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Rapid report

Cloning and functional characterization of a Na^+-independent, broad-specific neutral amino acid transporter from mammalian intestine

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Abstract

We have isolated a cDNA from a rabbit intestinal cDNA library which, when co-expressed with the heavy chain of the human 4F2 antigen (4F2hc) in mammalian cells, induces system L-like amino acid transport activity. This protein, called LAT2, consists of 535 amino acids and is distinct from LAT1 which also interacts with 4F2hc to induce system L-like amino acid transport activity. LAT2 does not interact with rBAT, a protein with a significant structural similarity to 4F2hc. The 4F2hc/LAT2-mediated transport process differs from the 4F2hc/LAT1-mediated transport in substrate specificity, substrate affinity, tissue distribution, interaction with D-amino acids, and pH-dependence. The 4F2hc/LAT2-associated transport process has a broad specificity towards neutral amino acids with K_t values in the range of 100–1000 μM, does not interact with D-amino acids to any significant extent, and is stimulated by acidic pH. In contrast, the 4F2hc/LAT1-associated transport process has a narrower specificity towards neutral amino acids, but with comparatively higher affinity (K_t values in the range of 10–20 μM), interacts with some D-amino acids with high affinity, and is not influenced by pH. LAT2 is expressed primarily in the small intestine and kidney, whereas LAT1 exhibits a much broader tissue distribution.

Keywords: Amino acid transport; System L; LAT2; 4F2 heavy chain; Primary structure; Functional expression; Rabbit

1. Introduction

The digestion products of dietary proteins are absorbed in the small intestine in the form of small peptides and free amino acids [1–3]. While the absorption of peptides is mediated predominantly by a single peptide transporter (PEPT1), the absorption of amino acids involves participation of several transport systems. The amino acid transport systems in the intestine differ in their substrate specificity and driving force. In recent years, several amino acid transporters have been cloned and functionally characterized [4–6]. However, most of these transporters have been cloned from non-intestinal tissues and these transporters may or may not be expressed in the intestine. The amino acid transporters that have been characterized at the molecular level and are known to be expressed in the intestine include ATB^0/ASCT2 [7], $b^{0,+}$ [5], and y^+ L [8,9]. ATB^0 is a Na^+-dependent neutral amino acid transporter that is expressed in the intestine [7] as well as in other non-intestinal tissues [10]. $b^{0,+}$ is a Na^+-independent

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amino acid transporter that is specific for neutral and cationic amino acids. It consists of at least two subunits, one of which (rBAT) has been cloned and characterized [5]. Genetic mutations in rBAT are responsible for a subgroup of patients with cystinuria, an intestinal and renal disorder associated with defective absorption of cationic and neutral amino acids and cystine [5,11]. y^+ L is a Na^+-independent transporter for cationic amino acids and Na^+-dependent transporter for neutral amino acids [5,6]. It also consists of two subunits, one of which is the heavy chain of the cell surface antigen 4F2 and the other is y^+ LAT1 [8,9,12]. Genetic mutations in y^+ LAT1 are responsible for lysinuric protein intolerance, an intestinal and renal disorder associated with defective absorption of cationic and neutral amino acids [13,14].

Recently, the amino acid transport system L has been characterized at the molecular level [15–17]. This is a Na^+-independent transporter for neutral amino acids. It also consists of two subunits, one of which is the heavy chain of the cell surface antigen 4F2 and the other is LAT1. Northern blot analysis has shown that LAT1 transcripts are not expressed in the small intestine. With an aim to determine whether the small intestine expresses any amino acid transporter that is structurally related to LAT1, we screened in the present study a rabbit intestinal cDNA library with human LAT1 cDNA as a probe. This screening resulted in the isolation of a cDNA that interacts with 4F2 heavy chain to induce a system L-like amino acid transport activity. Even though this newly identified transport system exhibits functional characteristics similar to the 4F2hc/LAT1 complex-induced system L activity, there are significant differences. Accordingly, we refer to this new transporter subunit as LAT2. Here we describe the structural features of LAT2 and the functional characteristics of the transport system induced by the 4F2hc/LAT2 complex.

2. Materials and methods

2.1. Isolation and sequence determination of rabbit LAT2 cDNA

The rabbit intestinal cDNA library [7] was screened with a 1.7-kbp fragment of human LAT1 cDNA [17] obtained by digestion of the full-length cDNA with *Kpn*I/*Pvu*II. The probe was labeled with [α-^{32}P]dCTP by random priming using the Ready-to-go oligolabeling beads (Amersham Pharmacia Biotech). The screening was done under low stringency conditions [18–20]. Positive clones were identified and the colonies purified by secondary screening. The clones were analyzed for restriction sites and partially sequenced at their 5′ ends. This analysis revealed that the clones isolated from the library consisted of two different types of cDNAs. One was found to be identical to the $b^{0,+}$ amino acid transporter cDNA that was reported recently from our laboratory [21]. The other represented a new cDNA that has not been previously described. There were four positive clones containing this cDNA insert. The size of the insert was, however, different in each clone. The clone with the longest cDNA insert was selected for further analysis. Both sense and antisense strands of the cDNA were sequenced by primer walking using *Taq* DyeDeoxy terminator cycle sequencing in an automated Perkin-Elmer Applied Biosystems 377 Prism DNA sequencer. The sequence was analyzed using the GCG sequence analysis software package GCG version 7.B (Genetics Computer Group, Madison, WI).

