Cystinuria-specific rBAT(R365W) mutation reveals two translocation pathways in the amino acid transporter rBAT-$b^{0,+}$AT

Marta PINEDA*[1], Carsten A. WAGNER†[1], Angelika BRÖER‡, Paul A. STEHBERGER§, Simone KALTENBACH§, Josep Ll. GELPÍ*, Rafael MARTÍN DEL RÍO‖ , Antonio ZORZANO*, Manuel PALACÍN*, Florian LANG§ and Stefan BRÖER‡§[2]

*Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Barcelona Science Park, Barcelona 08028, Spain, †Institute of Physiology, University of Zürich, 8057 Zürich, Switzerland, ‡School of Biochemistry and Molecular Biology, Australian National University, Canberra, ACT 0200, Australia, §Institute of Physiology, University of Tübingen, 72076 Tübingen, Germany, and ‖Servicio de Neurobiología, Hospital Ramón y Cajal, Crta de Colmenar Km 7, 28034 Madrid, Spain

Apical reabsorption of dibasic amino acids and cystine in kidney is mediated by the heteromeric amino acid antiporter rBAT/$b^{0,+}$AT (system $b^{0,+}$). Mutations in rBAT cause cystinuria type A, whereas mutations in $b^{0,+}$AT cause cystinuria type B. $b^{0,+}$AT is the catalytic subunit, whereas it is believed that rBAT helps the routing of the rBAT/$b^{0,+}$AT heterodimeric complex to the plasma membrane. In the present study, we have functionally characterized the cystinuria-specific R365W ($Arg^{365} \rightarrow Trp$) mutation of human rBAT, which in addition to a trafficking defect, alters functional properties of the $b^{0,+}$ transporter. In oocytes, where human rBAT interacts with the endogenous $b^{0,+}$AT subunit to form an active transporter, the rBAT(R365W) mutation caused a defect of arginine efflux without altering arginine influx or apparent affinities for intracellular or extracellular arginine. Transport of lysine or leucine remained unaffected. In HeLa cells, functional expression of rBAT(R365W)/$b^{0,+}$AT was observed only at the permissive temperature of 33 °C. Under these conditions, the mutated transporter showed 50 % reduction of arginine influx and a similar decreased accumulation of dibasic amino acids. Efflux of arginine through the rBAT(R365W)/$b^{0,+}$AT holotransporter was completely abolished. This supports a two-translocation-pathway model for antiporter $b^{0,+}$, in which the efflux pathway in the rBAT(R365W)/$b^{0,+}$AT holotransporter is defective for arginine translocation or dissociation. This is the first direct evidence that mutations in rBAT may modify transport properties of system $b^{0,+}$.

Key words: antiporter, cystinuria, epithelial transport, heteromeric amino acid transporter, membrane transport, structure–function relationship.

INTRODUCTION

Heteromeric amino acid transporters are a large family of antiporters in eukaryotes. They are composed of two different subunits: a heavy chain and a light chain (see [1–4] for reviews). The $b^{0,+}$ transporter is constituted by the rBAT heavy chain and the $b^{0,+}$AT light chain. The rBAT protein is a type II membrane protein with a single transmembrane helix and a large extracellular domain that has a sequence similar to bacterial glycosidases [2,5]. The $b^{0,+}$AT light chain is a typical polytopic membrane transport protein with 12 predicted transmembrane helices. For surface expression, both rBAT and $b^{0,+}$AT have to be expressed in the same cell [2,6]. Reconstitution experiments have shown that the $b^{0,+}$AT subunit catalyses the transmembrane movement of substrates in an antiport mechanism [7]. However, some studies have suggested that the rBAT heavy chain may alter functional properties of the rBAT/$b^{0,+}$AT heterodimer. First, mutation of the rat rBAT residue Cys^{664} (corresponding to human Cys^{666}) alters the NEM (*N*-ethylmaleimide) sensitivity of the transporter [8], and secondly kinetic constants of the $b^{0,+}$AT light-chain-mediated transport are altered when it interacts non-physiologically with 4F2hc owing to the overexpression in mammalian cells [9].

Functionally, rBAT and $b^{0,+}$AT constitute an Na^+-independent antiporter for neutral and dibasic amino acids with properties of system $b^{0,+}$ [10–12]. System $b^{0,+}$ is believed to be the major apical system for the reabsorption of cystine and dibasic amino acids in the renal proximal tubule as evident from the inherited disease cystinuria [1,2] and from co-immunoprecipitation studies in kidney [13]. Mutations in rBAT (*SLC3A1*) cause cystinuria type A, whereas mutations in the light-chain subunit $b^{0,+}$AT (*SLC7A9*) lead to cystinuria type B [10,14–16]. All cystinuria-causing rBAT mutations studied so far only affect the trafficking of the holotransporter to the plasma membrane [17,18].

When expressed in oocytes, the rBAT heavy chain interacts with an endogenous light-chain subunit that is functionally almost indistinguishable from its mammalian counterpart. The complex mediates the Na^+-independent uptake of neutral and dibasic amino acids and was therefore characterized as $b^{0,+}$-like [19,20]. Subsequently, the $b^{0,+}$ transporter has been shown to function as an obligatory amino acid exchanger [7,21–23]. Uptake of neutral amino acids in rBAT-expressing oocytes is accompanied by outward currents that are generated by the concomitant efflux of dibasic amino acids. Uptake of dibasic amino acids, conversely, is accompanied by inward currents, because of an antiport of neutral amino acids [21].

In the present study, we have functionally examined the rBAT mutation R365W ($Arg^{365} \rightarrow Trp$), previously found in an Italian [24] and two Spanish (M. Palacín, unpublished work) cystinuria type A patients. The mutant shows impaired trafficking to the plasma membrane and selectively affects efflux of intracellular arginine without altering apparent affinities. These results are the first strong experimental evidence that mutations in the rBAT heavy chain alter the functional properties of the system $b^{0,+}$, and provide the first insight into the question of how intensively heavy and light chains interact in the family of heteromeric amino acid transporters.

Abbreviations used: BCA, bicinchoninic acid; CFTR, cystic fibrosis transmembrane conductance regulator; GFP, green fluorescent protein; NEM, *N*-ethylmaleimide.

[1] These authors have contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail stefan.broeer@anu.edu.au).

EXPERIMENTAL

Oocytes and cRNA injections

Oocyte origin, management and injections were as described elsewhere [24]. Defolliculated stage VI *Xenopus laevis* oocytes were injected with 10 ng of cRNA of human rBAT wild-type [25], or the human rBAT(R365W) mutant. Synthesis of these cRNAs from rBAT cloned in vector pSPORT was performed as described in [24].

Site-directed mutagenesis and plasmids construction

For the rBAT(R365W) mutation, the Chameleon Double Stranded Site-Directed-Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used according to the manufacturer's instructions. The mutagenesis primer was antisense 5′-GGTCTGCC(A)GGAGCTGC-GGACAATGTA-3′. The success of mutagenesis was checked by sequencing. For expression in HeLa cells, rBAT, rBAT(R365W) and $b^{0,+}$AT were subcloned into vector pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) as described in [10].

Uptake and efflux measurements in oocytes

Influx rates of L-[^{3}H]arginine, L-[^{3}H]leucine and L-[^{35}S]cystine (Amersham Biosciences and Biotrend, Cologne, Germany) were measured in ND96 buffer (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, titrated with NaOH to pH 7.4). Measurements were performed at the indicated days after injection and under conditions where uptake increases proportional to the incubation time [22]. The cRNA-induced uptake was calculated by subtracting the uptake activity of non-injected oocytes from that of the corresponding cRNA-injected oocytes. Amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those of non-injected oocytes (results not shown). For each determination, groups of 8–10 cRNA- or non-injected oocytes were washed twice with 4 ml of ND96 buffer. They were then incubated at room temperature (24 °C) in a 5 ml polypropylene tube containing 100 μl of the same buffer containing 5–10 kBq of L-amino acid plus unlabelled substrates as indicated. Transport was stopped after the appropriate interval by washing the oocytes three times with 4 ml of ice-cold ND96 buffer. Single oocytes were placed in scintillation vials and lysed by the addition of 200 μl of 10 % SDS. After lysis, 3 ml of scintillation fluid was added, and the radioactivity determined by liquid-scintillation counting. For efflux experiments, oocytes were injected with the indicated concentration of L-[^{3}H]arginine or L-[^{14}C]leucine and incubated for 10 min. Subsequently, oocytes were washed twice with 4 ml of ND96 at room temperature. During this time, these amino acids were not significantly metabolized [26]. Efflux was determined in four groups of four oocytes in each experiment. Efflux was initiated by replacing the washing buffer by 0.5 ml of ND96 at room temperature containing amino acids as mentioned. Aliquots of 200 μl were removed from the supernatant for counting.

