Mutations of the basic amino acid transporter gene associated with cystinuria

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To investigate the function of a basic and neutral amino acid transporter-like protein (rBAT) which is a candidate gene for cystinuria, we analysed the rBAT gene in cystinuric patients. Patient 1 is a compound heterozygote with mutations in the rBAT gene causing a glutamine-to-lysine transition at amino acid 268, and a threonine-to-alanine transition at amino acid 341, who inherited these alleles from his mother (E268K) and father (T341A), respectively. Injection of T341A and E268K mutant cRNAs into oocytes decreased transport activity to 53.9 % and 62.5 % of control (L-cystine transport activity in oocytes injected with wild-type rBAT cRNA), respectively. Co-injection of E268K and T341A into oocytes strongly decreased amino acid transport activity to 28 % of control. On the other hand, co-injection of wild-type and mutant rBAT did not decrease transport activity. Furthermore, immunological studies have demonstrated that the reduction of amino acid transport is not due to a decrease in the amount of rBAT protein expressed in oocyte membranes. These results indicate that mutations in the rBAT gene are crucial disease-causing lesions in cystinuria. In addition, co-injection experiments suggest that rBAT may function as a transport activator or regulatory subunit by homo- or hetero-multimer complex formation.

INTRODUCTION

Amino acids are transported across the plasma membrane of cells via sodium-independent and sodium-dependent carriers [1]. Recently, cDNAs from rabbit and rat kidney cortex [named rBAT ($b^{0,+}$, amino acid transporter-related protein) and D2] have been cloned. Injection of rBAT or D2 into oocytes induces system $b^{0,+}$, a sodium-independent carrier for cationic and neutral amino acids (including L-cystine) [2–4]. Cystinuria is an autosomal recessive disorder characterized by excessive urinary excretion of cystine and the basic amino acids [5]. The major clinical manifestation of the disease is the development of cystine kidney stones resulting from the low solubility of cystine in urine, leading to obstruction, recurrent infections and ultimately renal insufficiency [5]. Cystinuria is thought to result from defective transport of cystine and dibasic amino acids through the epithelial cells of the renal tubules and intestinal tract [5]. Recently, Palacin and co-workers have identified six specific mutations in the rBAT gene, including homozygotes and compound heterozygotes, that segregate with the cystinuria phenotype [6]. These mutations nearly abolish the amino acid transport activity induced by rBAT in *Xenopus* oocytes [6]. The rBAT protein contains seven potential N-glycosylation sites near its putative extracellular C-terminus [2–4]. The presence of a leucine zipper motif at a C-terminus raises the possibility that the protein is associated with a second subunit [7]. The mutations found in patients with cystinuria are located in this C-terminal region of the rBAT protein [6]. However, it is still unclear how these mutations in the rBAT protein abolish amino acid transport activity. The predicted structure of the rBAT protein is not very hydrophobic and contains only one transmembrane-spanning domain, suggesting that it functions as a transport activator and regulatory subunit

[2–4]. In the present study, to analyse the function of rBAT, we analysed rBAT gene mutations in cystinuric patients.

MATERIALS AND METHODS

Patients and DNA preparations

Patient 1 was a 16-year-old man who was diagnosed with cystine kidney stones caused by the excretion of high urinary amounts of cystine. The urinary concentrations of various amino acids were as follows; cystine 1172 μmol/l (control, 30–88 μmol/l), lysine 3515 μmol/l (control, 11–380 μmol/l), ornithine 535 μmol/l (control, 7–21 μmol/l) and arginine 1359 μmol/l (control, 33–99 μmol/l). The patients father, subject 2 (44 years old), also demonstrated high concentrations of urinary cystine (1304 μmol/l) and basic amino acids (lysine, 9108 μmol/l) while his mother, subject 3 (41 years old), was demonstrated to have normal concentrations of urinary cystine (80 μmol/l), but high concentrations of urinary lysine (1863 μmol/l). We also analysed five other cystinuric patients. Blood was collected from these patients following informed consent. Lymphocytes were immortalized by transformation with the Epstein–Barr virus. DNA was prepared with a Wako-DNA extraction kit [8].

