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Contribution of Multidrug Resistance Protein 2 (MRP2/ABCC2) to the Renal Excretion of *p*-aminohippurate (PAH) and Identification of MRP4 (ABCC4) as a Novel PAH Transporter

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Abstract. *p*-Aminohippurate (PAH) is the classical substrate used in the characterization of organic anion transport in renal proximal tubular cells. Although basolateral transporters for PAH uptake from blood into the cell have been well characterized, there is still little knowledge on the apical urinary efflux transporters. The multidrug resistance protein 2 (MRP2/ABCC2) is localized to the apical membrane and mediates ATP-dependent PAH transport, but its contribution to urinary PAH excretion is not known. In this report, we show that renal excretion of PAH in isolated perfused kidneys from wild-type and Mrp2-deficient (TR^-) rats is not significantly different. Uptake of [^{14}C]PAH in membrane vesicles expressing two different MRP2 clones isolated from Sf9 and MDCKII cells exhibited a low affinity for PAH (Sf9, 5 ± 2 mM; MDCKII, 2.1 ± 0.6 mM). Human MRP4 (ABCC4), which has recently been localized to the apical membrane, expressed in Sf9 cells had a much higher affinity for PAH (K_m = 160 ± 50 μM). Various inhibitors of MRP2-mediated PAH transport also inhibited MRP4. Probenecid stimulated MRP2 at low concentrations but had no effect on MRP4; but at high probenecid concentrations, both MRP2 and MRP4 were inhibited. Sulfinpyrazone only stimulated MRP2, but inhibited MRP4. Real-time PCR and Western blot analysis showed that renal cortical expression of MRP4 is approximately fivefold higher as compared with MRP2. MRP4 is a novel PAH transporter that has higher affinity for PAH and is expressed more highly in kidney than MRP2, and may therefore be more important in renal PAH excretion.

The proximal part of the kidney nephron plays an important role in the renal excretion of organic anions. The cells of the proximal tubule are equipped with various transport systems for uptake of organic anions from blood across the basolateral membrane and subsequent excretion across the apical (brush-border) membrane into the urine (1). The mechanisms of renal organic anion transport have traditionally been characterized by using *p*-aminohippurate (PAH) as a model substrate because of its very efficient tubular secretion, weak binding to plasma proteins, and limited reabsorption. Until recently, our insight into the characteristics and driving forces of organic anion transport systems was largely based on studies with membrane vesicles isolated from the apical and basolateral membrane of proximal tubular cells. Knowledge of the molecular identity of renal organic anion transporters and their substrate specificity has increased considerably in the past decade by cloning of various carrier proteins. Uptake of PAH into the proximal tubule is mediated by the organic anion transporters 1 (OAT1) and 3 (OAT3) (2–6). The transport properties of OAT1 and OAT3 correspond well to those previously documented from studies with isolated basolateral membrane vesicles (7). In contrast, organic anion transporters at the apical membrane of the proximal tubule involved in PAH excretion have been less well characterized. The recently cloned pig kidney Oat_V1 (8) corresponds to the previously characterized apical voltage-dependent PAH transporter (9,10). Furthermore, NPT1 (SLC17A1) might represent the classical apical PAH/organic anion exchanger (9), but definite proof is lacking (11). The multidrug resistance proteins 2 (MRP2/ABCC2) and 4 (MRP4/ABCC4) are ATP-dependent organic anion transporters, and expression in the kidney is mainly restricted to the proximal tubule apical membrane (12,13,14). MRP2 transports a variety of conjugated and nonconjugated organic anions, including PAH (15), whereas substrates for MRP4 include xenobiotic (adefovir, ganciclovir) and endogenous (cAMP, cGMP) nucleosides, as well as prostaglandins (13,16–21). Because renal proximal tubule apical membrane vesicles are exclusively oriented right-side out (22) and therefore not suitable for studying ATP-dependent efflux transporters, the role

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of this type of transporters in the renal excretion of PAH may have been underestimated.

In this study, we investigated the contribution of MRP2 to the renal excretion of PAH by comparing wild-type perfused rat kidneys and kidneys from a mutant rat strain (TR^-) with an inherited defect in functional Mrp2 (23,24). Because substrate specificities of MRP2 and MRP4 partially overlap (13,15,17), we investigated whether MRP4 represents a novel transporter for PAH. Finally, we compared the relative mRNA and protein expression levels of MRP2 and MRP4 in human kidney.