Membrane isolation and Western blotting in *Xenopus* oocyte samples

For each preparation, 25 oocytes were lysed in 500 μl of homogenization buffer (10 mM NaCl/1 mM Pefabloc/50 mM Tris/HCl, pH 7.6) by trituration. The egg yolk and other debris were removed by centrifugation at 2000 ***g*** for 10 min at 4 °C. The clear part of the supernatant was transferred to a new tube and centrifuged at 140 000 ***g*** for 30 min at 4 °C. The membrane pellets were dissolved in 30–50 μl of SDS-containing homogenization buffer (10 mM NaCl/1 mM Pefabloc/4 % SDS/50 mM Tris/HCl, pH 7.6) and 40 μl of the dissolved membranes was loaded on to a 10 % SDS/polyacrylamide gel per lane and separated at constant voltage (200 V) for 1 h. Proteins were blotted for 2 h to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.). Membranes were treated overnight with blocking buffer [5 % (w/v) non-fat milk in PBS/0.1 % Tween 20, pH 7.4]. The rBAT protein was subsequently detected using affinity-purified primary antibodies (Ab124; 1:4000) [27] and a secondary antibody (sheep-anti-rabbit IgG coupled with horseradish peroxidase, 1:5000; Amersham Biosciences). Antibody Ab124, kindly supplied by Dr S. Tate (Cornell University, Ithaca, NY, U.S.A.) was raised against a peptide corresponding to residues 124–146 of rat rBAT [27]. Each antibody was incubated for 1 h at room temperature in 2.5 % non-fat milk in PBS and 0.15 % Tween 20. Subsequently, membranes were washed four times for 10 min each in PBS. For immunodetection, an enhanced chemiluminescence kit (Amersham Biosciences) was used, and the blots were exposed to Kodak X-OMAT film (Sigma, Castle Hill, Australia).

Electrophysiology in *Xenopus* oocytes

Two electrode voltage clamp recordings were performed 1–7 days after cRNA injection at room temperature as described previously [24]. The holding potential was -50 mV if not otherwise stated. The data were filtered at 10 Hz and recorded using a MacLab D/A converter and software for data acquisition and analysis. The external control solution (ND96) contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM Hepes (pH 7.4). The final solutions were titrated to the indicated pH using KOH or HCl. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath was reached within 10 s. The currents given are the maximal values measured during 30 s of substrate superfusion. The positively charged amino acids caused inward currents, which are given with a negative prefix ($-$), whereas the neutral amino acids caused outward currents given with a positive prefix (+). All chemicals were obtained from Sigma (Deisenhofen, Germany).

HeLa cell transfections

Transfections with human rBAT [25] and human $b^{0,+}$AT [10] cDNA were performed as described previously [14]. HeLa cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10 % (v/v) foetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin (D10) at 37 °C in a humidified atmosphere containing 5 % CO_2. Transfections were performed by standard calcium phosphate precipitation in 10-cm-diameter plates with a mixture of DNA containing 2 μg of pEGFP (where GFP stands for green fluorescent protein; ClonTech), 9 μg of pCDNA3-rBAT (wild-type or mutated rBAT) and 9 μg of pCDNA3-$b^{0,+}$AT. When rBAT or $b^{0,+}$AT was transfected alone the DNA transfection mixture contained 2 μg of pEGFP, 9 μg of pCDNA3-rBAT or pCDNA3-$b^{0,+}$AT and 9 μg of pCDNA3 empty vector. After overnight incubation with the DNA-calcium phosphate co-precipitate, cells were extensively washed with PBS and trypsinized. Then 150 000 cells in 1 ml of D10 were plated/well on a 24-well plate for uptake and efflux measurements, 300 000 cells on a 12-well plate for amino acid content measurement or 600 000 cells on a 6-well plate for Western-blot analysis. Cells were then kept at 33 °C or 37 °C for 48 h before experiments. Transfection efficiency was checked by analysing an aliquot of cells of each individual transfection

group for GFP expression by FACS using an EPICS XL Coulter cell sorter (Serveis Cientifico-Tècnics, Universitat de Barcelona, Spain). The percentage of positive cells was defined as the fraction beyond the region of 99.9 % of non-GFP-transfected cells. In different experiments, transfection efficiency of GFP ranged from 40 to 80 %. Transfections with an efficiency < 40 % were discarded.

Uptake and efflux measurements in HeLa-transfected cells

Before the start of the transport experiment, 24-well plates, with 1 ml of D10 medium/well, were placed in a dry incubator at 37 °C for 1 h. For uptake measurements, cells were washed twice with 1 ml of the transport medium [137 mM *N*-methyl-D-gluconate/2.8 mM $CaCl_2$/1.2 mM $MgSO_4$/5.4 mM KCl/10 mM Hepes (pH 7.5) equilibrated at 37 °C]. Subsequently, 200 μl of the transport medium with substrate {50 μM L-[^{3}H]arginine (2.5 μCi/ml) or 20 μM L-[^{35}S]cystine (2.5 μCi/ml) plus 5 mM glutamate} was added and cells were incubated for different periods. To determine linear transport rates, cells were incubated for 1 min (transport was linear up to 2 min; results not shown). After incubation, the uptake medium was removed and cells were washed three times in 1 ml of cold (4 °C) transport medium. Non-specific binding was assessed by addition and immediate removal of the uptake solution at 4 °C. After washing, cells were lysed by addition of 250 μl of 0.1 M NaOH/0.1 % SDS per well; 100 μl was used to count radioactivity and 20 μl was used to measure the protein content in the well using the BCA (bicinchoninic acid) Protein Assay kit (Pierce Biotechnology, Rockford, IL, U.S.A.). Radioactivity was measured in a β-scintillation counter (Beckman LS 6000TA; Beckman Instruments, Fullerton, CA, U.S.A.).

For efflux measurements, transfected HeLa cells were preloaded by incubation in transport medium containing 50 μM L-[^{3}H]arginine or L-[^{3}H]leucine (5 μCi/ml) for 20 min. The cells were then washed three times with ice-cold transport medium and incubated in the efflux solution in the presence or absence of unlabelled L-arginine (400 μM), L-leucine (400 μM) or L-cystine (200 μM). The radioactivity in the medium and the remaining radioactivity in the cells were measured. The efflux rate of radioactivity was linear up to 30 s (results not shown); consequently, efflux rates were measured at 20 s. The arginine or leucine efflux rates were expressed as released 'nmol/mg of protein' and were calculated as follows: (radioactivity released into the medium/total radioactivity in the system) × (intracellular content of arginine or leucine expressed in nmol/mg of protein), where (total radioactivity in the system) = (radioactivity remaining in cells) + (radioactivity released to the medium).

Determination of intracellular amino acid content

Groups of four oocytes in a propylene tube were washed three times with 1 ml of ice-cold PBS. For deproteinization, 100 μl of 15 % (w/v) sulphosalicylic acid and four glass beads (2 mm diameter) were added. The tubes were incubated for 10 min in a sonication bath. Then tubes were left to stand for 20 min at room temperature. For HeLa cells, 12-well plates were washed three times with 2 ml of ice-cold PBS. For deproteinization, 200 μl of 10 % sulphosalicylic acid was added per well. In both cases, 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 1 ml of 0.1 M NaOH for protein determination by using the BCA Protein Assay kit (Pierce Biotechnology). Total intracellular amino acid content was determined by cation-exchange chromatography on an automatic Amino Acid Analyser (Beckman model 6300). Amino acid content of the cells is expressed as nmol/mg of protein.

Western blotting of HeLa cell proteins

Cells, placed in 6-well plates, were washed three times in 2 ml of ice-cold PBS and incubated with 20 mM NEM in PBS for 5 min to prevent the formation of non-physiological disulphide bridges [28]. Lysis buffer (300 μl) containing 0.06 % Triton X-100, 0.06 % SDS, 0.03 % deoxycholic acid, 0.03 % BSA, 20 mM NEM in PBS and a cocktail of protein inhibitors (0.02 unit/ml aprotinin, 2 μM leupeptin, 1 mM PMSF and 2 μM pepstatin) were added per well. Cell extracts were obtained by trituration and incubation for 30 min at room temperature. Cell debris was removed by centrifugation at 10 000 ***g*** for 10 min at 4 °C. The supernatant was removed, and an aliquot was used to measure the protein concentration using a BCA Protein Assay kit (Pierce Biotechnology). Proteins (50 μg) in Laemmli sample buffer were separated by SDS/PAGE in the absence [7.5 % (w/v) acrylamide] or in the presence of 100 mM dithiothreitol (10 % acrylamide), and were then transferred to Immobilon (Millipore Iberica, Madrid, Spain). Membranes were then blocked with 5 % non-fat dry milk in PBS for 1 h at 37 °C. Human rBAT-251 Ab (directed against the N-terminus, [13]) and human P6–870 Ab [13] were used at a 1:500 and 1:1000 dilution respectively in 1 % non-fat dry milk in PBS (overnight incubation at 4 °C). Next, three washes were performed in PBS containing 0.3 % Tween 20 at 37 °C for 10 min each. Horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson Immunochemicals, West Grove, PA, U.S.A.) was used as a secondary antibody at 1:25 000 dilution in 1 % non-fat dry milk in PBS (1 h incubation at room temperature). Finally, membranes were washed three times in PBS containing 0.3 % Tween 20 at room temperature for 10 min each. Antibody binding was detected using ECL® Western blot-detection system (Amersham Biosciences).

