Human organic anion transporter MRP4 (ABCC4) is an efflux pump for the purine end metabolite urate with multiple allosteric substrate binding sites

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Human organic anion transporter MRP4 (ABCC4) is an efflux pump for the purine end metabolite urate with multiple allosteric substrate binding sites. Am J Physiol Renal Physiol 288: F327–F333, 2005. First published September 28, 2004; doi:10.1152/ajprenal.00133.2004.—The end product of human purine metabolism is urate, which is produced primarily in the liver and excreted by the kidney through a well-defined basolateral blood-to-cell uptake step. However, the apical cell-to-urine efflux mechanism is as yet unidentified. Here, we show that the renal apical organic anion efflux transporter human multidrug resistance protein 4 (MRP4), but not apical MRP2, mediates ATP-dependent urate transport via a positive cooperative mechanism (K_m of 1.5 ± 0.3 mM, V_max of 47 ± 7 pmol·mg^{-1}·min^{-1}, and Hill coefficient of 1.7 ± 0.2). In HEK293 cells overexpressing MRP4, intracellular urate levels were lower than in control cells. Urate inhibited methotrexate transport (IC_{50} of 235 ± 8 µM) by MRP4, did not affect cAMP transport, whereas cGMP transport was stimulated. Urate shifted cGMP transport by MRP4 from positive cooperativity (K_m and V_max value of 180 ± 20 µM and 58 ± 4 pmol·mg^{-1}·min^{-1}, respectively, Hill coefficient of 1.4 ± 0.1) to single binding site kinetics (K_m and V_max value of 2.2 ± 0.9 mM and 280 ± 50 pmol·mg^{-1}·min^{-1}, respectively). Finally, MRP4 could transport urate simultaneously with cAMP or cGMP. We conclude that human MRP4 is a unidirectional efflux pump for urate with multiple allosteric substrate binding sites. We propose MRP4 as a candidate transporter for urinary urate excretion and suggest that MRP4 may also mediate hepatic export of urate into the circulation, because of its basolateral expression in the liver.

Under normal conditions, urate is filtered at the kidney glomerulus, where it is almost completely reabsorbed (>90%) from the urine through the organic anion transporter URAT1 (8). Patients with inherited hypouricemia reabsorb <10% of urinary urate and have been shown to exhibit a nonfunctional URAT1 protein due to a mutation in the URAT1 coding sequence (8). Early studies with URAT1-defective patients have indicated the existence of a urate excretion pathway exceeding the glomerular filtration rate (17). Since proximal tubules hardly produce urate, a vectorial transport process from blood to urine must be involved. The first step most likely is mediated by the basolateral organic anion uptake transporters OAT1 and OAT3, which have recently been shown to transport urate (4, 13). A voltage-dependent urate antipporter has been suggested to participate in the urinary efflux, based on studies with human proximal tubule apical membrane vesicles (29). The human apical organic anion transporters NPT1 and hUAT1 have been proposed for this function (21, 38). However, the transport properties of NPT1 do not fit with the results from membrane vesicle studies, and there is no direct evidence that hUAT1 transports urate. So far, a candidate for the efflux step in human urinary urate excretion has not been identified.

Studies with mouse L1210 cells have identified multiple ATP-dependent export pumps for methotrexate (MTX), sharing urate as a substrate (12). Furthermore, in human erythrocytes an ATP-dependent urate transporter has been described (22). The characteristics of these transporters resemble members those of the multidrug resistance protein (MRP) family, of which MRP1, MRP4, and MRP5 are expressed in erythrocytes (18). MTX is a substrate for MRPs 1–4 (3, 20), and various endogenous (cAMP and cGMP) and xenobiotic nucleosides are substrates for MRP4 (6, 25, 40, 42, 43), MRP5 (15, 25, 42–44), and MRP8 (10). Since MRP2 and MRP4 are the only members expressed at the apical membrane of kidney proximal tubules (32, 40), we investigated the interaction of urate with MRP2 and MRP4. In this study, we show that human MRP4 mediates ATP-dependent urate transport through multiple allosteric binding sites, whereas urate is not a substrate for human MRP2.

