Membrane Proteins From A to Z

Heteromeric amino acid transporters. In search of the molecular bases of transport cycle mechanisms[1]

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Abstract

Heteromeric amino acid transporters (HATs) are relevant targets for structural studies. On the one hand, HATs are involved in inherited and acquired human pathologies. On the other hand, these molecules are the only known examples of solute transporters composed of two subunits (heavy and light) linked by a disulfide bridge. Unfortunately, structural knowledge of HATs is scarce and limited to the atomic structure of the ectodomain of a heavy subunit (human 4F2hc-ED) and distant prokaryotic homologues of the light subunits that share a LeuT-fold. Recent data on human 4F2hc/LAT2 at nanometer resolution revealed 4F2hc-ED positioned on top of the external loops of the light subunit LAT2. Improved resolution of the structure of HATs, combined with conformational studies, is essential to establish the structural bases for light subunit recognition and to evaluate the functional relevance of heavy and light subunit interactions for the amino acid transport cycle.

Introduction

Amino acid availability regulates cell physiology. The transfer of amino acids across the plasma membrane is mediated by specific transporter proteins that recognize, bind and transport these amino acids from the extracellular medium into the cell, or vice versa. In this review, we focus on the structural and mechanistically associated features of a particular family of amino acid transporters, namely the heteromeric amino acid transporters (HATs). These molecules comprise a heavy subunit and a light subunit linked by a conserved disulfide bridge [1]. HATs are particularly interesting in terms of structure–function relationships because they are the only known example in all kingdoms of life of solute transporters formed by two subunits linked by a disulfide bridge. The heavy subunits of HATs appeared in metazoans, whereas the light subunits can be traced to prokaryotes. Two homologous heavy subunits and ten light subunits belonging to the SLC3 and SLC7 protein families, respectively, have been identified in mammals [1]. Heavy subunits, rBAT (SLC3A1) [2] and 4F2 cell surface antigen heavy chain (4F2hc) (SLC3A2) [3], also known as CD98hc, are helper proteins required for trafficking the holotransporter to the plasma membrane [1], whereas the light subunit is the transporter, conferring substrate specificity [1]. Six of these light subunits heterodimerize with 4F2hc (LAT1, LAT2, y^+LAT1, y^+LAT2, asc1 and xCT) [4–9], two with rBAT ($b^{0,+}$AT and AGT1) [10,11]. For two [arpAT (SLC7A15) and asc2 (SLC7A12), not present in primates] [12,13], the heavy subunit has not been identified (Table 1).

The mammalian SLC7 family is part of the APC (amino acid, polyamine and organocation transporters) superfamily. SLC7 members are grouped into two families, namely cationic amino acid transporters (CATs) and the light subunits of HATs. CATs (SLC7A1–4), with the exception of the orphan transporter SLC7A14, are facilitated diffusers and the major route of entry in most cells for cationic amino acids, and they play key roles in macrophage activation and proliferation [14]. CAT members are N-glycosylated and have 14 putative transmembrane domains (TMs) with cytosolic N- and C-termini. The light subunits of HATs belong to the LAT family (system L amino acid transporters; LATs). It is worth

Key words: amino acid transporters, heterodimer, heteromeric amino acid transporter (HAT), modelling, structure, transport mechanism.

Abbreviations: CAT, cationic amino acid transporter; HAT, heteromeric amino acid transporter; LAT, L-amino acid transporters; LPI, lysinuric protein intolerance; TM, transmembrane domain.

[1]**In memory of Steve Baldwin, who put us on the right track for structural studies of membrane proteins.**

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Table 1 | SLC7 – human cationic amino acid transporters/L-type amino acid transporters

E, exchanger (antiporter); F, facilitator (uniporter); O, orphan (unknown transport activity). For detailed information about the SLC genes, please visit: http://www.bioparadigms.org. Adapted from Ref. [1]: Fotiadis, D., Kanai, Y. and Palacín, M. (2013) The SLC3 and SLC7 families of amino acid transporters. Mol. Aspects Med. **34**, 139–158.

