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Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines

Osamu Yanagida [a], Yoshikatsu Kanai [b,h,*], Arthit Chairoungdua [b], Do Kyung Kim [b], Hiroko Segawa [b,c], Tomoko Nii [d], Seok Ho Cha [b], Hirotaka Matsuo [b,e], Jun-ichi Fukushima [a], Yoshiki Fukasawa [b], Yoshiko Tani [d], Yutaka Taketani [d], Hiroshi Uchino [b,f], Ju Young Kim [b], Jun Inatomi [b], Isao Okayasu [g], Ken-ichi Miyamoto [c], Eiji Takeda [d], Tomoyuki Goya [a], Hitoshi Endou [b]

[a] *Second Department of Surgery, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan*
[b] *Department of Pharmacology and Toxicology, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan*
[c] *Department of Nutritional Chemistry, School of Medicine, Tokushima University, Kuramoto-Cho 3, Tokushima 770-8503, Japan*
[d] *Department of Clinical Nutrition, School of Medicine, Tokushima University, Kuramoto-Cho 3, Tokushima 770-8503, Japan*
[e] *First Department of Physiology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan*
[f] *Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan*
[g] *Department of Pathology, Kitasato University School of Medicine, 1-51-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan*
[h] *PRESTO, Japan Science and Technology Corporation (JST), 6-20-2 Shinkawa, Mitaka, Tokyo, 181-8611, Japan*

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Abstract

System L is a major nutrient transport system responsible for the transport of large neutral amino acids including several essential amino acids. We previously identified a transporter (L-type amino acid transporter 1: LAT1) subserving system L in C6 rat glioma cells and demonstrated that LAT1 requires 4F2 heavy chain (4F2hc) for its functional expression. Since its oncofetal expression was suggested in the rat liver, it has been proposed that LAT1 plays a critical role in cell growth and proliferation. In the present study, we have examined the function of human LAT1 (hLAT1) and its expression in human tissues and tumor cell lines. When expressed in *Xenopus* oocytes with human 4F2hc (h4F2hc), hLAT1 transports large neutral amino acids with high affinity (K_m = ~15–~50 μM) and L-glutamine and L-asparagine with low affinity (K_m = ~1.5–~2 mM). hLAT1 also transports D-amino acids such as D-leucine and D-phenylalanine. In addition, we show that hLAT1 accepts an amino acid-related anti-cancer agent melphalan. When loaded intracellularly, L-leucine and L-glutamine but not L-alanine are effluxed by extracellular substrates, confirming that hLAT1 mediates an amino acid exchange. hLAT1 mRNA is highly expressed in the human fetal liver, bone marrow, placenta, testis and brain. We have found that, while all the tumor cell lines examined express hLAT1 messages, the expression of h4F2hc is varied particularly in leukemia cell lines. In Western blot analysis, hLAT1 and h4F2hc have been confirmed to be linked to each other via a disulfide bond in T24 human bladder carcinoma cells. Finally, in in vitro translation, we show that hLAT1 is not a glycosylated protein even though an *N*-glycosylation site has been predicted in its extracellular loop, consistent with the property of the classical 4F2 light chain. The properties of the hLAT1/h4F2hc complex would support the roles of this

Abbreviations: 4F2hc, 4F2 heavy chain; LAT1, L-type amino acid transporter 1; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid

* Corresponding author. Fax: +81-422-79-1321. *E-mail address:* ykanai@kyorin-u.ac.jp (Y. Kanai).

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transporter in providing cells with essential amino acids for cell growth and cellular responses, and in distributing amino acid-related compounds.

Keywords: Amino acid transporter; System L; CD98; Essential amino acid; Malignant tumor; Melphalan

1. Introduction

Amino acid transport across the plasma membrane is mediated via amino acid transporters located on the plasma membrane. Among the amino acid transport systems described, system L is a major route for providing cells with large neutral amino acids including branched or aromatic amino acids [1]. System L, a Na^+-independent neutral amino acid transport agency, was originally identified in Ehrlich ascites carcinoma cells [2]. By expression cloning, we isolated, from C6 rat glioma cells, a cDNA encoding a Na^+-independent transporter LAT1 (L-type amino acid transporter 1), subserving the amino acid transport system L [3]. LAT1 preferentially transports large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine and histidine [3]. We and other researchers further demonstrated that LAT1 requires an additional protein, the heavy chain of the 4F2 antigen which forms a heterodimeric functional complex with the LAT1 protein [3–6].

The 4F2 antigen (CD98) was originally identified as a cell-surface antigen associated with lymphocyte activation [7,8]. It was later shown to be involved in various cellular activities such as cell proliferation, cell transformation and cell adhesion [7–11]. The 4F2 antigen is a heterodimeric complex composed of two subunits, an $\sim$80-kDa glycosylated heavy chain and a $\sim$40-kDa nonglycosylated light chain [7,8]. The 4F2 heavy chain (4F2hc) is an integral membrane protein with a single-membrane spanning domain, classified as a type II membrane glycoprotein [12–14]. Transporters subserving the amino acid transport systems L, y+L, x−C and asc have been shown to require 4F2hc for their functional expression, suggesting that these amino acid transporters are 4F2 light chains [3–6,15–21]. Furthermore, a protein structurally related to the 4F2hc-associated transporters has been determined to couple with the cystinuria-related type II membrane glycoprotein rBAT (related to $b^{0,+}$ amino acid transporter) and exhibit a function corresponding to that of system $b^{0,+}$, thereby establishing a family of amino acid transporters associated with type II membrane glycoproteins (LAT family) [22–24].

In transformed cells, it has been proposed that amino acid transporters are upregulated to support the high-level protein synthesis for continuous growth and proliferation [1]. It was previously reported that TA1/E16, a partial sequence corresponding to LAT1, was strongly expressed in rat transformed cell lines and the rat fetal liver whereas it was not detected in the adult liver [25]. TA1/E16 immunoreactivity was shown to be strong in human colon cancer tissues in vivo but barely detected in the surrounding normal colon tissues [26]. LAT1 expression has, therefore, been proposed to be related to the growth and proliferation of tumor cells and cells during tissue development [3,25,26]. Although the cDNA for human LAT1 was cloned and analyzed by transiently expressing it in human retinal pigment epithelial (HRPE) cells [27], its functional properties and substrate selectivity were not fully examined. In the present study, in order to investigate the role of LAT1 in human tumor cells, we isolated a cDNA encoding human LAT1 from PA-1 teratocarcinoma cells. We report here a more extensive characterization of its functional properties by expressing it in *Xenopus* oocytes and the protein characterization using antibodies raised against LAT1 and 4F2hc. We also report the expression of LAT1 and 4F2hc in human tumors cell lines by Northern blot analysis using human LAT1 cDNA as a probe.

2. Materials and methods

2.1. Functional characterization

Human LAT1 (hLAT1) cDNA (GenBank/EMBL/DDBJ accession no. AB018009) was isolated from the oligo dT-primed cDNA library prepared from human teratocarcinoma PA-1 cell poly(A)$^+$ RNA

(Clontech) using a fragment corresponding to nucleotides 1135–1529 of rat LAT1 cDNA (GenBank/EMBL/DDBJ accession no. AB015432) as a probe, as described elsewhere [16,21,28,29]. The amino acid sequence of hLAT1 was 100% identical to that of the human LAT1 published by Prasad et al. [27].

