Distinct classes of trafficking rBAT mutants cause the type I cystinuria phenotype

Paola Bartoccioni1,3,4, Mònica Rius1,2,3, Antonio Zorzano1,3, Manuel Palaciń1,3,4,† and Josep Chilarrón1,2,3,†,*

1Department of Biochemistry and Molecular Biology and, 2Department of Animal Physiology, Faculty of Biology, University of Barcelona, Barcelona, Spain, 3Institute for Research in Biomedicine, Barcelona Science Park, Barcelona, Spain and 4CIBERER, Barcelona, Spain

Received December 20, 2007; Revised and Accepted March 9, 2008

Most mutations in the rBAT subunit of the heterodimeric cystine transporter rBAT-b0,1AT cause type I cystinuria. Trafficking of the transporter requires the intracellular assembly of the two subunits. Without its partner, rBAT, but not b0,1AT, is rapidly degraded. We analyzed the initial biogenesis of wild-type rBAT and type I cystinuria rBAT mutants. rBAT was degraded, at least in part, via the ERAD pathway. Assembly with b0,1AT within the endoplasmic reticulum (ER) blocked rBAT degradation and could be independent of the calnexin chaperone system. This system was, however, necessary for post-assembly maturation of the heterodimer. Without b0,1AT, wild-type and rBAT mutants were degraded with similar kinetics. In its absence, rBAT mutants showed strongly reduced (L89P) or no transport activity, failed to acquire complex N-glycosylation and to oligomerize, suggesting assembly and/or folding defects. Most of the transmembrane domain mutant L89P did not heterodimerize with b0,1AT and was degraded. However, the few [L89P]rBAT-b0,1AT heterodimers were stable, consistent with assembly, but not folding, defects. Mutants of the rBAT extracellular domain (T216M, R365W, M467K and M467T) efficiently assembled with b0,1AT but were subsequently degraded. Together with earlier results, the data suggest a two-step biogenesis model, with the early assembly of the subunits followed by folding of the rBAT extracellular domain. Defects on either of these steps lead to the type I cystinuria phenotype.

INTRODUCTION

Cystinuria (OMIM 220100) is an autosomal recessive disease of renal reabsorption and intestinal absorption of cystine and dibasic amino acids. Cystine precipitates and forms calculi which ultimately produce renal insufficiency. Two cystinuria phenotypes are distinguished: type I, where the obligate heterozygotes have normal urinary excretion; and non-type I, where the obligate heterozygotes have moderate to high excess of urinary excretion of cystine and dibasic amino acids (1). All but one mutations in SLC3A1, encoding rBAT, cause type I cystinuria (2,3). Mutations in SLC7A9, encoding b0,1AT, cause non-type I phenotype and also some type I cases (3). A gene dosage effect can explain non-type I cystinuria, since SLC7A9+/− mice express already the phenotype (4). It is not known why type I heterozygotes are silent.

rBAT is a type II membrane glycoprotein with a large glucosidase-like extracellular domain. b0,1AT is a non-glycosylated, polytopic membrane protein. Their co-expression induces the transport system b0,1+-like, an obligatory exchanger that physiologically mediates the influx of cystine and dibasic amino acids and the efflux of neutral amino acids (5,6). Intracellular assembly of rBAT with b0,1AT to form a disulfide-linked heterodimer is required for functional expression. When expressed alone, rBAT remains endoglycosidase H sensitive and is degraded, whereas b0,1AT is stable (7,8). In contrast, b0,1AT expressed alone is stable and can be recovered in a functional form from its intracellular location (7–9). The rBAT-b0,1AT heterodimer oligomerizes into a stable heterotetramer expressed in the brush border membranes of the epithelial cells of the renal proximal...
System b0⁺-like is the main apical reabsorption system for cystine in kidney (6).

From the more than 60 rBAT mutations found in type I cystinuria patients, all are located in the extracellular domain, with the exception of the transmembrane mutants L89P and 1105R (3,11). The few pathogenic rBAT mutants which have been examined showed reduced or absent transport activity (8,12,13). For the M467T and M467K mutations expressed in Xenopus oocytes, a trafficking defect was suggested, although function was recovered with higher protein expression, specially for the M467T mutant (12), raising doubts about the situation in vivo. Only the R365W mutation has been expressed in a mammalian cell system and its traffic was also impaired (8). However, the mechanism underlying these putative trafficking defects has not been addressed.