PCR and single-stranded conformational polymorphism (SSCP) of rBAT

Sequences from the coding exon of rBAT were amplified by PCR and analysed by SSCP. Fourteen pairs of oligonucleotides designed to cover the rBAT coding region as shown in Figure 1, were synthesized prior to PCR; one oligonucleotide was radio-

ATG (1)   TAG (2055)

BAT1   BAT2R
|———————|

BAT3F   BAT11R
     |——|

BAT11F   BAT4R   BAT7F   BAT10R
|———————| |——|

BAT5F   BAT8R   BAT9F   BAT7R
|———————| |———|

BAT8F   BAT9R
     |———|

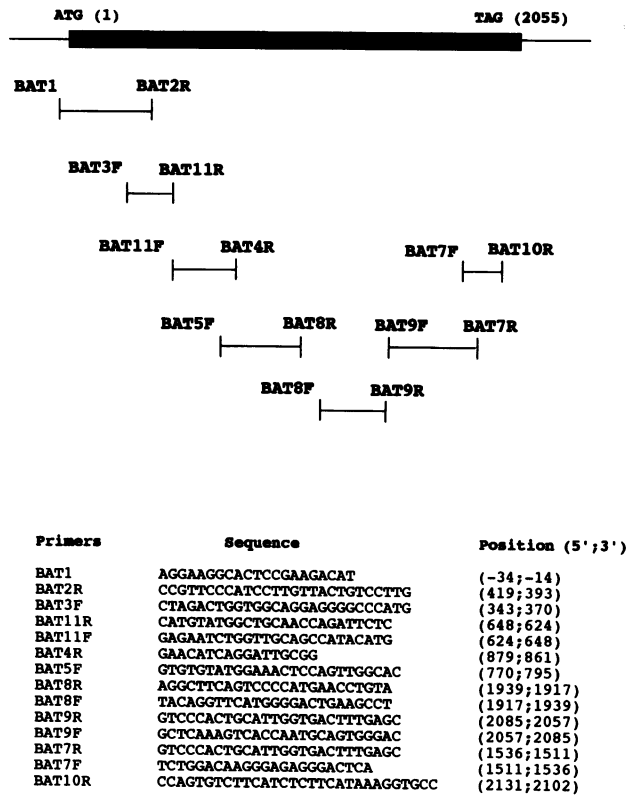| Primers	Sequence	Position (5';3')
BAT1	AGGAAGGCACTCCGAAGACAT	(-34;-14)
BAT2R	CCGTTCCCATCCTTGTTACTGTCCTTG	(419;393)
BAT3F	CTAGACTGGTGGCAGGAGGGCCCATG	(343;370)
BAT11R	CATGTATGGCTGCAACCAGATTCTC	(648;624)
BAT11F	GAGAATCTGGTTGCAGCCATACATG	(624;648)
BAT4R	GAACATCAGGATTGCGG	(879;861)
BAT5F	GTGTGTATGGAAACTCCAGTTGGCAC	(770;795)
BAT8R	AGGCTTCAGTCCCCATGAACCTGTA	(1939;1917)
BAT8F	TACAGGTTCATGGGGACTGAAGCCT	(1917;1939)
BAT9R	GTCCCACTGCATTGGTGACTTTGAGC	(2085;2057)
BAT9F	GCTCAAAGTCACCAATGCAGTGGGAC	(2057;2085)
BAT7R	GTCCCACTGCATTGGTGACTTTGAGC	(1536;1511)
BAT7F	TCTGGACAAGGGAGAGGGACTCA	(1511;1536)
BAT10R	CCAGTGTCTTCATCTCTTCATAAAGGTGCC	(2131;2102)

