Basalateral LAT-2 Has a Major Role in the Transepithelial Flux of L-Cystine in the Renal Proximal Tubule Cell Line OK

Abstract. During renal reabsorption, the amino acid transporters bo,+ and y,L have a major role in the apical uptake of cystine and dibasic amino acids and in the basolateral efflux of dibasic amino acids, respectively. In contrast, the transporters responsible for the basolateral efflux of the apically transported cystine are unknown. This study shows the expression of system L and y,L transport activities in the basolateral domain of the proximal tubule-derived cell line OK and the cloning of the corresponding LAT-2 and y,L-LAT-1 cDNAs. Stable transfection with a LAT-2 antisense sequence demonstrated the specific role of LAT-2 in the basolateral system L amino acid exchange activity in OK cells. This partial reduction of LAT-2 expression decreased apical-to-basolateral trans-epithelial flux of cystine and resulted in a twofold to threefold increase in the intracellular content of cysteine. In contrast, the content of serine, threonine, and alanine showed a tendency to decrease, whereas other LAT-2 substrates were not affected. This demonstrates that LAT-2 plays a major specific role in the net basolateral efflux of cysteine and points to LAT-2 as a candidate gene to modulate cystine reabsorption.

In the kidney, most glomerulus-filtered amino acids are reabsorbed in the early proximal tubule (1). The amino acids are taken up through the brush border membrane and exit the epithelial cells through the basolateral membrane into the interstitial space. Two heteromeric amino acid transport exchangers (systems bo,+ and y,L) have a major role in the reabsorption of cystine and dibasic amino acids (2–4). First, the heterodimer formed by rBAT and bo,+AT is the amino acid transporter bo,+ which mediates high-affinity uptake of cystine and dibasic amino acids coupled with the influx of neutral amino acids (5–8) at the apical membrane of epithelial cells of the proximal tubule (9). Indeed, mutations in the rBAT (SLC3A1) gene cause type I cystinuria, and mutations in the bo,+AT (SLC7A9) gene cause mainly non-type I and also type I cystinuria (recessive inherited aminoacidurias of cystine and dibasic amino acids) (6,10,11). Second, y,L-LAT-1 dimerizes with 4F2hc to form the amino acid transporter y,L which mediates the efflux of cationic amino acids coupled with the influx of neutral amino acids plus sodium (12–14). Mutations in y,L-LAT-1 (SLC7A7) cause lysinuric protein intolerance (15,16), an inherited aminoaciduria due to defective dibasic amino acid efflux from the basolateral membrane of proximal tubule epithelial cells (17). Thus, systems bo,+ and y,L explain the trans-epithelial transport of dibasic amino acids. In contrast, the amino acid transporters that play a major role in the basolateral efflux of the apically taken-up cystine remain to be identified.

Polarized OK cells, a proximal tubule-derived cell line from the American opossum, has been extensively used as a model for transport studies in renal epithelial cells. Indeed, OK cells express rBAT-associated system bo,+ transport activity in the apical pole (18). In these cells, like it is believed to occur during renal reabsorption (1), taken-up cystine is reduced to cysteine for efflux (19,20). LAT-2 is expressed in the basolateral plasma membrane of the renal epithelial cells of the upper part of the proximal tubule (S1 and S2 segments) (21), where most of amino acid reabsorption occurs (1). LAT-2/4F2hc co-expressed in Xenopus oocytes show exchange of neutral amino acids of any size (21–24). These characteristics suggest that LAT-2 may play a role in renal reabsorption, but which amino acids use this transporter for net efflux or influx in the context of a renal epithelial cell is unknown.

In the present study, we have demonstrated the functional expression of systems L and y,L in the basolateral domain of polarized OK cells and cloned opossum LAT-2 and y,L-LAT-1 cDNAs. We used an antisense strategy to elucidate the role of LAT-2 in the trans-epithelial flux of amino acids. The diminished basolateral LAT-2 transport activity resulted in a substantial reduction of cystine trans-epithelial transport and in a specific increase in the intracellular cysteine content. In contrast, the content of other amino acid substrates of LAT-2 tended to decrease or remained unaltered. Our results strongly support a major role of LAT-2/4F2hc transport activity in the renal reabsorption of cystine.
Materials and Methods