Materials and Methods

Materials

[^{14}C]PAH (1.9 GBq ·mmol^{-1}) was obtained from Perkin Elmer Life Sciences (Boston, MA). Creatine phosphate and creatine kinase were obtained from Roche (Mannheim, Germany). Primers for PCR experiments were obtained from Biolegio (Malden, The Netherlands). For real-time PCR experiments, probes 5'-labeled with tetra-chloro-6-carboxyfluorescein-5 (TET) and 3'-quenched with 6-carboxytetramethylrhodamine (TAMRA) were obtained from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). All other chemicals were obtained from Sigma (Zwijndrecht, The Netherlands).

Kidney Isolation and Perfusion

Rat kidneys of Wistar-Hannover (WH) and TR^- rats were isolated and perfused as described in detail previously (25,26). Research was undertaken under the auspices of a protocol approved by the local university animal care and usage committee. Briefly, male rats (225 to 275 g) were anesthetized intraperitoneally with pentobarbital (6 mg/100 g). Rats were injected with heparin (125 IU/100 g) and with furosemide (1 mg/100 g) to prevent deterioration of the distal nephron. The renal artery of the right kidney was cannulated via the mesenteric artery without interruption of the blood flow, and a cannula was inserted into the ureter. The excised kidney and the perfusate reservoir were placed in a fluid bath with a constant temperature of 37.5°C, and the perfusion fluid (for content, see (25), with the exception that Synthamin 14 was replaced with 8.5% Travasol [Baxter, Clintech Benelux NV/SA, Brussels]) was gassed with 95% O2/5% CO2. Pluronic F-108 was used as oncotic agent in the perfusion medium, and inulin (100 μg/ml) was added to determine the GFR. The perfusion medium was recirculated at a constant flow rate of 15 ml·min^{-1} under pressure of approximately 90 mmHg. After perfusion for 30 min to stabilize the kidney, the perfusion fluid was replaced by fluid containing additional 0.5 mM PAH. Over an experimental period of 150 min, urine samples were collected at 10-min intervals, and perfusate samples were drawn at the midpoint of each urine collection interval. Samples were analyzed by HPLC to determine PAH content, according to a previously described method (27).

Isolation of a Full-Length Human MRP2 *cDNA*

Total RNA was isolated from human Caco-2 cells with the acid guanidinium isothiocyanate-phenol-chloroform method and reverse transcribed in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, 2.5 mM dNTP mix (2.5 mM each) with 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Breda, The Netherlands) using the reverse primer MRP2-R1 (5'-CTAGAATTTTGTGCTGTTCACATT-3', stop codon underlined). For amplification of the *MRP2* cDNA, the forward primer MRP2-F1 (5'-GAATCATGCTGGAGAAGTTCTGCA-3', start codon underlined) and primer MRP2-R1 were used in a PCR reaction that used Advantage-2 Polymerase (Clontech, Leusden, The Netherlands) according to the manufacturer. The resulting PCR product (4.6 kb) was ligated into pGEM-T Easy resulting in pGTE-MRP2 (Promega, Leiden, The Netherlands). Sequencing of this human *MRP2* cDNA clone revealed six nucleotide changes compared with the published sequence (accession number NM_000392). Of these nucleotide changes, three led to the following amino acid changes: H648R, V1024A, and K1058N.

Expression of Human MRP2 and MRP4 in Sf9 Cells

For cloning of the *MRP2* cDNA into pFastBac1 (Invitrogen, Breda, The Netherlands), an *Aat*II linker fragment was introduced into the *Xba*I site of the polylinker, resulting in pFB-*Aat*II. From pGTE-MRP2, an *Aat*II-*Sal*I fragment was ligated into the *Aat*II and *Sal*I sites of pFB-*Aat*II (pFB-MRP2). *Escherichia coli* DH10Bac cells were transformed with pFB-MRP2, and recombinant bacmid DNA was isolated from positive clones. Sf9 cells were transfected with recombinant bacmid with CellFECTIN reagent (Invitrogen, Breda, The Netherlands), and after 5 d, recombinant baculovirus was harvested from the culture medium. Recombinant baculovirus was subjected to two amplification rounds in which Sf9 cells were infected with virus for 5 d before harvest. The resulting viral stock was used to infect Sf9 cells for protein production (28). Sf9 cells infected with recombinant baculovirus, expressing human MRP4, were prepared as described (13). A baculovirus containing the β-subunit of the rat gastric H^+-K^+ ATPase was used in control experiments (13). Expression of human MRP4 or MRP2 in Sf9 cells was verified with immunoblot analysis that used an affinity-purified polyclonal antibody directed against the linker region of MRP4 (amino acids 612 to 676) (13) or MRP2 (amino acids 839 to 904), respectively. Briefly, glutathione S-transferase (GST) fusion proteins were expressed in DH5α *Escherichia coli* cells and isolated by glutathione-Sepharose 4B (Amersham, Uppsala, Sweden) affinity chromatography. Rabbits were immunized and boosted three times with fusion protein (400 and 3 × 200 μg, respectively), followed by collection of serum, which was affinity purified with a column of immobilized GST and then a column of immobilized GST fusion protein.

