaglandins effluxed within the first 2 min (data not shown). Within the time limits of the assay, the efflux appeared to be independent of MRP4, because efflux was equivalent in parental and MRP4 overexpressing HEK293 cells (data not shown), similar to the rapid loss of prostaglandins from vesicles after inhibition of MRP4 (Fig. 3).

To verify that the decreased prostaglandin accumulation in the MRP4-transfected cells was indeed caused by MRP4, we investigated the accumulation of prostaglandins after reduction of MRP4 expression via an MRP4-specific RNAi construct. This construct strongly decreased MRP4 levels in both transient and stable transfections (Fig. 5*A*), resulting in an increase in prostaglandin accumulation by cells expressing PGT (Fig. 5 *B* and *C*). In transient transfections, RNAi-mediated inhibition of MRP4 expression and concomitant elevations in prostaglandin accumulation increased as a function of time after transfection (data not shown), and was also dependent on the amount of RNAi construct used (Fig. 5*B*), both in HEK293 and HEK293/4.63 cells. Similarly, HEK293/4.63 cells stably transfected with the same RNAi construct also showed an increase in prostaglandin accumulation (Fig. 5*C*) that was inversely proportional to the amount of MRP4 detected in the cells by Western blot (Fig. 5*A*).

Discussion

To act as signaling molecules, prostaglandins must be released from the cells where they are synthesized. Here we show that MRP4, a recently characterized membrane pump, could be involved in this process, because it transports PGE_1 and PGE_2. This is an example of prostaglandin transport by a carrier poised to mediate prostanoid release rather than uptake. Several mem-

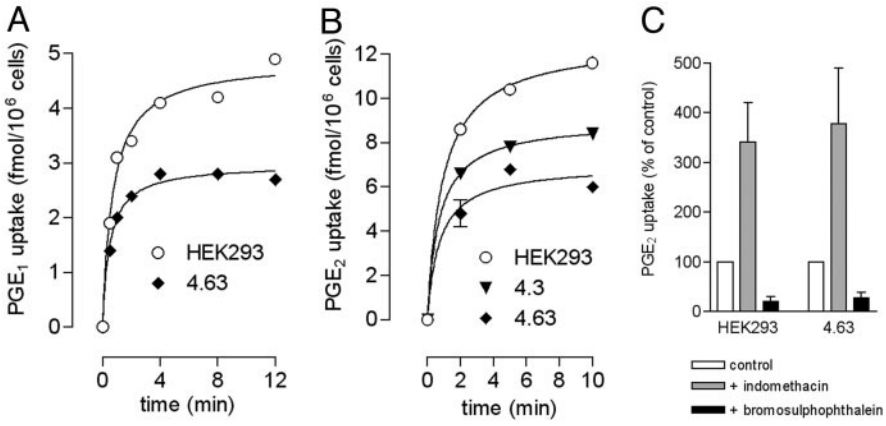

Fig. 4. Effect of MRP4 expression on PGE_1 and PGE_2 accumulation in HEK293 cells expressing PGT. HEK293 parental cells and those stably overexpressing MRP4 (4.3, medium overexpression; 4.63, high overexpression) were transfected with a PGT construct. On day 4 after transfection, cells were incubated with [^{3}H]PGE_1 (*A*) or [^{3}H]PGE_2 (*B*), and intracellular prostaglandin was determined at the indicated times (a representative of three independent experiments is shown). The effect of 25 μM indomethacin or 50 μM bromosulfophthalein on PGE_2 accumulation (*C*) was determined after 10 min and is expressed as the percentage of PGE_2 accumulated in the absence of these compounds (values represent the mean ± SE of three independent experiments).