Cell Culture

OK cell line (25) clone 3B/2, between passages 5 and 30, was subcloned from the parental line for its better adaptability to a low phosphate medium and inhibition of phosphate transport by parathyroid hormone. Cells were grown in Dulbecco modified Eagle medium/Nutrition Mix F-12 medium supplemented with 10% fetal calf serum, as described (18). Subcultures were prepared by trypsinization and reseeding at high density (approximately 1 × 10⁶ cells/ml). For transport polarity studies, OK cells were seeded (4 × 10⁵ cells/filter) on polycarbonate filters (12-mm diameter, 3-mm pore size; Costar). Before seeding the cells, each polycarbonate filter was pretreated with growth medium (without fetal calf serum) containing 2 mg of collagen type I from rat-tail (Upstate Biotechnology). After 16 to 20 h of incubation at 37°C, the collagen was aspirated and filters were allowed to dry. The cells were cultured in standard conditions. The formation of polarized monolayers was assessed by measuring trans-epithelial resistance with Millicell-Electrical resistance System (Millipore). Monolayers were considered optimal for transport polarity studies when the trans-epithelial resistance exceeded 300 Ohm/cm (2) after 19 d of culture. In addition, we also performed the test of mannitol permeability to assess the cell monolayer integrity: 7 nmols of [³H]-Mannitol were added (0.25 μCi/ml per filter) to the apical medium of filters containing or not the cells after 19 d of culture. After 3 h, the content of [³H]-Mannitol was 0.37 ± 0.01 and 0.04 ± 0.001 nmol in the basolateral medium of transwells with filters without or with cells, respectively (data are mean ± SEM; n = 3). Thus, approximately 5% of mannitol passed through the filters, whereas only approximately 0.5% passed through the cell monolayer and the holding filter.

cDNA Cloning

To obtain the cDNA sequence of opossum LAT-2 (oLAT-2) and y⁺LAT-1 (oy⁺LAT-1), first-strand cDNA from total RNA (5 μg) was synthesized using random primers and Superscript II kit (Life Technologies). This cDNA was used for PCR amplification of an oLAT-2 fragment with a forward 5'-CGGAGTAGCCCTGAAGAAAG-3' primer (b2c2OK2R) deduced from two conserved cDNA regions from the human, mouse (forward primer 5'-GGCAATGC-3'), and opossum (reverse primer 5' untranslated regions of both cDNAs) and opossum (reverse primer from the known partial opossum sequence; see reference 12) y⁺LAT-1 primer. PCR conditions were: 30 cycles of 95°C, 5 s; 55°C, 15 s; 72°C, 90 s. The resulting amplified fragment was sequenced with the same primers. The rest of the coding region of oLAT-2 was obtained with the SMART RACE cDNA amplification kit (Clontech) using a 5'-GGGTTAGATCTAGCAGGCATC-3' primer (b2c2OK5R). The cDNA amplification of oy⁺LAT-1 was performed with primers derived from the human, mouse (forward primer 5'-GGCAATGCGAAGAAGAAG-3' primer (b2c2OK2R) deduced from two conserved cDNA regions from the human LAT-2, and AA285581 and AA571992 mouse ESTs. Amplification was carried out in a Perkin-Elmer 9600 thermocycler in the following conditions: 35 cycles of 95°C, 5 s; 55°C, 15 s; 72°C, 90 s. The resulting amplified fragment was sequenced with the same primers. The sense oLAT-2 construct was made by directional cloning of the first 643 bp of its cDNA with EcoRI (5') and NotI (3') linkers into the EcoRI and NotI restriction sites of the eukaryotic vector pCDNA 3.1 (+) (Invitrogen). The antisense oLAT-2 construct was made by cloning the same sequence with reversed restriction linkers into the pCDNA 3.1 (+) vector.

oLAT-2 Constructs and Stable Transfection of OK Cells

The sense oLAT-2 construct was made by directional cloning of the first 643 bp of its cDNA with EcoRI (5') and NotI (3') linkers into the EcoRI and NotI restriction sites of the eukaryotic vector pCDNA 3.1 (+) (Invitrogen). The antisense oLAT-2 construct was made by cloning the same sequence with reversed restriction linkers into the pCDNA 3.1 (+) vector.

OK cell monolayers were transfected with sense or antisense oLAT-2 cDNA fragments by co-precipitation with CaPO₄. The stably transfected clones were selected as described (18). Selection of clones with a lower expression of the oLAT-2 transcript was performed by Northern blot analysis. The monolayer integrity was tested in the same way as for wild type OK cells.

Northern Blot Analysis

Total RNA was isolated from OK cell monolayers using a RNeasy Mini Kit (Qiagen). RNA was analyzed on a 1.2% agarose/formaldehyde gel and transferred to nylon membranes (Hybond N, Amersham) by capillarity in 10× SSC (0.15 M NaCl, and 0.015 M sodium citrate, pH 7.0). The 653 bp of 5' end of oLAT-2 cDNA was labeled with [α-³²P]dCTP (Amersham Pharmacia Biotech) using a random oligonucleotide-priming labeling kit (Amersham Pharmacia Biotech) and used as a probe. The prehybridization and hybridization solution was supplied by Clontech. Final wash conditions included 0.1× SSC with 0.1% SDS at 65°C. The blots were exposed to X-Omat film (Agfa film) for 12 to 60 h at −80°C with one intensifying screen.