Quantification of MRP2 and MRP4 in Renal Cortical Brush Border Membranes

Brush border membranes were isolated from a cortex sample of a healthy human kidney as described previously (13). Several identical immunoblots (data not shown), containing increasing amounts of MRP2-GST, MRP4-GST, and GST were incubated with either the MRP2 or the MRP4 antibody to estimate the GST background signal for each antibody, as well as the relative affinity of the antibodies for the epitope they are directed against. This made it possible to exclude both the background GST signal and take into account that the signal from a specific antibody reaction depends on its affinity for the epitope in question. Densitometric analysis was performed by Image Pro Plus 3.0 (Media Cybernetics, Sliver Spring, MD).

Isolation of Crude Membrane Vesicles from Sf9 and MDCKII Cells

Infected Sf9 cells were resuspended in ice-cold homogenization buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, 100 μM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM E64) and shaken on ice for 1 h. Lysed cells were centrifuged at 100,000 × *g*, and the resulting pellet was homogenized in ice-cold TS buffer (10 mM Tris-HEPES, 250 mM sucrose, pH 7.4) with a tight-fit Dounce glass tissue grinder. The homogenate was centrifuged at 500 × *g*, and

the resulting supernatant was centrifuged at 100,000 × *g*. The resulting pellet was resuspended in TS buffer and passed 30 times through a 27-gauge needle. Aliquots of crude membrane vesicles were frozen in liquid nitrogen and stored at −80°C until use.

MDCKII cells overexpressing human MRP2 were obtained from P. Borst (Dutch Cancer Institute, Amsterdam, The Netherlands). MDCKII cells were directly scraped in homogenization buffer and centrifuged at 100,000 × *g* to collect cells and debris. The pellet was homogenized in TS buffer, and the procedure described for Sf9 cells was followed.

Transport Studies

Uptake of radiolabeled substrate into membrane vesicles was performed as described (13). Briefly, membrane vesicles were prewarmed at 37°C and added to prewarmed transport buffer (10 mM $MgCl_2$, 10 mM Tris-HEPES, 250 mM sucrose, pH 7.4) supplemented with an ATP-regenerating system (4 mM ATP, 10 mM creatine phosphate, 100 $\mu g \cdot ml^{-1}$ creatine kinase) and [^{14}C]PAH. In control experiments, ATP was replaced by 5'-AMP. Samples were taken at the indicated time points, diluted in 900 μl ice-cold transport buffer to stop the reaction and filtered through GF/F glass fiber filters (Whatman, Maidstone, UK) with a sampling manifold (Millipore, Billerica, MA). Filters were dissolved in scintillation fluid and assayed for radioactivity. ATP-dependent transport was calculated by subtracting values obtained in the presence of 5'-AMP from those in the presence of ATP.

Real-Time PCR

Primers and probes for both transporters were designed by PrimerExpress Software, version 1.5 (Applied Biosystems) and checked for dimer and loop formation by OLIGO 4 (MedProbe, Oslo, Norway). The primers used were 5'-GCTCACTGGATTGTCTTCATTTTC-3', RT_MRP4_For1; 5'-CTCGGTTACATTTCCTCCTCCAT-3', RT_MRP4_Rev1; 5'-TCCATCATCCATAGCTTCATTCC-3', RT_MRP2_For1; 5'-CCTGCGAACATCGGAAAGTCT-3', RT_MRP2_Rev1. Probe sequences were TET-5'-TGCTTCAAGATTGGTGGCTTTCATACTGG-3'-TAMRA (MRP4) and TET-5'-TTACCTACAGCTGGTATGACA-3'-TAMRA (MRP2). Total RNA was isolated from 100 mg healthy human kidney cortex tissue (this sample was from a different individual as was used for the isolation of brush border membranes) with Trizol reagent. First-strand cDNA was synthesized with M-MLV reverse transcriptase in a mix containing first-strand synthesis buffer (Invitrogen, Breda, The Netherlands), 1 mM dNTP, 0,1 M DTT, 20 units RNasin (Promega, Leiden, The Netherlands), and 5 μg random hexamer primer (Amersham Biosciences, Roosendaal, The Netherlands). cDNA amplification was performed with AmpliTaq Gold DNA Polymerase (Applied Biosystems), 5 pmol of TET-labeled probe, and 10 pmol (MRP4) or 11.25 pmol (MRP2) forward and reverse primer. The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 9 s at 95°C and 1 min at 60°C. PCR reactions were analyzed with ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with software version 1.6.3. In each individual experiment, baseline and threshold parameters were set following the manufacturer's instructions. Briefly, baseline was set from cycle number 3 to two cycles before the earliest amplification. The threshold was set in the linear phase of the exponential amplification and optimized with the replicate method described by the manufacturer; threshold was adjusted so that values from the individual duplicates differed no more than 0.5 C_T. For each experiment the ratio of MRP4 to MRP2 ($n_{MRP4/MRP2}$) was calculated by the equation $n_{MRP4/MRP2} = {}_2(C_{T\text{-}MRP4} - C_{T\text{-}MRP2})$.

