The *rBAT* Gene Is Responsible for L-Cystine Uptake via the b$^{0,+}$-like Amino Acid Transport System in a "Renal Proximal Tubular" Cell Line (OK Cells)*

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Several studies have shown that the cRNA of human, rabbit, or rat rBAT induces in *Xenopus* oocytes sodium-independent, high affinity uptake of L-cystine via a system b$^{0,+}$-like amino acid exchanger. We have shown that mutations in rBAT cause type I cystinuria (Calonge, M. J., Gasparini, P., Chillarón, J., Chillón, M., Gallucci, M., Rousaud, F., Zelante, L., Testar, X., Dallapiccola, B., Di Silverio, F., Barceló, P., Estivill, X., Zorzano, A., Nunes, V., and Palacín, M. (1994) *Nat. Genet.* 6, 420–425; Calonge, M. J., Volpini, V., Bisceglia, L., Rousaud, F., De Sanctis, L., Beccia, E., Zelante, L., Testar, X., Zorzano, A., Estivill, X., Gasparini, P., Nunes, V., and Palacín, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 9667–9671). Apart from oocytes, no other expression system has been used for transfection of functional rBAT activity. Furthermore, the b$^{0,+}$-like transport activity has not been clearly described in the kidney or intestine. Here, we report that a "proximal tubular-like" cell line derived from opossum kidney (OK cells) expresses an rBAT transcript. Poly(A)$^+$ RNA from OK cells induced system b$^{0,+}$-like transport activity in oocytes. This was hybrid-depleted by human rBAT antisense oligonucleotides. A polymerase chain reaction-amplified cDNA fragment (~700 base pairs) from OK cell RNA corresponds to an rBAT protein fragment 65–69% identical to those from human, rabbit and rat kidneys. We have also examined transport of L-cystine in OK cells and found characteristics very similar to the amino acid exchanger activity induced by rBAT cRNA in oocytes. Uptake of L-cystine was of high affinity, sodium-independent and shared with L-arginine and L-leucine. It was trans-stimulated by amino acids with the same specificity as rBAT-induced transport activity in oocytes. Furthermore, it was localized to the apical pole of confluent OK cells. To demonstrate that the rBAT protein is functionally related to this transport activity, we have transfected OK cells with human rBAT antisense and sense sequences. Transfection with rBAT antisense, but not with rBAT sense, resulted in the specific reduction of rBAT mRNA expression and b$^{0,+}$-like transport activity. These results demonstrate that rBAT is functionally related to the L-cystine uptake via system b$^{0,+}$-like in the apical pole of the renal OK cell line.

Human rBAT cDNA elicits high affinity sodium-independent transport of cystine, dibasic amino acids, and some zwitterionic amino acids via a b$^{0,+}$-like transport system in *Xenopus* oocytes (1, 2). Heteroexchange diffusion of these amino acids has been reported for this transport activity expressed by rabbit (3, 4), rat (5), and human[1] rBAT cRNA in oocytes. rBAT protein is expressed in the brush border plasma membrane of both the proximal straight tubules of the nephron and the small intestine (6, 7). Recent studies have demonstrated that mutations in the human *rBAT* gene cause cystinuria (8–12). Cystinuria is a common inherited aminoaciduria due to the defective transport of cystine and dibasic amino acids through the epithelial cells of the renal tubule and intestinal tract (13, 14). The clinical manifestation of cystinuria is the development of kidney cystine calculi resulting from the poor solubility of this amino acid (14). Three types of classic cystinuria have been described on the basis of the amino acid hyperexcretion of heterozygotes and the degree of the intestinal transport defect (14–16). Very recently, strong evidence has been offered suggesting that rBAT is only responsible for type I (10, 12).

Due to the role of the *rBAT* gene in type I cystinuria, the rBAT protein is considered to be responsible for the reabsorption of cystine and dibasic amino acids in the proximal straight tubule. Two aspects of this remain to be clarified. First, a complete picture of the b$^{0,+}$-like transport activity responsible for cystine reabsorption has not been described in the epithelial cells of kidney or intestine. In renal brush border membrane vesicles a high affinity system for cystine and dibasic amino acids, which shows heteroexchange diffusion between these substrates, has been demonstrated (17–19). This transport was shown to be defective in biopsies of intestinal mucosa from cystinuric patients (20–22). The sodium dependence of this reabsorption system is controversial, as is its interaction with neutral amino acids, since no hyperexcretion of neutral amino acids occurs in cystinuria (17–19, 22–24). Second, the role of rBAT in the b$^{0,+}$-like transport mechanism is unknown. Evi-

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dence from Tate and co-workers (25) suggests a four-membrane-spanning domain model for rBAT. This topology is unusual for metabolite transporters, which appear to contain 8–12 transmembrane domains (26). This fostered the idea that rBAT is a regulatory subunit of an oligomeric transporter rather than the transporter itself (27, 28). Indirect evidence suggested that rBAT forms a heterodimeric structure of 125 kDa with an unidentified protein of 40–50 kDa in renal brush border membranes and oocytes (29).[2] In addition, transient expression of rBAT in COS cells revealed either expression of rBAT in the cell surface without concomitant amino acid transport activity (30) or a protein product that does not reach the plasma membrane.[3] Thus, no cell system other than oocytes has shown expression of amino acid transport activity associated with rBAT. It thus remains an open question whether the amino acid transport activity associated with rBAT in the renal epithelial cells is the same as that elicited by rBAT expression in oocytes.

To this end we searched for the role of rBAT in the transport of cystine in an epithelial cell line derived from proximal tubules of the kidney of an American opossum, the cell line OK. These cells express an mRNA transcript that hybridizes with rBAT probes (27, 28), and they show high affinity sodium-independent cystine-dibasic amino acid transport in the apical pole (31). Here we demonstrate that OK cells express rBAT mRNA and that the transport of cystine in the apical pole of these cells is due to an amino acid transport system very similar to the $b^{0,+}$-like system elicited by rBAT in oocytes. Transfection of rBAT antisense sequences results in the specific reduction of rBAT mRNA expression and $b^{0,+}$-like transport activity. This demonstrates that rBAT is responsible for the high affinity sodium-independent L-cystine uptake shared with dibasic and neutral amino acids (*i.e.* system $b^{0,+}$-like) present in the apical pole of the renal cell line OK.

<div align="center">MATERIALS AND METHODS</div>

Cell Culture and Uptake Studies in OK Cells—Studies were performed with the OK cell line clone 3B/2, derived by selection from the original OK cells (32), between passages 16 and 21. This clone was originally subcloned from the parental line for maximal response of phosphate transport to low phosphate medium and to inhibition by parathyroid hormone. Cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum, as described (33). Subcultures were prepared by trypsinization and reseeding at high density (approximately 1×10^6 cells/ml). Only cells free of mycoplasma (Gene Probe Mycoplasma T.C. (Movaco) test kit) were studied.

