

Primary inherited aminoacidurias and structure-function relationship studies on heteromeric amino acid transporters

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Primary inherited aminoacidurias (PIAs) are rare diseases caused by defective amino acid transport activities, which affect renal reabsorption of amino acids and may also affect intestinal absorption of amino acids and transport function in other organs. Mutations in B0AT1 (*SLC6A19*) cause most cases of Hartnup disorder (Seow *et al*, 2004; Kleta *et al*, 2004). B0AT1 corresponds to system B0 and catalyses the Na⁺-dependent transport of most neutral amino acids. Heteromeric Amino Acid Transporters (HATs) are involved in PIAs because mutations in rBAT (*SLC3A1*) and b^{0,+}AT (*SLC7A9*) cause cystinuria (Calonge *et al*, 1994; Feliubadalo *et al*, 1999) and mutations in y⁺LAT1 cause Lysinuric Protein Intolerance (LPI; Torrents *et al*, 1999).

HATs are composed of a heavy subunit and a light subunit. Two homologous heavy subunits (HSHATs) from the SLC3 family have been cloned and are called rBAT (*ie*, related to b^{0,+} amino acid transport) and 4F2hc (*ie*, heavy chain of the surface antigen 4F2hc, also named CD98 or fusion regulatory protein 1; FRP1). Ten light subunits (LSHATs; SLC7 family members from SLC7A5 to SLC7A13) have been identified. Six of them are partners of 4F2hc (LAT1, LAT2, y⁺LAT1, y⁺LAT2, asc1, and xCT); one forms a heterodimer with rBAT (b^{0,+}AT); and three (asc2, AGT-1 and arpAT) seem to interact with as yet unknown heavy subunits.

Over the last 15 years our group has identified the first members of HATs (Betran *et al*, 1992 a,b), cloned half of the light subunits (Torrents *et al*, 1998; Pineda *et al*, 1999; Feliubadalo *et al*, 1999; Fernandez *et al*, 2005) and reported the role of genes *SLC3A1*, *SLC7A9* and *SLC7A7* in cystinuria Type I, non-I and LPI (Calonge *et al*, 1994; Feliubadalo *et al*, 1999; Torrents *et al*, 1999). At present, we continue to study the pathology of PIA (Chillarón *et al*, 1996; Dello Strologo *et al*, 2002; Font-Llitjos *et al*, 2005) with the aim of identifying new amino acid transporters in order to unravel the molecular bases of renal reabsorption and intestinal absorption of amino acids (Boday *et al*, 2005; Ristic *et al*, 2006). One of our main research interests is to decipher the atomic structure and the molecular mechanism of transport of HATs.

HATs show an oligomeric structure

Blue Native gel electrophoresis, cross-linking, and fluorescence resonance energy transfer *in vivo* indicate that system b^{0,+} is a heterotetramer [b^{0,+}AT/rBAT]₂, whereas xCT-4F2hc (system x_c⁻) does not stably or efficiently oligomerise. However, substitution of the heavy subunit 4F2hc for rBAT is sufficient to form a heterotetrameric [xCT/rBAT]₂ structure. The functional expression of concatamers of two light subunits (which differ only in their sensitivity to inactivation by a sulfhydryl reagent) suggests that a single heterodimer is the functional unit of systems b^{0,+} and x_c⁻ (Fernández *et al*, 2006).

The observation that the functional unit of system b^{0,+} is the heterodimer points to a complex mechanism of amino acid exchange for HATs. The light subunit of these transporters acts as the "catalytic" subunit (true transporter) in the heterodimer, as revealed by reconstitution experiments of b^{0,+}AT (Reig *et al*, 2002). Moreover, it has been shown that system b^{0,+} from chick small intestine shows a sequential exchange mechanism compatible with the formation of a ternary complex (Torrás-Llort *et al*, 2001). If this applies to the expressed b^{0,+}AT-rBAT complex, then export and import pathways should co-exist in the proposed functional unit (*ie*, the heterodimer) and, therefore, in a single b^{0,+}AT catalytic

ic subunit (Figure 3). To probe this mechanism of transport a deep knowledge of the atomic structure and the structure-function relationships of HATs is needed.

