The 4F2hc surface antigen is necessary for expression of system L-like neutral amino acid-transport activity in C6-BU-1 rat glioma cells: evidence from expression studies in *Xenopus laevis* oocytes

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Mammalian cells possess a variety of amino acid-transport systems with overlapping substrate specificity. System L is one of the major amino acid-transport systems in all non-epithelial cells. Its molecular structure is not known. To clone the neutral amino acid-transporter system L we followed an expression cloning strategy using *Xenopus laevis* oocytes. A cDNA library derived from C6-BU-1 rat glioma cells was used as a source, because high expression of system L activity could be demonstrated with polyadenylated RNA isolated from these cells, when injected into *Xenopus laevis* oocytes [Bröer, Bröer and Hamprecht (1994) Biochim. Biophys. Acta **1192**, 95–100]. A single clone (lLAT) was identified, the sense cRNA of which, on injection into *Xenopus laevis* oocytes, stimulated sodium-independent isoleucine transport by about 100-fold. Further characterization revealed that transport of cationic amino acids was also stimulated. Sequencing of the cDNA showed that the identified clone is the heavy chain of the rat 4F2 surface antigen, a marker of tumour cells and activated lymphocytes. Uptake of neutral and cationic amino acids was not stimulated by the presence of Na^+ ions. Antisense cRNA transcribed from this clone or antisense oligonucleotides, when co-injected with polyadenylated RNA from C6-BU-1 rat glioma cells, completely suppressed system L-like isoleucine-transport activity. We conclude that lLAT is necessary for expression of system L-like amino acid-transport activity by polyadenylated RNA from C6-BU-1 rat glioma cells.

INTRODUCTION

Mammalian cells possess a variety of amino acid-transport systems with overlapping substrate specificity [1]. The different systems are generally categorized by dependence on Na^+ ions, substrate spectrum, including analogues, and a few other criteria [2]. Structural and molecular data on these systems have been obtained only recently [3–5]. The cDNAs of several Na^+-dependent amino acid transporters have been cloned in the last 3 years, e.g. transporters with characteristics similar to systems X^-_{AG}, ASC and Gly as well as transporters for γ-aminobutyric acid and proline (for a review see ref. [6]). Cloning of System A was claimed recently [7], but further characterization led to its identification as a sugar transporter [8]. Two families of transporter cDNAs have emerged from attempts to clone Na^+-independent amino acid transporters. One is the family of y^+-like transporters, with cDNAs named CAT1 and CAT2 [4]. Cationic amino acids are the main substrates of these transporters. Some neutral amino acids such as homoserine and glutamine are also transported by system y^+, but only in the presence of Na^+. The other family comprises cDNAs named rbAT, NBAT and D2 which exhibit properties similar to system $b^{0,+}$ when injected into oocytes [9–11]. System $b^{0,+}$ is a broad-specificity transporter which translocates neutral and cationic amino acids in a Na^+-independent process [12]. It is thought to be mainly present in epithelial cells. The sequences of the rbAT, NBAT and D2 cDNA clones have low similarity to the heavy chain of the surface antigen 4F2 (4F2hc)[13,14]. This led to the idea that the 4F2hc antigen may also be involved in amino acid transport. Injection of cRNA encoding the human 4F2hc protein into *Xenopus laevis* oocytes elicited only a 2–3-fold increase in leucine transport. Transport characteristics resembled those of system y^+L [13,14]. System y^+L was first characterized in human erythrocytes [15], but was also shown to be present in intestine and placenta [16,17]. The Na^+-dependence of this transporter is determined by the substrate. Neutral amino acids are transported in a Na^+-dependent manner, whereas cationic amino acids do not require the presence of Na^+ for transport [15]. The very broad specificity and higher substrate affinity discriminates the y^+L system from the y^+ system. Because of the low expression of the human 4F2hc cRNA in oocytes, it was reasoned that it might encode a regulatory protein that activates an endogenous oocyte transporter. The 4F2hc antigen was originally described as a marker for tumour cells and activated lymphocytes [18]. It is a protein composed of two subunits linked by a disulphide bridge [19]. The monoclonal antibody raised against the 4F2 antigen is directed against the heavy chain. The structures proposed for the rbAT, NBAT, D2 and 4F2hc proteins are remarkably similar and contain only one typical transmembrane helix [9,20,21]. An alternative structure with three additional transmembrane helices, situated in slightly hydrophobic stretches, was proposed for the NBAT protein [10,22]. The unusual structure for a transport protein, showing only one clearly detectable transmembrane helix, raised the question of the function of the second light-chain subunit of antigen 4F2, the primary structure of which is not known. A recent hypothetical model [5] proposed that the light chain is the transporter subunit and the heavy chain a regulatory or modulatory subunit. It was further proposed that

Abbreviations used: MeAIB, methylaminoisobutyric acid; BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid.

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X89225.

the transporters rbAT, NBAT and D2 also consist of two subunits.

Several major mammalian amino acid-transport systems are still not defined on a molecular basis. In particular, the well-characterized and ubiquitous amino acid-transport systems A and L [23] have not been cloned up until now [24]. In the brain, system L is the major transport system of the blood–brain barrier [25,26] and of glial cells [27–29]. Primary cultures of astroglial cells consume large amounts of branched-chain amino acids [30]. Accordingly, we found strong expression of system L-like amino acid-transport activity after injection into oocytes of polyadenylated RNA isolated from primary astroglia-rich cultures [31]. Greater expression of system L-like amino acid-transport activity was achieved with polyadenylated RNA from the glial cell line C6-BU-1 [32]. Therefore this cell line was used for further experiments.