Computer Analysis

Amino acid sequence homology search and the prediction of transmembrane segments of opossum LAT-2 were performed as indicated elsewhere (12).

Transport Measurements in Transwell Chambers

For basolateral membrane uptake experiments, apical and basal medium were washed three times in preheated (37°C) uptake solution (10 mM HEPES, 5.4 mM KCl, 1.2 mM MgSO₄, 7 mM H₂O, 2.8 mM CaCl₂, 2 mM H₃O, 1 mM KH₂PO₄, and 137 mM NaCl or 137 mM N-methyl-glucamine [MGA], pH 7.4). Approximately 0.5 μl of uptake solution was left on the apical side of the filter during the experiment. We then added to the basal side 0.5 μl of MGA or Na⁺ uptake solution containing the amino acid at the indicated concentration and the corresponding L-[³H] labeled amino acid as a tracer (2 μCi/ml) in the presence or absence of cold amino acids as competitors. Uptake was stopped with cold STOP solution (uptake solution at 4°C) added to the basal side. Then, the filters were washed three times with the same solution from apical and basal sides. The filters were then left to dry, cut, and placed in a counter vial with 200 μl of 0.5% Triton X-100 and 100 mM of NaOH for 30 min at room temperature. Then, 3 ml of scintillation liquid was then added, and the radioactivity was counted in a beta scintillation counter (Beckman LS 6000TA; Beckman Instruments).

For basolateral membrane efflux experiments, cells were loaded with the desired amino acid through the apical and basolateral membrane for 5 min as described above. Uptake solution with the labeled amino acid was washed after loading from the basal medium. L-cystine (4 μCi/ml) was only loaded through apical membrane for 10 min. The apical medium with the radioactive amino acid was not removed throughout the efflux experiment. Efflux started after the addition of MGA or Na⁺ efflux solution (uptake solution) with or without trans-stimulating cold amino acid. Fifty-microliter samples
Measurement of Intracellular Amino Acids Content

The measurements were performed with polarized cells after 19 d of culture. The cells were washed three times in 1 ml of ice-cold PBS. For deproteinization, 100 µl of 10% sulfoacetic acid was added per filter, and the mixture was centrifuged at 12,000 × g for 5 min. The supernatant was removed and stored at −20°C before assay of intracellular amino acids. The pellet was dissolved in 100 µl of 0.5% Triton-X100 and 0.1 N NaOH for protein determination by using the BCA Protein Assay Kit (Pierce). To measure the intracellular cysteine content, 45 µl of deproteinized supernatant was added to 13 µl of 3.5 M H2BO3, pH 13.5, and 1.25 µl of 50 mM of ICH2CO2Na. The alkylation reaction of cysteine residues was performed for 30 min in the dark at room temperature. The excess of sodium iodoacetate was precipitated by adding 10 µl of C2H5OH at room temperature for 30 min. The amino acid content analysis was performed with a reverse-phase HPLC method after derivatization with o-phthalaldehyde.

Results

Opossum LAT-2 and y+ LAT-1 Proteins Belong to the LSHAT Family

To identify the oLAT-2 sequence, reverse transcription-PCR amplification of total RNA from OK cells was performed with complementary primers to the human LAT-2 cDNA and mouse LAT-2 ESTs (see Materials and Methods). The open reading frame starts at base 77 and continues until the first stop codon (TGA) at base 1685 coding for a protein of 536 amino acid residues with a predicted molecular mass of 58.7 kD. (accession number AF514299 submitted to the GenBank/EBI Data Bank). The S’ and 3’ RACE-PCR revealed about 500 bp and 2000 bp of 5’ and 3’-untranslated regions showing an mRNA transcript of 4.3 kb (see Figure 3). The PCR amplification to identify the oy+LAT-1 sequence was performed with complementary primers derived from the human, mouse, and opossum (partial) (12) y+LAT-1 cDNA sequences. The open reading frame starts at base 44 and continues until base 1579 coding for a protein of 512 amino acid residues with a predicted molecular mass of 56.3 kD (accession number AF514786 submitted to the GenBank/EBI Data Bank).