2.2. Functional expression of rabbit LAT2 cDNA

The vaccinia virus expression system was used to functionally characterize the cloned cDNA as described previously [17–19]. The cDNAs of rabbit LAT2, human 4F2hc and human rBAT were all cloned into pSPORT such that the sense transcription of the cDNAs is under the control of T7 promoter. The cDNAs were transfected into HRPE cells grown in 24-well tissue culture plates using lipofectin and the functional expression of the cDNA was analyzed 12 h later by measuring radiolabeled amino acid uptake. One microgram of the plasmid carrying the specific cDNA (LAT2, 4F2hc or rBAT) was used per well. Sister wells transfected identically with vector alone served as control. The DNA:lipofectin ratio was kept constant at 1:2.5 in all cases by including the empty vector DNA wherever necessary. The transport buffer was composed of 25 mM HEPES/Tris (pH 7.5), supplemented with 140 mM NaCl, 5.4

mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. When the effect of Na^+ on amino acid uptake was studied, the NaCl in the buffer was replaced with *N*-methyl-D-glucamine (NMDG) chloride. The pH of the transport buffer was varied by adjusting the concentrations of HEPES, MES, and Tris appropriately. Uptake was initiated by incubating the cell monolayers with radiolabeled amino acids in transport buffer for a suitable time period. Following the incubation, the medium containing the radiolabeled substrate was aspirated off and the cells were washed with 2×2 ml of ice-cold transport buffer. The cells were then solubilized in 0.5% SDS in 0.2 N NaOH, transferred to vials and radioactivity associated with the cells quantitated by liquid scintillation spectrometry. Initial experiments revealed that the cDNA-specific transport was linear at least up to 20 min. All subsequent experiments were therefore carried out using a 15-min incubation period. The experiments were repeated 2–3 times with independent transfections, each done in duplicate or triplicate. Data are presented as means ± S.E. of these replicate measurements.

2.3. Northern blot analysis

Poly(A)$^+$ RNA, isolated from different rabbit tissues (small intestine, kidney, heart, lung, liver, and skeletal muscle), was used for Northern blot analysis. The membrane filter containing the size-fractionated RNA was probed sequentially, first with LAT2 cDNA and then, following stripping, with cyclophillin cDNA. Both probes were labeled with [α-^{32}P]dCTP by random priming using the Ready-to-go oligolabeling kit. The hybridization and post-hybridization washings were done under high stringency conditions.

3. Results and discussion

3.1. Structural features of rabbit LAT2

The rabbit LAT2 cDNA (Genbank accession no. AF170106) is 4070 bp long with an open reading frame of 1608 bp (including the termination codon), encoding a protein of 535 amino acids (Fig. 1). The open reading frame is flanked by a 5′ non-coding sequence of 743 bp long and a 3′ non-coding sequence of 1719 bp long. The predicted molecular mass of the protein is 58 kDa. Hydrophobicity analysis of the predicted amino acid sequence using the algorithm of Kyle and Doolittle [22] with a window size of 17–23 amino acid residues indicates that rabbit LAT2 contains 12 putative transmembrane domains. Base on the inside-positive rule of membrane topology of integral membrane proteins, both the amino terminus and the carboxy terminus lie on the cytoplasmic side. The protein does not contain any *N*-linked glycosylation sites in putative extracellular domains. It does possess, however, one site for protein kinase A-dependent phosphorylation (Thr-363), three sites for protein kinase C-dependent phosphorylation (Thr-11, Thr-337, and Thr-487), and one site for tyrosine kinase-dependent phosphorylation (Tyr-109) in putative intracellular domains. Blast search indicates that rabbit LAT2 is most closely related to human LAT1 in amino acid sequence (52% identity). The sequence comparison between rabbit LAT2 and human LAT1 is given in Fig. 1.

