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An Intracellular Trafficking Defect in Type I Cystinuria rBAT Mutants M467T and M467K*

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From the ‡Department of Biochemistry and Molecular Biology, Faculty of Biology, Universitat de Barcelona, Avda. Diagonal 645, Barcelona 08028, Spain and the §Institute of Physiology, Eberhard-Karls-Universität Tübingen, Gmelinstrasse 5, D-72076 Tübingen, Germany

The human rBAT protein elicits sodium-independent, high affinity obligatory exchange of cystine, dibasic amino acids, and some neutral amino acids in *Xenopus* oocytes (Chillarón, J., Estévez, R., Mora, C., Wagner, C. A., Suessbrich, H., Lang, F., Gelpí, J. L., Testar, X., Busch, A. E., Zorzano, A., and Palacín, M. (1996) *J. Biol. Chem.* 271, 17761–17770). Mutations in rBAT have been found to cause cystinuria (Calonge, M. J., Gasparini, P., Chillarón, J., Chillón, M., Galluci, M., Rousaud, F., Zelante, L., Testar, X., Dallapiccola, B., Di Silverio, F., Barceló, P., Estivill, X., Zorzano, A., Nunes, V., and Palacín, M. (1994) *Nat. Genet.* 6, 420–426). We have performed functional studies with the most common point mutation, M467T, and its relative, M467K, using the oocyte system. The K_m and the voltage dependence for transport of the different substrates were the same in both M467T and wild type-injected oocytes. However, the time course of transport was delayed in the M467T mutant: maximal activity was accomplished 3–4 days later than in the wild type. This delay was cRNA dose-dependent: at cRNA levels below 0.5 ng the M467T failed to achieve the wild type transport level. The M467K mutant displayed a normal K_m, but the V_{max} was between 5 and 35% of the wild type. The amount of rBAT protein was similar in normal and mutant-injected oocytes. In contrast to the wild type, the mutant proteins remained endoglycosidase H-sensitive, suggesting a longer residence time in the endoplasmic reticulum. We quantified the amount of rBAT protein in the plasma membrane by surface labeling with biotin 2 and 6 days after injection. Most of the M467T and M467K protein was located in an intracellular compartment. The converse situation was found in the wild type. Despite the low amount of M467T protein reaching the plasma membrane, the transport activity at 6 days was the same as in the wild type-injected oocytes. The increase in plasma membrane rBAT protein between 2 and 6 days was completely dissociated from the rise in transport activity. These data indicate impaired maturation and transport to the plasma membrane of the M467T and M467K mutant, and suggest that rBAT alone is unable to support the transport function.

The heterologous expression of rBAT in *Xenopus* oocytes elicits the sodium-independent, high affinity transport of cystine, dibasic amino acids, and some neutral amino acids ($b^{0,+}$-like activity) (1–3) by an obligatory exchange mechanism that accounts for the accumulation of such substrates in the oocytes (4, 5). The rBAT protein is located in the brush border of the epithelial cells of the proximal straight tubule and the small intestine (6, 7). Very recently, our group has demonstrated that rBAT is expressed in the "proximal tubule" cell line OK. An antisense sequence against rBAT abolishes *in vivo* the $b^{0,+}$-like activity expressed apically in these cells (8). All these data indicate a role of rBAT in cystinuria. This common inheritable disorder is due to the defective transport of cystine and dibasic amino acids through the epithelial cells of the renal tubule and the intestinal tract (9–11): the low solubility of cystine leads to the development of cystine calculi in the kidney. Three types of classic cystinuria have been described, but rBAT is responsible only for type I, as has been demonstrated by mutational and genetic analysis (12, 13).

Type I individuals who inherit one mutant rBAT gene are completely normal and do not hyperexcrete cystine or dibasic amino acids. Only the homozygotes for mutant rBAT genes display the above mentioned phenotype. Several mutations of rBAT have been described (12, 14–16). Among them, the most common point mutation is M467T, found in 26% of type I cystinuria chromosomes so far analyzed. This is also the only mutation that has been found in homozygosis in one Spanish family (12). Its relative, M467K, has been found as a compound heterozogous with the L678P mutant in one Italian patient (14). The methionine at position 467 is completely conserved among all the species in which rBAT sequences are known (1–3, 8). In the 4-transmembrane domain model proposed by Tate and co-workers (17) this residue lies in the third transmembrane domain, very close to the cytosol. There is little information regarding the dysfunctions provoked by rBAT mutations. Calonge and co-workers (12) reported preliminary experiments on the M467T mutant, showing a decrease in transport activity. Miyamoto and co-workers (18) obtained similar results with the E268K and T341A mutants. However, they do not provide any explanation for the reduced function.

The exact role of rBAT in the amino acid transport of system $b^{0,+}$-like is unknown. This is largely due to its unusual topology. Experimental evidence obtained using site-directed antibodies suggests a 4-transmembrane domain model for rBAT (17). This contrasts with common metabolite transporters, which appear to contain 8–12 transmembrane domains (19). Thus, it has been suggested that rBAT is only part of the

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functional transport unit (2, 3). rBAT is expressed in kidney and intestine as a 94-kDa protein in reducing conditions and as a 125-kDa complex in nonreducing conditions, suggesting a disulfide-linked heterodimer with another protein of 35–50 kDa (20, 21). This complex has also been detected in total membranes of oocytes injected with rBAT cRNA (20).

