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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Kidney amino acid transport

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Abstract Near complete reabsorption of filtered amino acids is a main specialized transport function of the kidney proximal tubule. This evolutionary conserved task is carried out by a subset of luminal and basolateral transporters that together form the transcellular amino acid transport machinery similar to that of small intestine. A number of other amino acid transporters expressed in the basolateral membrane of proximal kidney tubule cells subserve either specialized metabolic functions, such as the production of ammonium, or are part of the cellular housekeeping equipment. A new finding is that the luminal Na^+-dependent neutral amino acid transporters of the SLC6 family require an associated protein for their surface expression as shown for the Hartnup transporter B^0AT1 (SLC6A19) and suggested for the L-proline transporter SIT1 ($IMINO^B$, SLC6A20) and for B^0AT3 (XT2, SLC6A18). This accessory subunit called collectrin (TMEM27) is homologous to the transmembrane anchor region of the renin–angiotensin system enzyme ACE2 that we have shown to function in small intestine as associated subunit of the luminal SLC6 transporters B^0AT1 and SIT1. Some mutations of B^0AT1 differentially interact with these accessory subunits, providing an explanation for differential intestinal phenotypes among Hartnup patients. The basolateral efflux of numerous amino acids from kidney tubular cells is mediated by heteromeric amino acid transporters that function as obligatory exchangers. Thus, other transporters within the same membrane need to mediate the net efflux of exchange substrates, controlling thereby the net basolateral amino transport and thus the intracellular amino acid concentration.

Keywords Amino acid transport · Kidney · Proximal tubule · Transporter · Ammonia · Angiotensin

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Different kidney amino acid transporter functions

Amino acids play central roles in the metabolism of all organisms, in particular the 20 proteogenic amino acids that are building blocks of proteins. Amino acids also play an important role as substrates for energy production, as substrates for the synthesis of other molecules, such as compatible osmolytes and also as neurotransmitters. For mammals, approximately half of the proteogenic amino acids are essential, meaning that they need to be taken up from the diet, since they cannot be synthesized in the organism. Because free amino acids present in the blood plasma are filtered at the level of the kidney glomeruli in substantial amounts, namely more than 50 g per day in humans, their reabsorption by the kidney proximal tubules that prevents their loss in the urine is a very important function from the point of view of the organism homeostasis [56]. This transepithelial transport from the kidney tubular lumen into the extracellular space is therefore a much conserved function of the proximal tubule. A second amino acid transport function within the kidney which has a direct impact on organism homeostasis

is the cellular import and/or export of amino acids that are metabolized by kidney cells. In that regard, an important regulated transport function is the basolateral uptake of glutamine along the more distal segments of the proximal tubule which is required for the production of ammonia, a substrate used by kidney tubular cells for the secretion of protons [32]. Other amino acid transport functions observed in tubular cells relate presumably either to housekeeping and/or to additional specialized functions. Although this has not been studied in detail, it appears meaningful that it is another subset of mostly basolateral transport proteins that accounts for these functions. Thus, proximal kidney cells express, next to their housekeeping amino acid transporters, a subset of specialized transporters involved in metabolic functions (i.e., ammonium and glutathione synthesis) and another subset that composes the specialized proximal tubule amino acid reabsorption machinery (Fig. 1). A further degree of complexity is given by the fact that the specialized functions are not distributed uniformly along the proximal tubule axis.

From structural reductionism to integrative function: complementary perspectives

Over the past decades, amino acid transport functions have been investigated using always the newly available techniques to measure how these hydrophilic solutes cross the lipophilic membranes. A first wave of information came with the description of so-called transport systems that was made possible by the availability of radio-labeled amino acids and analytical quantification techniques used to perform transport studies first in tissues and cells and then using membrane vesicles [14, 33, 45]. A second wave of information started during the 1990s when the molecular identification of amino acid transporters was made possible by complementary DNA cloning and cellular expression techniques [8, 24, 25, 34, 55]. These new approaches allowed the characterization of the function of amino acid transporters in expression systems, the measurement of their messenger RNA (mRNA) expression levels in tissues and the production of antibodies to localize the protein. A third wave of insight in amino acid transporter function has started a few years ago with the determination of transport protein structures [58, 59].

