Apical reabsorption of dibasic amino acids and cystine in kidney is mediated by the heteromeric amino acid antiporter rBAT/b0⁺AT (system b0⁺). Mutations in rBAT cause cystinuria type A, whereas mutations in b0⁺AT cause cystinuria type B. b0⁺AT is the catalytic subunit, whereas it is believed that rBAT helps the routing of the rBAT/b0⁺AT heterodimeric complex to the plasma membrane. In the present study, we have functionally characterized the cystinuria-specific R365W (Arg365 → Trp) mutation of human rBAT, which in addition to a trafficking defect, alters functional properties of the b0⁺-transporter. In oocytes, where human rBAT interacts with the endogenous b0⁺-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)/b0⁺-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)/b0⁺-AT holotransporter was completely abolished. This supports a two-translocation-pathway model for antiporter b0⁺, in which the efflux pathway in the rBAT(R365W)/b0⁺-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 b0⁺.

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 b0⁺-transporter is constituted by the rBAT heavy chain and the b0⁺-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 b0⁺-AT light chain is a typical polytopic membrane transport protein with 12 predicted transmembrane helices. For surface expression, both rBAT and b0⁺-AT have to be expressed in the same cell [2,6]. Reconstitution experiments have shown that the b0⁺-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/b0⁺-AT heterodimer. First, mutation of the rat rBAT residue Cys664 (corresponding to human Cys666) alters the NEM (N-ethylmaleimide) sensitivity of the transporter [8], and secondly kinetic constants of the b0⁺-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 b0⁺-AT constitute an Na⁺-independent antiporter for neutral and dibasic amino acids with properties of system b0⁺ [10–12]. System b0⁺ 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 b0⁺-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 b0⁺-like [19,20]. Subsequently, the b0⁺-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 (Arg365 → 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 b0⁺, 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-Mutation 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 b0+AT were cloned into vector pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) as described in [10].

Uptake and efflux measurements in oocytes

Influx rates of L-[3H]arginine, L-[3H]leucine and L-[35S]cystine (Amersham Biosciences and Biotrend, Cologne, Germany) were measured in ND96 buffer (96 mM NaCl/2 mM KCl/1.8 mM CaCl2/1 mM MgCl2/5 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 lyzed 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-[3H]arginine or L-[3H]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 MgCl2, 1.8 mM CaCl2, 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 b0+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 % CO2. 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-b0+AT. When rBAT or b0+AT was transfected alone the DNA transfection mixture contained 2 µg of pEGFP, 9 µg of pCDNA3-rBAT or pCDNA3-b0+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. 30 000 cells on a 12-well plate for amino acid content measurement or 60 000 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 Científico-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-gluc/2.8 mM CaCl2/1.2 mM MgSO4/5.4 mM KCl/10 mM Hepes (pH 7.5) equilibrated at 37 °C]. Subsequently, 200 μl of the transport medium with substrate [50 μM L-[3H]arginine (2.5 μCi/ml) or 20 μM L-[35S]cystine (2.5 μCi/ml) plus 5 μM 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 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 the well using the BCA Protein Assay kit (Pierce Biotechnology). Total intracellular amino acid content was measured 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 10000 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, 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 1:100 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 \( I = I_{\text{max}} [S]^{h} / ([S]^{h} + K_{0.5}^{h}) \) to the data, where \( h \) and \([S] \) are the Hill coefficient and the amino acid concentration respectively, \( I_{\text{max}} \) 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 \( I_{\text{max}} \) 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
was detected in all lanes. The fastest migrating band is unspecific as it consistent with similar uptake activities. The MG form of rBAT was present at similar levels in rBAT- and rBAT(R365W)-expressing oocytes, confirming the tightly coupled 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 the mature-glycosylated (MG) rBAT protein were detected. In contrast, a significant accumulation of the CG form of rBAT was detected in samples from 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 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>
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<tr>
<th>Amino Acid</th>
<th>Wild-type</th>
<th>R365W</th>
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<tr>
<td>L-Arginine</td>
<td>36 ± 3</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>30 ± 3</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>41 ± 11</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>129 ± 16</td>
<td>74 ± 10</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>378 ± 18</td>
<td>345 ± 65</td>
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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, Imax for analysed amino acids was 70–110 % compared with rBAT-injected oocytes. All curves were fitted using the Hill equation to obtain K0.5 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 independent experiments). K0.5 values of the mutant were not significantly different from those of the wild-type.

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

(A) Uptake of [14C]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 < 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 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.

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) or L-arginine (1 mM). 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-arginine 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.
antiiporter mechanism performed by system b\(^{\text{a}}\). 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 were performed at low (0.5 mM, endogenous) and high (10.5 mM after injection) intracellular arginine concentration. 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 mV 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\(^{\text{a}}\) [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\(_{\text{m}}\) values, whereas V\(_{\text{max}}\) 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.

The rBAT(R365W)/b\(^{\text{a}}\) 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\(^{\text{a}}\) AT light-chain subunit in HeLa cells. In contrast with oocytes, no uptake activity above background was observed in R365W/b\(^{\text{a}}\) 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\(^{\text{a}}\) AT were higher in cells grown at 33 °C.