3.2. Functional characteristics of rabbit LAT2

Since the newly cloned transporter bears structural similarity to LAT1 that functions as a heterodimer with 4F2hc to induce system L-like amino acid transport activity, we investigated the ability of LAT2 to mediate amino acid transport with or without the co-expression of 4F2hc in the human retinal pigment epithelial (HRPE) cell line. Several amino acids were initially tested as possible substrates. These studies showed that the 4F2hc/LAT2 complex mediated the transport of several neutral amino acids (glutamine, serine, alanine, threonine, leucine, isoleucine, phenylalanine, and tryptophan), but not of anionic amino acids and cationic amino acids (data not shown). Fig. 2 describes the characteristics of the transport of glutamine as a representative of the transportable neutral amino acids. In the presence of Na^+, neither 4F2hc nor LAT2 mediated glutamine transport (Fig. 2A). Similarly, the transport activity remained unaltered, even when the two proteins were co-expressed. However, when measured in the absence of Na^+, the transport of glutamine was much higher in cells co-expressing the two proteins than in cells expressing either of the proteins independently

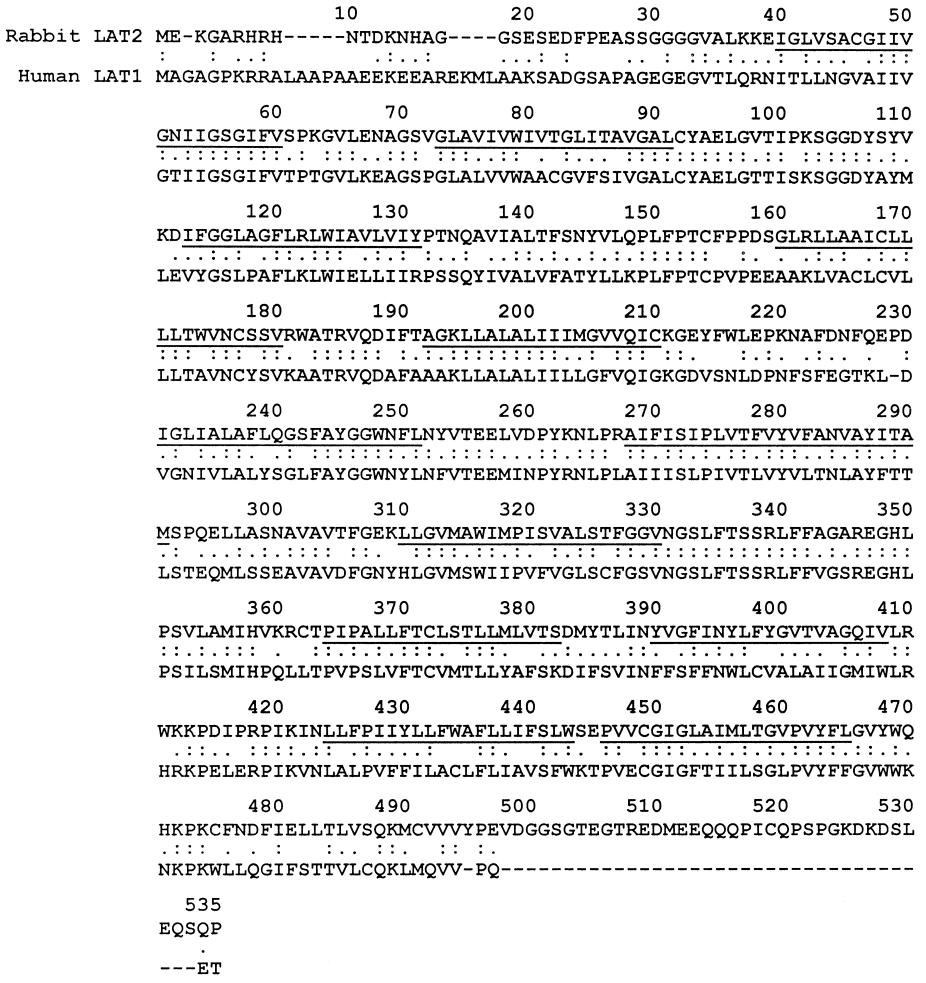

Fig. 1. Primary structure of rabbit LAT2. The sequence of rabbit LAT2 is compared with that of human LAT1. The putative transmembrane domains in rabbit LAT2 are underlined.

(Fig. 2B). These data show that the 4F2hc/LAT2 complex mediates Na^+-independent transport of glutamine. It is of some interest that the complex-mediated glutamine transport could not be detected when measured in the presence of Na^+. A Na^+-independent transport process is expected to be detectable both in the presence and absence of Na^+. It is possible that the high endogenous glutamine transport activity seen in the presence of Na^+ masks the 4F2hc/LAT2 complex-mediated transport activity in this experimental system. Since 4F2hc is structurally related to rBAT [5], we then examined if LAT2 is capable of interacting with rBAT to induce the Na^+-independent glutamine transport activity. In the presence as well as absence of Na^+, rBAT and LAT2 failed to induce glutamine transport either independently or together (Fig. 2C,D).

Since the transport activity of the 4F2hc/LAT2 complex was much higher for glutamine and serine than for other neutral amino acids, we examined the interaction of these two substrates with the 4F2hc/LAT2 complex in detail. Fig. 3 describes the saturation kinetics of Na^+-independent transport of glutamine (Fig. 3A) and serine (Fig. 3C) in control cells transfected with vector alone or in cells co-expressing 4F2hc and LAT2. The transport was saturable in each case. Fig. 3B and D describe the saturation kinetics of the 4F2hc/LAT2 complex-specific transport of glutamine and serine. The Michaelis–Menten constant (K_t) for the 4F2hc/LAT2 complex-mediated transport was 316 ± 67 μM for glutamine and 204 ± 17 μM for serine.