Light subunits

In recent years we have published two studies about the membrane topology (Gasol *et al*, 2004) and relevant residues for transport activity of the HAT light subunit xCT (LAT family of transporters - L-system amino acid transporter; Jimenez-Vidal *et al*, 2004). In the first study, and based on the accessibility of single cysteines to 3-(N-maleimidylpropionyl) biocytin, we proposed a topological model for xCT of 12 transmembrane domains with the N and C termini located inside the cell. The location of these termini was confirmed by immunofluorescence. Studies of biotinylation and accessibility to sulfhydryl reagents revealed a re-entrant loop within intracellular loops 2 and 3. Residues His110 and Thr112, facing outside, are located at the apex of the re-entrant loop. Biotinylation of H110C was blocked by xCT substrates, by the nontransportable inhibitor (S)-4-carboxyphenylglycine, and by the impermeable reagent (2-sulfonatoethyl) methanethiosulfonate, which produced inactivation of H110C that was protected by L-glutamate and L-cysteine with an IC₅₀ similar to the Km. Protection was independent of temperature. Our data indicate that His110 lies close to the substrate binding/permeation pathway of xCT. The membrane topology of xCT could serve as a model for other light subunits of HATs (Figure 1).

In the second study we measured sensitivity to thiol modification of the heteromeric glutamate/cystine transporter 4F2hc/xCT expressed in *Xenopus* oocytes. p-Chloromercuribenzoate (pCMB) and p-chloromercuribenzenesulfonate (pCMBS) rapidly blocked transport activity. Cys327, located in the middle of the eighth transmembrane domain of the light subunit (xCT), was the main target of inactivation. Cysteine, an impermeant reducing reagent, reversed the effects of pCMB and pCMBS only when applied from the extracellular medium. L-Glutamate and L-cysteine, but not L-arginine, protected against inactivation, with an IC₅₀ similar to the Km. Protection was not temperature-dependent, suggesting that it did not depend on large substrate-induced conformational changes. Mutation of Cys327 to Ala and Ser slightly modified the Km and a C327L mutant abolished transport function without compromising transporter expression at the plasma membrane. These results indicate that Cys327 is a functionally important residue accessible to the aqueous extracellular environment and that it is structurally linked to the permeation pathway and/or the substrate binding site.

To further increase our knowledge of the structure-function relationship of the light subunits of HATs, we initiated a line of research on the prokaryotic homologues of these subunits. We have identified an orphan protein from *Bacillus* sp as a new member of the LAT family of amino acid transporters. This protein shares an amino acid sequence identity of ~30% with the human light subunits of HATs. The purified protein from *E. coli* membranes was reconstituted in proteoliposomes and showed sodium-independent obligatory exchange activity of L-serine and L-threonine. Kinetic analysis supports a sequential mechanism of exchange characteristics of HAT transporters. Freeze-fracture analysis of the purified and active transporter in proteoliposomes, and Blue Native-polyacrylamide gel electrophoresis and transmission electron microscopy (TEM) of detergent-solubilised purified transporter indicate that the transporter exists in a monomeric form. To our knowledge, this is the first functional characterisation of a prokaryotic member of the LAT family and the first structural data about a transporter of the APC superfamily. This new transporter represents an excellent model to study the molecular architecture of light subunits of HATs and other APC transporters. Currently this and other homologous prokaryotic transporters of the

