The heteromeric amino acid transporters \( b^{0,+}AT-rBAT \) (apical), \( y^+LAT1-4F2hc \), and possibly \( LAT2-4F2hc \) (basolateral) participate to the (re)absorption of cationic and neutral amino acids in the small intestine and kidney proximal tubule. We show now by immunofluorescence that their expression levels follow the same axial gradient along the kidney proximal tubule (S1>S2>S3). We reconstituted their co-expression in MDCK cell epithelia and verified their polarized localization by immunofluorescence. Expression of \( b^{0,+}AT-rBAT \) alone led to a net reabsorption of L-Arg (given together with L-Leu). Coexpression of basolateral \( y^+LAT1-4F2hc \) increased L-Arg reabsorption and reversed L-Leu transport from (re)absorption to secretion. Similarly, L-cystine was (re)absorbed when \( b^{0,+}AT-rBAT \) was expressed alone. This net transport was further increased by the coexpression of \( 4F2hc \), due to the mobilization of LAT2 (exogenous and/or endogenous) to the basolateral membrane. In summary, apical \( b^{0,+}AT-rBAT \) cooperates with \( y^+LAT1-4F2hc \) or \( LAT2-4F2hc \) for the transepithelial reabsorption of cationic amino acids and cystine, respectively. The fact that the reabsorption of L-Arg led to the secretion of L-Leu demonstrates that the implicated heteromeric amino acid transporters function in epithelia as exchangers coupled in series and supports the notion that the parallel activity of unidirectional neutral amino acid transporters is required to drive net amino acid reabsorption.

Free amino acids need to be transported from the lumen of the intestine as well as from the urinary filtrate of kidney tubules into the extracellular space. This transeellular (re)absorption involves the passage across both the luminal and the basolateral membrane of epithelial cells. Most neutral amino acids are taken up through the luminal membrane of these cells by a \( Na^+ \)-dependent electrogenic transport system that was named \( B^{0} \) or B for kidney and intestine, respectively, and that is not molecularly defined as yet (1, 2).

The import of cationic amino acids and of L-cystine depends on the expression of a heteromeric transporter that is composed of a multitransmembrane span catalytic subunit (glycoprotein-

associated amino acid transporter, light chain) named \( b^{0,+}AT^{1} \) (broad specificity, \( Na^+ \)-independent neutral and cationic amino acid transporter) and the covalently associated type II glycoprotein (heavy chain) rBAT (related to \( b^{0,+} \) amino acid transporters) (3, 5). Genetic studies have shown that defects in the genes encoding either subunit lead to cystinuria (4, 7, 8). The function of this transporter has been characterized in Xenopus oocytes first by investigating the function of exogenous rBAT associated with an endogenous \( b^{0,+}AT \) and then, upon identification of the mammalian \( b^{0,+}AT \) subunit, by measuring the function of the mammalian heterodimer expressed as fusion protein in Xenopus oocytes or in COS-7 cells (3, 5, 6, 9–11). These studies indicated that \( b^{0,+}AT-rBAT \) functions as an obligatory exchanger and suggested that its major mode of transport is, at a normal membrane potential, the uptake of extracellular cationic amino acids or L-cystine that are exchanged against intracellular neutral amino acids. These neutral amino acids could in turn be recycled into the cell by system B(\( \beta \)). Recently, we have characterized the biosynthesis, localization and uptake function of \( b^{0,+}AT-rBAT \) expressed in MDCK cells (12). This study has shown in an epithelial context that the surface expression of \( b^{0,+}AT \) and rBAT depends on their association, which is necessary for the maturation and stabilization of rBAT. This study has also confirmed that \( b^{0,+}AT-rBAT \) displays extracellularly a high apparent affinity for L-cystine > cationic amino acids (L-Arg) > large neutral amino acids (L-Leu).

The basolateral efflux of amino acids is as yet less well understood than the apical influx. The two heteromeric amino acid transporters, composed of the glycoprotein subunit (heavy chain) \( 4F2hc \) (CD98) and an associated catalytic subunit, \( y^+LAT1 \) or LAT2, that are highly expressed in the proximal kidney tubule and the small intestine, were shown in Xenopus oocytes to function as obligatory exchangers (13–17). The physiological function of \( y^+LAT1-4F2hc \) appears to be the electro-neutral efflux of intracellular cationic amino acids that are exchanged for extracellular large neutral amino acids together with \( Na^+ \). This mode of transport is based on the results of expression experiments made in Xenopus oocytes and is compatible with the phenotype of patients carrying the genetic disease lysinuric protein intolerance that was shown to be due to a defect in the corresponding gene (13, 14, 18, 19). As this system needs to function in the context of the (re)absorption of all amino acids, we postulate that an additional export system that recycles the imported neutral amino acids needs to be expressed in the basolateral membrane.