Here, we report the molecular basis for the defect seen in the M467T mutation and its relative M467K. There is no significant difference in the functional properties of the M467T mutant compared with the wild type, but the M467K mutant shows a clear V_{max} defect. The two mutants are expressed only as an endo H[1]-sensitive band that remains mostly inside the cell. The lack of correlation between the rBAT protein in the plasma membrane and the expressed transport activity suggests that rBAT is necessary but not sufficient for the amino acid transport activity of system $b^{0,+}$-like.

EXPERIMENTAL PROCEDURES

Uptake Experiments and Electrophysiological Studies—Oocyte management, injections, uptake measurements, and electrophysiological studies were as described elsewhere (1, 22). *Xenopus laevis* (H. Kähler, Institut für Entwicklungsbiologie, Hamburg, Germany) oocytes were defolliculated by collagenase (Boehringer Mannheim) treatment. Only healthy looking stage VI oocytes were used. The uptake of the labeled amino acids (DuPont NEN) was measured in 7–8 oocytes per individual data point at 25 °C for 5 min when initial rates were measured or for 3 h for accumulation experiments. The data are expressed as the difference between the uptake in rBAT-injected oocytes and the uptake in uninjected oocytes (induced uptake). Uptake values in uninjected oocytes are the same as in water-injected oocytes. For electrophysiological measurements the oocytes were each injected with 1 ng of cRNA, and two-electrode voltage and current clamp recordings were performed 3–8 days later. Recordings were performed at 22 °C using a Geneclamp amplifier (Axon Instruments, Foster City, CA) and MacLab D/A converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia). Amino acid-induced voltage changes or currents were filtered at 10 Hz. Experiments were repeated with two batches of oocytes; in all repetitions, qualitatively similar data were obtained. The external control solution (ND96 medium) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES at pH 7.5. The holding potential was −50 mV, but this was varied in one set of experiments to analyze the voltage dependence of amino acid-induced currents. The solution flow was adjusted to 20 ml/min, which guaranteed a complete solution exchange in the recording chamber within 10–15 s. The maximal current amplitude induced during amino acid superfusion was measured. Inward currents are shown with the prefix −. All data are given as means ± S.E. where n is the number of oocyte measurements.

Site-directed Mutagenesis, cRNA Synthesis, and Injection—The construction of the M467T mutant in pSPORT-1 has been described elsewhere (12). For the construction of the M467K mutant we used the general procedure of Kunkel and co-workers (23) with additional steps described by Yan and Maloney (24). The mutagenic oligonucleotide was 5′-AAGC[T]TGTTCATCACGTT-3′ (antisense strand; the mutated nucleotide (position 1400; Ref. 1) is indicated between brackets). Mutants were identified by their acquisition of an *Alu*I restriction site (12), and a cassette between *Nco*I and *Bst*EII sites was completely sequenced. Then, this cassette was ligated into pGEM4Z containing the cDNA from human rBAT that had been cut with the same enzymes. Finally, the whole cassette was sequenced. The three plasmids were isolated using a miniprep kit (Promega), linearized by *Xba*I restriction endonuclease digestion, and transcribed *in vitro* using T7 RNA polymerase (Promega) for the wild type and the M467T mutant and SP6 RNA polymerase (Pharmacia Biotech Inc.) for the M467K in the presence of ^{7}mGpppG (NEB) as described elsewhere (1). Oocytes were injected (Inject+Matic-System, J. A. Gabay, Geneva, Switzerland) with 50 nl of cRNA at the concentrations given in the figures.

Kidney Brush Border and Oocyte Membranes—Brush border membranes from rat kidney cortex were obtained as described (6). Total oocyte membranes were purified as described elsewhere (25). Briefly 30–50 oocytes were homogenized in 10 μl/oocyte buffer A (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.5, plus 5 μg/ml leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) by 20 strokes of an Eppendorf Teflon-glass homogenizer. The homogenate was centrifuged twice at 1,000 × *g* for 10 min at 4 °C to eliminate the yolk, and the supernatant was pelleted (100,000 × *g*, 90 min, 4 °C) and resuspended in 2 μl/oocyte buffer A. Aliquots were used to quantify proteins by the method of Bradford (26). The membranes were stored at −20 °C until use.

Endoglycosidase H Treatment—50–100 μg of total membranes or rat kidney brush borders was denatured by boiling for 5 min in the presence of 100 mM DTT and 0.54% SDS, and incubated in a NaP_i buffer (50 mM NaP_i, pH 5.5, 0.36% SDS, 0.5 mM phenylmethylsulfonyl fluoride). 10 milliunits of endo H (Boehringer Mannheim) was added. The mixture was incubated for 18 h at 37 °C, and the reaction was stopped by the addition of Laemmli sample buffer (27) with DTT to 100 mM. Samples were boiled for 5 min and stored at −20 °C until use.