Human gene name	Protein name	Aliases [assoc. with]	Predominant substrates	Transport type/coupling ions[a]	Link to disease
SLC7A1	CAT-1	ATRC1, system y^+	Cationic L-amino acids	F[b] (non-obligatory E)	
SLC7A2	CAT-2 (A or B)	ATRC2, system y^+	Cationic L-amino acids	F	
SLC7A3	CAT-3	ATRC3, system y^+	Cationic L-amino acids	F	
SLC7A4	CAT-4			O	
SLC7A5	LAT1	[4F2hc], 4F2lc, system L	Large neutral L-amino acids, T_3, T_4, L-DOPA, BCH	E (similar intra- and extracellular selectivity, lower intracellular apparent affinity)	Cancer
SLC7A6	y^+LAT2	[4F2hc], system y^+L	Na^+ indep.: cationic amino acids; Na^+/large neutral amino acids	E (preferentially intracellular cationic amino acid against extracellular neutral amino acid/Na^+)	
SLC7A7	y^+LAT1	[4F2hc], system y^+L	Na^+ indep.: cationic amino acids; Na^+/large neutral L-amino acids	E (preferentially intracellular cationic amino acid against extracellular neutral amino acid/Na^+)	LPI
SLC7A8	LAT2	[4F2hc], system L	Neutral L-amino acids, T_3, T_4, BCH	E (similar intra- and extracellular selectivity, lower intracellular apparent affinity)	
SLC7A9	$b^{0,+}$AT	[rBAT], system $b^{0,+}$	Cationic amino acids, large neutral amino acids	E (preferentially extracellular cationic amino acid and cystine against intracellular neutral amino acid)	Cystinuria and isolated cystinuria
SLC7A10	Asc-1	[4F2hc], system asc	Small neutral amino acids	Preferentially E	
SLC7A11	xCT	[4F2hc], system x_c^-	Cystine (anionic form), L-glutamate	E (preferentially extracellular cystine against intracellular glutamate)	
SLC7A13	AGT-1	[rBAT]	L-Aspartate, L-glutamate and cystine	E	
SLC7A14				O	

mentioning that other system L-amino acid transporters like LAT3 and LAT4 belong to another protein family (SLC743) and do not form heterodimers with SLC3 members [15]. LATs are antiporters covering a full range of amino acid substrates (Table 1). They are non-N-glycosylated and have 12 transmembrane domains [1,14].

Several human pathologies highlight the physiological roles of HATs. Two transporters of this family are responsible for inherited aminoacidurias – mutations in either of the two genes coding for the subunits of system $b^{0,+}$ (rBAT and $b^{0,+}$AT) lead to cystinuria (MIM 220100) [10,16], whereas mutations in y^+LAT1 (a 4F2hc-associated system y^+L) result in lysinuric protein intolerance (LPI) (MIM 222700) [17]. In addition, xCT (the 4F2hc-associated system x_c^-), which mediates cystine uptake and glutamate efflux, is essential for Kaposi's sarcoma-associated Herpesvirus infection [18]. This transport system is also involved in cocaine relapse through the control of the basal levels

of extra-synaptic glutamate [19]. Finally, as is also the case for xCT, LAT1 (a 4F2hc-associated system L) is overexpressed in many human tumours, thereby suggesting that these amino acid transporters are essential for tumour cell survival and progression [20,21]. In this regard, two approaches for anticancer therapies involving both xCT and LAT1 have been proposed. First, the capacity of these transporters to mediate the uptake of several amino acid-derived anticancer drugs has been reported, thereby supporting the notion that these proteins are involved in the cellular internalization of these antineoplasic drugs [20,21]. Second, a novel strategy based on the inhibition of xCT and LAT1 activity has recently been put forward as a means to reduce tumour proliferation and progression [20,21]. Along these lines, system x_c^- (4F2hc/xCT) sustains cystine uptake for glutathione synthesis, assuring cell viability by preventing ferroptosis [22], and system L (4F2hc/LAT1) has been proposed to form part of the leucine sensor of mTOR [23,24]. The targeting of amino acid transporters in cancer is under development; however, there are few specific inhibitors available. In this regard, the development of better drugs to inhibit these transporters will challenge the scenario. In this regard, X-ray crystallography analysis of plasma membrane transporters is the most powerful tool for the intelligent design of more active and specific therapeutic molecules, as well as for a better understanding of the mechanisms underlying substrate binding and translocation. In this regard, prokaryotic transporter proteins have been shown to be excellent structural paradigms of their mammalian homologues, providing key insights into substrate pocket design and translocation mechanisms [25,26].