Statistical Analyses

Data were analyzed with GraphPad Prism version 3.03 for Windows (GraphPad Software, San Diego, CA). For statistical comparison, one-way ANOVA with Bonferroni's correction was used.

Results

To investigate the role of MRP2 in renal PAH excretion, clearance studies were done in isolated perfused kidneys from wild-type WH and TR$^-$ rats. The viability of the kidneys was assessed by measuring GFR, urine flow, fractional water reabsorption, and renal perfusion pressure (Table 1). No significant differences were observed between kidneys from WH or TR$^-$ rats. After 60 min of perfusion, the clearance of PAH corrected for GFR reached equilibrium (Figure 1), and the average value over the time period 60 to 120 min was not significantly different between WH and TR$^-$ rats (10 ± 3 *versus* 10 ± 2).

Human MRP4 has recently been localized to the apical

Table 1. Functional parameters of isolated rat kidneys perfused with medium containing 0.5 mM PAH[a]

Parameter	WH	TR$^-$
GFR ($\mu l \cdot min^{-1}$)	290 ± 40	280 ± 60
diuresis ($\mu l \cdot min^{-1}$)	21 ± 2	25 ± 9
FR_{water} (%)	92.7 ± 0.7	91.3 ± 1.6
RPP (mmHg)	77 ± 3	74 ± 2

[a] Values are presented as mean ± SD of four experiments over the period 30 to 120 min. Abbreviations: GFR, glomerular filtration rate; FR, fractional reabsorption; RPP, renal perfusion pressure.

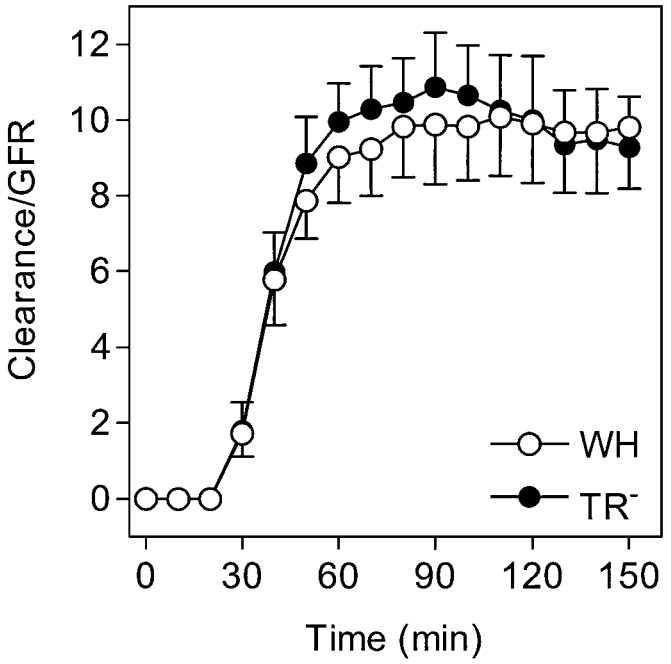

Figure 1. Renal clearance over GFR of *p*-aminohippurate (PAH) as a function of time. Kidneys from control (WH) and multidrug resistance protein (Mrp) 2–deficient (TR$^-$) rats were perfused with 0.5 mM PAH. Data are means ± SEM of four experiments.

membrane of renal proximal tubules and was shown to have a partially overlapping substrate specificity with human MRP2 (13,17,29). To investigate whether MRP4 transports PAH similar to MRP2, we analyzed uptake of [^{14}C]PAH into membrane vesicles isolated from Sf9 cells expressing human MRP4. For comparison, membrane vesicles from Sf9-MRP2 cells were used. Expression of MRP2 (160 kD) or MRP4 (140 kD) in the membrane vesicles was confirmed by immunoblot analysis (Figure 2A). ATP-dependent [^{14}C]PAH transport by both transporters was time dependent, and linear up to 15 min for MRP2 and 5 min for MRP4, with an initial transport rate of 3 and 25 pmol/mg protein per minute, respectively (Figure 2B). Furthermore, ATP-dependent [^{14}C]PAH transport was concentration dependent (Figure 3) with apparent K_m values of 160 ± 50 μM for MRP4 and 5 ± 2 mM for MRP2, and V_{max} values of 80 ± 6 and 160 ± 43 pmol/mg protein per minute, respectively. Because of the high K_m value as compared with the previously described K_m for MRP2 expressed in HEK293 cells (33), we also evaluated PAH transport in membrane vesicles isolated from MDCKII cells expressing MRP2 (30). An apparent K_m value of 2.1 ± 0.6 mM was determined for ATP-dependent [^{14}C]PAH transport in these vesicles (data not shown).

