Relació estructura-funció en la família de transportadors d'aminoàcids heteromultimèrics. Identificació d'una nova família de transportadors lisosomals

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RELACIÓ ESTRUCTURA-FUNCIÓ EN LA FAMÍLIA DE TRANSPORTADORS D'AMINOÀCIDS HETEROMULTIMÈRICS IDENTIFICACIÓ D'UNA NOVA FAMÍLIA DE TRANSPORTADORS LISOSOMALS

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channel activity of the potassium independent amino acid exchange via ASCT1 (627) and via the Glu404Asp EAAT2 mutant (266) points to this intermediate. As discussed above for the neurotransmitter transporter superfamily, only a small fraction of the expressed GAT-1 and NET cotransporters behaves as ion channels in the presence of the ligands (GABA or norepinephrine and Na^+) (82, 160). This could be interpreted as an extremely low open probability of the channel (reviewed in Ref. 609) or as a low fraction of the transporters interacting with the endogenous silent channels. In our view, reconstitution of the sodium/amino acid-gated chloride channel activity in proteoliposomes containing expressed and purified EAAT or ASCT1 transporters may be the final demonstration of the intrinsic channel activity of these transporters. More simply, it will be interesting to know the relationship between amino acid transport rate and the chloride conductance at different levels of transporter expression.

For two members of the present superfamily, ASCT2 and ATB°, no chloride channel activity has been described, but to our knowledge, this has not been properly tested (269, 568). Very recently, the amino acid-evoked current associated with ATB° has been reported to reverse at −30 to −40 mV (270). At present, there is no clear explanation for this E_{rev} value in terms of an associated chloride conductance. Data from two different labs (Hediger's and Stoffel's groups) are in apparent contradiction with the chloride channel activity associated with EAAT1 and EAAT3. L-Glutamate-induced current due to rat EAAT1 expression in oocytes does not reverse up to +80 mV (289), and that due to rabbit and human EAAT3 approaches asymptotically zero at +50 mV (246, 249). Is this a consequence of a differential behavior of different species counterparts (E_{rev} for human EAAT1 is +9 mV, see Table 7; Ref. 582), or does it reflect differential experimental protocols? In the latter sense, it is worth mentioning that Hediger's group (246) did not show currents at depolarization potentials over +50 mV, and Amara and Kavanaugh and co-workers (582) measured an E_{rev} of +38 mV for human EAAT3.

Although the mechanism of the uncoupled chloride conductance during the transport cycle remains unknown, there is compelling evidence for a chloride conductance in parallel with sodium/glutamate cotransport in photoreceptors and bipolar cells, where the ligands increase the rate of opening of the chloride channel (137, 138, 175, 176, 295, 471, 531). The retinal EAAT5 transporter, with a putative synaptic localization (see sect. IIC1), may be responsible for this chloride conductance. Pre- and postsynaptic glutamate-gated chloride conductances may have physiological roles in vertebrate retina. *1*) The light response mediated by cones in depolarizing bipolar cells in the perch retina is due to the closing of a postsynaptic chloride conductance that has properties of the glutamate transporter (i.e., similar pharmacology and ionic dependence) (175). *2*) Presynaptically, the activation of a chloride conductance concomitant with glutamate transport would provide a potential mechanism to offset the depolarizing action of transmitter reuptake and reduce cell excitability. Thus, in salamander cone photoreceptors, a glutamate-evoked chloride conductance, with properties similar to the glutamate transporters, responds as an inhibitory signal (hyperpolarization) to the release of glutamate from the same cell (421). The physiological relevance of the thermodynamically uncoupled chloride conductance of the glutamate transporters in several cell types (retinal and pituitary cells) has recently been reviewed by Sonders and Amara (507).

6. *Protein structure*

The main common structural features (Figs. 5 and 6) among the mammalian members of this family are as follows: *1*) the absence of a cleavable signal sequence, suggesting a cytosolic localization of the NH_2 terminus; *2*) the absence of an SOB motif, identified in a variety of sodium/solute cotransporters; *3*) the presence of the sequence motif AA(I,V,L)FIAQ, probably located in a membrane-spanning domain, which is conserved throughout the evolutionary diversity of glutamate transporters from prokaryotes to mammals, and also in the zwitterionic amino acid transporters of this family; *4*) a higher level of conservation in the COOH-terminal half of the proteins which exceeds the level of conservation in the NH_2-terminal half by a factor of at least three; *5*) the presence of six highly conserved putative membrane-spanning domains in the NH_2-terminal half of the proteins; *6*) the presence of two cannonical sites for *N*-linked glycosylation on a presumably extracellular hydrophilic loop EL2 between TM domains III and IV; and *7*) a similar appearance of EAAT1–3 glutamate transporters (there is no available data for EAAT4 or the zwiterionic transporters) as broad electrophoretic bands of similar size (65–75 kDa) due to variable glycosylation (121, 298, 305, 461, 481).

Ever since the initial description of the first three members of this superfamily (EAAT1–3), the topology of these transporters in the plasma membrane has been controversial. Based on topology algorithms, Stoffel's lab (214, 518) for EAAT1 and ASCT1, Kanner's group (424) for EAAT2, and Hediger's lab (245) for EAAT3 agree on the presence of six classical α-helix TM domains in the first NH_2-terminal part of these proteins (see Fig. 5). Controversy appears in the COOH-terminal part, and this is an important issue since homology in this part of these proteins is very high (see Fig. 5) and several amino acid residues critical for transport activity have been described within this region (see sect. IIC7). There is a long hydrophobic stretch of amino acid residues with no clear tendency to show α-helix structures toward this end in any of the members of this superfamily (dashed line in

Fig. 5). Kanner's group (424) suggested the presence of two additional classical TM domains within this protein region for EAAT2, whereas Kanai and Hediger (245) included four additional classical TM domains. In contrast, Stoffel and co-workers (214, 518) suggested six classical TM domains and four hydrophobic β-sheets crossing the plasma membrane. In all cases, these models positioned the COOH terminus inside the cell. Very recently, two studies offered experimental evidence on the topology of EAAT1 (585) and the glutamate transporter GltT from *Bacillus stearothermophilus*, a prokaryote-related member of this superfamily (498). After these studies, the controversy remains. Stoffel and co-workers (214, 518) applied "reporter glycosylation scanning" (i.e., chimeras containing EAAT1 domains and an *N*-glycosylated reporter peptide), expressing chimeras in oocytes, to support a model for EAAT1 (GLAST-1) with 10 TM domains (see Fig. 6*B*). This model has NH_2 and COOH termini intracellular, six NH_2-terminal hydrophobic TM α-helices, and four COOH-terminal short hybdrophobic domains spanning the membrane bilayer as β-sheets. The six NH_2-terminal hydrophobic TM α-helices correspond to those suggested for all these transporters. Site-directed antibodies used in immunofluorescence studies with permeabilized cells confirmed the intracellular location of the NH_2 terminus of EAAT1 (585) and the COOH terminus of rat EAAT1 and EAAT2 (298), suggesting an even number of TM domains. Slotboom et al. (498) used alkaline phosphatase (PhoA) gene fusion technique (i.e., scanning chimeras containing GltT domains and alkaline phosphatase) to study the controversial COOH-terminal part of the prokaryotic GltT transporter. Extrapolation of their results to the eukaryotic members of the superfamily is warranted by the fact that all these transporters showed a very similar hydropathy profile in the COOH-terminal half of the protein (i.e., fragment comprised between amino acid residues 400 and 550 of the multialignment of EEAT1–4 isoforms and GltT from *E. coli*, *Bacillus subtillis*, and *B. stearothermophilus*) (498). The GltT topology model proposed the presence of four additional TM α-helices in the COOH-terminal half of the protein (see Fig. 6*A*) (498).

Both strategies have been used to study the membrane topology of several proteins and are considered a good technical standard. In our view, however, both studies (498, 585) lack clarity, contain inconsistent data, and in addition used an objective experimental strategy to favor previous subjective topology models. Konings' group (498) based their model on the expression of the PhoA activity toward the periplasmic space if the particular chimera positioned PhoA extracellularly. The expression of low PhoA activity is interpreted as an intracellular location of the Phoa domain in the chimeras. In all cases, they demonstrate that low PhoA is not caused by a low expression level of the particular chimera, but they do not attempt to demonstrate that these chimeras are expressed in the plasma membrane and not as inclusion bodies. In addition, the model proposed is very rigid and needs three very small loops (EL4, IL4, and EL5) which is difficult to apply to mammalian members of this superfamily because of the presence of charged residues at the extremes of TM domains VII, IX, and X (see Fig. 6*A*). Stoffel's model is based on very clear data for the first NH_2-terminal part of the protein (TM domains I-VI); all the chimeras constructed showed glycosylation of the reporter protein domains (they used an endogenous *N*-glycosylated domain of EAAT1 that corresponds to a large portion of the EL2 loop) when connected to loops EL1, EL2, and EL3. Conversely, the reporter protein domain is not glycosylated when located in loops IL1, IL2, and IL3 (see Fig. 6, *A* and *B*). This part of the model is confirmed by the following evidence: *1*) the NH_2 terminus is intracellular since EAAT1-specific antibody immunofluorescence signal is only obtained with permeabilized cells (298, 585). *2*) For EAAT1, Stoffel's group showed by peptide sequencing, endoglycosidase F treatment, and site-directed mutagenesis that Asn-206 and Asn-216 residues are the only ones in the whole protein sequence that are *N*-glycosylated (116, 481); these residues are located in the extracellular loop EL2. *3*) The Ser-113 residue of the glutamate transporter EAAT2 is phosphorylated in vivo by protein kinase C (83); this agrees with the intracellular location of the IL1 loop (see Fig. 6*A*).

In contrast, Stoffel's model of the topology for the COOH-terminal part of EAAT1 (585) is based on data that appear to be inconsistent. The "reporter glycosylation scanning" data obtained with chimeras constructed with residues located in the proposed intracellular loops IL3 and IL4 and the COOH-terminal domain are clearly consistent with the model, but those with residues located in the proposed extracellular loops EL4 and EL5 are controversial. *1*) The latter chimeras produce only ~50% of the protein with the reporter domain glycosylated. In addition, the fusion protein of the reporter glycosylation domain at a residue located in the proposed EL4 loop is not glycosylated at all in the reporter domain when expressed in oocytes. To explain these results, the authors need to invoke reorientation or steric hindrance for the translocation of the COOH-terminal reporter domain to the lumen of the endoplasmic reticulum because of the moderate size and hydrophobicity of TM domains VII and IX acting as anchoring sequences (see Fig. 6*B*). *2*) A new *N*-glycosylation site produced by site-directed mutagenesis in the center of the proposed extracellular loop EL5 is not glycosylated when expressed either in oocytes or in a translation system in vitro (585). *3*) Three GltT-PhoA fusion proteins in amino acid residues within GltT protein regions that are homologous to the extracellular loops EL4 and EL5, proposed by Stoffel's group, gave rise to a very low periplasmic PhoA activity (498).

Finally, it is interesting to notice that the two groups gave differing interpretations to results that are consistent with each other. For instance, the higly conserved motif AA(I,V,L)FIAQ is placed in Stoffel's β-sheet TM domain IX and in Konings' α-helix TM domain VII. This is based on *1*) a nonglycosylated reporter domain and a low periplasmic PhoA activity when the reporter domain is fused to EAAT1 Glu-406 residue (498) or to its homologous residue in GltT (585), *2*) a low periplasmic PhoA activity when the fusion involves the GltT residue corresponding to EAAT1 Ile-413, and *3*) a partial glycosylated reporter domain and a high periplasmic PhoA activity when the reporter domain is fused to EAAT1 Gln-425 residue (498) or to the GltT residues that are homologous to the EAAT1 421 and 426 residues (585). This is used by Stoffel's group to propose a β-sheet (residues 407–416 of EAAT1) and by Konings' group to propose an α-helix (corresponding to residues 410–427 in the EAAT1 sequence) spanning the plasma membrane, respectively. Stoffel's group argues that in their studies, most probably, there is no room for an α-helix between the EAAT1 residues 407 and 425. Konings' group argues that detailed studies with the lactose permease LacY and the melibiose carrier MelB from *E. coli* have demonstrated that the NH_2-terminal half of an outgoing TM helix is sufficient to export the PhoA domain fused to a membrane protein, whereas the NH_2-terminal half of an ingoing TM helix is sufficient to prevent the export of the PhoA moiety to the periplasm (77, 428).

It is patently clear that the topology of these transporters stands in need of further research. The two models are quite different and could be tested with alternative strategies. Studies with limited proteolysis and peptide-directed specific antibodies could be informative. Notice that the exposed loops in the COOH-terminal half of these transporters in Stoffel's model are very conspicuous, whereas in Konings' model they are very limited (see Fig. 6, *A* and *B*). Alternatively, vectorial labeling of cystine residues reintroduced in the borders of the proposed TM domains of a cystineless transporter may also help. The establisment of the membrane topology of the COOH-terminal half of these transporters is an important issue because of the high level of homology in this region for all the members of this superfamily, and because several studies have shown that residues within this region are critical for substrate binding or translocation (see sect. IID). Finally, to date, β-sheet TM domains have been proposed for several eukaryotic and prokaryotic membrane proteins like the acetylcholine receptor, the VDAC ion channel, and the lac permease (6, 52, 438), but they have only been demonstrated by X-ray analysis of the bacterial porins (118).

7. *Structure-function relationship*

Our knowledge of the structure-function relationship is based on studies with glutamate transporters of this superfamily, using chimeric proteins (between the human homologs of EAAT1 and EAAT2; Ref. 571), site-directed mutagenesis (for rat EAAT1 and EAAT2 transporters; Refs. 83, 115, 116, 426, 630), on the conformational changes associated with the transport step (for rat EAAT2; Refs. 179, 266, 583), or on the homomultimerization of these transporters (for rat EAAT1–3; Ref. 199). Part of these studies has been recently reviewed (254).

In an elegant study, Kavanaugh, Amara, and co-workers (571) prepared a human EAAT1-2-1 chimera, in which 76 amino acid residues of EAAT2, comprising most of the highly conserved long hydrophobic stretch (see Figs. 5 and 6*A*) were exchanged within the EAAT1 sequence. This EAAT2 protein segment, in which only 18 amino acid residues are different in the two isoforms, corresponds to part of the IL3 loop, TM domain VII, EL4 loop, and most of TM domain VIII in the 10 α-helix TM domain model (498) (see Fig. 6*A*). This segment in the EAAT1-2-1 chimera, when expressed in oocytes, confers sensitivity to inhibition by the nontransported competitive analog KA to both glutamate transport (K_i in the micromolar range, characteristic of EAAT2 isoform) and to the uncoupled glutamate-independent sodium leak current, characteristic of EAAT1 isoform. Kinetic analyses are compatible with inhibition of both processes by binding of KA to a single site (571). Interestingly, other transport characteristics of EAAT1 isoform are unchanged in the EAAT1-2-1 chimera, like the apparent affinity for the substrate analog SOS (see Table 7) and the E_{rev} of the glutamate- and sodium-induced current [E_{rev} = ~10 mV for EAAT1-2-1 (461); compare with the E_{rev} for EAAT1 and EAAT2 in Table 7]. This suggests that the kinetic parameters for substrate translocation and the uncoupled chloride channel activity are determined by the EAAT1-derived sequences.

Most of the amino acid residues critical for the transport function of EAAT1 and EAAT2 transporters revealed by site-directed mutagenesis are within or near this highly conserved COOH-terminal part of glutamate transporters, which confers sensitivity to KA. Conradt and Stoffel (115) analyzed the effect of substitution of three positively charged residues (Arg-122, Arg-280, and Arg-479) and one polar residue (Tyr-405) in rat EAAT1 transporter, which are conserved in the glutamate transporters and substituted by apolar residues in the zwitterionic transporters (see Fig. 5). Mutations Arg122Ile and Arg280Val (and both together) reduce the apparent affinity for L-aspartate without affecting the kinetic parameters for L-glutamate transport, and mutants Tyr405Phe and Arg479Thr, within the highly conserved COOH-terminal part of these transporters, completely abolished the intrinsic EAAT1 transport activity (see below) (115). Kanner and co-workers (426) analyzed the role in transport of five negatively charged residues of rat EAAT2 located in hydrophobic surroundings and highly conserved within the glutamate transporter family (Asp-398, Glu404, Glu461, Asp462, and Asp-470). Only three of these residues (Asp-398, Glu-404, and Asp-470; indicated in Fig. 6*A*) are critical for intrinsic transport activity, which could not be explained by protein expression level or defects in trafficking to the plasma membrane. Interestingly, defective transport cannot be attributed to the mere requirement of a negative charge at this residues (i.e., transport is also affected by substitution of the corresponding charged residue, either Glu or Asp) (426).

The rat EAAT2 Glu404Asp (this residue is located in the TM domain VII of the 10 α-helix TM domain model; see Fig. 6*A*) mutant has been revealed as a powerful tool to address structure-function studies. This mutant conserves most of D/L-aspartate transport (~80%), but only a small part of L-glutamate transport (<20%) (426). The defective Glu404Asp L-glutamate transport is not because of defective binding (i.e., high-affinity L-glutamate inhibition of D/L-asparatate transport is conserved). This allows the authors to propose that the Glu-404 (conserved in all the glutamate transporter isoforms but absent from the zwitterionic transporters of this transporter family; see Fig. 5) determines the amino acid substrate permeation pathway of the glutamate transporters. The Glu-404 residue in EAAT2 together with residues Arg-122 and Arg-280 within the NH_2-terminal part of EAAT1 (see above) are those already identified, which are involved in substrate specificity discrimination (115, 426). Very recent data obtained from collaboration between Kanner's and Kavanaugh's groups (266) showed that Glu-404 residue also influences the potassium transport coupling (either binding or translocation), and the rat mutant Glu404Asp EAAT2 catalyzes obligatory exchange of coupled amino acid substrate and sodium through the plasma membrane. *1*) The sodium/D-aspartate transport via Glu404Asp EAAT2 is electroneutral in oocytes, *2*) external potassium does not reverse transport through Glu404Asp in oocytes, and *3*) in the liposome, reconstituted mutant influx and efflux of radiolabeled D-aspartate are dependent on *trans*-sodium/amino acid substrate but not on *trans*-potassium. In contrast, wild-type EAAT2 catalyzes *trans*-potassium-dependent influx and efflux of amino acid substrate in the presence of sodium. This is a consequence of the countertransport of potassium in the transport mechanism of these glutamate transporters (255, 529; see sect. II*C4*). Because the Glu404Asp mutant is locked in an exchange mode of transport, either potassium binding or permeation, or sodium binding is affected (i.e., a significant increase in sodium binding will displace potassium binding and force the transporter toward amino acid/sodium exchange) (266). The former possibility seems to be true because apparent sodium affinity is unchanged in the Glu404Asp mutant. From all this, it is not surprising that the sodium-dependent transient currents produced by voltage jumps in human and rat EAAT2 expressing oocytes (266, 583) are hardly affected by the mutant (266). These transient currents are thought to be a reflection of either sodium binding or a subsequent conformational change of the transporter. In agreement with the EAAT1–2-1 chimera studies discussed above (571), the Glu404Asp mutant, located within the KA-binding/sensitive determining domain, does not affect the uncoupled amino acid substrate/sodium-induced chloride channel activity of the transporter (266), suggesting that this protein region does not influence this channel activity. It is remarkable that Glu-404 residue is in between two other conserved residues in all glutamate transporters of the family, comprising the sequence Tyr-Glu-Ala (see Fig. 5). Interestingly, the Glu404Asp homologous mutation in human EAAT3 also abolishes potassium-dependent efflux (266). In contrast, the zwitterionic amino acid transporters of this family have the conserved sequence Phe-Gln-Cys (see Fig. 5). Interestingly, mutation of this conserved Tyr residue to Phe (as in the zwitterionic transporters of this family) in rat EAAT1 (Tyr405Phe) abolished the intrinsic glutamate transport activity (115), and in rat EAAT2 (Tyr403Phe) abolished interaction with potassium, and resulted in an increased sodium affinity (629a). Very recently, Zerangue and Kavanaugh (627) offered evidence that ASCT1 transporter has an electroneutral exchange mode of transport for the amino acid substrate and sodium through the membrane; this mechanism of transport has also been suggested for ATB° (162). It is therefore tempting to speculate that Glu-404 within these residues (located in the VII TM domain in the 10 α-helix topology model, see Fig. 6*A*) confers coupled cotransport of sodium and countertransport of potassium, whereas its lack determines an exchange mode of transport coupled with sodium. Unfortunately, for human ASCT2 transporter, which also contains the conserved sequence Phe-Gln-Cys, the mechanism of transport and potassium dependence has not been addressed in depth. In summary, the hydrophobic, topologically controversial, and highly conserved domain located toward the COOH terminus of the glutamate transporters is involved in kainate binding and amino acid and ion (potassium coupling) permeation pathways.