Here we report the basic features of the biogenesis of the rBAT-b0⁺-AT heterodimer in mammalian cells. We show that, without b0⁺-AT, rBAT is an ERAD (Endoplasmic Reticulum-Associated Degradation) substrate. Maturation of the rBAT-b0⁺-AT heterodimer requires interaction with the calnexin chaperone system, suggesting that folding of rBAT is not completed before assembly. We compare the fate of wild-type and type I cystinuria rBAT mutants L89P, T216M, R365W, M467K and M467T, with or without wild-type b0⁺-AT. T216M and M467T are the most common rBAT missense mutations, representing 6.4 and 26.4% of the alleles, respectively, of the International Cystinuria Consortium (3). The transmembrane domain L89P mutant is assembly-defective, but not folding-defective. The extracellular domain rBAT mutants are degraded after efficient assembly with b0⁺-AT, indicating misfolding, but not assembly impairment.

**RESULTS**

Our aim was to examine the early biogenesis of wild-type human rBAT, b0⁺-AT and the rBAT-b0⁺-AT heterodimer and compare it with type I cystinuria rBAT mutants. HeLa and MDCK cells are good models to study these proteins (7–10,14,15). We used previously documented stable MDCK cell lines expressing wild-type rBAT, b0⁺-AT or both proteins together (9). Only cells co-expressing rBAT and b0⁺-AT elicited cystine transport activity (9).

**Unassembled rBAT is an ERAD substrate**

We set-up pulse-chase experiments followed by immunoprecipitation with specific antibodies [6,15] and see Materials and Methods [directed against N-terminal peptides of rBAT and b0⁺-AT. In MDCK cells expressing only rBAT, this protein ran as a ~90 kDa endoglycosidase-H sensitive band (consistent with its intracellular localization) (7,8), which, after a lag phase of ~1 h, disappeared with a half-life of \( \tau_{1/2} = 54 \pm 4 \text{ min} \) (Fig. 1). In contrast, in MDCK cells expressing only b0⁺-AT, more than 60 and 30% of the pulse-labeled ~40 kDa band of b0⁺-AT was detected after 8 and 24 h of chase, respectively (data not shown). We further characterized rBAT degradation. The proteasome inhibitors MG132 (Fig. 1) and lactacystin (not shown) delayed degradation, and so did the ER-mannosidase inhibitor dMNJ (Fig. 1). Therefore, rBAT disposal is mediated, at least in part, by the proteasome and ER-mannosidase-dependent ERAD pathway (16,17). The lag phase suggests that rBAT is initially retained in the calnexin chaperone system (16). Castanospermine inhibits glucose trimming mediated by the ER-glucosidases I and II (see the mobility shift of the rBAT band in Fig. 1), avoiding the generation of monoglucosylated N-glycans required for interaction with calnexin/calreticulin. The drug accelerated disposal (Fig. 1, see the chase point 2 h), suggesting that interaction with the calnexin chaperone system delays rBAT degradation (16). However, the lag phase was not reduced (Fig. 1, see the chase point 1 h). Neither the lysosomal degradation inhibitor leupeptin nor the secretory pathway inhibitor brefeldin A had any effect on rBAT disposal (not shown).

![Figure 1](image-url)
Assembly and maturation of rBAT and b0,+AT

Pulse-chase experiments were performed in MDCK cells co-expressing rBAT and b0,+AT. The proteins were immunoprecipitated with anti-rBAT and anti-b0,+AT antibodies separately and run under reducing and non-reducing conditions (Fig. 2A). Immediately after the pulse, rBAT ran as a ~90 kDa endoglycosidase H sensitive band under reducing conditions (7,8) (Fig. 2A, +DTT panel). This band co-precipitated with the anti-b0,+AT antibody, indicating that assembly took place within the ER and that it was a fast event (Fig. 2A; +DTT panel, anti-b0,+AT IP, time zero lane). Assembly was mediated by a disulfide bridge (Fig. 2A; −DTT panel; the ~135 kDa heterodimer band).