The oLAT-2 and oy+LAT-1 proteins are light subunits of heteromeric amino acid transporters (LSHAT) (Figure 1). The oLAT-2 amino acid sequence shows identity of 85% to rat and mouse and 86% to human and rabbit LAT-2. The oy+LAT-1 amino acid sequence shows identity of 87% and 85% with its human and mouse counterparts, respectively. In agreement with the previous LSHAT sequences, 12 putative transmembrane domains with both cytoplasmatic N- and C-terminal segments are predicted (HMMTOP version 1.1 algorithm [26]). The cysteine residue, which is conserved in all LSHAT sequences and participates in the disulfide bridge with the corresponding heavy subunit (27), corresponds to residue 154 for oLAT-2 and residue 151 for oy+LAT-1.

OK Cells Express LAT-2 and y+ LAT-1 Related Transport Activities at the Basolateral Membrane

The Na+-independent uptake of L-leucine and L-alanine was analyzed to determine the expression of LAT-2 transport in the basolateral membrane of polarized OK cells. The following criteria were used to detect LAT-2 activity: (1) L-alanine is transported via LAT-2, asc-1, and asc-2 (22,23,28,29), but it is not transported via LAT-1 (30,31); (2) transport of L-alanine via LAT-2, but not via asc-1 and asc-2, is inhibited by BCH and L-tyrosine (23); (3) L-leucine is transported via LAT-1 and LAT-2 (30–31,21–23), but it is not transported via asc-1 and asc-2 (28,29); (4) transport of L-leucine via LAT-2 is inhibited by the amino acid analog B2 (2-aminobicyclo (2,2,1)-heptane-2-carboxylic acid) and by L-alanine (22,23). The LAT-2 transport activity was measured 3 and 19 d after seeding the cells on filters. At day 3, transport of 50 µM L-[3H] alanine (41 ± 2.9 pmol/filter · 30 s) was diminished to 9.6 ± 1.0 pmol/filter · 30 s and 9.9 ± 0.9 pmol/filter · 30 s by 100 fold excess of L-tyrosine or 400 fold excess of BCH respectively. At day 19, transport of 50 µM L-[3H] alanine (37 ± 8 pmol/filter · 30 s) was diminished to 1.06 ± 0.2 pmol/filter · 30 s and 3.0 ± 0.6 pmol/filter · 30 s by 100 fold excess of L-tyrosine or 400 fold excess of BCH respectively. Thus, LAT-2 transport activity was already detected at day 3, and thereafter this transport activity was further characterized after 19 d of culture when the cell monolayers reached the maximal electrical resistance (see Materials and Methods). At day 19, transport of 50 µM L-[3H] leucine (104 ± 9 pmol/filter · 30 s) was diminished to 36 ± 3 pmol/filter · 30 s and 46 ± 12 pmol/filter · 30 s by 200-fold excess of L-alanine or BCH, respectively, or by the combination of 200-fold excess of these two amino acids (19 ± 4 pmol/filter · 30 s).

To check the amino acid exchanger activity of the LAT-2-related L-system (21,22,24), polarized cells were loaded on the basolateral and apical membranes with the radioactive substrate (L-[3H] leucine or L-[3H] alanine), and the efflux across the basolateral membrane was measured. The efflux of L-leucine or L-alanine trans-stimulated by LAT-2 substrates was mostly sodium independent. Thus, the efflux of L-leucine trans-stimulated by L-alanine was 3493 ± 152 and 2306 ± 90 cpm/filter · min in the absence (MGA medium) or in the presence of 137 mM sodium respectively (mean ± SEM; n = 3). Similarly, the efflux of L-alanine trans-stimulated by L-leucine was 1399 ± 270 and 906 ± 154 cpm/filter · min in the absence or in the presence of 137 mM sodium respectively (mean ± SEM; n = 3). Although the L-leucine efflux was significant in non-trans-stimulated conditions, it increased threefold by LAT-2 amino acid substrates like L-leucine, L-alanine, or BCH at 2 mM concentration (Figure 2A). L-alanine efflux was also trans-stimulated twofold by 2 mM L-alanine, L-leucine, or L-tyrosine (Figure 2B). The efflux of another LAT-2 amino acid substrate, L-isoleucine, was also trans-stimulated twofold by external L-alanine (efflux was 1170 ± 152 and 2306 ± 69 cpm/filter · min in none and trans-stimulated conditions respectively; data are mean ± SEM; n = 3).

Kinetic analysis of L-alanine uptake, examined over a range of concentration from 2.5 µM to 5 mM, showed apparent Km and Vmax values of 2.4 ± 0.1 mM and 0.73 ± 0.01 nmol/filter · 30 s, respectively (mean ± SEM; n = 3). This Km value is in the range reported for this amino acid via the transport activity
elicited by human LAT-2 and 4F2hc cRNAs in Xenopus oocytes (22).