Data analysis and statistics

In oocyte experiments, kinetic constants were determined by fitting the Hill equation $I = I^*_{max}[S]^h/([S]^h + K^h_{0.5})$ to the data, where h and [S] are the Hill coefficient and the amino acid concentration respectively, I_{max} is the extrapolated maximal current and $K_{0.5}$ is the apparent concentration needed for half-maximal current. Data sets from each oocyte were analysed separately and the values obtained for $K_{0.5}$ and I_{max} were then used for statistical analysis. In flux experiments, the term K_m is used to indicate half-saturation. All data are means ± S.E.M. and n represents the number of experiments performed. The magnitude of the currents or uptake activities varied depending on the time period after cRNA injection and on the batch of oocytes (from different animals). Therefore, throughout the paper, we show experimental results obtained from the same day and the same batch of oocytes for each specific experiment. All experiments were repeated with at least two or three batches of oocytes; in all repetitions qualitatively similar results were obtained. In all experiments, both in oocytes and HeLa cells, data were tested for significance using paired or unpaired Student's t test.

RESULTS

rBAT(R365W) displays a weak trafficking defect in *Xenopus* oocytes

Initial experiments showed that after 4 days expression in *Xenopus* oocytes, the transport activity of rBAT(R365W) was similar to the wild-type, although the mutant was previously found

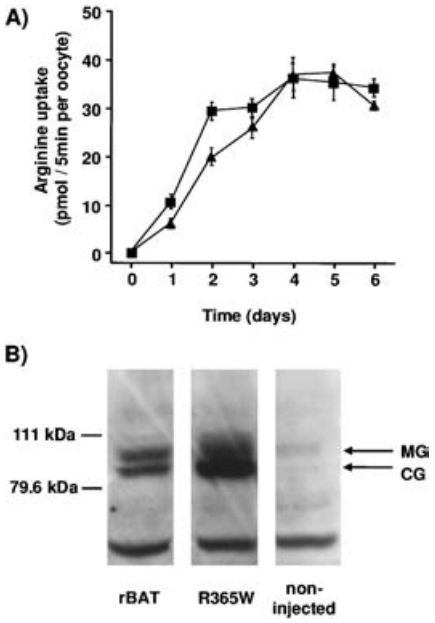

Figure 1 Expression time course of rBAT wild-type and rBAT(R365W) in oocytes

(**A**) Uptake of [^{14}C]arginine (100 μM) was determined for a 5 min incubation period for 1–6 days after injection of rBAT (■) or rBAT(R365W) (▲) ($n = 2$ independent experiments). The transport activity of non-injected oocytes was subtracted. The curves are significantly different ($P \leqslant 0.05$) by two-way ANOVA test (GraphPad PRISM program). (**B**) Western blotting of the rBAT protein in oocytes expressing rBAT, rBAT(R365W), and in non-injected oocytes. Two specific bands of core-glycosylated (CG) and the mature-glycosylated (MG) rBAT protein were detected (arrows). The MG form of rBAT was present at similar levels in rBAT- and rBAT(R365W)-expressing oocytes consistent with similar uptake activities. The fastest migrating band is unspecific as it was detected in all lanes.

in three cystinuria probands [29]. A more detailed analysis of the transport activity induced in R365W-expressing oocytes over a period of 6 days showed a slightly delayed expression of the mutant (Figure 1A). In some experiments, the delay was more pronounced, but usually wild-type-like activity was reached after 4–5 days of expression. Further support for a trafficking defect was provided by Western blots of oocyte membranes (Figure 1B). In the wild-type, equal amounts of core-glycosylated (CG) and mature-glycosylated (MG) rBAT were detected. In contrast, a significant accumulation of the CG form of rBAT was detected in samples from rBAT(R365W) expressing oocytes.

The rBAT(R365W) mutant has an arginine-specific efflux defect

When the rBAT(R365W) mutant was expressed in oocytes, the $K_{0.5}$ values for uptake of neutral and dibasic amino acids were not or only slightly reduced (< 3-fold) (Table 1). To analyse possible changes on the intracellular face of the transporter, L-arginine (1 mM)- and L-leucine (3 mM)-induced currents were measured before and after a 15 min superfusion with either L-leucine (3 mM) or L-arginine (1 mM) (Figure 2). Wild-type rBAT and rBAT(R365W) displayed a similar behaviour in oocytes preloaded with L-leucine (Figure 2A). In contrast, preloading with L-arginine resulted in a subsequent decrease in both L-leucine- and L-arginine-induced currents in the mutant (Figure 2B). These results suggested that efflux of arginine, particularly at increased intracellular concentrations, was impaired in the R365W mutant.

To investigate efflux more directly, we injected oocytes with carrier-free labelled arginine or leucine, resulting in final intracellular concentrations of approx. 0.5 and 0.05 mM respectively. These are the estimated endogenous concentrations of these amino acids [30], assuming that oocytes have an average volume of 400 nl [31]. Figure 3 shows the efflux of arginine and leucine trans-stimulated by saturating concentrations of arginine (1 mM) and leucine (3 mM) in a representative experiment. To discriminate between a general transport defect and a specific efflux defect, the uptake activity of the mutant was matched to that of the wild-type (Figure 3, legend). No efflux was observed in both the wild-type and the mutant in the absence of extracellular substrates (results not shown). Addition of extracellular arginine or leucine induced efflux of arginine in rBAT wild-type- and rBAT(R365W)-injected oocytes, confirming the tightly coupled

Table 1 Kinetic parameters of oocytes expressing rBAT wild-type and the rBAT(R365W) mutant

Substrate-induced currents were determined at concentrations between 0.003 and 3 mM for neutral amino acids and between 0.001 and 1 mM for dibasic amino acids. In R365W-expressing oocytes, I_{max} for analysed amino acids was 70–110 % compared with rBAT-injected oocytes. All curves were fitted using the Hill equation to obtain $K_{0.5}$ values. Hill coefficients ranged between 0.8 and 1.2. All two-electrode voltage clamp experiments were performed at least on five oocytes ($n = 2$ experiments). $K_{0.5}$ values of the mutant were not significantly different from those of the wild-type.

	$K_{0.5}$ (μM)	
	Wild-type rBAT	R365W
L-Arginine	36 ± 3	30 ± 3
L-Ornithine	29 ± 3	16 ± 3
L-Lysine	41 ± 11	15 ± 2
L-Histidine	129 ± 16	74 ± 10
L-Leucine	378 ± 18	345 ± 65

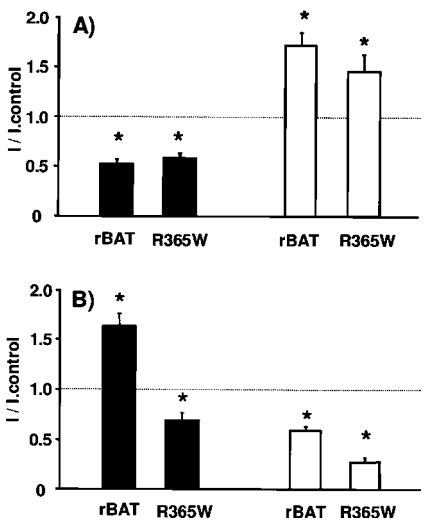

Figure 2 Altered properties of exchange in the rBAT(R365W) mutant

Electrogenic heteroexchange of dibasic and neutral amino acids as measured by two-electrode voltage clamp. Currents induced by L-leucine (3 mM, black bars) and L-arginine (1 mM, open bars) were measured before and after preloading with either L-leucine [3 mM, (**A**)] or L-arginine [1 mM, (**B**)]. Currents were normalized to the activity measured before preloading (·····). In the wild-type, an increase in the currents was observed when preloading was performed with an amino acid of charge different from the substrate. In the rBAT(R365W) mutant only preloading with L-leucine increased L-arginine currents, whereas preloading with L-arginine decreased L-leucine currents. Initial arginine-induced currents varied from −22 to −79 nA and from −39 to −94 nA in the wild-type and the R365W mutant respectively. Leucine-induced currents varied from 10 to 94 nA and from 22 to 65 nA in the wild-type and the R365W mutant respectively. The preloading-induced change was calculated from paired experiments for each oocyte separately. *$P < 0.05$ indicates significant difference to currents before preloading.

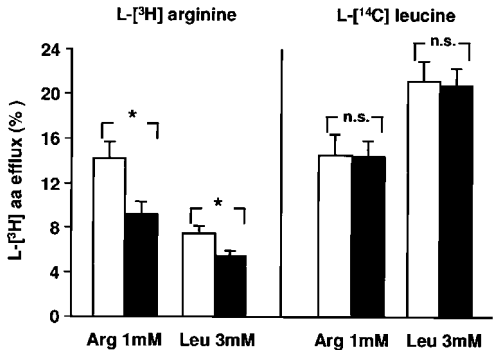

Figure 3 Impaired arginine but not leucine efflux in the rBAT(R365W) mutant

Oocytes expressing rBAT or rBAT(R365W) were injected with carrier-free L-[^{3}H]arginine or carrier-free L-[^{14}C]leucine. After washing, samples were taken from the medium after 5 min in the absence of extracellular amino acids or in the presence of 1 mM L-arginine (Arg) or 3 mM L-leucine (Leu). Trans-stimulated efflux of L-[^{3}H]arginine or L-[^{3}H]leucine was calculated by subtracting efflux in the absence of extracellular amino acids and is given as percentage of preloaded radioactivity. Results are means $\pm$ S.E.M. from a representative experiment of seven (arginine efflux) and four (leucine efflux) independent experiments. Both experimental groups showed the same surface expression as indicated by the arginine uptake activity (50 μM arginine) of the mutant, which on average amounted to 99 ± 2% of the wild-type ($n = 7$ independent experiments). The uptake activity for leucine (100 μM) in the mutant was 100 ± 10% of the wild-type activity ($n = 4$ independent experiments). *$P < 0.05$ indicates significant difference.

antiport mechanism performed by system $b^{0,+}$. In agreement with the electrophysiological data, we found that arginine release was impaired in the R365W mutant, whereas leucine efflux in the R365W mutant matched the activity of the wild-type (Figure 3).