Fig. 4 describes the specificity of the transport process mediated by the 4F2hc/LAT2 complex as

evidenced from the ability of various unlabeled amino acids to compete with radiolabeled glutamine and serine for the transport process. Only the 4F2hc/LAT2 complex-specific transport was analyzed. The transport of [^{3}H]glutamine (1 μM) was markedly inhibited by 5 mM unlabeled glutamine, leucine, alanine, serine, cysteine, phenylalanine, tryptophan, threonine, and the system L-specific amino acid BCH. In contrast, the anionic amino acids glutamate and aspartate, the cationic amino acids arginine and lysine, and the imino acid proline did not compete with [^{3}H]glutamine. Similarly, cystine also failed to inhibit [^{3}H]glutamine transport. The inhibition pattern of [^{3}H]serine transport was exactly the same as that of [^{3}H]glutamine transport. This substrate specificity is the characteristic of system L. The inability to interact with cationic amino acids eliminates the possible identity of the 4F2hc/LAT2 complex-mediated transport process with systems y^+ L and $b^{0,+}$. The dose–response relationship for the inhibition of the 4F2hc/LAT2 complex-mediated transport of [^{3}H]glutamine by seven different neutral amino acids is given in Fig. 5. The IC_{50} values (i.e. concentration of unlabeled amino acid at which the inhibition was 50%) for these amino acids were as follows: threonine, 79 ± 5 μM; phenylalanine, 181 ± 23 μM; tryptophan, 296 ± 17 μM; serine, 306 ± 16 μM; glutamine, 318 ± 38 μM; leucine, 324 ± 7 μM; alanine, 791 ± 106 μM. The IC_{50} values for glutamine and serine to inhibit [^{3}H]glutamine transport (318 ± 38 μM and 306 ± 16 μM, respectively) agreed closely with the corresponding K_t values calculated directly