Figure 1   Positions of rBAT cDNA primers for SSCP analysis

Position 1 corresponds to the adenine of the ATG initiation codon. Nucleotides 1–2055 encoded one exon and an open reading frame of rBAT cDNA. Primer sequences and their positions on the rBAT cDNA are shown. Blood was collected from cystinuric patients and extracted DNA was used for SSCP analysis.

labelled with T4 polynucleotide kinase and [^{32}P]ATP (Amersham). PCR was performed using a DNA thermocycler (Intermed) with 50 ng of genomic DNA in a total volume of 20 μl [9]. Thirty-five amplification cycles were carried out; each cycle consisted of 1 min at 94 °C, and 1 min at the optimally determined annealing temperature for each oligonucleotide pair. After PCR, 2 μl aliquots were mixed with 8 μl of sample buffer (95 % formamide, 20 mM EDTA, 0.05 % Bromophenol Blue, 0.05 % xylene cyanol FF) and boiled for 5 min. Samples (2 μl) were loaded on to 6 % polyacrylamide gels, and electrophoresis was performed in TBE (90 mM Tris/90 mM boric acid/2.5 mM EDTA) buffer at 30 W at 4 °C. The gel was dried on filter paper and exposed to XAR5 film at −70 °C for 12–24 h with an intensifying screen [8].

PCR product sequencing

PCR amplification products were subcloned into pBluescript II KS$^+$ (Stratagene) and sequenced by the chain termination method using T7 DNA polymerase (Pharmacia). No less than six clones were sequenced to ensure against Taq polymerase artifacts and to determine the sequence of both alleles.

cDNA cloning

Human rBAT clones were isolated from a human kidney library in phage λgT-10 [10,11]. A ^{32}P-labelled probe (rabbit rBAT cDNA) was prepared from a PCR fragment. Three clones were subcloned into pBluescript II and sequenced, the human rBAT

sequence data was identical, except for minor differences, with that reported by Bertran et al. [2].

Site-directed mutagenesis and in vitro transcription

We constructed a plasmid containing the complete human rBAT cDNA in pBluescript II as a template for E268K and T341A mutant rBAT construction by site-directed mutagenesis. The mutagenesis primers (anti-sense strand) were 5'-TGGCACTTT-GACAAAGTGCGAAC-3' (E268K) and 5'-CCGGACACGG-TCGCACAATACTCG-3' (T341A) respectively. rBAT mutagenesis was performed as previously described [9]. rBAT cDNA clones were linearized by digestion with XhoI and transcribed into rBAT complementary RNA (cRNA) using T3 RNA polymerase (Promega) [10].

Oocyte injections and transport assay

Xenopus laevis females were obtained from Hamamatsu Jikkenn (Sizuoka, Japan). Small clumps of oocytes were treated twice for 90 min with collagenase at 2 mg/ml in a Ca^{2+}-free solution (ORII solution: 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes/ Tris, pH 7.5) to remove the follicular layer [9,10]. To measure the uptake of L-[^{3}H]arginine and L-[^{35}S]cystine (Amersham), six or seven oocytes were used for each time point. Oocytes were first washed for 30 s in solution A (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes/ Tris, pH 7.5). After extensive washing, first with ORII solution and then with modified Barth's solution [88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM Hepes/Tris, pH 7.5), the oocytes were maintained in modified Barth's solution overnight at 18 °C. Healthy stage-V oocytes were then injected with cRNA (dissolved in water at a concentration of 0.2 mg/ml) or water using a manual injector (Narishige, Tokyo, Japan). After incubation with 50 μM amino acids (labelled at 20 μCi/ml) at 25 °C, the oocytes were washed three times with cold Na$^+$-free uptake solution containing 5 mM amino acids. Each oocyte was then transferred to a scintillation vial, dissolved in 0.2 ml of 10 % SDS, and counted in 5 ml of scintillation fluid [11].

