1-Type Amino Acid Transporter-1 Expressed in Human Astrocytomas, U343MGa

Shinji ASANO,⁎, a Megumi KAMEYAMA, b Ayako OURA, a Anna MORISATO, a Hideki SAKAI, b Yoshiaki TABUCHI, c Arthit CHAIROUNGDUA, d Hitoshi ENDOU, d and Yosikatsu KANAI d

a Department of Molecular Physiology, College of Information Science and Engineering, Ritsumeikan University; 1–1–1 Noji-Higashi, Kusatsu, Shiga 525–8577, Japan; and b Graduate School of Medical and Pharmaceutical Sciences, University of Toyama; c Life Science Research Center, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan; and d Department of Pharmacology and Toxicology, Kyorin University School of Medicine; 6–20–2 Shinkawa, Mitaka, Tokyo 181–8611, Japan. Received August 29, 2006; accepted December 19, 2006; published online December 21, 2006

LAT1 (t-type amino acid transporter 1), one of the t-type amino acid transporters, transports the branched and aromatic amino acids. LAT1 requires the heavy chain of 4F2 antigen (4F2hc) for the functional expression as an amino acid transporter. The expression of this transporter is up-regulated in tumor cells and rapidly-growing cells to support their proliferation. Here, we studied the expression of LAT1 and 4F2hc in human cultured cells by real-time PCR and Western blot, and found that human brain astrocytomas, U343MGa, highly expressed LAT1 and 4F2hc mRNAs and proteins. The uptake of [14C]leucine by U343MGa cells is Na+ -independent and inhibited by BCH (2-amino-2-norbornane carboxylic acid), and branched and aromatic amino acids, indicating that the LAT1 is expressed at the cell surface. Pulse chase labeling and surface labeling experiments of this cell line indicate that the protein synthesis of LAT1 and 4F2hc is slow, however, the heterodimeric complex assembled in the cells is very stable, and that the disulfide bond between the LAT1 and 4F2hc is not directly involved in the stability of the heterodimer.

Key words amino acid transporter; system L; astrocytoma

Among amino acid transporters, system L is a Na+-independent large and neutral amino acid transport agency.1) LAT1 (t-type amino acid transporter 1) is one of the t-type amino acid transporters, and transports the branched and aromatic amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, histidine, and methionine.2,3) Human LAT1 (SLC7A5) with a molecular mass of 55 kDa is a membrane protein with twelve transmembrane segments, and is directly involved in amino acid transport.4) LAT1 requires an additional integral membrane protein, the heavy chain of 4F2 antigen (4F2hc, SLC3A2) for the functional expression as an amino acid transporter.2,3) The 4F2 antigen, CD98, was originally found as a cell surface antigen that is up-regulated on lymphocyte activation.5,6) 4F2hc is a glycoprotein with its molecular mass being 72 kDa and 94 kDa for unglycosylated and glycosylated forms, respectively,7) and contains a single transmembrane segment, with its amino terminus located in the intracellular space.8,9) 4F2hc associates not only with LAT1 but also with light chains of other amino acid transporters, LAT2,9–11) y-LAT1, y-LAT2,12,13) asc-114) and xCT,15) which represent transport systems L, yL, aspartate/glutamate, and y-glutamate, respectively.16) LAT1 and 4F2hc form a heterodimeric functional complex via a disulfide bond between Cys109 of human 4F2hc and Cys116 of human LAT1.16) LAT1 is expressed at the cell surface only in the presence of 4F2hc in Xenopus oocytes as well as in COS cells, indicating that 4F2hc is essential for the cell surface expression of LAT1.17)

LAT1 expression is up-regulated in tumors cells and established cell lines, and seems to supply the cells with more essential amino acids to support their rapid proliferation. The LAT1 mRNA was reported to be expressed in T24 bladder carcinoma cells, RERF-LC-MA lung small-cell carcinoma cells, and HeLa uterine cervical carcinoma cells.18,19) In this study, we examined to compare the expression of LAT1 and 4F2hc in different kinds of human cultured cells by real-time PCR and Western blot, and found that human brain astrocytomas, U343MGa,20) highly express LAT1 and 4F2hc mRNAs and proteins. We studied the properties of amino acid transport of this cell line, and found that LAT1 is functionally expressed at the cell surface.