EXPERIMENTAL PROCEDURES

Materials. [1^{4}C]urate (53 Ci/mol), [2,8-^{3}H]cAMP (21.9 Ci/mmol), [8-^{3}H]cGMP (6.8 Ci/mmol), [3^{H}]MTX (15 Ci/mmol), and Bis(piv-aloxyoxymethyl)-9-(2-phosphonylmethoxyethyl)adenine (Bis-POMMAE) were from Moravek Biochemicals (Brea, CA). [3^{H}]PAH (3.25 Ci/mmol) was from New England Nuclear (Boston, MA).

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Creatine phosphate and creatine kinase were from Boehringer Mannheim (Almere, The Netherlands). All other chemicals were from Sigma (Zwijndrecht, The Netherlands).

Cell lines. Suspension cultures of Spodoptera frugiperda (Sf9) cells (100-ml spinner flasks) were infected with a recombinant baculovirus expressing human MRP2, MRP4 or, as a control, Opsine as described previously (41). HEK293 cells overexpressing human MRP4 (HEK293/4.63) (26) and HEK293/4.63 cells stably expressing an MRP4-specific RNA interference (RNAi) construct (HEK293/RNAi-53) (26) were obtained from P. Borst (Dutch Cancer Institute, Amsterdam, The Netherlands). The knock-down of MRP4 in these cells is not complete, but MRP4 levels are strongly decreased and comparable to parental HEK293 cells (26). Cells were grown in DMEM supplemented with 10% fetal calf serum and 100 U penicillin/streptomycin/ml at 37°C under 5% CO2-humidified air.

Isolation of membrane vesicles from Sf9 cells. Sf9 cells expressing human MRP2, MRP4, or opsin (control) 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 mM E64) and shaken on ice for 1 h. Lysed cells were centrifuged at 100,000 g for 1 h, and the resulting pellet was homogenized in ice-cold TS buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4) using a tightly fit douncer. The homogenate was centrifuged at 500 g for 10 min, and the resulting supernatant was centrifuged at 100,000 g for 1 h. 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. The uptake of various substrates into membrane vesicles was studied using a rapid filtration method as described previously (41). Sidedness of the isolated membrane vesicles was assessed as described previously (41) and revealed an equal distribution of inside-out and right-side-out configuration.

Fig. 1. Urate is a substrate for human multidrug resistance protein 4 (MRP4) but not MRP2. A: transport of [14C]urate (50 μM) in membrane vesicles from Spodoptera frugiperda (Sf9) cells expressing MRP4 in the presence of 5′-AMP or ATP. B: ATP-dependent transport of [14C]urate (50 μM) in membrane vesicles from Sf9 cells expressing MRP4 or opsin (control). C and D: ATP-dependent transport of [14C]urate at a concentration of 200 μM (C) or 1 mM (D) for 20 min in membrane vesicles from Sf9 cells expressing MRP4, MRP2, or opsin (control). All values are the means ± SE of 3 experiments performed in triplicate. *P < 0.05 compared with control and MRP2.
500 μM [¹⁴C]urate or [³H]PAH/well at 37°C. At the indicated time points, the medium was removed and the cells were washed twice with ice-cold HBSS. Cells were solubilized in 1 M NaOH, neutralized with HCl, and the associated radioactivity was quantified in a liquid scintillation counter.

Kinetic analysis. Curve fitting was done by nonlinear regression analysis with the computer program GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA). Values are presented as means ± SE. To determine allostery, data from concentration-dependent transport assays were analyzed according to the Hill equation

\[
v = \frac{V_{\text{max}} \cdot [S]^n}{[K_m]^n + [S]^n}
\]

where \(v\) = rate, \(V_{\text{max}}\) = maximum velocity, \([S]\) = initial substrate concentration, \(K_m\) = the substrate concentration at half-maximum velocity, and \(n = \text{Hill coefficient}\). The data were then compared with a fit to a one-binding site version of Eq. 1 (\(n = 1\)), the Michaelis-Menten equation.