The function of the second heteromeric exchanger, LAT2-
4F2hc, has been extensively studied in Xenopus oocytes (15–17, 20). This transporter preferentially exchanges middle-sized and large neutral amino acids across the membrane. Importantly, its apparent affinities for various amino acids is much lower inside compared with outside of the cells (with an exception for Gly), suggesting that its activity depends on the intracellular amino acid availability (15). It was suggested that its physiological role is to equilibrate the relative concentrations of the various intracellular neutral amino acids, in particular by transporting some amino acids out of the cell that would not be substrates of the putative unidirectional efflux pathway (20). Because LAT2-4F2hc efficiently exchanges intracellular L-Cys against other extracellular neutral amino acids, this transporter was proposed to play a major role in the basolateral efflux of this amino acid, a part of which is produced intracellularly by the reduction of L-cystine (16, 17, 20).

To investigate the function of the basolateral exchangers y′LAT1-4F2hc and LAT2-4F2hc, alone, together and in conjunction with the apical exchanger b0,+AT-RBAT in the context of transepithelial transport, we expressed them in MDCK cells, a recipient cell line that forms a tight epithelium and does not express a high amount of endogenous epithelial amino acid transporters itself.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK cells (strain II) were cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (cat. 41965, Invitrogen, Basel, Switzerland) with 10 × 105 units/liter penicillin, 50 mg/liter streptomycin, 2 mM l-glutamine, 1% non-essential amino acids (cat. 11140-035, Invitrogen) and 10% fetal calf serum. Phoenix amphotropic retrovirus producer cells, kindly provided by Dr. G. Nolan (Baxter Laboratory for Genetic Pharmacology, Dept. of Microbiology and Immunology, Dept. of Molecular Pharmacology, Stanford University, Stanford, CA), were cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (cat. 41966, Invitrogen, Basel, Switzerland) with 1 × 105 units/liter penicillin, 100 mg/liter streptomycin, 2 mM l-glutamine, 1% non-essential amino acids (cat. 11140-035, Invitrogen) and 10% fetal calf serum.

cDNA Constructs, Transfection, and Retroviral Transduction—The MDCK cells transfected with hrBAT and mb0,+AT were previously described (12). The bF2hc, mLAT2 and my′LAT1 cDNA (13, 20) coding sequences were subcloned in the vector LRZSpBMMZ-X (kindly provided by Dr. G. Nolan) in place of the lacZ sequence. Production of supernatants containing the pseudoviruses and subsequent transduction of MDCK target cells was performed according to protocols provided by Dr. G. Nolan (www.stanford.edu/group/nolan/protocols/pro_helper_free.html), which were adapted from Ref. 21. Briefly, Phoenix amphotropic retrovirus producer cells were transfected with the above-mentioned constructs using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer’s protocol. 16 h after transfection, the medium was exchanged, and after another 48 h at 32 °C, the supernatant was harvested and filtered. MDCK target cells were plated at 5–10% confluency, and viral supernatants containing 4 µg/ml polybrene (Sigma) were added 24 h later. The freshly transduced cells were centrifuged for 30 min at 1000 rpm and 32 °C immediately thereafter. After 24 h at 32 °C the medium was exchanged, and cells were further cultivated in normal medium at 37 °C. This procedure was repeated 5–10 times to achieve a maximal expression of the transport proteins to be investigated.

Antibodies—Polynomial rabbit antibodies were raised against synthetic peptides corresponding to the NH2 terminus of human rBAT, MAEDKSKRDSIEMMSKGCC, the COOH terminus of mouse b0,+AT, CHLAMkLMEVYTDKPE, the NH2 terminus of mouse y′LAT1, QHEAD- VGFLDQY (the COOH terminus of mouse y′LAT1, QHEAD- GELSKQDPKSK, and the COOH terminus of mouse LAT2, CPIFIFKPT- PVKDPDDEEQP, coupled to keyhole limpet hemocyanin (Eurogentech, Seraing, Belgium). The monoclonal anti-human 4F2hc antibody used was previously described in Ref. 22.

Double Immunofluorescence Staining of Cotransfected MDCK Cells—Cells were seeded on filters (24-mm Corning Costar Transwell filters, cat. Nr. 3412) at 100% confluence and cultivated for 7 days preceding experiments. rBAT expression was induced 3 days prior to experiment with 1 µM dexamethasone. Filters were washed three times using phosphate-buffered saline. Cells were fixed using 3% paraformaldehyde and 0.2% Triton X-100 for 15 min at room temperature. Filters were rinsed three times, and cut into squares. 4F2hc, y′LAT1-4F2hc, and LAT2-4F2hc double immunofluorescence was performed with a mix of the respective antibodies 4F2hc (1:1000), rBAT (SZS64; 1:50), b0,+AT (SZS57; 1:500), y′LAT1 (SZS98; 1:200), LAT2 (SZS60; 1:200) in phosphate-buffered saline containing 0.5% bovine serum albumin overnight at 4 °C. After washing, filter pieces were incubated for 6 h at room temperature with fluorescein isothiocyanate-labeled anti-rabbit-IgG antibody (Sigma) and CY3-labeled anti-mouse-IgG antibody (Sigma). After another round of washing, the filters were mounted in DAKO-glycerel (DAKO, Glostrup, Denmark) containing 2.5% 1,4-diazabicyclo (2, 2, 2) octane (DABCO) as fading retardant. Confoal images were taken using a Leica laser scan microscope (TCS-SSP, Wetzlar, Germany) equipped with a ×63 oil immersion objective. The appropriate controls were performed without the first and/or secondary primary antibodies.