For transport studies, cells were used 5 days after plating (90–100% confluence) on 35-mm diameter dishes, except for antisense studies, in which 16-mm diameter wells were used. Growth medium was removed, and dishes were washed three times in 3 ml of MGA[4] medium (*i.e.* 137 mM *N*-methyl-D-glucamine (MGA), 5.4 mM KCl, 2.8 mM CaCl$_2$, 1.2 mM MgSO$_4$, 10 mM HEPES; pH 7.4 at 37 °C) prewarmed to 37 °C. MGA was replaced by 137 mM NaCl (sodium medium) in those experiments in which the sodium-dependence was studied. Uptake media were prepared by adding the labeled amino acid (L-[³H]arginine and L-[³H]leucine from DuPont NEN or L-[³⁵S]cystine from Amersham Corp; final concentration, 0.5–1 μCi/ml) to MGA or sodium media. When cystine was present, the uptake medium contained 5 mM diamide as an oxidizing agent. Uptake was started by the addition of 1 ml (0.5 ml for 16-mm diameter wells) of uptake medium (at 37 °C) to the plate and terminated by removing uptake medium from the plate and washing it five times in cold stop solution (132 mM NaCl, 14 mM Tris/HCl, 5 mM L-arginine, 5 mM L-leucine; pH 7.4 at 4 °C). For the three amino acids used, uptake periods were previously assessed for all concentrations studied and consequently the uptake period used was 30 s. Nonspecific

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[4] The abbreviations used are: MGA, *N*-methyl-D-glucamine; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); BCH, 2-amino-2-norbornane-carboxylic acid.

binding was assessed by measuring zero-time uptake, which was achieved by adding the uptake medium and immediately removing it and stopping the uptake. Cell lysates were obtained by adding 1 ml of 0.5% Triton X-100/plate (0.5 ml in 16-mm diameter wells). 150 μl of this lysate was removed for scintillation counting in 3 ml of EcoLite^{+TM} scintillation fluid (ICN), and 25 μl was used for protein determination by the Lowry method (34). The zero point was subtracted from the 30-s value, and uptake is expressed as nmol/mg protein·min. Previous studies demonstrated that after subtracting the zero value, the best fit line passed through the origin (data not shown).

For trans-stimulation experiments, cells in a confluent 3.5-cm diameter plate were preloaded for 2 min with 50 μM L-[³H]arginine or L-[³H]leucine (0.5 μCi/ml). Afterward, they were washed in MGA medium and 1 ml of MGA prewarmed medium was added to the plate. From 15 s to 10 min, the efflux was monitored by removing aliquots (50 μl) periodically from the medium. The radioactivity contained in each aliquot was quantified by liquid scintillation counting. Results are expressed as (counts/min)/mg of protein/min. Efflux was linear for up to 1 min. Thin layer chromatography of the efflux medium in silica gel using butanol/acetic acid/water (4:1:1, by volume) as a solvent, as described elsewhere (35), revealed that ~95% of the efflux radioactivity corresponds to L-arginine.

For transport polarity studies, OK cells were grown on Handmade transwell filters (Nucleopore Corp. filtration products). A 1-cm diameter polycarbonate tube was cut in 1-cm sections, and polycarbonate filter membrane (0.1-μm pore size, 12-cm diameter, Nucleopore) was glued with 1:1 cyclohexanone:chloroform to one side of each section. The filters were dried, sterilized in 70% ethanol for at least 2 h, dried overnight, and collagen-coated with (50 μl/filter) of rat tail collagen (R-type, Serva; 0.5 mg/ml in 50% ethanol). Cells were seeded at high density 1/1.3 from the final trypsinized volume (20 ml of cell suspension). 500 μl of medium was added to the basal surface. Cells were refed 1 day before the uptake, which was carried out 3 days after the seeding, when cells had reached confluence. Transport measurements were performed using the uptake medium described above for plastic cell dishes. Both sides of the plastic transwell filters were thoroughly washed in MGA medium. For apical transport, in either the presence or absence of sodium, basal side was immersed in MGA medium. Uptake was initiated by the addition of 500 μl of the uptake medium to the corresponding side of the filter. For basal transport, the same procedure was followed, *i.e.* the apical side was immersed in MGA medium. Uptake was stopped by aspirating the uptake solution and washing the filters five times in the stop solution mentioned above. 100 μl of 0.5% Triton X-100 was added to each filter, and, 30 min later, total radioactivity incorporated into the monolayer was measured by liquid scintillation counting of the whole filter immersed in 9 ml of scintillation fluid. Results are expressed as pmol/30 s/filter.

Oocyte Studies—Oocyte management, injections, and amino acid uptake measurements were described elsewhere (1). *Xenopus laevis* (H. Kähler, Institut für Entwicklungsbiologie, Hamburg, Germany) oocytes were defolliculated by collagenase D (Boehringer Mannheim), and healthy-looking stage VI oocytes were injected (Inject+Matic system, J. A. Gabay, Geneva, Switzerland) with 50 nl of poly(A)$^+$, cRNA, or water, as described elsewhere (1). RNA was extracted from OK cells by the guanidinium-thiocyanate-phenol-chloroform method as described (36). Oligo(dT)-cellulose (Boehringer Mannheim) for the purification of poly(A)$^+$ RNA was used following the manufacturer's protocol.

For hybrid depletion experiments, OK cell mRNA (0.7–1.0 mg/ml) or human rBAT cRNA (0.01 mg/ml), prepared as described elsewhere (1), were denatured at 65 °C for 5 min in a solution containing 50 mM NaCl and 20 μM of a 20-mer sense or a 21-mer antisense oligonucleotide complementary to the human (and rat) rBAT mRNA sequence (sense: 5′-TGC CCA AGG AGG TGC TGT TC-3′, starting at base 203 of the coding region; antisense: 5′-GAA CAG CAC CTC CTT GGG CAT-3′, starting at base 222 of the coding region) and further incubated at 42 °C for 30 min prior to oocyte injection. In these conditions, human rBAT cRNA-induced amino acid transport activity was specifically hybrid depleted by >80% by the above mentioned antisense oligonucleotide (data not shown).