Expression cloning using *Xenopus laevis* oocytes has been used successfully to clone new transport systems [33]. We have employed this technique to clone the system L amino acid transporter from C6-BU-1 rat glioma cells.

In the present paper we provide evidence that the rat antigen 4F2hc, which has a sequence identity of only 76% with human 4F2hc, is necessary for expression of system L-like amino acid-transport activity. An expression cloning attempt was started from polyadenylated RNA which induced system L-like transport when injected into oocytes. A clone was isolated and identified as the rat homologue of the cloned mouse and human antigen 4F2hc. Sense cRNA transcribed from this clone increased Na^+-independent isoleucine-transport activity in oocytes up to 100-fold. Co-injection of antisense cRNA from this clone or antisense oligonucleotides together with polyadenylated RNA from C6-BU-1 rat glioma cells completely suppressed system L-like transport activity in oocytes.

EXPERIMENTAL

Materials

L-[U-^{14}C]Isoleucine was purchased from Amersham Buchler, Braunschweig, Germany, L-[U-^{14}C]arginine from Biotrend Chemikalien, Cologne, Germany and [[^{35}S]thio]dATP from Hartmann Analytic, Braunschweig, Germany. Oligo(dT)–cellulose and 7 mG(5′)ppp(5′)G RNA cap structure analogue were purchased from New England Biolabs, Schwalbach, Germany. The Superscript plasmid system for the construction of cDNA libraries, restriction enzymes and RNA polymerases were from Life Technologies, Gaithersburg, MD, U.S.A., the Sequenase sequencing system and nylon filters (Hybond N) from Amersham Buchler and the 'Wizard' plasmid purification system and Erase-a-base system from Promega, Madison, WI, U.S.A. 2-Aminobicyclo[2,2,1]heptane-1-carboxylic acid (BCH), methylaminoisobutyric acid (MeAIB) and Sephacryl S-500 HR were purchased from Sigma, Deisenhofen, Germany, collagenase (EC 3.4.24.3; 0.6–0.8 units/mg from *Clostridium histolyticum*) from Serva, Heidelberg, Germany, gentamicin from Boehringer, Mannheim, Germany and Ultima Gold scintillation cocktail from Canberra Packard, Frankfurt, Germany. All other chemicals were of analytical grade and supplied by E. Merck, Darmstadt, Germany, Roth, Karlsruhe, Germany or Boehringer.

Isolation and fractionation of polyadenylated RNA

Rat glioma cells C6-BU-1 were cultured as described by Amano et al. [32]. Total RNA was isolated from the cultures by the method of Chomczynski and Sacchi [34]. Polyadenylated RNA was then isolated by two passages over an oligo(dT)–cellulose column [35] and size-fractionated by gel filtration on Sephacryl S-500 HR as described previously [31]. The fraction inducing the highest transport activity when injected into *Xenopus laevis* oocytes was used to construct the cDNA library.

Construction and screening of a C6-BU-1 rat glioma cell cDNA library

The cDNA library was constructed in the plasmid vector pSPORT 1 following the manufacturer's Superscript protocol. Size-selected polyadenylated RNA (5 μg) was reverse-transcribed using 'Superscript reverse transcriptase'; resulting cDNAs larger than 1.5 kb were selected and directionally cloned into the *Sal*I and *Not*I sites of the vector. The suspension of transformed bacteria was spread on agar plates to reach a density of 500 clones per dish. The library consisted of 2.3×10^4 independent clones. Replicas were made on nylon filters. The nylon filters were placed in sterile plastic tubes (50 ml volume) containing 10 ml of Luria–Bertani medium supplemented with 100 μg/ml ampicillin. Cultures were grown overnight at 37 °C in a roller drum. Plasmid pools from each culture were isolated by using the 'Wizard' plasmid purification system. For screening, plasmid DNA was linearized with *Not*I and transcribed *in vitro* with T7 RNA polymerase in the presence of a cap analogue. The protocol supplied with the polymerase was followed except that all nucleotides and the cap analogue were used at twice the concentration (1 mM) to increase the yield of cRNA. Template plasmids were removed by digestion with RNase-free DNase. The cRNA was purified by phenol/chloroform extraction and this was followed by precipitation with 0.5 vol. of 7.5 M ammonium acetate to remove unincorporated nucleotides. After determination of the amount of cRNA by measuring A_{260}, integrity of the transcript was checked by denaturing agarose-gel electrophoresis; 20 ng of cRNA was injected into each oocyte.

Oocytes and injections

Xenopus laevis females were generously supplied by Dr. P. Hausen (Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany). Oocytes (stages V and VI) were isolated as described [31] and allowed to recover overnight. They were microinjected with 10–50 nl of either water or polyadenylated RNA or cRNA in water at a concentration of 1 μg/μl, using a microinjection device (Bachhofer, Reutlingen, Germany). In the case of the lLAT clone, 20 ng of cRNA was routinely injected for transport measurements if not indicated otherwise.