Surface Biotin Labeling and SDS-PAGE—The procedure for biotin labeling of oocytes was adapted from that described in Ref. 28. 50–75 oocytes, which were injected with wild type or M467T rBAT cRNA, were washed five times in OR-2 medium (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES adjusted to pH 7.5 with NaOH) and then placed into an agarose-coated Petri dish filled with 2 ml of OR-2 medium. The fresh membrane-impermeant reagent NHS-LC-biotin stock (2 mg/500 μl of OR-2) was carefully added to the dish. After 10 min at room temperature the reaction was stopped by adding 1 ml of 500 mM glycine, pH 7.4. The oocytes were then washed three times in 500 mM glycine, pH 7.4, followed by two washes in OR-2. The oocytes were then transferred to a microcentrifuge tube, and 0.5 ml of lysis buffer (2% Nonidet P-40, 150 mM NaCl, 2 mM $CaCl_2$, 20 mM Tris, pH 7.4, plus 2 μM leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) was added. The oocytes were passed 20 times through a 200-μl pipette, and the homogenate was centrifuged twice (1,000 × *g*, 10 min, 4 °C) to remove the yolk. The supernatant was sonicated for 1 min and then centrifuged again at 1,000 × *g* (10 min, 4 °C). The supernatant was dialyzed overnight at 4 °C against streptavidin buffer (SAv buffer) (0.3% Nonidet P-40, 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris, pH 8, plus 2 μM leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) and then centrifuged for 30 min at 14,000 rpm at 4 °C to remove insoluble material. The supernatant was incubated overnight with 75 μl of streptavidin-agarose bead suspension (previously washed three times in SAv buffer). Supernatant and pellet were separated by low speed (2,000 rpm) centrifugation. The supernatant was precipitated with 5% trichloroacetic acid and resuspended in 150 μl of Laemmli sample buffer. One-half was separated, and DTT was added to 100 mM. Then the two halves (reduced and nonreduced) were boiled for 5 min and stored. The pellets were eluted by adding 100 μl of Laemmli sample buffer without DTT and boiling for 5 min. Then one-half was separated, and DTT was added to 100 mM. Finally, the two halves were boiled once again and stored.

SDS-PAGE was performed on total membrane proteins (endo H-treated or not) and the supernatants and pellets from the biotin-labeling experiments according to Laemmli (27). For rBAT Western blotting, reduced samples were transferred to Immobilon (Millipore). Following the transfer the filters were blocked with 5% non-fat dry milk and 0.02% sodium azide in phosphate-buffered saline for 1 h at 37 °C and incubated with the polyclonal antibody anti-rBAT MANRX (6) at 1:100 dilution in 1% non-fat dry milk and 0.004% sodium azide in phosphate-buffered saline overnight at room temperature. Detection of the immune complex was accomplished using ^{125}I-protein A (ICN). For β1-integrin Western blotting, nonreduced samples were transferred as above, blocked in 10% non-fat dry milk and 0.05% Tween-20 in phosphate-buffered saline for 30 min at 37 °C, and incubated with the monoclonal antibody 8C8 (29) at 1:10 dilution in the blocking solution overnight at room temperature. Antibody binding was detected using the ECL Western blot detection system (Amersham Corp.). Blots were quantified by scanning densitometry. Immunoblots were performed under conditions in which autoradiography detection was in the linear response range. All electrophoresis reagents were obtained from Bio-Rad. NHS-LC-biotin was from Pierce, and streptavidin-agarose beads were from Sigma.

RESULTS

We introduced the naturally occurring mutations M467T and M467K in the rBAT cDNA and, upon transcription *in vitro*, injected them into *Xenopus* oocytes. As illustrated in Fig. 1, L-Arg-induced transport was clearly reduced in both M467T

[1] The abbreviations used are: endo H, endoglycosidase H; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum.

and M467K compared with the wild type at 1.5 days after the injection. For 2.5 ng the uptake values were 21.4 ± 1.6 pmol/5 min/oocyte for the wild type group, 4.3 ± 1 for the M467T, and 2.3 ± 0.4 for the M467K; for 0.5 ng the values were 12.2 ± 0.9, 2.6 ± 0.8, and 0.7 ± 0.4; and for 0.05 ng the values were 4.4 ± 0.6, 0.1 ± 0.4, and 0 ± 0.4, respectively. Surprisingly, 7 days after the injection of 2.5 ng, the M467T achieved the same level of expression as the wild type (47.7 ± 4 pmol/5 min/wild type oocyte *versus* 42.2 ± 0.6 pmol/5 min/M467T oocyte). This recovery was dose-dependent since it was not observed at the lower doses (72% recovery at 0.5 ng and only 40% at 0.05 ng), suggesting that at physiological levels of rBAT mRNA there is no recovery (Fig. 1). The M467K-induced uptake was also time- and cRNA dose-dependent: at 0.05 ng the uptake was undetectable 1.5 days after the injection and was 13% of the wild type at 7 days; at 2.5 ng the uptake rose from 11 to 25% of wild type values. In other experiments the uptake induced in the M467K-injected oocytes was between 5 and 35% of control values. Similar results were obtained with current measurements (data not shown). Attempts to determine transport over a longer period failed because of the increased oocyte mortality, so we cannot preclude the possibility that the M467K (or the M467T at low cRNA doses) could reach the wild type uptake values. We also performed experiments in which 25 ng was injected and obtained similar results (see Fig. 3).

To assess whether the decrease in function was due at least in part to changes in the K_m for the different substrates we performed kinetic analysis of transport. The induced uptake was studied both by tracer measurements and by electrophysiological techniques: 3–4 days after injection by the former method and 8 days after injection by the latter (Table I). The wild type and the M467T were indistinguishable after 8 days of expression with regard to their similar K_m (in the 100 μM range, as reported in Refs. 1, 3, 22, and 30) and V_{max} for both L-Arg and L-Leu. Only after a shorter time of expression, *i.e.* 3–4 days, was a V_{max} decrease found for the three substrates used (65% for L-cystine, 54% for L-Leu, and 59% for L-Arg). The difference in the K_m of L-Arg and L-Leu observed only in the electric measurements could be due to the voltage clamp of the oocytes at −50 mV; more positive voltages led to a reduction of the L-Leu K_m without changing the L-Arg K_m (22). At day 6 and with 20 ng of cRNA injected, the M467K also showed a V_{max} decrease (65% for L-Arg) without any change in the K_m (27 ± 6 μM for the M467K and 40 ± 7 μM for the wild type). Kinetic analysis of the M467K mutant by electric measurements was also attempted, but the low induced currents precluded accurate estimations of the K_m and V_{max}.