So far, there has been no report about the biosynthetic process and turnover of this transporter. Here, we studied the time-course of biosynthesis and degradation of LAT1, 4F2hc, and their heterodimer (HD) in U343MGa cells by pulse-chase labeling and surface biotinylation. We also studied the role of disulfide bond between the LAT1 and 4F2hc on the stabilization of each subunit.

MATERIALS AND METHODS

Cells U343MGa cells from human brain astrocytomas, T24 cells from human bladder carcinoma, HeLa cells from human uterine cervical carcinoma, RERF-LC-MA, cells from human lung small-cell carcinoma, HT-29 and WiDr cells from human colon carcinomas, HEK293 cells from human embryonic kidney, MKN1, MKN28, MKN45, MKN74, SCH15 cells from human gastric carcinomas were obtained from RIKEN Bioresource Center (Tsukuba, Japan). Cells were cultured in the growth medium recommended by the provider (in D-MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin for U343MGa cells) in a 5% CO2 humidified atmosphere.

Antibodies An anti-human LAT1 antibody was prepared against the N-terminal peptide MAGAGPKRRALA (corresponding to amino acids of 1—12). An anti-human 4F2hc antibody was prepared against the peptide, HKNQKD (corresponding to amino acid residues 164—175 of human 4F2hc). An anti-human LAT2 antibody was pre-
pared against the C-terminal peptide, EEAEDMEEEQQQ (corresponding to amino acids of 505—516). The C-terminal cysteine residue was introduced for conjugation with keyhole limpet hemocyanin. Anti-peptide antibodies were prepared as described previously, and affinity-purified with each peptide conjugated on the column.21) An anti-human LAT1 monoclonal antibody was prepared against the N-terminal portion of human LAT1. An anti-h4F2hc (FRP-1) monoclonal antibody (4.5.1) was a kind gift from Prof. Ito (Mie University of School of Medicine).22)

**Real-Time Quantitative RT-PCR** Total RNA samples were prepared from cells cultured in a 60-mm dish using an RNeasy Mini kit (QIAGEN) with the treatment of RNase-free DNase (QIAGEN) following the manufacturer’s instructions. The total RNA samples were reverse-transcribed by using an oligo d(T)6 primer to prepare cDNA samples with an Omniscript RT kit (QIAGEN). Real-time quantitative PCR was performed using TaqMan Universal PCR Master Mix and the following oligonucleotides, by the use of Sequence Detection System ABI Prism 7700, following the manufacturer’s instructions (Applied Biosystems Japan). For human LAT1, the forward and reverse primers were 5'-CATCCTGCTGCTTGCT-3' and 5'-AGTTTGGTGCCCTCAATAGAAG-3'; respectively, and the probe was 5'-FAM-AGATCGGGAAGGTATGTTGCTCAATC-TAMRA-3'. For human 4F2hc, the forward and reverse primers were 5'-TTGGCTGATGTCGAAAAATATCA-3' and 5'-GAATCAGTTAGGCTCAGATGCT-3'; respectively, and the probe was 5'-FAM-TGCCCAGACTAATCTCCGACC-TAMRA-3'. For human glycerolaldehyde 3-phosphate dehydrogenase (G3PDH), the forward and reverse primers were 5'-AAGACTCATGACACAGCTCAT-3' and 5'-CCATCAGCCCACAGTTTCC-3'; respectively, and the probe was 5'-FAM-CATCTACTGCGCCCCAGAGACTGTG-TAMRA-3'. RT-PCR for LAT1, 4F2hc, and G3PDH was verified by agarose gel electrophoresis of PCR samples. The mRNA level of each cell line was normalized to the mRNA level of a housekeeping gene, G3PDH.

**Membrane Preparation** Crude membrane fractions were prepared as described previously.23) Cells cultured in a 100 mm culture dish were collected, washed with 2 ml of phosphate buffered saline, and incubated with 2 ml of low ionic salt buffer, 0.5 mM MgCl2, 10 mM Tris–HCl, pH 7.4 at 100 mm culture dish were collected, washed with 2 ml of cellulose membrane, Hybond ECL (GE Bioscience). The membranes were blocked with 4% Block Ace solution, followed by incubation with an anti-LAT1 or anti-4F2hc antibody. After incubation with a horseradish peroxidase-conjugated secondary antibody, the bands were visualized with the ECLplus kit (GE Bioscience) and scanned with a luminescent imaging analyzer LAS-1000plus (Fuji Film, Tokyo).