Translocation mechanism

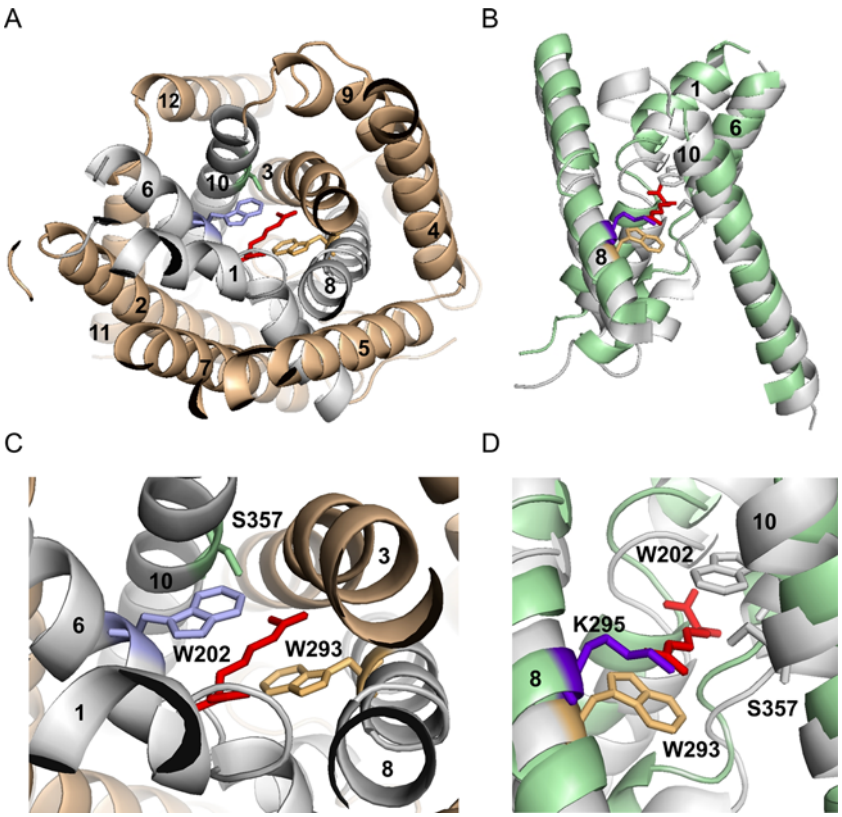
The prokaryotic amino acid transporters ApcT, AdiC and GadC, which belong to the APC superfamily, as do mammalian LATs, are the closest mammalian LAT homologues [amino acid sequence identity (AAI) ranging from 14% to 20%] whose atomic structure has been solved [27–32]. These transporters share the protein fold named LeuT with more distant homologues of this superfamily, with less than 10% AAI with mammalian LATs. Proteins with the LeuT-fold (also named 5 + 5 inverted repeat fold), although differing in the total number of TM helices in different transporters, are characterized by two structurally similar repeats, each containing a core of five consecutive TMs, by a pseudo-two-fold symmetry axis located in the plane of the membrane. As a hallmark of this fold, the first TM in each of the two inverted repeats (TM1 and 6) is discontinuous and consists of two short alpha-helices connected by a highly conserved unwound segment [25]. This structural feature is important for the transport mechanism of transporters with the LeuT-fold as it is part of the substrate coordination site in crystallized amino acid transporters with this fold [25,30,31]. In addition, atomic structures of various conformational states of the same transporter, as well as of several transporter proteins with the LeuT-fold, led to the proposal that structural changes are associated with substrate binding and translocation. Along these lines, substrates would translocate across the cell membrane via the alternating access transport mechanism. In this well-established mechanism, transporters with the LeuT-fold transit through a range of outward- and inward-facing conformational states (with and without substrate) and substrate-bound occluded states. In the particular case of AdiC, three distinct conformations have been determined: outward open arginine-free, outward open arginine-bound, and outward occluded arginine-bound [27,28,30,31]. Unfortunately, there is no information about the inward-facing conformations of AdiC, which is essential