Several residues within the NH_2-terminal part of these transporters have been shown to be involved in their transport activity or expression (83, 116, 630), in addition to the above-mentioned EAAT1 Arg-122 and Arg-280 residues (115). Stoffel and co-workers (116) demonstrated that the deglycosylated rat EAAT1 (*N*-glycosylation occurs in 2 canonical sites within the loop EL2; see Fig. 6*A*) is fully active, and none of its kinetic parameters is affected. Kanner and co-workers (630) examined the effect of substitution of the only two positively charged residues (Lys-298 and His-326 in EAAT2; see Figs. 5 and 6*A*) conserved in all members of this transporter family and located within putative α-helix TM domains (TM domains V and VI in the 10 α-helix TM domain topology model; see Fig. 6*A*). Replacement of these residues by small hydrophilic or positively charged amino acids produces in Lys-298 mutants a partial plasma membrane targeting defect and partial intrinsic transport defect of EAAT2; His-326 mutants have an almost complete impairment of their intrinsic transport activity without a trafficking defect toward the plasma membrane (630). Zhang et al. (630) suggested two possible roles for the conserved His-326 residue. In analogy with structure-function studies of the proton-coupled lactose permease of *E. coli*, His-326 could either form ion pairs with negatively charged residues within TM domains that stabilize the transporter (133, 283, 466) or participate in the mechanism of hydrogen transport (241, 406). The same group (426) examined the first possibility by constructing double mutants with three conserved negatively charged residues that are critical for the transport activity of EAAT2 (Asp-398, Glu-404, Asp-470; see above). None of the double mutants (i.e., His326Asn with Asp398Asn, Glu404Asn, or Asp470Asn) regained activity, and therefore, there is no evidence for these ionic pairs within EAAT2 transporter.

A very interesting line of research is the stimulation of EAAT2 by protein kinase C. In loop IL1 of EAAT1–4 glutamate transporters isoforms, there is a protein kinase C canonical site (see Fig. 6*A*). Giménez and co-workers (84) showed that phorbol esters increased V_{max} of sodium-glutamate cotransport in cultured glial cells. Later, these authors in collaboration with Kanner's group (83) demonstrated the following: *1*) protein kinase C phosphorylates, in serine residues, pig brain purified glutamate transporter; *2*) phorbol esters increase in parallel glutamate transport activity (2-fold) and phosphorylation of immunoprecipitated EAAT2 in C6 glial cells; and *3*) rat EAAT2 transfected in HeLa cells is stimulated by phorbol esters, and mutation of Ser-113 to Asn abolished this stimulation without affecting transport activity expression. This is the first direct demonstration of regulation of a neurotransmitter or amino acid transporter by phosphorylation. The nature of the upstream event that stimulates glutamate transport via EAAT2 through protein kinase C is at present unknown. The authors hypothesize that elevation of the extracellular glutamate concentration would stimulate NMDA receptors in astrocytic processes, resulting in activation of the phosphatidylinositol cycle and protein kinase C activation, and therefore in a more efficient clearance of the extracellular glutamate. To our knowledge, neither this hypothesis nor the mechanism of EAAT2 stimulation has been addressed experimentally.

Conformational changes of EAAT2 have been revealed through its transport cycle (179, 266, 583). Sodium-dependent transient currents of the expressed EAAT2 transporter suggested conformational changes associated with binding of sodium (266, 583). More directly, limited proteolysis studies demonstrated conformational changes of purified EAAT2 associated with glutamate and sodium, or potassium binding; these studies suggest that EAAT2 transporter has at least two conformation states and that the transition between them is associated with the transport step (179).

Finally, EAAT1–3 glutamate transporters form homomultimers (dimers and trimers), as revealed by chemical cross-linking in intact brain membranes and solubilized transporters, or after reconstitution in liposomes (199). The original EAAT2 purification studies by Kanner and co-workers (120, 121) revealed that the monomeric 73-kDa band of the transporter correlated with glutamate transport activity. In addition, it is interesting that the fully deglycosylated EAAT1, obtained either by deletion of the two glycosylation sites or after endoglycosidase F treatment, does not homodimerize in electrophoretic gels, and it is fully active (116). These data suggest that dimerization of glutamate transporters does not affect their transport activity. In contrast, radiation inactivation studies suggest that the minimal funtional unit corresponds to an oligomer of the rat EAAT2 transporter (199). It is therefore clear that further research is needed on this issue.

8. *Physiological role of the glutamate superfamily transporters*

It is believed that the transporters in this superfamily have a role both in the termination of transcription in the synapsis and also in the supply of nutrients to brain and peripheral tissues (205, 244, 247, 252, 269, 568, 627). The overall process of synaptic transmission, except for acetylcholine, is terminated by high-affinity sodium-dependent transport of neurotransmitters (e.g., GABA, L-glutamate, glycine, dopamine, serotonin, and norepinephrine; see reviews in Refs. 205, 252, 253, 394). The concentration of L-glutamate, the predominant excitatory neurotransmitter of the mammalian central nervous system, is typically four orders of magnitude higher in the nerve terminals than in the cleft (estimations of 10 mM in neurons, low millimolar range in glial cells and submicromolar to low micromolar range in the glial extracellular fluid; Refs. 40, 74, 262, 458); therefore, energy input is required. The Na^+-K^+-ATPase generates an inwardly directed electrochemichal sodium gradient that drives uphill the the sodium- and potassium-coupled glutamate transport in neurons and glial cells (210, 262, 394). This role of glial and neuronal glutamate transport, in maintaining a low extracellular neurotransmitter concentration (<1 μM), has been postulated to be critical to protect against exocitotoxic cell damage (63). Glutamate transport blockers, which are nonselective for isoforms and the different transport activities detected in brain (454), raise extracellular glutamate, alter postsynaptic potentials, and result in neurotoxicity both in vitro (33, 226, 359, 453, 460, 470) and in vivo (326, 402, 403). This effect is blocked by non-NMDA glutamate receptor antagonists (459, 460) and is most probably because of the excessive calcium influx through NMDA receptor channels (for review, see Refs. 205, 252, 394).

Knockout studies of EAAT glutamate transporter isoforms (416, 458, 544) revealed that the glial transporters (EAAT2 and EAAT1) rather than the neuronal transporter EAAT3 control the extracellular glutamate levels in brain. Very recently, studies on the null knockout mice for EAAT2 and EAAT3 have been published (416, 544). To our knowledge, the null knockout EAAT1 mouse has not been reported. Tanaka et al. (544) studied the knockout of the widely distributed astrocytic glutamate transporter EAAT2 (also named GLT-1). These mice show lethal spontaneous epileptic seizures, selective neuronal degeneration in hippocampus, and increased susceptibility to acute cortical injury. In these mice, the estimated peak concentration and time course of free glutamate in the synaptic cleft is elevated. This indicates that glial EAAT2 is an important determinant of the clearance of free glutamate from the synaptic cleft. Thus, in the absence of EAAT2 transport activity, glutamate levels rise enough to cause epilepsy and cell death. In contrast to this, null knockout EAAT3 mice, obtained by Stoffel and co-workers (416) show, in addition to the renal phenotype (see below), a nonconspicuous brain phenotype, only characterized by reduced locomotor activity. Rothstein and Kuncl (458) addressed the contribution of the three EAAT1–3 isoforms described in rat to the maintenance of global extracellular glutamate concentrations in the cerebrospinal fluid, as well as the histological and behavioral consequences of their specific partial knockouts (in vitro and in vivo intraventricular phosphorothioate antisense administration). At present, the cerebellar EAAT4 isoform, described in humans (63) and suspected to maintain extracellular glutamate concentrations below excitotoxic levels, has not been isolated from rat tissues. The partial knockout of EAAT1–3 isoforms (458) showed that both glial transporters (EAAT1–2) contribute largely to the maintenance of the tonic cerebrospinal glutamate concentration and that the impact of the EAAT2 isoform was more conspicuous. In contrast, the contribution of the neuronal EAAT3 is negligible. In parallel, the EAAT isoform-specific partial knockout showed that glial glutamate transport sites (EAAT1–2) are more conspicuous than the EAAT3 transport sites (binding of radiolabeled D-aspartate inhibitable by DL-threo-β-hydroxyaspartate to membranes) in the two structures studied, striatum and hippocampus. This is in agreement with greater expression of these transporters in comparison with the neuronal isoform. The EAAT2 isoform is present in astrocytes throughout the brain and spinal cord (121, 305, 461). In comparison with EAAT isoforms 1–3, partial knockout of EAAT2 resulted in the largest decrease in the glutamate transporter sites in striatum and hippocampus (458), purification through functional reconstitution from rat brain resulted in the identification of EAAT2 (120, 121, 424), and immunoprecipitation studies suggest that EAAT2 isoform is the most prevalent glutamate transporter in brain (199). The brain phenotype of knockout EAAT2 mice and the very low residual glutamate transport activity in cortical crude synaptosomes from these mice (544) have confirmed this suggestion. In agreement with this, in the sporadic form of amyotrophic lateral sclerosis (ALS), there is a specific marked reduction (up to 95%) of the expression of EAAT2 in the motor cortex and the spinal cord (463). In parallel, there is also a marked decrease in the V_{max} of high-affinity glutamate uptake in synaptosomes from those brain structures and an increased cerebrospinal fluid concentration of L-glutamate and L-aspartate in ALS patients (462) (see sect. III).

The above-mentioned knockout studies showed that the glial transporters (EAAT1–2) are the more conspicuous transporters in brain, and they have a crucial role in the maintenance of the tonic extracellular glutamate concentration. Thus the tonic increase in extracellular glutamate because of EAAT1–2-specific partial knockouts explains the progressive paralysis and neurodegeneration in these rats (458). As discussed by Rothstein and Kuncl (458), glial cells have a considerably lower estimated intracellular glutamate concentration (in the micromolar range) than neurons, which suggests that EAAT1–2 transporters operate far from equilibrium, most probably due to the rapid metabolization to glutamine by glutamine synthetase, which is absent in neurons. Therefore, in addition to its larger expression, the operation of EAAT1–2 far from equilibrium may explain why the phenotype obtained after total or partial knockout of EAAT1–2 is clearer than that given by knockout of the EAAT3 isoform (416, 458, 544). It is worth mentioning that the proposed lack of role for the EAAT3 transporter in the tonic extracellular glutamate levels (458) does not imply that this transporter has no role in excitotoxicity. Reversal of glutamate transport has been proposed as a mechanism of excitotoxicity under conditions of energy failure, as in cerebral ischemia (hypoxia, stroke; Refs. 23, 246, 394). The nonlimiting transport flux via EAAT3 running in reverse could produce a significant local increase in the extracellular concentration of glutamate (i.e., >350 μM as demonstrated in salamander retinal glia cells and EAAT3 expressed in oocytes; Refs. 63, 246).

Null knockout EAAT2 mice (544) confirmed the hypothesis (394) that astroglial glutamate transporters contribute to the reuptake/termination of the glutamate synaptic transmission. Thus total loss of EAAT2 transport activity (i.e., as in the homozygous null knockout mice), but not its partial loss (i.e., as in the heterozygous null knockout mice or in chronic antisense administration to rat brain), produces epilepsy (458, 544) and increases the time course of free glutamate in the synaptic cleft (544). On the other hand, Amara and Kavanaugh and co-workers

(583) and Kanner and co-workers (120) estimated a transport cycling time of 70–700 ms for human EAAT2 expressed in oocytes and rat purified EAAT2. This is significantly slower than the 1- to 2-ms time constant of glutamate decay estimated in hippocampal synapses (104, 112). This suggests that glutamate diffusion and "fast" binding to EAAT2 transporters (583) dominates the synaptic concentration decay kinetics.

What is the role of the neuronal EAAT3 glutamate transporter in the termination of synapsis? In contrast to the glial glutamate transporters, the partial knockout of EAAT3 protein produced no changes in extracellular glutamate and only mild neurotoxicity and motor phenotype, but consistent epileptic seizures (458). It is believed, although this is not completely clear (628), that the neuronal glutamate carrier EAAT3 operates at or near equilibrium, and its expression is confined to pre- and postsynaptic elements (461). It is somehow expected that the partial reduction of a plasma membrane transport activity, which is working at equilibrium (i.e., flux through this transporter does not limit the overall metabolic handling of the neuronal glutamate) and confined to the synapsis, has little or no impact in the global extracellular glutamate concentration, as the partial knockout studies showed (458). The epileptic phenotype of the partial knockout of EAAT3 suggests that a moderate rise in the intrasynaptic glutamate concentration, without global concentration changes, may cause persistent depolarization or alteration of the presynaptic transmitter release (458). In addition to glutamatergic neurons, EAAT3 has also been located in inhibitory GABAergic neurons, and because glutamate is a precursor for GABA synthesis, transport via EAAT3 could have a role in GABA neurotransmission (205, 461). Superstimulation of excitatory glutamatergic neurons and blockade of inhibitory GABAergic neurons are known to produce epilepsy (157). Unfortunately, the null knockout EAAT3 mouse model only reproduces the locomotor, but not the epileptic, phenotype (416) of the antisense-depleted EAAT3 rat model (458). It seems that overexpression of the glial glutamate transporters does not occur in the knockout EAAT3 mice (517). These apparently contradictory results raise doubts as to the contribution of the EAAT3 transporter to the termination of glutamate synapsis.

Expression of the glutamate transporter EAAT3 in GABAergic neurons (205, 461), its strong transcript expression in the small intestine, and at a lower levels in kidney, liver, and heart (245), suggest a metabolic role for this transporter. In epithelial cells, system X^-_{AG} has been described mainly in the apical plasma membrane (483, 513), and therefore, it is believed that EAAT3 mediates net absorption of glutamate and aspartate in kidney and intestine (205). This role is demonstrated by the dicarboxylic aminoaciduria developed by null knockout EAAT3 mice (416). In addition, this suggests that mutations in EAAT3 may cause dicarboxylic aminoaciduria, an inherited disease due to defective glutamate transport in kidney and intestine (see sect. III). Recent results showed that system X^-_{AG} transport activity is increased (V_{max} effect) by hypertonic stress in the bovine renal cell line NBL-1 (148). Concomitantly, the EAAT3 transcript levels increase, suggesting that this glutamate isoform is responsible for system X^-_{AG} in these cells, and indicating a direct effect of hypertonic stress in the expression of this transporter isoform (148). Whether hypertonic stress increases EAAT3 gene transcription and/or mRNA stability in these cells has not been reported. This regulation of EAAT3 might be due to a role of this glutamate transporter in glutamine metabolism and pH regulation in renal cells.

The physiological role of ASCT1–2 and ATB° zwitterionic transporters is at present unclear. It is necessary to clarify whether mouse ASCT2 corresponds to human ATB°, or whether they code for different transport activities. In addition, a more precise description of the mechanism of transport for these transporters is needed. If, finally, ASCT transporters mediate concentrative uptake of their substrate coupled with the transmembrane gradient of sodium and amino acids, ASCT1–2 might be assigned as variants of the almost ubiquitious ASC system. It will be also necessary to explain the molecular basis of the hepatic ASC system, which as discussed above does not appear to be represented by either one of these ASCT isoforms. Studies with anti-ASCT1–2 antibodies and knockout experiments will be needed to estimate the role of these transporters in the macroscopic flux of amino acids in cells expressing them.

The ATB° transporter (269) might correspond to system B°, the most conspicuous sodium-dependent uptake system for zwitterionic amino acids. This apical transport system is thought to play a major role in reabsorption of zwitterionic amino acids in kidney and small intestine (see Refs. 483, 513). Elucidation of the transport mechanism and demonstration of the apical localization of ATB° in epithelial cells may reaffirm the assignation of ATB° as system B° transporter. Finally, demonstration of the responsibility of ATB° in Hartnup disease, an inherited neutral hyperaminoaciduria (see sect. III), may confirm that ATB° plays a role in amino acid nutrition and renal reabsorption and system B° activity. In contrast to this view, the recent description of an amino acid exchange mechanism of transport for ATB° (162) questions the participation of this transporter in the active renal and intestinal absorption of neutral amino acids and its role in Hartnup disease.