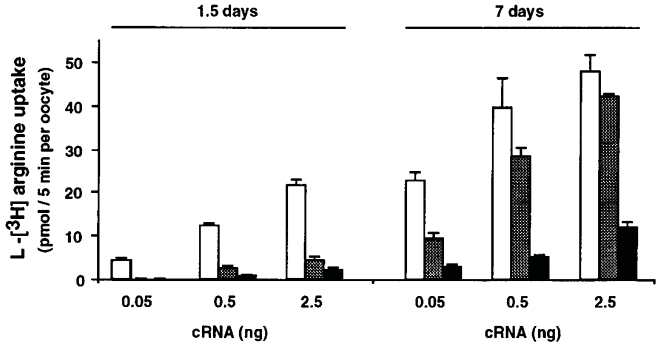

FIG. 1. **Time and cRNA dose dependence of the induced amino acid transport in wild type and mutant-injected oocytes.** Oocytes were injected with the indicated doses of cRNA of the wild type rBAT (*open bars*), M467T (*shaded bars*), or M467K (*closed bars*). The uptake of 50 mM L-Arg was determined for 5 min 1.5 and 7 days after the injection. Each bar is the mean ± S.E. of the induced uptake values measured in 6–8 oocytes. Another independent experiment gave similar results.

rBAT mediates an obligatory exchange of amino acids; no transport of amino acids occurs in the absence of amino acids on any side of the cell. This exchange accounts for accumulation of the substrates in the oocyte and it fully explains the rBAT amino acid-induced currents (4, 5). The L-Arg-induced currents measure the L-Arg inward-L-neutral amino acid outward mode of exchange, and the L-Leu-induced currents measure the reverse mode. Voltage modification leads to changes in these currents, reflecting, at least in part, changes in the modes of exchange; for instance, hyperpolarization reduces L-Leu-induced currents and increases L-Arg-induced currents. Therefore, we examined the possibility of a defect in the exchange by assaying the voltage dependence of L-Arg- and L-Leu-induced currents at day 8 after injection; as shown in Fig. 2 there was no difference between wild type and M467T-injected oocytes. The efflux of amino acids from the oocyte in the absence of amino acids in the extracellular medium was undetectable in either wild type or M467T-injected oocytes, as expected from an obligatory exchange (data not shown). Lastly, we examined the accumulation level of amino acids in both wild type and M467T-injected oocytes, and no differences were found (data not shown). In all, the data indicate no functional difference in the M467T mutant at high cRNA doses but a clear V_{max} decrease in the M467K.

Given that the M467T-injected oocytes displayed no functional defect despite the clear delay in the expression of transport, we next examined rBAT protein expression in the oocytes using the previously characterized polyclonal antibody MANRX in Western blot experiments (6). We injected a large dose of cRNA (25 ng) to ensure high levels of protein expression and measured uptake (Fig. 3*A*) and protein present in total membranes (Fig. 3*B*). The wild type protein appeared as a doublet, with one band of 94 kDa (band I) and one of higher mobility (85 kDa, band II). In contrast, only band II was present in the M467T mutant (Fig. 3*B*). This was also the case for

TABLE I

Kinetic parameters of the wild type (wt)- and M467T (MT)-induced amino acid transport activity

Kinetic parameters for the uninduced activity of transport of L-Arg, L-Leu, and L-cystine are given. Oocytes were prepared and injected with 2.5 ng of cRNA. K_m values are given in μM. V_{max} values are given in pmol/5 min/oocyte and in nA for tracer (3–4 days after injection) and electric (8 days after injection) measurements, respectively. Data (mean ± S.E.) correspond to representative experiments in which induced transport was measured varying the substrate concentration between 10 and 250 μM. 7–8 oocytes per triplicate were used for each data point. Different batches of oocytes from two different laboratories were used for the tracer and the electrophysiological studies. Therefore, the K_m values obtained are not comparable between the tracer and the electrophysiological studies.

J		3–4 days		8 days	
		wt	MT	wt	MT
L-Cystine	V_{max}	9.2 ± 0.6	3.2 ± 0.4	nd	nd
	K_m	43 ± 6	40 ± 10	nd	nd
L-Arginine	V_{max}	196 ± 16	81 ± 6	170 ± 4	181 ± 4
	K_m	93 ± 13	51 ± 8	30 ± 2	27 ± 2
L-Leucine	V_{max}	54 ± 4	24 ± 2	139 ± 3	133 ± 5
	K_m	78 ± 9	88 ± 14	213 ± 20	151 ± 24

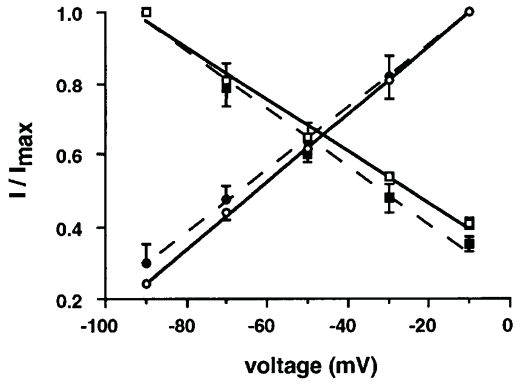

FIG. 2. **Voltage dependence of the wild type- and M467T rBAT-induced currents.** Currents were induced by superfusing wild type (*solid lines*, *open symbols*)- and M467T (*dashed lines*, *closed symbols*)-injected oocytes at the given holding potentials between −90 and −10 mV with L-Leu (1 mM, *circles*) or L-Arg (0.1 mM, *squares*) for 30 s. For the graph the currents were normalized against the maximal induced currents in each current-voltage experiment. The L-Leu- and L-Arg-induced currents were always the largest at −10 and −90 mV, respectively. When not visible, error bars are smaller than symbols.