The physiological question of the transport function remains, however, the central issue, at the molecular, cellular, organ, and systemic levels. Characterization of the functional role of amino acid transporters is necessary for understanding life processes and for identifying dysfunctions and corresponding drug targets. The multiple currently available approaches and future techniques are all required to address these important questions using in particular gene-modified animal models and also human subjects.

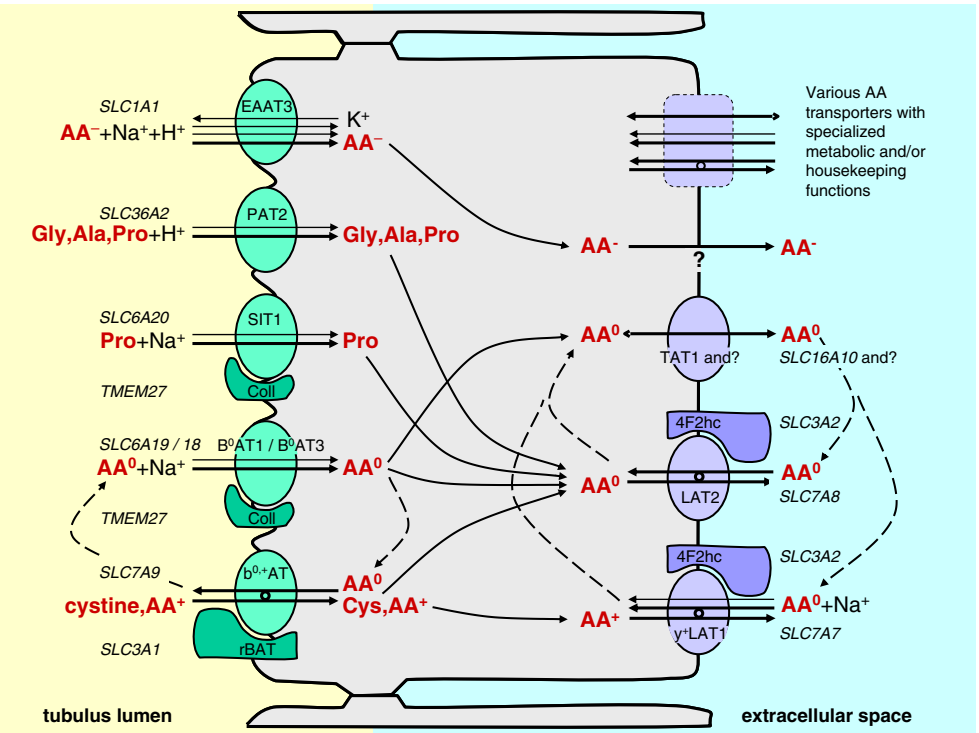

Fig. 1 Cellular model for the reabsorption of amino acids across a proximal kidney tubule cell. Names of the involved transporters and their human gene are indicated. Most of them have been shown to be expressed mainly in the early segments of mouse proximal tubule (S1 and S2), with the exception of SIT1 that is expressed all along the proximal tubule and of B^0AT3 and EAAT3 that are expressed mainly in the later proximal tubule (S2 and S3). AA^0 neutral amino acid, AA^- anionic amino acid, AA^+ cationic amino acid, *Coll* collectrin/TMEM27