**Uptake Measurements in U343MGa Cells** The [14C]-leucine uptake experiments were performed as described previously.19) The cells were seeded on 24-well plates (1×10⁴ cells/well) in the fresh growth medium. The uptake measurements were performed at 72 h after seeding. After the removal of the growth medium, the cells were washed three times with 500 μl of the Na⁺-containing uptake solution (125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 25 mM HEPES–Tris, 1.2 mM KH₂PO₄, 5.6 mM glucose, pH 7.4) or Na⁺-free uptake solution (125 mM choline Cl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 25 mM HEPES–Tris, 1.2 mM KH₂PO₄, 5.6 mM glucose, pH 7.4) and preincubated for 10 min at 37 °C. Then, the medium was replaced by 200 μl of the uptake solution containing 20 μM of [14C]-leucine. The uptake reaction was terminated by removing the uptake solution followed by washing three times with 500 μl of ice-cold uptake solution. Then, cells were lysed with 500 μl of 0.1 N NaOH and the radioactivity was counted by liquid scintillation spectrometry. Aliquots of each lysate were taken to determine the protein content using the BCA method (Pierce), and the values of [14C]-leucine uptake are expressed as pmol/mg protein/min. For the measurement of uptake of [14C]-leucine, three to four wells of U343MGa cells were used for each data point. To confirm the reproducibility of the results, three separate experiments were performed for each measurement. Kₘ and Vₘₐₓ values were determined using Eadie–Hofstee plots based on the [14C]-leucine uptake measured for 1 min at 1, 3, 10, 20, 30, 100, 300, and 1000 μM. For the inhibition experiments, the uptake of 20 μM [14C]-leucine was measured in the presence or absence of 2 μM non-labeled amino acids.

**Pulse-Chase Labeling** Pulse-chase labeling of U343MGa cells was performed by the method described previously.26) U343MGa cells cultured on 6-well plates were washed and incubated at 37 °C for 30 min in methionine-free medium. Cells were labeled for indicated period (1—24 h) with [35S]-Met, Cys Labeling mixture (Express) (Perkin Elmer), and chased in the complete minimum essential medium for indicated periods. Cells were washed with a lysis buffer composed of 125 mM NaCl, 0.5 mM EDTA, and 50 mM Tris–HCl, pH 7.4, and incubated in 500 μl of lysis buffer containing 1% Triton X-100 at 4 °C for 30 min. After centrifugation of the sample at 16000×g for 20 min, the supernatant was centrifuged at 10000×g for 90 min. The pellet was re-suspended in a solution composed of 250 mM sucrose and 5 mM Tris–HCl, pH 7.4.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting** SDS-polyacrylamide gel electrophoresis was performed as described elsewhere.25) Protein samples were incubated in a sample buffer composed of 2% SDS, 10% glycerol, 10 mM Tris–HCl, pH 6.8 in the presence or absence of 100 mM DTT at 100 °C for 3 min, and separated on the SDS-polyacrylamide gel. Western blotting was performed as described elsewhere.25) The proteins were transferred to a nitrocellulose membrane, Hybond ECL (GE Bioscience). The bands were visualized by digital autoradiography of dried gels using Bio Imaging Analyzer BAS 2000 (Fuji Photo Film, Tokyo).
**Cell Surface Biotinylation** Cells grown in 6 well tissue culture plates were washed with phosphate-buffered saline containing 0.1 mM CaCl\(_2\) and 1 mM MgCl\(_2\) (PBS(++)), incubated with PBS(++) containing 0.5 mg/ml sulfo-NHS-LC-biotin or sulfo-NHS-SS-biotin (Pierce) at 4 °C for 30 min. The labeling was quenched by incubation with PBS(++) containing 2 mg/ml BSA, 50 mM glycine for 5 min three times. The cells were cultured in normal growth medium at 37 °C for indicated period, followed by solubilization in the lysis buffer containing 1% Triton X-100. The lysate was centrifuged at 16000 \( \times g \) at 4 °C for 20 min, and the supernatant was incubated with avidin-conjugated agarose beads (Sigma) at 4 °C for 1 h with continuous rotation. The precipitated beads were washed with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 three times and with PBS. Proteins were eluted from the beads by incubation with SDS-PAGE sample buffer, and separated on an SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, and the LAT1 and 4F2hc were detected with an anti-LAT1 or anti-4F2hc antibody, and the bands were visualized with the ECLplus kit (GE Bioscience) and luminescent imaging analyzer LAS-1000plus (Fuji Film, Tokyo) as reported in the Western blot.