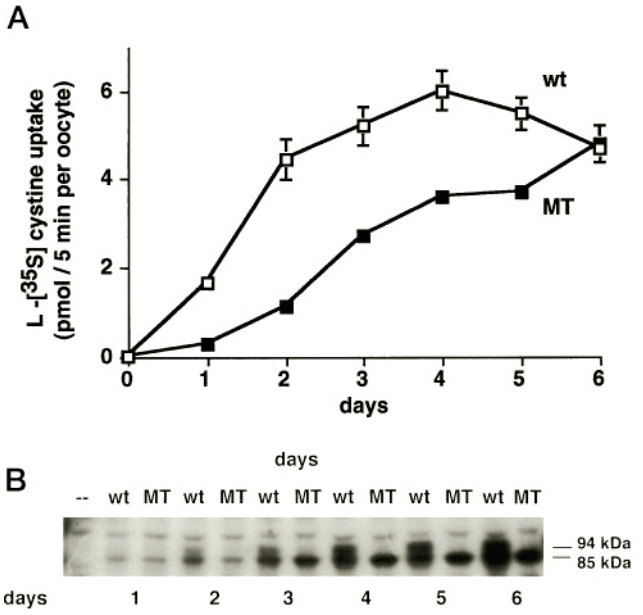

FIG. 3. **Time course of the induced amino acid transport and of the rBAT protein content in total membranes from wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng of cRNA. *A*, the induced uptake of 50 mM L-cystine was measured every day until day 6. Data are the mean ± S.E. of 6–8 oocytes. L-Cystine uptake in uninjected oocytes was 0.83 ± 0.08 pmol/5 min/oocyte at day 1 and 0.81 ± 0.05 pmol/5 min/oocyte at day 6. When not visible, error bars are smaller than symbols. *B*, total membrane proteins of oocytes were collected as described under "Experimental Procedures." 50 μg of reduced samples were run in a 7.5% PAGE system, transferred, incubated with MANRX polyclonal antibody against rBAT, and revealed with ^{125}I-protein A. Notice that the lower mobility band seen in the lanes also appeared in uninjected oocytes. Therefore, it is considered unspecific. --, uninjected oocytes.

the M467K (see Fig. 4). Quantification of the bands obtained in four experiments (data not shown) demonstrated that the total amount of wild type protein compared with the M467T was not significantly different at any day after injection and increased continuously until day 6 without any apparent saturation, despite uptake saturation. The amount of M467K protein was also similar to that of the wild type (data not shown). Electric measurements of the time course gave similar results. Thus, the different profiles of uptake observed for the wild type and M467T cannot be explained by the amount of rBAT protein present in total membranes.

From the Western blot in Fig. 3*B* it appeared that on the day after the injection only band II was detected in wild type-injected oocytes, suggesting that it could be a precursor of band I, most likely a non-fully glycosylated form of the protein. In fact, it is known from other membrane proteins, such as the cystic fibrosis transmembrane conductance regulator (31), low density lipoprotein receptor (32), sucrase-isomaltase (33), α_{IIb} integrin (34), and others, that some of their naturally occurring mutations produce only the endo H-sensitive form of the protein. This indicates intracellular accumulation of the mutant protein (probably in the ER) and failure to reach its normal location. The results of endo H digestions performed with total membranes of wild type-, M467T-, and M467K-injected oocytes are depicted in Fig. 4. Band II (85 kDa), the only one present in M467T and M467K, and the high mobility band of the wild type were endo H-sensitive, producing a new band of 71 kDa. This size agrees with the 72 kDa reported for the rBAT-expressing tunicamycin-treated oocytes (1). Band I shifted to 92 kDa when digested with endo H. rBAT from renal brush borders showed a similar shift, suggesting a similar glycosylation pattern for mature rBAT in oocytes and proximal tubule cells. The failure to detect band I in the M467T mutant suggests that the acquisition of the endo H-resistant condition is not necessary to its activity. This has also been demonstrated for other proteins expressed in oocytes, such as the *Torpedo* acetylcholine receptor γ and δ subunits (35) and glycophorin A (36).

The endo H sensitivity of band II suggested an intracellular location. To examine this hypothesis we performed surface labeling of intact oocytes with the membrane-impermeant reagent NHS-LC-biotin. We also attempted to determine whether the time course of the uptake correlated with the amount of wild type and M467T protein in the plasma membrane. Biotin labeling in oocytes has been successfully carried out in several studies (28, 37, 38). We opted for the method described by Müller and co-workers in Ref. 28 because it provided us with β1-integrin as an internal control for our biotinylation experiments. β1-integrin of *Xenopus* oocytes is expressed as two species of different molecular mass: one of 100 kDa (the pre-β1 form), endo H-sensitive, not biotin-labeled, and presumed to be localized to the cortical ER; and one of 115 kDa (the mature β1), endo H-resistant, biotin-labeled, and localized to the plasma membrane (28). Fig. 5 shows a representative biotin-labeling experiment of oocytes expressing wild type or M467T rBAT 2 or 6 days after the injection of 25 ng of cRNA. Labeled oocytes were lysed, and the biotinylated proteins were isolated by adsorption to streptavidin-agarose beads (see "Experimental Procedures") and probed for the presence of β1-integrin and rBAT. As expected, only the mature β1-integrin was biotinylated. In these conditions, most of the M467T protein remained in an intracellular compartment, whereas most of the wild type rBAT protein reached the oocyte surface. Densitometric measurements from three independent experiments showed that, at day 2 and day 6 in M467T-injected oocytes, 88% of the rBAT protein was in the supernatant. This value was only 35% in wild type-injected oocytes. As for the M467T, the M467K protein remained mostly intracellular, and only very small amounts reached the oocyte surface (data not shown). For the wild type, band II was detected in the intracellular fraction, and its presence in the plasma membrane was scarce; in some experiments it was not detectable, as shown in Fig. 5. In contrast, band I appeared at the plasma membrane, and its presence in the internal membranes was hardly detectable, suggesting an efficient biotin labeling. In agreement with the data obtained with total oocyte membranes (Figs. 3*B* and 4), band I was never observed in M467T-injected oocytes, even in the