Luminal transporters and their associated proteins

Quantitatively, neutral amino acids represent >80% of the free plasma amino acids and are all transported by the luminal B^0AT1 transporter (SLC6A19), though with different apparent affinities (Fig. 1). This Na^+-neutral amino acid cotransporter (symport) with broad selectivity has been identified a few years ago among previously orphan members of the SLC6 family of sodium (and chloride)-dependent neurotransmitter and amino acid transporters [7]. It is expressed in the luminal brush border membrane of the early segments of the kidney proximal tubule and similarly along the small intestine [39]. Its defect has been shown to cause Hartnup disorder, an autosomal recessive condition which is characterized by a urinary loss of neutral amino acids [26, 42]. In some cases, this aminoaciduria is accompanied by pellagra-like light-sensitive rashes, cerebellar ataxia, and/or other central nervous system (CNS) manifestations. The skin and CNS symptoms appear to be mostly compensated by niacin (nicotinamic acid/vitamin B3) treatment and are thus considered to be due to a defect in intestinal L-tryptophan uptake that leads to a consecutive defect in endogenous niacin production [8].

Sodium-driven reuptake of neutral amino acids by the proximal kidney tubule is analogous to the reuptake of D-glucose. Unlike in intestine, in kidney uptake of luminal D-glucose is mediated by two transporters, the low affinity SGLT2 that is expressed along the two first convoluted segments of the proximal tubule (S1 and S2) and the high affinity SGLT1 that is expressed essentially in the later straight proximal tubule segment S3 [57]. This sequential distribution of a low affinity and a high affinity transporter along the proximal tubule appears meaningful in order to recover a maximum of glucose from the filtrate and it is interesting to observe that such a sequential arrangement of a low and a high apparent affinity transporter is also present for the reuptake of di- and tripeptides, namely with the low affinity PEPT1 in the early proximal tubule and the higher affinity PEPT2 in the late proximal tubule [17].

In the case of neutral amino acid transporters, it is B^0AT1 (SLC6A19) that displays a relatively low apparent affinity for its substrates [4, 11] and that is expressed in the early proximal tubule segments. Interestingly, the structurally related transporter XT2 displays along the proximal tubule an axial complementary arrangement with B^0AT1 and its gene *SLC6A18* is arranged in tandem with *SLC6A19* (B^0AT1) on chromosome 5p15 in human [39]. In a knock-out mouse model of *Slc6a18*, a clear defect in glycine uptake was observed at the level of the late proximal tubule [36]. Together with our unpublished data on Slc6a18 (XT2) expression and on the *Slc6a18* null mouse reanalysis, this indicates that Slc6a18 has in mice the function of a high affinity neutral amino acid transporter analogous to that of SGLT1 for glucose and PEPT2 for peptides. Thus, we propose to rename XT2 B^0AT3 [36] (Fig. 1). The functional importance of this transporter in humans is, however, questioned by the fact that a substantial proportion of the population displays a nonsense single nucleotide polymorphism in this gene with which no phenotype has as yet been associated [20].

The expression of B^0AT1, as well as that of B^0AT3 and SIT1 (see below), has been shown recently to depend in mouse proximal tubule on the expression of collectrin (Tmem27), a relatively short type I transmembrane protein (25 kD) that is approximately 40% identical with the membrane anchor region of the renin–angiotensin system enzyme ACE2 [19, 29]. That collectrin is associated with B^0AT1 in the kidney brush border membranes of wild-type mice has been shown by co-immunoprecipitation [19]. In *collectrin* knock-out mice, the lack of this associated protein leads to the quasi absence of B^0AT1 from the luminal membrane and to a very strong reduction of B^0AT1 at the cellular level, suggesting that association with collectrin stabilizes B^0AT1 and allows its transfer to the luminal brush border membrane. Functionally, the lack of collectrin leads to a massive urinary loss of neutral amino acids that even leads to osmotic diuresis. It is relevant to notice that *collectrin* knock-out mice lose a substantial amount of L-proline in the urine and that their urinary glycine is also very high. These findings suggest that the defect in collectrin affects also transporters participating to the reabsorption of L-proline (SIT1, see below) and glycine (B^0AT3, Slc6a18).