Immunoprecipitation of [^{35}S]methionine-labelled rBAT oocytes

Oocytes were injected with wild-type or mutant rBAT cRNA in a final volume of 50 nl (50 ng). After 30 h [^{35}S]methionine (1 μCi in 50 nl of DEPC-treated water, ICN) was injected, and the oocytes were incubated for 24 h at 18 °C in 0.5 ml of modified Barth's solution. Single oocytes were titurated in PBS using a Gilson P-200 Pipetman. Oocyte plasma membrane 'ghosts' were separated from cytoplasmic contents and collected into a pipette. Plasma membranes then were washed by resuspension in PBS followed by centrifugation. An intact oocyte ghost contained 2.5–3.0 μg of plasma membrane protein (approximately 50000 c.p.m.). The pellets corresponding to membrane obtained from 15–20 oocytes were solubilized in buffer B (10 mM PBS, pH 7.4, 0.07 % Triton X-100, 0.07 % SDS/0.03 % deoxycholate, 0.03 % BSA and 1 mm PMSF). Samples were mixed with 10 μg of affinity-purified antibody [anti-(human rBAT) rabbit IgG against a CPRSFKDSDKDGNGD (human rBAT residues 125–138)] and 8 μl of Protein A-Sepharose [12]. The samples were then mixed at 4 °C overnight. The pellets were then washed twice with 600 μl of buffer B. Aliquots of these samples were used for electrophoresis. After electrophoresis, gels were dried and fluorography was performed [13].

RESULTS

rBAT gene analysis

Six patients with cystinuria were screened for mutations in the rBAT coding region by SSCP analysis. PCR primers were designed with allowed amplification of overlapping cDNA products encompassing the entire rBAT coding region of 685 amino acids (Figure 1). cDNAs from each cystinuria patient were analysed by SSCP for each of the seven overlapping PCR products. The corresponding PCR products were sequenced to determine the nature of each variation in SSCP. New PCR primers were then designed around each of the sequence alterations that allowed PCR amplification and sequence conformation analysis using genomic DNA as a template. Two missense point mutations of rBAT were found in genomic DNA from patient 1.

Two other polymorphisms, G204A and A198T, were detected in two other cystinuria patients. To verify that these substitutions were not due to PCR artifacts, direct sequencing of the amplification fragments was performed, which confirmed these transitions. Sequence analysis demonstrated that patient 1 was a compound heterozygote at the rBAT locus with a guanidine-to-adenine change at nt 802, changing the glutamine 268 residue to lysine (E268K) and an adenine-to-guanidine change at nt 1021 changing the threonine 342 residue to alanine (T341A). He has inherited the allele E268K from his mother and T341A from his father (Figure 2).

Characterization of the E268K and T341A mutants

To study the effect of the E268K and T341A changes on the L-arginine and L-cystine transport activity induced by human rBAT, corresponding substitutions were introduced into the rBAT cDNA by PCR-primer-directed mutagenesis and three rBAT cRNA were expressed and injected into Xenopus oocytes: a wild-type cRNA (rBAT-WT) and mutants carrying either the E268K or T341A mutations. cRNA were synthesized and injected into Xenopus oocytes, and the uptake of [^{35}S]cystine and [^{3}H]arginine were determined. Wild-type rBAT injection into oocytes resulted in the induction of system b$^{0,+}$-like amino acid transport; this system is responsible for sodium-independent uptake of L-arginine and L-cystine. Wild-type rBAT significantly stimulated the uptake of these two amino acids, as previously
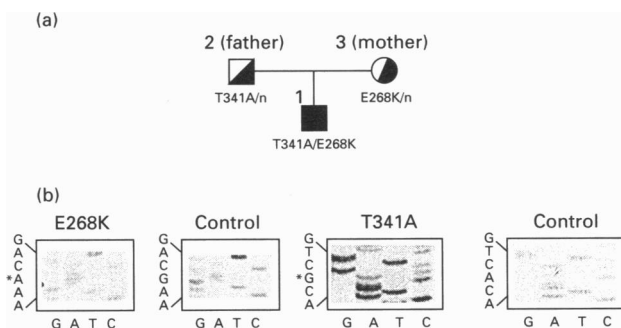
Figure 2   Identification of mutations E268K and T341A in a Japanese family with cystinuria

Two mis-sense point mutations of rBAT were found in genomic DNA from patient 1. Sequence analysis indicated that the mutation was present in a compound heterozygote with a G to A transition at nt 802, changing E to K at amino acid 268, and an A to G transition at nt 1021, changing T to A at amino acid 341. He has inherited these alleles from his mother (E268K) and father (T341A) respectively.