**RESULTS AND DISCUSSION**

**The Expression of LAT1 and 4F2hc in Human Tumor Cell Lines** LAT1 mRNA is highly expressed in the brain, placenta, testis, bone marrow, and fetal liver samples.\(^2,4,19\) It is also up-regulated in tumors and tumor cell lines of various organs, and seems to supply cells with more essential amino acids to support their rapid proliferation. Yanagida et al.\(^{19}\) reported high expression of LAT1 mRNA in PA-1 teratocarcinoma cells, T24 bladder carcinoma cells, RERF-LC-MA lung small-cell carcinoma cells, HeLa uterine cervical carcinoma cells, as well as many leukemia cell lines. They also studied the protein expression of LAT1 and 4F2hc in T24 cells.\(^{18,19}\) Shennan et al.\(^{27}\) reported the Na\(^+\)-independent BCH-sensitive L-leucine transport activity and expression of LAT1 mRNA in human breast cancer cells, MCF-7 and MDA-MB-231. Here, we studied to compare the expression levels of LAT1 and 4F2hc mRNAs and the proteins in several human tumor cell lines including gastrointestinal epithelial cell lines, with those in HeLa and T24 cells by real-time quantitative PCR and Western blotting, respectively.

Figure 1 shows the mRNA level of LAT1 and 4F2hc in several human cell lines. In the figure, the mRNA level was normalized with that of G3PDH. The expression level of LAT1 was quite different among tumor cell lines tested; the LAT1 expression was highest in human brain astrocytomas, U343MGa. The mRNA level of LAT1 in U343MGa cells was about 5-fold higher than that of T24 cells, in which Kim et al.\(^{19}\) found the transport activity of LAT1 in the previous study. U343MGa cells showed the highest mRNA expression of 4F2hc also. The mRNA level of 4F2hc in U343MGa cells was about 6-fold higher than that of T24 cells. Among these cell lines, there seems to be a relationship between the mRNA expression levels of LAT1 and 4F2hc except that MKN45 and MKN74 represent low LAT1 expression in spite of medium level of expression of 4F2hc.

Figure 1 also shows the Western blotting patterns of membrane fraction of the cells under the non-reducing condition, detected with anti-LAT1 and anti-4F2hc antibodies, respectively. Under the non-reducing condition, both LAT1 and 4F2hc were observed as a band with a molecular mass of 125 kDa, which represents the HD of LAT1 and 4F2hc.

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**Fig. 1.** (Upper Part) Quantitative Measurement of LAT1 and 4F2hc mRNAs by TaqMan Real-Time PCR

The mRNA level of each cell line was normalized to the mRNA level of G3PDH, and the values were shown as relative expression against the value for U343MGa cells. Values shown are the mean obtained from 2 independent triplicate experiments.

(Lower Part) Western Blots of Membrane Fractions from Human Cell Lines Detected with the Anti-LAT1 (N-Terminal) (Left) and the Anti-4F2hc Antibody (Right), Respectively

Thirty micrograms of membrane fractions were separated on an SDS-polyacrylamide gel in the absence of DTT, and transferred to a nitrocellulose membrane. The membrane was incubated with the anti-LAT1 and the anti-4F2hc antibody, respectively. HD, LAT1 (M), and 4F2hc (M) shown on the right side of the membranes represent the heterodimeric complex, LAT1 monomer, and 4F2hc monomer, respectively.
band of monomeric LAT1 with a molecular mass of 35—38 kDa was not observed in this condition except that a faint band of LAT1 monomer was observed in U343MGa cells. The expression levels of LAT1 and 4F2hc was also quite different among tumor cell lines. The expression level of HD of LAT1 and 4F2hc was highest in U343MGa, and HeLa cells. These cell lines show the extra bands with a molecular mass of 200 kDa detected by the anti-LAT1 antibody. MKN28 and MKN45 cells showed low protein expression for LAT1.