To decide which of the two models best fit the data, the extra sum-of-squares \(F\)-test was used. For visual inspection, Hill plots were generated according to the following equation, the slope of which represents the Hill coefficient

\[
\log\left(\frac{v}{V_{\text{max}} - v}\right) = n \log [S] - n \log [K_m]
\]

RESULTS

MRP2 and MRP4 are both localized to the apical membrane of renal proximal tubules and are candidate transporters for urate. To assess the interaction with urate, transport was investigated from membrane vesicles from Sf9 cells expressing human MRP2, MRP4, or opsin (control). In the presence of ATP, transport of 50 μM [¹⁴C]urate in Sf9-MRP4 membrane vesicles increased with time, although there was a small uptake of [¹⁴C]urate in the presence of 5'-AMP (Fig. 1A). ATP-dependent [¹⁴C]urate transport in Sf9-MRP4 membrane vesicles was time dependent and about twofold higher compared with control membrane vesicles, which exhibited a profound endogenous transport (Fig. 1B). We observed a similar difference between control and Sf9-MRP4 membrane vesicles for ATP-dependent transport of [¹⁴C]urate at 0.2 (Fig. 1C) and 1 mM (Fig. 1D). ATP-dependent [¹⁴C]urate transport in Sf9-MRP2 membrane vesicles was not significantly different from control either at a low (not shown) or high (0.2 and 1 mM) urate concentration (Fig. 1, C and D).

ATP-dependent [¹⁴C]urate transport in control- and MRP4-expressing membrane vesicles increased with increasing urate concentrations (Fig. 2A). Due to the limited solubility of urate in TS-buffer, we were unable to use concentrations >2.5 mM. Although saturation did not occur under this condition, over-

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**Table 1. Kinetic parameters of MRP4-mediated, ATP-dependent transport of urate and cGMP**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m), μM</th>
<th>(V_{\text{max}}), pmol/mg⁻¹·min⁻¹</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[¹⁴C]urate</td>
<td>1,550±270</td>
<td>47±7</td>
<td>1.7±0.2*</td>
</tr>
<tr>
<td>[³H]cGMP</td>
<td>180±20</td>
<td>58±4</td>
<td>1.4±0.1*</td>
</tr>
<tr>
<td>[³H]cGMP + urate</td>
<td>2,180±860</td>
<td>276±52</td>
<td>0.9±0.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 separate experiments. MRP, multidrug resistance protein. The kinetic parameters of MRP4-mediated, ATP-dependent transport of [¹⁴C]urate and [³H]cGMP, in the absence or presence of urate, were determined as described in Figs. 2B and 5. Data points were fitted to the Hill equation to obtain values for \(K_m\), \(V_{\text{max}}\), and the Hill coefficient (\(n\)). *Significantly better fit compared with a single binding site with \(n=1\) (\(F\)-test, \(P<0.05\)). †Significantly different from unity.

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**Fig. 2.** Kinetic analysis of urate transport by MRP4. A: ATP-dependent transport of [¹⁴C]urate (0.05–2.5 mM) for 5 min in membrane vesicles from Sf9 cells expressing MRP4 (●) or opsin (○; control). B: MRP4-specific ATP-dependent [¹⁴C]urate transport (0.05–2.5 mM) fit to the Hill equation. The solid curve represents positive cooperative allostery (\(n = 1.7 ± 0.2\)), and the dotted curve a 1-binding site (\(n = 1\)) interaction. C: Hill plot of the data in B where the slope represents the Hill coefficient. Values are means ± SE of representative experiments performed in triplicate.
expression of MRP4 clearly increased ATP-dependent $[^{14}C]$urate transport compared with control. After correction for endogenous ATP-dependent $[^{14}C]$urate transport, MRP4 showed a positive cooperative allosteric interaction with urate (Fig. 2B). The curve for an allosteric interaction was significantly better compared with a simple single-binding site model (Table 1). Positive allosterism was also visualized by the slope of the Hill plot, which was significantly $>1$ (Fig. 2C). This indicates that MRP4 exhibits more than one positively cooperating binding site for urate.