PCR Amplification and DNA Sequencing—cDNA was synthesized in a 20-μl reaction volume from a 1-μl RNA aliquot (poly(A)$^+$ RNA from OK cells at 1–3 μg/μl) and primed with 2.5 μM oligo(dT)$_{16}$ (GeneAmp RNA PCR kit; Perkin-Elmer). Incubation was for 60 min at 42 °C. Reverse transcriptase was inactivated at 99 °C for 5 min. The 20-mer oligonucleotides C4D (sense) and C5R (antisense), complementary to the the human rBAT cDNA sequence, elsewhere described (8), were used as primers for the PCR amplification that comprised: (*a*) an initial denaturation at 95 °C for 3 min; (*b*) 5 cycles with denaturation at 95 °C

for 35 s, annealing at 46 °C for 40 s, and extension at 74 °C for 70 s (after annealing, temperature increased at a ramp rate of 12 °C/min); and (*c*) 29 cycles with denaturation at 94 °C for 25 s, annealing at 56 °C for 25 s, and extension at 74 °C for 70 s. The reaction buffer was Perkin-Elmer Roche N808-0006 with 200 μM each dNTP, 0.6 μM of each primer, 2.5 mM MgCl$_2$, 10 μl of the reverse transcriptase reaction, and 2 units of *Taq* polymerase (Perkin-Elmer Roche) in 100 μl. This amplification yielded a fragment of ~700 bp as expected. The PCR product was run in a 4% nondenaturing polyacrylamide gel, and the 700-bp band was cut and eluted with 20 μl of distilled water. In order to obtain large amounts of this fragment, 5 μl of the eluted product was used as a template in a second round of PCR with the same primers and at high stringency conditions for 34 cycles. After purification with Qiagen columns (Qia Quick Spin PCR purification kit) this product was used for automatic DNA sequencing and as a DNA probe for Northern analysis.

The purified C4D-C5R PCR-fragment was sequenced with the same primers as for amplification and the internal primers C4R and C5D, described elsewhere (8), using an automatic DNA sequencer (Applied Biosystems model 373A) and *Taq* DyeDeoxy™ terminator cycle sequencing kit.

Permanent Transfection of OK Cells—A 669-bp *Eco*RI/*Cla*I (Klenow blunt-ended) fragment from the full-length human rBAT cDNA (1), comprising 43 bp of the 5′ noncoding region, the ATG translation initiation codon and 626 bp of the coding sequence (including two bases of the codon for the amino acid residue Ile-209), was inserted in pcDNA3 mammalian expression vector (Invitrogen Corp.) polylinker previously cut with (*a*) *Bam*HI (Klenow blunt-ended)/*Eco*RI for antisense orientation or (*b*) *Xho*I (Klenow blunt-ended)/*Eco*RI for sense orientation. The expression of both constructs was under the control of the constitutive cytomegalovirus promoter. After transformation, antisense construct clones and sense construct clones were isolated and constructions were checked by restriction analysis.

For transfections and clone selection, confluent OK cell monolayers (day 0) were trypsinized and seeded 1/7 in two 25-cm² flasks. On day 1 (40–50% of confluence) cells were washed three times and then covered with 3 ml of serum-free medium. Cells were then transfected by adding dropwise 120 μl of DNA-Lipofectin mixture to each flask and swirling gently. DNA-Lipofectin mixture (1:1, v/v) was prepared with human rBAT sense or antisense constructs (45 μg/60 μl) and Lipofectin (30 μg/60 μl), following supplier's protocol (Life Technologies, Inc.). Cells were incubated with DNA-Lipofectin-containing medium for 16 h in a humidified atmosphere of 5% CO$_2$, 95% air at 37 °C. Medium was then removed and replaced by complete medium (*i.e.* with 10% serum). Cells were grown to confluence, trypsinized, and seeded in the presence of 0.4 mg/ml Geneticin (G418; Life Technologies, Inc.) to a very low density (1/200) so that single clones could be isolated by picking the clones with sterile trypsin-embedded cotton-sticks. G418-resistant clones were continuously grown in the presence of G418 (0.4 mg/ml). Screening of positive clones expressing the sense or antisense human transcript was performed by Northern blot analysis using *Bam*HI fragment of human rBAT cDNA cloned in pSPORT1 (1) .

Northern Blot Analysis—Total RNA from OK cells was transferred to nylon membranes (Hybond N, Amersham) by capillarity in 10 × standard saline citrate (SSC; 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) after size separation on a 1.2% agarose/formaldehyde gel. RNA was visualized with ethidium bromide to ensure that it was intact and loaded in similar amounts and to confirm proper transfer. Blots were prehybridized in 5 × Denhardt's (1 × Denhardt's: 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.1% SDS, 5 × SSPE (1 × SSPE: 0.15 M NaCl, 1 mM EDTA, 10 mM NaH$_2$PO$_4$, pH 7.4), 200 μg/ml salmon sperm DNA, and 50% formamide, and hybridized in the same solution containing 10% dextran sulfate at 42 °C. After hybridization, blots were washed in 2 × SSC for 10 min at room temperature and then twice for 20 min in 0.4 × SSC, 0.1% SDS at 55 °C. Two cDNA probes were used, either a 2.2-kb *Bam*HI fragment lacking 148 bp at the 5′ end of the complete human rBAT cDNA (13) purified from a 1% agarose gel using the PCR preps kit (Promega), or the PCR-amplified OK cell rBAT fragment (C4D-C5R; see above) cut from a 1% low melting agarose gel and purified by phenol/chloroform extraction and ethanol precipitation. Hybridizations were visualized by autoradiography (Agfa film, Currix RP2) using standard protocols. Probes were labeled with [α-³²P]dCTP (1 × 10⁶ cpm/ml) by random oligonucleotide priming (Boehringer Mannheim).

RESULTS

OK Cells Express the rBAT Gene—OK cells mRNA showed a transcript of ≥2.3 kb that hybridized with a human rBAT
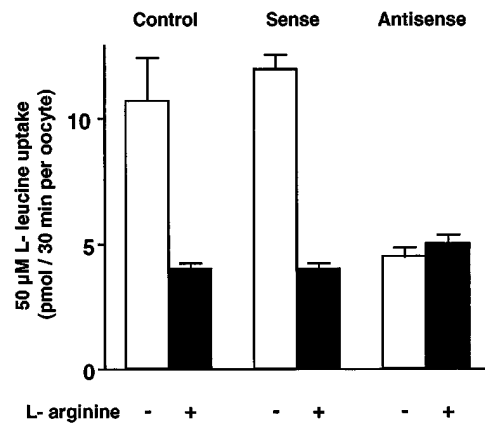

FIG. 1. **rBAT antisense hybrid depletion of the L-arginine-inhibitable L-leucine uptake induced by OK cells mRNA in oocytes.** Oocytes were injected with water containing 0 or 35 ng of OK cell mRNA. Prior to injection, mRNA was incubated alone (*Control*) or in the presence (20 μM) of a human rBAT antisense or sense oligonucleotide. Five days after injection, uptake of 50 μM L-leucine was determined for 30 min incubations and either in the absence or in the presence of 5 mM L-arginine. Each data point is the mean ± S.E. of the values (pmol/30 min/oocyte) of OK cell mRNA-induced L-leucine uptake, calculated by subtracting the uptake values obtained in water-injected oocytes from the uptake values in poly(A)⁺ RNA-injected oocytes. Uptake of L-leucine in water-injected oocytes were 7.3 ± 0.5 and 7.4 ± 0.3 pmol/30 min/oocyte in the absence or in the presence of L-arginine, respectively. Results were obtained with 6–8 oocytes per condition in a representative experiment. Another independent experiment showed similar results. The antisense effect and the L-arginine inhibition in the control and sense groups, but not in the antisense groups, were statistically significant (Student's *t* test; $p \leq 0.01$).