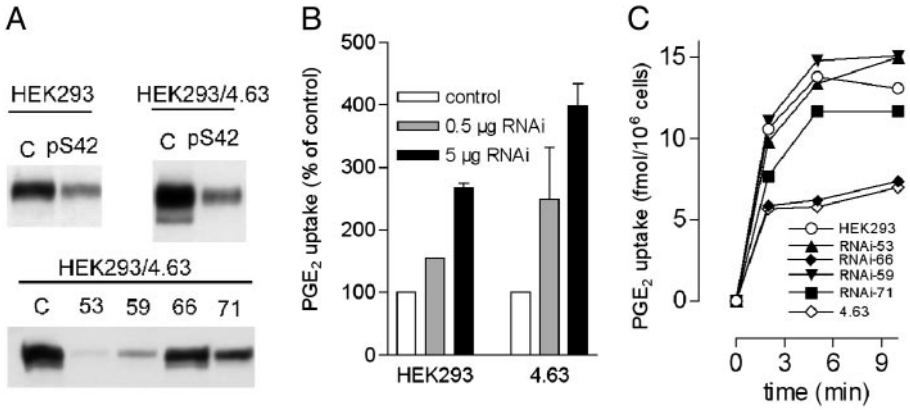

Fig. 5. Effect of RNAi on MRP4 expression and PGE_2 accumulation. (*A*) The effect of RNAi expression on the level of MRP4. (*Upper*) MRP4 expression in HEK293 (30-min exposure) and HEK293/4.63 (5-min exposure) cells 3 days after transfection with 7.5 μg of control pS vector (C) or RNAi construct pS42. (*Lower*) HEK293/4.63 cells were transduced with pRS or RNAi construct pRS42 and, after selection with puromycin, vector (C) and RNAi clones (53, 59, 66, and 71) were analyzed for MRP4 expression. (*B*) Cells were cotransfected with PGT and pS42, and 4 days after transfection the accumulation of PGE_2 was determined after 10 min and is expressed as a percentage of PGE_2 accumulated in the vector control. (*C*) Accumulation of PGE_2 in HEK293/4.63 cells and clones stably transfected with pRS or pRS42 was determined (a representative experiment is shown).

bers of the OAT polypeptide (OATP) and OAT families have been shown to transport prostaglandins (8, 35). The best characterized is PGT, which mediates high-affinity uptake of primary prostaglandins when expressed in oocytes or cultured cells (36), with affinities corresponding to those found in studies that modeled the kinetics of prostaglandin clearance by the lung, a tissue in which PGT is expressed (37). PGT can catalyze prostaglandin/lactate exchange, and, because the lactate gradient is outwardly directed in most cells, PGT most likely mediates uptake rather than release of prostaglandins (9). Similarly, members of the human OAT/OCT (SLC22A) family have been shown to transport PGE_2 and $PGF_{2\alpha}$ (38, 39), but these transporters are also believed to mediate uptake.

The release of prostaglandins from their site of synthesis was thus far thought to occur via passive diffusion through the plasma membrane of the producing cell, and Schuster has argued that the high intracellular $[H^+]$ and the electronegative cell interior can drive prostaglandins out of the cell (8). The rate of diffusion cannot be high, however, because prostaglandins are fairly water soluble and negatively charged at physiological pH. Indeed, the very fact that PGT can be used to accumulate PGE_1 and PGE_2 50-fold in cells (ref. 42 and Fig. 4) shows that diffusion of PGE_1 and PGE_2 out of cells is relatively slow and is no match for an active uptake transporter. In the same vein, our vesicular uptake experiments show that MRP4 action can result in the accumulation of PGE_1 and PGE_2 in membrane vesicles, even though leakage is substantial. Also, under more physiological conditions, when synthesis of prostaglandins is stimulated in cells, substantial amounts of PGE_2 may accumulate (40), and prostaglandin release is clearly rate-limiting in the response. Other observations support this picture: In monolayers of cultured canine cortical collecting tubule cells, for example, PGE_2 crossed the monolayer slowly, at the same rate as the inulin used to measure imperfections in the monolayer (41). Similarly, human cell lines (32, 42) and MDCKII cells (43) were impermeable to prostanoids, with no accumulation (or flux) of prostaglandins or thromboxane unless PGT was heterologously expressed. The permeability of *Xenopus laevis* oocytes, a model system used in many studies of transport proteins, is also low for prostaglandins (32, 36, 42). Despite this low intrinsic permeability, the amount of label released from oocytes injected with $[^3H]PGE_2$ increased linearly with concentration, up to an intraoocyte concentration of 380 nM, suggestive of passive diffusion (42). However, because oocytes endogenously express numerous transport proteins, carrier-mediated efflux would provide an alternative explanation for this observation.