Uptake measurements

For each determination, groups of seven healthy-looking mRNA- or water-injected oocytes were washed twice with 4 ml of OR2 buffer without $CaCl_2$ ($OR2^-$: 82.5 mM NaCl; 2.5 mM KCl; 1 mM $MgCl_2$; 1 mM Na_2HPO_4; 5 mM Hepes; final pH 7.6) before incubation at room temperature in a 5 ml polypropylene tube containing 70 μl of the same buffer supplemented with 7 kBq of radioactive substrate (e.g. isoleucine) and different amounts of unlabelled substrate. In Na^+-free buffer, NaCl was replaced by choline chloride, in K^+-free buffer, KCl was replaced by NaCl, in Cl^--free buffer NaCl, KCl and $MgCl_2$ were replaced by sodium gluconate, KH_2PO_4 and $MgSO_4$ respectively. Transport was stopped after different times by washing oocytes three times with 4 ml of ice-cold $OR2^-$ buffer. Single oocytes were placed into scintillation vials and lysed by addition of 200 μl of 10% SDS. After lysis 3 ml of scintillation fluid was added and radioactivity determined in a liquid-scintillation counter (LKB, Freiburg, Germany). All transport activities are given as pmol of

substrate/h per oocyte independent of the length of the incubation.

Hybrid-depletion experiments

Antisense cRNA was synthesized *in vitro* from plasmids containing the cDNA encoding the amino acid transporter rbAT [13] (kindly provided by Professor H. Murer, Institute of Physiology, University of Zürich, Zürich, Switzerland) or the chloride channel β-ClC2 [36] (kindly provided by Professor T. Jentsch, ZMNH, University of Hamburg, Hamburg, Germany). Sense cRNA (1 mg/ml) or polyadenylated RNA (1 mg/ml) was mixed with an equal amount of antisense cRNA before injection of 50 nl into each oocyte. In the case of oligonucleotides we followed the protocol of Snutch et al. [37]. For this, polyadenylated RNA (2 mg/ml) was heated to 65 °C for 3 min and then mixed with oligonucleotide solution (2 mg/ml) followed by incubation at 42 °C for 10 min. After cooling on ice, 50 nl of the mixture was immediately injected into each oocyte to avoid degradation of the cRNA. The following oligonucleotides were used in the experiments: (i) oligonucleotide 1, AAA GAT GTG GAG CTG AAC GAG (bases 148–168 of the cDNA sequence) and the corresponding antisense oligonucleotide; (ii) oligonucleotide 2, TGA TCC CGA TTT AGG CTC CC (bases 639–658) and the corresponding antisense oligonucleotide; (iii) oligonucleotide 3, AAG TCC TGT AAG CCT CAA CA (bases 1302–1321).

Sequencing of the cDNA clone

The nucleotide sequence of the transporter cDNA clone was determined by the dideoxy chain-termination method [38] using the Sequenase DNA sequencing kit. Both strands of the clone were sequenced using M13/pUC and T7 sequencing primers. Subclones containing progressive unidirectional deletions were generated using exonuclease III [39] as outlined in the Erase-a-base protocol by the manufacturer. Gaps between the nested deletions were sequenced using synthetic oligonucleotides derived from adjacent sequences as primers.

Calculations

Standard deviations are given for all values. Gauss' law of error propagation was applied when values had to be subtracted. Each experiment presented was performed at least twice with similar results.

RESULTS

Isolation of a cDNA responsible for expression of Na^+-independent isoleucine transport

The cDNA library of 2.3×10^4 independent clones was subdivided into plates containing 400–500 colonies each. Each of these pools was transcribed *in vitro* into capped cRNA and injected into oocytes. One pool reproducibly elicited a 1.5-fold increase in Na^+-independent isoleucine transport after expression in *Xenopus* oocytes. The signal increased with further subfractionation (Table 1), leading to a single clone (lLAT). cRNA prepared from this clone induced an almost 100-fold increase in Na^+-independent isoleucine-transport activity when 20 ng of cRNA was injected per oocyte.

Characterization of the cDNA responsible for expression of Na^+-independent isoleucine transport

Sense cRNA transcribed from lLAT cDNA strongly induced Na^+-independent isoleucine transport when injected into oocytes.

Table 1 Isolation of the lLAT cDNA clone

Sense cRNA from cDNA-library subfractions generated by *in vitro* transcription was injected into each oocyte. Transport activity was determined after 5 days of expression. Isoleucine uptake was measured for 1 h. The mean uptake by ten oocytes is given for each pool.