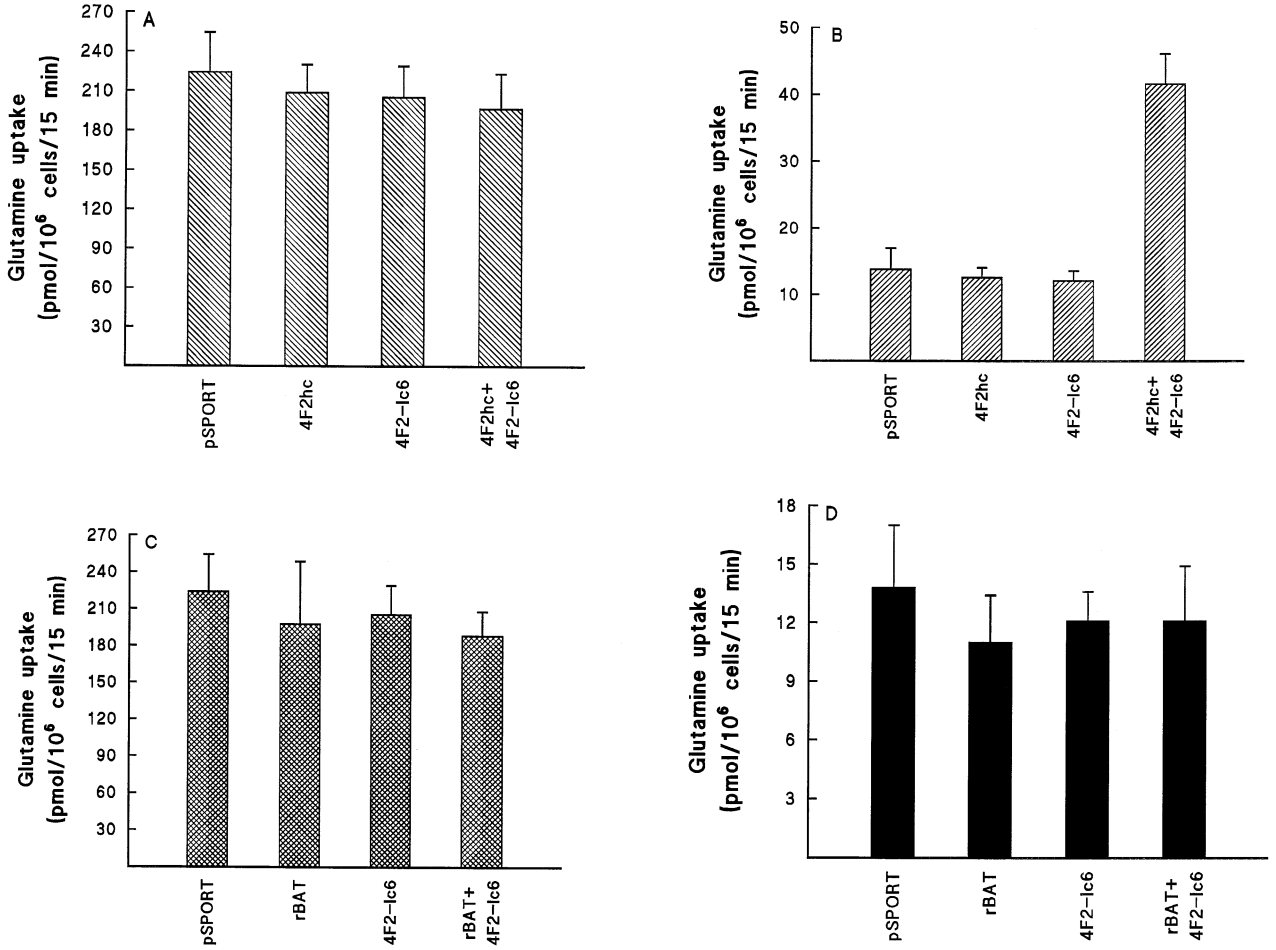

Fig. 2. Characteristics of glutamine transport associated with LAT2. HRPE cells were transfected with pSPORT vector, human 4F2hc cDNA, human rBAT cDNA, and rabbit LAT2 cDNA either independently or together. Transport of 1 μM [^{3}H]glutamine was measured in these cells with a 15-min incubation in the presence (A,C) or absence (B,D) of Na^+.