To investigate the interaction of urate with MRP4 at the cellular level, HEK293 cells overexpressing MRP4 (HEK293/4.63) and stably MRP4 RNAi-transfected HEK293/4.63 cells (HEK293/RNAi-53) were preloaded with $[^{14}C]$urate, and subsequently intracellular $[^{14}C]$urate was determined with time. Such an approach has recently been conducted to show that MRP4 reduces intracellular prostaglandins after cell preloading and therefore mediates prostaglandin efflux (26). As shown in Fig. 3A, intracellular $[^{14}C]$urate was lower in HEK293/4.63 cells compared with HEK293/RNAi-53 cells. Similarly, intra-

![Fig. 4](http://ajprenal.physiology.org/)

**Fig. 4. Effect of urate on methotrexate (MTX), cAMP, and cGMP transport by MRP4.** A: ATP-dependent transport (pmol·mg protein$^{-1}$·min$^{-1}$) of 1 mM $[^{3}H]$MTX, 1 mM $[^{3}H]$cAMP or 10 mM $[^{3}H]$cGMP by MRP4 was determined in the absence (control) or presence of various concentrations of urate for 5 min. Data are expressed as means ± SE of 2 determinations in triplicate. B, MRP4-mediated, ATP-dependent transport (pmol·mg protein$^{-1}$·min$^{-1}$) of 1 mM $[^{3}H]$cGMP or $[^{14}C]$urate alone, or 1 mM $[^{3}H]$cGMP in the presence of 1 mM $[^{14}C]$urate (cis) for 5 min. ATP-dependent $[^{3}H]$cGMP transport was also determined for 5 min after preincubation with 1 mM unlabeled urate for 15 min (trans). *$P < 0.05$ compared with ATP-dependent $[^{3}H]$cGMP transport in the absence of urate.
cellular \([^{3}H]PAH\), a recently discovered MRP4 substrate (35), was lower in HEK293/4.63 cells compared with HEK293/RNAi-53 cells (Fig. 3B).

Since reabsorption of urate in the renal proximal tubule may lead to high intracellular urate concentrations, we investigated the effect of urate on transport of the endogenous nucleosides and MRP4 substrates cAMP and cGMP as well as the MRP4 substrate MTX. Urate inhibited ATP-dependent \([^{3}H]MTX\) transport by MRP4 in a concentration-dependent fashion with an apparent IC\(_{50}\) of 235 ± 8 \(\mu\)M, whereas urate had no effect on ATP-dependent \([^{3}H]cAMP\) transport (Fig. 4A). In contrast, ATP-dependent \([^{3}H]cGMP\) transport by MRP4 was stimulated 1.5-fold at the highest concentrations tested (Fig. 4A). Since intracellular levels of these nucleosides can rise up to millimolar concentrations (7, 9, 23, 36), we further investigated the interaction between urate and cAMP or cGMP under saturated conditions (Fig. 4B). ATP-dependent transport of 1 mM \([^{3}H]cAMP\) by MRP4 in the presence of 1 mM \([^{14}C]urate\) occurred at a similar transport rate compared with that of \([^{3}H]cAMP\) or \([^{14}C]urate\) alone (not shown). ATP-dependent transport of 1 mM \([^{3}H]cGMP\) by MRP4 was cis-stimulated by 1 mM \([^{14}C]urate\) from 35 ± 2 to 64 ± 4 pmol mg\(^{-1}\) min\(^{-1}\), whereas ATP-dependent \([^{14}C]urate\) transport remained unaffected in the presence of \([^{3}H]cGMP\). ATP-dependent transport of 1 mM \([^{3}H]cGMP\) by MRP4 was also trans-stimulated by 1 mM urate.