For functional expression in *Xenopus* oocytes, cRNAs for hLAT1 and human 4F2hc (h4F2hc) (GenBank/EMBL/DDBJ accession no. AB018010) were obtained by in vitro transcription using T3 RNA polymerase for hLAT1 in pBluescript II SK$^-$ (Stratagene) linearized with *Xho*I and T7 RNA polymerase for human 4F2hc (h4F2hc) in pZL1 (Life Technologies Inc.) linearized with *Bam*HI, as described elsewhere [30]. *Xenopus* oocyte expression studies and uptake measurements were performed as described previously [28,31] with minor modifications. *Xenopus* oocytes were injected with 17.6 ng of hLAT1 cRNA and 7.4 ng of h4F2hc cRNA at a final molar ratio of 1:1. To express solely hLAT1 or h4F2hc in *Xenopus* oocytes, 17.6 ng and 7.4 ng of cRNAs were injected for hLAT1 and h4F2hc, respectively. Two days after injection, the uptake of ^{14}C-labeled amino acids was measured in the Na^+-free uptake solution (100 mM choline-Cl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM Tris, pH 7.4) containing 0.5–2.0 μCi/ml [^{14}C]amino acids (DuPont NEN) as described elsewhere [28].

For the Na^+-containing uptake solution, choline-Cl in the Na^+-free uptake solution was replaced with NaCl. For the Cl^--free uptake solution, Cl^- in the Na^+-containing uptake solution was replaced with gluconate anion. Preliminary experiments to determine the time-course of L-[^{14}C]leucine (20 μM) uptake into oocytes expressing hLAT1 indicated that the uptake was linearly dependent on incubation time up to 45 min (data not shown); thus, for all the subsequent experiments, amino acid uptake rate was measured for 30 min and expressed as pmol/oocyte/min.

K_m and V_{max} of amino acid substrates were determined using the Eadie–Hofstee equation based on the hLAT1-mediated amino acid uptakes measured at 1, 3, 10, 30, 100 and 300 μM, except for those of glutamine and asparagine, the uptakes of which were measured at 0.03, 0.1, 0.3, 1, 3 and 10 mM. To measure the K_i value of melphalan, the hLAT1-mediated transport of L-[^{14}C]leucine was measured in the Na^+-free uptake solution with varying concentrations of L-[^{14}C]leucine (3, 5, 10, 30, 100 and 300 μM) with or without melphalan (100 μM). The K_i values were determined by double reciprocal plot analysis [32,33]. The hLAT1-mediated amino acid uptakes were calculated as the differences between the means of uptakes of the oocytes injected with hLAT1 and h4F2hc cRNAs and those of the control oocytes injected with water.

For the efflux measurement, 100 nl (2 nCi) of L-[^{14}C]-labeled amino acids (~400 μM) was injected into oocytes using a fine-tipped glass micropipette as described elsewhere [19,21,33]. Oocytes were individually incubated for 5 min in the ice-cold Na^+-free uptake solution, and transferred to the Na^+-free uptake solution with or without 20 μM nonradiolabeled L-leucine maintained at room temperature (18–22°C). The radioactivity of the medium and the residual radioactivity of the oocytes were measured. The efflux values were expressed as percent radioactivity (radioactivity of medium/(radioactivity of medium+radioactivity of oocytes)×100%) [19,21,33].

For the uptake and efflux measurements, seven to eight oocytes were used for each data point. Each data point in the figures represents the mean ± s.e.m. of uptake (n = 7–8). To confirm the reproducibility of results, three separate experiments using different batches of oocytes and in vitro transcribed cRNA were performed for each measurement except for K_m and V_{max} determination (see Table 1). Results from the representative experiments are shown in the figures.

2.2. Northern analysis

The *Sma*I fragment corresponding to 649–1128 bp of hLAT1 cDNA (GenBank/EMBL/DDBJ accession no. AB018009) and the *Pst*I fragment corresponding to 106–645 bp of h4F2hc cDNA (GenBank/EMBL/DDBJ accession no. AB018010) were labeled with [^{32}P]dCTP using a ^{T7}Quick prime kit (Pharmacia). Multiple Tissue Northern blots (Human, Human II, Human immune II: Clontech) were hybridized with the probes and processed following the manufacturer's instructions. Total RNA was prepared from human tumor cell lines using an RNA preparation kit (Isogen, Nippon-Gene, Japan) following the manufacturer's instructions. The total RNA (10

μg/lane) was separated on a 1% agarose gel in the presence of 2.2 M formaldehyde and was blotted onto a nitrocellulose filter (Schleicher and Schuell) [28,34]. Hybridization was performed for 20 h at 42°C as described elsewhere [28,34]. The filters were washed in 0.1×SSC/0.1% sodium dodecyl sulfate (SDS) at 65°C. Tumor cell lines were provided by the Health Science Research Resources Bank, Japan Health Sciences Foundation (Osaka, Japan) and Riken Cell Bank (Tsukuba, Japan).

2.3. Anti-peptide antibody generation

Oligopeptides [CQKLMQVVPQET] corresponding to amino acid residues 497–507 of hLAT1 and oligopeptides [EPHEGLLLRFPYAAC] corresponding to amino acid residues 516–529 of h4F2hc were synthesized. The N-terminal or C-terminal cysteine residues were introduced for conjugation with keyhole limpet hemocyanine. Anti-peptide polyclonal antibodies were generated as described elsewhere [22,35]. Antisera were affinity-purified as described elsewhere [36].

2.4. Western blot analysis

T24 cell membranes were prepared as described elsewhere [21,22,33,37], with minor modifications. Briefly, T24 cells were homogenized in 9 volumes of a solution containing 50 mM Tris–HCl (pH 7.5), 25 mM KCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and 0.25 M sucrose, with 15 strokes in a Dounce homogenizer. The homogenate was centrifuged for 10 min at 8000×g, and the supernatant was centrifuged further for 1 h at 100 000×g. After centrifugation, the membrane pellet was resuspended in a solution containing 0.25 M sucrose, 100 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris (pH 7.4). The protein samples were heated at 100°C for 5 min in the sample buffer either in the presence (reducing condition) or absence (nonreducing condition) of 5% 2-mercaptoethanol and then subjected to SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred electrophoretically to a Hybond-P PVDF transfer membrane (Amersham) that was then treated with nonfat dried milk and an affinity-purified anti-hLAT1 antibody (1:500 dilution) or anti-h4F2hc antiserum (1:20,000 dilution). The membrane was then treated with horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody (Jackson Immuno Research Laboratories). The signals were detected using an ECL plus system (Amersham) [21,22,33].

2.5. In vitro translation

In vitro translation of cRNA for hLAT1 was performed using a rabbit reticulocyte lysate system with or without a canine pancreatic microsome membrane (Promega) and endoglycosidase H (Boehringer Mannheim) as described elsewhere [3,16,38,39].