Pool size (colonies)	Amount cRNA injected (ng)	Transport activity (pmol of isoleucine/h per oocyte)
0	0	0.62 ± 0.13
Approx. 500	50	0.95 ± 0.09
Approx. 150	50	6.4 ± 0.7
20	20	12.4 ± 2.1
20	50	21.9 ± 3.1
1	20	50.7 ± 9.6

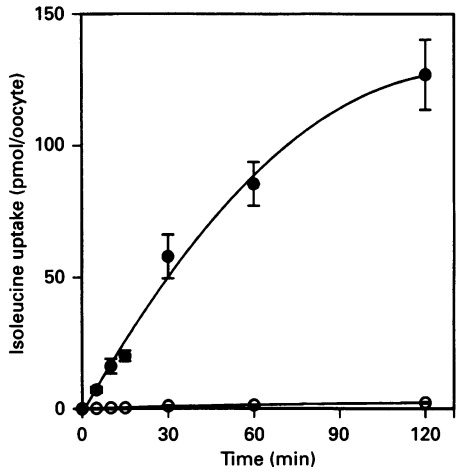

Figure 1 Uptake of labelled isoleucine by *Xenopus laevis* oocytes expressing lLAT

Each oocyte was injected with 20 ng of cRNA (1 μg/μl) (●) or 20 nl of water (○). After expression for 4 days, oocytes were washed and incubated for the indicated times in Na^+-free transport buffer containing 100 μM [^{14}C]isoleucine. Transport was stopped by three washing steps with ice-cold buffer. Each point represents the mean ± S.D. for seven oocytes.

Uptake was proportional to time for approx. 30 min (Figure 1). After 2 h, 125 pmol of isoleucine had been taken up by the oocytes. Assuming a volume of 500 nl/oocyte, this corresponds to a 2.5-fold accumulation. Isoleucine is metabolized only to a small extent in 2 h [31]. Expression of the transporter increased steadily for 4 days, suggesting that the cRNA was very stable (Figure 2). The K_m value for isoleucine transport was determined (Figure 3). When the data were plotted according to Eadie–Hofstee, two components became visible (Figure 3, inset). The low- and high-affinity components corresponded to K_m values of 175 μM (150 μM determined with a separate batch of oocytes) and 24 μM (in both oocyte batches) respectively.

Transport activity was independent of the ionic composition of the transport buffer. In the presence of Na^+ ions, a transport activity of 114 ± 5 pmol/h per oocyte was determined. Replacing NaCl by choline chloride resulted in a transport activity of 117 ± 8 pmol/h per oocyte; replacing K^+ ions by Na^+ ions resulted in a transport activity of 120 ± 4 pmol/h per oocyte and when Cl^- ions were replaced by gluconate ions a transport activity of 124 ± 14 pmol/h per oocyte was determined.

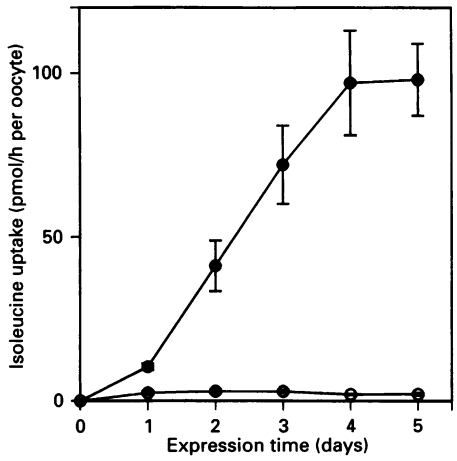

Figure 2 Time course of expression of ILAT cRNA in *Xenopus laevis* oocytes

Each oocyte was injected with 20 ng of cRNA (1 μg/μl) (●) or 20 nl of water (○) and transport activity was determined daily. Uptake was initiated at room temperature by the addition of transport buffer containing 100 μM labelled isoleucine and stopped after 20 min by washing with ice-cold transport buffer. Each point represents the mean ± S.D. for seven oocytes.

To investigate the substrate specificity of the transport process, different unlabelled amino acids (10 mM) were added to the transport assay at 100-fold excess (Table 2). Except for taurine and proline, all neutral amino acids strongly inhibited isoleucine transport. MeAIB, a representative methylated amino acid, was not recognized by the transporter. Medium-sized amino acids were preferentially taken up by the expressed transporter. Large

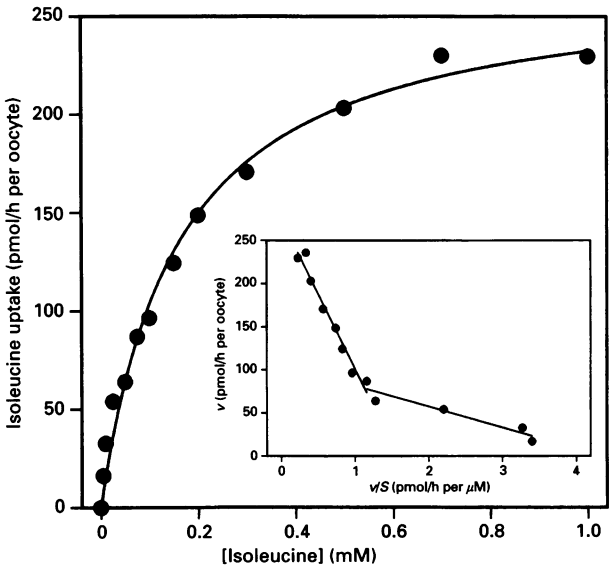

Figure 3 Determination of the K_m value for the expressed transporter

Each oocyte was injected with 20 ng of cRNA (1 μg/μl) or 20 nl of water. After expression for 4 days, oocytes were washed and incubated at room temperature for 20 min in Na^+-free transport buffer containing [^{14}C]isoleucine and unlabelled isoleucine at the indicated concentrations. Transport was stopped by three washing steps with ice-cold buffer. Each point represents the mean of values obtained with seven oocytes; transport activity of water-injected oocytes was determined at all isoleucine concentrations and subtracted from the transport activity achieved in cRNA-injected oocytes. Inset: Eadie–Hofstee transformation of the data.