to unravel the complete translocation mechanism. However, models of possible transition states for crystallized transporters based on internal protein symmetry have been successfully obtained for various transporter proteins sharing the LeuT-fold [33]. In this regard, symmetry-based models generated for the inward-facing conformation of AdiC might help elucidate the structural rearrangements associated with substrate translocation across the transporter protein [31]. Taking all this information together, a proposed translocation mechanism for AdiC (and probably extendable to both prokaryotic and eukaryotic LAT transporters) consists of initial structural shifts of TM2, TM6 and TM10 as a result of substrate binding (induced fitting), thus leading to the occlusion of the substrate. At this stage, proper coordination of the substrate with W293 and S357 is essential for subsequent transit to the occluded state (Figure 1). Once occluded, the substrate is blocked by a thin gate (W202 residue in *Escherichia coli* AdiC) and a thick gate (TMs 1, 6, 3 and 8), thereby preventing the release of the substrate to either side of the membrane. Transition from outward- to inward-facing states would result from the pivoting of the bundle (TM 1, 2, 6 and 7) and the hash (TM 3, 4, 8 and 9 are positioned in such a way as to mimic this symbol) domains, whereas TM 5 and 10 would present significant but minor changes. Nevertheless, the lack of crystal structures for LAT transporters makes it difficult to determine the exact amino acid residues that participate in the translocation mechanism of the members of this family of amino acid transporters. In this regard, elucidation of the structure of closely related prokaryotic homologues of human LAT transporters (AAI higher than 30%) or the generation of structural models from crystal structures and real-time techniques, such as single molecule FRET (smFRET) or EPR – as done for the neurotransmitter transporter homologue LeuT from *Aquifex aeolicus* – would contribute to unravelling the translocation mechanism used by LAT transporters [34,35].