Immunohistochemistry on Mouse Kidney Sections—Kidneys of anesthetized male mice (NMRI; RCC, Fullendorf, Switzerland) were fixed for 5 min by intravascular perfusion through the abdominal aorta as previously described (23). Coronal slices (1–2-mm thick) of the kidney were frozen in liquid propane and stored at −80 °C until use. Serial cryosection (4–5 µm) were cut and placed on Chromium(III) potassium sulfate-coated glass slides. Sections were preincubated for 10 min with 10% normal goat serum in phosphate-buffered saline, 2% bovine serum albumin. Afterward, sections were sequentially incubated with primary antibodies against LAT2 (SZS590) 1:1000, y′LAT2 (SZS595) 1:200, 500, and mb0,+AT (SZ400) 1:500 for 1 h at 4 °C. Binding sites of primary antibodies were revealed with Cy3-conjugated donkey anti-rabbit-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were studied by epifluorescence with a Polyvar microscope (Reichert Jung, Vienna, Austria). For controls, consecutive cryosections were incubated in preimmune serum. Images were acquired with a charge-coupled device camera (Visiarm 1280, Visirion System, Puching, Germany) and processed by Image-Pro Plus version 3.9 (Media Cybernetics, Silver Spring, MD) and Corel Photoshop software.

Filter Uptake Experiments—MDCK cells were passaged to 24-mm Corning Costar Transwell filters at 100% confluence and cultivated for 7 days. rBAT expression was induced 24 h prior to experiment with 1 µM dexamethasone. Integrity of the monolayer was checked by resistance measurement using the Millicell device (Millipore, Bedford, MA). Filters were washed three times with uptake buffer (150 mM NaCl, 10 mM HEPES pH 7.4, 1 mM CaCl2, 5 mM KC1, 1 mM MgCl2, 10 mM glucose) at 37 °C and incubated in uptake buffer for 30 min. The buffer was replaced unilaterally with buffer supplemented with amino acid at the indicated concentrations and the corresponding 3H-labeled L-amino acid as tracer (except for L-[14C]cystine); the contralateral compartment received the same solution without the labeled L-amino acid tracer. Uptake experiments were performed for the times indicated. DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) (100 µM) (Sigma) was added to the solution for experiments with L-cystine. The uptake was stopped by replacing the amino acid uptake solution with ice-cold uptake buffer and washed four times. The filters were excised and placed into scintillation vials containing scintillation fluid (Packard, Meriden, CT). After shaking overnight at room temperature, radioactivity was determined by scintillation counting.

RT-PCR—RT-PCR was performed to identify the dog LAT2 transporter. First-strand cDNA was synthesized from 100 ng of total RNA from wild-type MDCK cells, LAT2-transfected cells serving as the internal positive control, with or without MMRV reverse transcriptase (Promega, Madison, WI) and 50 pmol of random hexamer primers (Invitrogen). 1:10 of the first-strand cDNA was used as a template for PCR amplification using 50 µl of degenerate LAT2 primers (forward primer 5′-GTCAGYGYCTTGTTGATCA-3′, reverse primer 5′-GCAG- CACRATTGGGAGAAG-3′ (Mycosyn, Balgach, Switzerland)) and 2 units of recombinant Taq polymerase (Promega, Madison, WI). The cycling parameters were the following: 3 min 94 °C, 30 s 57 °C, 30 s 72 °C, followed by 10 min at 72 °C. PCR products were separated on agarose gel, the bands cut out and extracted with QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). DNA sequencing was performed at Microsyn (Balgach, Switzerland) using the above PCR primers.

Statistics—Data are expressed as means ± S.E. The difference between control and test values was evaluated using analysis of variance (one-way) with Bonferroni’s multiple comparison test.
Fig. 1. Localization of $b^{0,+}$AT, LAT2, and $y^{+}$LAT1 in the mouse kidney. Serial cryosection (4–5 μm) of mouse kidney were stained with antibody against $b^{0,+}$AT (left), $y^{+}$LAT1 (middle), and LAT2 (right). Staining of the kidney cortex (top) revealed that all three transporters are colocalized in proximal tubule cells, showing the same gradient of expression, from S1>S2>S3 segment. A larger magnification view (bottom) shows that expression of all three proteins commences directly after the glomerulus. The signal for $b^{0,+}$AT is localized in the brush border membrane, whereas both $y^{+}$LAT1 and LAT2 are found in the basolateral membrane of the same cells. Scale bar: top, 80 μm; bottom: 20 μm. G, glomerulus; D, distal tubule, S1, S1 segment of the proximal tubule.