Table 2 Substrate specificity of the expressed transporter

Each oocyte was injected with 20 ng of cRNA (1 μg/μl) or 20 nl of water. After expression for 4 days, oocytes were washed and incubated for 20 min in Na^+-free transport buffer containing 100 μM [^{14}C]isoleucine and the indicated amino acid (10 mM) . Transport was stopped by three washing steps with ice-cold buffer. Each value is the mean ± S.D. determined in seven oocytes. The transport activity of water-injected oocytes has already been subtracted. During the time course of the experiment (10 h) the rate of transport of the uninhibited control increased from 55.6 pmol/h per oocyte to 95.4 pmol/h per oocyte, therefore data are given as a percentage of the respective control.

Added amino acid (10 mM)	Transport activity (%)
None	100.0 ± 10.2
Taurine	128.0 ± 15.5
Proline	103.3 ± 7.4
Glutamate	98.8 ± 12.9
MeAIB	93.2 ± 8.6
BCH	25.5 ± 2.7
Glycine	23.2 ± 1.7
Lysine	14.6 ± 2.0
Arginine	11.7 ± 2.0
Tryptophan	6.9 ± 1.6
Valine	5.8 ± 0.3
Alanine	5.0 ± 1.2
Serine	4.3 ± 0.5
Glutamine	3.9 ± 1.0
Methionine	3.4 ± 1.2
Threonine	3.0 ± 1.1
Citrulline	2.6 ± 0.9
Phenylalanine	2.5 ± 0.5
Leucine	2.4 ± 0.8
Histidine	2.0 ± 0.8

Table 3 Arginine-transport activity induced by expression of ILAT and competition by isoleucine

Each oocyte was injected with 20 ng of cRNA. After 4 days of expression oocytes were washed three times in transport buffer with or without Na^+. Mean ± S.D. of seven oocytes is given for each determination. Transport was initiated by addition of transport buffer with or without Na^+ containing 100 μM labelled arginine and 10 mM unlabelled isoleucine if indicated and stopped after 20 min by washing with ice-cold transport buffer. Values in parentheses are percentages.

Conditions	Transport activity (pmol of arginine/h per oocyte)
$-Na^+$	138.1 ± 14.5 (100)
$-Na^+ + 10$ mM isoleucine	73.4 ± 6.8 (53)
$+Na^+$	124.5 ± 5.2 (91)
$+Na^+ + 10$ mM isoleucine	57.3 ± 2.7 (42)

substrates such as tryptophan or the model substrate BCH inhibited isoleucine transport less than medium-sized amino acids. Surprisingly, cationic amino acids also exerted a strong inhibition of isoleucine transport when added at 100-fold excess. As two kinetic components were observed in the plot of transport velocity against substrate concentration, competition assays were performed at different substrate concentrations. At 10 μM isoleucine, transport was inhibited by 70% after addition of 1 mM BCH and by 85% after addition of lysine at the same concentration. At 200 μM isoleucine, addition of 20 mM BCH inhibited transport by 62% and addition of 20 mM lysine resulted in 76% inhibition. Cationic amino acids were not only inhibitors of isoleucine transport, but also substrates for the transport process itself (Table 3). Inhibition of arginine transport by

Table 4 Hybrid-depletion experiments with antisense cRNA and oligonucleotides

Sense cRNA or polyadenylated RNA was mixed with antisense cRNA or oligonucleotides before injection into oocytes. After 4 days of expression transport activity was determined. Mean transport activity of seven oocytes is given for each determination. Transport was initiated by addition of transport buffer containing 100 μM labelled isoleucine and stopped after 1 h by washing with ice-cold transport buffer. Data were taken from different oocyte batches and therefore normalized to the uninhibited control. Only experiments with high expression were used. Numbers of oligonucleotides are the same as given in the Experimental section.

Poly(A)$^+$ RNA or sense cRNA	Antisense cRNA or oligonucleotide	Transport activity (%)
C6-BU-1 (20/30/40 ng)	–	100.0
C6-BU-1 (20 ng)	Anti lLAT (20 ng)	0.0
C6-BU-1 (40 ng)	Anti lLAT (10 ng)	5.2
C6-BU-1 (20 ng)	Anti β-ClC2 (20 ng)	103.0
C6-BU-1 (30 ng)	Antisense oligo 1 (22 ng)	4.0
C6-BU-1 (30 ng)	Sense oligo 1 (22 ng)	122.0
C6-BU-1 (30 ng)	Antisense oligo 2 (22 ng)	0.0
lLAT (10/33 ng)	–	100.0
lLAT (10 ng)	Anti lLAT (10 ng)	4.5
lLAT (10 ng)	Anti rbAT (10 ng)	69.0
lLAT (33 ng)	Antisense oligo 2 (20 ng)	7.1
lLAT (33 ng)	Antisense oligo 3 (20 ng)	8.9

isoleucine was less strong than vice versa and did not depend on the presence of Na^+ (Table 3). The inhibition of isoleucine transport by cationic amino acids was not seen during the expression studies with polyadenylated RNA from C6-BU-1 cells [31], which was used as the source of the cDNA library. We repeated these experiments and still could not detect inhibition of Na^+-independent isoleucine transport by arginine or lysine with oocytes expressing polyadenylated RNA from C6-BU-1 rat glioma cells (results not shown).