Substrate binding site design

Understanding the conformational changes that occur during each transport cycle is only one of the important steps towards the elucidation of the transport mechanism. In this regard, substrate binding represents another additional key aspect. Comparison of the crystal structures of amino acid transporters with the LeuT-fold, like ApcT and AdiC, reveals

Figure 1 | Residues involved in substrate recognition in prokaryotic amino acid transporters AdiC and SteT

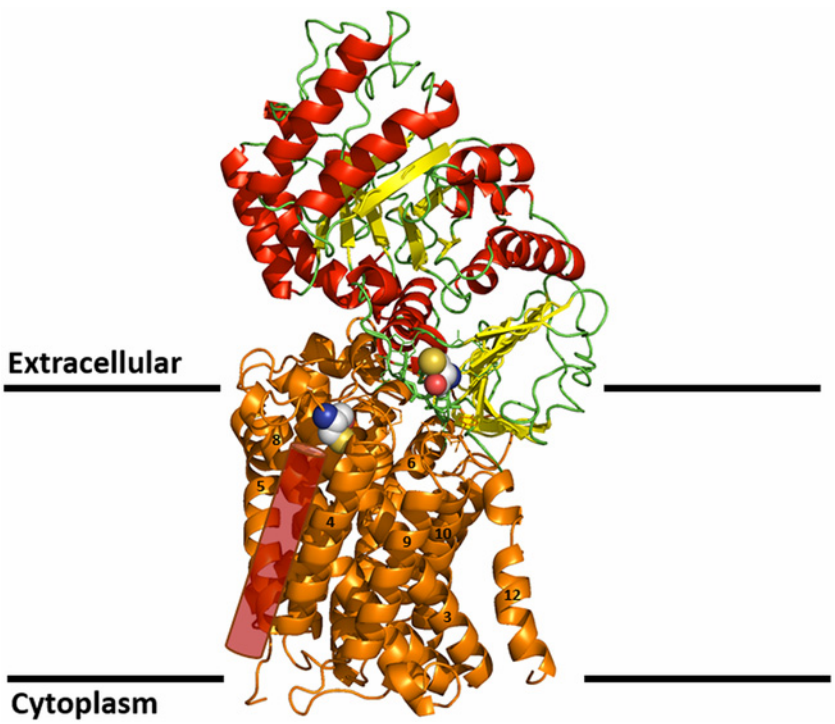
(**A**) Structure of the arginine–agmatine exchanger AdiC outward-facing in complex with arginine occluded (3L1L). Relevant residues for substrate (arginine, red) recognition (S357 in TM10, pale green and W293 in TM8, light orange) and occlusion (W202 in TM6, light blue) are depicted. TMs involved in substrate recognition are shown in grey, whereas the rest of TMs are shown in brown. (**B**) Alignment of 3L1L (grey) with AdiC-based serine–threonine exchanger (SteT) model (pale green). Equivalent residue to W293 (light orange) in AdiC is shown for SteT model (K295, purple). AdiC substrate arginine (red) and AdiC residues W202 (grey) and S357 (grey) are shown as a reference for the substrate binding site location. (**C**) Magnification of substrate binding site from panel A. (**D**) Magnification of substrate binding site from panel B.



the conservation of the substrate binding site location [27–31]. Nevertheless, although the location of this binding site and that of the key residues involved in both substrate recognition and translocation appear to be conserved, the composition of the residues coordinating the substrates varies significantly [27–31]. In support of this idea, LAT family members range from transporters with very broad substrate specificity to highly specific transporters (e.g., LAT2 compared with xCT) (Table 1), thereby suggesting that although similar in design, substrate recognition relies on particular residues strategically located in the binding site. In this regard, amino acid substitutions in particular residues would result in completely different substrate selectivity. On this point, Jocelyn and Steve Baldwin generated the first structural model of a bacterial LAT, the *Bacillus* Ser/Thr exchanger SteT [36]. Mutation of the lysine 295, homologous with the main substrate attractor residue W293 in AdiC, to cysteine (or glycine) in TM8 markedly broadened the amino acid profile of inhibitors and the transporter substrate selectivity [36] (Figure 1). In addition, functional characterization of cystinuria and LPI mutants in b^{0+}AT and y^+LAT1, respectively, revealed specific residues involved in transport activity in these two human LAT transporters [37,38]. In this regard, when mutated to methionine (T123M), residue T123, located in TM3 of $b^{0,+}$AT within the putative substrate binding pocket, causes isolated cystinuria without urine hyperexcretion of dibasic amino acids. This observation would suggest that this residue is involved in substrate recognition [37]. In all, these data support the notion that residues in TM3 and TM8 are relevant for the recognition of the lateral chain of the amino acid substrates of LATs. Thus, conservation of substrate binding design, regardless of residues involved in substrate coordination, would allow the generation of relatively strong and reliable models of the substrate binding site of human LAT transporters. Nevertheless, there is no crystal structure currently available for any LAT transporter, and models generated using distant homologues (AdiC or ApcT e.g.) must be confirmed by site-directed mutagenesis and functional studies in the modelled transporters [36]. However, a first approach was recently reported whereby