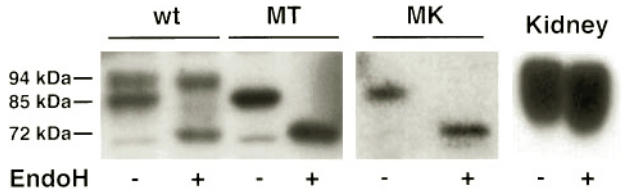

FIG. 4. **Sensitivity of wild type (*wt*), M467T (*MT*), and M467K (*MK*) rBAT proteins to the treatment with endo H.** Oocytes were injected with 25 ng of cRNA. Four days later, total membrane proteins were obtained. 50–100 μg of membrane proteins were digested or not with endo H and immunoblotted with MANRX. An equivalent amount of protein was loaded in each lane, except in kidney lanes, where rat kidney brush border membranes were loaded as a control. Another experiment was performed that gave the same results. The high mobility band seen below 72 kDa in the wild type and mutant lanes is not specific, since it also appeared in uninjected oocytes (not shown).

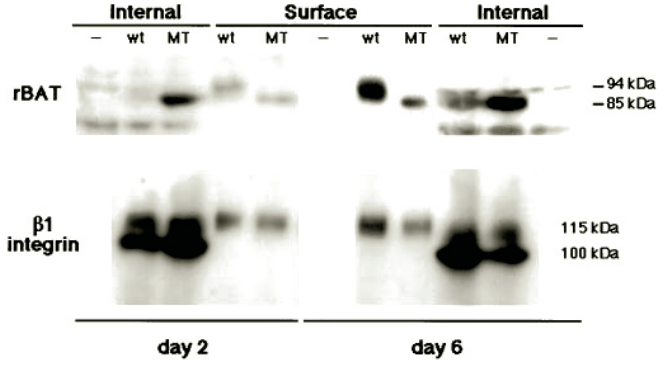

FIG. 5. **Biotin labeling of wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng of cRNA. Biotinylation was performed 2 and 6 days later as described under "Experimental Procedures." A representative experiment is shown. Times of exposure for 2 and 6 days are different (lower for 6 days). In the rBAT panel reduced samples were subjected to SDS-PAGE, probed with MANRX polyclonal antibody against rBAT, and revealed with ^{125}I-protein A. An equivalent of 16.5 oocytes was loaded in the precipitates (*Surface*), and 5 oocytes were loaded in the supernatants (*Internal*). The bands detected above and below the 85-kDa band in the internal fraction of the wild type and mutant lanes at days 2 and 6 are not specific, since they also appeared in uninjected oocytes (—). In the β1-integrin panel nonreduced samples were subjected to SDS-PAGE, probed with 8C8 monoclonal antibody against the oocyte β1-integrin, and revealed with the ECL system (Amersham Corp.) using an anti-mouse IgG coupled to peroxidase. An equivalent of 5 oocytes was loaded both in precipitates and supernatants. (—, uninjected oocytes).

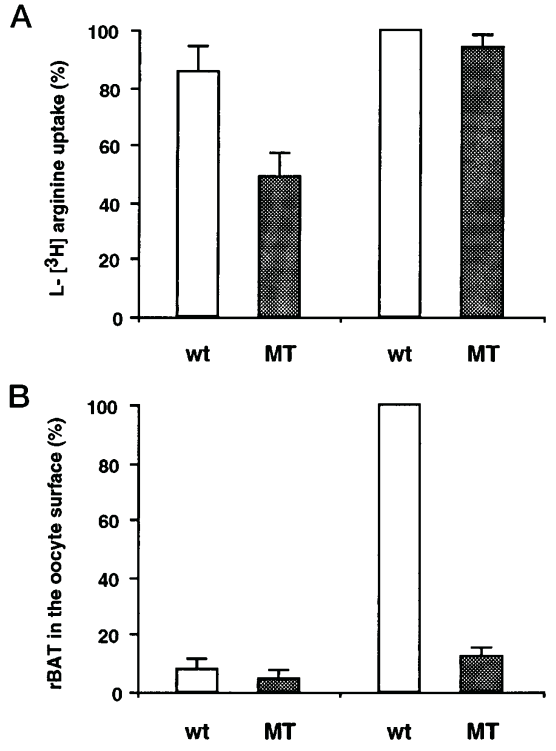

FIG. 6. **Induced amino acid transport and surface rBAT protein in wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng. *A*, 50 μM L-Arg-induced uptake was measured at 2 and 6 days. The wild type-induced uptake at day 6 was adjusted to 100. We present data (mean ± S.E.) of five experiments. *B*, in parallel, oocytes were treated as in Fig. 5. The densitometric quantification of the precipitates corrected for their β1-integrin control precipitates is depicted. The value for wild type at day 6 was adjusted to 100. Data (mean ± S.E.) of three experiments for 2 days and five experiments for 6 days are presented.

highly purified fraction corresponding to the biotin-labeled proteins (surface) (Fig. 5).