Hybrid-depletion experiments

As we detected a difference between transport characteristics induced by polyadenylated RNA from rat glioma cells and the cRNA from the lLAT clone isolated from this source, we tested whether lLAT mRNA was responsible for system L-like activity in the polyadenylated RNA pool from C6-BU-1 rat glioma cells. For this a mixture of equal amounts of antisense cRNA from lLAT and polyadenylated RNA from C6-BU-1 cells was injected into oocytes. After expression for 4 days, no Na^+-independent isoleucine-transport activity could be detected (Table 4). To rule out non-specific competition effects of antisense cRNA, for example at ribosomes, an unrelated antisense cRNA corresponding to the chloride channel ClC-2 [36] was mixed with polyadenylated RNA from C6-BU-1 cells. Expression of isoleucine transport remained unaltered (Table 4). When an equal mixture of sense and antisense lLAT cRNA was injected into oocytes, no expression of Na^+-independent isoleucine-transport activity could be detected (Table 4), demonstrating the efficacy of the hybrid-depletion assay. When antisense cRNA, transcribed from the weakly related neutral amino acid transporter clone rbAT, was injected together with sense cRNA from lLAT, expression of isoleucine transport was only partially inhibited. In addition to hybrid depletion by antisense cRNA we also used several antisense oligonucleotides to inhibit translation of lLAT in the polyadenylated RNA pool from C6-BU-1 rat glioma cells. This method has been successfully used to suppress translation of specific mRNA molecules (see, for instance, ref. [37]). Both antisense oligonucleotides strongly suppressed development of Na^+-independent isoleucine-transport activity. A corresponding sense oligonucleotide did not suppress transport activity (Table 4). In oocytes injected solely with sense or antisense oligonucleotides, transport rates were similar to those of water-injected oocytes (not shown).

DNA and amino acid sequence

The sequence of the cDNA clone was determined (Figure 4). The cDNA was 1907 bp long. The poly(A) tail consisted of 43 bases with a preceding polyadenylation signal. The largest open reading frame consisted of 1581 bp. It uses the first ATG which is surrounded by a reasonable consensus sequence for initiation codons [40]. Comparison with the EMBL database revealed that the cloned transporter was the rat homologue of the heavy chain of the mouse and human antigen 4F2 [20,21,41]. However, the lLAT amino acid sequence is only 76% identical with the human 4F2hc, yet it is 91% identical with the mouse sequence, which has not been functionally characterized (Figure 5). Similarly to the other two sequences, only one transmembrane helix is predicted by hydropathy analysis (Figure 4). No signal sequence was found at the N-terminus. The protein sequence contains eight putative N-glycosylation sites, all located on the C-terminal side of the transmembrane helix (Figure 4), suggesting a cytosolic N-terminus and an extracellular C-terminus.

DISCUSSION

Progress has been made recently in the molecular identification of several mammalian amino acid-transport systems. In particular, expression cloning has led to the discovery of several transporters, which had not been previously purified [33]. Recently we found strong expression of system L-like amino acid-transport activity after injection into oocytes of polyadenylated RNA isolated from C6-BU-1 cells or primary astroglia-rich cultures [31]. Using expression cloning, we identified a clone that produced high expression of Na^+-independent isoleucine-transport activity. To our surprise this clone could be identified as the heavy chain of the rat 4F2 surface antigen (r4F2hc). We suggest the more functional name lLAT (*l*inked to system *L a*mino acid *t*ransport) for this clone, following a proposal of Christensen et al. [42]. Several lines of evidence strongly suggest that r4F2hc is necessary for expression of system L-like amino acid-transport activity in C6-BU-1 rat glioma cells. (i) Expression cloning started from a size-selected polyadenylated RNA pool which strongly induced system L-like transport activity when expressed in *Xenopus laevis* oocytes. (ii) cRNA transcribed from the isolated clone induced a 100-fold stimulation of Na^+-independent isoleucine transport after expression in oocytes. Isoleucine transport was strongly inhibited by BCH, an amino acid analogue that is fairly specific for system L [43]. (iii) Antisense cRNA and antisense oligonucleotides strongly suppressed expression of system L-like transport activity when first hybridized to polyadenylated RNA from C6-BU-1 rat glioma cells and then injected into oocytes. The last argument is most important, since we detected differences in substrate specificity between the transport activity induced in oocytes by polyadenylated RNA from C6-BU-1 rat glioma cells and cRNA of the isolated clone. When polyadenylated RNA from C6-BU-1 cells was expressed, isoleucine transport was not inhibited by lysine or arginine as expected for system L-like transport activity. Isoleucine transport induced by the isolated clone, however, was strongly inhibited by arginine and lysine.