To assess whether the intracellular arginine concentration affects the efflux defect of rBAT(R365W), paired experiments at low (0.5 mM, endogenous) and high (10.5 mM after injection) intracellular arginine concentration were performed. Arginine efflux (homologous exchange) at low intracellular arginine concentration reached 70 ± 5% of the wild-type activity ($n = 7$ independent experiments). At high intracellular arginine concentration, transport activity amounted to 63 ± 4% of the wild-type activity ($n = 7$ paired experiments). Thus increasing intracellular arginine concentration does not modify the arginine efflux defect of rBAT(R365W).

To investigate whether rBAT(R365W) has a generalized defect for efflux of dibasic amino acids, lysine efflux trans-stimulated by arginine was studied. In contrast with arginine efflux, lysine efflux was not affected by the mutant either at high (10.5 mM) or at low (endogenous, 0.5 mM; [30]) (results not shown) intracellular lysine concentration.

In the wild-type, arginine efflux in exchange against leucine (3 mM) reached 57 ± 5% ($n = 5$) of the exchange against arginine in the presence of 100 mM NaCl (see also Figure 3). Depolarizing oocytes from -40 ± 7 to -16 ± 3 mV by addition of 50 mM KCl resulted in an increase in arginine efflux against leucine to 89 ± 7% of the velocity of arginine–arginine exchange. This suggests that arginine efflux in exchange against leucine occurred against the prevailing membrane potential. In contrast, arginine efflux against leucine in the R365W mutant amounted to 40 ± 4% of the wild-type activity at normal resting potential and reached only 57 ± 7% under depolarizing conditions, indicating that the R365W defect is independent of the membrane potential.

The defect of arginine efflux can be explained by three possible mechanisms: (i) the affinity of the intracellular binding site for arginine is decreased; (ii) the translocation velocity and/or substrate dissociation of the transporter complex is decreased when intracellular arginine binds to the transporter; and (iii) the extracellular affinity is decreased when arginine binds to the cytosolic binding site. The last mechanism may occur as a result of the proposed simultaneous mechanism (also referred to as sequential-binding mechanism) of system $b^{0,+}$ [32]. A simultaneous mechanism requires the formation of a ternary complex with the intra- and extracellular substrate bound to the transporter. As a result, binding of an intracellular substrate may affect binding of the extracellular substrate. To discriminate between the three possibilities, we determined the kinetic constants of arginine efflux and influx (the latter at an intracellular arginine concentration of 10 mM; Table 2). No significant differences were observed in the intracellular and extracellular K_m values, whereas V_{max} values of efflux and influx were decreased significantly. Thus at high intracellular concentration of arginine both efflux and influx of arginine are defective in rBAT(R365W). Therefore the rBAT(R365W) mutant is characterized by a decreased translocation velocity and/or dissociation of substrates when arginine binds to the endofacial side of the transporter.

Table 2 Extracellular and intracellular kinetic parameters for arginine in oocytes expressing rBAT and rBAT(R365W) mutant

To determine extracellular kinetic parameters, oocytes were injected with L-arginine at a final concentration of 10 mM. The uptake of L-[^{3}H]arginine was subsequently determined at concentrations between 0 and 0.5 mM. Kinetic parameters for intracellular arginine were determined in oocytes injected with L-[^{3}H]arginine at final concentration ranging from 0 to 10 mM. In these oocytes, L-[^{3}H]arginine efflux against 1 mM L-arginine was measured in four groups of four oocytes for each condition. Trans-stimulated efflux of L-[^{3}H]arginine was calculated by subtracting the induced efflux in non-injected oocytes. The estimated V_{max} values of rBAT (100 %) for arginine in the uptake and efflux experiments were 39 ± 2 and 136 ± 8 pmol · (5 min)$^{-1}$ · (oocyte)$^{-1}$ respectively.

	K_m (μM)		V_{max} (%)	
	rBAT	R365W	rBAT	R365W
Uptake (extracellular arginine)	42 ± 6	39 ± 5	100 ± 5	74 ± 3*
Efflux (intracellular arginine)	1004 ± 197	1294 ± 286	100 ± 6	73 ± 5*

* $P \leqslant 0.05$ indicates significant difference from the wild-type.

The rBAT(R365W)/$b^{0,+}$AT shows defect in trafficking and arginine efflux in transfected HeLa cells

The mild trafficking defect of human R365W mutant expressed in *Xenopus* oocytes (Figure 1) became more pronounced when it was co-expressed with the human $b^{0,+}$AT light-chain subunit in HeLa cells. In contrast with oocytes, no uptake activity above background was observed in R365W/$b^{0,+}$AT-expressing cells grown at 37 °C (Figure 4A). However, when cells were grown at lower temperature (33 °C) a partial rescue of the defect was observed, enabling the mutant to transport cystine (Figure 4B, 49 ± 4% of the wild-type activity in 11 independent experiments). Similarly, transport rates of wild-type rBAT/$b^{0,+}$AT were higher in cells grown at 33 °C.

The observation of the effect of temperature on trafficking of the rBAT/$b^{0,+}$AT and rBAT(R365W)/$b^{0,+}$AT complexes was confirmed by Western-blot analysis of cell extracts under reducing and non-reducing conditions (Figure 5). Expression of $b^{0,+}$AT, rBAT and rBAT(R365W) was increased by growing cells at the permissive temperature of 33 °C (Figure 5). Co-transfection of the light-chain $b^{0,+}$AT improved expression of wild-type rBAT and facilitated the acquisition of mature glycosylation (i.e. endoglycosidase H resistant; results not shown) at both 33 and 37 °C

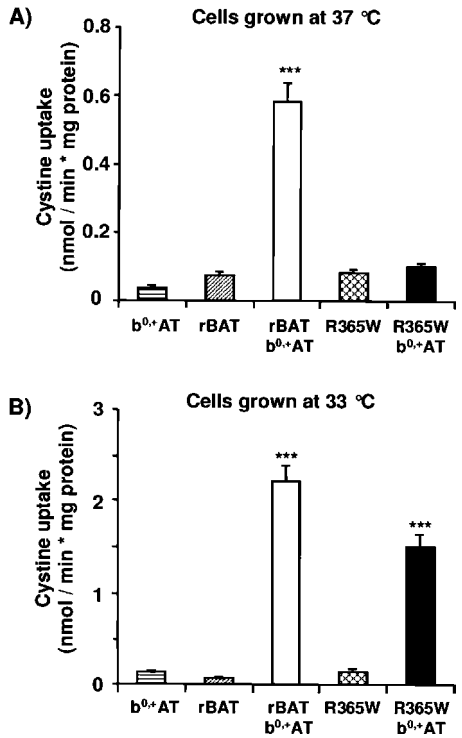

Figure 4 Temperature sensitivity of rBAT(R365W) and $b^{0,+}$AT expression in transfected HeLa cells

HeLa cells were transfected with rBAT or rBAT(R365W) alone or in combination with $b^{0,+}$AT. Subsequently, cells were incubated either at 33 °C or at 37 °C for 48 h before uptake experiments. Decrease in temperature partially rescued the defect observed in 20 μM [^{35}S]cystine uptake in the mutant. No significant cystine uptake was observed in cells grown at 37 °C. Results from an independent experiment with four replicas per each group are shown. ***$P \leqslant 0.001$ indicates significant difference compared with control conditions (i.e. rBAT- or R365W-transfected cells).

(Figure 5A, cf. lanes 2/4 and 8/10 in the rBAT panel). The mature glycosylated rBAT displayed a band of similar mobility to rBAT in human brush-border membranes (Figure 5A, rBAT panel lane 6 and [13]). This confirms earlier observations that both subunits are required to translocate the holotransporter into the plasma membrane [10]. In cells co-transfected with rBAT(R365W) and $b^{0,+}$AT, in contrast, increased expression of rBAT(R365W) and its mature glycosylated form (i.e. endoglycosidase H-resistant; results not shown) was only observed at the permissive temperature of 33 °C (Figure 5A, cf. lanes 5 and 11 in the rBAT panel). These results suggest that the light-chain subunit $b^{0,+}$AT is essential for the translocation to the plasma membrane and that it protects wild-type rBAT against degradation. However, $b^{0,+}$AT-promoted trafficking and stability of the rBAT(R365W) mutant was only observed at the permissive lower temperature. Expression of the $b^{0,+}$AT subunit (lanes 4, 5 and 10, 11) in the same samples is shown in the right panel of Figure 5(A).

The formation of the rBAT-$b^{0,+}$AT heterodimer (approx. 130 kDa) could be observed under non-reducing conditions (Figure 5B). Again, expression of the heterodimer was strongly induced at 33 °C in the wild-type and in the mutant. However, the majority of the proteins appear to form a complex of 250 kDa, suggesting that a dimer of heterodimers may be the prevailing form of this transporter complex. When rBAT(R365W) was co-expressed with $b^{0,+}$AT, no high-molecular-mass complexes were observed at 37 °C (Figure 5B, lane 11, both panels). However, at the permissive temperature of 33 °C, significant amounts of the mutated complex were formed (Figure 5B, lane 5, both panels). In the wild-type both, the 130 and the 250 kDa complexes, were formed at both temperatures (Figure 5B, lanes 4 and 10, both panels).

