

Expression of the surface antigen 4F2hc affects system-L-like neutral-amino-acid-transport activity in mammalian cells

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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 of non-epithelial cells. By expression cloning we have recently demonstrated that the surface antigen 4F2hc (CD98) is a necessary component for expression of system-L-like amino acid-transport activity in C6-BU-1 rat glioma cells [Bröer, Bröer and Hamprecht (1995) *Biochem. J.* **312**, 863–870]. 4F2hc mRNA was detected in CHO cells, COS cells, activated lymphocytes isolated from mouse spleen and primary cultures of astrocytes. In all these cell types,

Na⁺-independent isoleucine transport was mediated by system L. No contribution of system y⁺L to isoleucine or arginine transport was detected in C6-BU-1 cells. In lymphocytes, both system-L-like amino acid-transport activity and 4F2hc mRNA levels increased after treatment with phorbol ester plus ionomycin. Antisense oligonucleotides caused modest inhibition of Na⁺-independent isoleucine transport in C6-BU-1 cells and primary cultures of astroglial cells, whereas arginine transport was unaffected. Overexpression of 4F2hc cDNA in CHO cells resulted in an increase in Na⁺-independent isoleucine transport.

INTRODUCTION

Mammalian cells possess a variety of amino acid-transport systems with overlapping substrate specificity [1–3]. The different systems are generally categorized by their dependence on Na⁺ ions, specificity for substrates and their analogues, and a few other criteria [4]. Several amino acid transporters have been identified on a molecular basis in recent years [3]. Four families of proteins can so far be discriminated. One is the family of Na⁺- and Cl⁻-dependent neurotransmitter transporters [5]; another family consists of transporters similar to the brain glutamate transporters [6], which also includes transporters with characteristics similar to system ASC [5,7,8]. The third is comprised of transporters for cationic amino acids, named CAT, with characteristics of system y⁺ [9–11]. The last group consists of proteins belonging to the rbAT/NBAT/D2, 4F2hc family [12–17]. The cDNAs of all members of this family have been isolated by expression cloning with *Xenopus laevis* oocytes and show unusual features for transport proteins: (i) analysis of the hydropathy plots indicates only one transmembrane helix; (ii) cRNA of all members can be efficiently expressed in *Xenopus laevis* oocytes but DNA expression in cultured mammalian cells does not result in elevated amino acid transport [14,18]. However, the rbAT protein has been shown to be necessary for uptake of lysine and cystine in kidney [19] and OK cells [20]. Therefore the proteins of this family are usually considered to be activators of amino acid transport, in the case of the rbAT/NBAT/D2 protein amino acid-transport system b^{0,+} [18]. This process transports neutral and basic amino acids independently of Na⁺ [21]. In contrast, there is considerable debate on the transport system activated by the 4F2hc protein, which has an amino acid sequence similarity of 30% to that of the rbAT/NBAT/D2 protein. The 4F2hc protein has been implicated in Na⁺/Ca²⁺ exchange [22], in system-y⁺-related amino acid transport [12,16], in system-y⁺L-like amino acid transport [23] and finally in system-L-like amino acid-transport activity [17]. Most of these data have been obtained in experiments using *Xenopus laevis* oocytes. Analysis in this system is hampered because the 4F2hc and rbAT/

NBAT/D2 proteins are probably not the transport proteins themselves [18], and endogenous proteins might interfere with their expression in *Xenopus laevis* oocytes [24]. It has been shown by immunoprecipitation experiments that a heavy and a light chain exist for the 4F2 surface antigen [25,26]. Similar results have recently been presented for the NBAT protein [24]. The possible effect of this second subunit on transport has not so far been determined. Hybrid depletion experiments were performed in *Xenopus laevis* oocytes to identify the amino acid-transport system that is activated by 4F2hc. In C6-BU-1 cells, expression of the whole mRNA pool in oocytes led to the induction of system-L-like amino acid-transport activity, which was suppressed by antisense oligonucleotides against 4F2hc [17,27]. Expression of the whole mRNA pool from human chorioncarcinoma cells in oocytes led to induction of system-y⁺L-like amino acid transport [23], which could also be suppressed by 4F2hc antisense oligonucleotides. Contrasting observations were made by Yao et al. [28], who found that y⁺L-like amino acid transport expressed by mRNA from rat jejunum was not inhibited by antisense oligonucleotides directed against 4F2hc. System y⁺L is defined as a Na⁺-dependent transport process for neutral amino acids and Na⁺-independent transport process for cationic amino acids [29]. The system has therefore been regarded as a high-affinity variant of system y⁺ with a lower substrate specificity. However, there is no sequence homology between the CAT sequences and the 4F2hc sequences.