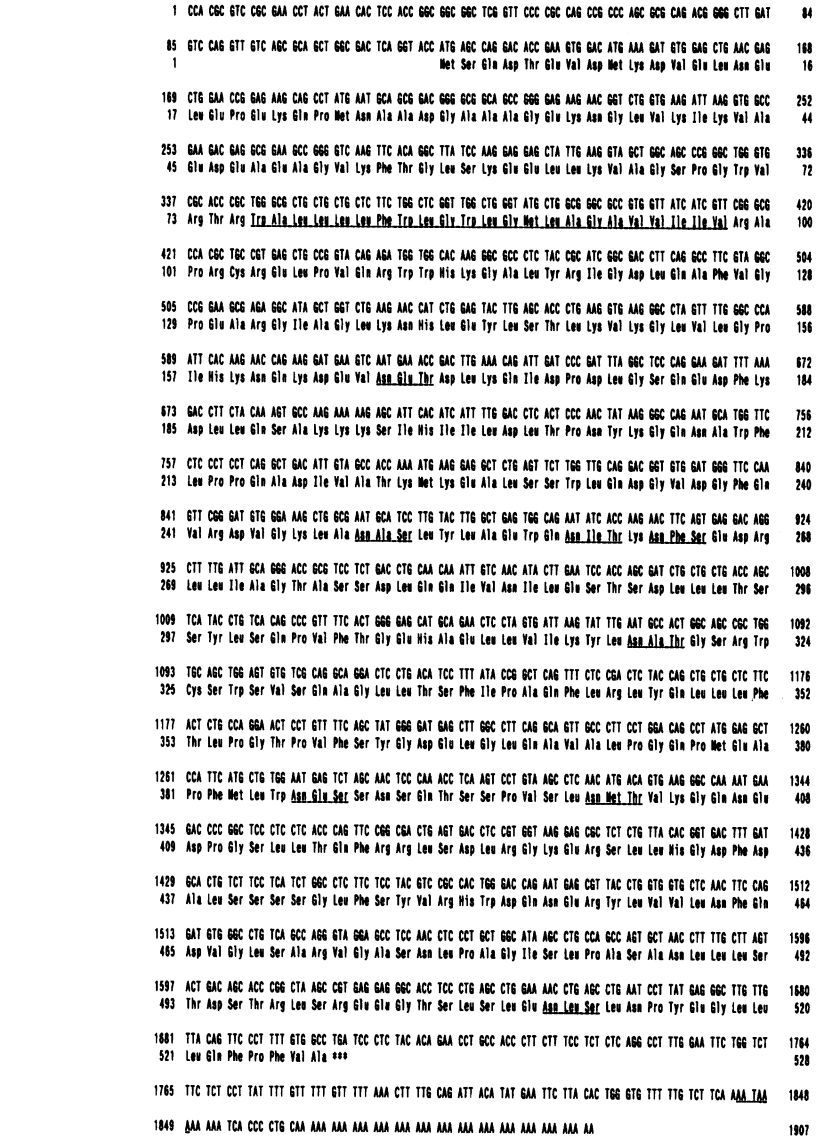

Figure 4 Nucleotide sequence and deduced amino acid sequence of the lLAT cDNA clone

Nucleotides are numbered in the 5′ to 3′ direction. The start codon of the longest open reading frame lies within a consensus initiation sequence [40]. The putative single transmembrane helix (amino acid residues 76–98), the eight possible N-glycosylation sites [Asn-X-(Ser/Thr)] and the possible polyadenylation signal (AATAAA) are underlined.

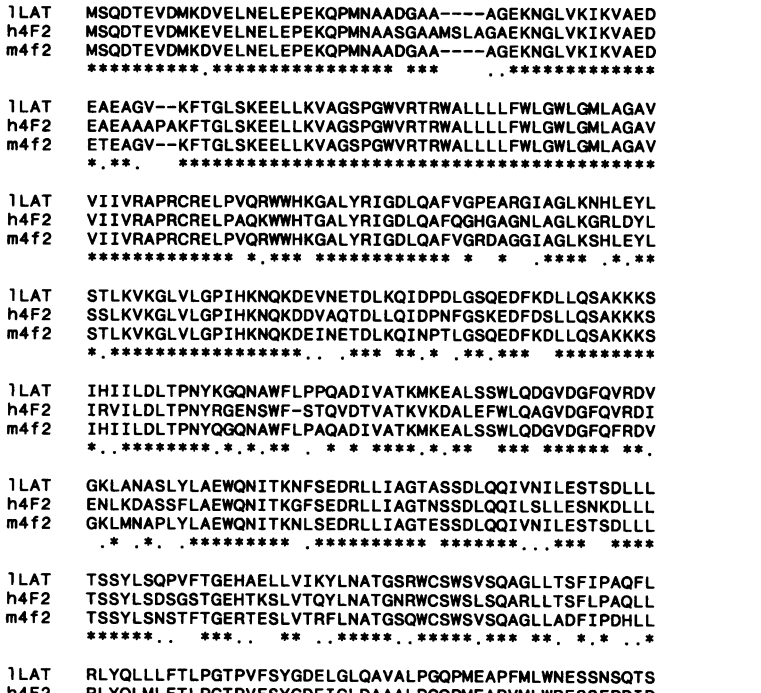

Figure 5 Alignment of the mouse, rat and human 4F2hc amino acid sequences

Comparison of the deduced amino acid sequences of rat 4F2hc (lLAT), mouse 4F2hc (m4F2) and human 4F2hc (h4F2). Multiple sequence alignment was performed by using the CLUSTAL V program. Identical amino acids in all three proteins are indicated by asterisks, and conservative replacements by dots.