Expression of the wild-type-rBAT/$b^{0,+}$AT heterodimer at 33 °C allowed the functional characterization of the mutant in HeLa cells. The intracellular content of arginine and lysine in cells transfected with wild-type rBAT plus $b^{0,+}$AT and supplied with a medium containing all proteinogenic amino acids was 5–6-fold higher when compared with that in cells transfected with rBAT alone. The intracellular content of L-ornithine was almost 2-fold higher under the same conditions. The content of neutral amino acids, in contrast, reached levels similar to those in cells transfected with rBAT alone (Figure 6). This demonstrates that system $b^{0,+}$ (rBAT/$b^{0,+}$AT) preferentially mediates the exchange of extracellular dibasic amino acids against intracellular neutral amino acids. The intracellular content of dibasic amino acids dropped to approximately half in cells transfected with rBAT (R365W)/$b^{0,+}$AT when compared with rBAT/$b^{0,+}$AT-transfected cells.

To monitor efflux of arginine, HeLa cells were preloaded with 50 μM L-[^{3}H]arginine during a 20 min incubation. At this time of incubation, accumulation of radiolabelled arginine matches well with the intracellular arginine content. Figures 7(A) and 7(B) show the time course of L-[^{3}H]arginine uptake and the intracellular content of L-arginine after a 20 min incubation period. As expected, rBAT(R365W)/$b^{0,+}$AT showed a lower accumulation capacity of arginine than wild-type-rBAT/$b^{0,+}$AT. Subsequently, trans-stimulation of arginine efflux was measured in the presence of L-cystine (200 μM), L-arginine (400 μM) or L-leucine (400 μM). Wild-type-rBAT, together with $b^{0,+}$AT, mediated exchange of intracellular L-arginine with the tested extracellular amino acids. In contrast, the rBAT(R365W) mutant together with $b^{0,+}$AT showed no significant efflux over background conditions [i.e. wild-type-rBAT- or rBAT(R365W)-transfected cells; Figure 7C]. Thus arginine efflux is impaired in the mutant heterodimer expressed in HeLa as in *Xenopus* oocytes.

To analyse leucine efflux, cells were incubated with 50 μM L-[^{3}H]leucine during 20 min. After incubation, uptake of L-[^{3}H]leucine fits well with the intracellular content of leucine (Figure 8A). In contrast with arginine, leucine is neither accumulated in rBAT/$b^{0,+}$AT- nor in rBAT(R365W)/$b^{0,+}$AT-transfected cells. As shown in Figure 8(B), cells expressing rBAT/$b^{0,+}$AT or rBAT(R365W)/$b^{0,+}$AT mediated significant leucine efflux in the presence of extracellular arginine (400 μM). However, leucine efflux is significantly lower in rBAT(R365W)/$b^{0,+}$AT group when compared with that in rBAT/$b^{0,+}$AT, as expected for the partial uptake activity induced by rBAT(R365W)/$b^{0,+}$AT-transfected cells grown at 33 °C (Figure 4B).

DISCUSSION

Physiological function

System $b^{0,+}$ has been found to function as an obligatory amino acid exchanger for dibasic and neutral amino acids [7,21–23,32]. The hyperexcretion of dibasic but not of neutral amino acids in cystinuria [18,33] suggests that its major mode of transport is the uptake of dibasic amino acids in exchange for neutral amino acids. This was confirmed by expression of the rBAT/$b^{0,+}$AT in mammalian cells, which resulted in the specific accumulation of dibasic but not of neutral amino acids, although both are substrates of system $b^{0,+}$. The maintenance of the asymmetric exchange in a non-epithelial cell line expressing rBAT and $b^{0,+}$AT demonstrates that this direction of exchange is an inherent property of the

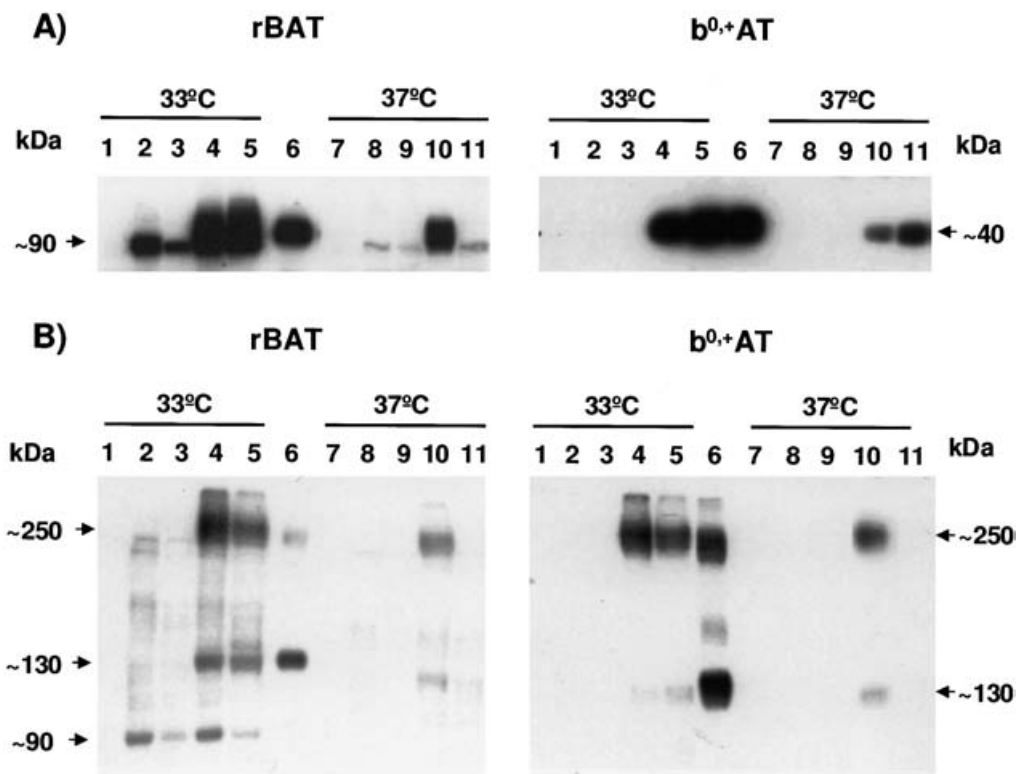

Figure 5 Synthesis and assembly of the rBAT/$b^{0,+}$AT complex at 33 and 37 °C

Detection of rBAT and $b^{0,+}$AT by Western blotting in membranes prepared from transfected HeLa cells in the presence (**A**) or in the absence of dithiothreitol (**B**). Lanes 1 and 7, non-transfected cells; lanes 2 and 8, rBAT-transfected; lanes 3 and 9, rBAT(R365W)-transfected; lanes 4 and 10, rBAT- plus $b^{0,+}$AT-co-transfected; lanes 5 and 11, rBAT(R365W) plus $b^{0,+}$AT-transfected cells. Lane 6, proteins as detected in human kidney brush-border membranes served as a control. (**A**) rBAT (left panel) and $b^{0,+}$AT (right panel) are detected as immunoreactivity at molecular masses of approx. 90 and 40 kDa respectively. Two specific bands for rBAT are found representing the core glycosylated (lower band, molecular mass of approx. 90 kDa) and the mature glycosylated forms (upper band, molecular mass of approx. 98 kDa) [17]. At 37 °C, the mature glycosylated form of rBAT wild-type occurred only in the presence of $b^{0,+}$AT and was absent in rBAT(R365W)/$b^{0,+}$AT-transfected cells (upper left panel). When cells were grown at 33 °C, the mature R365W form was produced in rBAT(R365W)/$b^{0,+}$AT-transfected cells (lane 5). The $b^{0,+}$AT subunit is detectable regardless of the co-expression of wild-type or rBAT(R365W) (upper right panel). (**B**) The fully assembled heterodimeric complex was detected at a molecular mass of 130 kDa by rBAT-specific antibodies (lower left panel) and by $b^{0,+}$AT-specific antibodies (lower right panel). Immunoreactivity was also detected by both antibodies at approx. 250 kDa, most probably representing a dimer of heterodimers. At 37 °C, the heterodimer was only detected when wild-type rBAT and $b^{0,+}$AT were co-expressed.

rBAT/$b^{0,+}$AT heterodimeric complex in the plasma membrane of cells cultured under standard conditions. Depolarization of the membrane potential abolishes the prevalence of arginine homoexchange over the heteroexchange of leucine (influx) for arginine (efflux) in oocytes. This indicates that the membrane potential contributes to the preferential exchange mode of system $b^{0,+}$: antiport of extracellular dibasic amino acids against the intracellular neutral amino acids.