D. Putative Subunits of Sodium-Independent Cationic and Zwitterionic Amino Acid Transporters

The last protein family related to plasma membrane amino acid transport in mammals is composed by the

TABLE 8. *Putative subunits of sodium-independent cationic and zwitterionic amino acid transporters*

Putative Transporter Subunits (Gene Name)	Accession Numbers (Origin of Human Clones)	Origin of First Clones and Other Names	Human Chromosome	Human Protein Amino Acid Length	Other Clones
rBAT (SLC3A1)	L11696 (kidney) (45) M95548, M95298 (kidney) (297) D82326 (kidney) (367)	Rat kidney (NAAT, NBAT) (549) Rabbit kidney (rBAT) (46) Rat kidney (D2) (598)	2p16.3-21 (79, 616, 629)	685	rBAT long transcript (rabbit kidney) (344) rBAT short transcript (rabbit kidney and OK cells) (110, 374)
4F2hc (SLC3A2)	J02939, M17430, M18811, M21904 (lymphocytes) (327, 435, 554) J03569 (fibroblasts) (327)	Human lymphocytes (4F2hc) (435, 553) (corresponds to CD98)	11q12-13 (174)	529	Mouse brain (413), hematopoietic stem cells (592), and rat glioma cells (69)

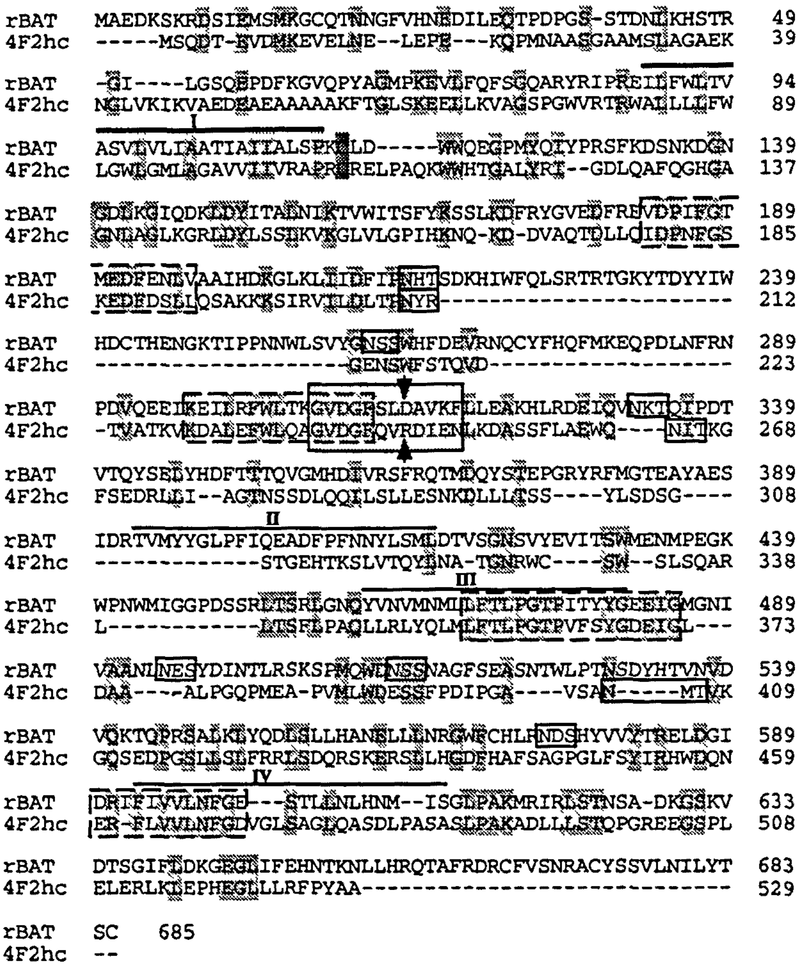
Accession numbers for human rBAT and 4F2hc cDNAs are indicated. Alternative names for other cDNA are shown. Reference numbers are given in parentheses.

protein rBAT and the heavy chain of the cell surface antigen 4F2 (4F2hc) (see Table 8). Amino acid transport expression in *Xenopus* oocytes was used independently in three labs to clone cDNA of a putative transporter from rabbit, rat, and human kidney; homology between these proteins is very high (~85% identity) (45, 46, 110, 297, 549, 598). A partial rBAT cDNA sequence from OK cells has also been reported (374). The three labs gave different names to these cDNA: NBAT (Udenfriend and Tate's group), D2 (Hediger's group), and rBAT (ourselves). For clarity, the name rBAT will be used for all these cDNA and proteins in this review. The cDNA of human 4F2hc was cloned using a monoclonal antibody designed against a cell surface antigen from lymphoblastoid cells (327, 435, 553), and its mouse counterpart was identified by homology (413). The biological role of this antigen was unknown. The deduced rBAT protein amino acid sequences have ~30% identity (~50% similarity) with the heavy chain of the cell surface antigens 4F2 (4F2hc) (69, 413, 435, 553). Figure 7 shows the sequence homology between the human rBAT and 4F2hc proteins, and in Figure 8, the amino acid residues conserved in all rBAT and 4F2hc proteins known are indicated. Consistent with rBAT and 4F2hc being members of the same family, within the open reading frame of human rBAT and 4F2hc, introns 1 and 2 have identical locations, intron 3 in 4F2hc corresponds to intron 4 in rBAT, and intron 8 in 4F2hc to intron 9 in rBAT (174, 434, 431) (see Fig. 8). Given the homology between rBAT and 4F2hc, cRNA from 4F2hc was tested in oocytes for expression of amino acid transport activity. Expression of 4F2hc resulted in an amino acid transport activity (system y^+L- like) different from that elicited by rBAT (system $b^{0,+}$-like) (42, 599) (see Table 9). Interestingly, expression cloning in oocytes after a zwitterionic amino acid transport signal (68) resulted in the isolation of rat 4F2hc (named lLAT in this study for linked to L amino acid transport) (69). It is worth mentioning that rat and mouse 4F2hc proteins are very similar (91% amino acid sequence identity), whereas the human protein is only 76% identical to the rat and mouse proteins (69). More recently, using an antibody that induces apoptosis in hematopoietic progenitor cells and homotypic aggregation of lymphoid progenitor cells as a screening tool in transiently transfected COS-1 cells, the mouse 4F2hc was cloned again (592). As discussed in section II*D5*, 4F2hc might have multiple functions.

The relevance of rBAT in the reabsorption of cystine and dibasic amino acids in kidney and intestine has been demonstrated by the involvement of the *rBAT* gene (named SLC3A1 in Gene Data Bank) in cystinuria (for recent reviews, see Refs. 170, 408, 467). This is an inherited aminoaciduria due to defective renal and intestinal reabsorption of cystine and dibasic amino acids; the poor solubility of cystine causes the formation of renal cystine calculi (351, 487). Surprisingly, rBAT and also 4F2hc are not very hydrophobic, and they seem to be unable to provide an aqueous translocation pathway in the plasma membrane. This prompted the hypothesis that rBAT and 4F2hc are subunits or modulators of the corresponding amino acid transporters. In this sense, it has been suggested or demonstrated that there is an association of rBAT and 4F2hc, respectively, with a corresponding light subunit of ~40 kDa. Here attention is focused on the hypothesis that both rBAT and 4F2hc are subunits of the actual amino acid transporters corresponding to system $b^{0,+}$-like and y^+L-like. Structural and functional evidence in favor of this is discussed. The role of the *rBAT* gene in cystinuria is described in section III.

1. *Tissue expression*

The rBAT mRNA is expressed in the kidney and the mucosa of the small intestine (45–47, 297, 598, 617). Consistent with this, hybrid depletion with rBAT antisense oligonucleotides blocks expression of system $b^{0,+}$-like by renal and intestinal poly(A)$^+$ RNA in oocytes (45, 338, 598). Northern blot analysis of human, rat, and rabbit renal and intestinal RNA revealed two rBAT transcripts:



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rBAT   MAEDKSKRDSIEMSMKGCQTNNGFVHNEDILEQTPDPGS-STDNLKHSTR   49
4F2hc  -----MSQDT-EVDMKEVELNE--LEPE---KQPMNAASGAAMSLAGAEK   39

rBAT   -GI----LGSQEPDFKGVQPYAGMPKEVLFQFSGQARYRIPREILFWLTV   94
4F2hc  NGLVKIKVAEDEAEAAAAAKFTGLSKEELLKVAGSPGWVRTRWALLLLFW   89

rBAT   ASVLVLIAATIAIIALSPKCLD-----WWQEGPMYQIYPRSFKDSNKDGN  139
4F2hc  LGWLGMLAGAVVIIVRAPRCRELPAQKWWHTGALYRI--GDLQAFQGHGA  137

rBAT   GDLKGIQDKLDYITALNIKTVWITSFYKSSLKDFRYGVEDFREVDPIFGT  189
4F2hc  GNLAGLKGRLDYLSSLKVKGLVLGPIHKNQ-KD-DVAQTDLLQIDPNFGS  185

rBAT   MEDFENLVAAIHDKGLKLIIDFIPNHTSDKHIWFQLSRTRTGKYTDYYIW  239
4F2hc  KEDFDSLLQSAKKKSIRVILDLTPNYR-----------------------  212

rBAT   HDCTHENGKTIPPNNWLSVYGNSSWHFDEVRNQCYFHQFMKEQPDLNFRN  289
4F2hc  --------------------GENSWFSTQVD-------------------  223

rBAT   PDVQEEIKEILRFWLTKGVDGFSLDAVKFLLEAKHLRDEIQVNKTQIPDT  339
4F2hc  -TVATKVKDALEFWLQAGVDGFQVRDIENLKDASSFLAEWQ----NITKG  268

rBAT   VTQYSELYHDFTTTQVGMHDIVRSFRQTMDQYSTEPGRYRFMGTEAYAES  389
4F2hc  FSEDRLLI--AGTNSSDLQQILSLLESNKDLLLTSS----YLSDSG----  308

rBAT   IDRTVMYYGLPFIQEADFPFNNYLSMLDTVSGNSVYEVITSWMENMPEGK  439
4F2hc  -------------STGEHTKSLVTQYLNA-TGNRWC----SW--SLSQAR  338

rBAT   WPNWMIGGPDSSRLTSRLGNQYVNVMNMLLFTLPGTPITYYGEEIGMGNI  489
4F2hc  L------------LTSFLPAQLLRLYQLMLFTLPGTPVFSYGDEIGL---  373

rBAT   VAANLNESYDINTLRSKSPMQWDNSSNAGFSEASNTWLPTNSDYHTVNVD  539
4F2hc  DAA----ALPGQPMEA-PVMLWDESSFPDIPGA----VSAN-----MTVK  409

rBAT   VQKTQPRSALKLYQDLSLLHANELLLNRGWFCHLRNDSHYVVYTRELDGI  589
4F2hc  GQSEDPGSLLSLFRRLSDQRSKERSLLHGDFHAFSAGPGLFSYIRHWDQN  459

rBAT   DRIFIVVLNFGE---STLLNLHNM--ISGLPAKMRIRLSTNSA-DKGSKV  633
4F2hc  ER-FLVVLNFGDVGLSAGLQASDLPASASLPAKADLLLSTQPGREEGSPL  508

rBAT   DTSGIFLDKGEGLIFEHNTKNLLHRQTAFRDRCFVSNRACYSSVLNILYT  683
4F2hc  ELERLKLEPHEGLLLRFPYAA-----------------------------  529