These results indicate that U343MGa cells highly express LAT1 both in the mRNA and protein levels. However, there is no clear relationship between the mRNA and protein levels of LAT1 and 4F2hc. It should be noted that HeLa cells showed high protein expression level of LAT1 and 4F2hc, which is almost comparable with that in U343MGa cells in spite of its low mRNA expression level of LAT1 and 4F2hc. MKN28 cells showed little protein expression in spite of its moderate mRNA expression level of LAT1 and 4F2hc. All these cell lines do not express a different L-type amino acid transporter, LAT2. In the Western blot with an anti-LAT2 antibody, no band representing LAT2 was detected in the membrane fraction of these cell lines (data not shown). Hereafter, we focus on the behavior of LAT1 and 4F2hc in U343MGa cells.

Figure 2 shows the Western blotting pattern of the membrane fraction of U343MGa cells in the presence and absence of 100 mM DTT, detected with the anti-LAT1 or anti-4F2hc antibody. Under the non-reducing condition, LAT1 was mainly observed as a band with a molecular mass of 125 kDa as shown in Fig. 1. This band was also observed with the anti-4F2hc antibody in the same non-reducing condition. These results indicate that this band represents the HD of LAT1 and 4F2hc. Under the reducing condition, LAT1 was mainly observed as a band with a molecular mass of 35—38 kDa, and 4F2hc was observed as a band with a molecular mass of 85 kDa although a small amount of the HD was observed. The molecular mass of monomeric LAT1 estimated in the SDS-PAGE was lower than that expected from the amino acid sequence, 55 kDa. However, this band was observed either with an anti-LAT1 amino-terminal antibody or an anti-LAT1 carboxy-terminal antibody (data not shown). The molecular mass of 4F2hc (85 kDa) was lower than that reported previously (94 kDa). It may be due to the difference in modification of carbohydrate chains among different cells. Similar finding was reported for the gastric proton pump β-subunit which contains seven N-linked carbohydrate chains. The molecular mass of β-subunit in native gastric mucosae is about 60—90 kDa, whereas that the molecular mass of the β-subunit expressed in HEK-293 cells is 60—70 kDa. The gel pattern presented here is almost comparable with that of T24 cells in the previous study.

**The Properties of [14C]-L-Leucine Uptake by U343MGa Cells**

Hereafter, we examined the properties of [14C]-L-leucine transport in U343MGa cells because of its high expression of LAT1 in the membrane fraction. The level of [14C]-L-leucine uptake was almost same in the presence and absence of Na⁺ in the uptake medium (data not shown), indicating that the uptake was not dependent on Na⁺. Therefore, in the subsequent uptake measurements, the [14C]-L-leucine uptake was examined in the absence of Na⁺. To determine the time course of [14C]-L-leucine uptake by U343MGa cells, the uptake level was measured in the incubation period from 0.5 to 30 min. As the uptake of [14C]-L-leucine exhibited a linear dependence on the incubation time up to 1 min (data not shown), all the subsequent uptake measurements were performed in the incubation period of 1 min. Figure 3 shows that [14C]-L-leucine uptake was dependent on the concentration of L-leucine. The uptake was saturable and followed Michaelis–Menten kinetics with its $K_m$ value being 160 μM. This pattern was quite different from those found in other L-type amino acid transporters, the LAT3 and LAT4, which showed high and low affinity components; 1024 and 8.4 μM of $K_{m1}$ and $K_{m2}$ for LAT3, and 4690 and 178 μM of $K_{m1}$ and $K_{m2}$ for LAT4, respectively. $K_m$ value found in U343MGa cells is slightly lower than that of MCF-7 (179 μM), slightly higher than that reported for T24 (100 μM), and 4-fold higher than that of MDA-MB-231 cells (46 μM). The uptake of [14C]-L-leucine (20 μM) was inhibited by a System L-specific inhibitor, BCH in a concentration dependent manner with its IC₅₀ value of 150 μM as shown in Fig. 4, indicating that the [14C]-L-leucine uptake by U343MGa cells...
is mediated by the system L. In order to examine which amino acids interact with l-leucine uptake by U343MGa cells, the [14C]-l-leucine uptake (20 μM) was measured in the presence of 2 mM non-labeled amino acids (Fig. 5). The [14C]-l-leucine uptake was highly inhibited by cysteine, methionine, isoleucine, valine, phenylalanine, tryptophan, and histidine. On the other hand, the uptake was not inhibited by glycine, aspartic acid, glutamic acid, lysine, arginine, and proline. The amino acid specificity presented here is almost comparable with that reported for LAT1 amino acid transporter found in T24 cells and expressed in Xenopus oocytes. These results indicate that LAT1 is highly expressed in U343MGa cells, and seem to transport hydrophobic amino acids with bulky side chains at the cell surface. Recently, the expression of LAT1 in the human primary astrocytic tumor tissues was studied. Nawashiro et al. reported that high LAT1 expression correlated with poor survival of astrocytic tumor patients. They also found that BCH inhibited mortality of rats treated with C6 glioma cells, indicating that LAT1 could be one of the molecular targets in glioma therapy. U343MGa cells were established from a human primary malignant astrocytoma, and express the EGF receptor at the high level. Retinoic acid treatment of this cell line inhibits proliferation, alters morphology, and increases the expression of a glial differentiation marker, glial fibrillary acidic protein. Therefore, U343MGa may be a good system to study the amino acid transport by LAT1 transporter in human brain astrocytoma.