Taken together, these observations indicate that efflux of prostaglandins by passive diffusion may not be sufficiently rapid under some physiological conditions, and that a PGT may be required to increase the rate of efflux, just as PGT is required to increase the rate of influx. The situation may be analogous to the passage of fatty acids through membranes, long thought to be caused by passive diffusion, until a family of fatty acid transporters was discovered (44, 45) speeding up the rate of passage 10- to 100-fold. ABC transporters have been found to transport amphipathic drugs and phosphatidyl choline from cells (reviewed in ref. 46). The use of the ABC transporter MRP4 for PGE_1 and PGE_2 excretion therefore does not come as a surprise. Although it has proven difficult to determine the intracellular concentration of free prostaglandins, the micromolar affinities of MRP4 for PGE_1 and PGE_2 are similar to the affinity of the COX enzymes for the PGE_2 precursor arachidonic acid (47), and are compatible with a physiological role for MRP4 in prostaglandin transport.

Although the presence of MRP4 can result in accumulation of PGE_1 and PGE_2 in membrane vesicles, the accumulated prostaglandin is rapidly lost as soon as uptake is blocked (Fig. 3). This is not simply leak from poorly sealed vesicles, because the loss of accumulated $E_2 17\beta G$ from the vesicles was much lower. It is possible that the rapid release of PGE is caused by diffusion, but we cannot rule out the involvement of transporters. It is worth noting that the accumulation of PGE_2 in PGT-containing HEK293 cells increases substantially when export transporters are inhibited with indomethacin (Fig. 4*C*) or an RNAi construct reducing MRP4 levels (Fig. 5*B*), even though the endogenous levels of MRP4 in these cells are low and the inhibition with RNAi is not complete (Fig. 5*A*). Likewise, we cannot exclude that the Sf9 insect cells and the HEK293 cells used to make vesicles contain endogenous OAT able to transport PGE. Such transporters of the OAT and OATP type are widespread in nature (8). Attempts to block PGE release from these vesicles failed (unpublished results), but that negative result does not prove that release is by diffusion only.

Multiple cell types in a wide variety of tissues produce prostaglandins, and it is intriguing that MRP4 mRNA is detectable in most tissues (48). Moreover, MRP4 protein is present at high levels in kidney and prostate, organs involved in the metabolism and clearance of prostaglandins and their metabo-

lites. The expression of PGT in polarized kidney cells results in transport of PGE through the monolayer (43). PGT is present predominantly in the apical membrane of these cells, resulting in apical uptake and basolateral release of PGE_2. PGT was detected in many cell types in the kidney, but not in the cells of the proximal tubule (49), which takes up and secretes PGE_2 (50). Because the proximal tubule is also the site of MRP4 expression in the kidney (22), MRP4 might provide a means for PGE_2 secretion into the urine. The inhibition of proximal tubule PGE_2 secretion by indomethacin (50) is consistent with such a role of MRP4. Further studies are required to determine whether MRP4 and the prostaglandin-synthesizing machinery are expressed in the same cells, and the extent to which MRP4 contributes to vectorial transport of prostaglandins across epithelia.

Our experiments provide evidence that MRP4 is inhibited by indomethacin and other NSAIDs at physiologically relevant concentrations. Although MRP4 was substantially more sensitive than MRP1 to several of these drugs, neither pump was strongly inhibited by the COX-2-specific inhibitors rofecoxib and celecoxib. Nevertheless, the sensitivity of MRP4 to some NSAIDs might affect blood tests used to determine the COX activity of newer NSAIDs. These tests often measure release of TxB_2 and PGE_2 from blood cells in which COX activity is stimulated (51). Our results suggest that such assays might be confounded by inhibition of prostanoid transport mechanisms, as seen in HEK293 cells expressing PGT.

In summary, we have found that MRP4 transports PGE_1 and PGE_2 with high affinity, and that other prostaglandins are high-affinity inhibitors of MRP4 function. This further broadens the physiological substrate specificity of MRP4, which was previously shown to transport cyclic nucleotides, folates, and steroid conjugates (19–21). The recently generated MRP4 knockout mice (J. D. Schuetz, personal communication) will provide an important tool to ascertain the physiological importance of prostaglandin transport by MRP4, and to test whether PGE_1 and PGE_2 are substrates of other ABC transporters.