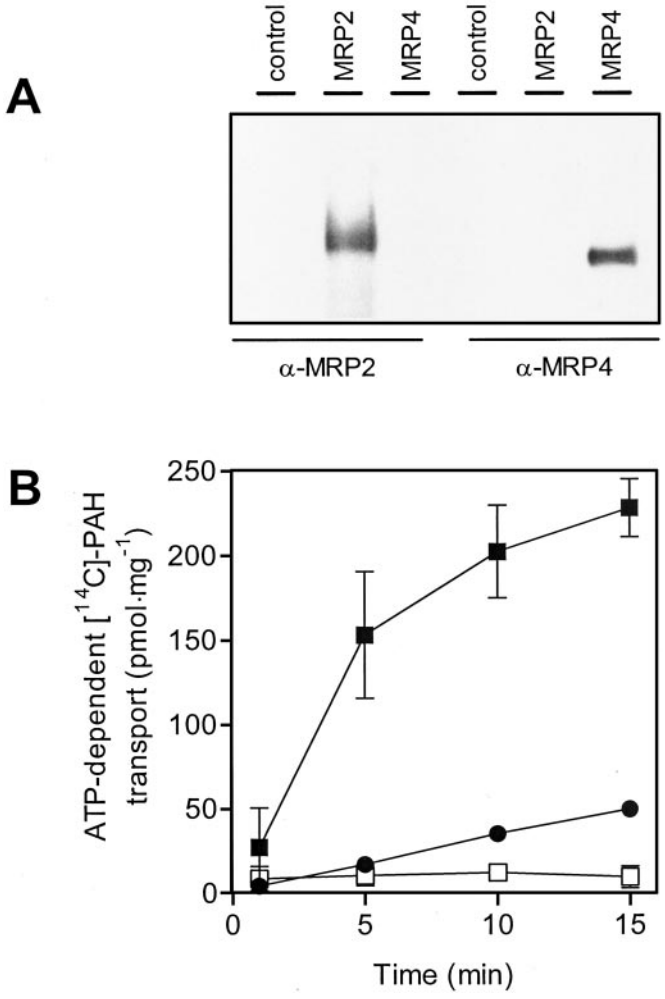

Figure 2. ATP-dependent transport of [^{14}C] *p*-aminohippurate (PAH) by human multidrug resistance protein (MRP) 4 and MRP2. (A) Sf9 cells were infected with baculovirus encoding human MRP4, MRP2, or the β-subunit of gastric H^+-K^+ ATPase (control). Crude membrane vesicles were isolated and subjected to immunoblot analysis by means of an affinity-purified polyclonal antibody raised against MRP2 or MRP4. (B) Membrane vesicles expressing MRP4 (■), MRP2 (●), or control (□) protein were incubated with 100 μM [^{14}C]PAH. Samples were taken at the indicated time points. Data are the means of three experiments ± SE performed in triplicate.

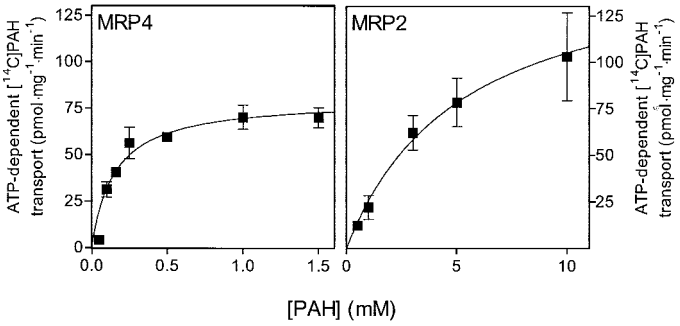

Figure 3. Kinetic analysis of multidrug resistance protein (MRP) 4- and MRP2-mediated ATP-dependent [^{14}C] *p*-aminohippurate (PAH) transport. Crude membrane vesicles from Sf9 cells infected with baculovirus encoding MRP4 or MRP2 were incubated for 5 min with increasing concentrations of [^{14}C]PAH. Data are means of three experiments ± SE performed in triplicate.