To further explore the effect of urate on ATP-dependent cGMP transport by MRP4, we analyzed concentration-dependent \([^{3}H]cGMP\) transport in the absence and presence of 1 mM urate (Fig. 5). In the absence of urate, MRP4 showed a positive cooperative allosteric interaction with cGMP. The curve for an allosteric interaction was significantly better compared with a single-binding site model (Table 1). In the presence of 1 mM urate, MRP4 showed a single-binding site interaction with cGMP with a 12-fold decreased affinity but a 5-fold increased \(V_{\text{max}}\) (Fig. 5 and Table 1). Furthermore, the Hill plot shifted from sigmoidal (significantly better fit compared with linear regression, \(F\)-test \(P < 0.05\)) to linear (Fig. 5).

**DISCUSSION**

Urate transport in the kidney proximal tubule is a bidirectional process. Although the urate reabsorption pathway has been elucidated recently, there is little knowledge about the excretory route. The basolateral organic anion transporters OAT1 and OAT3 presumably mediate uptake from blood into the cell, but for apical export from the cell into urine no candidate transporter has been identified. In this study, we show that the apical organic anion transporter MRP4 mediates ATP-dependent urate transport in isolated membrane vesicles and exports urate from transfected cells. Furthermore, the complex interaction pattern of urate with the MRP4 substrates MTX, cAMP, and cGMP indicates that MRP4 is an organic anion transporter with multiple allosteric binding sites.

Isolated apical and basolateral membrane vesicles from kidney proximal tubule have been used widely to study organic anion transport mechanisms, mainly using PAH or urate as a substrate (31). In contrast to basolateral membrane vesicles, results from studies with apical membrane vesicles so far have been difficult to match with cloned urate transporters, not in the least due to differences between species. Human, rat, monkey, and most breeds of dogs favor urate reabsorption, mediated by URAT1 (28). In contrast, rabbit, pig, snake, and Dalmatians are urate-excreting species, probably due to the lack of URAT1 (28). Nevertheless, all species exhibit an apical voltage-dependent urate efflux transporter, which might be represented by the recently cloned pig OAT1 (16). However, a human OAT,1 ortholog does not seem to exist, and human NPT1 does not qualify based on its transport properties. OAT4 may be a candidate, but it is unknown whether urate is a substrate (5). Completely opposite to the expectations of previous membrane vesicle studies, two ATP-dependent organic anion transporters, MRP2 and MRP4, were reported to be expressed at the apical membrane of renal proximal tubules (32, 40). In this study, we show that MRP4, but not MRP2, is a low-affinity, ATP-dependent export pump for urate. Our finding is in agreement with previous reports on the presence of an ATP-dependent urate transporter in erythrocytes as well as in the L1210 cell line. We propose that MRP4, as an apical export pathway in the kidney, requires a low affinity for urate due to the high intracellular urate concentrations.

Our study with HEK293 cells indicated a substantial efflux of urate, which was enhanced by overexpression of MRP4. At this point, we do not know the nature of the endogenous transporter(s), but MRP5 or MRP8 may be possible candidates. Endogenous expression of MRP5 in HEK293 cells has been documented, and the release of urate from HEK293/RNAi-53
cells was inhibited by PMEA (Van Aubel RAMH, unpublished observations), which is a substrate for MRP5 and MRP8 (10, 44). Evidence of an endogenous ATP-dependent urate transporter was also found in Sf9 cells. Although there are very limited data available on genomic sequences of Sf9 cells, an MRP-like transporter might be involved since we previously identified a profound ATP-dependent PAH transporter in these cells (39).