Table 1   Functional analysis of rBAT mutants in Xenopus oocytes

Mutant rBAT cDNA clones were linearized by digestion with XhoI and transcribed into rBAT complementary RNA using T3 RNA polymerase. Healthy stage-V oocytes were injected with 10 ng of cRNA (dissolved in DEPC-treated water at 0.2 mg/ml). Measurements of uptake of L-[^{3}H]arginine and L-[^{35}S]cystine (50 μM) used six or seven oocytes at each concentration. After incubation for 3 days, the oocytes were washed, dissolved in SDS and counted in scintillation fluid as described in the Materials and methods section. *$P < 0.01$, wild-type versus mutant rBAT.

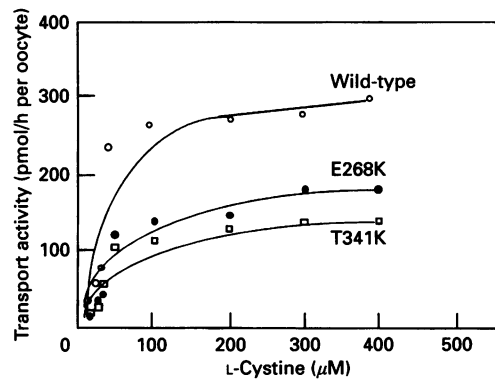|	Amino acid uptake (pmol/3 min per oocyte)	
Mutants	L-Arginine	L-Cystine
Wild-type	14.4 ± 0.9	8.5 ± 2.2
E268K	8.9 ± 1.2*	4.4 ± 1.9*
T341A	7.8 ± 1.5*	3.6 ± 0.8*
Water	0.4 ± 0.1	0.06 ± 0.02

Figure 3   Dependence of mutant rBAT cRNA-induced L-cystine uptake on L-cystine concentration

Oocytes injected with wild-type or mutant rBAT cRNA were assayed 3 days after injection for uptake of radiolabelled L-cystine at concentrations ranging from 5 to 400 μM.

reported by several groups [2–4]. For comparison, the relative residual activities in Xenopus oocytes injected with cRNA are shown in Table 1. The L-cystine uptake activity in oocytes injected with T341A and E268K rBAT cRNAs were significantly lower (53.9 ± 5.8 % and 62.5 ± 13.3 % of control, respectively) than the activity in wild-type rBAT-injected oocytes. The relative L-arginine transport activity in oocytes injected with E268K or T341A mutant rBAT was similarly decreased. No significant difference was found between the relative amino acid uptake in G204A and A198T rBAT cRNA mutants and wild-type rBAT cRNA-injected oocytes (results not shown).

We also determined the kinetics of L-cystine uptake in oocytes injected with each mutant rBAT. Uptake of L-cystine, as shown on a Michaelis–Menten plot, was saturable (Figure 3). The K_m for rBAT-induced cystine uptake were 30 ± 7.8 μM for wild type, 38 ± 8.7 μm for T341A and 41 ± 9.6 μm for E268K respectively. On the other hand, the V_{max} values for mutant rBAT-induced cystine uptake were significantly different from that for wild-type uptake; 310 ± 21 pmol/h per oocyte for wild-type, 134 ± 14 pmol/h per oocyte for T341A and 178 ± 16 pmol/h per oocyte for E268K.