To study inhibitor specificity, various organic anions were analyzed for their effect on MRP2- and MRP4-mediated ATP-dependent [^{14}C]PAH transport. MK571 and the glucuronide conjugates of α-naphthol, *p*-nitrophenol, and phenolphthalein, which have been shown to interact with MRP2 (28), also inhibited MRP4 (Table 2 and Figure 4). Low-concentration probenecid (10 μM) stimulated MRP2- and MRP4-mediated transport (although MRP4 was not statistically significant), whereas at 1 mM, both transporters were inhibited. A similar profile of stimulation and inhibition as compared with control was observed with ATP-dependent [^{14}C]PAH transport in membrane vesicles isolated from MDCKII-MRP2 cells (data not shown). In contrast, sulfinpyrazone only stimulated MRP2, but inhibited MRP4. The MRP2 substrate indocyanine green (31) also interacted with MRP4; the MRP4 inhibitor dipyridamole (13) also inhibited MRP2 (Table 2). The purine metabolite uric acid and the nucleoside drugs AZTMP and acyclovir, but not PMEA and PMPA, inhibited MRP4 (Table 2).

Because MRP4 and MRP2 show overlap in renal localization, ability to transport PAH, and inhibitory properties, we compared their expression levels in human kidney with real-time PCR analysis and Western blot. Figure 5A shows the amplification plot *versus* the cycle number, and expression of *MRP4* mRNA was calculated to be approximately fivefold higher (5.2 ± 1.1, $n = 4$) in comparison with *MRP2* mRNA. By means of Western blot analysis of human BBM and the GST fusion proteins of MRP2 and MRP4 (used to create antibodies), we studied protein expression levels of MRP2 and MRP4 in human kidney cortex samples (Figure 5B). After

Table 2. Effect of various compounds on ATP-dependent [^{14}C]PAH transport by MRP2 and MRP4[a]

Compound	Concentration (μM)	MRP2 (% of control)	MRP4 (% of control)
No inhibitor		100[b]	100[b]
MK571	10	51 ± 20	66 ± 16
	100	10 ± 27**	47 ± 12**
	1000	1 ± 21***	12 ± 13***
Sulfinpyrazone	10	178 ± 17*	69 ± 6*
	100	119 ± 22	59 ± 4***
Dipyridamole	1	42 ± 19*	103 ± 6
	25	68 ± 21	50 ± 5***
Probenecid	10	170 ± 15*	121 ± 8
	100	121 ± 18	107 ± 4
	1000	68 ± 14	53 ± 2***
Uric acid	1000	n.d.	70 ± 5*
PMPA	1000	n.d.	88 ± 5
PMEA	1000	n.d.	71 ± 9
AZTMP	100	n.d.	59 ± 4***
Acyclovir	100	n.d.	39 ± 12***
Indocyanine green	100	n.d.	8 ± 4***

[a] Sf9-MRP4 and Sf9-MRP2 crude membrane vesicles were incubated for 5 min with 100 μM [^{14}C]PAH, in the absence (control) or presence of various compounds. Data are means ± SE from at least three experiments performed in triplicate. Results were tested using one-way ANOVA with Bonferroni's correction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* control (no inhibitor). Abbreviations: MK571, (E)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethyl amino)-3-oxopropyl]thio]methyl]thio]-propanoic acid; PMEA, 9-(2-phosphorylmethoxyethyl) adenine; PMPA, (R)-9-(2)-phosphonylmethoxypropyl) adenine; AZTMP, 3′-azido-2′,3′-dideoxythymidine-5′-monophosphate; acyclovir, 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6H-purin-6-one, n.d., not determined.

[b] Transport rates in the absence of inhibitor: 2.5 ± 0.3 (MRP2) and 8.0 ± 0.5 pmol · mg^{-1} · min^{-1} (MRP4).

correction for background GST signal and the difference in affinity of the antibodies, the ratio of MRP4 *versus* MRP2 was estimated to be 4.8 ± 1.7 ($n = 3$).

Discussion

Renal tubular secretion of the classical organic anion PAH is a two-step process, comprising concentrative uptake from blood at the basolateral membrane into the proximal tubule, followed by efflux across the apical membrane into the urine. MRP2 has recently been identified as an apical export pump in the kidney with transport affinity for PAH, but in this study, we show that excretion of PAH in Mrp2-deficient (TR$^-$) rat kidney is not different from wild-type rat kidney. Furthermore, we show that human MRP2 expressed in Sf9 cells exhibits a low affinity for PAH (K_m of approximately 5 mM), which is lower than previously reported values for rabbit Mrp2 (K_m of approximately 2 mM) (32) and human MRP2 (K_m approximately 1 mM) (33). Possible explanations for this can be the expression system used or the nucleotide changes in our clone. By means of an independent MRP2 clone expressed in MDCK cells, we found a K_m value (approximately 2 mM) closer to the previously described values, but a similar profile of stimulation and inhibition by other compounds as with Sf9-MRP2.