Most of the labeled rBAT was co-precipitated with the anti-b0,+AT antibody (Fig. 2A; compare the rBAT bands with both immunoprecipitating antibodies in the +DTT panel). Consistently, rBAT was mainly found in the ~135 kDa band (Fig. 2A, −DTT panel, anti-rBAT IP, time zero lane). During the chase, virtually all rBAT matured to a ~96 kDa endoglycosidase H resistant band (7,8) in a precursor-product fashion (Fig. 2A, +DTT panel). The maturation half-time was ~1 h. Maturation of the heterodimer could also be observed (Fig. 2A, −DTT panel). In spite of the similar amounts of rBAT detected with anti-rBAT and anti-b0,+AT antibodies, a stronger b0,+AT signal was seen with anti-b0,+AT (Fig. 2A, +DTT panel), indicating that much of the precipitated b0,+AT is not associated with rBAT, and that b0,+AT is present in great excess over rBAT. Moreover, the signal of the co-precipitated b0,+AT was much less intense than the rBAT signal, suggesting that newly synthesized rBAT was associated mainly with unlabelled b0,+AT (Fig. 2A; anti-rBAT IP, +DTT panel). The group of Verrey observed a similar scenario (7).

Next, we used castanospermine to examine the relevance of glucose trimming in assembly and maturation of rBAT-b0,+AT (Fig. 2B). When added during all the experiment, labeled rBAT molecules remained untrimmed (Fig. 2B, Cast. all; see the mobility shift). Inhibition of glucose trimming did not affect assembly of the heterodimer via a disulfide bridge (Fig. 2B, Cast. all, and data not shown), and did not induce degradation of rBAT (compare with Fig. 1, Cast.). When castanospermine was added only during the chase (Fig. 2B, Cast. chase), no labeled untrimmed rBAT molecules were detected. In these conditions, we can measure the effect of inhibition of the glucosidase II-mediated trimming of the last glucose of N-glycans. This trimming facilitates dissociation of glycoproteins from calnexin/calreticulin (18). Maturation of rBAT was delayed (Fig. 2B; observe the persistence of the endoglycosidase H sensitive rBAT band in the Cast. chase compared to the Control group), suggesting that the folding of rBAT is not complete before assembly of both subunits, and that it is facilitated by the calnexin chaperone system.

Type I cystinuria rBAT mutants are trafficking-defective

We used transient transfections in HeLa cells to study the biogenesis of type I cystinuria rBAT mutants localized either to the transmembrane or the TIM-barrel domains (Fig. 3A). We have already used these cells to analyze the transport activity of the rBAT-b0,+AT heterodimer, its functional and structural units, and to report initial data on the R365W rBAT mutant (8,10,15).

Figure B. Assembly and maturation of the rBAT-b0,+AT heterodimer. MDCK cells stably expressing human rBAT and b0,+AT were labeled for 15 min and chased as above. (A) Immunoprecipitations (IP) were performed with anti-rBAT (left) and anti-b0,+AT (right) antibodies, and the precipitates were run both under reducing (+ DTT, upper panel) and non-reducing (− DTT, bottom panel) conditions. In reducing conditions, core-glycosylated rBAT (rBATc; ~90 kDa), mature rBAT (rBATm; ~96 kDa) and the b0,+AT band (b0,+AT; ~40 kDa) are observed. In non-reducing conditions, core-glycosylated (rBAT, b0,+AT) and mature (rBAT, b0,+AT) heterodimers are also distinguished, and unassembled rBAT, is observed disappearing during the chase. (B) Here only the co-precipitated rBAT bands with the anti-b0,+AT antibody are shown in reducing conditions. The results with the anti-rBAT antibody are similar (not shown). Pulse-chase experiments were as above but castanospermine (1 mM) was included in all the experiment (Cast. all) or only in the chase (Cast. chase). Observe the mobility shift of rBAT (Cast. all; Glc3rBATc), indicating that the trimming of the synthesized rBAT has been blocked. Representative data of at least n = 4 experiments are shown.

We confirmed the main results obtained in MDCK cells. In the absence of b0,+AT, rBAT was degraded, after a lag phase of ~2 h, with a half life of T1/2 = 106 ± 18 min, and degradation was at least in part mediated by the proteasome (Supplementary Material, Figure A). We monitored assembly of rBAT and b0,+AT with transfection conditions which allow that almost all pulse-labeled rBAT co-precipitates with the anti-b0,+AT antibody (see Materials and Methods). The results were also comparable to MDCK cells. The endoglycosidase H resistant rBAT band was detected as a smear just above the endoglycosidase H sensitive band (Supplementary Material, Figure B and also Fig. 8). For this reason, maturation was followed directly with endoglycosidase H assays. After 8 h, all the pulse-labeled rBAT had complex N-glycosylation. The maturation half-time was ~2 h (Supplementary Material, Figure B). Inhibition of glucose trimming with castanospermine did not disturb assembly (not shown).