Because collectrin is not expressed in substantial amounts in small intestine and B^0AT1 is normally highly expressed in the brush border membrane of enterocytes of *collectrin* knock-out mice, others and we postulated that another protein can substitute in intestine for the role that collectrin plays in kidney tubule [19, 28]. Having demonstrated that the carboxypeptidase ACE2 colocalizes with B^0AT1 in small intestine and can be coprecipitated with B^0AT1, we tested whether ACE2 absence impacts on the expression of B^0AT1. Indeed, B^0AT1 turned out to be absent from the brush border membrane of *ace2* null mice [12]. Thus, ACE2 is the accessory subunit of these SLC6 transporters in small intestine playing an equivalent role to that of collectrin in kidney. This suggests the possibility that during evolution, a gene duplication of ACE2 allowed the separation of the carboxypeptidase function of ACE2 from its amino acid transporter subunit function allowing independent regulation of the two functions in kidney.

Another member of the same SLC6 amino acid transporter cluster, SLC6A20 in human, has been identified as the molecular correlate of system IMINO, mediating the cotransport of L-proline presumably with two Na^+ and one Cl^- [27, 46]. This transporter called SIT1 or $IMINO^B$ was

shown to localize to the brush border membrane of the proximal tubule in mice by immunofluorescence [39]. As expected for system IMINO, it transports also hydroxy-L-proline and betaine with a relatively high apparent affinity but does not transport glycine. In rodents, a relative recent gene duplication has led to the expression of XT3 ($IMINO^K$, *Slc6a20b*), a very similar transporter that localizes as well to the kidney proximal tubule brush border membrane but the function of which has not yet been characterized. As mentioned above, SIT1/$IMINO^B$ appears to require, as B^0AT1 and B^0AT3, the association with collectrin for its surface expression in kidney. This is at least suggested by the low brush border membrane expression of SIT1/$IMINO^B$ in *collectrin* knock-out mice and by the large urinary L-proline loss in these animals [19].

Besides the sodium-dependent SLC6 family transporters mentioned above, proton cotransporters are also expressed in the kidney brush border membrane. The characterization of their function has been rendered more difficult by the fact that the proton gradient across the luminal membrane of proximal tubule cells depends on the activity of the sodium–proton exchanger NHE3, such that in many assays, their transport function first appeared to be Na^+ dependent. The first kidney H^+-dependent transporters to be identified were the peptide transporters PEPT1 and PEPT2 [18]. Their function in epithelia is complementary to that of amino acid transporters and, in the case of PEPT1, quantitatively very important for the absorption of amino acids across small intestine enterocytes. Within these epithelial cells, di- and tripeptides are mostly hydrolyzed and then released as free amino acids into the extracellular space [16]. More recently, transporters corresponding to the already previously suggested imino acid carrier that cotransport imino acids or glycine with a proton were identified and named PAT1 and PAT2 [1, 5]. Interestingly, PAT1 was first identified in neurons where it functions as a lysosomal amino acid transporter and was correspondingly first named LYAAT [41]. Functionally, the imino acid carrier is expected to be localized in the early segments of the proximal tubule where a recent report by Australian groups just localized PAT2 [9, 13, 23].

In contrast to the neutral amino acids discussed above, cationic amino acids and the disulfide-linked L-cysteine dimer L-cystine enter the epithelial cells along their (electro)-chemical gradient via the cystinuria transporter ($b^{0,+}AT$). This transporter is made of a catalytic subunit belonging to the SLC7 family and a disulfide linked accessory subunit referred to as heavy chain and called rBAT (SLC3A1). Similarly to the basolateral heterodimeric exchangers of the SLC7/SLC3 family (see below), it functions as an obligatory antiporter and specifically exchanges its cationic substrates or L-cystine against intracellular neutral amino acids. How mechanistically this transporter can preferentially perform a heteroexchange of cationic amino acids or cystine against neutral amino acids is still a matter of debate [49]. The hypothesis that we prefer is that heteroexchange is favored by the cooperation of two heterodimeric transporters forming a heterotetramer [21].