Our results show that MRP4, which has recently been localized to the apical membrane next to MRP2 (13), transports PAH with a much higher affinity than MRP2. In addition, *MRP4* transcript is expressed fivefold higher in human kidney cortex compared with *MRP2* and a similar ratio was observed for the expression of MRP4 protein in cortical brush border membranes as compared with MRP2. During completion of our study, two other groups reported that mRNA expression of *MRP4* is higher than *MRP2* in human (34) and rat kidney (35,36). On the basis of its higher expression on both mRNA and protein level and higher affinity for PAH, we propose that MRP4 has more influence on renal PAH excretion than MRP2. Although this also seems to provide a logical explanation for the lack of PAH transport characteristics in the TR$^-$ rat, we cannot exclude the notion that another compensatory mechanism for PAH transport is present in the kidney other than MRP4 or that the reserve capacity of both transporters for PAH is large enough to compensate in the event of the loss of one transporter.

In the liver, MRP2 is crucial for the biliary excretion of organic anions and its absence in humans and rats leads to inherited jaundice (24). However, renal function seems unimpaired in TR$^-$ rats, which may be explained by a relatively minor role for Mrp2 in renal physiology as a result of the presence of various other compensatory organic anion transporters. On the other hand, we recently found that renal excretion of certain exogenous bulky organic anions in TR$^-$ kidney is either reduced (calcein, fluo-3) or delayed (lucifer yellow) (26). Furthermore, under the influence of drug treatment, MRP2 function may be altered. We found that ATP-dependent PAH transport by human MRP2 is stimulated by probenecid and sulfinpyrazone, which is in agreement with previous findings that used estradiol-17β-D-glucuronide (37), N-ethylmaleimide glutathione (38), or saquinavir as a substrate (39). Furthermore, expression of Mrp2 in rat kidney is induced upon treatment with cisplatin or dexamethasone (40,41). This indicates that during drug treatment, the role of MRP2 in renal organic anion excretion may be enhanced through induction of protein expression and/or function.

The phenomenon of stimulation of MRP2- and MRP4-mediated PAH transport by other compounds at low concentration and inhibition at higher concentrations can be explained by the existence of at least two independent binding sites: one site that transports PAH, and another site that is able to allosterically stimulate the transport site. At low concentration, the compound enhances PAH transport by binding predominantly to the stimulatory site, whereas at higher concentrations, it inhibits transport through competition with the transporter site. This type of allosteric interaction seems a general characteristic of MRP and was described in more detail for MRP2 and MRP3 (42,43).

Human MRP4 transports a number of MRP2 substrates, such as methotrexate (13,29), estradiol-17β-D-glucuronide (13,17), and PAH (this study). Our finding that the glucuronide