Figure 2 | Docking model of the human 4F2hc-ED/LAT2 complex

4F2hc, with a bulky ectodomain (4F2hc-ED), is linked by a conserved disulfide bridge to the corresponding light subunit, which are polytopic proteins with 12 transmembrane domains (orange). The corresponding position numbers of the LAT2 transmembrane domains are indicated. Cys residues involved in the intersubunit disulfide bridge, Cys^{109} (4F2hc) and Cys^{154} (LAT2) are shown as sphere models (C atoms, grey; O atoms, red; N atoms, blue; S atoms, yellow). The structure of 4F2hc-ED is similar to that of α-glycosidases, including two domains: a TIM-barrel $(\beta/\alpha)_8$ and a C-terminal domain with eight antiparallel β-sheets. α-helices and β-sheets present in 4F2hc-ED are shown in red and yellow respectively. The indicated position of 4F2hc transmembrane domain (TM), illustrated by a transparent red cylinder, is a supposition based on compatibility with the generation of the disulfide bridge between the two subunits. Residue Cys^{109} (4F2hc) would be closer to 4F2hc TM and to Cys^{154} (LAT2) due to the flexibility of the N-terminus of 4F2hc-ED. This model of 4F2hc-ED/LAT2 complex is based on docking analysis, together with transmission electron microscopy and cross-linking experiments. A hypothetical position of plasma membrane is indicated based on 4F2hc-ED/LAT2 docking data [51]. The long N-terminus of 4F2hc is not shown.



a human LAT1 model was generated using AdiC and ApcT as templates and successfully used for structure-based ligand discovery assays [39]. Amino acid transporters are emerging as promising targets for the treatment of a relatively wide range of diseases. In this regard, the discovery of innovative leads is expensive and time-consuming. In this regard, structure-based virtual screening is becoming a routine procedure in both pharmaceutical companies and academic groups for early-stage drug discovery purposes. In this respect, the generation of reliable models from human LAT transporters would contribute to the rapid design of more specific drugs with clinical relevance.

The heavy subunits of HATs

The heavy subunits (~80–90 kDa) are type II membrane N-glycoproteins with a single TM, an intracellular N-terminus, and a large (~50–60 kDa) extracellular C-terminus [1]. The cysteine residue involved in the intersubunit disulfide bridge is located a few residues away from the TM domain, interacting with the corresponding cysteine placed in the extracellular loop between TM3 and TM4 of the light subunits [1] (Figure 2). The amino acid sequence of the ectodomains of rBAT (rBAT-ED) and 4F2hc (4F2hc-ED) share identity with bacterial α-amylases [40]. Moreover, the crystal structure of the human 4F2hc-ED at 2.1 Å (1 Å = 0.1 nm) resolution (PDB code 2DH2) is similar to that of bacterial α-amylases, showing domain A, a triose phosphate isomerase [TIM] barrel [$(\alpha/\beta)_8$], and domain C, eight antiparallel β-strands (Figure 2) [41]. The structure of rBAT-ED has been predicted based on 4F2hc-ED and prokaryotic glycoside hydrolase structures [42], showing domains A and C, and an additional domain B (a $\alpha_2\beta_3$ loop) between $\beta 3$ and $\alpha 3$ of domain A. 4F2hc-ED lacks key catalytic residues for α-amylase function and consequently shows no enzymatic activity [41], whereas possible α-amylase activity associated with rBAT-ED has not been demonstrated.