3. Results

3.1. Transport activities

As shown in Fig. 1A, hLAT1 requires h4F2hc for its functional expression in *Xenopus* oocytes. For all subsequent experiments, therefore, hLAT1 was coexpressed with h4F2hc in *Xenopus* oocytes for functional analyses. The uptake of L-[^{14}C]leucine mediated by hLAT1 was saturable and followed the Michaelis–Menten kinetics with a K_m value of

Table 1
Kinetic parameters of amino acid substrates

Amino acid	K_m (μM)[a]	V^a_{max}
L-Leucine	19.7[b]	(1.00)
L-Isoleucine	25.1	1.18
L-Phenylalanine	14.2	0.91
L-Methionine	20.2	1.28
L-Tyrosine	28.3	0.86
L-Histidine	12.7	0.80
L-Tryptophan	21.4	0.65
L-Valine	47.2	1.35
L-Glutamine	1,640	1.61
L-Asparagine	2,150	0.73
D-Leucine	74.8	1.11
D-Phenylalanine	121	1.32

[a] K_m and V_{max} values were determined as described in Section 2. The data were obtained from six separate experiments using different batches of oocytes. In each experiment, L-leucine uptake was measured to compare V_{max} values between experiments. The V_{max} value of each amino acid was normalized to that of L-leucine in the same experiment.
[b] K_m for L-leucine is the mean of four measurements (mean ± S.E.M. = 19.7 ± 4.1 μM).

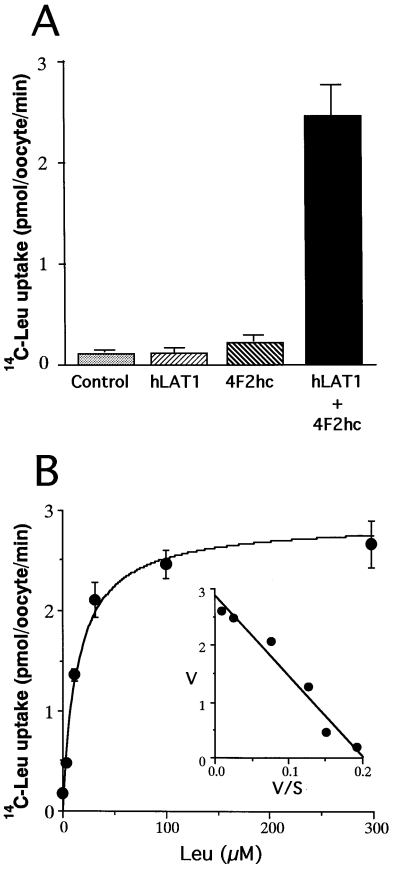

Fig. 1. Functional expression of hLAT1 in *Xenopus* oocytes. (A) Coexpression of hLAT1 and h4F2hc. The uptake of L-[^{14}C]leucine (50 μM) by *Xenopus* oocytes injected with water, hLAT1 cRNA (labeled as 'hLAT1'), 4F2hc cRNA ('4F2hc'), and both hLAT1 cRNA and 4F2hc cRNA ('hLAT1+4F2hc') was measured in the Na^+-free uptake solution. The coexpression of hLAT1 and 4F2hc resulted in a high uptake of L-[^{14}C]leucine. (B) Concentration dependence of hLAT1-mediated L-[^{14}C]leucine uptake. The hLAT1-mediated L-[^{14}C]leucine uptake in oocytes coexpressing hLAT1 and h4F2hc was measured at 1, 3, 10, 30, 100, and 300 μM L-leucine in the Na^+-free uptake solution, and plotted against L-leucine concentration. The uptake was saturable and fit the Michaelis–Menten curve. Inset: Eadie–Hofstee plot of the L-leucine uptake from which kinetic parameters were determined.

19.7 ± 4.1 μM (mean ± s.e.m. of four separate experiments) (Fig. 1B). The L-[^{14}C]leucine uptake was not dependent on Na^+ or Cl^- (data not shown).

3.2. Substrate selectivity

The substrate selectivity of hLAT1 was investigated in the inhibition experiments in which 20 μM L-[^{14}C]leucine uptake was measured in the presence of an amino acid at 2 mM. The L-leucine uptake was strongly inhibited by L-isomers of isoleucine, phenylalanine, methionine, tyrosine, histidine, tryptophan, valine and a conventional system L-specific inhibitor, namely, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) (Fig. 2A). All the natural amino acids were confirmed to be transported by hLAT1 using radiolabeled compounds (Fig. 2C). The K_m values for these substrate amino acids were between ~15 and ~50 μM, indicating a high-affinity transport (Table 1). Basic amino acids, such as lysine and arginine, and acidic amino acids, such as glutamate and aspartate, did not inhibit L-[^{14}C]leucine uptake (Fig. 2A). D-Isomers of leucine, phenylalanine and methionine strongly inhibited the hLAT1-mediated L-[^{14}C]leucine uptake, whereas hLAT1 was highly stereoselective for tyrosine, histidine, tryptophan, valine and isoleucine (Fig. 2B). Using radiolabeled compounds, D-leucine and D-phenylalanine were shown to be transported with high affinity by hLAT1 (Fig. 2C and Table 1). Although the level of inhibition of L-[^{14}C]leucine uptake by glutamine and asparagine was low, they were in fact transported with low affinity (Table 1).

3.3. Interaction of amino acid-related compounds with hLAT1

The hLAT1-mediated L-[^{14}C]leucine uptake (1 μM) was measured in the presence of 100 μM nonlabeled L-Dopa, triiodothyronine, thyroxine and melphalan. As shown in Fig. 3A, L-[^{14}C]leucine uptake was markedly inhibited by these amino acid-related compounds. Triiodothyronine exerted the strongest inhibition of hLAT1-mediated L-[^{14}C]leucine uptake (Fig. 3A). As shown in Fig. 3B, the inhibition of L-[^{14}C]leucine uptake by melphalan was shown to be competitive in a double reciprocal plot analysis with a K_i value of 56 μM.

3.4. Amino acid exchange mediated by hLAT1

As shown in Fig. 4, L-leucine applied extracellularly induced the efflux of a preloaded high-affinity substrate L-[^{14}C]leucine and a low-affinity substrate L-[^{14}C]glutamine but not a nonsubstrate L-[^{14}C]alanine from the oocytes expressing hLAT1. L-