Despite the lower levels of M467T protein on the oocyte surface the recovery of the induced transport was total at 6 days. In Fig. 6*B* we present the densitometric quantification of the precipitates for three experiments (day 2) or five experiments (day 6). To obtain a better quantification, the signals in the rBAT precipitates were corrected for their β1-integrin content. The values of transport activity are also shown for comparison (Fig. 6*A*). Although there were no differences in the uptake, M467T surface rBAT protein was only 12% of the amount observed in wild type-injected oocytes at 6 days. Moreover, a 10-fold increase in plasma membrane rBAT protein was measured between 2 and 6 days in wild type-injected oocytes, despite an almost saturated transport at 2 days. Thus, there was a clear dissociation between protein in the plasma membrane and uptake: high increases in surface rBAT protein were not reflected in higher uptake rates. On the other hand, the difference between wild type and M467T surface rBAT protein at 2 days might correlate with the uptake values. The same is true of the increase in M467T rBAT plasma membrane protein from 2 to 6 days. In all, biotin-labeling experiments indicate that, in contrast to the wild type protein, M467T protein is mostly located in an intracellular compartment. A lack of correlation between surface rBAT protein and transport activity was also observed, suggesting the presence of rBAT molecules in the plasma membrane that are not functional. So, as we and others have proposed (1, 2, 20, 21), rBAT may be an essential but not a sufficient component of a complex of two or more proteins that mediates transport function.

DISCUSSION

In this paper we describe the molecular basis for the reduced transport observed in the rBAT point mutations M467T and M467K. Both mutants display an intracellular trafficking defect that impairs their transport to the oocyte surface.

Functional studies showed that transport was impaired in both mutants, the defect being more severe in the M467K. This may be caused by the more drastic amino acid change (from a nonpolar residue to a polar residue in the M467T and to a positively charged residue in the M467K). The magnitude of the defect, however, was dependent on the time after cRNA injection and on the amount of cRNA injected, especially for the M467T mutant. Thus, at high levels of cRNA the M467T mutant achieved the same levels as those induced by the wild type; this was not the case at lower levels. This "cRNA dose and time effect" may be related to the level of saturation of the quality-control machinery in the oocyte: only when the system is saturated (*i.e.* with high cRNA doses or after a longer time) does some M467T rBAT protein reach the surface. The dependence on the cRNA level might be important for the epithelial cell of the tubule *in vivo*; an rBAT mRNA level below the amount injected in the oocytes is conceivable.

The recovery of function is difficult to explain if the mutation affects only the amino acid translocation across the transporter, and it is better supported by an impairment of the intracellular transport of the molecule to its normal localization in the cell. An alteration of the transporter's turnover rate is also difficult to apply in this case because the total amount of wild type protein compared with the M467T is similar. Whole oocyte transport assays indicated no intrinsic functional defect in the M467T mutant: the K_m for the different substrates remained the same and the exchanger activity, measured as the voltage dependence of amino acid-induced currents and the efflux of amino acids in the absence of any amino acid in the extracellular medium, was not different from that of the wild type. Then, as expected from the above data, the amino acid accumulation level in the M467T-injected oocytes was similar to that in wild type-injected oocytes. The K_m of the M467K-induced transport was also the same as that of the wild type, but a V_{max} decrease was observed. This may reflect an additional intrinsic defect in the translocation pathway of amino acids. Nevertheless, the magnitude of this decrease is, as for the M467T but to a lesser extent, dependent on the time and on the cRNA injected, again suggesting a trafficking defect.

The normal rBAT protein accumulated in the oocyte in two forms: an endo H-sensitive form (band II) of 85 kDa and an endo H-resistant form (band I) of 94 kDa. Thus, rBAT followed the general pathway of membrane proteins, passing first through the endoplasmic reticulum and then through the Golgi complex, where it acquired the endo H-resistant condition, on its way to the plasma membrane. Band I was by far the major rBAT protein found in the plasma membrane in wild type-injected oocytes, as revealed by surface labeling with biotin. In contrast, both mutants remained in an endo H-sensitive form. We had no evidence that the two rBAT mutants were ever transported to the Golgi complex. However, we detected the presence of the endo H-sensitive form in the plasma membrane. This also occurred with the M467K mutant (data not shown). The mutants may traverse the Golgi complex without being processed; it has been shown that some naturally occurring mutants of the sucrase-isomaltase gene are endo H-sensitive and are missorted to the basolateral membrane of the epithelial cells of the jejunum. The authors suggested that conformational and/or structural alterations in the protein prevented the acquisition of the endo H-resistant condition (33).

It is worth mentioning the method we used to localize rBAT in the oocyte. Initial experiments with an oocyte membrane subfractionation protocol proposed by Thomas and co-workers (25), based on an earlier method (39), showed, surprisingly, the same localization for both the normal and the M467T mutant, in the "plasma membrane" fraction. Control Western blots with the oocyte β1-integrin showed the endo H-sensitive and the endo H-resistant forms of β1-integrin in the plasma membrane fraction, but neither form was detected in the intracellular fractions (data not shown). β1-integrin has been immunolocalized to the plasma membrane and to the cortical ER (28), which extends around the cortical granules and contacts the plasma membrane of the oocyte (40). The fractionation method may lead to the copurification of the plasma membrane and the cortical ER. Thus, the information so obtained should be interpreted with caution.