However, it is not clear whether the T341A and E268K substitutions are the crucial disease-causing mutations in patients

Table 2 Effect of co-injection of rBAT mutant cRNAs on amino acid uptake in Xenopus oocytes

E268K/T341A represents injection of 10 ng of rBAT cRNAs (5 ng of E268K mutant cRNA and 5 ng of T341A mutant cRNA) into Xenopus oocytes. Wild-type/E268K and T341A represent injection of 10 ng of rBAT cRNAs (5 ng of wild-type cRNA and 5 ng of E268K or T341A mutant cRNA). Transport assay conditions are as described in the legend to Figure 3. *$P < 0.01$, wild-type versus mutant rBATs.

	Amino acid uptake (pmol/3 min per oocyte)	
Mutants	L-Arginine	L-Cystine
Wild-type	15.4 ± 2.5	9.8 ± 2.4
E268K/T341A	$4.3 \pm 1.6^*$	$2.6 \pm 1.1^*$
Wild-type/E268K	11.1 ± 2.4	8.3 ± 1.8
Wild-type/T341A	10.6 ± 2.6	8.2 ± 2.1
Water	0.3 ± 0.1	0.05 ± 0.02
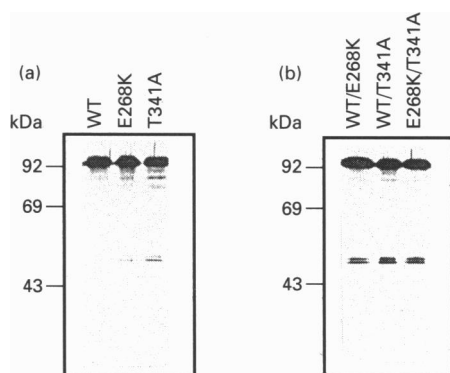
Figure 4 Immunoprecipitation analysis of rBAT protein in Xenopus oocytes

Oocytes were injected with water or 50 ng of mutant rBAT cRNA in a final volume of 50 nl. After 30 h [^{35}S]methionine (1 μCi in 50 nl of DEPC-treated water; ICN) was injected, and the oocytes were incubated for 24 h at 18 °C in 0.5 ml of modified Barth's solution. Oocytes were then harvested and used for immunoprecipitation experiments as described in the Materials and methods section.

1, because oocytes injected with the mutant rBAT cRNA still had about 50 % amino acid transport activity. To address this question, we co-injected a two-mutant cRNA mixture into Xenopus oocytes. As shown in Table 2, co-injection of E268K and T341A cRNA mutants strongly decreased amino acid transport activity compared with wild-type rBAT-injected oocytes. These results indicate that mutations in the rBAT gene are crucial disease-causing lesions in cystinuria.

To rule out the possibility that the reduction of transport activities in mutant rBAT-injected oocytes was caused by reduced rBAT synthesis or insertion into the cell membrane, oocytes were radiolabelled and rBAT levels analysed by immunoprecipitation with anti-rBAT antibody. As shown in Figure 4, radiolabelled rBAT was present at similar levels in oocytes injected with wild-type and mutant cRNAs. As expected, rBAT was barely detectable in oocytes injected with water, indicating that endogenous expression of rBAT by Xenopus oocytes was very limited (results not shown). Furthermore, we analysed cRNA expression in Xenopus oocytes co-injected with E268K and T341A cRNA by Northern blotting. There was no difference in the level of cRNA

expression between wild-type- and mutant-injected oocytes (results not shown).

DISCUSSION

In this study, we have characterized a novel mutant allele that is associated with cystinuria in a Japanese patient. Amino acid residues 341T and 268E are highly conserved in human, rat and rabbit rBAT [2–4]. Recently, Tate and co-workers have demonstrated that rBAT has four transmembrane domains using site-directed polyclonal antibodies directed against rBAT [14]. This proposed structure is distinct from that of other known mammalian transporters which, in general, are more complex proteins presumed to contain from 8 to 14 transmembrane domains [14]. E268K and T341A are present in the first transcellular loop. Our results suggest that these mutations significantly affect the function of rBAT as an amino acid transporter. Interestingly, we also found that co-injection of both rBAT mutants (T341A and E268K) into oocytes strongly reduced amino acid uptake. These results indicate that T341A and E268K substitutions may be crucial disease-causing mutations in cystinuria.