To determine the expression of $y^+L$ exchange activity in the basolateral domain of OK cells, the efflux of L-arginine was measured in several trans-stimulated conditions 19 d after seeding the cells on the filters. As shown in Figure 2C, 1 mM L-leucine plus Na$^+$ in the basal medium trans-stimulated L-$[{}^3\text{H}]$ arginine efflux to the same extent as did 1 mM L-arginine in the presence or absence of Na$^+$. The trans-stimulation effect of L-leucine was blunted in the absence of Na$^+$. This activity is similar to $y^+L$-LAT-1/4F2hc activity when both are coexpressed in oocytes (12–14). Basolateral efflux of L-arginine via system $y^+L$ was already detected 3 d after seeding the cells on the filters. Thus, efflux of L-arginine trans-stimulated by L-leucine was 450 ± 54 and 4270 ± 100 cpm/filter · min in the absence (MGA medium) or in the presence of 137 mM sodium, respectively (mean ± SEM; n = 3).

**LAT-2 Contributes to the Transport of Neutral Amino Acids across the Basolateral Plasma Membrane**

To assess the contribution of LAT-2 to the transport of small and large zwitterionic amino acids in the basolateral membrane of the OK cells, we used a LAT-2 cDNA antisense strategy. After transfection with 5′-end fragments of opossum LAT-2 cDNAs, antisense (AS) or sense (S) cell clones (see Materials and Methods) were selected according to reduced LAT-2 transcript expression by Northern analysis. In comparison with control (i.e., untransfected cells) the LAT-2 mRNA levels were lower in four antisense clones (18%, 21%, 10%, and 6% of control values for AS1, AS4, AS10, and AS12, respectively) and unaffected in the sense clones (S1, S3) (Figure 3A). To screen the basolateral LAT-2–related transport activity, the exchange of L-alanine and L-leucine was measured. Thus, the cell clones were polarized and loaded with L-$[{}^3\text{H}]$ alanine from the apical and basolateral media. The Na$^+$-independent efflux across the basolateral membrane was carried out in the pres-
ence or absence of 2 mM L-leucine. The percentage of L-
alanine efflux trans-stimulated by L-leucine (i.e., percentage of the increment over non-trans-stimulated conditions) was 10% in the AS1, 22% in the AS4, 6% in the AS10, and 30% in the AS12 antisense clones (Figure 3B). For S1 and S3 sense clones, this efflux was trans-stimulated 85% and 123%, respectively (Figure 3B); trans-stimulation in these conditions was 103 ± 8% in the wild-type OK cells (mean ± SEM; n = 9).

AS10 and S3 cell clones were used for further experiments in which LAT-2 activity was studied. Figure 4A shows that the basolateral influx of L-[3H] alanine was 73% lower in the AS10 and S3 clones compared to wild-type OK cells.
for 5 min from both apical and basolateral sides. Basal medium was then washed three times in pre-warmed uptake Na\(^+\) medium. Efflux was started by adding at the basal side 1 ml of Na\(^{137}\) mM L-cysteine \((\text{Leu})\) or not (none) 1 mM of cold leucine. For B and C, 50 \(\mu\)M L-cystine, S3 and AS10 cell clones were loaded with 200 \(\mu\)M L-[\(^3\)H]-arginine, and efflux was measured (Figure 4C). Efflux was very low in the absence of amino acids but was increased 2.5-fold by 1 mM L-leucine in the presence of 137 mM Na\(^+\) in the basal efflux medium. Trans-stimulation was similar for both clones.

**LAT-2 Contributes to the Transepithelial Flux of L-Cystine**

To test the participation of LAT-2 in the trans-epithelial flux of L-cystine, S3 and AS10 cell clones were loaded with 200 \(\mu\)M L-[\(^3\)S]-cystine in the apical medium, and the appearance of radioactivity in the basal medium was monitored in the absence or presence of LAT-2 amino acid substrates. In the presence of L-leucine, the efflux of radioactivity was trans-stimulated 108\% in the S3 clone, whereas trans-stimulation fell to 34\% in the AS10 clone (Figure 5). In the presence of L-alanine the trans-stimulation percentage reached 86\% and 27\% in the S3 and AS10 clones, respectively (trans-stimulated efflux was 1840 ± 210 and 448 ± 80 cpm/filter·min in S3 and AS10 clones, respectively; mean ± SEM; \(n = 9\)).