Therefore, surface labeling with biotin was used to quantify the amount of rBAT protein in the oocyte surface, using β1-integrin as a control in the biotinylation procedure. We observed a lower amount of M467T and M467K proteins at the plasma membrane and higher amounts located intracellularly. The converse situation was true for the wild type. Moreover, the overall rate of accumulation at the surface was higher for the normal protein. All these data strongly suggest a defect in the delivery of the mutants to the plasma membrane due to their retention in an intracellular compartment, probably the ER. The residence time in the ER is expected to be longer for both mutants. The intracellular retention could be due to an improper folding in the ER, as shown for the ΔF508 CFTR in cystic fibrosis (41) or α_1-antitrypsin deficiency (42). Met-467 lies on the third transmembrane domain according to the model of Tate and co-workers (17). The change to threonine would only slightly impair the folding of the protein, but the introduction of a lysine in the highly hydrophobic environment of the transmembrane domain would have a stronger effect. The complete carrier may be constituted only if rBAT is assembled with other proteins; this assembly might be impaired (see below). Assembly of proteins into native homo- or heterooligomers is essential for their transport out of the ER (43, 44). In eukaryotic cells, proteins that fail to fold or to assemble properly usually follow an ER-associated degradation pathway: ΔF508 CFTR (45) and the subunits of the T-cell receptor (44) are good examples. The slow rate of this pathway at low temperatures (46) makes its involvement in the defect displayed by these mutants unlikely, at least in oocytes, because they are continuously kept at 18 °C. Actually, the ΔF508 CFTR does not appear to be degraded in oocytes and cell lines cultured at low temperature (47–49) but is degraded in cells cultured at 37 °C (45). Whatever the cause of the retention in the ER, we propose that type I cystinuria should be added to the list of putative human protein-folding diseases given by Thomas and co-workers (50).

Here we have shown a clear lack of correlation between the amount of rBAT protein in the oocyte surface and the induced amino acid transport. The simplest explanation is that rBAT alone is unable to sustain its induced amino acid transport activity. So the question arises: what is the functional unit for the rBAT-induced $b^{0,+}$-like activity? Several findings support the hypothesis that it is a complex formed by rBAT and another protein. First, all the cloned metabolite and neurotransmitter transporters contain 8–12 transmembrane domains (19), which is in contrast to the 4-transmembrane domain model proposed for rBAT (17). This suggests that rBAT may not be the carrier but a modulator of it (*i.e.* delivering silent transporters to the cell surface, like the β subunit of the Na^+/K^+ ATPase (51)). Second, rBAT is expressed in kidney and intestine as a 94-kDa band in reducing conditions and as a 120–130-kDa band in nonreducing conditions (6, 20, 21), indicating a disulfide-linked heterodimer with a 30–40-kDa protein. Furthermore, this 120–130-kDa band is the only one detected upon cross-linking of kidney brush border membranes followed by reducing Western blot (20). Third, the rBAT homologous protein 4F2hc induces in oocytes a y^+L-like amino acid exchanger activity (2, 5, 52) and is expressed in different tissues as a disulfide-linked heterodimer of 120 kDa. Its 40-kDa component has been detected by ^{125}I surface labeling followed by immunoprecipitation and reduction of the complex. Unfortunately, only the 85-kDa component (4F2hc) has been cloned (53, 54). We have been unable to immunoprecipitate the rBAT 120–130-kDa band, most probably due to a steric masking of the epitope produced by the tight aposition of the other protein.[2] Fourth, so far the only heterologous system in which rBAT has been successfully expressed is the *Xenopus* oocyte. rBAT transiently expressed in COS cells is unable to reach the plasma membrane (21) or, if it arrives, no amino acid transport induction is detected (55). This correlates with the absence of the 120–130-kDa band in nonreducing Western blots from these cells (21).

[2] R. Estévez and M. Palacín, unpublished results.

Finally, Wang and Tate have recently shown that in nonreducing conditions, several high molecular weight rBAT-specific complexes (*i.e.* not present in uninjected oocytes) are detected in oocyte total membranes (20). In preliminary experiments, we detected such complexes in biotin-labeled oocytes (data not shown). Among them, only the 125-kDa complex was exclusively found at the surface, and its relative amount in wild type and M467T oocytes may correlate with transport function (data not shown). Its identical electrophoretic mobility in wild type and M467T oocytes, however, is surprising; perhaps a very small fraction of M467T protein matures (but so small as to be undetectable, even in overexposed films of the highly purified surface fraction) or, more likely, the endogenous subunit may display an anomalous mobility that renders the same size when it binds to either band I or II. Interestingly, the rBAT homologous protein 4F2hc, when it is bound to its very hydrophobic subunit (4F2 light chain), has an abnormal electrophoretic mobility (54).

In conclusion, the M467T and M467K mutants display a trafficking defect that maintains them in an intracellular location, probably the ER. A slower folding or assembly in the ER could be responsible for this defect. Thus, misfolding or a folding delay of the mutants may decrease the assembly rate with the endogenous subunit. The present data do not allow us to distinguish between these two possibilities. In any case, this phenotype can explain type I cystinuria. Direct confirmation of this requires analysis of the rBAT protein from tissue samples of patients carrying these mutations. A 125-kDa complex composed of rBAT and an endogenous oocyte protein may be the functional unit of transport. However, reconstitution experiments are needed to demonstrate that only a complex of rBAT with another protein is able to mediate transport. These studies await the cloning of this putative protein.

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