We transfected wild-type rBAT and type I cystinuria rBAT mutants in the presence of b0,+AT in HeLa cells and measured cystine transport. Only the L89P mutant showed a significant transport function (Fig. 3B). Loss of function was not due to
decreased expression levels of the mutants compared to the wild-type (Fig. 4A). The percentages of transfected cells (see Materials and Methods) and b0,þAT expression (data not shown) were similar. However, most of the mutant proteins remained endoglycosidase H sensitive, consistent with retention in an intracellular location (most likely the ER) (Fig. 4B). Only a small but significant amount of the L89P rBAT acquired endoglycosidase H resistance (Fig. 4B); and, in some experiments, we observed the same for the M467T mutant. The rBAT mutants formed heterodimers with b0,þAT (Fig. 4C) as judged by the presence of the ~135 kDa band (rBAT/b0,þAT) under non-reducing conditions. To our surprise, this band was clearly diminished in the L89P mutant. A ~250 kDa band appeared also under non-reducing conditions, which may correspond to the heterotetramer [rBAT-b0,þAT]2 (6,10). This band was not detected in the mutants (with the exception of the L89P mutant and, in some experiments, the M467T mutant), suggesting failure to oligomerize (Fig. 4C). We measured directly the amount of heterotetrameric rBAT-b0,þAT by blue native-PAGE followed by western blot, where [rBAT-b0,þAT]2 runs at ~500 kDa (10). We detected this band in the wild type and also in L89P, but at much lower amounts. Even less heterotetramer was present in the R365W and M467T lanes (Fig. 5). All mutants were detected as aggregates (operationally defined as material that did not enter the stacking gel and/or that ran as a smear at the top of the running gel), with the exception of the M467T. We suggest that these aggregates contain both unassembled rBAT and single heterodimers, since: (i) bands with a size consistent with these species are not detected in blue native gels (data not shown and 10); (ii) they are observed in SDS–PAGE western blots (Fig. 4C); and (iii) unassembled rBAT is also seen as aggregates in blue native gels (not shown). Assembly and oligomerization might dissolve rBAT and b0,þAT aggregates [which contain functional b0,þAT (9)], similar to the association of the chaperone Shr3p with the General Amino Acid Permease from yeast (19).

The L89P mutant assembles inefficiently with b0,þAT

We seek to find the molecular mechanism for the trafficking defects. We examined the degradation kinetics of the different rBAT species in the absence of b0,þAT and found no significant differences between the wild type and the mutants (Fig. 6). Notice that there is no mobility change in the T216M mutant (Fig. 6, the asterisks). T216 lies in an N-glycosylation consensus site. The result indicates that this site is not used in vivo. The structural alignment of rBAT with the X-ray structure of the extracellular domain of the 4F2hc homologue shows that T216 is an internal residue located very close to the putative active cleft, explaining why this site is not N-glycosylated (20). Next, we measured the assembly of wild-type and mutant rBAT with b0,þAT. Under our experimental conditions, more than 80% of the rBAT and b0,þAT expressed is immunoprecipitated with the specific antibodies (not shown). We compared the amounts of pulse-labeled rBAT immunoprecipitated with the specific antibodies (not shown). We compared the amounts of pulse-labeled rBAT immunoprecipitated by the anti-rBAT antibody with that co-precipitated by the anti-b0,þAT antibody, under reducing conditions (Fig. 7A). In HeLa cells, there is a great excess of b0,þAT over rBAT and most
of the labeled rBAT is associated with unlabeled $b^0\text{-AT}$ (data not shown), as in MDCK cells (Fig. 2A). Therefore, under non-reducing conditions most of the radioactivity associated with the $\sim135$ kDa heterodimer band comes from labeled

![Figure 4](image)