In contrast with the still ill-defined y⁺L system, system-L-like amino acid transport has been well characterized in a variety of cell types. System L is expressed in all non-epithelial cells investigated so far. In epithelial cells it is restricted to the basolateral side [30]. Usually system L is considered to be a constitutive transport system. However, there is some regulation by the availability of leucine [31]. Lymphocytes, in contrast, possess an inducible system L, which can be activated by phorbol esters [32,33]. The regulation of the 4F2hc gene and protein in lymphocytes has been well characterized [34–36] and also shown to be induced by phorbol esters.

In this study we present evidence that expression of the 4F2hc

surface antigen is linked to amino acid transport by system L in mammalian cells. The mRNA is present in cells known to express system L. Transport activity and 4F2hc expression increased simultaneously in phorbol ester-activated lymphocytes. Antisense experiments and overexpression of 4F2hc resulted in a decrease and increase respectively of Na⁺-independent neutral amino acid transport.

EXPERIMENTAL

Materials

L-[U-¹⁴C]isoleucine, L-[4,5-³H]isoleucine and the Sequenase sequencing system were purchased from Amersham Buchler, Braunschweig, Germany; L-[U-¹⁴C]arginine was from Biotrend Chemikalien, Cologne, Germany and [³⁵S]dATP from Hartmann Analytic, Braunschweig, Germany. Oligo(dT)-cellulose was purchased from New England Biolabs, Schwalbach, Germany. Superscript reverse transcriptase, restriction enzymes and RNA polymerases were from Life Technologies, Eggenstein, Germany, the SureClone Ligation Kit from Pharmacia, Uppsala, Sweden, and RNasin and the Wizard plasmid purification system from Promega, Madison, WI, U.S.A. 5-Bromo-2'-deoxyuridine, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) and α -(methylamino)isobutyric acid (MeAIB) were purchased from Sigma, Deisenhofen, Germany, and Ultima Gold scintillation cocktail from Canberra Packard, Frankfurt, Germany. PMA and ionomycin were purchased from Calbiochem, Bad Soden, Germany. All other chemicals were of analytical grade and supplied by E. Merck, Darmstadt, Germany, Roth, Karlsruhe, Germany or Boehringer, Mannheim, Germany.

Cell culture

Rat glioma cells C6-BU-1 were seeded on 60 mm culture dishes (0.25×10^6 cells in 5 ml of medium) and cultivated in 90% Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS)/0.1 mM 5-bromo-2'-deoxyuridine in a humidified atmosphere of 10% CO₂ in air until reaching a density of 4×10^6 cells after 4 days. CHO cells and COS-7 cells were grown under the same conditions as C6-BU-1 cells except that 5-bromo-2'-deoxyuridine was omitted and a nucleoside mixture (final concentrations 3.5 mg/l each adenosine, cytidine, uridine and guanosine and 1.2 mg/l thymidine) was added to the growth medium for CHO cells. Astroglia-rich primary cultures, derived from brains of neonatal Wistar rats, were prepared and cultured as described by Hamprecht and Löffler [37]. For the preparation of lymphocytes, spleens from adult NMRI mice were aseptically removed and a suspension of splenic cells was generated, which was centrifuged through Ficoll-Paque according to the manufacturer's protocol (Pharmacia). Recovered resting lymphocytes were cultured at a density of 10^6 cells/ml in DMEM with 5% FCS containing 50 μ M 2-mercaptoethanol. The FCS was dialysed three times for 5 h against a 10-fold volume of PBS before use. Cells were stimulated by the addition of 5 ng/ml PMA and 0.5 μ g/ml ionomycin.