cDNA probe (data not shown). This agrees with previous studies that reported a transcript of similar size detected by Northern blot with rabbit or rat rBAT cDNA probes (27, 28). Cross-reaction with the homologous cell surface antigen 4F2hc transcript seems to be ruled out, since no hybridization signal was obtained in OK cells mRNA using a human 4F2hc cDNA (37) probe (data not shown). Poly(A)⁺ RNA from OK cells expressed b$^{0,+}$-like amino acid transport activity upon injection in *Xenopus* oocytes. Thus, injection of mRNA from OK cells in oocytes resulted in sodium-independent L-leucine uptake, which was inhibited (>60%) by L-arginine (Fig. 1). Similarly, oocytes injected with mRNA from OK cells showed expression of sodium-independent uptake of 50 μM L-cystine (induced activity over background of 2.6 ± 0.7 pmol/30 min per oocyte) and 50 μM L-arginine (induced activity over background of 33.0 ± 4.3 pmol/30 min/oocyte) uptake, which were inhibited (>60%) by a 100-fold excess of L-leucine (data not shown). Hybrid depletion of OK mRNA with an antisense oligonucleotide complementary to the human (and rat) rBAT cDNA specifically blocked (*i.e.* the sense oligonucleotide had no effect) expression of sodium-independent L-leucine uptake to the level inhibited by a 100-fold excess of L-arginine (Fig. 1). Similarly, OK mRNA-expression of sodium-independent L-arginine uptake inhibitable by L-leucine was hybrid-depleted (>90%) specifically by the same antisense oligonucleotide in oocytes (data not shown). These results demonstrate the functional expression of rBAT mRNA in OK cells. To provide further evidence for such expression, we searched for a partial sequence of rBAT cDNA in OK cells. RT-PCR from OK cell RNA, using oligonucleotides (C4D, sense; C5R, antisense; described in Ref. 8) complementary to the human rBAT cDNA, yielded a specific fragment of ~700 bp (data not shown). The nucleotide sequence of this fragment shows significant homology to human (74%), rabbit (74%), and rat (70%) rBAT cDNA (data not shown). The predicted amino acid sequence of this OK cell fragment covers amino acid residues 373–593 of the predicted human rBAT

protein and the second and third putative transmembrane domains in the topology model proposed by Tate and co-workers (25) (Fig. 2). Homology of this predicted protein fragment to human, rabbit, and rat rBAT cDNA ranged between 70% and

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OK                       ..... REPGRYRFMGTEARNQESIEK
Human  373                     T              -YA    DR
Rabbit                                 L       -YA    DR
Rat                                            V-SA   T R

        TMMYYGKPFVQEADFPFNFYLTEMSTISGTTIFDV
393     V    L   I        N      SMLD V  NSVYE
        R    LS  I               F TLD L  N VYE
        V    LS  I        K      ATLD L  H VYEA

        IKSWMKNMPEGKWPNWMIGGPDIMRVTSRIGKEYV
428     T    E                   SS  L    L NQ
        TA   E              T     T  L    L NQ
        T    E                   ETS L    V S

        NVMNMLILTLPGTPITYYGEEIGMEAISAANVNES
463          LF                  GN  V    L
        I    LF                  GN  L T  L
        A    LF                  GD   IT L  R

        YA--TLFSKSPMQWDNSSNAGFSEGNQTWLPTNKD
498     DIN  R              NSS      ASN       S
        DVN  L                       H         SS
        DTNA L                     T A H       S

        YQSLNVDVQTTQATSPLKLYQQLSSLRSSELLLSR
533     HTV      K   PR A      D  L HAN      N
        HTV      K   P  A      A  L HAN
        HTV      K   PS A      D  L HAR

        GWLCHVWHDADLVVYTRELDGLDRAF .....
568     F    LRN SHY          I   I
        F    LLRN GRVL        I   V
        F    LLRD NHS         I KV
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FIG. 2. **Comparison of the predicted and partial amino acid sequence of OK cell rBAT protein with the human, rabbit, and rat counterparts.** The first line shows the OK cells rBAT amino acid sequence. Below, only substituted amino acids in the sequences of human (*second line*), rabbit (*third line*), and rat (*fourth line*) rBAT proteins are shown. Amino acid gaps in the sequence are indicated by *dashes*. This fragment of the OK cell rBAT protein is 220 amino acid residues long and in this alignment starts at the amino acid residue 373 of the human rBAT protein. This fragment of the OK cells rBAT protein is 72%, 71%, and 70% identical (85%, 86%, and 84% similarity) to the human, rabbit, and rat rBAT protein fragment, respectively. Two putative transmembrane domains, corresponding to the second and third domains of the four-transmembrane-domain model for the rBAT protein proposed by Tate and co-workers (27) are *underlined* in the OK cell protein sequence. Two (OK cell protein) and three (human, rabbit, and rat proteins) potential *N*-glycosylation sites are *boxed*.
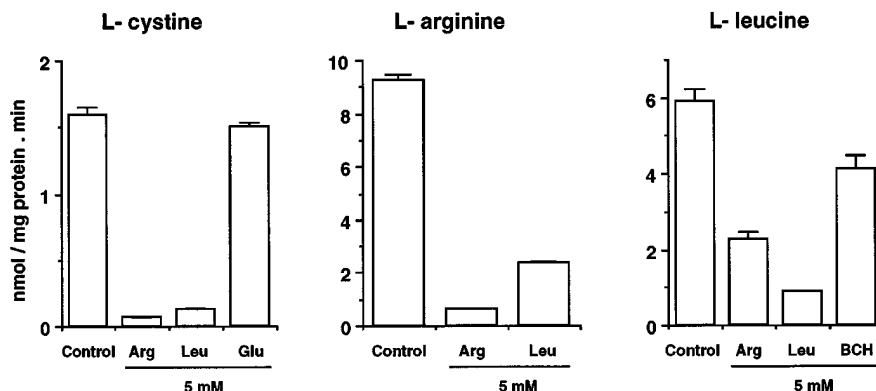
72% identity (84–86% similarity). Two out of three potential *N*-glycosylation sites present in the human, rabbit, and rat rBAT protein are conserved in the predicted OK cell rBAT protein fragment (Fig. 2). Regions with maximal homology between the four predicted protein fragments are those corresponding to the second and third putative transmembrane domains and around the second *N*-glycosylation site of the protein fragments. The region comprising the second putative transmembrane domain corresponds to one of the fragments highly conserved between rBAT and 4F2hc predicted proteins (38). These results demonstrate that OK cells express the *rBAT* gene.