**Figure 4.** Wild-type and mutant rBAT expression and maturation in HeLa cells. In (A) and (C), aliquots of the same cells used for the transport experiment shown in Figure 3 were used for western blot analysis. Total membranes from the transfected cells were loaded for SDS–PAGE. Ten micrograms of protein were loaded per lane. Proteins were transferred to PVDF membranes and decorated with the anti-rBAT antibody. (A) Comparison of the expression levels of wild-type and mutant rBAT, in the presence and absence of $b^0\text{-AT}$, under reducing conditions. The asterisk (*) marks the mobility shift of the wild-type rBAT in the presence of $b^0\text{-AT}$, indicating maturation of N-glycans. rBATc: $\sim90$ kDa core-glycosylated rBAT. (B) Endoglycosidase H (endo H) assays of wild-type and mutant rBAT in the presence of $b^0\text{-AT}$. Total membranes were obtained from HeLa cells transfected with $b^0\text{-AT}$ and the wild-type and mutant rBAT species. Ten micrograms of protein were digested or not with the enzyme, and run in SDS–PAGE under reducing conditions. Proteins were transferred to PVDF membranes and decorated with the anti-rBAT antibody. A representative experiment (of at least $n = 4$ for all the rBAT species) is shown. rBATDG: de-glycosylated rBAT. (C) The same samples of (A) (with $b^0\text{-AT}$) were run under non-reducing conditions. The arrow marks the few amount of L89P mutant in the heterodimeric form. rBAT/b$^0\text{-AT}$: $\sim135$ kDa heterodimer. Observe the $\sim250$ kDa band. In (A) and (C), all lanes belong to the same gel and membrane. WT, wild-type rBAT; MT, M467T; MK, M467K; LP, L89P; RW, R365W; TM, T216M.

![Figure 5](image)

**Figure 5.** Blue native-PAGE analysis of wild-type and mutant rBAT. HeLa cells were transfected with $b^0\text{-AT}$ and wild-type or mutant rBAT species. After 36 h, total membranes were solubilized with digitonin and 30 $\mu$g were loaded in 4–20% linear blue native gels. Proteins were transferred and decorated with anti-$b^0\text{-AT}$ (not shown) and anti-rBAT antibodies. The 3% stacking gel is also presented. Data are from a representative experiment of $n = 4$. The exposure time to detect rBAT alone (asterisk) is longer (>10-fold) than for the rest of the gel. Wild-type and mutant rBAT are abbreviated as in Figure 4.

![Figure 6](image)

**Figure 6.** Decay kinetics of wild-type and mutant rBAT in the absence of $b^0\text{-AT}$. HeLa cells transiently expressing wild-type or mutant rBAT were labeled for 30 min with $^{35}$S-Met/Cys and chased with excess Met/Cys up to 8 h. Equivalent amounts of radioactivity incorporated into protein were immunoprecipitated with the anti-rBAT antibody. The precipitates were run under reducing conditions. The core-glycosylated rBAT band (rBATc, $\sim90$ kDa) is observed. The asterisks mark the identical size of wild-type rBAT and the T216M mutant. Representative experiments of at least $n = 4$ for each rBAT species are shown.
rBAT extracellular domain. In striking contrast, the transmembrane domain of rBAT either delay or disrupt post-assembly folding of the extracellular domain, suggesting that mutants of the TIM-barrel domain of rBAT either delay or disrupt post-assembly folding of the extracellular domain. These results suggest that the TIM-barrel domain of rBAT are degraded after assembly with b0,AT. The most relevant interaction (related to degradation) between the calnexin chaperone system and rBAT during the lag phase may occur later in MDCK cells as compared with HeLa cells.

rBAT degradation is blocked by heterodimerization in the ER. This step is fast. Some labeled rBAT associates with b0,AT after a 2.5 min pulse (not shown). This time is within the range needed to synthesize full-length rBAT (24), suggesting that it can assemble co-translationally with already synthesized b0,AT. Assembly might be independent of the calnexin chaperone system, as it is not impaired by the inhibition of the formation of monoglucosylated N-glycans. In contrast, preventing the deglucosylation of monoglucosylated N-glycans, which slows dissociation of glycoproteins from calnexin/calreticulin, delays maturation of rBAT-b0,AT (Fig. 2B). This result indicates that maturation of rBAT within the heterodimer depends on interactions with the lectins. We propose that there are some post-assembly folding steps of the extracellular domain of rBAT, which are

DISCUSSION

Biogenesis of the wild-type transporter

We measured for the first time the turnover rates and maturation half-times of the human subunits of the system b0,+,AT together with wild-type or mutant rBAT were pulse-labeled for 30 min and lysed. Equivalent amounts of radioactivity incorporated into protein were immunoprecipitated with the anti-rBAT (IP, r) and the anti-b0,AT antibodies, and run under reducing (A) and non-reducing conditions (B; here only the anti-rBAT immunoprecipitations are presented). Representative experiments are shown. (A) For quantification, the rBATb band signal in the anti-b0,AT immunoprecipitation (rBATb) was multiplied by 100 and divided by the rBATc band signal in the anti-rBAT immunoprecipitation (rBATc). The results (mean ± SEM of at least n = 4 experiments for each rBAT species) are given as the percentage of total pulse-labeled rBATc co-precipitated with the anti-b0,AT antibody. (B) For quantification, the rBATb0,AT (~135 kDa) band signal was multiplied by 100 and divided by the total rBAT signal (rBATc plus rBATb0,AT bands) in the anti-rBAT immunoprecipitation. The results (mean ± SEM of at least n = 4 experiments for each rBAT species, with the exception of T216M, n = 1) are given as the percentage of total rBAT found in the rBATb0,AT band (assembled rBAT). See text for further explanations. Wild-type and mutant rBAT are abbreviated as in Figure 4.

