Expression Cloning and Characterization of a Transporter for Large Neutral Amino Acids Activated by the Heavy Chain of 4F2 Antigen (CD98)*

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A cDNA was isolated from rat C6 glioma cells by expression cloning which encodes a novel Na+-independent neutral amino acid transporter designated LAT1. For functional expression in Xenopus oocytes, LAT1 required the heavy chain of 4F2 cell surface antigen (CD98), a type II membrane glycoprotein. When co-expressed with 4F2 heavy chain, LAT1 transported neutral amino acids with branched or aromatic side chains and did not accept basic amino acids or acidic amino acids. The transport via LAT1 was Na+-independent and sensitive to a system L-specific inhibitor 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; kb, kilobase pair(s).

The organic nutrients such as sugars and amino acids are provided to cells via transporters situated on the plasma membrane (1, 2). The transport of large neutral amino acids with branched or aromatic side chains are mediated by amino acid transport system L (1, 3). System L is a Na+-independent neutral amino acid transport agency and thought to be a major route to provide cells with branched or aromatic amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, histidine, and methionine (1). The molecular nature of system L has not been characterized. It has, however, been indicated recently that the knockout of 4F2 heavy chain (4F2hc)1 by antisense oligonucleotides reduced the system L activity in rat C6 glia cells (4).

4F2 antigen (CD98) is a heterodimeric protein composed of two subunits, a 80-kDa glycosylated heavy chain and a 40-kDa nonglycosylated light chain (5, 6). The 4F2 antigen has been identified originally as a cell surface antigen associated with lymphocyte activation (5, 6). Although the function of 4F2 antigen has not been clarified, it has attracted investigators, because it is involved in variety of cellular activity such as cell activation, cell growth, and cell adhesion (5–8). 4F2hc is an integral membrane protein with a single membrane-spanning domain classified as type II membrane protein (9). The 4F2 light chain, however, has not been identified by molecular cloning.

When 4F2hc was expressed in Xenopus oocytes, it induced the transport of neutral and basic amino acids with the property of system y+L, which is in agreement with the fact that 4F2hc exhibits amino acid sequence similarity to the type II membrane protein D2/rBAT, a cystinuria-associated putative amino acid transport activator (10–12). Therefore, it was supposed that 4F2hc, as well as D2/rBAT, associates with unidentified amino acid transporters to activate them (12). As mentioned above, in mammalian cells, 4F2hc was proposed to activate neutral amino acid-specific transport system L based on the knockout of 4F2hc by antisense oligonucleotides (4). In the present study to identify system L transporter, we have, therefore, performed expression cloning by co-expression of 4F2hc and rat C6 glia cell cDNA library. We have isolated a cDNA encoding a novel Na+-independent transporter for large neutral amino acids, which requires 4F2hc for its functional expression.

EXPERIMENTAL PROCEDURES

Co-expression of 4F2hc and Poly(A)+ RNA—Xenopus laevis oocyte expression studies and uptake measurements were performed as described elsewhere (13, 14). Defolliculated oocytes were injected with in vitro transcribed cRNA (5 ng) of 4F2hc (GenBank™/EBI/DDBJ accession number AB015433) and poly(A)+ RNA (45 ng) obtained from C6 glia cells. Two days after injection, the uptake of 4[14C]L-leucine was measured for 30 min in Na+-free uptake solution (choline-Cl, 100 mM; KCl, 2 mM; CaCl2, 1 mM; MgCl2, 1 mM; HEPES, 10 mM; Tris, 5 mM, pH 7.4) containing 50 μM 4[14C]L-leucine (1.0 μCi/ml).

Expression Cloning—Expression cloning using the Xenopus oocyte expression system was performed as described (15–17). Four-hundred μg of C6 glia poly(A)+ RNA was size-fractionated (17). RNA from each fraction (45 ng) was co-expressed with 4F2hc cRNA (5 ng) in Xenopus oocytes. Positive fractions showing peak stimulation of 4[14C]L-leucine (50 μM) uptake when co-expressed with 4F2hc were used to construct a directional cDNA library. cRNA synthesized in vitro from

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pools of ~500 clones was injected with 4F2hc cRNA into Xenopus oocytes (17). A positive pool was serially subdivided and analyzed until single clone (LAT1) was identified. The cDNA was sequenced in both directions by dideoxy termination method and dye terminator cycle sequencing method by Applied Biosystems.