OK Cells Express $b^{0,+}$*-like Amino Acid Transport Activity in the Apical Pole*—OK cells, grown on plastic, showed uptake of L-cystine, L-arginine, and L-leucine in the absence of sodium (Fig. 3). Most of the 50 μM L-cystine uptake was sodium-independent (~80%) (MGA medium: 1.39 ± 0.07 nmol/mg protein·min, $n = 8$; sodium medium: 1.87 ± 0.05 nmol/mg protein·min, $n = 8$). This is in full agreement with a previous report of L-cystine uptake in OK cells (31). Approximately half (~55%) of the L-leucine uptake was sodium-independent (MGA medium: 6.37 ± 0.09 nmol/mg protein·min, $n = 3$; sodium medium: 11.5 ± 0.6 nmol/mg protein·min, $n = 3$). Uptake of L-arginine was completely sodium-independent (MGA medium: 11.2 ± 0.5 nmol/mg protein·min, $n = 5$; sodium medium: 9.6 ± 1.1 nmol/mg protein·min, $n = 5$).

The sodium-independent uptake of L-cystine, and part of the L-arginine and L-leucine uptake, showed a pattern of substrate inhibition very similar to the $b^{0,+}$-like transport activity elicited by rBAT in oocytes (27). Thus, L-cystine uptake was almost completely abolished by a 100-fold excess of L-arginine and L-leucine in the uptake medium; in contrast, L-glutamate did not affect L-cystine transport, precluding expression of system X_{C^-} (Fig. 3). Similarly, the uptake of L-arginine was strongly inhibited (70–80%) by a 100-fold excess of L-leucine (Fig. 3), and the uptake of L-leucine was partially inhibited (60–70%) by a 100-fold excess of L-arginine (Fig. 3). The non-metabolizable amino acid analog BCH, a substrate model for system L, partially inhibited L-leucine uptake (20–40%). This suggests that part ([mteq]30%) of L-arginine and L-leucine uptake is due to transport activities resembling systems y^+ and L, respectively. On the other hand, interaction of L-leucine with sodium-independent L-arginine uptake suggests system $b^{0,+}$-like or system y^+L transport activities (27, 38, 39). Inhibition of 50 μM L-arginine uptake by varying concentrations of L-leucine (100 μM to 10 mM) was identical in the absence and in the presence of sodium (data not shown). This strongly indicates that the up-

FIG. 3. **Sodium-independent uptake of amino acids in OK cells.** Uptake of 50 μM L-[^{35}S]cystine, L-[^{3}H]arginine, or L-[^{3}H]leucine by OK cells were measured in the absence (*Control*) or in the presence of the indicated unlabeled L-amino acids at 5 mM concentration in the uptake MGA medium. Uptake values (nmol/mg protein·min) are the mean ± S.E. from 5–12 determinations from two to four independent experiments. All the inhibitions shown were statistically significant (Student's *t* test at least $p \leq 0.01$) with the exception of L-cystine transport in the presence of L-glutamate.
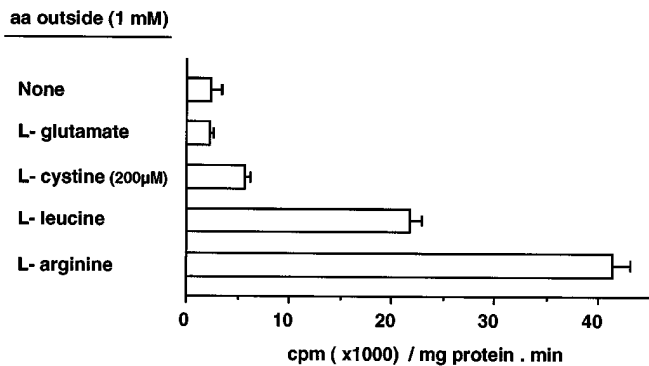
aa outside (1 mM)

FIG. 4. **Trans-stimulation of arginine efflux in OK cells.** Cells were preloaded with 50 μM L-[^{3}H]arginine for 2 min, then the medium was washed out and L-[^{3}H]arginine efflux was measured for 45 s in media containing no amino acids (none) or the indicated L-amino acids at 1 mM, except for L-cystine, which was present at 200 μM. Efflux rates (mean $\pm$ S.E.) are counts/min ($\times$ 1000) measured in the medium/mg protein·min from triplicates from a representative experiment. Efflux rates in the L-cystine, L-leucine, and L-arginine groups were statistically different (Student's *t* test; $p \leq 0.05$) from those of the none and L-glutamate groups. Another five independent experiments gave similar results.

FIG. 5. **Polarity of the arginine-inhibitable cystine uptake in OK cells.** Uptake of 50 μM L-[^{35}S]cystine by OK cells grown on filters was measured through the apical or basolateral poles, and in the absence (*open bars*) or in the presence (*filled bars*) of 5 mM unlabeled L-arginine. Uptakes were determined in the absence of sodium (MGA medium). Uptake values (pmol/min/filter) are the mean $\pm$ S.E. from triplicates in a representative experiment. Amino acid uptake inhibitions in the apical pole were statistically significant (Student's *t* test; $p \leq 0.01$). Another independent experiment showed similar results.

take of L-arginine is not due to y$^+$L activity. Studies using L-cystine as inhibitor are difficult to perform due to the low solubility of this amino acid. For this reason we analyzed the effect of L-cystine on the uptake of L-arginine and L-leucine at low concentration. The uptake of 5 μM L-arginine (1.28 $\pm$ 0.06 nmol/mg protein·min) was inhibited (23%) by 450 μM L-cystine (0.99 $\pm$ 0.02 nmol/mg protein·min) and to a similar extent by 450 μM L-leucine (1.00 $\pm$ 0.09 nmol/mg protein·min). Similarly, the uptake of 5 μM L-leucine (0.73 $\pm$ 0.07 nmol/mg protein·min) was inhibited (27%) by 450 μM L-cystine (0.53 $\pm$ 0.01 nmol/mg protein·min). In all, these results show that, in OK cells, uptake of L-cystine is mostly due to the system b$^{0,+}$-like activity, and that the uptake of L-arginine and L-leucine in the absence of sodium is due both to system b$^{0,+}$-like and, probably, systems y$^+$ and L, respectively.

Kinetic analysis of L-cystine uptake, examined over a range of concentration from 5 μM to 450 μM, showed apparent K_m and $V_{\max}$ values of 370 $\pm$ 40 μM and 13.6 $\pm$ 1.0 nmol/mg protein·min, respectively (data not shown). In agreement with a previous report on OK cells (31), one high affinity system appears to be present in OK cells. Kinetic analysis of L-arginine and L-leucine was performed over the range of 5 μM to 1000 μM. For arginine, one single kinetic component was observed with apparent kinetic parameters of K_m 211 $\pm$ 48 μM and $V_{\max}$ 59.9 $\pm$ 6.6 nmol/mg protein·min for L-arginine (data not shown). For the uptake of L-leucine inhibitable by 5 mM L-arginine, the apparent kinetic parameters were: K_m 175 $\pm$ 56 μM and $V_{\max}$ 20.8 $\pm$ 2.5 nmol/mg protein·min (data not shown). These low apparent K_m values for L-cystine, L-arginine, and L-leucine are of the same order (slightly higher) as those reported for these amino acids via the b$^{0,+}$-like amino acid transport system elicited by human, rabbit, and rat rBAT cRNA in *Xenopus* oocytes (1, 2, 27, 28, 40).