RESULTS

Axial Gradient of Apical and Basolateral Heteromeric Amino Acid Transporters along the Mouse Kidney Proximal Tubule—

The fact that $b^{0,+}$AT, LAT2 and $y^{+}$LAT1 are colocalized in the proximal tubule of the kidney in vivo is shown in Fig. 1. As yet, the in vivo localization of $y^{+}$LAT1 had not been published. Interestingly, the present images of serial kidney sections indicate that the localizations of $b^{0,+}$AT, LAT2, and $y^{+}$LAT1 along the proximal tubule are superimposable, all showing the same axial gradient along the proximal tubule segments (S1>S2>S3). Thus, in view of the fact that all $b^{0,+}$AT expressed in the kidney was shown to be associated with rBAT (24), the three heteromeric transporter $b^{0,+}$AT-rBAT, LAT2-4F2hc, and $y^{+}$LAT1-4F2hc colocalize along the kidney proximal tubule with a very similar axial gradient. Only for $y^{+}$LAT1, the staining along the S1 and S2 segments appears to terminate earlier. Interestingly, some LAT2 staining is visible along the distal tubules as well.

Basolateral Localization of the Heteromeric Transporters $y^{+}$LAT1-4F2hc and LAT2-4F2hc in Transduced MDCK Cells—To perform a functional analysis of coexpressed heteromeric amino acid transporters of the proximal tubule and intestine, the MDCK cell line that is of distal nephron origin was chosen as recipient, because it does not express a high baseline transepithelial amino acid transport activity. Nevertheless, for the interpretation of the data obtained on transfected/transduced MDCK cells, one has to keep in mind that they do express some endogenous transporters. For instance, experiments performed in the eighties suggested the presence of endogenous system A and L (mostly basolateral), ASC (basolateral and apical), and of an apical Na$^+$/amino acid symporter with a broad substrate selectivity (25).

The MDCK cell line already expressing the apical heteromeric transporter $b^{0,+}$AT-rBAT as well as untransfected MDCK cells were sequentially transduced with amphotropic pseudoretrovirus encoding the subunits of the heteromeric transporters $y^{+}$LAT1-4F2hc and/or LAT2-4F2hc. The steady-state localization of these gene products was then analyzed by immunofluorescence confocal microscopy. Fig. 2, A and B shows that 4F2hc, when expressed with LAT2 in $b^{0,+}$AT-rBAT-expressing cells, localizes mainly to the lateral membrane. Only little intracellular and no apical 4F2hc staining can be seen. At the cell surface, there is also no overlap with rBAT (panel A, apical staining) or $b^{0,+}$AT (panel B, intracellular and apical staining).

LAT2 and 4F2hc co-localize in the basolateral membrane (Fig. 2C), as expected from their localization in mouse kidney and small intestine (20). In this figure, made at a stage at which only ~50% of the cells expressed both 4F2hc and LAT2, some cells exhibited LAT2 staining only, that, interestingly, was localized both intracellularly and at the lateral membrane. Because LAT2 was previously shown to necessitate association with 4F2hc for functional surface expression in Xenopus oocytes, the partial membrane localization of LAT2 in the absence of visible 4F2hc indirectly suggests the presence of some endogenous canine 4F2hc that is not recognized by the species-specific monoclonal antibody. The fact that coexpression of exogenous 4F2hc leads to an essentially basolateral immunolocalization of LAT2 confirms the hypothesis that 4F2hc is the limiting factor for LAT2 surface expression in MDCK cells.

The subcellular localization of $y^{+}$LAT1 has been visualized

Fig. 2. Colocalization of rBAT, $b^{0,+}$AT, LAT2, or $y^{+}$LAT1 with 4F2hc in transfected MDCK cells. Images taken parallel to the filter (X-Y plane) at the level of the nucleus are shown in the square panels. The rectangular panels represent corresponding Z-Y reconstitutions. Scale bar: 10 μm. A, in MDCK cells transfected with $b^{0,+}$AT-rBAT and LAT2-4F2hc, rBAT (green) shows a strict apical localization, while 4F2hc (red) is found in the basolateral membrane. B, visualization of $b^{0,+}$AT (green) and 4F2hc (red) in the same cells show the same basolateral localization for 4F2hc, while $b^{0,+}$AT is found intracellularly in large excess and in the apical membrane. C, in cells coexpressing LAT2 (green) and 4F2hc (red), both proteins colocalize to the basolateral membrane, as seen by the yellow staining (Z-Y reconstitution). D, cells coexpressing $y^{+}$LAT1 (green) and 4F2hc (red) show the same basolateral colocalization of both proteins (colocalization yellow). Note that in panels C and D some cells exhibit, besides intracellular staining, a basolateral staining for LAT2/y$^{+}$LAT1 in the absence of 4F2hc staining, suggesting the presence of low amounts of endogenous 4F2hc.
as yet only in Xenopus oocytes and in non-polarized transfected HEK293 cells (26, 27). Here we demonstrate that exogenous y’ LAT1 behaves as LAT2, namely that it colocalizes with exogenous 4F2hc in the basolateral membrane of MDCK cells (Fig. 2D) and that in the absence of exogenous 4F2hc, only a small fraction of it appears at the basolateral surface (putatively associated with endogenous 4F2hc).