Functional Characterization—Xenopus oocytes were injected with 15 ng of LAT1 cRNA and 10 ng of 4F2hc cRNA giving the mole ratio of 1:1. Two days after injection the uptake of [3H]-L-leucine amino acids was measured as described above in the Na"-free uptake solution containing 0.6 μCi/ml radiolabeled compounds. For Na"-uptake solution, choline-Cl in the Na"-free uptake solution was replaced by NaCl. For Cl"-uptake solution, Cl" in the Na"-uptake solution was replaced by gluconate anion. For the efflux measurement, [3H]-L-leucine (20 μM; 2 μCi/ml) was preloaded by incubating the oocytes for 30 min. Then, the individual oocytes were transferred to Na"-free uptake solution with or without 100 μM nonradioabeled L-leucine (18). The radioactivity in the medium and the remaining radioactivity in oocytes were measured. Because the [3H]-L-leucine (20 μM) uptake into oocytes expressing LAT1 was linearly dependent on incubation time up to 60 min (data not shown), so for all the experiments uptakes were measured for 30 min, and the values were expressed as picomoles/oocyte/min.

For the uptake measurements in the present study, six to nine oocytes were used for each data point. Each data point in the figures represents the mean ± S.E. of uptake (n = 6–9). To confirm the reproducibility of the results, three separate experiments using different batches of oocytes and in vitro transcribed cRNA were performed for each measurement. Results from the representative experiments were shown in the figures.

In Vitro Translation—Procedure for in vitro translation have been described elsewhere (19, 20). In vitro translation of cRNAs for LAT1 and 4F2hc was performed by using a rabbit reticulocyte lysate system with described elsewhere (19, 20). The size fractionation of the C6 glioma cell poly(A) RNA (3 μg/lane) isolated from rat tissues and tumor cell lines was separated on 1% agarose gel in the presence of 2.2% formaldehyde and blotted onto a nitrocellulose filter (Schleicher & Schuell) (14, 21). The BamHI fragment of LAT1 cDNA corresponding to 1135–1529 base pairs was labeled with 32P using a QuickPrime kit (Amersham Pharmacia Biotech). Hybridization was for 20 h at 42 °C in 50% formamide. The final stringent wash of the filter was in 0.1 × SSC, 0.1% SDS at 65 °C for 3 × 20 min (14, 21). Tumor cell lines were provided by Health Science Research Resources Bank, Japan Health Sciences Foundation.

RESULTS

When poly(A)RNA from rat C6 glioma cells was expressed in X. laevis oocytes, the synergistic augmentation of [3H]-L-leucine uptake was detected by co-expression with 4F2hc (Fig. 1A). The size fractionation of the C6 glioma cell poly(A)RNA revealed that the fraction of 2.8–3.8 kb contained the peak activity for [3H]-L-leucine uptake when co-expressed with 4F2hc. From this fraction, a cDNA library was constructed and screened for [3H]-L-leucine uptake by co-expression with 4F2hc in Xenopus oocytes. A 3.5-kb cDNA was isolated, which encodes a protein designated LAT1 (L-type amino acid transporter 1). As shown in Fig. 1B, LAT1 by itself did not induce [3H]-L-leucine transport. 4F2hc when solely expressed induced low levels of L-leucine transport, probably due to the activation of oocyte endogenous transporters. The co-expression of LAT1 and 4F2hc resulted in the large leucine uptake, indicating that 4F2hc is indispensable for the functional expression of LAT1.

The functional characteristics of LAT1 were examined by co-expression with 4F2hc in Xenopus oocytes. The uptake of [3H]-L-leucine was saturable and followed Michaelis-Menten kinetics with a Km value of 1.8 ± 0.3 μM (mean ± S.E., n = 4), which is not shown. The substrate selectivity of LAT1 was investigated by inhibition experiments in which 20 μM [3H]-L-leucine uptake was measured in the presence of 2 mM amino acids. The L-leucine uptake was highly inhibited by l-isomers of isoleucine, phenylalanine, methionine, tyrosine, histidine, tryptophan, valine, and a classical system L-specific inhibitor 2-amino-3-nobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) (Fig. 2A). These amino acids were confirmed to be transport substrates of LAT1 by the uptake of radiolabeled compounds (Fig. 2B). Basic amino acids lysine and arginine and acidic amino acids glutamate and aspartate did not inhibit [3H]-L-leucine uptake (Fig. 2A) (12, 22). Interestingly, LAT1 was less stereoselective for leucine, phenylalanine, and methionine, whereas it was highly stereoselective for tyrosine, histidine, tryptophan, and valine (Fig. 2C).