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1. Funk, C. D. (2001) *Science* **294,** 1871–1875.
2. Warner, T. D. & Mitchell, J. A. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 13371–13373.
3. Vane, J. R. (2000) *J. Physiol. Pharmacol.* **51,** 573–586.
4. Prescott, S. M. & Fitzpatrick, F. A. (2000) *Biochim. Biophys. Acta* **1470,** M69–M78.
5. Gupta, R. A. & Dubois, R. N. (2001) *Nat. Rev. Cancer* **1,** 11–21.
6. Breyer, M. D. & Breyer, R. M. (2000) *Am. J. Physiol.* **279,** F12–F23.
7. Harris, S. G., Padilla, J., Koumas, L., Ray, D. & Phipps, R. P. (2002) *Trends Immunol.* **23,** 144–150.
8. Schuster, V. L. (2002) *Prostaglandins Other Lipid Mediators* **68/69,** 633–647.
9. Chan, B. S., Endo, S., Kanai, N. & Schuster, V. L. (2002) *Am. J. Physiol.* **282,** F1097–F1102.
10. Jedlitschky, G., Leier, I., Buchholz, U., Center, M. & Keppler, D. (1994) *Cancer Res.* **54,** 4833–4836.
11. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P., Deeley, R. G. & Keppler, D. (1994) *J. Biol. Chem.* **269,** 27807–27810.
12. Muller, M., Meijer, C., Zaman, G. J., Borst, P., Scheper, R. J., Mulder, N. H., de Vries, E. G. & Jansen, P. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 13033–13037.
13. Cui, Y., Konig, J., Buchholz, J. K., Spring, H., Leier, I. & Keppler, D. (1999) *Mol. Pharmacol.* **55,** 929–937.
14. Wijnholds, J., Evers, R., van Leusden, M. R., Mol, C. A., Zaman, G. J., Mayer, U., Beijnen, J. H., van der Valk, M., Krimpenfort, P. & Borst, P. (1997) *Nat. Med.* **3,** 1275–1279.
15. Akimaru, K., Kuo, M. T., Furuta, K., Suzuki, M., Noyori, R. & Ishikawa, T. (1996) *Cytotechnology* **19,** 221–227.
16. Ishikawa, T., Akimaru, K., Nakanishi, M., Tomokiyo, K., Furuta, K., Suzuki, M. & Noyori, R. (1998) *Biochem. J.* **336,** 569–576.
17. Evers, R., Cnubben, N. H., Wijnholds, J., van Deemter, L., van Bladeren, P. J. & Borst, P. (1997) *FEBS Lett.* **419,** 112–116.
18. Evers, R., Kool, M., van Deemter, L., Janssen, H., Calafat, J., Oomen, L. C., Paulusma, C. C., Oude Elferink, R. P., Baas, F., Schinkel, A. H. & Borst, P. (1998) *J. Clin. Invest.* **101,** 1310–1319.
19. Chen, Z. S., Lee, K. & Kruh, G. D. (2001) *J. Biol. Chem.* **276,** 33747–33754.
20. Zelcer, N., Reid, G., Wielinga, P. R., Kuil, A., van der Heijden, I., Schuetz, J. D. & Borst, P. (2003) *Biochem. J.* **371,** 361–367.
21. Chen, Z. S., Lee, K., Walther, S., Raftogianis, R. B., Kuwano, M., Zeng, H. & Kruh, G. D. (2002) *Cancer Res.* **62,** 3144–3150.
22. van Aubel, R. A., Smeets, P. H., Peters, J. G., Bindels, R. J. & Russel, F. G. (2002) *J. Am. Soc. Nephrol.* **13,** 595–603.
23. Rindler, M. J., Bashor, M. M., Spitzer, N. & Saier, M. H., Jr. (1978) *J. Biol. Chem.* **253,** 5431–5436.
24. Heasley, L. E. & Brunton, L. L. (1985) *J. Biol. Chem.* **260,** 11514–11519.
25. Wielinga, P. R., van der Heijden, I., Reid, G., Beijnen, J., Wijnholds, J. & Borst, P. (2003) *J. Biol. Chem.* **278,** 17664–17671.