rBAT   SC   685
4F2hc  --
```

FIG. 7. Amino acid sequence comparison of human rBAT and 4F2hc proteins. Thick horizontal line over sequences indicates hydrophobic segment that corresponds to first putative TM domain, whereas thin horizontal lines indicate amphipathic TM domains II-IV proposed by Tate and co-workers (378). Solid frame box in gray indicates residues that resemble catalytic site of homologous glycosidases. Here, arrows indicate position of proposed catalytic residue (aspartate or glutamate) of these glucosidases; this residue is substituted by arginine in human 4F2hc and by asparagine in rabbit rBAT (42). In gray boxes are indicated amino acid residues present in human rBAT and 4F2hc proteins. Four dash frame boxes indicate segments of 12–17 amino acid residues with high homology between rBAT and 4F2hc proteins. Solid frame boxes indicate potential *N*-glycosylation sites, 6 for rBAT and 2 for 4F2hc. Dash indicates gaps for sequence multialignment obtained with all known rBAT and 4F2hc sequences with Clustal Sequence Alignment from Baylor College of Medicine.

~2.3 kb (which corresponds to the above-mentioned cDNA) and ~4 kb. A cDNA corresponding to the long rBAT transcript was identified by expression cloning in oocytes and represents an alternative polyadenylation of the same gene (344). In situ hybridization and immunolocalization studies have demonstrated that rBAT localizes to the microvilli of the small intestinal mucosa and the epithelial cells of the proximal straight tubules of the nephron (159, 248, 422). Interestingly, the expression of rBAT is developmentally regulated in rat kidney; rBAT transcripts appear after birth, and the onset of the protein expression coincides with postnatal nephron maturation (159). Clear rBAT transcripts are also visible in human pancreas; the significance of rBAT expression in pancreas is unknown (45). In addition to kidney and intestine, brain tissues show a transcript of ~5 kb that hybridizes with rBAT cDNA probes (45, 46, 617). This long transcript is almost ubiquitous, but with a substantially lower abundance in tissues other than brain (45). The RNA protection assay studies and Western blot analysis with some but not all anti-rBAT peptide antibodies suggested that this long transcript corresponds to the expression of a gene that is homologous to rBAT (422, 617). Moreover, rBAT immunoreactivity in hypothalamus is intracellular, and it is not located in the plasma membrane as in kidney and intestine (212). One antibody directed against a peptide of the rBAT sequence labeled intracellular structures of magnocellular neurons of the supraoptic and paraventricular nuclei.

In contrast to rBAT, 4F2hc mRNA is almost ubiquitous in mouse tissues, with a higher expression level in testis, lung, kidney, brain, and spleen and without a clear pattern of developmental regulation (413). Studies previous even to the cloning of 4F2hc showed that this protein is induced after activation of human and mouse lymphocytes (reviewed in Ref. 413; see sect. *IID5*). In fibroblasts (NIH 3T3 and BALB/c 3T3 cells), 4F2hc expression is induced during cell activation and maintained high throughout the cell cycle in exponentially growing cells (413). This suggests that 4F2hc plays a role in proliferating and quiescent cells. The amino acid transport activities associated with 4F2hc, as described here, may be relevant for both situations.

2. Transport properties

The characteristics of the amino acid transport activity associated with rBAT and 4F2hc expression have been

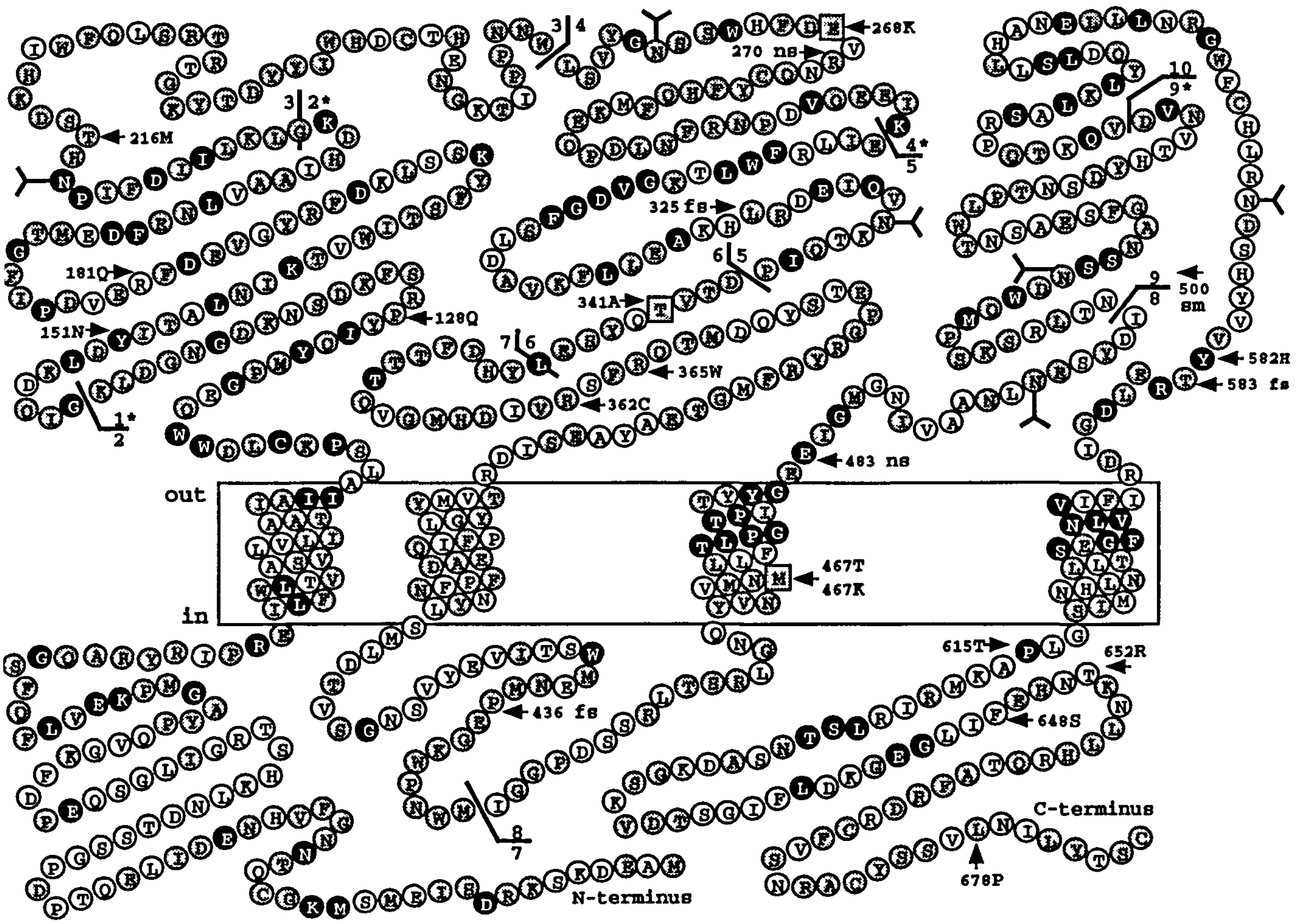

FIG. 8. Cystinuria-specific mutants in 4 TM domain topology model of human rBAT protein. This model has been roposed by Tate and co-workers (378) (see text for details). Amino acid residues conserved in all rBAT proteins including OK cell rBAT protein fragment corresponding to human residues 373–593) are indicated in gray circles and hose present also in 4F2hc proteins in white letters on a black circle. Notice that cysteine residue 114, after first TM lomain, is conserved in all these proteins. Twenty-one cystinuria-specific mutations are indicated by arrows. Number hows amino acid residue involved. For 15 missense mutations, substitution amino acid is indicated. Most of these nutations occur in conserved amino acid residues, with exception of R181Q, T652R, and F648S. ns, fs, and sm denote ionsense, frame shift, and splice mutations, respectively. In addition, four large deletion mutations have been described vith the following approximate boundaries: 114–1306, 198–1575, 430–765, and a mutation affecting exons V to X; osition 1 corresponds to 5′-position of first ATG codon. All mutations referred to here have been reviewed in References 70 and 408. Four of these mutations (E268K, T341A, M467T, and M467K; amino acid residues indicated by an square) iave been analyzed in oocytes and show defective transport expression (78, 91, 366). Six potential *N*-glycosylation sites Y) are indicated in first and second putative extracellular loops. Drawing of extracellular and intracellular loops and IH_2 and COOH terminals do not indicate any type of structure. Exon-intron boundaries of human rBAT are shown as eported in References 434 and 431. Boundaries conserved in open reading frame of human 4F2hc (174) are indicated y an asterisk.

tudied mainly in oocytes. rBAT induces, through the oo- yte plasma membrane, transport of cystine (up to >100- old over background) and dibasic and zwitterionic amino cids (up to 50-fold over background). This is a high- ffinity transport with K_m values in the micromolar range or amino acids such as L-cystine, L-arginine, L-lysine, L- rnithine, L-leucine, and L-histidine. Kinetic and cross-in- ibition studies offered convincing evidence that rBAT iduces a single amino acid transport system in *Xenopus* oocytes, at least in sodium-free medium (see below) (46), which is not present in stage VI oocytes (42, 46, 354). This transport activity is sodium independent, and it is very similar to the amino acid transport system $b^{0,+}$ defined by Van Winkle et al. (577) in mouse blastocysts, as a sodium-independent high-affinity system for dibasic and zwitterionic amino acids. In contrast to the transport system associated with rBAT, the blastocyst $b^{0,+}$ system does not transport L-cystine (L. J. Van Winkle, personal communi-

TABLE 9. *Tissue distribution and transport characteristics of expressed rBAT and 4F2hc proteins*

Transporter Subunit	Tissue Distribution (Transcript Size)	Expression System	Substrates (K_m, μM)	Cotransported Ligands	Amino Acid Exchange	Accumulation of Substrates
rBAT	Epithelial cells (mainly kidney and small intestine)[a] (~2.4 kb, ~4 kb)	Oocytes[c] Partial knockout in OK cells (374)	System b$^{o,+}$-like* substrates (~50)[e] L-Cystine, aa$^+$ and some aa^o (e.g., L-Leu)	None (75, 110)	Homo- and heteroexchange of aa$^+$ and aa^o[g] Favored exchange (90): 1 aa$^+$ (influx)/1 aa^o (efflux)	~50-fold (90)
4F2hc	Ubiquitous[b] (~2 kb)	Oocytes[d] Antisense in blood cells (88)	System y$^+$L-like* substrates (~50)[f]: aa$^+$ and aa^o plus Na$^+$	Na$^+$ with aa^o[f]	Favored exchange (90): 1 aa^o plus Na$^+$ (influx)/1 aa$^+$ (efflux)	~30-fold (90)

Transcript sizes of rBAT are similar in human, rat, and rabbit tissues, and 4F2hc transcript size is similar in human and mouse tissues. * Amino acid transport activity of system b$^{o,+}$-like for rBAT expression in oocytes and OK cells, and of system y$^+$L-like for 4F2hc expression in oocytes described by Tate's, Hediger's, Ganapathy's and our groups (see references below) are shown. Others proposed induction of sodium-dependent histidine transport in rBAT-injected oocytes (4) and sodium-independent transport for both dibasic and zwitterionic amino acids (69) (see text for details). Values of substrate accumulation in oocytes were obtained at 50 μM radiolabeled susbtrates. aa^o, Zwitterionic amino acids; aa$^+$, dibasic amino acids. References are as follows: a) 45, 46, 297, 344, 598, 617; b) 413, 435, 553; c) 3, 45, 46, 75, 78, 90, 91, 109, 110, 297, 338, 344, 374, 367, 549, 598; d) 42, 69, 90, 142, 146, 338, 599; e) 4, 45, 46, 75, 91, 110, 297, 338, 344, 367, 374, 598, 617; f) 42, 142, 146, 599; g) 3, 75, 90, 91, 109, 110. Other references are given in parentheses.

cation). For this reason, we named our human and rabbit cDNA clones rBAT, as an acronym for "related to b$^{o,+}$ amino acid transporter"). Further characterization of the rBAT/system b$^{o,+}$-like transport activity showed that it was independent of external potassium and chloride (75), changes in the external pH (Palacín, unpublished data), and internal ATP (110). Cystine and dibasic and zwitterionic amino acid transport with the characteristics of system b$^{o,+}$-like have been described in renal and intestinal plasma membrane preparations (see sect. IID5).

Ahmed et al. (4) and Taylor and co-workers (420) propose that the expression of rBAT induces several amino acid transport systems: *1*) a NEM-resistant sodium-independent transport for cationic and zwitterionic amino acids, equivalent to system b$^{o,+}$-like (R. Estévez and M. Palacín, unpublished data), and in brush-border membrane preparations of chicken jejunum (560); *2*) sodium-independent transport activities (perhaps two), which are sensitive to NEM treatment, and with overlapping specificties for cationic and zwitterionic amino acids; and *3*) a sodium-dependent transport for L-histidine, which has a pH dependence compatible with the transport of this substrate in the nonprotonated form. Then, either as a consequence of the overexpression of rBAT in oocytes or reflecting a true mechanism of activation, rBAT induces several amino acid transport activities in the oocyte (see sect. IID4). The fact that partial knockout of rBAT in OK cells results in a specific, partial decrease in the apical system b$^{o,+}$-like activity (374), and the finding that mutations in the rBAT gene cause cystinuria (for review, see Refs. 170, 408, 467) demonstrate the role of rBAT in the high-affinity reabsorption system of cystine (system b$^{o,+}$-like) (see sect. IID5). In this context, the physiological relevance of the other amino acid transport activities induced in rBAT-expressing oocytes remains to be demonstrated.

In contrast to rBAT, the cRNA of human 4F2hc induces an amino acid transport activity (e.g., up to 10-fold over background for radiolabeled L-arginine), which is sodium independent with high affinity (micromolar range) for L-dibasic amino acids, but with high affinity for L-zwitterionic amino acids only in the presence of sodium; in the absence of sodium, the affinity for L-zwitterionic amino acids is dramatically reduced (46, 599). This transport activity, which does not transport L-cystine, is very similar to the system y$^+$L, initially described in human erythrocytes by Devés et al. (127), and later described in brush-border membrane vesicles from human placenta (135); a recent review (126) describes the transport characteristics of system y$^+$L. In the same line, Ganapathy and co-workers (146) have shown that poly(A)$^+$ RNA from a human choriocarcinoma cell line expresses y$^+$L transport activity in oocytes that is hybrid depleted by 4F2hc antisense oligonucleotides. Very recently, similar data have been obtained with rat lung poly(A)$^+$ RNA (142). In contrast to this, Broër et al. (69) showed a clear induction by rat 4F2hc in oocytes of a high-affinity uptake for cationic and zwitterionic amino acids, both in the absence of sodium. The induced transport activity has combined characteristics of system b$^{o,+}$-like and L-like, but not of system y$^+$L-like (see sect. IID4).

The fact that mutations in the rBAT gene cause cystinuria (see sect. III), a defect in the renal and intestinal reabsorption of cystine and dibasic amino acids, raised an important question: how does a sodium-, potassium-, proton-, and ATP-independent transporter such as the b$^{o,+}$-like system associated with rBAT participate in an active process, like the reabsorption of cystine and dibasic amino acids? The answer came from the study of the electrical activity of system $b^{0,+}$-like. Busch et al. (75) studied this activity of the system $b^{0,+}$-like expressed by rBAT in oocytes; as expected, in oocytes expressing rBAT, but not in control oocytes injected with water, the presence of L-arginine in the medium produces an inward positive current, most probably due to the positive charge of arginine at neutral pH. Surprisingly, exposure of rBAT-expressing oocytes to L-leucine produced an outward positive current through the plasma membrane of the oocyte. The participation of ions (e.g., Na^+, K^+, Cl^-) in these currents was ruled out. These results prompted the hypothesis that the $b^{0,+}$-like/rBAT transporter exchanges amino acids through the plasma membrane; the outward positive current produced by zwitterionic amino acids (e.g., L-leucine) would be due to the concomitant exit of dibasic amino acids from the oocyte. This was demonstrated in several laboratories by testing the dependence of amino acid efflux from oocytes expressing rBAT on the external amino acids; the efflux of L-[^{3}H]arginine and L-[^{3}H]leucine is totally dependent of the presence of amino acids in the medium (3, 90, 110). In fact, Coady et al. (110) have isolated a renal rabbit rBAT cDNA by expression of the electric activity of system $b^{0,+}$-like/rBAT in oocytes. Additional data confirmed that system $b^{0,+}$-like is an obligatory exchanger, which acts as a tertiary active transporter (90).

1) Only the system $b^{0,+}$-like substrates elicited efflux via system rBAT/$b^{0,+}$-like in oocytes.

2) The exchange mechanism is able to accumulate amino acid substrates in oocytes expressing rBAT; ~50-fold intracellular accumulation of 50 μM extracellular radiolabeled L-arginine, L-leucine, or L-cystine. This level of accumulation is not due to metabolism of the radiolabeled substrate in the oocytes, and it is significantly higher than that obtained in noninjected oocytes, or in oocytes expressing the cationic amino acid transporter CAT1 (system y^+ activity, which shows *trans*-stimulation but is not an exchanger; see sect. IIA*2*).

3) The active transport due to rBAT/system $b^{0,+}$-like expression in oocytes has a limit of accumulation, which coincides with the amount of intracellular free amino acid substrates in the oocyte (these cells contain a very high intracellular concentration of amino acids that has been estimated to be ~2,500 μM zwitterionic amino acids and 750 μM dibasic amino acids; Ref. 550).

4) As a consequence of this, prolonged incubations of the rBAT-expressing oocytes in the presence of an rBAT/system $b^{0,+}$-like substrate results in the complete exchange of this amino acid within the oocyte. In this situation (homogeneous exchange), and in voltage-clamp conditions, when homoexchange is forced (e.g., L-arginine influx and efflux or L-leucine influx and efflux), the electric activity of rBAT/system $b^{0,+}$-like disappears. In contrast, when homogeneous heteroexchange is forced (e.g., L-arginine influx and L-leucine efflux, or vice versa), the current elicited by the substrates in oocytes expressing rBAT is maximal, and with a direction corresponding to the exchange. At a defined membrane potential (e.g., −50 mV), the exchange L-arginine (influx)/L-leucine (efflux) is favored versus the reverse exchange. This demonstrated that the exchange of substrates via rBAT/system $b^{0,+}$-like is the only electric activity of this transporter, in agreement with previous data obtained with the cut-open oocyte model (110). In addition, both influx and efflux require substrates on both sides; no electric activity is evoked by substrates in rBAT-expressing cut-open oocytes, which is entirely due to heteroexchange of substrates, if no substrates are present on the *trans*-side (109, 110).

5) In conditions of homogeneous exchange, either homo- or heteroexchange, radiolabeled substrate efflux equals influx. This together with estimations of Hill coefficients of approximately one both in radiolabeled and electric amino acid transport measurements (45, 47, 75, 90) indicates a stoichiometry of exchange of one amino acid (influx)/one amino acid (efflux).

The tightness of the obligatory exchange mechanism of rBAT/system $b^{0,+}$-like is at present unknown. The fact that cationic amino acid-evoked currents occur in rBAT-expressing cut-open oocytes only when zwitterionic amino acids are present on the *trans*-side favors a very tight coupling of exchange (109). With the assumption of a theoretical absolute requirement of the transporter to be occupied by a substrate at either side (intra- or extracellularly) to translocate (this is thermodynamically impossible) the amino acid, accumulation curves via rBAT/system $b^{0,+}$-like have been modeled (90). If the transport model assumes that the velocity constants of translocation of the empty (no substrate bound at either side) transporter is ~30-fold lower than for the amino acid-transporter complex, the experimental accumulation curves may still be reproduced by the model (J. L. Gelpí and M. Palacín, unpublished data); the sensitivity of the transport studies of radiolabeled substrates in oocytes expressing rBAT precludes a more precise determination. The mechanism of exchange of rBAT/system $b^{0,+}$-like (sequential or concerted substrate binding at both sides) is at present unknown. Interestingly, when the analog aminoisobutyric acid is used as a substrate, the amino acid exchange via rBAT/system $b^{0,+}$-like shows a variable stoichiometry of exchange: the aminoisobutyric acid-induced currents (i.e., efflux of the positively charged cationic amino acid substrates) is higher than the concomitant aminoisobutyric acid radiolabeled transport flux (109). This suggests that aminoisobutyric acid "locks" the transporter in a conformation that enables free translocation of the transporter in a fraction of transport cycles. Accumulation studies with aminoisobutyric acid (predicted to be lower than with physiological substrates) have not been reported.

The exchange mechanism of rBAT/system $b^{0,+}$-like is not an oocyte artifact. It has also been shown to occur in renal cells that naturally express rBAT; the gene is expressed, and the system $b^{o,+}$-like is present in the "renal proximal tubular" OK cell line (374). *1*) In the apical pole, the transport of cystine and most of the L-arginine transport (~80%) has the substrate specificity of system $b^{o,+}$-like. *2*) The substrate efflux via system $b^{o,+}$-like shows complete dependence on external amino acid substrates. *3*) This transport activity is due to the expression of the rBAT gene; stable transfection with antisense rBAT sequences results in the partial and specific loss of system $b^{o,+}$-like activity. This demonstrated that the exchange mechanism for rBAT/system $b^{o,+}$-like also occurs in epithelial renal cells. This is in full agreement with heteroexchange of cystine and dibasic amino acids observed in renal brush-border membrane vesicles (352). The sodium-dependent L-histidine transport induced by rBAT in oocytes (4) is currently being studied in OK cells to assess the physiological relevance of this induction.

Interestingly, the y^+L-like transport activity induced by 4F2hc in oocytes also behaves as an exchanger (90). *1*) Efflux of radiolabeled L-arginine via 4F2hc/system y^+L-like requires the extracellular presence of its substrates (e.g., cationic amino acids in sodium-free medium or zwitterionic amino acids in sodium-containing medium). *2*) As a consequence of this exchange mechanism, oocytes expressing 4F2hc are able to accumulate system y^+L-like substrates at higher levels than noninjected oocytes or CAT1/system y^+-injected oocytes. *3*) The exchange of amino acids via 4F2hc/system y^+L-like is asymmetric. Efflux of radiolabeled L-leucine is not observed in oocytes expressing 4F2hc even in the presence of extracellular substrates; this is interpreted as showing that the interaction of zwitterionic amino acids with 4F2hc/system y^+L-like at the low intracellular sodium concentration is very weak and therefore not visible in radiolabeled uptake studies. This suggests that exchange via 4F2hc/system y^+L-like favors the efflux of cationic amino acids and sodium-dependent influx of zwitterionic amino acids. The erythrocyte/placental system y^+L shows marked *trans*-stimulation, compatible with a ratio of velocity constants for the translocation of the occupied and empty carrier of at least 25 (14, 127, 135). It is therefore also possible that system y^+L may indeed be acting as an exchanger. In this sense, as discussed previously for the modeling of the accumulation of substrates in oocytes via rBAT/system $b^{o,+}$-like, the level of *trans*-stimulation of system y^+L would allow transient accumulation of substrates similar to those observed in 4F2hc-expressing oocytes (90). It would be interesting to discern whether the asymmetric exchange (efflux of cationic amino acids/influx of zwitterionic amino acids plus Na^+) observed via system y^+L-like activity in 4F2hc-injected oocytes also happens in the erythrocyte/placental y^+L system. What is the nature of the sodium dependence of L-leucine efflux from plasma membrane preparations in conditions in which other transport activities are blocked (i.e., NEM-treated vesicles to inhibit system y^+ and in the presence of intravesicular BCH, a system L inhibitor)?