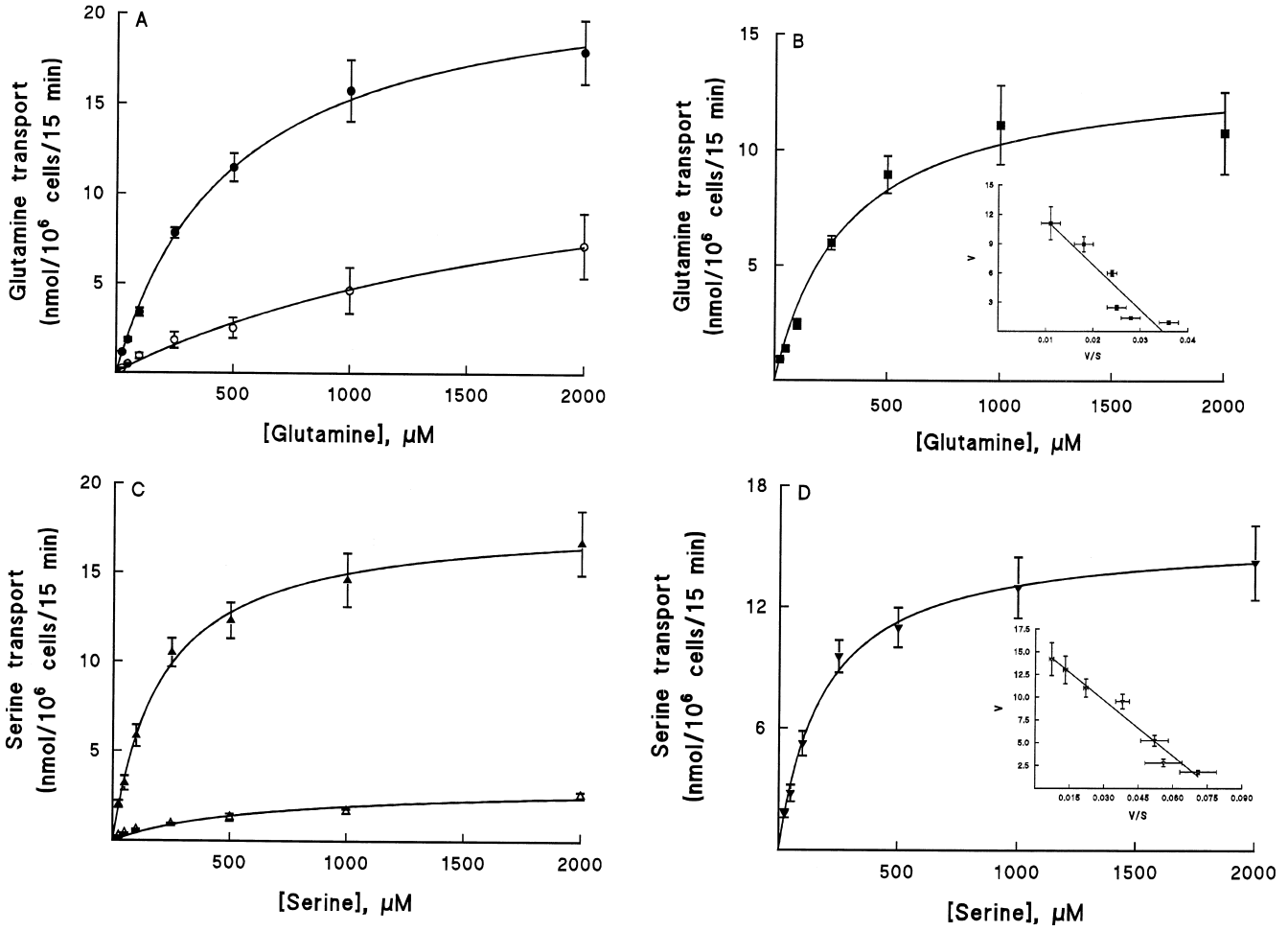

Fig. 3. Saturation kinetics of glutamine transport (A) and serine transport (C), measured in the absence of Na^+, in cells transfected with vector alone (○) and in cells co-expressing 4F2hc and LAT2 (●). Saturation kinetics of glutamine transport (B) and serine transport (D) specific for the 4F2hc/LAT2 complex, given either as substrate concentration versus transport or as an Eadie–Hofstee plot (inset).

from the transport of these two amino acids (316 ± 67 μM and 204 ± 17 μM, respectively).

The functional characteristics of LAT1 cloned from rat and human tissues have been studied in detail [15,17]. Comparison of the characteristics of LAT1 with those of LAT2 reveals important differences in substrate selectivity and affinity. LAT1 interacts preferentially with large neutral amino acids. Short chain neutral amino acids show comparatively less affinity for LAT1. In contrast, LAT2 has a much broader substrate selectivity, interacting with short chain as well as large neutral amino acids with comparable affinity. In terms of substrate affinity, LAT1 is a high-affinity transporter with K_t values for transportable substrates, such as tryptophan, in the range of 10–20 μM. In contrast, LAT2 is a relatively low-affinity transporter with K_t values for transportable substrates being several-fold higher than observed in the case of LAT1.

A unique characteristic of LAT1 is its ability to interact with D-amino acids [15]. D-Isomers of leucine, phenylalanine, and methionine are recognized as substrates by LAT1, even though D-isomers of valine, histidine, tyrosine, and tryptophan are not. Since the present study has already revealed significant differences between LAT1 and LAT2 in terms of substrate selectivity and affinity, we compared the interaction of these two transporters with D-leucine and D-valine. Human LAT1 and rabbit LAT2 were co-expressed with human 4F2hc and the activities of

the 4F2hc/LAT1 and 4F2hc/LAT2 complexes were measured with [^{3}H]tryptophan and [^{3}H]serine as appropriate substrates, respectively. The ability of D-leucine and D-valine to inhibit these transport activities was compared (Fig. 6A). As observed earlier [15], the LAT1-associated [^{3}H]tryptophan transport was inhibited markedly by D-leucine (IC_{50}, 34 ± 4 μM). D-valine was about 200-fold less potent (IC_{50}, 7.0 ± 1.1 mM). The LAT2-associated [^{3}H]serine transport was also inhibited by D-leucine, but with an IC_{50} value of >10 mM. D-valine was almost totally ineffective. Thus, the affinity of D-leucine for LAT2 appears to be at least 300-fold lower than for LAT1. Thus, LAT2 differs from LAT1 in its interaction with D-leucine.