Figure 7. Assembly of wild-type and mutant rBAT with b0,AT. HeLa cells transiently transfected with b0,AT together with wild-type or mutant rBAT were pulse-labeled for 30 min and lysed. Equivalent amounts of radioactivity incorporated into protein were immunoprecipitated with the anti-rBAT (IP, r) and the anti-b0,AT antibodies, and run under reducing (A) and non-reducing conditions (B; here only the anti-rBAT immunoprecipitations are presented). Representative experiments are shown. (A) For quantification, the rBATb band signal in the anti-b0,AT immunoprecipitation (rBATb) was multiplied by 100 and divided by the rBATc band signal in the anti-rBAT immunoprecipitation (rBATc). The results (mean ± SEM of at least n = 4 experiments for each rBAT species) are given as the percentage of total pulse-labeled rBATc co-precipitated with the anti-b0,AT antibody. (B) For quantification, the rBATb0,AT (~135 kDa) band signal was multiplied by 100 and divided by the total rBAT signal (rBATc plus rBATb0,AT bands) in the anti-rBAT immunoprecipitation. The results (mean ± SEM of at least n = 4 experiments for each rBAT species, with the exception of T216M, n = 1) are given as the percentage of total rBAT found in the rBATb0,AT band (assembled rBAT). See text for further explanations. Wild-type and mutant rBAT are abbreviated as in Figure 4.
facilitated by the calnexin chaperone system. Whether b0,þAT is mandatory for post-assembly folding of rBAT remains an open question. b0,þAT folding seems to be independent of rBAT, because it is functional in its absence (9). Our data are compatible with models proposed for other oligomeric protein complexes, such as potassium channels and acetylcholine receptors, where biogenesis proceeds through interspersed folding and assembly steps (25,26).

Biogenesis of type I cystinuria rBAT mutants

Wild-type biogenesis provides the framework to study type I cystinuria rBAT mutations. In Xenopus oocytes, the M467T, M467K and T216M mutants showed reduced transport activity (12,13). Amino acid uptake increased and even reached wild-type levels upon higher expression (12), raising doubts about the situation in vivo. Instead, the R365W mutant was not functional in HeLa cells [(8) and Fig. 3B]. Here we demonstrate that the mutants tested lack function in human cells and none of them acquires complex N-glycosylation (but see below). Therefore, the absence of transport activity is primarily due to strong trafficking defects.

The heterotetramer [rBAT-b0,þAT]2 is the structural unit of this transporter at the plasma membrane and the single heterodimer is the functional unit (10). The small, but significant, transport measured in the L89P mutant correlates with the few heterotetramers and mature L89P molecules detected. The even lower levels of M467T heterotetramers and mature molecules may be not enough to detect significant uptake (Figs 4B and 5). In fact, all the mutants tested fail, to different extents, to form stable heterotetramers. They are instead detected as aggregates in blue native gels (with the exception of M467T). Our solubilization conditions may allow resolution only of heterotetramers, but not heterodimers or monomers. Aggregation could be also due to the presence of high molecular weight complexes containing ER-resident chaperones or ERAD machinery (27,28). In any case, the aggregation suggests a folding and/or assembly defect that hinders formation of mutant heterotetramers. Dimerization of the heterodimer may be needed for ER-exit. Oligomerization can be one of the final quality control steps of the biogenesis of rBAT-b0,þAT, as shown for other proteins (29,30).