The uptake of L-leucine was not dependent on Na+ or Cl" (Fig. 1C). Because LAT1-induced amino acid transport was apparently cumulative despite its independence on Na+ or Cl" we tested whether LAT1-mediated transport is an amino acid exchange that could drive the transport. As shown in Fig. 1D, L-leucine applied outside the oocytes induced the efflux of preloaded [3H]-L-leucine, suggesting LAT1 is an amino acid exchanger.

The LAT1 cDNA (3455 base pairs) contains a single open reading frame encoding a putative 512-amino acid protein with a predicted molecular mass of 56 kDa (Fig. 3A). The first ATG,
which is in the Kozak consensus initiation sequence for translation (23) (GAGAGCATGG), was predicted to be the start for translation. Kyte-Doolittle hydropathy analysis (24) indicated that LAT1 is an integral membrane protein with putative 12 membrane-spanning domains (Fig. 3B).

In vitro translation of LAT1 showed a band of 44-kDa protein (Fig. 3C). Although 4F2hc was glycosylated by canine pancreatic microsomes, LAT1 was not glycosylated (Fig. 3C).

The search of protein data bases (April 1998) revealed that LAT1 sequence is novel and exhibits relatively low but significant homology to those of mammalian Na\(^+\)-independent cationic amino acid transporters (e.g. 30% identity to mouse CAT2 (25)) and amino acid permeases of bacteria and yeasts (e.g. 29% identity to Saccharomyces cerevisiae methionine permease MUP1 (26)). Therefore LAT1 denotes a new and distinct member of the APC superfamily, which includes prokaryote and eukaryote Na\(^+\)-independent transporters for amino acids, polyamines, and choline (27).

The Northern blot analysis indicated that a 3.8-kb message is expressed at high level in brain, spleen, and placenta and at low level in testis and colon (Fig. 4A). In placenta, an additional 2.6-kb message was also detected. LAT1 was expressed at high levels in C6 glioma, hepatoma (dRLh-84), and hepatocarcinoma (FAA-HTC1) cell lines, whereas normal liver did not express LAT1 (Fig. 4B). A high level of LAT1 expression was also detected in human tumor cell lines such as stomach signet ring cell carcinoma (KATOIII), malignant melanoma (G-361), and lung small cell carcinoma (RERF-LC-MA) by Northern blot analysis (data not shown).
Amino Acid Transporter Activated by Type II Membrane Protein

DISCUSSION

By co-expression of 4F2hc and rat C6 glioma cell cDNA library in Xenopus oocytes, we have isolated a cDNA encoding a novel Na+-independent transporter LAT1. Because it prefers neutral amino acids with branched or aromatic side chains and is inhibited by a system L-specific inhibitor BCH, we conclude that LAT1 is a transporter corresponding to classically characterized neutral amino acid transport system L (1, 3).

For the functional expression in Xenopus oocytes, LAT1 requires co-expression of 4F2hc. This is in agreement with the previous report showing that the antisense oligonucleotide for 4F2hc reduced the system L activity in C6 glioma cells (4). Although the manner of interaction between the two proteins is not clarified at present, it is indicated in the present study that the interaction is essential for the transporter to be functional. The 4F2 antigen is a heterodimeric protein. Its light chain has been reported to be a 40-kDa nonglycosylated protein (5, 6). Our in vitro translation results are consistent with the properties of 4F2 light chain. It is, therefore, suggested that LAT1 is at least one of the proteins previously referred to as 4F2 light chain (5, 6, 12).

Our Northern blot showed that LAT1 is expressed in some restricted tissues. Because system L transporter should be present in every tissue for cellular nutrition and in kidney and small intestine for epithelial transport (1), it is proposed that the other isoforms exist in tissues which lack LAT1. In fact, heterogeneity in the properties of system L has been reported (28–31). It is interesting to know whether other unidentified isoforms are also coupled to 4F2hc, which is expressed ubiquitously (32). Furthermore, it should be clarified whether other transporters of the APC superfamily require 4F2hc or other related proteins for their functional expression. When 4F2hc was solely expressed in Xenopus oocytes, it induces the activity of neutral and basic amino acid transport system yL but not system yL.

We have identified a system L amino acid transporter LAT1 and showed that 4F2hc is essential for LAT1 to be functional. The cloning of LAT1 is expected to facilitate the research on the protein–protein interaction in the transporter field and to provide a clue to the search for still unidentified amino acid transporters.

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