26. Assaraf, Y. G., Sierra, E. E., Babani, S. & Goldman, I. D. (1999) *Biochem. Pharmacol.* **58,** 1321–1327.
27. Reid, G., Wielinga, P. R., Zelcer, N., de Haas, M., van Deemter, L., Wijnholds, J., Balzarini, J. & Borst, P. (2003) *Mol. Pharmacol.* **63,** 1094–1103.
28. Wijnholds, J., Mol, C. A., van Deemter, L., de Haas, M., Scheffer, G. L., Baas, F., Beijnen, J. H., Scheper, R. J., Hatse, S., De Clercq, E., *et al*. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7476–7481.
29. Wielinga, P. R., Reid, G., Challa, E. E., van der Heijden, I., van Deemter, L., de Haas, M., Mol, C. A., Kuil, A. J., Groeneveld, E., Schuetz, J. D., *et al*. (2002) *Mol. Pharmacol.* **62,** 1321–1331.
30. Zelcer, N., Saeki, T., Reid, G., Beijnen, J. H. & Borst, P. (2001) *J. Biol. Chem.* **276,** 46400–46407.
31. Bakos, E., Evers, R., Sinko, E., Varadi, A., Borst, P. & Sarkadi, B. (2000) *Mol. Pharmacol.* **57,** 760–768.
32. Lu, R., Kanai, N., Bao, Y. & Schuster, V. L. (1996) *J. Clin. Invest.* **98,** 1142–1149.
33. Brummelkamp, T. R., Bernards, R. & Agami, R. (2002) *Science* **296,** 550–553.
34. Borst, P. & Oude Elferink, R. (2002) *Annu. Rev. Biochem.* **71,** 537–592.
35. Hagenbuch, B. & Meier, P. J. (2003) *Biochim. Biophys. Acta* **1609,** 1–18.
36. Kanai, N., Lu, R., Satriano, J. A., Bao, Y., Wolkoff, A. W. & Schuster, V. L. (1995) *Science* **268,** 866–869.
37. Schuster, V. L. (1998) *Annu. Rev. Physiol.* **60,** 221–242.
38. Enomoto, A., Takeda, M., Shimoda, M., Narikawa, S., Kobayashi, Y., Yamamoto, T., Sekine, T., Cha, S. H., Niwa, T. & Endou, H. (2002) *J. Pharmacol. Exp. Ther.* **301,** 797–802.
39. Kimura, H., Takeda, M., Narikawa, S., Enomoto, A., Ichida, K. & Endou, H. (2002) *J. Pharmacol. Exp. Ther.* **301,** 293–298.
40. Horton, J. K., Williams, A. S., Smith-Phillips, Z., Martin, R. C. & O'Beirne, G. (1999) *Anal. Biochem.* **271,** 18–28.
41. Garcia-Perez, A. & Smith, W. L. (1984) *J. Clin. Invest.* **74,** 63–74.
42. Chan, B. S., Satriano, J. A., Pucci, M. & Schuster, V. L. (1998) *J. Biol. Chem.* **273,** 6689–6697.
43. Endo, S., Nomura, T., Chan, B. S., Lu, R., Pucci, M. L., Bao, Y. & Schuster, V. L. (2002) *Am. J. Physiol.* **282,** F618–F622.
44. Schaffer, J. E. & Lodish, H. F. (1994) *Cell* **79,** 427–436.
45. Hirsch, D., Stahl, A. & Lodish, H. F. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8625–8629.
46. Borst, P., van Meer, G. & Oude Elferink, R. P. (2003) in *ABC Proteins from Bacteria to Man*, eds. Holland, I. B., Cole, S. P., Kuchler, K. & Higgins, C. F. (Academic, London), pp. 461–478.
47. Brash, A. R. (2001) *J. Clin. Invest.* **107,** 1339–1345.
48. Kool, M., de Haas, M., Scheffer, G. L., Scheper, R. J., van Eijk, M. J., Juijn, J. A., Baas, F. & Borst, P. (1997) *Cancer Res.* **57,** 3537–3547.
49. Bao, Y., Pucci, M. L., Chan, B. S., Lu, R., Ito, S. & Schuster, V. L. (2002) *Am. J. Physiol.* **282,** F1103–F1110.
50. Irish, J. M., III (1979) *Am. J. Physiol.* **237,** F268–F273.
51. Pairet, M. & van Ryn, J. (1998) *Inflamm. Res.* **47,** Suppl. 2, S93–S101.