3. Protein structure

rBAT and 4F2hc proteins have no membrane leader sequence, similar hydrophobicity plots (reviewed in Ref. 407), and four regions (12–17 amino acid residues long) highly conserved (67–80% identity) (see Fig. 7). Both proteins also have a domain with significant homology with a protein family of prokaryotes and insect α-amylases and α-glucosidases (42, 46, 598). Interestingly, the catalytic site of these glucosidases is not totally conserved in rabbit rBAT or human 4F2hc; this is consistent with the fact that expression of rBAT in oocytes does not show α-amylase or maltase activity (598). In contrast to the well-known membrane multispanning structure of membrane transporters of substrates of polar nature (607), rBAT and 4F2 (4F2hc) are less hydrophobic and contain, depending on prognosis based on hydrophobicity algorithms, a single TM domain (i.e., a type II membrane glycoprotein; Refs. 46, 435, 553, 598) or four TM domains, with intracellular NH_2 and COOH termini; Ref. 549); the more NH_2-terminal hypothetical TM domain is the only one showing a clear prognosis as a TM domain (see Fig. 7). Surprisingly, these structures induce amino acid transport activity via system $b^{o,+}$-like and y^+L-like in *Xenopus* oocytes, respectively, and the involvement of rBAT in cystinuria demonstrates a role for rBAT in renal and intestinal reabsorption of amino acids. The apparent inability of these proteins to provide an aqueous translocation pathway in the plasma membrane, due to their low hydrophobicity, prompted the hypothesis that they may be modulators of transporters with a heteromeric structure (42, 46, 598).

Biochemical and immunochemical studies have demonstrated that rBAT and 4F2hc are integral membrane *N*-glycoproteins. The experimental evidence for rBAT can be summarized as follows: *1*) in vitro translation. Addition of microsomes to the reticulocyte translation system increases (<20 kDa) the molecular mass of the protein product synthesized from rBAT cRNA (344, 598). *2*) rBAT is expressed in oocytes. The protein product (~90 kDa) from rBAT cRNA in oocytes, shown by metabolic labeling with [^{35}S]methionine, is an integral *N*-glycoprotein. Thus the product is not solubilized from oocyte membranes by sodium carbonate treatment. The treatment of the oocytes with tunicamycin reduces the size of the protein to ~72 kDa, compatible with the mass of the deduced protein from the cDNA ($M_r < 79 \times 10^3$) (45). *3*) Studies with the native protein have been done. Western blot analysis using specific anti-rBAT antibodies revealed a protein band of 90–95 kDa in membrane preparations from kidney and mucosa from the small intestine (159, 379). The size of this band is reduced to ~72 kDa after endoglycosidase F treatment of renal brush-border membranes (J. Chillarón and M. Palacín, unpublished data). Because of the lack of leader peptide and because the *N*-glycosylation sites are toward the COOH terminus from the location of the most evident putative transmembrane domain (see Fig. 7), it was proposed that rBAT and 4F2hc were type II membrane glycoproteins (i.e., cytosolic NH_2 terminus and extracellular COOH terminus) (46, 435, 553, 598). In contrast, Tate and co-workers (378) have proposed that rBAT crosses the plasma membrane at least four times, with the first transmembrane domain already mentioned and three additional amphipathic transmembrane domains (Fig. 8). This is based on studies of limited proteolysis and peptide-specific antibody detection of permeabilized cells expressing the rBAT protein (378). These highly interesting results on the rBAT protein await confirmation with different approaches; no similar studies have been conducted with 4F2hc. In any case, it seems that one or four transmembrane domains are not enough to conform a polar pore for the movement of amino acids through the plasma membrane.

rBAT and 4F2hc might be components of heteromeric amino acid transporters (42, 46, 599): rBAT and 4F2hc may be "activators" of silent $b^{o,+}$-like and y^+-like transporters of the oocyte, respectively. A possible mechanism for this activation could be the constitution of holotransporters with subunits present in the *Xenopus* oocytes. This hypothetical mechanism would be similar to the activation of the oocyte α-catalytic subunits of the Na^+-K^+-ATPase by the expression of foreign β-subunits of the Na^+-K^+-ATPase (166); a similar mechanism has been described for multimeric channels (32, 206, 469). In this sense it is very interesting that the cell surface antigen 4F2 is a heterodimer (~125 kDa) composed of a heavy chain of 85 kDa (4F2hc, i.e., the homologous protein to rBAT) and a light chain of 40 kDa linked by disulfide bridges (204, 209). Unfortunately, this light subunit evidenced by ^{125}I labeling and immunoprecipitation has not been microsequenced or cloned. In a similar way, Wang and Tate (591) have reported the presence of these complexes in brush-border preparations from kidney and intestine. In our hands, renal rBAT is immunodetected in Western blot studies in nonreducing conditions as complexes of ~240 and ~125 kDa; in two-dimensional gels (first in nonreducing conditions, then in reducing conditions), the 240-kDa and the 125-kDa bands contribute to the ~90 kDa seen in reducing conditions (408). Interestingly, in membranes obtained in the presence of NEM from oocytes expressing rBAT, complexes similar in size to those observed in kidney have been reported (591). All this suggests that similarly to 4F2 antigen, rBAT forms a heterodimeric structure (125 kDa) of a "heavy chain" (~90 kDa) linked by disulfide bridges to a putative "light chain" of 40–50 kDa.

4. *Structure-function relationship*

The studies on structure-function relationship on rBAT/system $b^{o,+}$-like and 4F2hc/system y^+L-like are scarce, and limited to, the defect in four cystinuria-specific rBAT mutants (78, 91, 366), the effect of a COOH-terminal deletion of rBAT (367), and indirect evidence that supports the hypothesis that the functional units of the systems $b^{o,+}$-like and y^+L-like are heterodimeric structures of rBAT and 4F2hc with their corresponding putative "light subunits" (see below; see Refs. 408, 409, 548).

Four human cystinuria-specific rBAT missense mutations have been tested for amino acid transport expression in oocytes (Met467Thr and Met467Lys, Refs. 78 and 91; E268K and T341A, Ref. 366) (see location of these amino acid residues in Fig. 8). All four show partially defective amino acid transport when expressed in oocytes. Expression in oocytes of Met467Thr, the most frequent cystinuria type I mutant known worldwide (it represents 26% of the type I cystinuria chromosomes explained; see sect. III), and the mutant Met467Lys results in a reduced V_{max} of the induced system $b^{o,+}$-like, without substantial effect on the apparent K_m; this is not due to defects in the synthesis or degradation of the transporter (78, 91). A deeper study on the transport defect associated with Met467Thr and Met467Lys mutants revealed a plasma membrane trafficking defect. These mutants express only an endoglycosidase H-sensitive protein band in the oocytes, and the protein reaches the oocyte plasma membrane slowly and inefficiently, as revealed by surface biotinylation studies (91). Long oocyte expression periods (>3 days after injection) and injection of oversaturating amounts of mutant rBAT cRNA result in total (Met467Thr) or partial (≤20% activity of the wild type for Met467Lys) recovery of the induced amino acid transport (91); it is interpreted that these conditions overcome the protein quality control machinery of the oocyte. Interestingly, when the amino acid transport activity induced by Met467Thr mutant is recovered, the amount of Met467Thr on the oocyte surface is only <10% of the corresponding wild-type protein; this suggests that an oocyte "factor" limits the expression of system $b^{o,+}$-like activity when oversaturating amounts of rBAT cRNA are expressed (91).

In an interesting study, Miyamoto et al. (367) showed that a COOH-terminus deletion (Δ511–685) on human rBAT, which eliminates the fourth putative TM domain as well as the fourth segment of high homology between rBAT and 4F2hc (see Figs. 7 and 8), induces in oocytes a decreased amino acid transport activity (radiolabeled amino acid transport studies) that resembles that of 4F2hc/system y^+L-like (i.e., sodium-independent transport of dibasic amino acids and sodium-dependent transport of zwitterionic amino acids); expression of longer deletions in the COOH terminus of rBAT renders no transport function in oocytes. This suggests that rBAT and 4F2hc are modulators or subunits of the complete transporters, in which the substrate specificity of systems $b^{o,+}$-like and y^+L-like resides. In addition, this study suggests that the COOH terminus is relevant for the interaction with the putative transporter or subunit. There is one concern in this interpretation of these results. Δ511–685 rBAT expresses substantial substrate-evoked currents (15–20 nA by 50 μM L-arginine or L-leucine at −50 mV) in the oocytes (367). In contrast, substrate-evoked currents by 4F2hc in oocytes are very small (≤1 nA by 50 μM L-arginine or L-leucine at −50 mV) (90). In agreement with this, placental y^+L activity is largely insensitive to membrane potential (135). This is interpreted as the reflection of the cotransport of sodium with zwitterionic amino acids in exchange with cationic amino acids via y^+L, resulting in no electric activity (90). At present, there is no explanation as to why Δ511–685 rBAT induces in oocytes an amino acid transport activity that is identical to 4F2hc/system y^+L-like when transport is measured with radiolabeled substrates but differs in its electrical activity.

Additional evidence also points to rBAT and 4F2hc as modulators or subunits of the amino acid transporter. Transient expression of rBAT in COS cells resulted in the production of a glycosylated rBAT form that either does not reach the plasma membrane (our experiments; Ref. 408) or does so (Tate's group experiments; Ref. 378) but, in both cases, with no amino acid transport expression. Interestingly, in nonreducing conditions, the renal and intestinal characteristic ~125 kDa rBAT complex is not present; it might be that the putative "light subunit" of rBAT is not expressed in COS cells, precluding transport expression (408). Recently, we obtained additional functional evidence for the need of the putative light subunit in the 4F2hc-induced expression of system y^+L-like (142): *1*) there is dissociation between oocyte surface 4F2hc protein and induced amino acid transport activity (saturation of induced amino acid transport occurs at very low amounts of injected cRNA, 0.01–0.1 ng 4F2hc cRNA/oocyte); expression of larger amounts of cRNA results in more 4F2hc on the surface without increment in the induced uptake. *2*) In addition, there is coexpression of system y^+L-like activity upon injection of saturating doses of 4F2hc plus rat lung mRNA or plus rat lung size-fractionated mRNA; 4F2hc is necessary for this coexpression since 4F2hc antisense oligonucleotides specifically hybrid-deplete the coexpression of system y^+L-like activity (Estévez and Palacín, unpublished data).

In summary, the studies with the Met467Thr rBAT mutant, the COOH-terminal deletion of rBAT and the coexpression of 4F2hc and rat-lung mRNA strongly suggest that oocyte light subunits together with expressed rBAT or 4F2hc are responsible for the expression of systems $b^{o,+}$-like and y^+L-like, respectively. This, together with the heterodimeic structure demonstrated for 4F2hc or indirectly evidenced for rBAT, suggests that the functional unit of these transporters is a heterodimer (rBAT or 4F2hc plus the corresponding putative light subunit). As already mentioned, two labs recently showed induction of additional amino acid transport activities with rBAT and 4F2hc. Ahmed et al. (4) showed sodium-dependent histidine uptake induced by rBAT in oocytes, in addition to the induction of system $b^{o,+}$-like activity. Bröer et al. (69) showed induction by rat 4F2hc in oocytes of a type of system L-like transport activity (sodium-independent transport for cationic and some zwitterionic amino acids). This is in contrast to others who showed induction of system y^+L-like activity (sodium-independent transport for cationic and sodium-dependent transport for some zwitterionic amino acids) by both human and rat 4F2hc (46, 142, 146, 599). The physiological relevance of the induction of sodium-dependent uptake by rBAT and a system L-like is at present unknown. Knockout studies similar to that reported for rBAT in OK cells (374) are necessary to assess the physiological relevance of these amino acid inductions. Nevertheless, this opens the possibility that several putative light subunits in combination with rBAT and 4F2hc, or unidentified homologous proteins, constitute different amino acid transport systems. If the hypothesis of the heterodimeric holotransporters for rBAT and 4F2 is valid, the amino acid transport systems $b^{o,+}$-like and y^+L-like will be the first examples of heteromeric transporters for organic substrates in mammals. Knowledge of the structure-function relationship of rBAT and 4F2hc urgently needs the isolation and cloning of the light subunit of 4F2 and the putative light subunit of rBAT. Purification of the ~125-kDa rBAT complex by classical biochemical ways and coexpression cloning strategies are currently in progress in several labs in an attempt to identify these subunits.

5. *Physiological role of rBAT and 4F2hc*

Our knowledge of the physiological role of rBAT is clearly greater than that of 4F2hc (see Ref. 408 for a recent review). The involvement of rBAT in classic cystinuria demonstrates the role of rBAT in the renal and intestinal reabsorption of cystine and dibasic amino acids. Because of the cellular localization of the rBAT protein and its mechanism of exchange (tertiary) active transport (see above), we proposed a model for the physiological role of transporter $b^{o,+}$-like in the renal reabsorption of cystine and dibasic amino acids (90). In this model, the function of the transporter is directed toward apical reabsorption of cystine and dibasic amino acids, dissipating the intracellular gradient of zwitterionic amino acids. The negative membrane potential and the intracellular reduction of cystine to cysteine should favor this direction of the exchange. The zwitterionic amino acids released to the tubular lumen should be reabsorbed via active transporters (e.g., the sodium-dependent system neutral brush border) located in the apical plasma membrane of tubular epithelial cells. Is this model valid? The fact that mutations in the rBAT gene cause cystinuria, aminoaciduria of cystine and dibasic amino acids, but not of zwitterionic amino acids, clearly favors this model.

In addition to the cystine and dibasic amino acid reabsorption defect of classic cystinuria, the present knowledge of cystine reabsorption in kidney and intestine is very confused (for review, see Refs. 487 and 496). Work with brush-border membrane preparations from rat kidneys showed that L-cystine reabsorption is less sodium dependent than that of other zwitterionic amino acids (153, 352, 353, 449). Because of the weak sodium dependence of L-cystine reabsorption, cystine and dibasic amino acids may be accumulated across the apical membrane of kidney epithelial cells partly because of the intracellular reduction of cystine to cysteine and the negative membrane potential, respectively; basolateral transport systems would mediate the efflux of these amino acids (496). Segal and co-workers (352, 486) have provided evidence that renal brush-border membrane vesicles show two cystine transport systems: one with high-affinity (K_m in the micromolar range), shared with dibasic amino acids that shows heteroexchange diffusion, and the other with low affinity and unshared. In addition, several authors have found inhibition by zwitterionic amino acids of cystine uptake, measured at low concentration (micromolar range) in renal brush-border preparations or perfused tubules, suggesting that the high-affinity system is also shared by zwitterionic amino acids (153, 158, 449, 479). In contrast to renal preparations, cystine transport in brush border from mucosa of the small intestine shows a single kinetic transport system of high affinity, shared with dibasic amino acids (404). Therefore, this high-affinity system, present in kidney and intestine, may be the system that is defective in cystinuria (111, 555). Microperfusion studies showed that this cystine high-affinity transport system is present in the proximal straight tubule (S3 segment), whereas the low-affinity system is present in the proximal convoluted tubule (S1-S2 segments) (479). Recently, Riahi-Esfahani et al. (449) reported that luminal membrane vesicles from the pars recta ("outer medulla") of rabbit kidney show a conspicuous component of cystine transport of high affinity (K_m values of $\sim$30 μM); interestingly, cystine transport in the pars recta is less sodium dependent and more sensitive to inhibition by micromolar concentrations of zwitterionic amino acids than in the pars convoluta (i.e., in apical membranes isolated from the "outer cortex").

More recently, it was demonstrated that cystine is transported through the apical pole of the "renal proximal tubular" cell line OK via system $b^{o,+}$-like (i.e., sodium-independent, high-affinity transport system, shared with dibasic and zwitterionic amino acids), and it is due to the expression of rBAT; expression of antisense rBAT sequences specifically reduces this amino acid transport activity in OK cells (374). System $b^{o,+}$-like activity has also been described in brush-border membrane vesicles from chicken jejunum (560) and in Caco-2 cells (557). The specific expression of rBAT in the microvilli of the S3 segment of the nephron and the cystinuria-specific mutations found in the rBAT gene allows us to propose that system $b^{o,+}$-like (associated with rBAT) participates in the renal and intestinal reabsorption of cystine and dibasic amino acids of high affinity, most probably with a tertiary active transport mechanism (see above). Because of the location of rBAT in the S3 segment of the nephron, where only a part of the cystine reabsorption occurs (496), system $b^{o,+}$-like could be envisaged as a low-capacity high-affinity system of physiological relevance as revealed by its alteration in cystinuria. In conclusion, most probably rBAT/system $b^{o,+}$-like corresponds to the high-affinity reabsorption system of cystine described in renal ("pars recta") and intestinal preparations (see above). In contrast, the proteins responsible for the high-capacity low-affinity reabsorption of cystine in the proximal convoluted tubule are unknown.

We are far from establishing the physiological role of 4F2hc. It is even possible to imagine a multifunctional role for this protein. Before the linkage between 4F2hc and amino acid transport, it was implicated in calcium movement through the plasma membrane: *1*) an anti-4F2hc monoclonal antibody (44D7) inhibited sodium/calcium exchanger activity in cardiac and skeletal muscle sarcolemmal vesicles (for review, see Ref. 303). *2*) An anti-4F2hc antibody on parathyroid cells produces an increase in cytosolic free calcium concentration at low extracellular calcium levels (427). Recently, 4F2hc has also been implicated in cell fusion (396) and regulation of cell survival/death control (592). Fusion regulatory protein (FRP-1) regulates virus-mediated cell fusion and fusion of monocytes. Purification and partial sequencing of human FRP-1 revealed a strong homology of the NH_2 terminus with human 4F2hc (it corresponds to the cluster of differentiation CD98) (11 of 15 amino acid residues are identical); both proteins show cross-reactivity with different antibodies, and the expression of both proteins is induced by concanavalin A or interleukin-2 treatment (396). To us, it seems that FRP-1 and 4F2hc are highly homologous, although not identical. To our knowledge, more extended sequences of FRP-1 have not been reported. In addition, treatment of monocytes with anti-4F2hc antibodies resulted in cell fusion and formation of multinucleated giant cells of Cd^+U2ME-7, a $CD4^+$U97 cell line transfected with HIVgp160 gene, whereas other anti-4F2hc antibodies suppress these induced fusion events (396, 397). Similarly, anti-FRP-1/4F2hc antibodies suppress human parainfluenza virus type 2-induced cell fusion (398). In a search for cell surface markers expressed on hematopoietic stem cells, Palacios and co-workers (592) found that Joro 177 monoclonal antibody stained these cells. A cDNA library search with this antibody resulted in the cloning of mouse 4F2hc. Interestingly, this antibody stimulates tyrosine phosphorylation of an unidentified 125-kDa protein, induces homotypic aggregation of progenitor lymphoid cells, inhibits cell survival/growth of hematopoietic cells, induces apoptosis, and prevents the generation of lymphoid, myeloid, and erythroid lineage cells. This study suggests that 4F2hc might act as a membrane receptor involved in the control of cell survival/death of hematopoietic cells (592).