The luminal influx of anionic amino acids requires another transporter that has been identified as being EAAT3 (EAAC1, SLC1A1) [25]. This transporter has been shown to cotransport its substrates with three Na^+ ions and one proton in exchange for one K^+ ion [60]. Its expression at the luminal membrane of proximal tubule cells follows a gradient toward the late part of the proximal tubule [43]. Unlike the SLC6 and SLC7 transporters mentioned above, no SLC1 associated transmembrane protein has been identified so far. However, the GTRAP3-18 protein that associates to EAAT3 C terminus appears to be expressed also in kidney [10].

ASCT2 is a Na^+-dependent exchanger of neutral amino acids that belongs to the SLC1 family and has been shown to be expressed at the mRNA level in kidney. One report has suggested that it localizes to the brush border membrane [2, 52]. Its potential role in epithelial transport is not clear and warrants further study.

Cooperation of basolateral transporters for a controlled amino acid efflux

It is well established that the basolateral heterodimeric obligatory amino acid exchanger y^+LAT1-4F2hc plays an important role for the transepithelial transport of its cationic substrates which it transports out of the cells in exchange for extracellular neutral amino acids and Na^+ [35, 50]. The functional importance of this electroneutral exchange for transcellular cationic amino acid transport is demonstrated by the autosomal recessive disease lysinuric protein intolerance that is caused by mutations of the y^+LAT1 gene (*SLC7A7*) [6, 51]. Lysinuric protein intolerance is characterized next to the urinary loss of L-arginine, L-ornithine, and L-lysine also by a poor intestinal absorption of these amino acids. This leads to low plasma concentrations of these amino acids and to an impaired function of the urea cycle and hyperammonemia [47]. The other basolateral heterodimeric obligatory amino acid exchanger belonging to the amino acid absorption machinery is LAT2-4F2hc [40]. It shares with y^+LAT1-4F2hc the same accessory subunit (4F2hc) and also shows the same localization along the proximal kidney tubule [3]. Furthermore, it apparently displays similarly high levels of mRNA and protein expression. As yet, no genetic condition with impaired LAT2 function has been identified, but knock down experiments of LAT2 performed in the proximal tubule OK cell line model have shown a reduction of transepithelial L-cysteine transport [22].

Considering that the function of the proximal tubule amino acid reabsorption machinery is a vectorial transepithelial

transport, whereas these heterodimeric transporters only exchange amino acids, it is necessary to postulate the presence of parallel transporters within the same membrane that can unidirectionally recycle influx substrates of these exchangers back out of the cell. It is interesting to consider that from a global amino acid transport point of view, these directional transporters can control the rate of net amino acid transport, whereas the exchangers only modulate the relative concentration of individual amino acids [54].

One transporter that fulfils the criteria to be considered a basolateral directional amino acid efflux pathway is TAT1 (*SLC16A10*). This transporter has been shown to colocalize in the proximal kidney tubule with the two exchangers mentioned above (Fig. 1) [37]. It has been shown in the *Xenopus laevis* oocyte expression system to function as facilitated diffusion pathway (uniport) for aromatic amino acids [38]. It's recycling function and cooperation with LAT2-4F2hc has also been demonstrated in the same expression system [37]. Indeed, its coexpression enabled LAT2-4F2hc to release endogenous amino acids from oocytes to the exterior in the absence of amino acids in the buffer. This indicates that aromatic amino acids that exit the cell via TAT1 can be recycled back into the cell interior in exchange for intracellular amino acids, before diffusing away from the membrane. This very efficient substrate recycling motivated us to investigate the possibility that TAT1 not only functionally cooperates with LAT2-4F2hc but that it would also physically interact. However, all attempts of co-immunoprecipitation and cross-linking failed to reveal such a physical interaction [37].