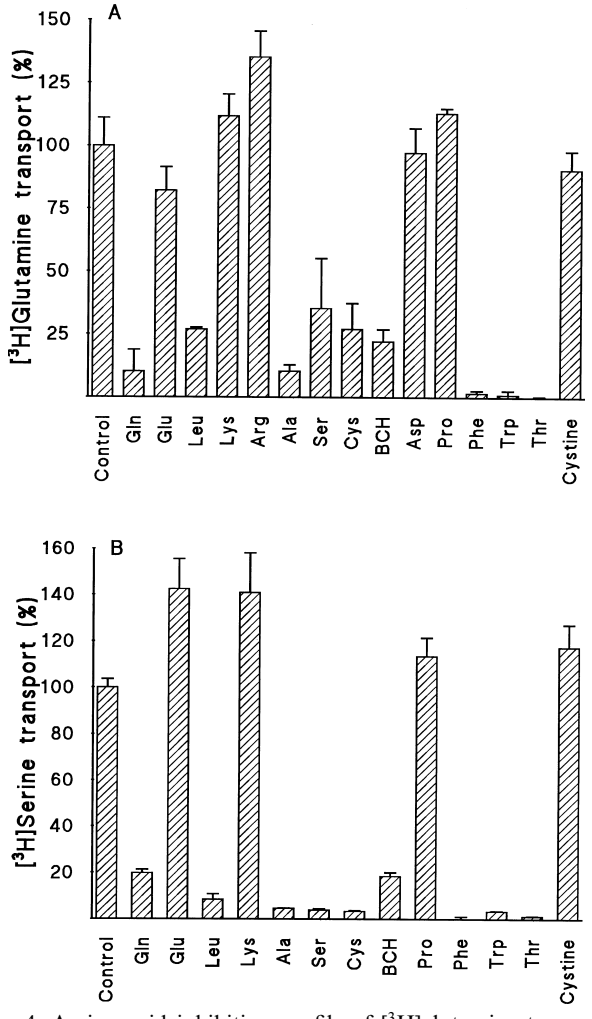

Fig. 4. Amino acid inhibition profile of [^{3}H]glutamine transport (A) and [^{3}H]serine transport (B) mediated by the 4F2hc/LAT2 complex. Transport of [^{3}H]glutamine (1 μM) and [^{3}H]serine (1 μM) was measured with a 15-min incubation in the absence of Na^+ in cells transfected with vector alone and in cells co-expressing 4F2hc and LAT2 in the presence or absence of indicated amino acids (5 mM). Transport in vector-transfected cells was subtracted from corresponding transport in cells co-expressing 4F2hc and LAT2 to calculate the 4F2hc/LAT2 complex-specific transport. Results are given as percent of control transport measured in the absence of inhibitory amino acids.

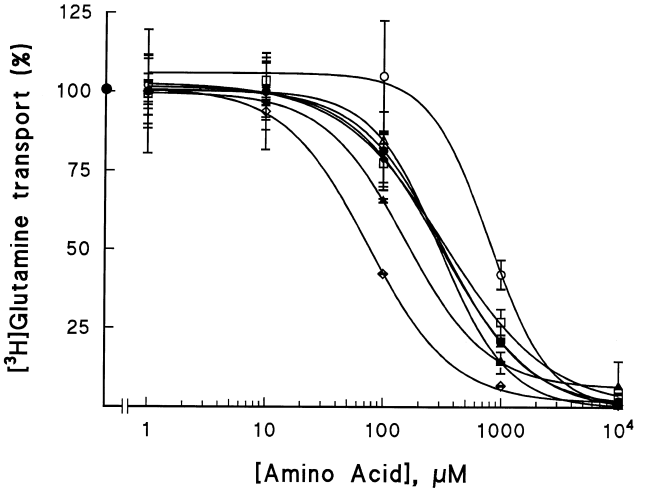

Fig. 5. Inhibition of the 4F2hc/LAT2 complex-specific [^{3}H]glutamine transport by alanine (○), leucine (●), glutamine (□), tryptophan (△), phenylalanine (▲), threonine (◇), and serine (◆). Transport of 1 μM [^{3}H]glutamine was measured with a 15-min incubation in the absence of Na^+ in cells transfected with vector alone and in cells co-expressing 4F2hc and LAT2 in the presence of varying concentrations of unlabeled amino acids. Transport in vector-transfected cells was subtracted from corresponding transport in cells co-expressing 4F2hc and LAT2 to calculate the 4F2hc/LAT2 complex-specific transport. Results are given as percent of control transport measured in the absence of inhibitory amino acids.

We also analyzed the pH-dependence of LAT2 in the present study because our earlier work [17] has shown that LAT1 activity is not influenced by pH in the range of 5.5–8.5. The present studies show that the transport activity of LAT2 is significantly influenced by pH in this range (Fig. 6B). The activity is much higher at pH 6.5 than at pH 8.5. With glutamine as the substrate, the activity increases almost 100% when the pH is changed from 8.5 to 6.5. The corresponding increase is about 70% with serine as the substrate. The difference between the transport activities at pH 8.5 and 6.5 is statistically significant in both cases ($P < 0.05$). We have shown earlier that system L transport activity in human placental choriocarcinoma cells is stimulated by acidic pH [23,24],