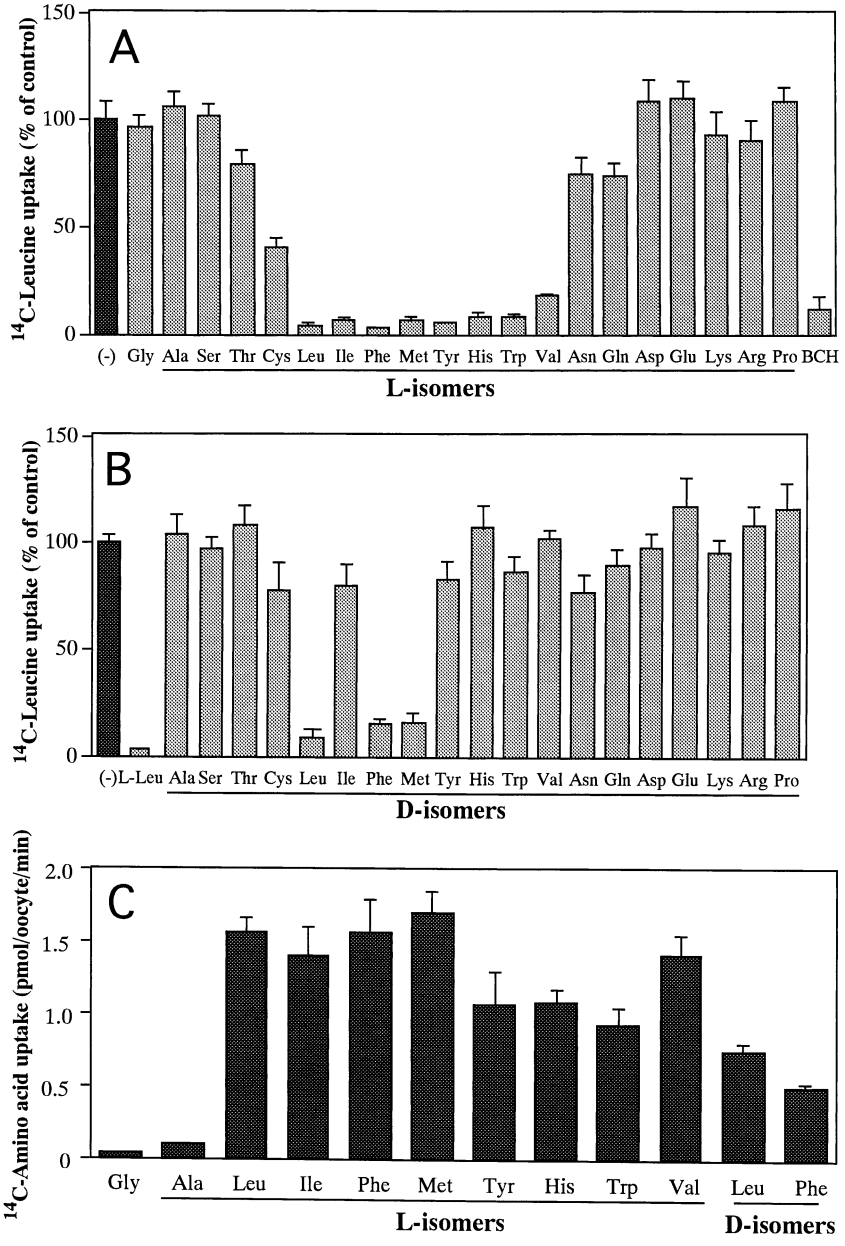

Fig. 2. Substrate selectivity of hLAT1. (A) Inhibition of hLAT1-mediated L-[^{14}C]leucine uptake by glycine, L-amino acids and BCH. The hLAT1-mediated L-[^{14}C]leucine uptake (20 μM) was measured in the presence of 2 mM nonradiolabeled glycine, L-amino acids and system L-specific inhibitor BCH, and expressed as percent of the control L-[^{14}C]leucine uptake in the absence of inhibitors ('(−)'). The L-leucine uptake was highly inhibited by BCH and neutral amino acids with branched or aromatic side chains. BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid. (B) Inhibition of LAT1-mediated L-[^{14}C]leucine uptake by D-amino acids. The LAT1-mediated L-[^{14}C]leucine uptake (20 μM) was measured in the presence of 2 mM nonradiolabeled D-amino acids and expressed as percent of the control L-[^{14}C]leucine uptake in the absence of inhibitors ('(−)'). D-Leucine, D-phenylalanine and D-methionine strongly inhibited hLAT1-mediated L-[^{14}C]leucine uptake. (C) hLAT1-mediated uptakes of 20 μM L-[^{14}C]- and D-amino acids were measured. hLAT1 transported neutral L-amino acids with branched or aromatic side chains. hLAT1 also transported D-leucine and D-phenylalanine.

leucine did not induce the efflux of preloaded ^{14}C-labeled amino acids from the control oocytes, confirming that the amino acid efflux was mediated by hLAT1 (Fig. 4).

3.5. Tissue distribution and expression in tumor cell lines

The distribution of hLAT1 and h4F2hc mRNAs in human tissues was analyzed by Northern blot analysis. A single mRNA band of 4.8 kb was detected in samples of brain, placenta, testis, peripheral blood leukocytes, bone marrow, fetal liver, lymph node and thymus (Fig. 5A). The blots from the analysis were also hybridized with a ^{32}P-labeled h4F2hc probe. The h4F2hc signal was detected ubiquitously. The placenta, kidney and testis showed a relatively higher expression level of 4F2hc mRNA (Fig. 5B). The hLAT1 and h4F2hc mRNAs were detected in the human carcinoma cell lines such as PA-1 teratocarcinoma cells, T24 bladder carcinoma cells, RERF-LC-MA lung small-cell carcinoma cells and HeLa uterine cervical carcinoma cells (Fig. 6A). In human leukemia cell lines, hLAT1 was expressed in all the cell lines examined, whereas the expression level of h4F2hc varied among the cell lines (Fig. 6B). The h4F2hc signal was not detected in Daudi Burkitt lymphoma cells, CCRF-SB acute lymphoblastic leukemia (B-lymphoblastoid leukemia) cells and P30/OHK non-T non-B acute lymphoblastic leukemia cells by Northern blot analysis (Fig. 6B).

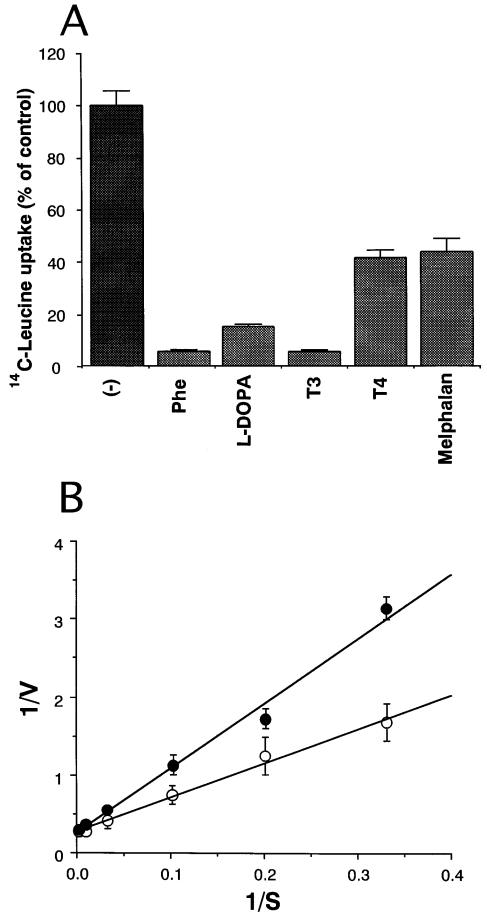

Fig. 3. Interaction of hLAT1 with amino acid-related drugs. (A) The hLAT1-mediated L-[^{14}C]leucine uptake (1 μM) was measured in the presence of 100 μM nonradiolabeled compounds, and expressed as percent of the control L-[^{14}C]leucine uptake in the absence of inhibitors ('(−)'). The uptakes in the presence of 0.1% dimethyl sulfoxide were measured. T3, triiodothyronine; T4, thyroxine. (B) Inhibitory effect of melphalan on hLAT1-mediated L-[^{14}C]leucine uptake. The hLAT1-mediated uptake of L-[^{14}C]leucine (3, 5, 10, 30, 100 and 300 μM) was measured in the Na^+-free uptake solution in the presence (●) or absence (○) of 100 μM melphalan. Double reciprocal plot analyses were performed. The K_i value of melphalan was calculated to be 56 μM.