To test the role of LAT-2 in the flux of amino acids across the basolateral plasma membrane of OK cells, the intracellular content of several amino acids was measured in LAT-2 sense and antisense clone cells cultured in growth medium at both sides of the monolayer (Figure 6). If LAT-2 contributes to the net influx of a particular amino acid, the partial decrease in LAT-2 expression will tend to decrease the intracellular content of this amino acid. In contrast, if LAT-2 contributes to the net efflux of this amino acid, its intracellular concentration will tend to increase when LAT-2 expression is decreased. As expected, the intracellular content of dicarboxylic amino acids like glutamate or aspartate, dibasic amino acids like L-arginine, and taurine, which are not substrates of LAT-2, was similar in both S3 and AS10 cell clones. No significant differences were then washed three times in pre-warmed uptake Na\(^+\) medium. Efflux was started by adding at the basal side 1 ml of Na\(^+\) medium containing (Leu) or not (none) 1 mM of cold leucine. For B and C, 50 \(\mu\)M of samples were taken from the basal medium at indicated periods of time and their radioactivity counted. Data are mean ± SEM from three filters per efflux condition in two representative experiments. (S: sense cell clone; AS: antisense cell clone).
found for glutamine, histidine, tyrosine, or glycine intracellular content. Interestingly, intracellular alanine, serine, and threonine were 26%, 40%, and 22% lower in the AS10 than in the S3 cell clones (Figure 6). In contrast, intracellular cysteine was 2.5-fold higher in the AS10 than in the S3 cell clones (Figure 6). The effect of LAT-2 antisense on the intracellular amino acids content was also checked in two additional clones. Thus, the intracellular cysteine content was comparable between AS10 (111 ± 11 pmols/filter; see Figure 6), AS1 and AS12 cell clones (125.2 ± 12.1 and 121.1 ± 33.6 pmols/filter, respectively; mean ± SEM; n = 5) and increased approximately twofold in respect to wild-type OK cells and S3 cell clone cysteine content (60.6 ± 11.6 and 40.35 ± 10.8 pmols/filter; mean ± SEM; n = 5). In contrast, the content of other neutral amino acids was not changed in the AS1 and AS12 antisense cell clones. Only the content of serine was significantly decreased in AS12 (t test; P = 0.05), but not in AS1, compared with wild-type OK cells and S3 cell clone (998 ± 133 and 1262 ± 137 pmols/filter for AS12 and AS1 cell clones, respectively, and 1763 ± 118 pmols/filter for OK cells and 1555 ± 94 pmols/filter for S3 cell clone; mean ± SEM; n = 3).

Discussion

In this study, we showed that the renal tubular-related cell line OK express system L (LAT-2 related) and y+L (most probably y+LAT-1 related) exchange activities in the basolateral plasma membrane. Partial depletion of LAT-2 transport activity resulted in the decrease of the trans-epithelial flux of apical L-cystine and the specific increase of the intracellular content of L-cysteine. These results demonstrate that LAT-2 has an important role in the vectorial trans-epithelial flux of L-cystine.

The process of renal reabsorption of cystine and dibasic amino acids is only partially understood. System b6+ is the main, if not the unique, apical transport system involved in the reabsorption of cystine and a major player in dibasic amino acid reabsorption. In the proximal tubule, the heterodimer b6+AT/rBAT constitutes the apical system b6+ (9), and mutations in either of its subunits resulted in cystinuria (6,10). Cystinuria patients usually show renal cystine reabsorption close to zero, whereas a substantial reabsorption of dibasic amino acids remains active (32). The apical amino acid transporters responsible for this residual reabsorption are unknown. Dibasic amino acids leave renal epithelial cells across the basolateral domain via system y+ L. The main support for this is the fact that mutations in y+LAT-1 (SLC7A7) cause lysinuric protein intolerance, a disease characterized by dibasic amonicaciduria (15,16). Transport activity reminiscent of system y+ L has been described in the basolateral membrane of rat enterocytes (33). To our knowledge, basolateral y+ L transport activity in OK cells is the first description of this transport activity in renal epithelial cells.

Which are the basolateral transporters involved in the renal reabsorption of cystine? The present study offers direct evidence for the preferential involvement of LAT-2/4F2hc transporter in the apical-to-basolateral trans-epithelial flux of cystine in OK cells: (1) half of the flux of radioactivity from apical L-[35S] cystine to basolateral medium is mediated by LAT-2; (2) partial depletion of basolateral LAT-2 transport activity resulted in a twofold to threefold increase in the intracellular content of cystine. In contrast to dibasic amino acids, the intracellular fate of apically absorbed cystine is more complex. Cystine became reduced to cysteine inside renal epithelial cells (1). In OK cells, transported L-[35S] cystine is reduced to cysteine, which contributes to glutathione synthesis or leaves the cell (19,20). The LAT-2/4F2hc transporter is a good candidate to mediate the basolateral efflux of cysteine in proximal tubule epithelial cells and in other epithelia: (1) LAT-2/4F2hc transports cysteine and not cystine, and the former is one of the preferred intracellular substrates of the transporter when expressed in oocytes (24); (2) the expression of LAT-2 protein or transport activity has been described in the basolateral plasma membrane of placenta, enterocytes, and the renal epithelial cells of the proximal tubule (34–36,21). Therefore, our study, in full agreement with the known characteristics of the renal reabsorption of cystine, strongly supports a crucial role of LAT-2/4F2hc transporter in this process.