Since all antisense experiments were unambiguous, the conclusion is irresistible that lLAT is necessary for expression of system L-like transport activity in C6-BU-1 glioma cells. We are aware of the fact that the amino acid-transport systems of C6-BU-1 cells, a cell line of glial origin, have not been studied thoroughly in cell culture. Primary cultures of glial cells, which consist of several cell types, have been studied in much more detail and strong system L-like transport activity could be demonstrated in these cultures [27–29]. Further studies are necessary to correlate 4F2hc activity with transport activity in several well-characterized cell types. In this respect it is interesting to note that polyadenylated RNA from CHO cells, when injected into oocytes, induced Na^+-independent leucine transport which could also be inhibited by lysine and arginine [44]. The Na^+-independent transport of neutral amino acids has been characterized thoroughly in these cells and is solely due to system L [45].

The 4F2hc antigen has been cloned from mouse (m4F2hc) [41] and man (h4F2hc) [20,21]. The h4F2hc has recently been proposed to be involved in amino acid transport [13,14]. Expression in *Xenopus* oocytes was very low and transport characteristics resembled those of system y^+L [15]: e.g. neutral amino acids were transported at a high rate in the presence of Na^+, but hardly at all in its absence; inhibition of arginine transport by neutral amino acids also depended on the presence of Na^+. No comparison with transport activity induced by polyadenylated RNA from the source of the clone was performed in the expression studies. In contrast with the low expression of the human clone, lLAT (rat 4F2hc) cRNA induced a 100-fold stimulation of transport activity when injected into oocytes. With lLAT we could not detect any effect of Na^+ on transport of isoleucine or on the inhibition of arginine transport by isoleucine.

This difference in transport characteristics between human and rat 4F2hc could be due to the low expression of the human cRNA [13,14] or be a consequence of the 24% diversity of amino acid residues between the two clones. This is rather high in view of the fact that homologous proteins of rat and man usually have only about 5–10% different amino acid residues. It is also possible that system y^+L has not been identified up to now, because antisense oligonucleotides against 4F2hc could not suppress y^+L-like transport induced by rat jejunal polyadenylated RNA [17].

In contrast with expression of system L-like transport by polyadenylated RNA from C6-BU-1 cells, transport characteristics induced by cRNA of the isolated clone, when expressed in oocytes, resembled those of system $b^{0,+}$ [46]. cDNA clones inducing $b^{0,+}$-like transport activity in *Xenopus* oocytes have recently been isolated by several groups [9–11]. These clones, named rbAT, NBAT and D2, show significant similarity to the lLAT clone (27% identical amino acid residues). When expressed in oocytes the major difference between lLAT and rbAT, NBAT and D2 transport activity is the sensitivity to inhibition by BCH. Only weak competition was detected in the case of NBAT and rbAT, whereas strong competition was detected with lLAT, as expected for system L-like transport. Common to lLAT (4F2hc), rbAT, NBAT and D2 proteins is the structure predicted by hydropathy analysis. Only one typical transmembrane helix is found in all cases. This led to the proposal that the members of this family are not transporters by themselves, but activators of endogenous oocyte transporters. The observed discrepancy in substrate specificity of the transport activities elicited by expression of polyadenylated RNA from glioma cells on the one hand and cRNA from the isolated clone on the other could be explained by activation of such an endogenous transporter. Nevertheless, lLAT was necessary for expression of system L-like transport activity, as indicated by the antisense experiments. Interesting in this context is the fact that immunoprecipitation of the 4F2hc resulted in the coprecipitation of a smaller protein (called the light chain) of M_r 41000, which is bound to the heavy chain by a disulphide bridge [18]. It has been hypothesized that this small subunit could be the transporter subunit [5]. A different model with four transmembrane helices has been proposed for NBAT [10]. Recently, tentative evidence in favour of this model has been obtained by using epitope-specific antibodies [22]. Thus it is still possible that these proteins are themselves the transporters.

The unexpected finding of two transport components with different affinities is especially interesting with respect to system L. System L has been subdivided into subtypes L_1 and L_2, which appear at different ages in primary cultures of hepatocytes [47]. The subtypes are characterized by their different K_m values. As we expressed only one cDNA in oocytes, it would appear possible that one transport system can phenotypically display two K_m values. Two kinetic components were also observed after expression of cRNA for system y^+ in *Xenopus* oocytes [48].

The evidence presented here that the 4F2hc surface antigen is intimately involved in system L-like transport activity fits with the following known properties of these proteins. (i) Antigen 4F2hc has a ubiquitous tissue distribution and also occurs in the brain [41,49], as expected for system L. (ii) It was first detected on lymphocytes. System L is the major amino acid-transport system of these cells [50]. (iii) Expression of antigen 4F2hc is stimulated by treatment of lymphocytes with phorbol esters [51]; similarly, expression of system L is stimulated by treatment of chronic leukaemic B-lymphocytes with phorbol esters [52].

All Na^+-independent amino acid transporters can now be grouped into two families: (1) the lLAT(4F2hc)/NBAT/rbAT/D2 family of cDNAs which express transport systems with broad substrate specificity characteristic of systems L, $b^{0,+}$ and perhaps y^+L, and (2) the CAT family of cDNAs encoding transporters for cationic amino acids with the characteristics of system y^+.

The most important unresolved question, whether the lLAT protein is the transporter itself or a regulator, is currently being addressed in our laboratory.

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