Cooperation of Apical and Basolateral Heteromeric Amino Acid Transporters for L-Arg Transport—To test the role and the cooperation of b0,+ AT-rBAT and/or y’ LAT1-4F2hc on transepithelial cationic amino acid transport, we added l-Arg and L-Leu together to both sides of the epithelia, to allow amino acid exchange to proceed in the absence of a transepithelial concentration gradient. L-Arg was chosen because it is, on the one hand, a good influx substrate for the apical transporter b0,+ AT-rBAT that exchanges preferentially extracellular cationic amino acids or l-cysteine against intracellular neutral amino acids and, on the other hand, a good efflux substrate for the basolateral transporter y’ LAT1 known to preferentially exchange intracellular cationic amino acids against extracellular neutral amino acids plus Na+ (13, 14). L-Leu was taken as second substrate because it is a good influx substrate for y’ LAT1 and a suitable efflux substrate for b0,+AT-rBAT (5, 13, 14).

Radioactive tracer of either amino acid was added on separate filter cultures to each side of the epithelia and the amount of labeled amino acid in the cell and at the contralateral side was measured after a 2-h incubation. Because of interexperimental variation in absolute transport levels, amounts of accumulated amino acids were arbitrarily normalized for each experiment to the amount of L-Arg taken up from the apical side by b0,+AT-rBAT-expressing cells (mean: ~0.8 nmol × cm⁻² × 2h⁻¹).

MDCK cells expressing no b0,+AT-rBAT exhibited almost no apical uptake of L-Arg and displayed only a small apical-to-basolateral (A to B) flux (Fig. 3A). In contrast, when b0,+ AT-rBAT was expressed at the apical membrane, the intracellular amount of L-[³H]Arg increased by a factor of ~30. With epithelia expressing no exogenous basolateral transporter, an amount corresponding to 2.7× the intracellular L-Arg was accumulated in the basolateral chamber within 2 h, suggesting that there is an endogenous basolateral efflux pathway for L-Arg in MDCK cells. However, the additional expression of basolateral y’ LAT1-4F2hc led to a further increase in basolateral L-[³H]Arg accumulation and to a slight decrease in its intracellular level such that the ratio of basolateral/intracellular L-[³H]Arg was increased by a factor of two, indicating that y’ LAT1-4F2hc mediates the basolateral efflux of L-Arg.

Wild-type MDCK cells displayed an efficient basolateral uptake of L-Arg, probably via a y’-type transporter. However, the apical L-Arg accumulation was small, indicating that there is no efficient endogenous apical efflux pathway for L-Arg. The expression of the basolateral transporter LAT2-4F2hc and/or y’ LAT1 in wild-type MDCK cells did not change the basolateral uptake of L-Arg.

All cells expressing apical b0,+AT-rBAT displayed a higher basolateral-to-apical flux of L-Arg that is compatible with the hypothesis that b0,+AT-rBAT transports some L-[³H]Arg apically out of the cells, possibly by homoechange against unlabeled apical L-Arg. The fact that there was also an increase in cellular L-[³H]Arg imported from the basolateral side can be attributed to a transstimulatory effect of the apically imported unlabeled L-Arg at the level of the endogenous basolateral y⁺ transporter (homoeexchange). This hypothesis is supported by the fact that in the cell lines that express basolateral y’ LAT1 (that prefers heteroexchange of intracellular L-Arg for extracel-

![Fig. 3. Cooperation of apical and basolateral heteromeric amino acid transporters for L-Arg and L-Leu transport.](https://example.com/fig3.png)

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Footnotes:

1. **p < 0.001, ***p < 0.001.
Epithelial Heteromeric Amino Acid Transporters

Cooperation of Apical and Basolateral Heteromeric Amino Acid Transporters for L-Cystine Transport—The role of b₀,−AT-rBAT and of its cooperation with basolateral LAT2-4F2hc and/or y⁺LAT1-4F2hc for transepithelial transport of L-cystine was tested by adding to both sides the amino acid pair L-cystine + L-Leu (Fig. 4A). The transport of L-cystine was tested because it is its tubular accumulation that leads to most clinical problems found in cystinuria. Concerning the basolateral efflux of L-cystine, it is believed that its reduction products (two L-Cys), which is of the same range as that measured for L-Arg, indicates that this transporter cooperates with apical b₀,−AT-rBAT for a transepithelial exchange of L-Arg against L-Leu. The effect of LAT2-4F2hc on net L-Leu transport does not correspond to an increase in apical-to-basolateral L-Arg flux and can thus be explained by a basolateral exchange of extracellular L-Cystine against endogenous neutral amino acids.

The resulting net L-Arg transport is in the apical-to-basolateral direction. The effect of b₀,−AT-rBAT expression is such that more L-Arg is being transported into the cell and thus also extruded through the basolateral membrane. This extrusion is increased by the presence of y⁺LAT1-4F2hc in the basolateral membrane.