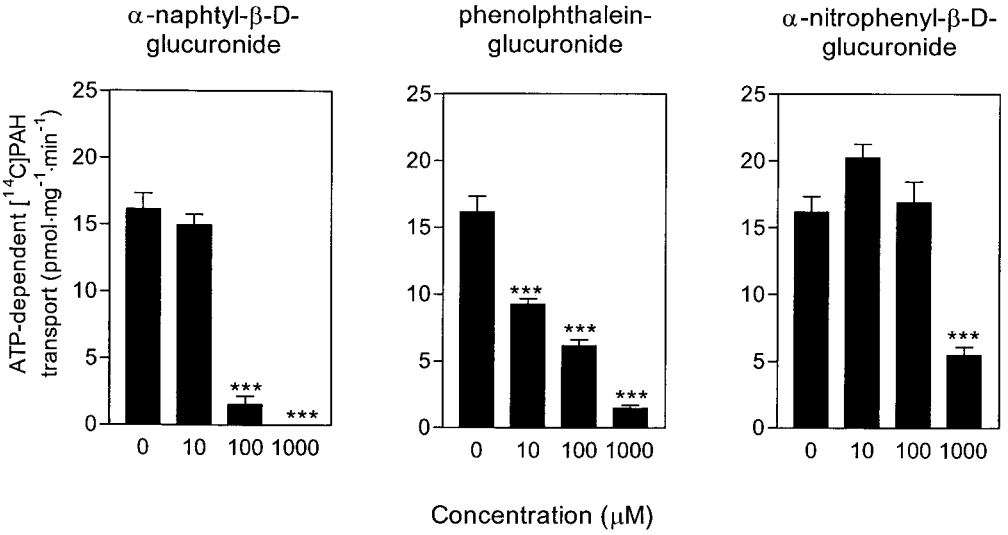

Figure 4. Effect of glucuronide conjugates on ATP-dependent [^{14}C] *p*-aminohippurate (PAH) transport by multidrug resistance protein (MRP) 4. Sf9-MRP4 crude membrane vesicles were incubated with 100 μM [^{14}C]PAH and 10, 100, or 1000 μM α-naphthyl-β-D-glucuronide, phenolphthalein-glucuronide, or *p*-nitrophenyl-β-D-glucuronide for 10 min. Data are means ± SEM of a representative experiment performed in triplicate. Results were tested by one-way ANOVA with Bonferroni's correction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* control (no inhibitor).

conjugates of α-naphthol, *p*-nitrophenol, and phenolphthalein interact with MRP4 and that renal excretion of α-naphthyl-β-D-glucuronide is not reduced in TR$^-$ kidney (44) suggests a role for MRP4 in the renal excretion of these conjugates. The substrate specificity of MRP4 can be distinguished from that of MRP2 by its ability to transport nucleosides and prostaglandins (13,16–21). The renal excretion of prostaglandins is inhibited by probenecid, indomethacin, and PAH (45), which all interact with MRP4, suggesting a role for MRP4 in urinary export of these hormones (19). However, inhibition of the basolateral uptake of substrates may also account for a decreased apical excretion.

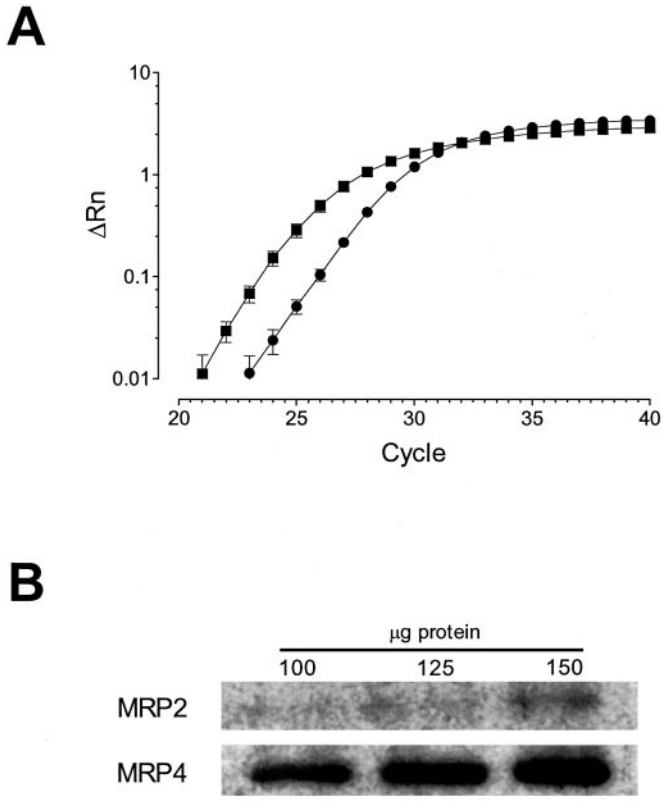

Figure 5. (A) Quantitative expression analysis of *MRP2* and *MRP4* mRNA in human kidney. RNA isolated from a human kidney cortex sample was reverse transcribed and amplified with specific primers and probes for detection of *MRP2* (●) and *MRP4* (■). Data are the means ± SEM of four experiments performed in duplicate. (B) Immunoblot showing MRP2 and MRP4 protein in human brush-border membranes. Membranes were isolated from a different human kidney cortex sample as for mRNA analysis, and 100, 125, and 150 μg of protein was used. MRP, multidrug resistance protein.

Various organic anion transporters with affinity for PAH have been identified in the apical membrane of renal proximal tubule cells, including MRP2, MRP4, the antiporters NPT1 (11) and OAT4 (46), and the voltage-dependent channel Oat_v1 (8). The large number of different transporters with overlapping substrate specificity emphasizes the importance of renal organic anion excretion for the body. As compared with MRP2, MRP4 may contribute to a larger extent to the renal excretion of PAH, but its specific role relative to the other apical organic anion transporters remains to be elucidated. To study this, (multiple) knockout models of the genes encoding these proteins may be required because of the high redundancy within this group of transporters.

In summary, we have shown that MRP4 is a novel renal PAH efflux transporter, which, in comparison with MRP2, transports PAH with higher affinity and is expressed at a higher level in kidney.

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