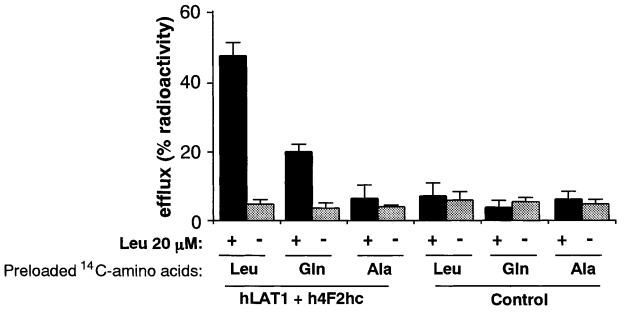

Fig. 4. Efflux of intracellularly injected [^{14}C]amino acids via hLAT1. Oocytes coexpressing hLAT1 and h4F2hc (labeled as 'hLAT1+h4F2hc') or control oocytes injected with water instead of cRNAs ('Control') were preloaded with ^{14}C-labeled L-leucine, L-glutamine and L-alanine by microinjection (~400 μM [^{14}C]amino acids; 100 nl (2 nCi)/oocyte). Efflux of the preloaded radioactivity in the presence (closed column) or absence (open column) of 20 μM of L-leucine in the Na^+-free uptake solution was measured. The values are expressed as a percent of the total radioactivity microinjected into the oocytes.

3.6. Association of hLAT1 and h4F2hc in T24 cells

Western blot analysis was performed on the membrane fractions prepared from T24 human bladder carcinoma cells. The antibodies raised against hLAT1 (Fig. 7, left) and h4F2hc (Fig. 7, right) both recognized the 125-kDa-protein band under

the nonreducing condition, whereas this band shifted to the 38-kDa-protein band for hLAT1 (Fig. 7, left) and 85-kDa-protein band for h4F2hc under the reducing condition (Fig. 7, right).

3.7. In vitro translation of hLAT1 cRNA

In vitro translation of hLAT1 cRNA showed a band of a 42-kDa protein (Fig. 8). hLAT1 was not glycosylated by canine pancreatic microsomes under the condition in which 4F2hc is glycosylated [3] (Fig. 8).

4. Discussion

It was previously reported that rat, mouse, *Xenopus* and human LAT1s are associated with 4F2hc [3,4,6,27]. We confirmed this for hLAT1 by showing that hLAT1 requires h4F2hc for its functional expression in *Xenopus* oocytes (Fig. 1A). When coex-

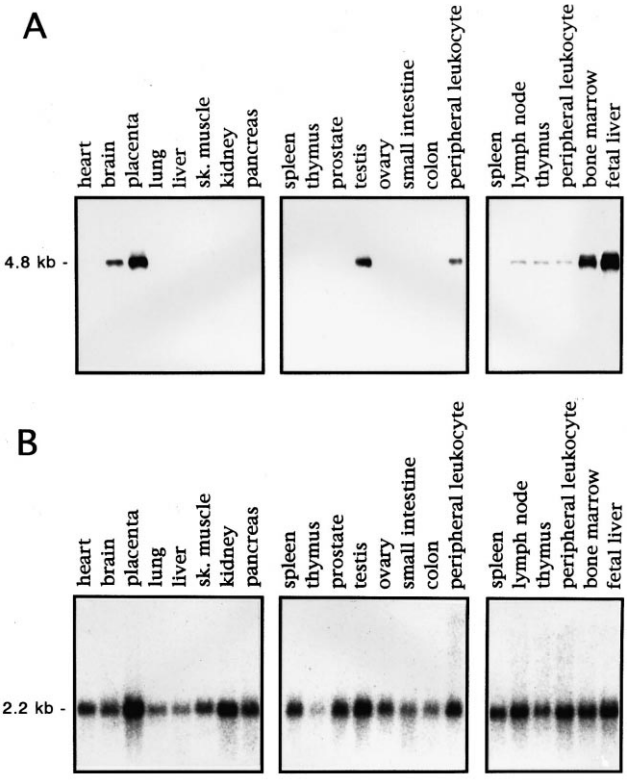

Fig. 5. Tissue distribution of expression. High-stringency Northern blot analysis of poly(A)$^+$ RNA from human tissues using probes for hLAT1 (A) and h4F2hc (B). For hLAT1, strong hybridization signals were detected in the brain, placenta, testis, bone marrow and fetal liver. Weak signals were detected in peripheral leukocytes, lymph node and thymus (A). Although the expression of h4F2hc was basically ubiquitous, a higher level of expression was detected in the placenta, kidney and testis (B).

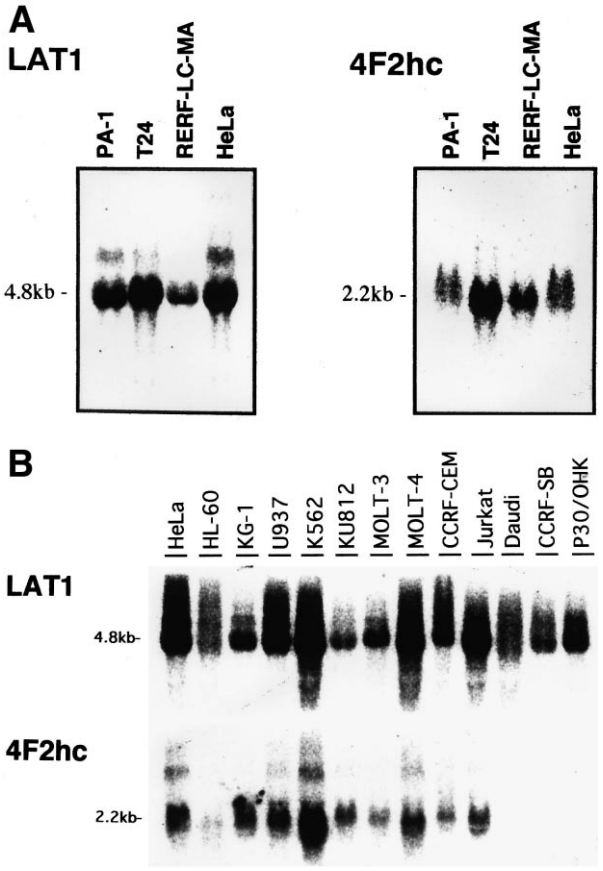

Fig. 6. Expression of hLAT1 and h4F2hc in human tumor cell lines. (A) High-stringency Northern blot analysis of total RNA from human carcinoma cells (PA-1 teratocarcinoma cells, T24 bladder carcinoma cells, RERF-LC-MA lung small-cell carcinoma cells, and HeLa uterine cervical carcinoma cells) using probes for hLAT1 (left) and h4F2hc (right). Strong hybridization signals were detected both for hLAT1 and h4F2hc. (B) High-stringency Northern blot analysis of total RNA from human leukemia cells using probes for hLAT1 (upper) and h4F2hc (lower). HeLa cell RNA was included to compare the expression levels with those shown in A. HL-60, acute promyelocytic leukemia cells; KG-1, acute myeloblastic leukemia cells; U937, diffuse histiocytic lymphoma cells; K562, chronic myelogenous leukemia (blast crisis) cells; KU812, chronic myeloid leukemia cells; MOLT-3, acute lymphoblastic leukemia cells; MOLT-4, acute lymphoblastic leukemia cells; CCRF-CEM, acute lymphoblastic leukemia cells; Jurkat, T-lymphocyte-like cells; Daudi, Burkitt lymphoma cells; CCRF-SB, acute lymphoblastic leukemia (B-lymphoblastoid leukemia) cells; P30/OHK, non-T non-B acute lymphoblastic leukemia cells.