Since the transport substrates of TAT1 are high affinity uptake substrates for LAT2-4F2hc and y^+LAT1-4F2hc, TAT1 could theoretically suffice to support their function in kidney proximal tubule. To address this possibility, we have analyzed the plasma and urinary amino acid concentration in TAT1 (*Slc16a10*) null mice. These experiments revealed that the fractional reabsorption of most amino acids, including that of most obligatory exchanger substrates of LAT2-4F2hc is normal (Ramadan et al., unpublished results). From these results, we deduce that at least one additional transporter capable of recycling exchanger substrates, probably another uniport, is or can be expressed at a sufficiently high level in the same basolateral membrane to drive net amino acid transport.

Amino acid transporters involved in uptake/release of amino acids metabolized in proximal kidney tubule cells

Next to its transport functions, the proximal tubule exerts also many metabolic functions. The ones leading to substantial amino acid fluxes and thus transports are (a) the production of ammonia and bicarbonate from L-glutamine, (b) gluconeogenesis mostly also using as amino acid substrate L-glutamine, (c) L-arginine production from citrulline, (d) L-tyrosine production from L-phenylalanine, and (e) L-serine production from glycine [15, 31, 48, 53].

The regulation of amino acid transporters in the context of acidosis has been investigated, since it was expected that the transport of the ammonia precursor L-glutamine would be induced [32, 44]. In a broad study, we analyzed the potential regulation of amino acid transporters in the proximal kidney tubule of mice rendered acidotic with NH_4Cl. The basolateral L-glutamine transporter SNAT3 (*SLC38A3*) was shown to be strongly upregulated by this treatment. Interestingly, not only its total amount was increased but also its localization was extended from the late proximal S3 segment into the earlier S2 portion [32]. It is not surprising that the regulated uptake of L-glutamine as precursor of a metabolic function is through the basolateral membrane, since the availability of this substrate should not depend on its concentration in the urinary filtrate from which it is removed already to a large extent in the early S1 segment. The only other amino acid transporter next to SNAT3 that was substantially regulated by acidosis is the cationic amino acid efflux pathway y^+LAT1 that was downregulated at the protein level.

Conclusions and future directions

Our current model of transcellular amino acid reabsorption by the proximal tubule cells is shown in Fig. 1 and lacks only few elements to be potentially complete. The luminal influx of amino acids is mediated by a series of transporters with uphill transport function, which is fuelled by the cotransport of Na^+ or H^+. An exception to this rule is represented by $b^{0,+}$AT-rBAT which functions as an obligatory exchanger transporting cationic amino acids and cystine along their electrical and chemical gradients into the cells, respectively, in exchange for intracellular neutral amino acids. An open question is the mechanism that allows $b^{0,+}$AT1 to preferentially perform heteroexchange of cationic amino acids or cystine with neutral amino acids [21, 49].

The description of the function of B^0AT3 (*SLC6A18*, formerly XT2) allows us to attribute to this transporter the function of a high affinity broad selectivity neutral amino acid transporter that complements the function of B^0AT1 along the later segments of the proximal tubule (Singer et al., submitted). Whereas in mice the absence of B^0AT3 leads to a substantial aminoaciduria, a frequent nonsense mutation (Y319X) leading presumably to the absence of functional B^0AT3 transporter in a substantial percentage of the Japanese population has as yet not been associated with

any pathological finding [20]. More detailed analysis of the phenotype of homozygous carriers will reveal whether B^0AT3 still has a recognizable amino acid transport function in humans or not.

Another open question is the precise relative role of the two H^+-dependent transporters PAT1 (SLC36A1) and PAT2 (SLC36A2). It is possible that they represent low (PAT1) and high affinity (PAT2) transporters and a recent study suggests that the high affinity transporter PAT2 (SLC36A2) is localized to the early proximal tubule whereas PAT1 (SLC36A1) would be the intestinal transporter [9].