The above-mentioned roles of 4F2hc in cell fusion and aggregation might involve integrin function. Very recently, 4F2hc has been implicated in the regulation of integrin function (146): *1*) expression of 4F2hc (i.e., CD98) complements dominant suppression due to the overexpression of an integrin β_1-cytoplasmic domain, *2*) 4F2hc coimmunopreipitates with active β_1-integrins, and *3*) antibody-mediated cross-linking of 4F2hc stimulated β_1-integrin-dependent cell adhesion. In this sense, anti-β_2- and anti-β_1-integrin antibodies blocked anti-FRP/4F2hc antibody-induced cell aggregation and antibody-induced polykaryocyte formation, respectively (530). In any case, this issue is not yet clear because other proteins also associate with 4F2hc. Thus FRP-1/4F2hc and cytoskeletal proteins (e.g., actomyosin, vimentin, and heat shock cognate protein 70) are coimmunoprecipitated by anti-FRP-1/4F2hc antibodies (522), and anti-FRP-1/4F2hc antibodies change the immunofluorescence pattern of these cytoskeletal proteins (522). It is therefore difficult at present to ascertain whether anti-FRP-1/4F2hc antibody-mediated cell fusion events are due to direct or indirect effects via changes in the cell surface distribution or conformation of other proteins. The role of the interaction of 4F2hc (or FRP-1) with other proteins (e.g., cystoskeletal proteins) in any of the putative functions of 4F2hc is also unknown.

As mentioned before, several labs have observed induced amino acid transport activity in oocytes injected with 4F2hc. One important question to resolve is the amino acid transport activity associated physiologically with 4F2hc in the cells that express 4F2hc naturally. In this sense, 4F2hc was originally described as a marker for tumor cells and activated lymphocytes (204). Stimulated lymphocytes (e.g., by concanavalin A, interleukin-2, or phytohemagglutinin) have a larger increment (~60-fold) of 4F2hc in the plasma membrane (for review, see Ref. 303); in some instances, this is due to increased transcript stability (554). Boyd and co-workers (88) addressed this question (88): *1*) phytohemagglutinin induces in lymphocytes cationic amino acid transport with system y^+ characteristics; and *2*) transfection of antisense oligonucleotide sequences of human 4F2hc and CAT-1 (system y^+; see sect. IIA), singly or in combination, inhibits the phytohemagglutinin-induced system y^+ activity in human peripheral blood mononuclear cells. These results suggest a shared responsibility of 4F2hc and CAT-1 in system y^+ activity. Unfortunately, all our attempts to coexpress system y^+ transport activity by coinjecting human 4F2hc and mouse CAT-1 or CAT2-a failed (142). Therefore, at present, we do not known which specific cationic transport activity is physiologically related to 4F2hc [i.e., systems similar to y^+L (42, 146, 599), L (69), or y^+ (88)]. In any case, the obligatory exchange of amino acids via system y^+L-like, associated with 4F2hc expression in oocytes, which has been discussed in the previous sections, might have important physiological consequences. It has been reported that efflux across the basolateral membrane is the rate-limiting step in the intestinal absorption of dibasic amino acids (86, 383). Furthermore, leucine at low micromolar concentration increases (6- to 10-fold) the transepithelial flux of lysine (86, 87). Countertransport between lysine (outward) and leucine (inward) or allosterism was considered to be responsible for this process. System y^+L can sustain lysine-leucine exchange with an apparent K_m for leucine of ~10 μM in the presence of sodium (127). If such a system is found in the basolateral membranes of intestinal or renal epithelial cells, the hypothesis that system y^+L can affect countertransport will be supported (14). The surface antigen 4F2hc has a basolateral localization in renal epithelial cells from the proximal tubule (436). System y^+L-like, associated with 4F2hc expression, could be responsible for the active release of dibasic amino acids through the basolateral membrane of epithelial cells. The fact that the direction of exchange that is favored is L-arginine (outward) with low micromolar concentration of leucine (inward) in the presence of sodium strongly supports this hypothesis (90). Further research is needed to elucidate the mechanism (e.g., a weak interaction of zwitterionic amino acids from inside due to the low intracellular concentration of sodium) responsible for this asymmetric exchange.

III. INHERITED DISEASES OF PLASMA MEMBRANE AMINO ACID TRANSPORT

This section deals with the inherited pathology due to defective amino acid transport in the plasma membrane of human cells. Table 10 summarizes the characteristics of the defective amino acid transport systems and the candidate genes for eight (including subtypes) of these diseases. They are all aminoacidurias and therefore affect the tubular reabsorption of specific amino acids. For background information (clinical, genetic, biochemistry, and physiology) about these diseases, see Reference 476 and OMIM (On-line Mendelian Inheritance in Men; http://www3.ncbi.nlm.nih.gov/omim/). Only one human amino acid transporter gene, rBAT (also named SLC3A1, for solute carrier family 3, member 1; OMIM no. 104614), has been shown to be responsible for one of these inherited diseases, cystinuria type I (see below). Very recently (49,

TABLE 10. *Inherited diseases of plasma membrane amino acid transport*

	Defective Amino Acid Transport System			Candidate Gene			
Disease	Tissue affected	Characteristics of transport	Chromosome locus	Name	Amino acid transport system	Chromosome locus	Mutations
Cystinuria							
Classic							
Type I	Kidney and intestine	For CssC and aa$^+$ (luminal) (high affinity)	2p16.3-21	rBAT	Exchanger b$^{0,+}$-like	2p16-3-21	25 mutations in humans
Type II	Kidney and intestine	For CssC and aa$^+$ (luminal)	19q13.1 (?)		For CssC and aa$^+$ (?)		
Type III	Kidney (intestine?)	For CssC and aa$^+$ (luminal)	19q13.1		For CssC and aa$^+$ (low affinity) (?)		
Isolated	Kidney (intestine?)	For CssC (?)			For CssC (?)		
LPI	Kidney, intestine fibroblasts, hepatocytes	Efflux for aa$^+$ (antiluminal)	14q		For aa$^+$ (?)		
Hartnup disorder	Kidney and intestine	Na$^+$ dependent for aa^0 (luminal)		ATB^0 (?)	Na$^+$ dependent for aa^0 (luminal?)	19q13.3	
				hph2 (?)	Na$^+$ dependent for aa^0 (?)	11q13	hph2 mice
Iminoglycinuria	Kidney	Gly, Pro, OH-pro (high affinity) (?)					
Dicarboxylic aminoaciduria	Kidney (intestine?)	For aa$^-$ (luminal)		EAAT3 (?)	Na$^+$ and K$^+$ dependent for aa$^-$ (luminal?)	9p24	KO mice

KO mice refers to null EAAT3 knockout reported by Stoffel and co-workers (416). CssC, cystine; aa$^+$, dibasic amino acids; aa^0, zwitterionic amino acids; aa$^-$, dicarboxylic amino acids. LPI, lysinuric protein intolerance. Question marks indicate ascription not well established; dashes indicate almost complete lack of information.

296, 595), cystinuria type III (perhaps also type II) and LPI have been linked to chromosomes 19q13.1 and 14q, respectively. The tissue distribution and the transport characteristics associated with the expression of the sodium- and potassium-dependent zwitterionic amino acid transporter ATB^0 and the sodium- and potassium-dependent anionic amino acid transporter EAAT3 (see sect. II) make them good candidates for Hartnup disorder and dicarboxylic aminoaciduria, respectively (see Table 10). In addition, the dicarboxylic aminoaciduria developed by the null knockout EAAT3 mice (416) reinforces the putative role of EAAT3 in this inherited disease. For the rest of aminoacidurias due to defective renal reabsorption (i.e., isolated cystinuria, hyperdibasic aminoaciduria 1, isolated lyinuria, and iminoglycinuria), neither obvious candidate genes nor chromosomal location is known.

In addition to the above-mentioned inherited diseases, it is worth mentioning the putative responsibility of the EAAT2 glutamate transporter in the sporadic form of ALS. Amyotrophic lateral sclerosis is a chronic degenerative neurologic disorder characterized by the death of motor neurons in the cerebral cortex and spinal cord. About 90% of ALS is sporadic, and only 10% is familial; mutations in the superoxide dismutase-1 gene have been found in 15–20% of all familial cases (see entry no. 105400 in OMIM). Although the etiopathology of sporadic ALS is not known, it is hypothesized that glutamate excitotoxicity participates in the selective motor neuron degeneration of the disease (457). Amyotrophic lateral sclerosis is characterized by increased cerebrospinal fluid concentration of L-glutamate and L-aspartate and a marked and specific decrease (~70%) in the V_{max} of high-affinity glutamate uptake in synaptosomes from motor cortex and spinal cord (462). In this sense, decreased D-aspartate binding sites have been reported in the spinal cord of ALS specimens (492), suggesting a decreased number of glutamate transporters. Rothstein et al. (463) showed that this defect is specific to the glial EAAT2 transporter, the expression of which is reduced up to 95% in the motor cortex and spinal cord of postmortem samples from ALS patients. Despite the large loss of EAAT2 protein in those brain structures, the transcript levels of EAAT2 in motor cortex are not altered in ALS (66). The first mutational analysis study showed no mutations in the EAAT2 mRNA sequence of ALS patients (347). In contrast, Rothstein found aberrant EAAT2 RNA, including exon-skipping and intron-retension species, in 65% of sporadic ALS specimens (J. D. Rothstein, personal communication). An intron-retention species has a dominant negative effect on the stability of wild-type EAAT2 protein when expressed in COS cells. At present, the origin of these aberrant RNA species is unknown, and more likely, they are not genomic but rather due to aberrant RNA processing. Further research is needed to understand the mechanism underlying the aberrant EAAT2 RNA processing in the brain of ALS patients.

1. Cystinuria

Classic cystinuria (OMIM no. 220100) is an inherited hyperaminoaciduria of cystine and dibasic amino acids (for review, see Refs. 351, 487), discovered by Wollaston (604), and described as one of the first four "inborn errors of metabolism" by Garrod (163). Cystinuria is an autosomal recessive disease with an overall estimated prevalence of 1 in 7,000 neonates; prevalence estimations range between 1 in 2,500 neonates in Israeli Jews of Libyan origin and 1 in 100,000 in Sweden (487). In our opinion, these numbers are overestimations in screening programs, because of the nonsilent hyperaminoaciduria phenotype of cystinuria types II and III (see below). Because of the poor solubility of cystine, it precipitates to form kidney calculi that produce obstruction, infection, and ultimately renal insufficiency. Three types of classic cystinuria have been described (456): type I heterozygotes present normal aminoaciduria, whereas types II and III present high and moderate hyperaminoaciduria of cystine, lysine, and, to a lesser extent, arginine and ornithine. As a consequence of the intestinal amino acid transport defect, type I and II homozygotes do not show increase in the plasma levels of cystine after an oral administration of the amino acid. In contrast, type III homozygotes show a nearly normal increase in the plasmatic levels of cystine after the oral dose. This suggests that the amino acid transport system affected in cystinuria type III is not expressed or is not very conspicuous in the intestine. Others (197, 377) divide cystinuria into two types: type I, or true recessive, and type II, or incomplete recessive (this includes the types II and III of Rosenberg; Ref. 456).

Dent and Rose (124) postulated that cystinuria may result from the defective function of a common uptake system for cystine and dibasic amino acids. Milne et al. (361) demonstrated a reduced intestinal absorption of dibasic amino acids in patients with cystinuria. Finally, transport studies in vitro demonstrated a defective accumulation of cystine and dibasic amino acids in biopsies of patients with cystinuria (111, 555). Interestingly, patients with cystinuria show no malabsorption of arginine when given in a peptide form; this suggested normal apical absorption of peptides in cystinuria and positioned the disease-associated transport defect at the apical membrane of the intestinal epithelium (21). As discussed in section II*D5*, there is an apical high-affinity amino acid transport system for cystine and dibasic amino acids that also shows interaction (*cis*-inhibition and heteroexchange) with zwitterionic amino acids, in the brush-border membranes of the epithelial cells of the proximal straight tubules of the nephron and of the small intestine. It is believed that this is defective in cystinuria (for review, see Refs. 408, 487). As described in section II, the transport characteristics of rBAT/$b^{o,+}$-like system and its tissue and subcellular distribution suggested the participation of rBAT in a high-affinity reabsorption system of cystine and dibasic amino acids in kidney and intestine and postulated rBAT as a good candidate gene for cystinuria. Mutational analysis of the rBAT gene of patients with cystinuria initially revealed six missense cystinuria-specific mutations; for one of these mutations (Met467Thr; see Fig. 8), defective amino acid transport activity was shown (see sect. II); this demonstrated that mutations in rBAT cause cystinuria and that rBAT/system $b^{o,+}$-like participates in the renal reabsorption of cystine and dibasic amino acids (78). Genetic analysis demonstrated linkage of cystinuria with chromosome 2p microsatellite markers (430), which colocalize with the rBAT gene locus in 2p16.3 (79). Further mutational analysis by several groups (for review, see Refs. 170, 408, 409) of the rBAT gene in Italian, Spanish, Middle Eastern, Eastern European, Canadian, Japanese, and United States populations revealed a growing number of cystinuria-specific mutations in the rBAT gene (25 mutations have been described, including missense, nonsense, splice-junction, deletions, and insertions; see Fig. 8). Four cystinuria-specific rBAT mutants have been shown to express defective amino acid transport activity (see sect. II*D4* and Fig. 8; Refs. 78, 91, 366). Mutations Met467Thr and R270X [stop codon at arginine residue 270; this eliminates two-thirds of the protein toward the COOH terminus; Miyamoto et al. (367) reported that deletions affecting the COOH terminus result in defective rBAT-expressed amino acid transport activity] represent approximately one-half of the cystinuric chromosomes where mutations have been detected. These mutations have been found in homozygosis in several patients and in compound heterozygotes with other mutations.

Clinical and physiological evidence suggested heterogeneity in cystinuria (see above). *1*) The oral cystine test may be indicative that in type III cystinuria the intestinal defect is not very conspicuous. *2*) Most of renal reabsorption of cystine occurs in segments S1-S2 of the nephron (i.e., in a tubular region other than that in which rBAT is expressed). Thus other cystine reabsorption system(s) not present (or not very conspicuous) in the small intestine may also be cystinuria genes (see sect. II*D5*). Mutational analysis suggested that only patients with type I cystinuria carried mutations in the *rBAT* gene (164, 217). Genetic linkage analysis with markers of the genomic region of rBAT in chromosome 2 and intragenic markers of rBAT have demonstrated genetic heterogeneity for cystinuria (80). The *rBAT* gene is linked to type I cystinuria, but not to type III (OMIM no. 600918). A wide search through the genome, carried out independently by two groups, localized type III (and perhaps also type II) cystinuria gene in patients from Italy and Israeli Jews with a Lybian origin to 19q13.1 (49, 595). We are currently analyzing this locus for the identification of a new cystinuria gene. In these studies, the phenotype classification of cystinuria (types I, II, and III) was based on the urine excretion values of cystine and dibasic amino acids in the obligate heterozygotes.

Cystinuria type I, the most frequent worldwide (i.e., >60% of the cases), is due to mutations in the *rBAT* gene. As discussed in section IID, this gene codes for a protein that most probably participates as a subunit of a heterodimeric $b^{o,+}$-like transporter. This activity is responsible for the high-affinity cystine and dibasic reabsorption in the S3 segment of the nephron and in the small intestine with a tertiary active transport mechanism coupled with the exchange of neutral amino acids. Interestingly, in the Italian and Spanish patients with cystinuria type I subjected to study, we have identified mutants only in ~50% of the cases. In the future, this figure may increase through the analysis of deletions in the *rBAT* gene (we are currently studying 3 putative new deletions), but it is possible that mutations in the *rBAT* gene will not explain all cystinuria type I chromosomes. In this sense, the putative light subunit of rBAT could also be envisaged as a type I cystinuria gene. From the cystinuria loading test (see above), we can speculate that the type III cystinuria gene would have a low expression in the small intestine. The transport system responsible for the high-capacity low-affinity reabsorption of cystine in segments S1-S2 of the nephron may be defective in cystinuria type III. In contrast, there is no obvious candidate gene for type II cystinuria. This is a rare (≤5% of the cystinuria cases) type of the disease, and its ascription to the 19q13.1 locus is at present not definitive, since this linkage, although significant, is based on a small number of cases (49) (see Table 10).

Whether mutations in rBAT (chromosome 2p16.3) and in the new type III cystinuria locus (chromosome 19q13.1) lead to a full-blown type I/type III cystinuria phenotype is still an open question. Initial mutational analysis suggested this genotypic/phenotypic interaction (164, 217). Linkage analysis with both cystinuria loci is currently in progress. Preliminary data suggest that cases of type III heterozygotes within the lower range of cystine and dibasic hyperexcretion values of these carriers (173) may be due to mutations in the *rBAT* gene.

Finally, Brodhel et al. (67) reported isolated cystinuria (OMIM no. 238200) in two siblings of unrelated parents (see Table 10), in which urinary hyperexcretion of amino acids was restricted to cystine. This suggested that a cystine renal transporter not shared with dibasic amino acids was defective in these patients (for review, see Ref. 487). Biochemical evidence for this transporter has not been obtained in renal or intestinal transport studies (496). It is therefore possible that a rare allele either of the rBAT gene or of the cystinuria gene in 19q13.1 may be responsible for this phenotype (i.e., a mutant affecting cystinuria transport but not dibasic amino acid transport). To our knowledge, linkage and/or mutational analysis of the *rBAT* gene and the 19q13.1 cystinuria locus in isolated cystinuria has not been reported.

2. Other dibasic aminoacidurias

There are four diseases in which a cationic amino acid transport defect is suspected (for a review, see Ref. 497): *1*) cystinuria (see above); *2*) LPI, hyperdibasic aminoaciduria type 2, or familial protein intolerance (OMIM no. 222700); *3*) hyperdibasic aminoaciduria type 1; and *4*) isolated lysinuria. Lysinuric protein intolerance is an autosomal recessive trait. Almost one-half of the known LPI patients (~100) are from Finland, where the prevalence of the disease is 1 in ~60,000. In contrast, hyperdibasic aminoaciduria type 1 (autosomal dominant trait) and isolated lysinuria have been described only in one French Canadian pedigree and in a Japanese patient, respectively. Lysinuric protein intolerance was first described by Perheentupa and Visakorpi (418). In addition to hyperdibasic aminoaciduria, the clinical symptoms of LPI are failure to thrive, protein aversion, short stature, hepatomegaly, osteoporosis, hyperammonemia, common interstitial lung disease, and renal damage, and occasionally moderate mental retardation. It is believed that the disease is caused by a defective dibasic amino acid transport that is expressed at the basolateral membrane of the renal and intestinal epithelia, and in nonepithelial cell types (e.g., culture fibroblasts, hepatocytes) (for review, see Ref. 497). An oral loading administered to LPI patients with the dipeptide lysyl-glycine increased plasma glycine concentrations properly, but plasma lysine remained almost unchanged; this indicated unaffected apical peptide absorption and cellular hydrolysis and suggested the basolateral location of the defective cationic amino acid transport (443). In agreement with this, transport studies with jejunal LPI biopsy samples showed that the transport defect is situated at the basolateral plasma membrane (125).