Recently, it has been reported that system b$^{0,+}$-like expressed by rBAT in oocytes shows trans-stimulation, suggesting that this transport system is an amino acid exchanger (3–5). To provide further evidence for the presence of system b$^{0,+}$-like transport activity in OK cells, we searched for trans-stimulation of L-arginine efflux by different amino acids. Efflux was very low in the absence of amino acids ($\sim$1% of the total 2-min loading of L-[^{3}H]arginine) but was increased by amino acid substrates of system b$^{0,+}$-like in the external medium: 17-fold by 1 mM L-arginine, 6-fold by 1 mM L-leucine, and >2-
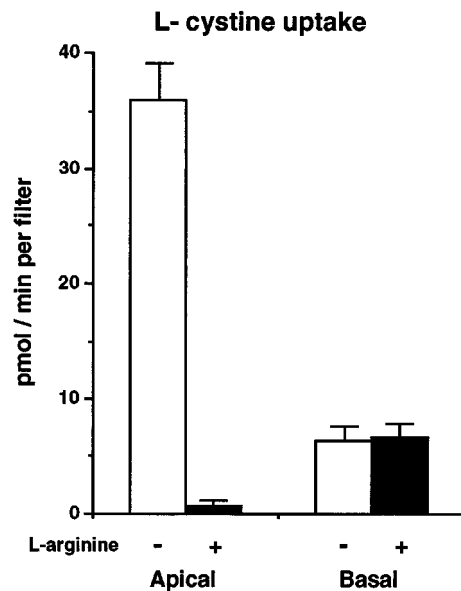
fold by 200 μM L-cystine (Fig. 4). In contrast, L-arginine efflux was not trans-stimulated by 1 mM L-glutamate in the external medium (Fig. 4). The finding that most ($\sim$70%) of the L-arginine uptake in OK cells was due to system b$^{0,+}$-like activity and that y$^+$L activity was not present in these cells indicates that trans-stimulation of L-arginine efflux by L-leucine is due to system b$^{0,+}$-like. Similarly, L-leucine efflux was trans-stimulated by 1 mM L-arginine (6-fold) and by 300 μM L-cystine (2.5-fold) (data not shown), also showing trans-stimulation with characteristics of system b$^{0,+}$-like.

Finally, we investigated the polarity of system b$^{0,+}$-like activity in OK cells grown on transwell filters. Sodium-independent L-cystine (Fig. 5) and L-arginine (data not shown) uptake were higher (6–8-fold) in the apical pole. This is in full agreement with a previous report of L-cystine uptake on OK cells (31). Interestingly, the uptake of L-cystine and L-arginine in the apical but not in the basolateral pole showed the characteristic inhibition pattern of system b$^{0,+}$-like; L-cystine uptake was abolished by a 100-fold excess of L-arginine (Fig. 5), and the uptake of L-arginine was strongly inhibited (<80% inhibition) by a 100-fold excess of L-leucine (data not shown). In all, these results demonstrate that OK cells express system b$^{0,+}$-like amino acid transport activity in the apical pole.

Transfection of rBAT Antisense Results in a Specific Decrease of System b$^{0,+}$-like Transport Activity in OK Cells—In order to demonstrate that the *rBAT* gene was responsible for system b$^{0,+}$-like amino acid transport activity in OK cells, we performed rBAT cDNA antisense expression in these cells. After transfection with 5′-end fragments of human rBAT cDNA antisense or sense and Geneticin selection, cell clones were searched for expression of the transfected constructs. Two clones were positive for antisense transcript expression, and two others for sense expression (data not shown). Expression of endogenous rBAT mRNA in these clones was studied with the PCR-amplified fragment rBAT cDNA from OK cells shown in Fig. 2 as a probe. In comparison with control (*i.e.* untransfected cells), the rBAT mRNA levels were lower in two antisense
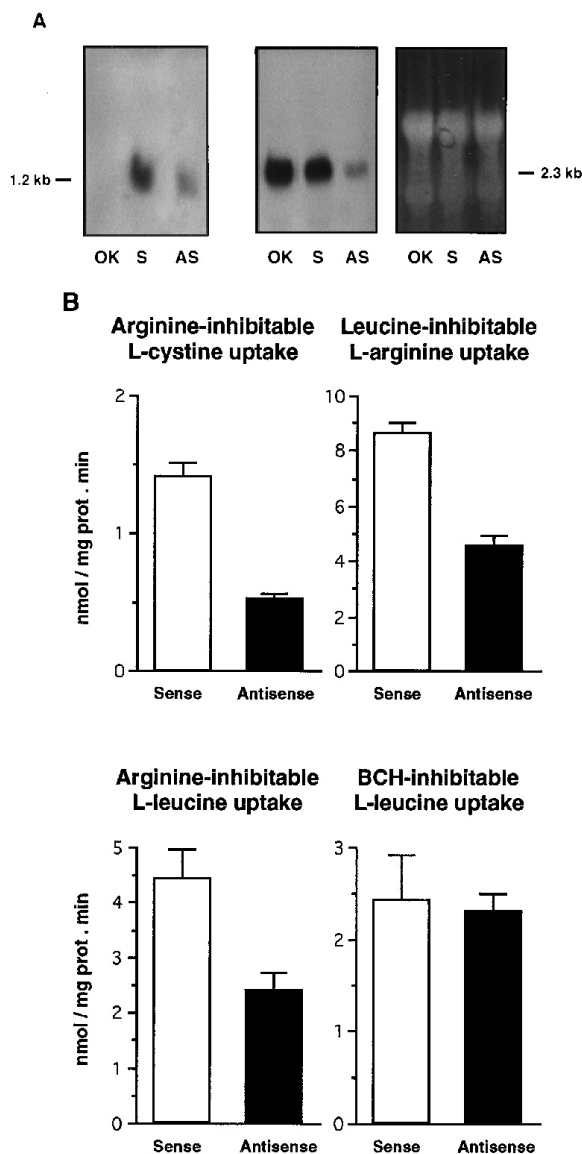
A