MRPs exhibit a broad substrate specificity, and multiple binding sites may play a role. In this study, we found that MRP4 mediates low-affinity, ATP-dependent transport of urate through a positive, cooperative allosteric interaction. Furthermore, urate inhibited MTX transport, stimulated cGMP transport, but had no effect on cAMP transport by MRP4. In addition to urate, cGMP transport by MRP4 is of low affinity and is also characterized by positive cooperativity. In line with our results, ATP-dependent cGMP transport in erythrocyte membrane vesicles, which probably is primarily mediated by MRP4, also displays an allosteric interaction (18). Furthermore, the reported affinity for ATP-dependent cGMP transport ($K_m$ 170 ± 50 μM) is in the same range as reported in this study for recombinant MRP4 ($K_m$ 180 ± 20 μM). Chen et al. (6) might have overestimated the affinity for cGMP ($K_m$ 10 ± 2 μM) by limiting their concentration range to 25 μM, whereas we used cGMP concentrations up to 1–3 mM.

Urate stimulated MRP4-mediated cGMP transport by increasing the $V_{max}$. From the Hill coefficients, we conclude that urate changes cGMP transport from an allosteric binding to a single binding site, suggesting that a cGMP binding site is displaced by urate. In a similar fashion, indomethacin and sulfaniltrate change estradiol-17-β-d-glucoronide transport by MRP2 from an allosteric to a single binding component (2, 46). In contrast to urate, the Hill plot for cGMP transport was sigmoidal, indicating that multiple cGMP binding sites are not occupied simultaneously, as described for oxygen binding to hemoglobin (19, 45).

The presence of multiple substrate binding sites for MRP4 also became apparent from our studies with two labeled substrates. Whereas cAMP and cGMP interact with each other’s transport (40), both compounds can be transported by MRP4 simultaneously with urate, even under saturated conditions. Furthermore, the effect of urate on cGMP transport likely is a trans-stimulatory mechanism, since preincubation of membrane vesicles with urate showed a similar stimulation of cGMP transport compared with cis-incubation. This suggests that under conditions of high intracellular cAMP or cGMP, MRP4-mediated urate efflux is unaffected and that extracellular urate may influence the transport properties of MRP4. However, it remains to be investigated whether stimulation of cellular cAMP or cGMP production also influences MRP4 function itself, e.g., by phosphorylation.

The interactions found for MRP4 are complex, and more than two distinct binding sites are required to explain our observations. The presence of multiple binding sites is consistent with findings with other multidrug resistance transporters, like MRP2 (2, 14, 46) and MDR1/P-glycoprotein (33). Analogous to MDR1 we propose a working model of two independent transport sites, one for cGMP and one for urate, and an allosteric site shared by cGMP and urate, which interacts in a positively cooperative manner with both transport sites. Through this allosteric site, cGMP and urate are able to stimulate their own transport sites. Apparently, urate displaces cGMP from the allosteric site, explaining why cGMP transport can be described by a single binding site in the presence of urate (Fig. 5) and why cGMP had no influence on urate transport (Fig. 4B). However, the increase in $V_{max}$ and decrease in affinity for cGMP by urate are rather unusual, as well as the sigmoidal Hill plot for cGMP transport alone, indicating a much more complex interaction than can be described by our working model. cAMP probably shares the cGMP transport site, but not the urate site, which is consistent with the finding that urate had no influence on cAMP transport, whereas cAMP is known to inhibit cGMP transport (40). Apparently, the inhibition of urate on MTX transport indicates that MTX is a substrate for the urate site of MRP4. It is clear that our working model is still incomplete, and further studies are needed to gain more insight into the nature of the different binding sites and the kinetics of their mutual interaction.

In summary, we show that human MRP4 mediates low-affinity ATP-dependent urate transport, whereas urate is not a substrate for human MRP2. Urate is able to activate its own transport, and a complex interaction pattern was observed with the MRP4 substrates cGMP, cAMP, and MTX, indicative of multiple allosteric binding sites. We propose MRP4 as a candidate transporter for urinary urate excretion and, given its basolateral expression in the liver (27), MRP4 may provide the blood circulation with urate through hepatic export.

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