Trafficking defect

In the present study, we functionally characterized the R365W mutation of human rBAT, which was found in three cystinuria patients [29]; additionally, a mutation to leucine at the same position (R365L) was reported in another patient [34]. Interestingly, the rBAT mutation R365W combines a trafficking defect with altered functional properties of the transporter $b^{0,+}$. The trafficking defect of rBAT(R365W) in HeLa cells suggests that the assembly of the functional complex is a prerequisite for stabilization of rBAT and trafficking beyond the endoplasmic reticulum/Golgi, which confirms earlier observations [10,12]. We cannot rule out the possibility that the rBAT(R365W)/$b^{0,+}$AT complex is formed, but is subsequently degraded owing to folding problems of rBAT. In any case, the fact that the lowering of temperature from 37 to 33 °C allowed the formation of detectable rBAT(R365W)/$b^{0,+}$AT heterodimers suggests a folding defect for this mutant. The permissive temperature of 33 °C clearly also increased the assembly of the wild-type rBAT/$b^{0,+}$AT heterodimer. Thus it appears that the wild-type heterodimeric complex is particularly sensitive to assembly/trafficking defects. This situation is reminiscent of the trafficking of the CFTR (cystic fibrosis transmembrane conductance regulator) protein. Only 20–40 % of nascent CFTR polypeptides are able to mature beyond the endoplasmic reticulum [35]. The ubiquitin/proteasome pathway degrades the remaining protein. Moreover, the ΔF508 mutant in CFTR is thought to be partially reverting its folding defect by decreased temperature, allowing the accumulation of the mutated channel at the cell surface [36]. Similarly, the rBAT(R365W) mutant also behaves as a temperature-sensitive folding mutant. In this sense, it is probable that the cystinuria phenotype associated with the R365W mutation is a consequence of a severe trafficking defect. It has been shown that most cystinuria-specific rBAT mutations studied in oocytes are trafficking mutants [17,37]. This is in agreement with the proposed role of rBAT in the routing of the holotransporter to the plasma membrane.

Transport mechanism

In the present study, we show that the rBAT mutation R365W, in addition to its trafficking phenotype, specifically alters an intrinsic

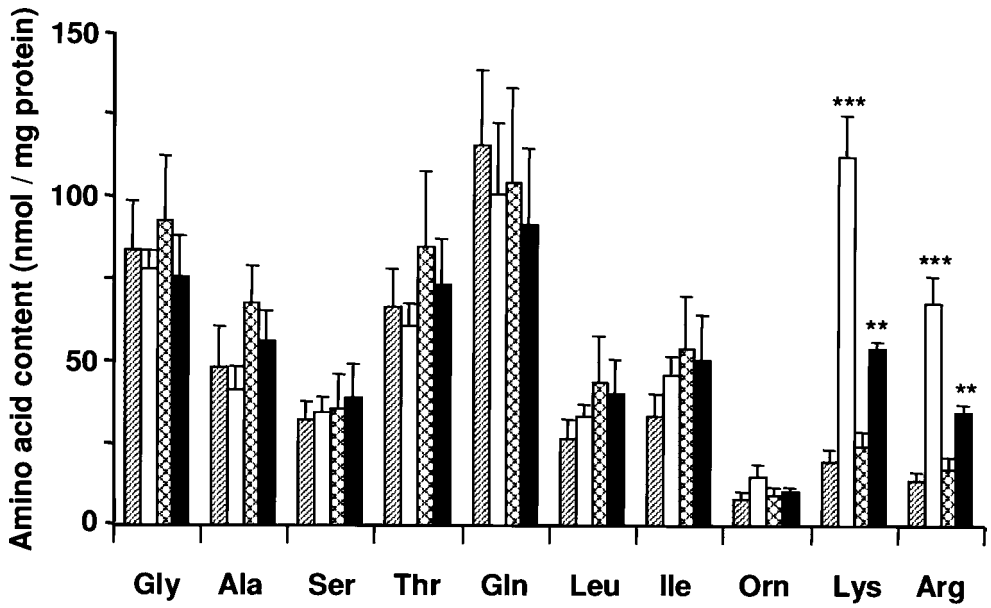

Figure 6 Defective accumulation of dibasic amino acids by rBAT(R365W) mutant with $b^{0,+}$AT in transfected HeLa cells

HeLa cells were transfected with rBAT (hatched bars) or rBAT(R365W) (cross-hatched bars) alone or in combination with $b^{0,+}$AT [open bars, rBAT(wt)/$b^{0,+}$AT; black bars, rBAT(R365W)/$b^{0,+}$AT]. The amino acid content was measured in triplicates from 3 to 4 independent transfection experiments. rBAT(wt)/$b^{0,+}$AT-transfected and rBAT(R365W)/$b^{0,+}$AT-transfected cells showed a higher intracellular content of lysine and arginine when compared with rBAT- or rBAT(R365W)-transfected cells (**$P \leqslant 0.01$; ***$P \leqslant 0.001$). This intracellular accumulation was lower in rBAT(R365W)/$b^{0,+}$AT-transfected than in rBAT/$b^{0,+}$AT-transfected cells (**$P \leqslant 0.01$). No significant differences were found in the intracellular content of neutral amino acids as depicted or that of taurine and acidic amino acids (results not shown). The medium contained 340 μM arginine, 666 μM lysine, 200 μM cystine, 4.0 mM glutamine, 399 μM glycine, 800 μM isoleucine, 800 μM leucine, 201 μM methionine, 400 μM phenylalanine, 400 μM serine, 798 μM threonine, 78 μM tryptophan, 397 μM tyrosine and 803 μM valine.

functional property of the rBAT/$b^{0,+}$AT holotransporter. The mutation decreases arginine efflux without affecting its influx or the apparent affinities for intracellular and extracellular arginine. Transport of leucine and lysine is unaffected in the mutant. This indicates that the mutated holotransporter has a defect in the translocation step of intracellular arginine (i.e. translocation and/or consequent dissociation of substrates). This strongly suggests that the rBAT/$b^{0,+}$AT heterodimeric complex has two translocation pathways. In the rBAT(R365W)/$b^{0,+}$AT holotransporter these two pathways would be asymmetric: one is used for influx, the other for efflux, the latter being defective in the translocation of intracellular arginine. We cannot completely exclude that uptake of arginine is also affected in the R365W mutant, because a partial decrease in uptake is difficult to discriminate from a decreased surface expression caused by the trafficking defect. However, we always observed a stronger defect in arginine efflux than in arginine uptake in both oocytes and cultured cells. This is exemplified by an arginine efflux defect at matched arginine uptake activities in oocytes; and a completely abolished arginine efflux at partially decreased arginine influx activities in cultured cells. The partially decreased uptake activity in cultured cells is readily explained by the trafficking effect, but it may disguise an additional intrinsic uptake defect. The behaviour of the rBAT mutation R365W in oocytes, compared with that in cultured cells, thus shows quantitative rather than qualitative differences. This difference might result from the presence of different light chains in the two expression systems. A 'two-translocation pathway' model for system $b^{0,+}$ is also supported by results of previous papers. First, Coady et al. [38] demonstrated that amino-isobutyric acid is not taken up by rBAT-expressing oocytes, but is capable of eliciting efflux of both neutral and dibasic amino acids. This suggests that amino-isobutyric acid blocks the influx pathway when applied in the bath, but that the efflux pathway remains open and is permeable to both groups of amino acids. Secondly, Torras-Llort et al. [32] reported kinetic patterns for chicken intestinal system $b^{0,+}$ that are compatible with the formation of a ternary complex as a consequence of simultaneous binding of one internal and one external arginine molecule to the carrier. Such a mechanism also requires two translocation pathways [39].

The 'efflux translocation pathway' of the rBAT(R365W)/$b^{0,+}$AT holotransporter seems to be specifically defective for arginine, but not for lysine and leucine. This suggests that either the guanidinium group of arginine or the size of its lateral chain (the largest of the three studied substrates) would be at the basis of this behaviour. The arginine efflux defect caused by rBAT(R365W) mutation is somehow surprising, because the light-chain subunit $b^{0,+}$AT is the catalytic subunit and it is fully functional in the absence of rBAT [7].

The simultaneous transport mechanism, proposed in the present study to explain the behaviour of the rBAT(R365W)/$b^{0,+}$AT transporter, necessitates that the substrate binding sites of the 'import' and 'export' translocation pathway preferentially face the extracellular and the intracellular medium respectively. This mechanism further implies that after release of both substrates, the antiporter returns to its initial conformation in substrate-free form. This model is based on the specific arginine efflux defect of rBAT(R365W)/$b^{0,+}$AT and therefore it might be specific for the mutated but not for the wild-type transporter. To our knowledge, such a model has not been proposed for any other antiporter, although it has been considered as a theoretical possibility in the transport cycle of the anion-exchanger AE-2 [40].