The proximal tubule-derived OK cell represents a good cell model to study renal reabsorption of amino acids. Polarized OK cells express the complete set of amino acid transporters known to be involved in the vectorial trans-epithelial flux of the amino acids hyperexcreted in cystinuria (Fig 7). OK cells express the rBAT-related system b6+ exchange activity in the apical pole of the cell (18), and as shown here, the basolateral system L isoform LAT-2/4F2hc and the basolateral system y+L. System y+L most probably represents the y+LAT-1/4F2hc transporter and not the y+LAT-2/4F2hc isoform be-
cause the former is expressed in OK cells (present study) and the latter is only weakly expressed in kidney (37). Moreover, among the heteromeric amino acid transporters that mediate exchange of neutral amino acids, only LAT-2 transport activity, but not LAT-1, asc-1 and asc-2, is expressed in the basolateral domain of OK cells. This is in full agreement with the expression of these transporters in the epithelial cells of the renal proximal tubule; LAT-1 is barely expressed in human and mouse kidney (38,39), asc-1 is not expressed in kidney (28), asc-2 is localized in collecting ducts in mouse kidney (29), and only LAT-2 is conspicuously expressed in the epithelial cells of the renal proximal tubule and the small intestine (21–23,40).

Systems \( \text{b}^{\circ,\text{+}} \), \( \text{y}^{\text{+}}\text{L} \), and \( \text{L} \) are exchangers (2–4). The direction of the exchange for systems \( \text{b}^{\circ,\text{+}} \) and \( \text{y}^{\text{+}}\text{L} \) is conditioned by the membrane potential and the gradient of sodium across the plasma membrane (Figure 7). Thus, vectorial electrogenic hetero-exchange of dibasic amino acids (influx) and neutral amino acids (efflux) via system \( \text{b}^{\circ,\text{+}} \) has a 1:1 stoichiometry (5) and is favored by the negative membrane potential in rBAT-expressing oocytes (41) and in \( \text{b}^{\circ,\text{+}}\text{AT} \)-reconstituted systems (42). Similarly, the electroneutral exchange mediated by \( \text{y}^{\text{+}}\text{L} \) (efflux of dibasic amino acids and influx of neutral amino acids plus sodium with an expected stoichiometry of 1:1:1 [5,14]) is driven by the chemical gradient of sodium. In contrast, exchange via system L isoforms (e.g., LAT-1/4F2hc and LAT-2/4F2hc) is always electroneutral and shows a 1:1 stoichiometry (24). Therefore, the direction of exchange via LAT-2/4F2hc for a given amino acid is not easily predictable and would depend on its concentration and affinity relative to other substrates of the transporter on either side of the plasma membrane. In kidney, LAT-2 is expressed in the epithelial cells of the upper part of the proximal tubule, close to the glomerulus (21,22). In this part of the proximal tubule the luminal amino acid concentration corresponds to that of the glomerulus filtrate, and it is close to that of the plasma. In conditions that mimic this situation, LAT-2 is a major and specific transporter for net cysteine efflux and may mediate the net influx of alanine, serine, and threonine in OK cells. This is evidenced by the dramatic increase in the intracellular content of cysteine in LAT-2 partially depleted cells. In contrast, this maneuver tended to decrease (e.g., alanine, serine, and threonine) or did not alter (e.g., histidine, tyrosine, and glycine) the intracellular content of other LAT-2/4F2hc substrates. Thus, LAT-2 limits the basolateral efflux of cysteine, but not that of other LAT-2/4F2hc substrates. This suggests that even in LAT-2 partially depleted conditions these amino acids are efficient substrates of this transporter, or other transporters or metabolic pathways determine the steady state of these amino acids. The different impact of partially depleted LAT-2 transport activity in the intracellular content of its amino acid substrates defines a “metabolic profile.” Amino acid transporters have characteristics not common for other transporters: a given amino acid transporter carries several amino acids, and a given amino acid is carried across the plasma membrane by a set of different transporters in a given cell (43). Partial depletion of different plasma membrane amino acid transporters in polarized OK cells would give different “amino acid metabolic profiles” that could be used to understand the role of these transporters in.