In an interesting study, Scriver, Simell, and co-workers (501) reproduced in LPI fibroblast cell lines the cationic amino acid transport defect; LPI fibroblasts showed a reduced *trans*-stimulated efflux of cationic amino acids. This defect showed gene-dosage effect (homozygotes more affected than heterozygotes). It is believed that the defective cationic amino acid transport activity corresponds to system y^+, but unfortunately, this has not been carefully characterized. Cationic amino acids are transported through the plasma membrane of human fibroblasts via systems y^+ and y^+L (see sect. I) (Torrents and Palacín, unpublished data).

Very recently, Simell, Aula, and co-workers (296) reported a locus on chromosome 14 for LPI in Finnish patients; linkage disequilibrium in markers within this locus suggests that LPI in these patients is due to one historical mutation. The hyperdibasic aminoaciduria characteristic of LPI has fostered studies on the involvement of the known cationic amino acid transporter in this disease. Unfortunately, none of the known proteins involved in cationic amino acid transport seems to be responsible for LPI. Indeed, human CAT-1 (chromosome 13q12–14), CAT-2 (chromosome 8p21.3–22), and CAT-4 (chromosome 22q11.2) (see Table 1) have been excluded from linkage to the LPI phenotype in Finnish patients (296). Similarly, mutational and linkage analysis excluded human CAT-1, CAT-2, and CAT-4 as LPI genes among Italian or Japanese LPI patients (128, 224, 485). The recently described rat CAT-3 (219), for which no human counterpart has been cloned (see Table 1), is expressed exclusively in brain and therefore does not represent a candidate gene for LPI. Two other proteins are known to be associated with cationic amino acid transport: rBAT and 4F2hc (see sect. IID). The *rBAT* gene is expressed in kidney and intestine, and it is associated with the cystinuria phenotype (see above). In contrast, the putative role of 4F2hc in the renal reabsorption and intestinal absorption (basolateral efflux of cationic amino acids by exchange with zwitterionic amino acids plus sodium; see sect. II*D5*) makes it a good candidate for LPI. In addition, 4F2hc is expressed in fibroblasts (Torrents and Palacín, unpublished data), where the LPI transport defect has been substantiated (501). 4F2hc does not seem to be directly involved in LPI, since, as indicated above, LPI gene localizes to chromosome 14q (296), and the human *4F2hc* gene localizes to chromosome 11q12–13 (174). However, a role of 4F2hc in LPI cannot be ruled out. As mentioned in section II*D*, there is evidence that the functional unit of 4F2hc/system y^+L-like transporter is composed by 4F2hc (heavy chain) plus an unidentified light subunit (142). This putative light subunit might be envisaged as an LPI gene. The identification of the LPI gene in the 14q locus (already restricted to 100 kb) and/or the cloning of the putative light subunit of 4F2 surface antigen may clarify this issue in the near future.

3. *Hartnup disorder*

This disorder (OMIM no. 234500) was first described by Baron et al. (35). It is transmitted as an autosomal recessive trait, and it is characterized by a pellagra-like light-sensitive rash (niacin deficiency), cerebellar ataxia, emotional instability, and aminoaciduria. This is a characteristic aminoaciduria that involves the zwitterionic amino acids (with the exception of cysteine/cystine, glycine, methionine, and the imino acid proline) and that occurs at a frequency of 1 in ~40,000 in urine amino acid screens (for review, see Ref. 304). Most of the hyperexcretors never display the niacin deficiency symptoms, and therefore, the Hartnup disorder is usually benign (477, 300). Urinary hyperexcretion occurs with normal amino acid plasma levels. Some patients have elevated fecal amino acid levels and secondary metabolites of the excess of tryptophan in the urine, plasma, and feces as well as reduced transient plasma levels increase after an oral load of zwitterionic amino acids. Then, the disorder appears to involve a renal reabsorption defect, and in some patients intestinal malabsorption of zwitterionic amino acids (for review, see Ref. 304). Studies with brush border of intestinal mucosa biopsies, but not with leukocytes or fibroblasts, from Hartnup patients showed defective zwitterionic amino acid transport (178, 493, 532, 545).

Scriver and co-workers (474, 477) have proposed that Hartnup disorder is an amino acid transport single gene defect affecting kidney and intestine, with a variant form affecting only the kidney; in contrast, the pathological state associated with the disease (niacin deficiency) seems to be multigenic. These authors suggest that other genes that control plasma amino acid homeostasis may influence the occurrence of clinical abnormalities with the Hartnup biochemical defect. The disease symptoms occur with low aggregate plasma amino acid levels and nutritional stress (malnutrition, diarrhea).

Very recently, Dove and co-workers (528) developed a mouse model for Hartnup disease (hyperphenylalaninemia 2; hph2) by *N*-ethyl-*N*-nitrosourea mutagenesis and screening for delayed plasma clearance of an injected load of phenylalanine. The hph2 is a recessive mutation that causes a deficient amino acid transport that is similar but not identical to Hartnup disease. Like Hartnup patients, the hph2 homozygotes show *1*) specific urinary hyperexcretion of many of the zwitterionic amino acids, while plasma concentrations of these amino acids are normal; *2*) a partial deficiency in the sodium-dependent uptake of glutamine in brush-border membrane vesicles; and *3*) a niacin-reversible syndrome influenced by diet and genetic background. In contrast to Hartnup patients, hph2 homozygotic mice show urine hyperexcretion of arginine, a mild urine hyperexcretion of tryptophan and valine, and significant urine hyperexcretion of methionine.

Dove and co-workers (527) mapped hph2 to a region of mouse chromosome 7 synthenic with human chromosome 11q13 (see Table 10). Interestingly, 4F2hc, the putative subunit of the amino acid transport system y^+L-like, also maps to this locus (see Table 1, rBAT). This amino acid transporter-related protein has been suggested as a candidate gene for the Hartnup disorder (304). In our opinion, the amino acid transport associated with 4F2hc expression in oocytes (systems y^+L-like or L-like, depending on the authors; see sect. II*D5*), and the almost ubiquitous tissue distribution and the renal basolateral localization of 4F2hc (see sect. II*D*) weakens the candidature of this gene for this disorder. In any case, because of the hitherto unclear physiological role of 4F2hc in amino acid transport (see sect. II) and the dissimilar aminoaciduria phenotypes (i.e., urine excretion values of arginine, tryptophan, valine, and methionine; see above) of the hph2 mice with Hartnup patients, it will be very informative to answer the following questions: *1*) What renal amino acid transport activity is defective in the the hph2 mice? *2*) Does the hph2 locus contain the mouse *4F2hc* gene? If the answers to these questions reinforce the candidature of *4F2hc* for the hph2 phenotype, it should be assessed directly.

The transport characteristics (sodium-dependent zwitterionic amino acid transport) and the epithelial distribution of ATB° (see sect. II*C*) fit those expected for the transporter responsible for the Hartnup disorder (Table 10). In contrast to the human synthenic locus of the hph2 mouse mutation (chromosome 11q13), the *ATB°* gene localizes to 19q13.3 (see Table 6). Therefore, *ATB°* does not seem to hold the hph2 mutation. In our opinion, because of the nonidentical aminoaciduria phenotype of Hartnup disorder and hph2 mutation, there is still room for a role of the *ATB°* gene in the Hartnup disorder. First, direct evidence for the apical localization of the ATB° transporter in renal and intestinal epithelia and for an active transport mechanism for the amino acid transport activity associated with ATB° should be offered. Then, direct genetic analysis of the *ATB°* gene (mutational and/or linkage studies) in Hartnup's aminoaciduria families should be addressed.

4. Iminoglycinuria

Familial iminoglycinuria (OMIM no. 242600) is a benign inherited defect of membrane transport (for review, see Ref. 89). It involves a glycine, L-proline, and hydroxy-L-proline transporter in the renal tubule and, in some cases, in the epithelial intestine. There are no reports of the prevalence of this disease, but it seems more frequent in Ashkenazim (see OMIM). As for other systems of renal reabsorption of amino acids, the reabsorption of these amino acids matures during the first months of life. The persistence of iminoglycinuria beyond 6 mo is considered abnormal. In addition to familial iminoglycinuria, this urinary hyperexcretion phenotype also occurs in familial hyperprolinemia and hyperhydroxyprolinemia, and in the generalized disturbance of membrane transport of the Fancony syndrome. In contrast to these, urine hyperexcretion of glycine, L-proline, and hydroxy-L-proline in familial iminoglycinuria is specific to these amino acids and occurs with normal levels of these amino acids in plasma. For glycine and these imino acids, the endogenous renal clearance rates are high, and the net reabsorption decreased in familial iminoglycinuria probands (reviewed in Ref. 89).

The iminoglycinuria phenotype is autosomal recessive, but in some pedigrees, there is an incomplete recessive phenotype; of 16 familial iminoglycinuria pedigrees reviewed by Chesney (89), in 9 pedigrees the obligate heterozygotes show hyperglycinuria without prolinuria. In addition, Greene et al. (177) reported a family in which the father and two sons had hyperglycinuria. The renal tubular titration curve for proline reabsorption in one of the sons was compatible with a mutation affecting the affinity of the proline transporter. This "K_m" variant has been designated iminoglycinuria type II (OMIM no. 138500). It is believed that all these variants are allelic: the same renal phenotype is observed in probands inheriting two recessive mutant alleles, two hyperglycinuric alleles, or two different alleles (475).

There is evidence of two sodium-dependent proline transport systems in the brush border of human renal cortex (154): a high-affinity system shared with glycine and a low-affinity system not shared with glycine. Studies in rat, dog, and rabbit kidneys (reviewed in Ref. 89) revealed two sodium-dependent transport systems for imino acids and glycine, one of high affinity and specific for these substrates, and the other with low affinity and with broad specificity with other zwitterionic amino acids. For glycine, two apical sodium-dependent transport systems have been described: a high-affinity low-capacity system located in the proximal straight tubules and a low-affinity high-capacity system in the proximal convoluted tubule. Notice the similarity with the proposed renal reabsorption systems for cystine (see sect. II*D5*). It is hypothesized that the defective transport system is low-capacity high-affinity for glycine and the two imino acids in the proximal straight tubule, but there is no direct proof of this (89). Ontogeny in humans also gives clues to the amino acid transport systems serving the renal reabsorption of these amino acids (reviewed in Ref. 89): *1*) maturation of the renal reabsorption of glycine and proline occurs at different times after birth. *2*) In contrast to controls, iminoglycinuria homozygotes have an almost complete absence of tubular reabsorption for proline and glycine; with maturation of the tubular function, reabsorption of proline and glycine appear independently. *3*) In rats, the postnatal prolinuria is associated with low activity of a high-affinity sodium-dependent nephron transport system. This and additional evidence suggest that ontogeny is associated with deficient activity of high-affinity systems for imino acids and glycine that does not include the system controlled by the familial iminoglycinuria gene (89).

Unfortunately, there is no genetic information of a chromosome locus for the familial iminoglycinuria phenotype. The transport characteristics of the expected amino acid transport system defective in this phenotype fit that of the IMINO and Proline transport systems (see sect. I). Three cDNA and their splice variants, which belong to the superfamily of sodium- and chloride-dependent neurotransmitter transporters, GLYT1, GLYT2, and PROT (see sect. II), transport glycine and/or proline with characteristics of these systems (see Table 5). At present, it seems that GLYT2 and PROT are specific to the CNS, and only a peripheral tissue distribution has been demonstrated for GLYT1 (see Table 5). The splice variant 1a of GLYT1 is expressed in kidney and other peripheral tissues; in lung, spleen, and liver, there is evidence that GLYT1–1a is expressed in macrophages and not in the parenchymal cells (62). To our knowledge, there are no data on the subcellular distribution of GLYT1–1a in kidney. If this transporter is expressed in the apical pole of the tubular epithelium, it would be a good candidate for the familial iminoglycinuria phenotype. It is worth mentioning that L-proline uptake in renal brush border is chloride dependent in addition to sodium dependent (478). The human *GLYT1* gene localizes to chromosome 1p31.3-p32 (see Table 4). Linkage studies of the familial iminoglycinuria phenotype would be the first step to contrasting this hypothesis.

5. *Dicarboxylic aminoaciduria*

Teijema et al. (552) reported the first case of dicarboxylic aminoaciduria (OMIM no. 222730) in a female child, most probably due to an anionic amino acid transport defect in kidney and intestine. To our knowledge, only two other cases have been reported (355, 526). Swarna et al. (526) found one of these by screening for amino acid disorders in 500 mentally retarded children in India. Melancon et al. (355) detected in a neonatal screening program a boy with massive glutamic and aspartic aminoaciduria. The boy was apparently healthy at the age of 3 years. Amino acid clearance studies revealed the presence of renal wastage of dicarboxylic amino acids. Intestinal transport and in vitro oxidation of dicarboxylic amino acids were found to be intact. The same group later reported (356) reduced uptake velocities of glutamate and aspartate in dicarboxylic aminoaciduria fibroblasts.

The neuronal and peripheral high-affinity glutamate transporter EAAT3 (see Tables 6 and 7) is an obvious candidate for the transporter defective in dicarboxylic aminoaciduria (see sect. IIC). This transporter is highly expressed in kidney and in epithelial small intestinal cells (19, 245). Indeed, EAAT3 is the only known anionic transporter in kidney and intestine (see Table 7). Finally, Stoffel and co-workers (416, 517) reported that null knockout EAAT3 mice develop dicarboxylic aminoaciduria. This clearly substantiates the role of EAAT3 transporter in the renal reabsorption of anionic amino acids and in addition suggests *EAAT3* gene (chromosome 9p24; see Table 6) as the immediate candidate for dicarboxylic aminoaciduria (Table 10). At this stage, because of the low number of disease cases described, the obvious next step is to search for mutations of the *EAAT3* gene in patients with dicarboxylic aminoaciduria.

IV. PROSPECTS

We are halfway toward the identification of the genes coding for the transporters that mediate the amino acid flux across the plasma membrane of mammalian cells. Relevant amino acid transport systems, like systems A, L, N, and x_c^-, have not been identified at the molecular level. We are just beginning to understand the molecular bases of the human inherited diseases of amino acid transport: mutations in the *rBAT* gene cause cystinuria type I. On the other hand, the first knockouts for amino acid transporters have been produced, by homologous recombination for the cationic amino acid transporter CAT-1 and for the glutamate transporters EAAT2 and EAAT3, and by antisense technology for the glutamate transporters EAAT1, EAAT2, and EAAT3 in brain and for the rBAT/system $b^{o,+}$-like in epithelial renal cells. We can envisage a final goal in this line of research: the ascription of every amino acid transporter and the cognate transport system to the macroscopic fluxes of amino acids across the plasma membrane of mammalian cells.

The amino acid transporters cloned can be grouped in four protein families, and for many amino acid transport systems, several transporter isoforms have been identified. This has revealed a high complexity in mammalian amino acid transport. A relevant question, then, is what are the key structural elements that explain amino acid transport mechanisms at the molecular level? After the cloning of the first mammalian amino acid transporters, a growing number of studies based on site-directed mutagenesis and chimera constructions are being reported. These studies, although valuable, show a weakness, the lack of knowledge of the three-dimensional structure of these transporters sitting in the plasma membrane. There is no doubt that an enormous challenge in this line of research is the resolution of the amino acid transporter structures at the A° scale, as for aquaporin-1 (587).

Finally, two amino acid transport systems, $b^{o,+}$-like and probably y^+L-like, could be a heterodimeric structure composed of rBAT or 4F2hc, respectively, plus the corresponding as yet unidentified subunit. If this hypothesis is proven, these transporters will be the first known transporters for organic solutes with a heteroligomeric structure.

In the present decade, molecular biology has reached mammalian amino acid transport; now we are on the way to explaining interorgan amino acid flux at the molecular level.

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Treball de revisió 4

Cystinuria calls for heteromultimeric amino acid transporters

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El doctorand va escriure les lectures recomenades, va participar de forma activa en la búsqueda d'informació i també en la correcció i revisió de l'article.

Cystinuria calls for heteromultimeric amino acid transporters

Manuel Palacín*, Raúl Estévez and Antonio Zorzano

The proteins rBAT (related to $b^{o,+}$ amino acid transporter) and 4F2hc (the heavy chain of the surface antigen 4F2) are homologous proteins that induce amino acid transport in *Xenopus* oocytes. The role of rBAT in amino acid transport is substantiated by the fact that mutations in the gene encoding it cause cystinuria, a heritable disease characterised by high concentrations of cystine in the urine. Structural and functional evidence supports the hypothesis that both rBAT and 4F2hc proteins form part of heterodimeric amino acid transporters. There is new evidence that the functional unit of system y^+L amino acid transporter is a disulfide bridge-dependent complex of 4F2hc with a *Xenopus* oocyte plasma membrane protein.

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Abbreviations

4F2hc	4F2 heavy chain
FRP-1	fusion regulatory protein-1
NEM	*N*-ethyl maleimide
rBAT	related to $b^{o,+}$ amino acid transporter

Introduction

Classic cystinuria is an inherited hyperaminoaciduria of cystine and dibasic amino acids. Because cystine is poorly soluble, it precipitates in the kidney tubules to form calculi that produce obstruction, infection and ultimately renal insufficiency. Cystinuria is a result of defective amino acid transport that also affects intestinal absorption of cystine and some other amino acids, including arginine (for an extensive review see [1]). In contrast, cystinuria patients show no malabsorption of arginine when given this amino acid orally in an oligopeptide form [2]. This shows that peptide absorption is not affected in cystinuria and points to a disease-associated transport defect of amino acids at the apical plasma membrane of the intestinal epithelium. Dent and Rose [3] were the first to postulate that cystinuria may result from the defective function of a common uptake system for cystine and dibasic amino acids. Segal's group then [4] described a high-affinity transport system for cystine and dibasic amino acids in renal brush-border plasma membrane vesicles. Full characterization of the defective amino acid transport system in renal or intestinal biopsies is still missing.

In 1992, three labs working independently identified by expression cloning a renal cDNA (named rBAT for the rabbit clone, and D2 or NBAT for the rat clones) that was also expressed in small intestine, and that induced high-affinity transport of cystine and dibasic amino acids upon expression in *Xenopus laevis* oocytes [5–7]. Two years later, six missense mutations of the rBAT gene (SLC3A1 in the genome database [GDB]) in patients with cystinuria were reported [8], as was genetic linkage between cystinuria and the rBAT chromosomal region [9]. Today, 32 cystinuria-specific mutations of rBAT have been described worldwide in patients with cystinuria (for review see [10] and for descriptions of new mutations see [11–13]). These mutations include include missense, splice site and nonsense mutations, as well as deletions and insertions (Figure 1). Defective amino acid transport has been reported for seven of these missense mutations when expressed in *X. laevis* oocytes [8,11,14•,15]. Therefore, mutations in the rBAT gene appear to be the genetic cause of cystinuria.