The observation of the effect of temperature on trafficking of the rBAT/b\(^{\text{a}}\) AT and rBAT(R365W)/b\(^{\text{a}}\) AT complexes was confirmed by Western-blot analysis of cell extracts under reducing and non-reducing conditions (Figure 5). Expression of b\(^{\text{a}}\) 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\(^{\text{a}}\) 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 3 Impaired arginine but not leucine efflux in the rBAT(R365W) mutant](image_url)

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

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<th>rBAT</th>
<th>rBAT(R365W)</th>
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<tr>
<td>K(_{\text{m}}) (µM)</td>
<td>42 ± 6</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>V(_{\text{max}}) (%)</td>
<td>100 ± 5</td>
<td>74 ± 3*</td>
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<tr>
<th></th>
<th>rBAT</th>
<th>rBAT(R365W)</th>
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<tr>
<td>Uptake (extracellular arginine)</td>
<td>1004 ± 197</td>
<td>1294 ± 286</td>
</tr>
<tr>
<td>Efflux (intracellular arginine)</td>
<td>100 ± 6</td>
<td>73 ± 5*</td>
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* P < 0.05 indicates significant difference from the wild-type.
that it protects wild-type rBAT against degradation. However, is essential for the translocation to the plasma membrane and uptake experiments. Decrease in temperature partially rescued the defect observed in 20 µM [35S]cysteine uptake in the mutant. No significant cysteine uptake was observed in cells grown at 37 °C. Results from an independent experiment with four replicas per each group are shown. ***P<0.001 indicates significant difference compared with control conditions (i.e. rBAT- or R365W-transfected cells).

The formation of the rBAT-b0+AT heterodimer at 33 °C demonstrated that this direction of exchange is an inherent property of the system b0+AT when compared with that in rBAT/b0+AT-transfected cells (Figure 4B). 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-[3H]leucine during 20 min incubation. At this time of incubation, accumulation of radio labelled arginine matches well with the intracellular arginine content. Figures 7(A) and 7(B) show the time course of L-[3H]arginine uptake and the intracellular content of L-arginine after a 20 min incubation period. As expected, rBAT(R365W)/b0+AT showed a lower accumulation capacity of arginine than wild-type-rBAT/b0+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 b0+AT, mediated exchange of intracellular L-arginine with the tested extracellular amino acids. In contrast, the rBAT(R365W) mutant together with b0+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 cells.

**DISCUSSION**

**Physiological function**

System b0+ 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/b0+AT in mammalian cells, which resulted in the specific accumulation of dibasic but not of neutral amino acids, although both are substrates of system b0+. The maintenance of the asymmetric exchange in a non-epithelial cell line expressing rBAT and b0+AT demonstrates that this direction of exchange is an inherent property of the

![Figure 4 Temperature sensitivity of rBAT(R365W) and b0+AT expression in transfected HeLa cells](image-url)
rBAT(R365W) mutant alters functional properties of b0+

Figure 5 Synthesis and assembly of the rBAT/b0+AT complex at 33 and 37 °C

Detection of rBAT and b0+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 b0+AT-co-transfected; lanes 5 and 11, rBAT(R365W) plus b0+AT-transfected cells. Lane 6, proteins as detected in human kidney brush-border membranes served as a control. (A) rBAT (left panel) and b0+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 b0+AT and was absent in rBAT(R365W)/b0+AT-transfected cells (upper left panel). When cells were grown at 33 °C, the mature R365W form was produced in rBAT(R365W)/b0+AT-transfected cells (lane 5). The b0+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 b0+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 b0+AT were co-expressed.

rBAT/b0+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 b0+: 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 b0+. 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)/b0+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)/b0+AT heteromers suggests a folding defect for this mutant. The permissive temperature of 33 °C clearly also increased the assembly of the wild-type rBAT/b0+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
than in rBAT/b0 content of lysine and arginine when compared with rBAT- or rBAT(R365W)-transfected cells (**P < 0.001). This intracellular accumulation was lower in rBAT(R365W)/b0-AT-transfected cells (**P < 0.001). 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, 597 µM tyrosine and 863 µM valine.

The 'efflux translocation pathway' of the rBAT(R365W)/b0-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 b0-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)/b0-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)/b0-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 (αβ)8-barrel structure (see [2,5] for reviews) that might cover the b0-AT subunit (of 487 amino acid residues). Therefore
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.

HeLa cells were transfected with rBAT(R365W) mutant alone or in combination with b\(^{0+}\)AT and grown at 33 °C for 48 h. 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 was higher in cells transfected with rBAT/b\(^{0+}\)AT- and rBAT(R365W)-transfected cells 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) (**P < 0.01) or with rBAT(R365W)/b\(^{0+}\)AT (++P < 0.001) 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 < 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 or 400 \(\mu\)M L-arginine (Arg) or L-leucine (Leu) at the indicated concentrations. The efflux trans-stimulated efflux of leucine was determined in a medium containing no amino acids or 400 \(\mu\)M L-arginine. The efflux trans-stimulated by extracellular arginine is shown (i.e. efflux rate in the presence of arginine minus in their absence).

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.

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. 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 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).

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