**Pulse Chase Experiments of LAT1 and 4F2hc Expressed in U343MGa Cells** Although LAT1 seems to be a candidate of target molecule for cancer therapy, there has been no report for the biosynthetic process and turnover of this transporter. Because U343MGa cells functionally express the LAT1 at the highest level among human tumor cell lines tested, we studied the expression and stability of LAT1 and 4F2hc using this cell line. To study the stability of LAT1 and 4F2hc proteins and the process of HD formation, pulse-chase labeling of U343MGa cells was performed. U343MGa cells were labeled with [35S]-methionine/cysteine for various incubation periods (1—24 h), followed by immunoprecipitation of the cell lysate with the anti-LAT1 and anti-4F2hc monoclonal antibodies, respectively. The precipitated proteins were separated on an SDS-polyacrylamide gel, and the radioactive bands were visualized by digital autoradiography (Fig. 6). Under the non-reducing condition, a faint 35-kDa...
band was observed after a 3 h labeling period. The mobility of this band is identical with that of LAT1 band which is observed in the 24-h pulse sample treated with DTT (Fig. 6A), indicating that this band represents the monomeric LAT1. The amount of this monomeric LAT1 band was low and almost constant between 3 and 24 h. The band representing the HD was observed after a 3 h labeling period. The amount of this HD band gradually increased within a 24 h labeling period. Under the reducing condition, the HD band disappeared, followed by appearance of the monomeric LAT1 and 4F2hc bands. In Fig. 6A, two intense bands (about 40 kDa) were also observed at just above the monomeric LAT1. However, these bands do not represent the monomeric LAT1 because the intensity of these bands was unchanged when the HD band disappeared under the reducing condition.

Under the non-reducing condition, a clear band representing the monomeric 4F2hc was not observed in a 24 h labeling period. However, the band representing the HD was observed after a 3 h labeling period. The amount of this band gradually increased within a 24 h labeling period (Fig. 6B). These results suggest that LAT1 is assembled with 4F2hc and forms the HD soon after the LAT1 is synthesized. There is no large pool of monomeric LAT1 and 4F2hc in U343MGa cells. Nawashiro et al. found that LAT1 was predominantly expressed on the plasma membrane of human astrocytoma.

After labeled with [35S]-methionine/cysteine for 24 h, U343MGa cells were incubated with non-labeled medium for indicated chase periods, and the cell lysate was immunoprecipitated with the anti-LAT1 monoclonal antibody (Fig. 7). The HD was stable in the chase period, and more than 50% of the labeled HD was present after a 36 h chase period. These results suggest that the protein synthesis of LAT1 and 4F2hc is slow, however, the HD complex formed is stable in the cell. This result was further supported by the cell surface biotinylation of U343MGa cells with a membrane-impermeable biotinylation reagent, sulfo-NHS-SS-biotin. After a 30 min labeling period at 4 °C, the HD with a molecular mass of 125 kDa was biotinylated and detected with the anti-LAT1 antibody. However, the monomeric LAT1 was not observed on the Western blot (Fig. 8), confirming that HD formation is essential for cell surface delivery of LAT1. After the cell surface biotinylation for 30 min, U343MGa cells were incubated with the normal medium for indicated chase periods, and the biotinylated HD was detected with the anti-LAT1 monoclonal antibody (Fig. 8). The band representing biotinylated HD gradually decreased in the chase periods. However, a dense band with a molecular mass of 125 kDa was observed after 36 h chase period. This result indicates that once the HD was formed, it was stable in the chase period at the cell surface.