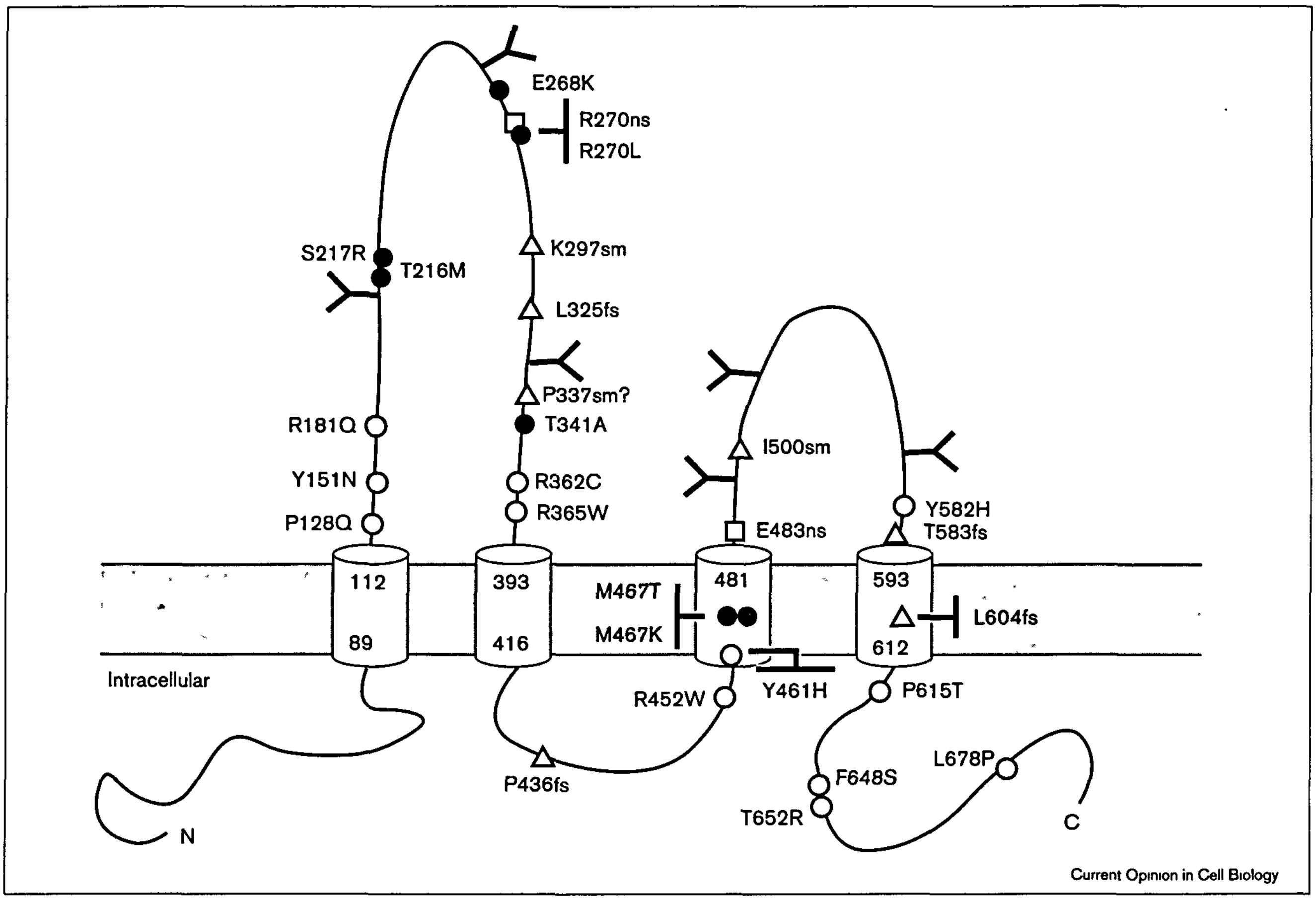
The surface antigen 4F2hc (also named CD98) is homologous to rBAT and also induces amino acid transport activity in *Xenopus* oocytes [16–18]. Here we discuss the evidence that supports the hypothesis that rBAT and 4F2hc form part of two different heterodimeric amino acid transporters.

Two related proteins with an unusual structure induce amino acid transport in oocytes

The homology between rBAT and the cell surface antigen 4F2hc encouraged expression studies of 4F2hc amino acid transport (Table 1; human rBAT and 4F2hc proteins show ~30% identity and ~50% similarity [19–22], and four out of nine exon–intron boundaries of the human rBAT gene are conserved in the human 4F2hc gene; for review see [10]). As expected from its protein sequence homology with rBAT, human 4F2hc induced amino acid transport activity in *Xenopus* oocytes [16,17]. Later, other groups confirmed and extended these results to rat 4F2hc [18].

Hydrophobicity algorithms suggest that rBAT and 4F2hc contain a single transmembrane domain towards the amino terminus of each protein [5,7]. Both proteins are *N*-glycosylated integral proteins of the plasma membrane [7,19,23,24], and are therefore suspected to be type II membrane glycoproteins (these have an intracellular amino terminus, a single transmembrane domain and a bulky extracellular carboxyl terminus). This is in contrast to the well-known multispanning structure of membrane transporters of substrates of polar nature. Tate's group [25], however, proposed that rBAT crosses the plasma membrane four times, with the first transmembrane domain

Figure 1



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This four transmembrane domain topological model of the rBAT protein is based on studies by Tate's group [25] (amino acid residue numbers shown inside the cylinders indicate the limits of the transmembrane segments). Thirty two cystinuria-specific mutations in the rBAT gene have been reported (for review see [10], and for newly described mutations see [12,13]). This includes 19 missense (○,●), two nonsense (□, ns), three splice (△, sm) and four frame shift (△, fs) mutations, and four large deletions (not shown): Δ38–436, Δ66–525, Δ144–255 (amino acid residue numbers indicate the limits of these deletions) and Δ298-? (the carboxy-terminal limit of this deletion is not yet known). The ● denotes the seven missense mutations for which defective amino acid transport activity in oocytes has been reported. Mutations are indicated using the single letter code for amino acids. P337sm might be a splice mutation but this has not been conclusively proven. The Y shape indicates six potential *N*-glycosylation sites, and the amino (N) and carboxyl (C) termini are labelled.

being the same as predicted by the hydrophobicity algorithms, but with three additional amphipathic transmembrane domains (Figure 1). This model is based on limited proteolysis studies and peptide-specific antibody detection of permeabilized cells expressing the rBAT protein. These results await confirmation with different approaches, and no similar studies have been conducted with 4F2hc. Whichever model is correct, however, it seems that rBAT and 4F2hc are not hydrophobic enough to provide a polar pathway for the movement of amino acids across the plasma membrane.

The low hydrophobicity of rBAT and 4F2hc proteins prompted the hypothesis that they may be subunits of heteromultimeric transporters [5,7,16]. Thus, induction of amino acid transport in *Xenopus* oocytes by expression of these proteins would be a result of constitution of holotransporters using additional 'silent' subunits already present in the oocytes. A similar mechanism of expression has been described for the Na^+/K^+ ATPase and multimeric channels [26–29]. Consistent with this hypothesis, rBAT and 4F2hc do form part of heterodimeric structures.

The surface antigen 4F2 is a heterodimer of ~125 kDa composed of a heavy chain of ~85 kDa (4F2hc, the rBAT-homologous protein) and a light subunit of ~40 kDa. The two subunits are linked by disulfide bridges [24]. This light subunit has not been microsequenced or cloned. Similarly, Wang and Tate [30] showed, in nonreducing conditions, high molecular weight complexes of rBAT in renal and intestinal brush border preparations and in *Xenopus* oocytes [30]. In our hands, renal rBAT is immunodetected as complexes of

Table 1

Comparison between human rBAT and 4F2hc genes and proteins, and their associated amino acid transport activities.

	Human rBAT	Human 4F2hc
Gene information		
Chromosome	2p16.3–p21	11q12–q13
Number of exons*	10	9
Protein information		
Amino acid residues†	685	529
Proposed transmembrane domains§	1 or 4	1
Electrophoretic mobility		
Reducing conditions	~90 kDa	~85 kDa
Nonreducing conditions	~125 kDa and >240 kDa	~125 kDa
Disulfide-bridge-linked 'light subunit'	Putative size of 40–50 kDa	~40 kDa
Tissue expression	Kidney (proximal straight tubule) and small intestine	Broad
Subcellular localization in epithelial cells	Brush-border plasma membrane	Basolateral plasma membrane
Induced amino acid transport activity in oocytes	System $b^{o,+}$-like (NEM-resistant)	System y^+L-like
	Na^+-independent transport for neutral and positively charged amino acids (NEM-sensitive)	System L-like
	Na^+-dependent transport for histidine	

References of the indicated characteristics of human rBAT and 4F2hc are quoted in a recent review [10]. *Four out of ten exon–intron boundaries of human rBAT gene are conserved in the human 4F2hc gene. †Human rBAT and 4F2hc proteins share ~30% identity (50% similarity). §Hydrophobicity plots of human rBAT and 4F2hc proteins are very alike, with a clear putative transmembrane domain towards the amino terminus of these proteins Tate's group [25] proposed three additional amphipathic transmembrane domains.

~240 kDa and ~125 kDa in nonreducing conditions; in two-dimensional gels these complexes contribute to the 90 kDa rBAT band seen in reducing conditions [31]. It seems, therefore, that like 4F2hc, rBAT forms a heterodimeric structure (~125 kDa) of a 'heavy chain' (90 kDa) linked by disulfide bridges to a putative 'light chain' of 40–50 kDa (Table 1). These heterodimeric structures might be the basic functional unit of these transporters. This may explain why all the attempts to express amino acid transport activity by transfecting rBAT into mammalian cells failed (COS cells [25,31]; MDCK cells, D Torrents and M Palacín, unpublished data). In these transfection experiments, the previously mentioned 125 kDa rBAT complex was not detectable, suggesting that the 'light subunit' of rBAT is needed for its transport function.

rBAT and 4F2hc induce multiple amino acid transport components in *Xenopus* oocytes

The characteristics of the amino acid transport activity associated with rBAT and 4F2hc have mainly been studied in *Xenopus* oocytes. Initially, we reported [5] that rBAT induces a single system of amino acid transport in oocytes: a high-affinity (micromolar range), sodium-independent transport system for cystine, dibasic amino acids and some zwitterionic amino acids, with characteristics similar to those of system $b^{o,+}$ (transport system with a broad [b] specificity for zwitterionic [o] and dibasic [+] amino acids), which was initially described by Van Winkle's group as occurring in mouse blastocysts [32]. Similarly, the first expression studies suggested that 4F2hc induces the expression of a single amino acid transport system in *Xenopus* oocytes with characteristics of system y^+L (transport system with typical substrates L-lysine [y+] and L-leucine [L]) [16,17,33]. This transport system, initially described by Devés *et al.* [34] as existing in erythrocytes, has a substrate specificity similar to that of system $b^{o,+}$: that is, a sodium-independent high affinity (micromolar range) for dibasic amino acids and sodium-dependent high-affinity (micromolar range) for zwitterionic amino acids. System y^+L (4F2hc) does not transport cystine in contrast to system $b^{o,+}$-like (rBAT).

In contrast with this initial view, several groups have reported induction of a different amino acid transport system upon expression of rBAT and 4F2hc in oocytes. Ahmed's and Taylor's groups [35,36•] proposed that, when expressed in *Xenopus* oocytes, rBAT induces the expression of the above-mentioned *N*-ethyl maleimide (NEM)-resistant system $b^{o,+}$, and an NEM-sensitive sodium-independent transport activity with overlapping specificities for dibasic and zwitterionic amino acids, as well as sodium-dependent transport for L-histidine. Similarly, Broër's group [18] showed that 4F2hc induces, in addition to system y^+L, a transport activity with characteristics of system L (sodium-independent transport for zwitterionic amino acids) in *Xenopus* oocytes. These results suggest that rBAT and 4F2hc do not have an intrinsic amino acid transport activity by themselves, and that interaction with oocyte transport subunits results in the induction of different amino acid transport activities upon expression of rBAT and 4F2hc.

The physiological role of rBAT

The multiple amino acid transport activities induced by rBAT and 4F2hc raise a new question: what is the physiologically relevant amino acid transport activity associated with these proteins? For rBAT this seems to be clear: it has a role in the $b^{o,+}$-like transporter system and in renal reabsorption and intestinal absorption of cystine and dibasic amino acids. This role is based on the following observations. Firstly, the rBAT gene is expressed mainly in kidney and intestine; in rat kidney the rBAT protein localizes to the brush-border plasma membrane of the epithelial cells of the proximal straight tubule [37,38]. Secondly, the $b^{o,+}$-like system induced by rBAT in oocytes is an obligatory exchanger with a high affinity for cysteine, as well as dibasic and some neutral amino acids, that preferentially mediates the influx of dibasic amino acids and the efflux of zwitterionic amino acids [39–41]. An amino acid transport system with similar characteristics has been described in the brush-border plasma membrane of the epithelial cells of the proximal straight tubule (see [10] for review). Thirdly, transfection of rBAT antisense sequences resulted in the partial and specific knockout of the system $b^{o,+}$-like amino acid transport activity present in the apical plasma membrane of the epithelial renal cell line OK (opossum kidney) [42]. Finally, mutational and linkage studies have demonstrated that mutations in the rBAT gene cause cystinuria (see Introduction). This all suggests that the amino acid transport systems other than system $b^{o,+}$-like that are induced by rBAT in oocytes may be due to an artifactual interaction between rBAT and silent oocyte amino acid transporters that are similar to system $b^{o,+}$-like.

Multiple physiological roles for 4F2hc?

In contrast to rBAT, our knowledge of the physiological role of 4F2hc is less clear. Attempts using antisense strategies to clarify the amino acid transport activity associated with 4F2hc in cells naturally expressing it were not conclusive (see [10] for review). In addition no human disease has yet been connected with the putative amino acid transport activities induced by 4F2hc in oocytes. It is possible that 4F2hc is associated with different amino acid transport activities that have overlapping substrate specificities (see Table 1). Thus, it might be that different 'light subunits' associated with 4F2hc by disulfide bridges confer different amino acid transport activities. This remains an unanswered question.

In addition to the above mentioned role of 4F2hc in amino acid transport, this protein may have other functions. In the past few years 4F2hc has also been implicated in cell fusion as fusion regulatory protein-1 (FRP-1) [43] and in the regulation of hematopoietic cell survival or death [44]. The role of 4F2hc in cell fusion might involve integrin function. Very recently 4F2hc has been implicated in the regulation of integrin function [45••]. Expression of 4F2hc (also named CD98) complements dominant suppression due to the overexpression of an integrin β1 cytoplasmic domain. Furthermore, 4F2hc co-immunoprecipitates with active β1 integrins, and antibody-mediated crosslinking of 4F2hc stimulates β1-integrin-dependent cell adhesion. Moreover, antibodies to β2 integrin blocked cell aggregation induced by antibodies to FRP/4F2hc, and antibodies to β1 integrin blocked polykaryocyte formation induced by antibodies to FRP/4F2hc [46]. This issue is not yet clear, however, because other proteins also associate with 4F2hc. Thus, FRP-1/4F2hc and cytoskeletal proteins (e.g, actomyosin, vimentin and heat shock cognate protein 70 [hsc70] are co-immunoprecipitated by anti-FRP-1/4F2hc antibodies [47], and anti-FRP-1/4F2hc antibodies change the immunofluorescence pattern of these cytoskeletal proteins [47]. It is therefore difficult at present to ascertain whether anti-FRP-1/4F2hc antibody-mediated cell fusion events are due to direct or indirect effects via changes in the cell surface distribution or conformation of other proteins. The role of the interaction of 4F2hc (FRP-1) with other proteins (e.g. cystoskeletal proteins) in any of the putative functions of 4F2hc is also unknown.

The analysis of the molecular defect of cystinuria calls for rBAT-associated proteins related to amino acid transport function

Defective amino acid transport resulting from the expression of seven human cystinuria-specific rBAT missense mutations has been reported for *Xenopus* oocytes [8,11,14•,15] (Figure 1). Some of these mutants show a very low residual activity (< 20% for Met467→Lys and Arg270→Leu [11,14•]), and for some of these mutants, the activity is dependent totally or partially on the amount of cRNA injected into the oocytes and the time of expression; that is, the higher the injected dose and longer the time of expression, the higher the residual transport activity. This is the case for Met467→Thr (the most frequent cystinuria Type I/I mutant (see below) known worldwide representing ~26% of the abnormalities seen in this type of patient; for review see [10]), Met467→Lys and Ser217→Arg [11,14•]. Two of these mutants (Met467→Thr and Met467→Lys) have been analyzed in depth. This study revealed a plasma membrane trafficking defect [14•]. These mutant proteins do not achieve a full maturation in the oocyte, as shown by endoglycosidase H digestion, and reach the plasma membrane slowly and inefficiently, as revealed by surface biotinylation studies. Long oocyte expression periods (more than three days after injection) and injection of oversaturating amounts of mutant rBAT cRNAs result in total (for Met467→Thr) or partial (≤20% activity of the wild-type protein for M467→Lys) recovery of the induced amino acid transport activity.

We believe that these conditions of long expression periods and saturating amounts of mutant rBAT cRNA overcome the protein quality-control machinery of the oocyte. Interestingly, when the amino acid transport activity induced by the Met467→Thr mutant is recovered, the amount of Met467→Thr on the oocyte surface is less than 10% of the corresponding wild-type protein; this suggests that an oocyte 'factor' (in our hypothesis, the rBAT 'light subunit') limits the expression of system $b^{o,+}$-like activity when oversaturating amounts of rBAT cRNA are expressed. Similar studies have not yet been conducted with other mutants, such as Ser217→Arg.

An interesting study with a mutant form of rBAT, which is not associated with cystinuria, also invokes oocyte 'factors' for rBAT-induced amino acid transport activity. Miyamoto and co-workers [48] showed that a carboxyl terminus deletion (Δ511–685) of human rBAT, which eliminates the fourth putative transmembrane-domain (Figure 1), induces in oocytes a decreased amino acid transport activity that resembles that of 4F2hc/system y$^+$L-like (that is, Na$^+$-independent transport of dibasic amino acids and Na$^+$-dependent transport of zwitterionic amino acids). This suggests that the carboxyl termini of rBAT and 4F2hc might be relevant for the interaction with the oocyte 'factor', (i.e. the putative transporter or subunit).

In 1995, genetic heterogeneity for cystinuria was demonstrated [49]: genetic linkage with the rBAT locus (chromosome 2p16.3-p21) is only positive for cystinuria families where the obligate heterozygotes have no hyperaminoaciduria (Type I/I patients), whereas linkage was excluded from cystinuria families in which obligate heterozygotes show hyperexcretion of cystine and dibasic amino acids (non-Type I/I patients). Last year, two groups reported independently a genetic locus in chromosome 19q for non-Type I/I cystinuria [50•,51•]. One is tempted to suggest that the cystinuria non-Type I/I gene might be the 'light subunit' associated with rBAT.

4F2hc needs associated proteins in order to induce amino acid transport in oocytes

Saturation of the induction of system y$^+$L-like activity occurs with very low expression of 4F2hc at the *Xenopus* oocyte surface, measured both by immunofluorescence and plasma membrane freeze-fracture studies. Further, increased expression of the protein at the cell surface, by increased cRNA doses or days of expression, does not result in higher induction of system y$^+$L-like activity (R Estévez *et al*, unpublished data). This shows that only part of the 4F2hc present in the oocyte plasma membrane is functional and suggests that amino acid transport activity is limited by an endogenous factor or factors. On the other hand inactivation studies by covalent modification of external cysteine residues show that 4F2hc is intimately associated with a membrane oocyte protein for the expression of system y$^+$L amino acid transport activity (R Estévez *et al.*, unpublished data). The 4F2hc-induced system y$^+$L is inactivated by direct covalent modification of external cysteine residue(s), and pre-treatment with reducing agents increases sensitivity to this inactivation. Interestingly, the y$^+$L activity induced by a cysteineless mutant of 4F2hc is still inactivated by direct covalent modification of external cysteine residue(s). Moreover, sensitivity to cysteine reagents is higher for the y$^+$L activity induced by the cysteineless mutant than by the wild type 4F2hc. These results indicate that 4F2hc is intimately associated by disulfide bridges with a membrane oocyte protein for the expression of system y$^+$L amino acid transport activity. To our knowledge, this is the first direct evidence for a heteromultimeric protein structure of an organic solute carrier in mammals.

Conclusions and future directions: identification of rBAT- and 4F2hc-associated subunits

As mentioned throughout this review there is structural and functional evidence that amino acid transporters associated with rBAT and 4F2hc are heterodimeric, with their subunits linked by disulfide bridges. Of these proteins only the 'heavy chains' are known (rBAT and 4F2hc) whereas the 'light subunits' have not been sequenced or cloned. Therefore the immediate research goal is to identify these subunits. Two obvious strategies are currently being pursued. First, purification of the heterodimeric complexes and then isolation of the 'light subunits' for microsequencing or development of antibodies and cloning of the gene. This strategy might be compromised if multiple light subunits were linked to 4F2hc. The second strategy is based on the induction of amino acid transport activity in oocytes by coexpression of both subunits. This approach was used first to identify the three subunits of the epithelial sodium channel [52]. Interestingly, at saturating doses, rat lung poly(A)$^+$ RNA and human 4F2hc cRNA overexpress system y$^+$L activity in oocytes (R Estévez *et al.*, unpublished data) indicating that this mRNA encodes subunits or activators of system y$^+$L. Screening of a rat lung cDNA library is currently in progress.

Finally, as mentioned above, the rBAT gene cannot be used to explain non-type I/I cystinuria, and a new cystinuria gene locus has been localized in chromosome 19q for these patients. The non-type I/I cystinuria gene might correspond with the rBAT light subunit. We hope that in the near future the light subunits of rBAT and 4F2hc will be identified after protein purification, coexpression cloning, positional cloning or serendipity (transporter-like cDNAs in gene databases might be waiting for identification as the putative rBAT or 4F2hc light subunits).

Note added in proof

The data referred to as R Estévez *et al.* have now been published [53•].

Acknowledgements

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