Functional oligomerization of the rBAT–$b^{0,+}$AT complex

The quaternary structure of rBAT–$b^{0,+}$AT has been characterized both in transfected cells and in brush border membranes of the kidney, where two disulfide-linked heterodimers form a noncovalent heterotetramer [43]. In this complex, two $b^{0,+}$AT molecules homodimerize within the heterotetramer, as demonstrated by fluorescence resonance energy transfer studies and cross-linking assays [43]. The rBAT–$b^{0,+}$AT oligomerization process might be determined by the heavy subunit rBAT and not by $b^{0,+}$AT. This notion is supported by experiments performed using the functional chimera formed by rBAT and the 4F2hc-associated light subunit xCT, where this transporter retained the capacity to form heterotetramers [43]. Although the oligomeric structure of rBAT–$b^{0,+}$AT is a heterotetramer, the single rBAT–$b^{0,+}$AT heterodimer is the functional transport unit [43]. Therefore, oligomerization may participate in aspects of rBAT–$b^{0,+}$AT biology other than the transport mechanism. The oligomerization of membrane proteins has been associated with their traffic through the secretory pathway [44]. In fact, many rBAT mutants associated with type I cystinuria show endoplasmic reticulum (ER)-retention and a degradation phenotype that correlates with the inability to form heterotetramers despite the presence of disulfide-linked heterodimers [42]. The heterodimerization of these transporters is strongly linked to their biogenesis. rBAT and $b^{0,+}$AT are interdependent for trafficking from the ER to the plasma membrane [45]. Assembly with $b^{0,+}$AT prevents ER-associated degradation (ERAD) of unassembled rBAT and is required for the oxidative folding of the rBAT–ED, which proceeds via the formation of three consecutive disulfide bonds (see 'The heavy subunits of HATs'), two of them – the domain B disulfide and the disulfide connecting domain C with the C-terminal tail – essential for biogenesis [46]. Recently, it has been reported that the N-glycan N575 of human rBAT is indispensable for early traffic, and the C-terminal loop of this heavy subunit is relevant for the stability and/or early traffic of rBAT–$b^{0,+}$AT [47]. A possible interaction between the N575 N-glycan and the C-terminal loop has been proposed to be important for the biogenesis of this heterodimer [47].

Structure–function relationship in 4F2hc-associated heterodimers

The native structure of the mammalian 4F2hc-associated transporters seems to be that of a single heterodimer [43]. A number of studies have provided some insight into the function of the various heavy chain domains. The cytoplasmic N-terminus and the transmembrane helix are essential for the interaction of 4F2hc with the light chains [48], but the function of the extracellular domain of 4F2hc is less well defined. Truncations of the 4F2hc carboxy terminus slow down trafficking of the LAT1 light chain to the plasma membrane but do not interfere with the transport function [49]. In contrast, a chimeric version of 4F2hc, where 4F2hc-ED is replaced by the ectodomain of CD69, another type II transmembrane protein, induces amino acid transport when co-expressed with LAT1 [50]. In addition, truncations of the 4F2hc-ED result in a total loss of function of LAT2 and y^+LAT2 [49], thereby reinforcing the essential role of the extracellular domain of 4F2hc in the stimulation of amino acid transport in HATs. Moreover, the structure of human 4F2hc/LAT2 obtained by 3D reconstruction from negatively stained complexes at 21 Å, together with docking analysis and cross-linking experiments, revealed that the extracellular domain of 4F2hc interacts with the extracellular loops of LAT2 [51] (Figure 2). This observation would thus support the relevance of 4F2hc-ED for light subunit recognition.

4F2hc is a dual-function protein involved in cell proliferation, homoeostasis, and also cancer [52–54]. In addition to its role as an ancillary subunit of various light subunits, it behaves as a co-receptor of β-integrins and enhances their downstream signalling [55]. Physical and functional interaction of 4F2hc with integrins has been mapped to the TM and cytosolic N-terminal domain [50,55]. The combination of covalent cross-linking, mass spectrometric protein identification, and co-immunoprecipitation showed 4F2hc/LAT1 as an integrant of a supercomplex formed by the heterodimer CD147-MCT (monocarboxylate transporters 1 and 4), the neutral amino acid transporter ASCT2, and the epithelial cell adhesion molecule (EpCAM) [56]. A strong positive association of CD147–4F2hc cell surface supercomplex with cell proliferation was demonstrated and suggested that this complex plays a critical role in energy metabolism, possibly by coordinating transport of lactate and amino acids [56].

A sub-nanometer resolution structure of a HAT is essential to achieve a detailed description of the structural bases for light subunit recognition and to evaluate the functional relevance of intersubunit interactions during the conformational changes associated with substrate translocation (see 'Translocation mechanism'). Moreover, it would be highly pertinent to characterize the structural bases for the 4F2h/CD147 and 4F2hc/β-integrin interactions. Recent developments in the field of cryo-electron microscopy have been crucial to solve the structure of proteins of only a few hundred kilodaltons at near-atomic resolution [57], thus paving the way to obtaining high-resolution structures of a metazoan HAT or of some of the complexes that include these transporters.

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