1.2 kb —   — 2.3 kb

OK S AS   OK S AS   OK S AS

B

Arginine-inhibitable L-cystine uptake

Leucine-inhibitable L-arginine uptake

Arginine-inhibitable L-leucine uptake

BCH-inhibitable L-leucine uptake

FIG. 6. *A*, Northern blot analysis for human rBAT-sense and antisense expression and for the endogenous rBAT mRNA in transfected OK cells. A human rBAT cDNA probe hybridized to a transcript of ~1.2 kb in length, which is present in the RNA from OK cells transfected with rBAT-sense or rBAT-antisense, but absent in nontransfected cells (*OK*) (*left panel*). 30 μg of total OK cell RNA was loaded in each lane. An OK cell rBAT cDNA probe, corresponding to the PCR-amplified cDNA fragment shown in Fig. 2, hybridized with a transcript of >2.3 kb present in non transfected OK cells (control) and in rBAT-sense and rBAT-antisense OK transfected cells total RNA (*middle panel*). 30 μg of OK cell total RNA was loaded per lane. The level of rBAT mRNA was reduced in the OK cells transfected with rBAT-antisense to ~20% of that in control and rBAT-sense groups, as revealed by scanning densitometry in two independent Northern blot analyses. Ethidium bromide staining of the Northern blot membrane shown in the *middle panel* (*right panel*). *B*, amino acid uptake in OK cells transfected with human rBAT sense or antisense. Uptake of 50 μM L-[^{35}S]cystine, L-[^{3}H]arginine, or L-[^{3}H]leucine by OK cells was measured in the absence or in the presence of the indicated unlabeled L-amino acids or the amino acid analog BCH at 5 mM concentration in the uptake MGA medium. Uptake values (nmol/mg protein·min) correspond to the uptake inhibited by the indicated amino acids or analog and are the mean ± S.E. from triplicates of a representative experiment. Total uptake values (*i.e.* in the absence of inhibitors) in the sense and antisense groups were, respectively: 1.50 ± 0.10 and 0.61 ± 0.03 nmol/mg protein·min for L-cystine uptake, 10.5 ± 0.3 and 6.0 ± 0.3 nmol/mg protein·min for L-arginine uptake, and 6.0 ± 0.5 and 4.6 ± 0.2 nmol/mg protein·min for L-leucine uptake. All the components shown by amino acid inhibition were statistically different from zero value (Student's *t* test; at least $p \leq 0.05$). Similar results were obtained in one to three more independent experiments.

clones (AS1 and AS2; ~20% and ~50% of control values, respectively) and unaffected in the two sense clones (S1 and S2). Expression of the transfected constructs and the rBAT mRNA levels in antisense AS1 and sense S1 clones, as well as in control cells (*i.e.* untransfected) are shown in Fig. 6*A*. An initial screening for amino acid transport activity in these clones revealed a decreased L-arginine transport activity in the antisense clones, which showed reduced rBAT mRNA expression. Thus, 50 μM L-arginine uptake in MGA uptake medium, expressed as nmol/mg protein·min, was: 11.0 ± 0.5 for control untransfected cells, 10.5 ± 0.3 and 9.5 ± 0.5 for S1 and S2 sense clones, and 6.0 ± 0.4 and 6.6 ± 0.3 for AS1 and AS2. Similar results were obtained for L-leucine uptake (data not shown).

Cell clones AS1 (antisense) and S1 (sense) were used for further experiments in which system b$^{0,+}$-like activity was studied. Consistently (*i.e.* from passage 20 to 22), b$^{0,+}$-like transport activity was lower (40–55%) in the AS1 antisense clone than in the S1 sense clone (Fig. 6*B*). System b$^{0,+}$-like transport activity was almost identical in the S1 sense clone and in control untransfected cells (Figs. 3 and 6*B*). The b$^{0,+}$-like transport activity expressed by rBAT cRNA in oocytes shows hetero-exchange between L-arginine and L-leucine (3–5). This has also been suggested for this transport activity in OK cells in the present study (Fig. 4). In order to demonstrate that rBAT is responsible for this activity via b$^{0,+}$-like transporter, trans-stimulation of L-arginine efflux by L-leucine was studied in AS1 antisense and S1 sense clones. This trans-stimulation was reduced to ~50% in the AS1 antisense clone. Thus, efflux (cpm/mg protein·min, corrected for the radioactivity loading after 2 min of 50 μM L-[^{3}H]arginine of 85,000 cpm/mg protein) was 1,300 ± 1,100 and 1,200 ± 600 in the presence of 1 mM L-glutamate in the medium for S1 sense and AS1 antisense clones, respectively. This efflux was trans-stimulated to 17,500 ± 900 and 9,200 ± 300 by 1 mM L-leucine in the medium of S1 sense and AS1 antisense cells, respectively (data are mean ± S.E. of triplicates from a representative experiment). In contrast to system b$^{0,+}$-like transport activity, the uptake of L-leucine inhibited by the amino acid analog BCH, model for system L, was unaffected in the AS1 antisense clone (Fig. 6*B*). Similarly, the sodium-dependent uptake of 50 μM L-leucine was not affected in the antisense AS1 clone (*i.e.* the uptake values from triplicates of a representative experiment were: 4.7 ± 1.4 and 4.0 ± 0.7 nmol/mg protein·min for the S1 sense and the AS1 antisense clones, respectively). This demonstrates that rBAT antisense expression in OK cells results in a specific decrease in system b$^{0,+}$-like transport activity. Kinetic analysis of L-cystine uptake showed that rBAT antisense expression reduces the apparent V_{max} (7.8 ± 0.8 and 3.5 ± 0.7 nmol/mg protein·min, for the S1 sense and the AS1 antisense clones, respectively) without a significant effect on the estimated apparent K_m values (227 ± 39 and 285 ± 50 μM, for the S1 sense and the AS1 antisense clones, respectively) (Fig. 7).

DISCUSSION

Here we have shown that system b$^{0,+}$-like is the major component of the transport of cystine in the apical pole of the opossum kidney cell line OK. This system is very similar to the exchanger activity of amino acids elicited by rBAT cRNA in oocytes. In addition, we have demonstrated that rBAT expression is necessary for this b$^{0,+}$-like amino acid transport activity in this renal epithelial cell line. Due to the role of human *rBAT* gene in type I cystinuria (8, 10, 12), a corollary of the present study is that the b$^{0,+}$-like amino acid exchanger would be defective in this type of cystinuria.