From the mutations studied here, L89P is in the transmembrane domain and the rest lie in the (b/α)8 TIM-barrel of the
extracellular domain ([20] and Fig. 3A). We do not know how these mutations affect the folding and/or assembly of rBAT. However, they do not lead to a new phenotype in the absence of b⁰⁺AT, since unassembled wild-type and mutant rBAT disappear with similar kinetics (Fig. 6). There are other examples of this behavior, as Kᵦ₆.2 mutations of Kᵥ₆.2 channels when expressed without the sulfonyleurea receptor (SUR) subunit (31). Only co-expression with b⁰⁺AT discriminates between wild-type rBAT and mutants. The mutants fail to exit the ER and are directed to disposal, strongly suggesting a defect in early biogenesis, like many other mutant proteins in the secretory pathway (17,32). Most of the L89P molecules do not stably assemble with b⁰⁺AT. L89 is the putative second residue of the transmembrane segment. The L89P mutant is fully N-glycosylated, suggesting a correct insertion in the membrane. Prolines may induce a kink in the transmembrane domain or directly interfere with interactions with b⁰⁺AT. The assembly defect of the L89P mutant agrees with the proposed dominant role of the N-terminal and transmembrane domains of rBAT in the association with b⁰⁺AT (33). Despite the assembly defect, some [L89P]rBAT-b⁰⁺AT heterodimers and heterotetramers form and are functional (Figs 3B, 5 and 9). This may be due to overexpression. However, the stability and functionality of the L89P heterodimers suggest that, in contrast to the TIM-barrel mutants (see below), the L89P mutant does not cause misfolding. The TIM-barrel mutants show a trafficking defect of different origin. They assemble efficiently with b⁰⁺AT, but are subsequently degraded, suggesting misfolding of the extracellular domain. It is interesting also to compare the ratio 135/~250 kDa bands between wild-type, L89P and M467T mutants in Figure 4C. This ratio is similar for the wild-type and the L89P mutant, but it clearly differs from that of the M467T mutant. This suggests that, once the heterodimer is formed, both the wild-type and the L89P mutant can readily form the heterotetramer. In contrast, for the M467T mutation, only a few heterodimers can oligomerize, arguing again for a folding defect. Therefore, at least two different classes of pathogenic rBAT mutants exist: assembly defective (L89P) and folding-defective (TIM-barrel mutants). At our level of analysis, these two classes are mutually exclusive, but lead to the same type I cystinuria phenotype. It is unlikely that the correlation between the position of the mutants within the rBAT protein and their molecular effects is just a mere coincidence.

Summary model

The data are consistent with a minimal working model for the biogenesis of the rBAT-b⁰⁺AT transporter. Fast interactions of the N-terminal and transmembrane domains of rBAT with folded b⁰⁺AT determine formation of the heterodimer, which is impaired in the L89P mutant. Assembly with b⁰⁺AT blocks rBAT degradation. These early steps do not require the calnexin chaperone system. After assembly, the rBAT extracellular domain folds within that chaperone system. Heterotetramerization proceeds immediately after completion of rBAT folding, or interspersed within the final folding steps. Only the heterotetramers exit the ER to the Golgi complex. Mutations of the extracellular domain of rBAT disrupt or delay the post-assembly folding of rBAT, hindering stable oligomerization and leading to its degradation.

MATERIALS AND METHODS

Reagents and antibodies

Reagents were purchased from Sigma unless otherwise indicated. Pro-mix l-[³⁵S] in vitro cell labeling mix (l-[³⁵S]Methionine and l-[³⁵S]Cysteine), D-MEM media without L-Methionine and L-Cystine, and Dialyzed FBS, were from GE Healthcare. Antibodies against the N-termini of human b⁰⁺AT and rBAT are described elsewhere (6,15).

cDNA constructs

The vectors for mammalian cell expression of human rBAT and b⁰⁺AT have been described elsewhere (15). The construction of the R365W, M467T and M467K rBAT mutants is detailed in (8,12). To express the M467T and M467K rBAT mutants in mammalian cells, the cDNAs in pSPORT were removed with EcoRI and XbaI and cloned into pCDNA3 cut with the same enzymes. The L89P and T216M mutants were obtained by site-directed mutagenesis (QuickChangeTM, Stratagene) of pCDNA3-rBAT, using the mutagenic oligonucleotides (only sense oligonucleotides are shown): 5’-ATACCTCGGGAGATCTCCCTCTGGCTCAGTG-3’, and 5’-CATACAAAAACACATGATGTAATACATTTGGTTTC-3’, respectively. All mutations were confirmed by DNA sequencing using the d-Rhodamine dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems). The sequence reactions were analysed with an Abi Prism 377 DNA Sequencer.