The apical extrusion of L-Leu (intracellular + basolateral accumulation) is significantly increased by the expression of b₀,−AT-rBAT, confirming that this exchanger does transport L-Leu also inwards, though less efficiently than it does L-Arg (Fig. 3B). This experiment also shows that MDCK cells display an efficient endogenous basolateral efflux pathway for L-Leu, since the ratio of labeled basolateral to intracellular L-Leu was high. It is noteworthy that neither the addition of LAT2-4F2hc nor of y⁺LAT1-4F2hc increased the basolateral efflux of L-Leu, an observation that is not surprising in view of the fact that L-Leu is not a good efflux substrate for either of these exchangers. The apical extrusion of L-Leu was increased by the expression of b₀,−AT-rBAT, demonstrating that L-Leu is an efflux substrate for this exchanger. The fact that this apical extrusion was increased by the expression of either basolateral exchanger (significant only in the case of coexpression of both transporters) strongly supports the notion that both LAT2-4F2hc and y⁺LAT1-4F2hc import L-Leu from the basolateral side.

The net L-Leu transport across the wild type MDCK monolayer is in an apical-to-basolateral direction (re)absorption, compatible with the presence of some apical Na⁺-neutral amino acid transport activity (25) and unchanged by the expression of either the apical or the basolateral heteromeric transporters. However, if a combination of apical b₀,−AT-rBAT and either basolateral transporter is expressed, the direction of the net L-Leu transport is reversed into the basolateral-to-apical direction. In the case of y⁺LAT1-4F2hc expression, the change in L-Leu net flux is of the same magnitude but opposite direction than that of L-Arg, indicating that this transporter cooperates with apical b₀,−AT-rBAT for a transepithelial exchange of L-Arg against L-Leu. The effect of LAT2-4F2hc on apical L-Leu efflux does not correspond to an increase in apical-to-basolateral L-Arg flux and can thus be explained by a basolateral exchange of extracellular labeled L-Leu against endogenous neutral amino acids.

Cooperation of apical and basolateral heteromeric amino acid transporters for L-cystine transport. Cells expressing combinations of heteromeric amino acid transporters, indicated by the individual abbreviations (key in A, bottom left panel), were cultivated on permeable supports. The filter cultures were incubated for 2 h with 100 μM L-cystine and L-Leu + Na⁺ on both sides of the monolayer, with the tracer L-[¹⁴C]cystine (A) or L-[³H]Leu (B) added either to the apical (1st row) or the basolateral (2nd row) compartment. After the 2-h period, aliquots of the contralateral compartment (apical (A) or basolateral (B)) were taken to determine the unidirectional transepithelial and the cells lysed to determine the cellular (C) accumulation. The net transport rate across the monolayer (3rd row) is the difference between the unidirectional transport rates. The amounts of transported amino acids in individual experiments were arbitrarily normalized to the amount of L-cystine accumulated intracellularly from the apical side by b₀,−AT-rBAT-expressing cells (mean: 0.8 nmol × cm⁻² × h⁻¹ L-Cys), n = 9; error bars, S.E.; **, p < 0.01; ***, p < 0.001.

Fig. 4. Cooperation of apical and basolateral heteromeric amino acid transporters for L-cystine and L-Leu transport.
the basolateral transporters. The expression of apical b0\(^{−}\)-AT-rBAT led to a 4-fold higher intracellular accumulation of L-cystine, as expected from our previous study (12). Its basolateral extrusion (probably as L-Cys) was increased as well, though by a lower factor. The coexpression of either basolateral transporter increased the basolateral extrusion of L-cystine/L-Cys. As mentioned above, this was expected for LAT2-4F2hc that can exchange intracellular L-Cys for extracellular L-Leu (15). However, y\(^{−}\)LAT1-4F2hc was not expected to mediate L-cystine or L-Cys efflux. Thus, we tested the possibility that y\(^{−}\)LAT1 was an efflux pathway for L-Cys in Xenopus oocytes but did not observe any transport (not shown). We then tested the hypothesis that MDCK cells express LAT2 endogenously. This would explain the increase in basolateral L-Cys efflux upon expression of exogenous 4F2hc, assuming that endogenous 4F2hc would limit (endogenous) LAT2 surface translocation. With a set of degenerate primers we identified a partial sequence from MDCK cells bearing 89% identity with the mouse, 90.5% identity with the rat, and 91.5% identity with the human sequences. This suggests that the expression of 4F2hc alone (or with y\(^{−}\)LAT1) induces the expression/activation of the endogenous LAT2 transporter, which is responsible for the observed L-Cys efflux. This hypothesis was corroborated by the fact that expression of 4F2hc alone increased cystine reabsorption in b0\(^{−}\)-AT-rBAT-expressing cells (not shown).

Because the basolateral-to-apical transport of L-cystine was not affected, in contrast to its apical-to-basolateral transport, by any of the exogenous transporters, net transport of L-cystine (that was close to zero in wild-type MDCK cells) was strongly increased in the apical-to-basolateral direction by the expression of apical b0\(^{−}\)-AT-rBAT and further increased by the coexpression of either basolateral exchanger.