Identification of the amino acid transport activity associated with rBAT in renal cells has so far been elusive. No functional
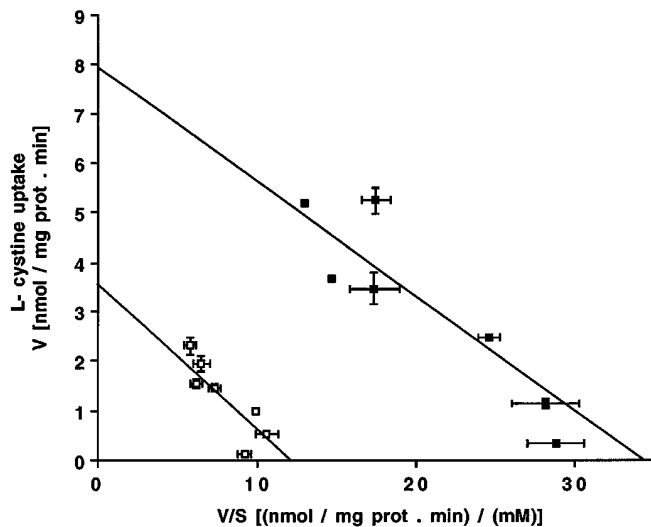
Fig. 7. **Kinetic analysis of L-cystine uptake in OK cells transfected with human rBAT sense or antisense.** Uptake of L-[35S]cystine was measured at varying concentrations in the absence of sodium in the uptake medium, both in OK cells transfected with rBAT-sense (*filled symbols*) or with rBAT-antisense (*open symbols*). Eadie-Hofstee transformations of 5 mM L-arginine-inhibitable L-cystine uptake are shown. Uptake values (nmol/mg protein·min) are the mean ± S.E. from triplicates of a representative experiment. When not visible, S.E. bars are smaller than symbols.

expression of the amino acid transport activity associated with rBAT has been obtained in mammalian cells (*e.g.* COS cells) (30). All the data previously reported on the amino acid transport activity associated with rBAT were obtained in *Xenopus* oocytes (reviewed in Ref. 41). In addition, the rBAT protein has a low predicted number of transmembrane domains (25, 27, 28), and indirect evidence suggested its association with an unidentified subunit of 35–50 kDa to give a putative functional complex of 125 kDa in kidney and in oocytes expressing rBAT (Ref. 29).[2] All this prompted the hypothesis that rBAT is not an amino acid transporter by itself, but rather full expression of the $b^{0,+}$-like exchanger can only be achieved by association with an endogenous subunit of the oocyte. In this situation, description of the amino acid transport activity associated with rBAT in renal cells was a clear prerequisite for the full understanding of the physiopathology of rBAT. The present study demonstrates that, as in oocytes, rBAT expression is necessary for the $b^{0,+}$-like transport activity present in the OK cell line. Whether rBAT acts as a modulator or as a catalytic component of this transport activity remains to be established through reconstitution experiments and identification of the putative subunit linked to rBAT.

To our knowledge the present study represents the first description in renal epithelial cells of $b^{0,+}$-like amino acid transport activity, defined as high affinity sodium-independent heteroexchange diffusion for cystine, dibasic (*e.g.* L-arginine) and neutral (*e.g.* leucine) amino acids. Only fragmental views of this amino acid transport activity have been described in kidney and intestine. Thus, functional studies indicated a high affinity reabsorption system for L-cystine in the proximal straight tubule of the nephron (23, 24) (*i.e.* S3 segment, where rBAT has been localized; Refs. 6 and 7). This was also shown to be present in the small intestinal mucosa and to be defective in biopsies from patients with cystinuria (20, 21, 42). This high affinity system is shared with dibasic amino acids and shows heteroexchange diffusion of dibasic amino acids and cystine (17–19, 42). Transport of L-cystine, examined at low (μM) concentration, is inhibited by some L-neutral amino acids, suggest-

ing a high affinity cystine transporter shared with neutral amino acids (18, 22–24). The sodium dependence of cystine reabsorption has been controversial for years; the high affinity cystine uptake in rat brush border membranes was first believed to be sodium-dependent and, later, sodium-independent (18, 43). After the molecular identification of rBAT and its role in type I cystinuria, it was necessary to characterize the amino acid transport activity associated with rBAT in renal epithelial cells and test their consistency with the previously reported data on renal cystine reabsorption. Among the different renal epithelial cell lines analyzed, the high affinity sodium-independent cystine-dibasic amino acid transport system evidenced in renal preparations was also substantiated in OK cells (14, 31). Here we have shown that this transport system is also shared by neutral amino acids, is due to rBAT expression, and is very similar, if not identical, to the $b^{0,+}$-like amino acid exchanger expressed by rBAT in oocytes.

All the foregoing suggests the following as a plausible description of the role of rBAT in L-cystine renal reabsorption. The sodium-independent $b^{0,+}$-like amino acid exchanger (*i.e.* associated to rBAT) is responsible for L-cystine reabsorption in the brush border of the epithelial cells of the S3 segment of the nephron; a defect in this reabsorption system causes type I cystinuria. In contrast to the S3 segment of the nephron, the bulk of cystine reabsorption occurs in the S1-S2 segment of the nephron (44), and it seems to be due to a low affinity, high capacity system that appears to be unshared with dibasic amino acids and which most probably is sodium-dependent and not detectable in the small intestine (17–19, 23, 24, 42, 45). Due to the genetic heterogeneity in cystinuria (11, 12) and the very mild, if any, alteration of cystine absorption in the small intestine in type III cystinuria, the putative low affinity, high capacity cystine reabsorption system is an obvious candidate for this type of cystinuria.

Given that there is no clear agreement on the sodium dependence of the renal reabsorption of cystine, it was suggested that the main driving force for this reabsorption may be the intracellular reduction to L-cysteine, which then leaves the cell by a basolateral transport system (44). The exchange of amino acids via system $b^{0,+}$-like in oocytes expressing rBAT (3–5), and also suggested for this transport activity in OK cells by the results presented here, offers an additional mechanism of accumulation for cystine and dibasic amino acids in the brush border membranes of the epithelial cells of the S3 segment of the nephron; exchange with intracellular neutral amino acids could drive the active reabsorption of cystine and dibasic amino acids. Indeed, rBAT-specific accumulation of cystine occurs in oocytes where reduction to cysteine is prevented by diamide.[1] Reabsorption of cystine and dibasic amino acids through this exchange may be favored by intracellular reduction of cystine to cysteine and the negative brush border membrane potential, respectively. This is consistent with the finding that patients with type I cystinuria have mutations in the *rBAT* gene, and therefore a defective $b^{0,+}$-like amino acid exchange activity, and show hyperexcretion of cystine and dibasic amino acids but not neutral amino acids. This heteroexchange mechanism of cystine and dibasic amino acid reabsorption could also explain the enigmatic secretion of cystine exceeding the glomerular filtration rate in dogs after lysine infusion *in vivo* (46, 47); the high concentration of lysine in the glomerular filtrate after infusion may block by competition the cystine reabsorption through system $b^{0,+}$-like in the epithelial cells of the S3 segment and stimulate the exchange through the brush border membrane of lysine (inward) and neutral amino acids (outward), including cysteine, which could be oxidized to cystine in the lumen. The final result would then be a cystine urinary

excretion higher than the cystine that reaches the kidney for glomerular filtration.

Finally, the OK cells could be envisaged as an ideal model with which to study the cell biology and regulation of this transport activity associated with rBAT. The $b^{0,+}$-like amino acid exchanger seems to be the only transport system for cystine in the apical pole of these cells. Aside from the ontogenic regulation of rBAT expression in kidney (6), little is known about the hormonal regulation of this reabsorption system for cystine and dibasic amino acids.

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