Cell culture and transfection

MDCK-IIJ and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal calf serum (heat-inactivated), 100 U/ml penicillin (Gibco) and 0.1 mg/ml streptomycin (Gibco), at 37°C in a humidified atmosphere containing 5% CO₂. Stably transfected MDCK-derived cell lines (9) were supplemented with the appropriate selection agent (400 μg/ml genetin and/or 100 μg/ml hygromycin B). Calcium phosphate transient transfection of HeLa cells was performed as described (12,15). The efficiency of transfection was above 70% in all experiments. For transient transfections, 10 cm diameter plates were incubated with a mixture of DNA containing 2 μg of pEGFP (green fluorescence protein; Clontech), 6 μg of pCDNA3-rBAT (wild-type or the different mutants) and 12 μg of pCDNA3-b⁰⁺AT as described (12,15). In these conditions, more than 80% of the labeled wild-type rBAT molecules in pulse-chase studies were assembled with b⁰⁺AT (data not shown). When rBAT or b⁰⁺AT were transfected alone, 12 or 6 μg of pCDNA3 were added, respectively.
Membrane preparation, SDS–PAGE, western blot and blue native gel electrophoresis

We have published detailed procedures for these methods elsewhere (6,10). Protein quantitation was performed with the BCA method (Pierce).

Transport measurements

Influx rates of 50 μM L-[3H]arginine (ARC) and 20 μM L-[35S]cysteine (Amersham) in transfected HeLa cells were performed as described (9,15).

Endoglycosidase H assay

The enzyme was obtained from New England Biolabs and was used following the manufacturer protocol.

Pulse-chase and immunoprecipitation protocols

Cells were transfected and seeded in 3.5 cm diameter plates at 60–70% confluence. The following day cells were incubated for 30 min in pre-warmed L-Methionine/L-Cystine free media containing 10% dialyzed FBS. Subsequently, cells were labeled for 15 (MDCK) or 30 min (HeLa) with a mixture of [35S]methionine/cysteine (120 μCi/ml) and, after removal of the labeling media, incubated with pre-warmed media supplemented with 5 mM unlabeled L-methionine/L-cysteine. At the indicated times, cells were washed twice with cold phosphate-buffered saline (PBS) and once with cold PBS containing 20 mM N-ethylmaleimide for 5 min. Cells were collected and lysed on a rotating wheel in 0.2 ml of NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 8.3) and twice with 40 mM HEPES, pH 8. Samples were run on SDS–PAGE under reducing or non-reducing conditions. Gels were stained with Coomassie brilliant blue to control for precipitating antibodies, dried and put on an intensifying screen for quantification with a Phosphoimager Typhoon 8600 (Molecular Dynamics).

Preliminary immunoprecipitation experiments were performed to control for the efficiency and the specificity of the immunoprecipitating antibodies. In our experimental cell system and pulse-chase conditions, the antibodies almost quantitatively immunoprecipitated all the pulse-labeled rBAT and b0,AT molecules (more than 80%; data not shown). Co-precipitation of rBAT and b0,AT with the anti-b0,AT antibody, and of b0,AT and rBAT with the anti-rBAT antibody did not occur when lysates from cells expressing rBAT alone and b0,AT alone were mixed before immunoprecipitation (Supplementary Material, Figure C).

Data analysis

The relative intensities of the labeled bands were determined using phosphorimaging, as follows: each band was outlined by a rectangle (as tightly-fitting to the band as possible) and a rectangle of identical size was drawn in the closest area without any band in the lane. The relative positions of band and background rectangles were maintained within the experiment and among similar experiments. The value for each rectangle was calculated using the Local Average Background Correction of the ImageQuant software. The final value of the band was the difference between the value of the rectangle band and the value of the rectangle background. The data were plotted as intensity values of the fraction remaining obtained by dividing by the zero time value, if not otherwise indicated. The decay data were fitted to an initial time in a Plateau followed by a single exponential function. The Plateau period and the half-life results are given as mean ± SEM. The maturation values were fitted to a four-parameter logistic equation (with bottom and top values set of 0 and 100, respectively).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Susanna Bial (Institute for Research in Biomedicine and CIBERER, Barcelona) for technical assistance and Robin Rycroft for editorial support.

Conflict of Interest statement. None declared.

FUNDING

This study was supported by the grants BFU2006-06788/BMC (to J.C.) and BFU2006-14600 (to M.P.); by the European Community project grant 502802 EUGINDAT, and by Generalitat de Catalunya grant 2005 SGR00947. P.B. was a recipient of a predoctoral fellowship from the Spanish Ministry of Education and Science, and is currently supported by the CIBERER (Centro de Investigación Biomédica en Red de Enfermedades Raras), Barcelona.
REFERENCES