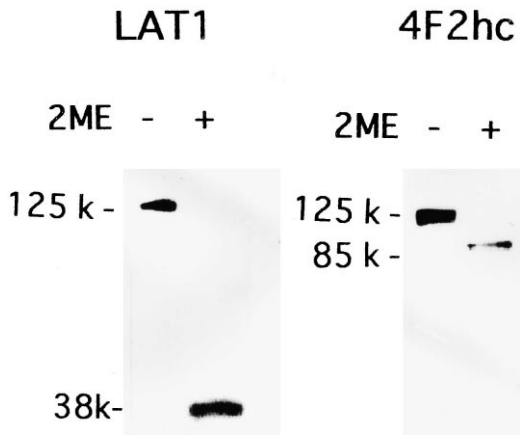

Fig. 7. Association between hLAT1 and h4F2hc proteins. Western blot analysis was performed on the membrane fraction prepared from human bladder carcinoma cell line T24 in the presence or absence of 2-mercaptoethanol using an anti-hLAT1 antibody and an anti-h4F2hc antibody. For LAT1 (left), the 125-kDa-protein band detected in the absence of 2-mercaptoethanol ('2ME −') was not detected in the presence 2-mercaptoethanol ('2ME +'), and a 38-kDa-protein band was detected instead. For 4F2hc (right), the 125-kDa-protein band detected in the absence of 2-mercaptoethanol ('2ME −') shifted to an 85-kDa-protein band by the treatment of 2-mercaptoethanol ('2ME +').

pressed with h4F2hc, hLAT1 exhibited BCH-sensitive Na^+-independent high-affinity transport of large neutral amino acids (Table 1), in agreement with the properties of system L and already reported properties of rat, mouse, *Xenopus* and human LAT1s [1–4,6,15,27]. In rat LAT1, we previously showed that D-isomers of leucine, phenylalanine and methionine strongly inhibited L-[^{14}C]leucine uptake [3]. The hLAT1-mediated transport was also inhibited by D-leucine, D-phenylalanine and D-methionine (Fig. 2B). In the present study, using ^{14}C-labeled D-leucine and D-phenylalanine, we demonstrated that these D-amino acids are in fact transported by hLAT1 with high affinity (Fig. 2C and Table 1). The absence of marked stereoselectivity seems to be a common characteristic of members of the LAT family. For example, amino acid transport mediated by LAT2 is also inhibited by D-isomers of some amino acids such as serine, cysteine and asparagine, although they are not transported by LAT2 [16]. Asc-1, which prefers small neutral amino acids, transports not only L-amino acids but also D-amino acids such as D-serine, D-alanine, D-threonine and D-cysteine [21,40]. It was shown that D-amino acids are endogenously present in mammalian tissues [41]. Thus, LAT1, as well as Asc-1, is proposed to play significant roles in the mobilization of D-amino acids in mammals.

It has been proposed that system L mediates the transport of amino acid-related compounds [1,42–44]. As shown in Fig. 3A, the hLAT1-mediated L-leucine uptake was inhibited by L-Dopa, triiodothyronine, thyroxine and melphalan. These amino acid-related compounds are proposed to be accepted by the binding site of hLAT1 and transported by hLAT1. In agreement with this, it has been shown that triiodothyronine and thyroxine were transported by *Xenopus* LAT1 (ASUR4) [43]. The anticancer agent melphalan was shown to be transported by system L in in vivo preparation [44]. The level of system L activity in tumor cells was reported to be correlated with melphalan sensitivity [45,46]. In the present study, we demonstrated that melphalan inhibited the hLAT1-mediated L-[^{14}C]leucine uptake in a competitive manner with a K_i value close to the K_m values of substrate amino acids, indicating that the binding site of hLAT1 accepts melphalan with an affinity similar to that of substrate amino acids (Fig. 3B). Because hLAT1 is upregulated in tumor cells, it is proposed as one of the major routes through which melphalan permeates the plasma membrane to enter tumor cells.

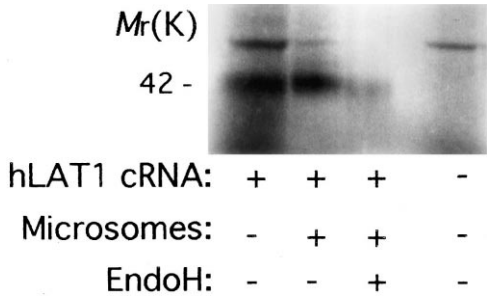

Fig. 8. In vitro translation of hLAT1 cRNA. The autoradiograph of SDS–polyacrylamide gel (10%) used to analyze the in vitro translation products obtained in the absence of pancreatic microsomes (first lane) and in the presence of microsomes (second lane) is shown. The third lane shows the products in the presence of microsomes after deglycosylation with endoglycosidase H. The fourth lane shows the reaction in the absence of hLAT1 cRNA, indicating that the upper ~50-kDa-protein band is not produced due to the translation of hLAT1 cRNA. Translation of hLAT1 cRNA in the absence of microsomes thus yielded translation products apparently of M_r 42 000. The hLAT1 translation product was not glycosylated by pancreatic microsomes.

For rat LAT1, we previously showed that the efflux of L-[^{14}C]leucine preloaded into oocytes by incubating them in the L-[^{14}C]leucine-containing medium was induced by adding L-leucine to the medium, suggesting that rat LAT1 mediated the amino acid exchange [3]. In the present study, we further examined this by microinjecting ^{14}C-labeled amino acids into oocytes expressing hLAT1. We showed that a high-affinity substrate L-leucine and a low-affinity substrate L-glutamine but not a nonsubstrate L-alanine were effluxed via hLAT1 by the application of L-leucine in the medium, confirming that hLAT1 is an amino acid exchanger (Fig. 4). This, furthermore, suggests that the substrate selectivity of the intracellular substrate binding site of hLAT1 is similar to that of the extracellular substrate binding site.

Northern blot analysis indicated that hLAT1 is heterogeneously expressed in human tissues with a high expression in the placenta, testis, bone marrow and brain, whereas the expression of h4F2hc is rather ubiquitous (Fig. 5A,B), consistent with the reports for mice and rats [3,6,16,47,48]. Interestingly, the fetal liver has a high expression level of the hLAT1 message whereas this expression was not detected in the adult liver (Fig. 5A), which is in agreement with the observation in rats using a cDNA probe for TA1/E16, a partial sequence corresponding to LAT1 [25]. TA1 (rat) was originally identified as a tumor-associated sequence with an oncofetal pattern of expression in the rat liver [25]. TA1 immunoreactivity was found to be strong in human colon cancer [26], suggesting that hLAT1 is upregulated in human cancer tissues. In the present study, we examined the expression of hLAT1 messages in human tumor cell lines using a hLAT1 cDNA probe. hLAT1 messages were detected in all the carcinoma and leukemia cell lines examined, consistent with previous results of the Northern blot analysis using rat LAT1 cDNA probes [3,25] (Fig. 6A,B).