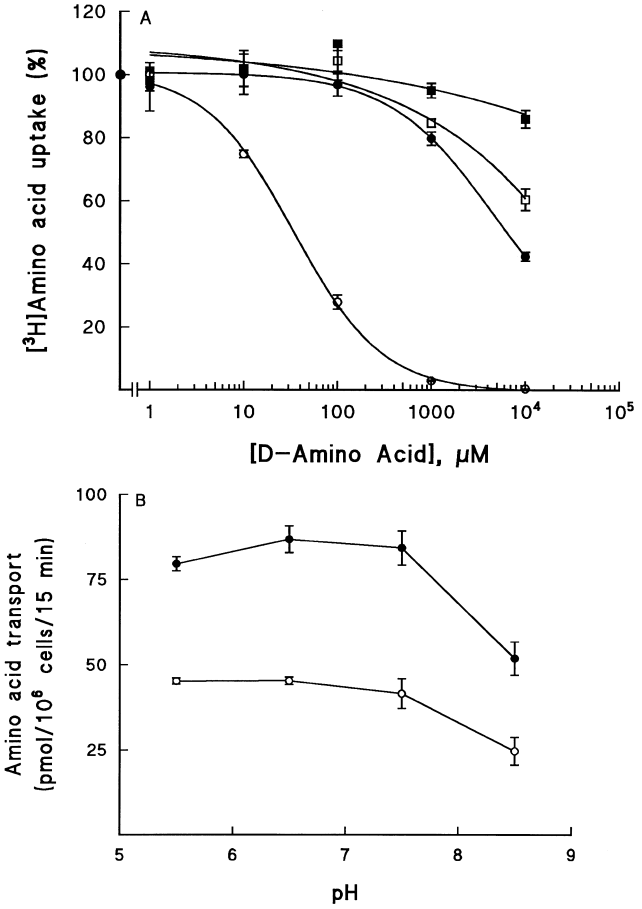

Fig. 6. (A) Comparison of the interaction of the 4F2hc/LAT2 complex and the 4F2hc/LAT1 complex with D-amino acids. Cells were transfected with vector alone, human 4F2hc cDNA plus rabbit LAT2 cDNA or human 4F2hc cDNA plus human LAT1 cDNA. Transport was measured with a 15-min incubation in the absence of Na^+. Transport of 1 μM [^{3}H]serine was measured to assess LAT2-associated activity (□,■) while transport of 1 μM [^{3}H]tryptophan was measured to assess LAT1-associated activity (○,●). In both cases, transport measured in vector-transfected cells was subtracted from corresponding transport measured in cells co-expressing 4F2hc/LAT2 or 4F2hc/LAT1 to calculate the transport specific for the 4F2hc/LAT2 complex (□,■) or for the 4F2hc/LAT1 complex, (○,●). Transport was measured in the presence of varying concentrations of D-leucine (○,□) or D-valine (●,■). Results are given as percent of control transport measured in the absence of inhibitory amino acids. (B) pH-Dependence of 4F2hc/LAT2-specific transport of glutamine (○) and serine (●). Transport of 1 μM [^{3}H]glutamine and 1 μM [^{3}H]serine was measured at different pH with a-15 min incubation in the absence of Na^+ in cells transfected with vector alone and in cells co-expressing human 4F2hc and rabbit LAT2. Data represent only the 4F2hc/LAT2 complex-specific transport calculated by subtracting the transport in vector-transfected cells from the corresponding transport in cells expressing the 4F2hc/LAT2 complex.

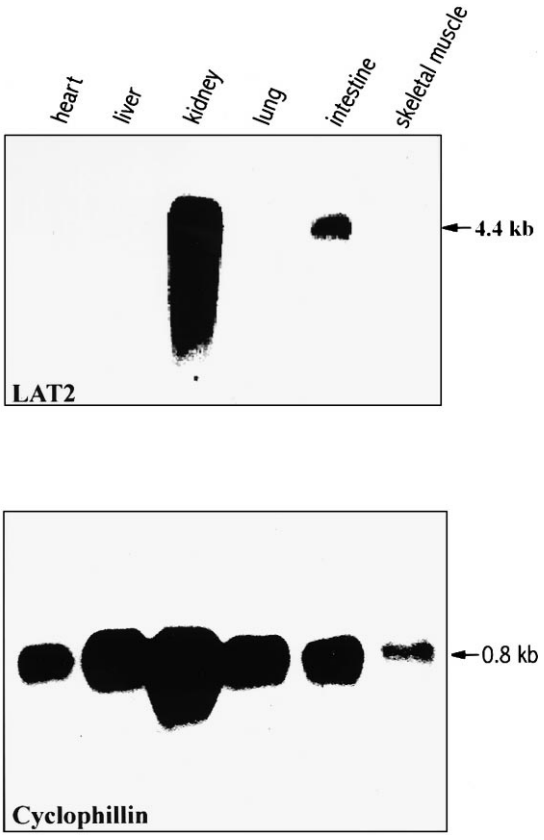

Fig. 7. Northern blot analysis of LAT2 mRNA expression in rabbit tissues. The blot was hybridized sequentially with ^{32}P-labeled rabbit LAT2 cDNA and cyclophillin cDNA. Each lane contained 5 μg of poly(A)$^+$ RNA isolated from the tissues indicated.

suggesting that the pH-responsive transport in these cells is likely to be mediated by LAT2. Thus, pH-dependence is an additional distinguishing characteristic between LAT1 and LAT2.

3.3. Expression pattern of LAT2 in rabbit tissues

The tissue distribution pattern of LAT2 was studied by Northern blot analysis (Fig. 7). In the rabbit, the LAT2-specific transcripts (4.4 kb in size) was detectable only in the small intestine and kidney. Heart, skeletal muscle, liver, and lung were negative. The tissue distribution pattern of LAT2 is clearly different from that of LAT1 which is expressed in most tissues except small intestine [15,17].

In summary, we have cloned and functionally characterized a new amino acid transport-associated protein which interacts with 4F2hc to induce system

L-like transport activity. This protein, called LAT2, is distinct from the previously described LAT1. Even though both LAT1 and LAT2 interact with 4F2hc to induce system L-like amino acid transport activity, the two systems differ significantly in substrate selectivity, substrate affinity, interaction with D-leucine, pH-dependence and tissue distribution. While this manuscript was in preparation, Segawa et al. [25] and Pineda et al. [26] reported the cloning and functional characterization of the rat and human homologs of rabbit LAT2.

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