In the same series of experiments, the transport of L-Leu was qualitatively affected in a similar way by the expression of heteromeric amino acid transporters as in the experiments in which it was given together with L-Arg (see above). However, due to the unfavorable ratio of exogenous to endogenous transport and the experimental variability, changes in net L-Leu transport did not reach the level of significance.

**DISCUSSION**

**Early Proximal Tubule as Major Expression Site of the Three Amino Acid Exchangers That Form a Cationic Amino Acid and Cystine Transport Module**—The present immunolocalization performed on mouse kidney sections demonstrates that the three gpaATs b0\(^{−}\)-AT, LAT2, and y\(^{−}\)LAT1 are coexpressed in cells of the proximal tubule, following exactly the same axial gradient (Fig. 1). They are strongly expressed in the S1>S2 segments and display a much weaker expression in S3. Former studies have already described the kidney brush border localizations of b0\(^{−}\)-AT and its subcellular colocalization with rBAT as well as the fact that rBAT displays an opposite axial distribution gradient along the proximal tubule (S3>S2>S1) (13). The basolateral localization of LAT2 and its colocalization with 4F2hc were also previously illustrated (20). We now show that y\(^{−}\)LAT colocalizes with LAT2 in the basolateral membrane of the same kidney proximal tubule cells that also express apical b0\(^{−}\)-AT and thus follows the same axial gradient. This precise coexpression of three gpaATs suggests that they function together as a building block of the transcellular amino acid reabsorption machinery for cationic amino acids and cystine. The parallelity of y\(^{−}\)LAT1 and LAT2 expression with that of b0\(^{−}\)-AT, and not with rBAT, implies that the reabsorption of these amino acids follows the same proximal to distal gradient, and suggests that the additional high level expression of rBAT in the S3 portion of the proximal tubule is not linked to an additional dibasic amino acid reabsorption. The fact that all kidney b0\(^{−}\)-AT appears to be associated with rBAT has been recently shown and supports the hypothesis of an additional rBAT function in the S3 segment (24).

**Serial b0\(^{−}\)-AT-rBAT and y\(^{−}\)LAT1-4F2hc Are a Functional Unit for Cationic Amino Acid (Re)absorption**—The expression of the b0\(^{−}\)-AT-rBAT transporter leads to apical L-Arg influx in MDCK cells, as previously demonstrated (12). In the absence of added basolateral transporter, this already leads to a net transepithelial transport of L-Arg (Fig. 3A). It appears likely that in this case the basolateral efflux of L-Arg is mediated by an endogenous y\(^{−}\)-LAT-type and not by a y\(^{−}\)LAT-type transporter, since L-Arg is not exchanged against extracellular L-Leu. L-Arg uptake by system y\(^{−}\)CAT transporters (is known to be transstimulated by intracellular cationic amino acids and thus obeys a non- obligatory exchange mode (uniport) (see Ref. 28 for review). Thus, at the low extracellular y\(^{−}\)-LAT1 substrate concentration used in this study (50 μM L-Arg), a favorable concentration gradient appears to overcome the electrical gradient and thus permits the net influx of L-Arg via a basolateral CAT transporter.

Here, we show that the additional expression of y\(^{−}\)LAT1-4F2hc in the basolateral membrane increases the transepithelial transport of L-Arg and, by the same amount, the opposite transport of L-Leu. This is the clear demonstration that the in-series arrangement of the two obligatory exchangers b0\(^{−}\)-AT and y\(^{−}\)LAT1 suffices to mediate transepithelial L-Arg (re)absorption, when an exchange substrate and Na\(^{+}\) are present. At the apical membrane, the influx of L-Arg is thus driven by the membrane potential and is coupled to the efflux of a neutral amino acid, in this case essentially L-Leu (the only neutral amino acid present in the extracellular milieu). However, the relatively low ratio of apical/intracellular labeled L-Leu suggests that L-Leu might not be a high affinity substrate for efflux via b0\(^{−}\)-AT-rBAT. L-Arg taken up apically by b0\(^{−}\)-AT is sequentially transported across the basolateral membrane via y\(^{−}\)LAT1 in exchange for L-Leu and Na\(^{+}\) (additionally to an efflux via endogenous y\(^{−}\)-LAT1; see above). This electroneutral transport is driven by the outward-directed concentration gradient of L-Arg and inward one of Na\(^{+}\). The fact that in the presence of y\(^{−}\)LAT1 some transepithelial L-Arg transport that is not compensated by an opposite L-Leu transport persists, although the driving forces at the basolateral side should favor the latter transport over the endogenous y\(^{−}\)-LAT1 one, can be explained by the fact that not all b0\(^{−}\)-AT-rBAT-expressing cells express a corresponding level of basolateral y\(^{−}\)LAT1-4F2hc (see inhomogeneity in Fig. 2).