Figure 6. Intracellular content of amino acids in polarized S3 and AS10 clones. The cells were grown in polycarbonate filters in DMEM/F12 supplemented with 10% fetal calf serum during 19 d. The amino acid content (nmol/filter or pmol/filter as indicated) was measured in two independent experiments with five replicas each. The accumulation of alanine, serine, and threonine was higher in S3 than AS10 cell clone (t test; \( P \leq 0.05 \)) whereas the accumulation of cysteine showed an opposite pattern (t test; \( P \leq 0.05 \)). No significant differences were found in the intracellular content of the other amino acids analyzed.
are then released through the basolateral membrane by y

\[ \text{AA}^{+} \text{CysC} \] coupled to the exchange of intracellular neutral amino acids (AA\text{\text{O}}), which enter the cell by Na\text{\text{\textsuperscript{+}}}–dependent transporters located at both apical and basolateral plasma membranes (T AA\text{\text{O}}) (only those at the apical side are depicted). Membrane potential favors the uptake of dibasic amino acids. Cystine uptake is favored by its reduction into cysteine (Cys) associated with glutathione oxidation (GSH → GssG). Dibasic amino acids are then released through the basolateral membrane by y\text{\textsuperscript{+}}LAT-1/4F2hc complex. This efflux is also coupled to the influx of neutral amino acids plus Na\text{\text{\textsuperscript{+}}}. At the basolateral membrane, 4F2hc/LAT-2 accounts for the efflux of neutral amino acids including the cystine derived cysteine. We show that when equal concentrations of neutral amino acids are applied on both sides of the epithelium, the direction of the exchange favors the net exit of cysteine and the net influx of alanine, serine or threonine. Another system (T) at the basolateral membrane would account for the net efflux of neutral amino acids. The heteromeric amino acid transporter cDNAs cloned from American opossum are shaded.

Figure 7. Model for the reabsorption of cystine, dibasic, and neutral amino acids in the opossum proximal tubule cell line OK. rBAT heterodimerizes with b\text{\textsuperscript{0},+} AT in the apical membrane of renal epithelial cells. The complex mediates the active reabsorption of dibasic amino acids (AA\text{\textsuperscript{+}}) and cystine (CysC) coupled to the exchange of neutral amino acids (AA\text{\text{O}}), which enter the cell by y\text{\textsuperscript{+}}-dependent transporters located at both apical and basolateral plasma membranes (T AA\text{\text{O}}) (only those at the apical side are depicted).

References


The crucial role of LAT-2 in the apical-to-basolateral trans-epithelial flux of cystine suggests that dysfunction of this transporter may lead to hyperexcretion of cysteine (cystine upon extracellular oxidation). This is reminiscent of the hyper-excretion of dibasic amino acids that occurs by dysfunction of basolateral system y\text{\textsuperscript{+}}L in lysinuric protein intolerance. In contrast to this view, screening for mutations in the 18% of cystinuria alleles not explained by mutations in the open reading frame of SLC7A8 (coding for LAT-2) (Font, Palacín, and Nunes; personal communication). This suggests that mutations in this gene do not cause cystinuria and/or they might be deleterious. Indeed, LAT-2 is expressed, in addition to renal and intestinal epithelia, in brain, placenta, and skeletal muscle (21–23). In this scenario, the crucial role of LAT-2 in the vectorial trans-epithelial flux of cystine fosters the hypothesis of SLC7A8 as a modulator gene for renal reabsorption of cystine. As shown here for OK cells, a partial depletion of LAT-2 transport activity may result in the intracellular accumulation of cystine in the epithelial cells of the proximal tubule that might increase backflow across the apical membrane, contributing to intraluminal cystine upon oxidation. Indeed, cystine secretion in urine occurs in some cystinuria patients (44). Control population and homozygotes and heterozygotes of cystinuria mutations, in addition to the degree of severity associated to a particular mutation, show a high degree of individual variability on urine hyperexcretion of cystine and dibasic amino acids (45,11). Association of the degree of amino acid urine hyperexcretion and LAT-2 polymorphisms in general population and in patients with cystinuria and their relatives is currently under study.

Acknowledgments

We are grateful to Ricardo Casaroli for help in measuring the trans-epithelial resistance of the cell monolayers and Judith García for technical assistance and Ramón Roca for computer support. We also thank Robin Rycroft for editorial help. EF is a recipient of a predoctoral fellowship from the University of Barcelona. DT was recipient of a predoctoral fellowship from the Ministerio de Educación, Cultura y Deporte. This research was supported by grant PM99/0172 from the Dirección General de Investigación Científica y Técnica, Spain, and the BIOMED2 CT98-BMH4–3514 EC grant and the support of the Comissionats per a Universitats i Recerca de la Generalitat de Catalunya (Catalonia, Spain) to MP.


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