Residue R365 is located in the extracellular domain of rBAT. It is suggested that this domain (of approx. 570 amino acid residues) has a $(\alpha\beta)$8-barrel structure (see [2,5] for reviews) that might cover the $b^{0,+}$AT subunit (of 487 amino acid residues). Therefore

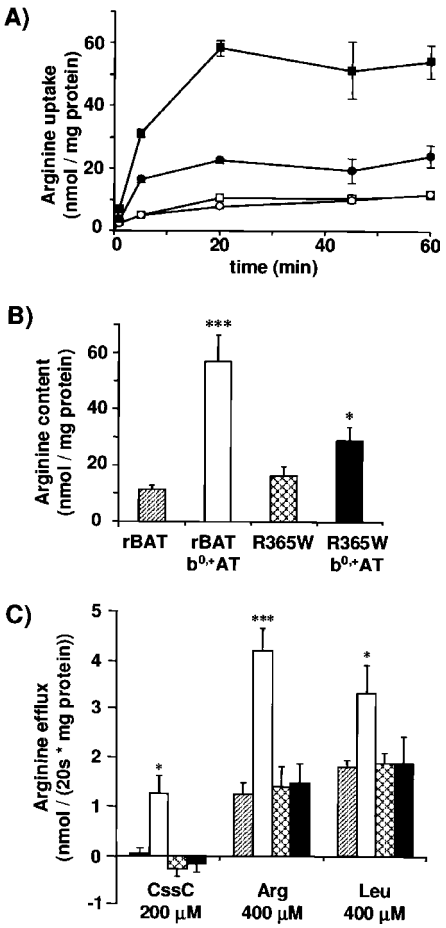

Figure 7 Defective exchange of intracellular arginine by rBAT(R365W) mutant together with $b^{0,+}$AT in transfected HeLa cells

HeLa cells were transfected with rBAT wild-type (□ or hatched bars) or the R365W mutant (○ or cross-hatched bars) alone or in combination with $b^{0,+}$AT [(■) or open bars, rBAT/$b^{0,+}$AT; (●) or closed bars, rBAT(R365W)/$b^{0,+}$AT] and grown at 33 °C for 48 h. (**A**) Time course of uptake of 50 μM L-[^{3}H]arginine. Accumulation of radiolabelled L-arginine over background conditions [rBAT- or rBAT(R365W)-transfected cells] was higher in rBAT/$b^{0,+}$AT-transfected cells than in rBAT(R365W)/$b^{0,+}$AT-transfected cells (***$P \leqslant 0.001$). The results expressed in nmol/mg of protein represent the means ± S.E.M. from four determinations in one representative experiment. Two additional experiments gave similar results. (**B**) Intracellular arginine content after 20 min incubation with 50 μM L-arginine. The intracellular arginine content parallels the plateau of accumulation of radiolabelled L-arginine in the four groups of transfected cells. The results, expressed in nmol/mg of protein, represent the means ± S.E.M. from three independent experiments with 3 to 4 replicas each. (**C**) To monitor exchange of intracellular arginine, cells were preloaded with 50 μM L-[^{3}H]arginine for 20 min. Subsequently, the efflux rate of arginine was determined in a medium containing no amino acids or L-cystine (CssC), L-arginine (Arg) or L-leucine (Leu) at the indicated concentrations. The efflux trans-stimulated by the extracellular amino acids is shown (i.e. efflux rate in the presence of extracellular amino acids minus the rate in their absence). The trans-stimulation of the efflux of arginine was significantly (*$P \leqslant 0.05$) higher in cells transfected with rBAT/$b^{0,+}$AT when compared with the efflux under background conditions [i.e. rBAT- and rBAT(R365W)-transfected cells]. Cells transfected with rBAT(R365W)/$b^{0,+}$AT did not show trans-stimulated efflux over background conditions. Results represent the means ± S.E.M. from 3 to 4 independent experiments with 3 to 4 replicas each. Efflux (nmol of arginine/mg of protein in 20 s) to medium containing no amino acids was: 1.4 ± 0.2 (rBAT-), 3.9 ± 0.3 (rBAT/$b^{0,+}$AT-), 1.9 ± 0.4 [rBAT(R365W)-], 2.8 ± 0.3 [rBAT(R365W)/$b^{0,+}$AT-transfected cells].

parts of the extracellular domain of rBAT might be in close contact with $b^{0,+}$AT. In this context, the changes produced by the rBAT(R365W) mutation in the $b^{0,+}$ holotransporter might be a consequence of: (i) a direct effect of the mutated residue, (ii) a conformational change induced in the rBAT subunit, or (iii) a conformational change transmitted to the catalytic $b^{0,+}$AT subunit.

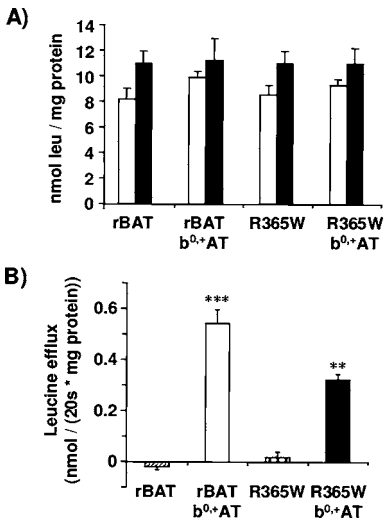

Figure 8 Exchange of intracellular leucine against extracellular arginine by rBAT(R365W) mutant together with $b^{0,+}$AT in transfected HeLa cells

HeLa cells were transfected with rBAT (wild-type or the R365W mutant), alone or in combination with $b^{0,+}$AT and grown at 33 °C for 48 h. (**A**) Uptake of radiolabelled L-leucine (black bars) and intracellular leucine content (open bars) after incubation with 50 μM L-leucine for 20 min. Among the four groups of transfected cells, differences were found neither in the intracellular content of leucine nor in its accumulation. The intracellular leucine content parallels the accumulation of radiolabelled L-leucine. Results represent means ± S.E.M. from two independent experiments with four replicas each. (**B**) To monitor exchange of intracellular leucine, cells were preloaded with 50 μM L-[^{3}H]leucine for 20 min. Subsequently, the efflux rate of leucine was determined in a medium containing no amino acids or 400 μM L-arginine. The efflux trans-stimulated by extracellular arginine is shown (i.e. efflux rate in the presence of arginine minus in their absence). The arginine trans-stimulated efflux of leucine was significantly higher in cells transfected with rBAT/$b^{0,+}$AT (***$P \leqslant 0.001$) or with rBAT(R365W)/$b^{0,+}$AT (**$P \leqslant 0.01$) when compared with the efflux under background conditions [i.e. rBAT- and rBAT(R365W)-transfected cells]. The arginine trans-stimulated efflux of leucine was significantly higher in cells transfected with rBAT/$b^{0,+}$AT than in rBAT(R365W)/$b^{0,+}$AT-transfected cells ($P \leqslant 0.01$). Results represent the means ± S.E.M. from two independent experiments with 3 to 4 replicas each. Efflux (nmol of leucine/mg of protein in 20 s) into medium containing no amino acids was 3.9 ± 0.5 (rBAT-transfected cells), 3.7 ± 0.8 (rBAT/$b^{0,+}$AT-transfected cells), 3.7 ± 0.8 [rBAT(R365W)-transfected cells] and 3.4 ± 0.6 [rBAT(R365W)/$b^{0,+}$AT-transfected cells].

At present, we cannot distinguish among these possibilities and therefore additional structure–function studies are needed. The goal of these studies would be the knowledge of the structure interactions between $b^{0,+}$AT and its heavy-chain rBAT, and the role of the latter on the $b^{0,+}$ holotransporter activity.

We thank B. Noll, J. Garcia and A. Latorre for technical assistance and Dr S. Tate for the gift of the rBAT antibodies (Ab124). The present study was supported by the following grants and agencies: EC grant CT 98-3514 to M. P. and F. L. (Dirección General de Investigación Científica y Técnica, Spain; grant PM99/0172); Instituto de Salud Carlos III network G03/054, Generalitat de Catalunya grant (support of the Comissionat per a Universitats i Recerca and grant 2001 SGR 00118) to M. P.; Federal Ministry of Education, Science, Research and Technology (grant Fö. 01KS9602), Interdisciplinary Center for Clinical Research (IZKF) grant to C. A. W. and F. L., Deutsche Forschungsgemeinschaft (Br1318/2-3) and ARC grant DP0208961 to S. B. M. P. is a recipient of a fellowship from the Comissió Interdepartamental de Recerca i Innovació Tecnològica (Catalonia, Spain).

REFERENCES

1 Wagner, C. A., Lang, F. and Broer, S. (2001) Function and structure of heterodimeric amino acid transporters. Am. J. Physiol. Cell Physiol. **281**, C1077–C1093
2 Chillaron, J., Roca, R., Valencia, A., Zorzano, A. and Palacin, M. (2001) Heteromeric amino acid transporters: biochemistry, genetics, and physiology. Am. J. Physiol. Renal Physiol. **281**, F995–F1018
3 Verrey, F., Jack, D. L., Paulsen, I. T., Saier, Jr, M. H. and Pfeiffer, R. (1999) New glycoprotein-associated amino acid transporters. J. Membr. Biol. **172**, 181–192