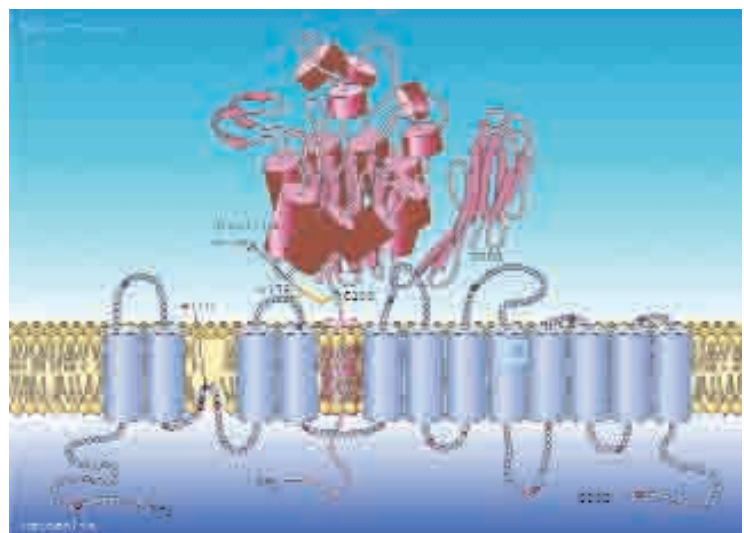


Figure 1. A heteromeric amino acid transporter. The heavy subunit (pink) and the light subunit (blue) are linked by a disulfide bridge (yellow) with conserved cysteine residues (cysteine 158 for the human xCT and cysteine 109 for human 4F2hc). The heavy subunits (4F2hc or rBAT) are type II membrane glycoproteins with an intracellular NH₂ terminus, a single transmembrane domain, and a bulky COOH terminal. This part of the protein shows homology with bacterial glycosidases. The membrane topology of the light subunit xCT, as model of the other light subunit, shows 12 transmembrane domains, with the NH₂ and COOH terminals located intracellularly and with a re-entrant loop-like structure in the intracellular loop IL2-3 (His110 corresponds to the apex of the loop; Gasol *et al*, 2004). Residues with external (black) or internal (red) accessibility are shown. Taken from Palacín *et al*, 2005.

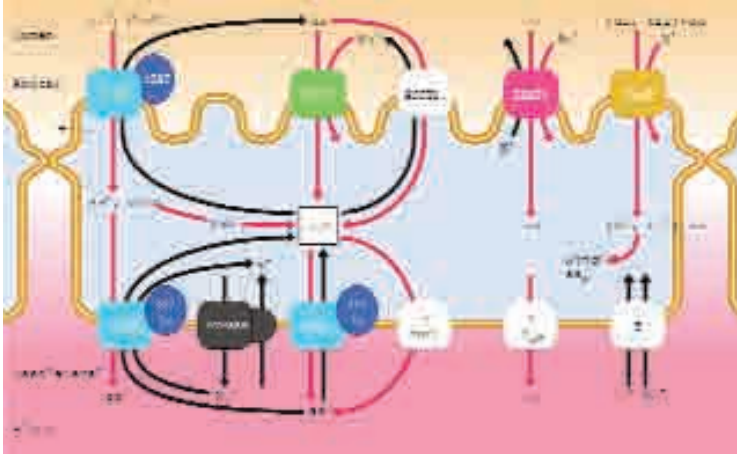


Figure 2. Transporters involved in the renal and intestinal reabsorption of amino acids. Transporters with proven roles in renal reabsorption or intestinal absorption of amino acids are shown in colour. Transporters present in the apical or basolateral plasma membrane of the epithelial cells of the proximal convoluted tubule or of the small intestine, but with no direct experimental evidence supporting their role in reabsorption, are shown in black and white. Fluxes of amino acids in the reabsorption direction are in red. PepT (H⁺-dependent peptide transporter PepT1 in small intestine and Pept2 in kidney) cotransports di- and tripeptides [(di,tri)-aa] with protons. Intracellular hydrolysis then renders single amino acids. The high intracellular concentration of neutral amino acids (aa⁰) due to the transport activity of apical (B0AT1) and basolateral (y+LAT1-4F2hc, T) transporters drives the active reabsorption of dibasic amino acids (aa⁺) and cystine (CysC), together with the membrane potential and reduction of CysC to cysteine (CysH). Basolateral efflux transporters for dicarboxylic amino acids are unknown (Taa⁻). T represents basolateral Na⁺-dependent transporters with undefined roles in reabsorption (systems A and ASC). Our group made a key contribution to the determination of the role of transporters rBAT/b⁰,+AT, 4F2hc/y+LAT1 and 4Fhc/LAT2 in renal reabsorption of amino acids. Taken from Palacin et al, 2005.

light subunits of HATs are being studied using 2D and 3D crystallisation.