LAT1 and 4F2hc form a heterodimeric functional complex via a disulfide bond. However, the functional role of the disulfide bond remains elusive. The disulfide bond between the LAT1 and 4F2hc is not qualitatively essential for cell surface delivery of LAT1 and the function of the amino acid transporter suggesting that non-covalent bonds seem to dominate the interaction between the LAT1 and 4F2hc. On the other hand, Wagner reported that the disulfide bond is important for the cation channel activity evoked by the expression of LAT1 and 4F2hc in Xenopus oocytes. It is also interesting that the LAT1 cannot be co-immunoprecipitated with the mutant 4F2hc (C103S mutant of mouse 4F2hc) which cannot form a disulfide bond with the LAT1. This result may suggest that the disulfide bond is important for stable association between the LAT1 and 4F2hc.

Here, we studied the roles of disulfide bond on the stability of LAT1 and 4F2hc in U343MGa cells incubated in the presence of DTT. These results suggest that the protein synthesis of LAT1 and 4F2hc is not qualitatively essential for cell surface delivery of LAT1. After the cell surface biotinylation for 30 min, U343MGa cells were incubated with the normal medium for indicated chase periods (0—60 h). The cells were solubilized in 500 μl of lysis buffer containing 1% Triton X-100, and the cell lysate was immunoprecipitated with the anti-LAT1 monoclonal antibody. The precipitated proteins were incubated in the presence (DTT+) or absence of 100 μM DTT (DTT−) at 100 °C for 3 min, separated on an SDS-polyacrylamide gel. The radioactive bands were visualized by digital autoradiography of the dried gel. HD, 4F2hc, and LAT1 shown on the right side of gel indicate the bands representing the heterodimeric complex, 4F2hc monomer, and LAT1 monomer, respectively. A representative result from three experiments is shown.

U343MGa cells were labeled with [35S]-methionine/cysteine for 24 h, followed by chase in the complete minimum essential medium for indicated periods (0—60 h). The cells were solubilized in 500 μl of lysis buffer containing 1% Triton X-100, and the cell lysate was immunoprecipitated with the anti-LAT1 monoclonal antibody. The precipitated proteins were incubated in the presence of DTT+ or absence of 100 μM DTT (DTT−) at 100 °C for 3 min, separated on an SDS-polyacrylamide gel. The radioactive bands were visualized by digital autoradiography of the dried gel. HD, 4F2hc, and LAT1 shown on the right side of gel indicate the bands representing the heterodimeric complex, 4F2hc monomer, and LAT1 monomer, respectively. A representative result from three experiments is shown.

U343MGa cells were cultured in the presence of 10 mM DTT for various periods. Proteins in the cell lysate were separated by SDS-polyacrylamide gel electrophoresis under non-reducing condition, followed by Western blotting with the anti-LAT1 and anti-4F2hc antibody, respectively (Fig. 9). Within 10 min incubation with DTT, the band representing the HD of LAT1 and 4F2hc disappeared (data not shown), and the bands representing the LAT1 and 4F2hc monomers were observed. The LAT1 and 4F2hc were stable within 24 h incubation of the cells with DTT. These results indicate that the LAT1 and 4F2hc are stable in the cells in the absence of disulfide bond formation. This result was further supported by the pulse chase labeling experiments. U343MGa cells were labeled with [35S]-methionine/cysteine for 24 h followed by cold chase in the presence of 10 mM DTT, and the...
In conclusion, we found that human brain astrocytomas, U343MGa, highly expressed LAT1 at the cell surface, is useful to study the amino acid transport by LAT1 transporter in human brain astrocytoma. In this cell line, the protein synthesis of LAT1 and 4F2hc was slow, however, the HD complex was very stable.

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