4 Deves, R. and Boyd, C. A. (2000) Surface antigen CD98(4F2): not a single membrane protein, but a family of proteins with multiple functions. J. Membr. Biol. **173**, 165–177
5 Broer, S. and Wagner, C. A. (2002) Structure–function relationships of heterodimeric amino acid transporters. Cell Biochem. Biophys. **36**, 155–168
6 Verrey, F., Meier, C., Rossier, G. and Kuhn, L. C. (2000) Glycoprotein-associated amino acid exchangers: broadening the range of transport specificity. Pflugers Arch. **440**, 503–512
7 Reig, N., Chillaron, J., Bartoccioni, P., Fernandez, E., Bendahan, A., Zorzano, A., Kanner, B., Palacin, M. and Bertran, J. (2002) The light subunit of system b(0,+) is fully functional in the absence of the heavy subunit. EMBO J. **21**, 4906–4914
8 Peter, G. J., Panova, T. B., Christie, G. R. and Taylor, P. M. (2000) Cysteine residues in the C-terminus of the neutral- and basic-amino-acid transporter heavy-chain subunit contribute to functional properties of the system b0,+-type amino acid transporter. Biochem. J. **351**, 677–682
9 Rajan, D. P., Huang, W., Kekuda, R., George, R. L., Wang, J., Conway, S. J., Devoe, L. D., Leibach, F. H., Prasad, P. D. and Ganapathy, V. (2000) Differential influence of the 4F2 heavy chain and the protein related to b(0,+) amino acid transport on substrate affinity of the heteromeric b(0,+) amino acid transporter. J. Biol. Chem. **275**, 14331–14335
10 Feliubadalo, L., Font, M., Purroy, J., Rousaud, F., Estivill, X., Nunes, V., Golomb, E., Centola, M., Aksentijevich, I., Kreiss, Y. et al. (1999) Non-type I cystinuria caused by mutations in SLC7A9, encoding a subunit (b0,+AT) of rBAT. International Cystinuria Consortium. Nat. Genet. **23**, 52–57
11 Pfeiffer, R., Loffing, J., Rossier, G., Bauch, C., Meier, C., Eggermann, T., Loffing-Cueni, D., Kuhn, L. C. and Verrey, F. (1999) Luminal heterodimeric amino acid transporter defective in cystinuria. Mol. Biol. Cell **10**, 4135–4147
12 Bauch, C. and Verrey, F. (2002) Apical heterodimeric cystine and cationic amino acid transporter expressed in MDCK cells. Am. J. Physiol. Renal Physiol. **283**, F181–F189
13 Fernandez, E., Carrascal, M., Rousaud, F., Abian, J., Zorzano, A., Palacin, M. and Chillaron, J. (2002) rBAT-b(0,+)AT heterodimer is the main apical reabsorption system for cystine in the kidney. Am. J. Physiol. Renal Physiol. **283**, F540–F548
14 Font, M. A., Feliubadalo, L., Estivill, X., Nunes, V., Golomb, E., Kreiss, Y., Pras, E., Bisceglia, L., d'Adamo, A. P., Zelante, L. et al. (2001) Functional analysis of mutations in SLC7A9, and genotype–phenotype correlation in non-Type I cystinuria. Hum. Mol. Genet. **10**, 305–316
15 Calonge, M. J., Gasparini, P., Chillaron, J., Chillon, M., Gallucci, M., Rousaud, F., Zelante, L., Testar, X., Dallapiccola, B., Di Silverio, F. et al. (1994) Cystinuria caused by mutations in rBAT, a gene involved in the transport of cystine. Nat. Genet. **6**, 420–425
16 Dello Strologo, L., Pras, E., Pontesilli, C., Beccia, E., Ricci-Barbini, V., de Sanctis, L., Ponzone, A., Gallucci, M., Bisceglia, L., Zelante, L. et al. (2002) Comparison between SLC3A1 and SLC7A9 cystinuria patients and carriers: a need for a new classification. J. Am. Soc. Nephrol. **13**, 2547–2553
17 Chillaron, J., Estevez, R., Samarzija, I., Waldegger, S., Testar, X., Lang, F., Zorzano, A., Busch, A. and Palacin, M. (1997) An intracellular trafficking defect in type I cystinuria rBAT mutants M467T and M467K. J. Biol. Chem. **272**, 9543–9549
18 Palacin, M., Borsani, G. and Sebastio, G. (2001) The molecular bases of cystinuria and lysinuric protein intolerance. Curr. Opin. Genet. Dev. **11**, 328–335
19 Bertran, J., Werner, A., Moore, M. L., Stange, G., Markovich, D., Biber, J., Testar, X., Zorzano, A., Palacin, M. and Murer, H. (1992) Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for cystine and dibasic and neutral amino acids. Proc. Natl. Acad. Sci. U.S.A. **89**, 5601–5605
20 Wells, R. G. and Hediger, M. A. (1992) Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. Proc. Natl. Acad. Sci. U.S.A. **89**, 5596–5600
21 Busch, A. E., Herzer, T., Waldegger, S., Schmidt, F., Palacin, M., Biber, J., Markovich, D., Murer, H. and Lang, F. (1994) Opposite directed currents induced by the transport of dibasic and neutral amino acids in *Xenopus* oocytes expressing the protein rBAT. J. Biol. Chem. **269**, 25581–25586
22 Chillaron, J., Estevez, R., Mora, C., Wagner, C. A., Suessbrich, H., Lang, F., Gelpi, J. L., Testar, X., Busch, A. E., Zorzano, A. et al. (1996) Obligatory amino acid exchange via systems b0,+-like and y+L-like. A tertiary active transport mechanism for renal reabsorption of cystine and dibasic amino acids. J. Biol. Chem. **271**, 17761–17770
23 Coady, M. J., Jalal, F., Chen, X., Lemay, G., Berteloot, A. and Lapointe, J. Y. (1994) Electrogenic amino acid exchange via the rBAT transporter. FEBS Lett. **356**, 174–178
24 Wagner, C. A., Friedrich, B., Setiawan, I., Lang, F. and Broer, S. (2000) The use of *Xenopus laevis* oocytes for the functional characterization of heterologously expressed membrane proteins. Cell. Physiol. Biochem. **10**, 1–12
25 Bertran, J., Werner, A., Chillaron, J., Nunes, V., Biber, J., Testar, X., Zorzano, A., Estivill, X., Murer, H. and Palacin, M. (1993) Expression cloning of a human renal cDNA that induces high affinity transport of L-cystine shared with dibasic amino acids in *Xenopus* oocytes. J. Biol. Chem. **268**, 14842–14849
26 Broer, S., Broer, A. and Hamprecht, B. (1994) Expression of Na^+-independent isoleucine transport activity from rat brain in *Xenopus laevis* oocytes. Biochim. Biophys. Acta **1192**, 95–100
27 Mosckovitz, R., Udenfriend, S., Felix, A., Heimer, E. and Tate, S. S. (1994) Membrane topology of the rat kidney neutral and basic amino acid transporter. FASEB J. **8**, 1069–1074
28 Wang, Y. and Tate, S. S. (1995) Oligomeric structure of a renal cystine transporter: implications in cystinuria. FEBS Lett. **368**, 389–392
29 Gasparini, P., Calonge, M. J., Bisceglia, L., Purroy, J., Dianzani, I., Notarangelo, A., Rousaud, F., Gallucci, M., Testar, X., Ponzone, A. et al. (1995) Molecular genetics of cystinuria: identification of four new mutations and seven polymorphisms, and evidence for genetic heterogeneity. Am. J. Hum. Genet. **57**, 781–788
30 Meier, C., Ristic, Z., Klauser, S. and Verrey, F. (2002) Activation of system L heterodimeric amino acid exchangers by intracellular substrates. Embo J. **21**, 580–589
31 Stegen, C., Matskevich, I., Wagner, C. A., Paulmichl, M., Lang, F. and Broer, S. (2000) Swelling-induced taurine release without chloride channel activity in *Xenopus laevis* oocytes expressing anion channels and transporters. Biochim. Biophys. Acta **1467**, 91–100
32 Torras-Llort, M., Torrents, D., Soriano-Garcia, J. F., Gelpi, J. L., Estevez, R., Ferrer, R., Palacin, M. and Moreto, M. (2001) Sequential amino acid exchange across b(0,+)-like system in chicken brush border jejunum. J. Membr. Biol. **180**, 213–220
33 Palacin, M., Goodyer, P., Nunes, V. and Gasparini, P. (2001) Cystinuria. In Metabolic and Molecular Basis of Inherited Diseases (Scriver, C. R., Beaudet, A. L., Sly, S. W. and Valle, D., eds.), pp. 4909–4932, McGraw-Hill, New York
34 Albers, A., Lahme, S., Wagner, C., Kaiser, P., Zerres, K., Capasso, G., Pica, A., Palacin, M., Lang, F., Bichler, K. H. et al. (1999) Mutations in the SLC3A1 gene in cystinuric patients: frequencies and identification of a novel mutation. Genet. Test. **3**, 227–231
35 Ward, C. L. and Kopito, R. R. (1994) Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. J. Biol. Chem. **269**, 25710–25718
36 Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E. and Welsh, M. J. (1992) Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature (London) **358**, 761–764
37 Saadi, I., Chen, X. Z., Hediger, M., Ong, P., Pereira, P., Goodyer, P. and Rozen, R. (1998) Molecular genetics of cystinuria: mutation analysis of SLC3A1 and evidence for another gene in type I (silent) phenotype. Kidney Int. **54**, 48–55
38 Coady, M. J., Chen, X. Z. and Lapointe, J. Y. (1996) rBAT is an amino acid exchanger with variable stoichiometry. J. Membr. Biol. **149**, 1–8
39 Palmieri, F., Indiveri, C., Bisaccia, F. and Kramer, R. (1993) Functional properties of purified and reconstituted mitochondrial metabolite carriers. J. Bioenerg. Biomembr. **25**, 525–535
40 Restrepo, D., Cronise, B. L., Snyder, R. B. and Knauf, P. A. (1992) A novel method to differentiate between ping-pong and simultaneous exchange kinetics and its application to the anion exchanger of the HL60 cell. J. Gen. Physiol. **100**, 825–846

Received 26 June 2003/9 October 2003; accepted 15 October 2003
Published as BJ Immediate Publication 15 October 2003, DOI 10.1042/BJ20030956