An interesting finding of the Northern blot analysis of the tumor cell line is that the expression level of h4F2hc is quite varied among tumor cell lines particularly in leukemia cell lines (Fig. 6A,B). We found three leukemia cell lines in which h4F2hc messages were not detected (Fig. 6B). In these cell lines that lack h4F2hc expression, hLAT1 was still expressed, suggesting different mechanisms of regulation in hLAT1 and h4F2hc gene expression. Consistent with this, it was shown that LAT1 and 4F2hc respond differently to amino acid availability in rat hepatic cells [49]. It was previously reported that the level of h4F2hc expression in leukemia cells is varied among leukemia patients even with the same clinical diagnosis [50,51]. Because h4F2hc is essential for hLAT1 to be functional, the level of h4F2hc expression would greatly affect the formation of functional system L transporters in the plasma membrane [3,4,52]. It would be interesting to determine how the h4F2hc expression level influences the rate of amino acid uptakes and eventually affects the growth and proliferation of tumor cells. The leukemia cell lines examined in the present study will be useful tools for investigating this issue.

In previous immunoprecipitation studies, LAT1 (or 4F2 light chain) was coprecipitated with 4F2hc using anti-4F2hc antibodies under the nonreducing condition [4–6]. LAT1 and 4F2hc dissociated under the reducing condition, indicating that LAT1 and 4F2hc are linked via disulfide bonds. As for ASUR4 (*Xenopus laevis* LAT1), a site-directed mutagenesis study revealed that Cys 164, which is conserved in different species, is responsible for the linking with 4F2hc [52]. These studies were performed by exogenously expressing LAT1 and 4F2hc in *Xenopus* oocytes. In order to examine whether hLAT1 and h4F2hc, which are endogenous to human cells, exist as a heterodimeric complex in the plasma membrane, we generated antibodies against hLAT1 and h4F2hc. We performed Western blot analysis on the membrane fractions prepared from T24 human bladder carcinoma cells, because T24 cells expressed both hLAT1 and h4F2hc at high levels (Fig. 6A). We showed that the 125-kDa-protein band corresponding to the heterodimeric complex of hLAT1 and h4F2hc detected under the nonreducing condition shifted to the 38-kDa- and 85-kDa protein bands corresponding to the hLAT1 and h4F2hc monomers, respectively, under the reducing condition (Fig. 7). This indicates that hLAT1 and h4F2hc proteins coexist as a heterodimeric complex linked via disulfide bonds in T24 cells. This is, in fact, the first demonstration of the band shift using an anti-LAT1 antibody. The role of 4F2hc in the functional expression of LAT1 has been proposed to assist the LAT1 protein to be sorted to the plasma membrane [4]. Because hLAT1 is linked to h4F2hc in T24 cells (Fig.

5), both proteins are expected to be localized in the plasma membrane. We confirmed that hLAT1 and h4F2hc immunoreactivity coexist on the plasma membrane of T24 cells by confocal immunofluorescence microscopic analysis [53] (data not shown).

Although the 4F2 light chain has been assumed to be a nonglycosylated protein [7,8], an *N*-glycosylation site is predicted in the putative extracellular loop of hLAT1, which is not conserved in rat LAT1 [27]. In rat LAT1, we previously showed that the LAT1 protein is not glycosylated in in vitro translation [3]. We, thus, examined, in in vitro translation, whether hLAT1 is glycosylated or not. The results indicated that hLAT1 is not glycosylated under the condition in which 4F2hc is glycosylated [3] (Fig. 8). Thus, it is confirmed that hLAT1 is a 4F2 light chain, i.e., a nonglycosylated subunit of the 4F2 antigen.

Regarding the regulation of the expression of hLAT1, there are some interesting findings. E16, a human partial sequence corresponding to hLAT1, was reported to be upregulated upon mitogenic stimulation of lymphocytes [54]. ASUR4, the *Xenopus* homolog of hLAT1 was identified to be upregulated upon stimulation of the A6 epithelial cell line by aldosterone [55]. Based on this highly regulated nature, together with its high levels of expression in tumor cells, fetal liver and bone marrow, it is speculated that hLAT1 expression is upregulated in order to provide cells with essential amino acids for high levels of protein synthesis associated with cell activation or hormonal stimulation and also to support rapid growth or continuous proliferation. In order for hLAT1 to fulfill these tasks, it appears a disadvantage for hLAT1 to be an amino acid exchanger. Amino acids are released via hLAT1 in exchange for the influx of amino acids, thus no net amino acid influx should be observed. We showed that L-glutamine, which is abundantly present in cells and generated intracellularly, is transported by hLAT1 albeit with low affinity (Table 1), consistent with a previous report for *Xenopus* LAT1 [4]. We further demonstrated that intracellularly loaded L-glutamine is effluxed in exchange for extracellularly applied L-leucine. Therefore, we propose that extracellular high-affinity LAT1 substrates, most of which are essential amino acids, are taken up by cells via hLAT1 driven by the exchange for intracellular L-glutamine, which results in the net influx of essential amino acids.

Acknowledgements

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References

[1] H.N. Christensen, Physiol. Rev. 70 (1990) 43–77.
[2] D.L. Oxender, H.N. Christensen, Nature 197 (1963) 765–767.
[3] Y. Kanai, H. Segawa, K. Miyamoto, H. Uchino, E. Takeda, H. Endou, J. Biol. Chem. 273 (1998) 23629–23632.
[4] L. Mastroberardino, B. Spindler, R. Pfeiffer, P.J. Skelly, J. Loffing, C.B. Shoemaker, F. Verrey, Nature 395 (1998) 288–291.
[5] B.A. Mannion, T.V. Kolesnikova, S.-H. Lin, N.L. Thompson, M.E. Hemler, J. Biol. Chem. 273 (1998) 33127–33129.
[6] E. Nakamura, M. Sato, H. Yang, F. Miyagawa, M. Harasaki, K. Tomita, S. Matsuoka, A. Noma, K. Iwai, N. Minato, J. Biol. Chem. 274 (1999) 3009–3016.
[7] B.F. Haynes, M.E. Hemler, D.L. Mann, G.S. Eisenbarth, J. Shelhamer, H.S. Mostowski, C.A. Thomas, J.L. Strominger, A.S. Fauci, J. Immunol. 126 (1981) 1409–1414.
[8] M.E. Hemler, J.L. Strominger, J. Immunol. 129 (1982) 623–628.
[9] H. Yagita, T. Masuko, Y. Hashimoto, Cancer Res. 46 (1986) 1478–1484.
[10] K. Hara, H. Kudoh, T. Enomoto, Y. Hashimoto, T. Masuko, Biochem. Biophys. Res. Commun. 262 (1999) 720–725.
[11] C.A. Fenczik, T. Sethi, J.W. Ramos, P.E. Hughes, M.H. Ginsberg, Nature 390 (1997) 81–85.
[12] S. Teixeira, S. Di Grandi, L.C. Kuhn, J. Biol. Chem. 262 (1987) 9574–9580.
[13] E. Quackenbush, M. Clabby, K.M. Gottesdiener, J. Barbo-