In amino acid excretion values in urine of the family members, the mother (bearing the E268K mutation) has normal urinary cystine levels. This is in contrast to the father (bearing the T341A mutation) and the child. Both mutations, however, cause a similar reduction in transport levels. In contrast, the mother has high urinary lysine levels. This may be due to a complex transport pathway for L-cystine in renal membranes [1]. Studies with brush-border-membrane vesicles indicate two transport systems for L-cystine, suggesting the existence in renal membranes of a high-affinity system which is shared with dibasic amino acids, and a low-affinity system which appears to be unshared [1].

Biochemical and immunological experiments have identified the $b^{0,+}$ co-transporter as a polypeptide of 90 kDa on SDS/PAGE reducing gels [12], and about 78 kDa in vitro translation studies [15]. However, information regarding the structure–function relationships of rBAT in situ in the native membrane is lacking. In the case of the Na$^+$-dependent glucose transporter, which contains multiple membrane-spanning regions, a high-energy electron radiation inactivation method indicated that this transporter functions in the membrane as a homotetramer composed of four independent subunits [16]. Similar studies on rBAT are now in progress. On the other hand, the potential for association of rBAT with other proteins is indicated by the presence of a leucine zipper motif and a conserved cystine residue at the external membrane interface [7]. We have confirmed the importance of interaction with other proteins at the external membrane interface, because a point mutation (L565P) in the leucine zipper motif of rBAT significantly decreased the uptake of L-arginine (K. Miyamoto, unpublished work). Interactions between members of the type-II membrane glycoprotein family, such as rBAT, with the transporter at the external membrane surface, may be necessary for stimulation of amino acid transport. However, microinjection of some point mutant (L565A and L565G) did not decrease L-arginine and L-cystine uptake in oocytes. Therefore, drastic amino acid changes in the leucine zipper motif are necessary to abolish transport activity (K. Miyamoto, unpublished work). Recently, we made several deletion mutants of rBAT, and injected them into Xenopus oocytes. One C-terminal deletion mutants (Δ511–685) stimulated a y$^+$-like system which is an endogenous amino acid transport system in Xenopus oocytes. The Δ511–685 mutant, which truncates the leucine-zipper motif, could stimulate amino acid transport system y$^+$, suggesting that stimulation of amino acid transport by Δ511–685 rBAT is not necessary for this motif. As

mentioned above, the C-terminal region (511–685) of rBAT is important for interactions with other proteins and for determining its specificity as a transport activator.

Furthermore, we could not find any rBAT mutations in other Japanese cystinuria patients. These observations suggest that mutations in other components of the amino acid transport system may cause cystinuria. Alternatively, the wild-type rBAT stimulated a $b^{0,+}$ system with broad substrate specificities for cystine and for basics and some neutral amino acids. If rBAT functions as an activator for an amino acid transport system, multiple rBAT subunits may interact with transporter proteins, because of the low transport activity in oocytes co-injected with rBAT mutants compared with those injected with each rBAT mutant alone.

In voltage- and current-clamp studies, Busch et al. have suggested that the system induced by rBAT is an amino acid exchanger [17]. rBAT-mediated transport of neutral and dibasic amino acids is associated with net outward or inward currents, respectively, which may be caused by an exchange of neutral with dibasic amino acids. However, it is not clear how an exchanger can perform its function in proximal renal tubular and intestinal amino acid absorption, because, in these epithelial cells, Na^+-coupled transport systems allow the cellular accumulation of neutral amino acids [17]. Thus the function of rBAT in amino acid transport is still unknown. Present studies indicate that rBAT is a candidate gene for cystinuria, and a loss of rBAT function could decrease the activity of the cystine transport system. Further studies are still needed to clarify the molecular basis of cystinuria.

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