Compared with the luminal amino acid influx step, the efflux step across the basolateral membrane is not as well known, actually for several reasons. One cause is that cellular influx measurements are technically easier to perform and interpret than efflux measurements. A second reason is that a reliable technique to produce luminal brush border membrane vesicles has allowed the study of the function of apical transport proteins independently of the basolateral transporters [33]. Practically, the luminal uptake of amino acids probably requires less physiological control than the basolateral efflux, since from a systemic point of view, luminal amino acids need to be taken up just as much as possible, whereas the basolateral efflux needs to maintain a relatively stable amino acid gradient across the cellular membrane. Indeed, whereas some amino acids display similar concentrations within and outside of the cells, some display an asymmetric distribution. This is in particular the case for the anionic amino acids which are present at much higher concentrations in the cytosol than in the extracellular space, despite the fact that their charge would predict just the opposite. On the other hand, cationic amino acids are not more concentrated in the cytosol compared with plasma, despite of their charge that would drive an intracellular accumulation. Thus, an important function of the basolateral efflux machinery is to preserve this transmembrane amino acid concentration asymmetry.

Two transporters that appear to play an important role for the control of the intracellular concentration of the spectrum of different neutral and cationic amino acids are the obligatory exchangers LAT2-4F2hc (exchange of neutral amino acids) and y^+LAT1-4F2hc (electroneutral exchange of intracellular cationic amino acids against extracellular neutral amino acids + Na^+). In parallel to these basolateral exchange transporters, (an) other transporter(s) that can function as unidirectional amino acid efflux pathway(s) needs to be present to allow a net vectorial transport. Which transporter(s) fulfil(s) this function is not yet fully established. We have shown, using the *X. laevis* oocyte expression system, that the aromatic amino acid transporter TAT1 that is present in the proximal kidney tubule and small intestine basolateral membranes is capable of fulfilling this function [37]. However, the TAT1 knock-out mouse does not show the dramatic change in urinary amino acid concentrations that would be expected, if TAT1 was the only transporter exerting the recycling function necessary to activate the efflux via the exchangers LAT2-4F2hc and y^+LAT1-4F2hc. Another candidate for this recycling function is LAT4 (Slc43a2) which we have also shown to be able to cooperate with LAT2 in the *X. laevis* oocyte expression system [37]. Clearly more work is required to fully understand the controlled efflux of amino acids through the basolateral membrane of transporting epithelia.

Yet another challenging question is how anionic amino acids are released from epithelial cells, maintaining the very high concentration gradient between the cells and the extracellular space. Transporters from two families have been proposed to potentially be involved in this controlled efflux. In particular is AGT1 (SLC7A13), a gene product belonging to the family of heterodimeric amino acid exchangers to which LAT2 and y^+LAT1 belong, but the putative heavy chain of which has not been identified. Its anionic amino acid transport function could only be revealed upon fusing it to the "heavy chains" rBAT or 4F2hc. However, the question whether it functions as obligatory exchanger has not been addressed [8, 30]. Also, a potential role of the two members of the SLC1 family EAAT1 (SLC1A3) and ASCT2 (SLC1A5) for the efflux of anionic amino acids has been proposed [56]. However, a clear concept as to how the net efflux of anionic amino acids through the basolateral membrane of proximal kidney tubule cells takes place and is controlled has not been put forward yet.

Considering the important progress that the identification and characterization of presumably most amino acid transporters represents, it appears now to be important to understand more quantitatively their function and cooperation within cells, also in respect of local amino acid concentrations. Importantly, transporters not only control the flux of amino acids across transporting epithelia such as that of the proximal kidney tubule but also between the organs of the organism. Thus, they control the availability of these substrates and protein building blocks that play a central role in cell growth control in physiology and pathophysiology. Increasing our knowledge about the mechanisms that govern amino acid transport across epithelia and also their homeostasis in the organism will require the development of additional new techniques to follow accurately their local concentrations. To integrate the resulting large amount of complex data on amino acid fluxes and on transporter kinetics and regulatory behavior, complementary modeling approaches will be required.

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