The serial b0\(^{−}\)-AT-rBAT and y\(^{−}\)LAT1-4F2hc exchangers thus represent a functional unit for the reabsorption of cationic amino acids that, in exchange, secretes neutral amino acids (in this case L-Leu). Thus, to assure a net reabsorption of neutral amino acids as well, this functional unit needs to be installed in parallel with a unidirectional transepical (re)absorptive pathway for neutral amino acids, in particular for the exchange substrates of b0\(^{−}\)-AT and y\(^{−}\)LAT1-4F2hc. The major apical transport system that corresponds to these criteria is b0\(^{−}\) that, however, has not been yet molecularly defined. At the basolateral membrane, the characteristics of an efflux pathway for neutral amino acids have not yet been described.

**Role of LAT2-4F2hc for L-cystinol-Cys Reabsorption**—For L-cystine (maintained extracellularly in oxidized form with DTNB and presumably reduced to two L-Cys intracellularly), as for L-Arg, the apical expression of b0\(^{−}\)-AT-rBAT not only increases the apical uptake, but also the net transepithelial flux, even in the absence of an additional basolateral transporter. It appears that an endogenous basolateral pathway mediates this basolateral L-Cys efflux in a unidirectional man-
ner, because there is no concomitant increase in basolateral L-Leu or L-cystine uptake (the only two amino acids present extracellularly in these experiments). The nature of the pathway that mediates this unidirectional efflux of L-Cys is not known. The existence of such a pathway could play a role in maintaining the cellular osmolyte level, because the intracellular reduction of L-cystine into two L-Cys increases the number of intracellular solute molecules.

The additional expression of 4F2hc alone or in combination with LAT2 or y’LAT1 yielded a small but significant increase in the basolateral extrusion of L-cystine/L-Cys in b0,+AT-RbAT-expressing cells. The fact that 4F2hc alone already had this effect (data not shown) suggests that 4F2hc activates an endogenous L-Cys efflux pathway. We tested by RT-PCR the hypothesis that LAT2 is the activated endogenous transporter because LAT2 is known from Xenopus laevis oocyte expression experiments to mediate the efflux of L-Cys when coexpressed with 4F2hc (15). The presence of a corresponding transcript in MDCK cells suggests indeed that exogenous 4F2hc increases the surface expression of endogenous LAT2 that otherwise is limited by the low level of endogenous 4F2hc.

Thus, although the present data lack the clarity of those obtained with an endogenous LAT2 and of the relatively high transport rates of L-Leu by endogenous transporter(s) that prevent a precise quantification of basolateral L-Cys - L-Leu exchange, they strongly suggest that LAT2 participates in basolateral L-Cys efflux in the context of transepithelial L-cystine/L-Cys transport. These data also support the notion that MDCK cells express a basolateral unidirectional efflux pathway for neutral amino acids that can function in parallel with LAT2-4F2hc and that is required for net directional amino acid transport across the basolateral membrane. Whether classical L-Cys transporters like asc1 (4F2hc-associated transporter) or ASC2 are involved in this efflux remains to be established.

Endogenous Transporters in MDCK Cells—As shown by RT-PCR and suggested by the functional experiments discussed above, MDCK cells possess endogenous LAT2, a part of which might be functionally expressed in the absence of exogenous 4F2hc. This would imply the presence of some endogenous 4F2hc, a possibility that is supported by the immunolocalization study that shows a partial basolateral membrane localization of y’LAT1 and/or LAT2 in the absence of exogenous 4F2hc.

The functional experiments further demonstrate that endogenous transport systems for influx and efflux of L-Leu exist in both the apical and basolateral membranes of MDCK cells. The (re)absorptive transport of L-Leu observed across untransfected MDCK epithelia is compatible with the previous observation of a Na+–dependent neutral amino acid transport that resembles B0 (25). The fact that most L-Leu taken up apically via b0,+AT-RbAT is extruded basolaterally also in the absence of exogenous basolateral transporter indicates that MDCK cells express an endogenous basolateral efflux system for L-Leu that has apparently a high capacity and possibly also a relatively high affinity. The molecular nature of the transporter that mediates this efflux of L-Leu and whether it is the same one that transports L-Cys remains to be established.

In conclusion, the present study demonstrates the cooperation of apical and basolateral heteromeric exchangers for the transepithelial transport of cationic amino acids and L-cystine and the fact that these transporters also function as exchangers in an epithelial context. The opposite direction of neutral amino acid transport (exchange) supports the notion that additional unidirectional transporters of neutral amino acids need to be expressed in both the apical and the basolateral membrane of directionally transporting epithelia to recycle the exchange substrates and thus drive the transport of neutral amino acids in the (re)absorptive direction. Such an apical unidirectional transport system has been functionally characterized in the kidney proximal tubule (B0) but not yet molecularly identified. The basolateral unidirectional efflux pathway, that appears to be expressed at a certain level also in MDCK cells, remains to be further characterized.
Functional Cooperation of Epithelial Heteromeric Amino Acid Transporters Expressed in Madin-Darby Canine Kidney Cells
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