Heavy subunits

Little is known about the structure of the heavy subunits of HATs (rBAT and 4F2hc). They have a molecular mass of ~90 and ~80 kDa for rBAT and 4F2hc, respectively, and are type II membrane N-glycoproteins with an extracellular COOH terminus (ectodomain) homologous to insect and bacterial glucosidases (Figure 1). Recently, we have solved the atomic structure of the human 4F2hc ectodomain at 2.1 Å resolution. This domain has a similar architecture to bacterial glucosidases with a triose phosphate isomerase (TIM) barrel [(αβ)⁸] and eight anti-parallel β-strands. Several features of this structure provide insight into the position of the ectodomain on the plasma membrane, potential interaction surfaces with the accompanying light subunits and motifs that might be involved in the multiple functions of 4F2hc in cell fusion, adhesion and transformation. Currently mutants of the 4F2hc ectodomain are being examined to identify the residues involved in the multiple functions of the protein.

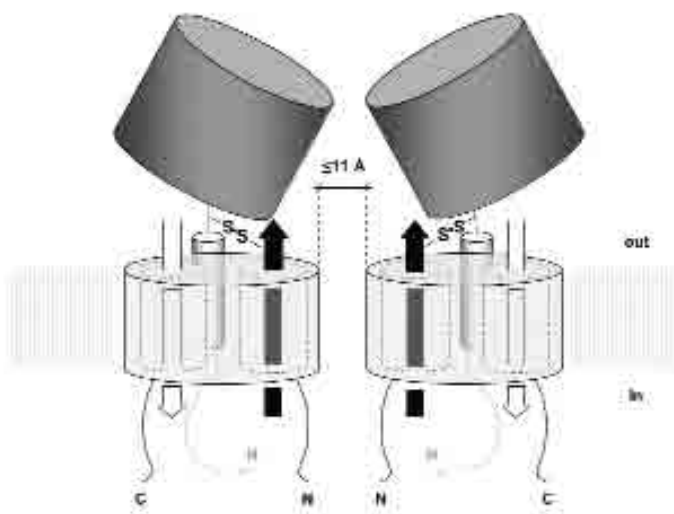


Figure 3. Heterotetrameric structure of system b⁰,+AT. Cross-linking experiments revealed that lysine residues in each light subunit b⁰,+AT are separated by no more than 11 Å. Functional studies revealed that the heterodimer (composed of rBAT and b⁰,+AT; the light wide cylinder) is the functional unit of the transporter (Fernández et al, 2006). In this functional model, two translocation pathways (one for influx and the other for efflux is present in each light ("catalytic") subunit (see text for details). -S-S-, disulfide bridge connecting rBAT and b⁰,+AT. In, cytosolic. Out, extracellular.

PUBLICATIONS

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RESEARCH NETWORKS OR GRANTS

Heteromeric amino acid transporters: structure, functional genomics and pathophysiology
MCYT, Plan Nacional, SAF2003-08940-C02-01: 2003-2006
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Project Coordinator: Manuel Palacín

Inherited metabolic diseases. Network REDEMETH
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Principal Investigator: Manuel Palacín

Role of 4F2hc in tumorigenesis
La Marató TV3: 2006-2008
Project Coordinator: Manuel Palacín

Heteromeric amino acid transporters: Structure, physiology and pathology
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Project Coordinator: Manuel Palacín

Molecular bases of megalencephalic leukoencephalopathy. Biochemical and electrophysiological study
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Project Coordinator: Raúl Estévez

OTHER FUNDING SOURCES

Development of new drugs for cystinuria
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COLLABORATIONS

Disorders of plasma membrane amino acid transporters
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Arginine transport in macrophages
Antonio Celada (IRB Barcelona, Spain)

Development of cystinuria drugs
Laboratories Rubió, Spain

Development of PIA animal models
Ingenium Pharmaceuticals AG, Germany

Metabolic syndrome
Genmedica Therapeutics, Spain

Molecular interactions with 4F2hc
Cristax, Spain



Manuel Palacin's group, March 2006.