sa, N.H. Jones, J.L. Strominger, S. Speck, J.M. Leiden, Proc. Natl. Acad. Sci. USA 84 (1987) 6526–6530.
[14] J.A. Lumadue, A.B. Glick, F.H. Ruddle, Proc. Natl. Acad. Sci. USA 84 (1987) 9204–9208.
[15] M. Pineda, E. Fernandez, D. Torrents, R. Estevez, C. Lopez, M. Camps, J. Lloberas, A. Zorzano, M. Palacin, J. Biol. Chem. 274 (1999) 19738–19744.
[16] H. Segawa, Y. Fukasawa, K. Miyamoto, E. Takeda, H. Endou, Y. Kanai, J. Biol. Chem. 274 (1999) 19745–19751.
[17] D. Torrents, R. Estevez, M. Pineda, E. Fernandez, J. Lloberas, Y.-B. Shi, A. Zorzano, M. Palacin, J. Biol. Chem. 273 (1998) 32437–32445.
[18] R. Pfeiffer, G. Rossier, B. Spindler, C. Meier, L. Kuhn, F. Verrey, EMBO J. 18 (1999) 49–57.
[19] Y. Kanai, Y. Fukasawa, S.H. Cha, H. Segawa, A. Chairoungdua, D.K. Kim, H. Matsuo, J.Y. Kim, K. Miyamoto, E. Takeda, H. Endou, J. Biol. Chem. 275 (2000) 20787–20793.
[20] H. Sato, M. Tamba, T. Ishii, S. Bannai, J. Biol. Chem. 274 (1999) 11455–11458.
[21] Y. Fukasawa, H. Segawa, J.Y. Kim, A. Chairoungdua, D.K. Kim, H. Matsuo, S.H. Cha, H. Endou, Y. Kanai, J. Biol. Chem. 275 (2000) 9690–9698.
[22] A. Chairoungdua, H. Segawa, J.Y. Kim, K. Miyamoto, H. Haga, Y. Fukui, K. Mizoguchi, H. Ito, E. Takeda, H. Endou, Y. Kanai, J. Biol. Chem. 274 (1999) 28845–28848.
[23] International Cystinuria Consortium, Nat. Genet. 23 (1999) 52-57.
[24] R. Pfeiffer, J. Loffing, G. Rossier, C. Bauch, C. Meier, T. Eggermann, D. Loffing-Cueni, L. Kuhn, F. Verrey, Mol. Biol. Cell 10 (1999) 4135–4147.
[25] J. Sang, Y.-P. Lim, M. Panzia, P. Finch, N.L. Thompson, Cancer Res. 55 (1995) 1152–1159.
[26] D.A. Wolf, S. Wang, M.A. Panzia, N.H. Bassily, N.L. Thompson, Cancer Res. 56 (1996) 5012–5022.
[27] P.D. Prasad, H. Wang, H. Huang, R. Kekuda, D.P. Rajan, F.H. Leibach, V. Ganapathy, Biochem. Biophys. Res. Commun. 255 (1999) 283–288.
[28] N. Utsunomiya-Tate, H. Endou, Y. Kanai, J. Biol. Chem. 271 (1996) 14883–14890.
[29] N. Utsunomiya-Tate, H. Endou, Y. Kanai, FEBS Lett. 416 (1997) 312–316.
[30] Y. Kanai, M.A. Hediger, Nature 360 (1992) 467–471.
[31] Y. Kanai, S. Nussberger, M.F. Romero, W.F. Boron, S.C. Hebert, M.A. Hediger, J. Biol. Chem. 270 (1995) 16561–16568.
[32] N. Apiwattanakul, T. Sekine, A. Chairoungdua, Y. Kanai, N. Nakajima, S. Sophasan, H. Endou, Mol. Pharmacol. 55 (1999) 847–854.
[33] D.K. Kim, Y. Kanai, A. Chairoungdua, H. Matsuo, S.H. Cha, H. Endou, J. Biol. Chem. 276 (2001) 17221–17228.
[34] Y. Kanai, M.G. Stelzner, W.-S. Lee, R.G. Wells, D. Brown, M.A. Hediger, Am. J. Physiol. 263 (1992) F1087–F1092.
[35] A. Altman, J.M. Cardenas, R.A. Houghten, F.J. Dixon, A.N. Theofilopoulos, Proc. Natl. Acad. Sci. USA 81 (1984) 2176–2180.
[36] S. Hisano, H. Haga, K. Miyamoto, E. Takeda, Y. Fukui, Brain Res. 710 (1996) 299–302.
[37] B. Thorens, H.K. Sarkar, H.R. Kaback, H.F. Lodish, Cell 55 (1988) 281–290.
[38] M.A. Hediger, J. Mendlein, H.-S. Lee, E.M. Wright, Biochim. Biophys. Acta 1064 (1991) 360–364.
[39] Y.J. Fei, Y. Kanai, S. Nussberger, V. Ganapathy, F.H. Leibach, M.F. Romero, S.K. Singh, W.F. Boron, M.A. Hediger, Nature 368 (1994) 563–566.
[40] J. Nakauchi, H. Matsuo, D.K. Kim, A. Goto, A. Chairoungdua, S.H. Cha, J. Inatomi, Y. Shiokawa, K. Yamaguchi, I. Saito, H. Endou, Y. Kanai, Neurosci. Lett. 287 (2000) 231–238.
[41] A. Hashimoto, T. Oka, Prog. Neurobiol. 52 (1997) 325–353.
[42] P. Gomes, P. Soares-da-Silva, Brain Res. 829 (1999) 143–150.
[43] J.W. Ritchie, G.J. Peter, Y.B. Shi, P.M. Taylor, J. Endocrinol. 163 (1999) R5–R9.
[44] E.M. Cornford, D. Young, J.W. Paxton, G.J. Finlay, W.R. Wilson, W.M. Pardridge, Cancer Res. 52 (1992) 138–143.
[45] J.M. Moscow, C.A. Swanson, K.H. Cowan, Br. J. Cancer 68 (1993) 732–737.
[46] N. Harada, A. Nagasaki, H. Hata, H. Matsuzaki, F. Matsuno, H. Mitsuya, Acta Haematol. 103 (2000) 144–151.
[47] M.S. Parmacek, B.A. Karpinski, K.M. Gottesdiener, C.B. Thompson, J.M. Leiden, Nucleic Acids Res. 17 (1989) 1915–1931.
[48] G. Rossier, C. Meier, C. Bauch, V. Summa, B. Sordat, F. Verrey, L.C. Kuhn, J. Biol. Chem. 274 (1999) 34948–34954.
[49] W.A. Campbell, D.E. Sah, M.M. Medina, J.E. Albina, W.B. Coleman, N.L. Thompson, J. Biol. Chem. 275 (2000) 5347–5354.
[50] H. Taskov, A. Pashov, E. ffmitrova, M. Yordanova, M. Serbinova, Leuk. Res. 20 (1996) 75–79.
[51] M. Nikolova, M. Guenova, H. Taskov, E. Dimitrova, M. Staneva, Leuk. Res. 22 (1998) 39–47.
[52] R. Pfeiffer, B. Spindler, J. Loffing, P.J. Skelly, C.B. Shoemaker, F. Verrey, FEBS Lett. 439 (1998) 157–162.
[53] H. Matsuo, S. Tsukada, T. Nakata, A. Chairoungdua, D.K. Kim, S.H. Cha, J. Inatomi, H. Yorifuji, J. Fukuda, H. Endou, Y. Kanai, Neuroreport 11 (2000) 3507–3511.
[54] H.W. Gaugitsch, E.E. Prieschl, F. Kaithoff, N.E. Huber, T. Baumruker, J. Biol. Chem. 267 (1992) 11267–11273.
[55] B. Spindler, L. Mastroberardino, M. Custer, F. Verrey, Pflugers Arch. 434 (1997) 323–331.