Transport experiments

For uptake experiments cells were grown to a density of $4 \times 10^6/60$ mm culture dish in a humidified atmosphere of 10% CO₂ in air at 37 °C in DMEM, supplemented with 10% FCS. All experiments were performed at 37 °C. Growth medium was aspirated, and cells were washed three times with 3 ml of Hanks buffered salt solution (HBSS: 136.6 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO₃, 2.7 mM Na₂HPO₄, 1 mM CaCl₂, 0.5 mM

MgCl₂, 0.44 mM KH₂PO₄, 0.41 mM MgSO₄, pH 7.8). To initiate transport, 3 ml of HBSS containing [¹⁴C]isoleucine and unlabelled isoleucine at a final concentration of 100 μ M and a specific radioactivity of 500 d.p.m./nmol was added to the cells. After 15 s, transport was stopped by aspirating the transport buffer followed by three washing cycles with 3 ml of ice-cold HBSS. When Na⁺-independent transport was determined, NaCl was replaced by choline chloride, and sodium phosphate was replaced by potassium phosphate in all buffers. Cells were lysed by addition of 1 ml of 0.1 M HCl. A 900 μ l aliquot of the resulting suspension was mixed with 3 ml of scintillation cocktail, and radioactivity was determined in a Rackbeta 1214 scintillation counter (LKB, Gräfelfing, Germany). A 100 μ l aliquot was used for protein determination using the Bio-Rad Protein assay (Bio-Rad Laboratories, München, Germany). For transport experiments with lymphocytes silicone oil centrifugation was used. Briefly, aliquots containing 1.5×10^6 lymphocytes were washed once with 1 ml of HBSS and then resuspended in 100 μ l of HBSS. To initiate transport the whole suspension was added to 10 μ l of $11 \times$ transport buffer (HBSS containing 1.1 mM isoleucine at a specific radioactivity of 6000 d.p.m./nmol). After an incubation time of 30 s, 100 μ l aliquots were subjected to silicone oil centrifugation as described elsewhere [38].

Transient expression of 4F2hc cDNA

To perform functional studies with 4F2hc cDNA a 1.8 kb *Eco*RI fragment containing the complete coding sequence and part of the pSPORT multicloning site was excised and cloned into the expression vector pCS2+; this construct was named pB19/3. Transient expression was achieved by electroporation of CHO cells. Briefly, the cells were harvested by trypsinization, washed twice in PBS, centrifuged, and resuspended in a solution, the composition of which mimicked the intracellular environment [39]. Aliquots containing 4×10^6 viable cells in 300 μ l were mixed with 10 μ g of the recombinant plasmid pB19/3 or with the same amount of pCS2+ or pSV- β -galactosidase and transferred to a 0.4 cm electroporation cuvette. The cuvette was chilled on ice for 1 min followed by electroporation with a Gene Pulser (Bio-Rad), using a voltage pulse of 300 V, a capacitance of 960 μ F and no pulse controller. Alternatively a voltage pulse of 1.6 kV and a capacitance of 25 μ F at a resistance of 100 Ω was used. After the pulse, cells from each cuvette were placed on ice for 10 min, then diluted into 5 ml of DMEM/Ham's F-12 (1:1) containing 2.5% FCS and 2.5% newborn calf serum and seeded in 60 mm culture dishes. Uptake experiments were performed between 48 and 72 h after electroporation. Transfection efficiency was checked for each electroporation protocol by using *in situ* detection of β -galactosidase activity [40].

Oligonucleotides and plasmids

For the cloning and detection of homologues of the 4F2hc and rbAT/NBAT/D2 cDNAs by reverse transcriptase (RT)-PCR, two degenerate oligonucleotides were constructed that were complementary to conserved regions present in both families. Oligonucleotide lat7s had the sequence 5'-(AG)(AC)C GGT GTG GA(CT)GG(AGCT) TTC-3' and oligonucleotide lat8a had the sequence 5'-GGA GTT CCT GG(AGCT)A(AG) (AGCT) GT(AG) AA-3'. Oligonucleotide lat7s corresponded to nt 820–837 and lat8a to nt 1174–1193 of the published rat 4F2hc sequence [17]. In the antisense experiments a phosphorothioate oligonucleotide with the sequence 5'-GGG AGC CTA AAT CCG GAT-3' was used, corresponding to nt 641–659. The β -galactosidase vector was purchased from Promega. Plasmid pCS2+ was a gift from Dr. Ralf Rupp (Max-Planck Institut

für Entwicklungsbiologie, Tübingen, Germany) and has been described previously [41].

RT-PCR

Total RNA was isolated from cell cultures by the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi [42]. For reverse transcription, 0.5 µg of oligo(dT)₁₅ was added to 1–2 µg of total RNA in a total volume of 12 µl. The mixture was incubated for 10 min at 65 °C and then chilled on ice. Dithiothreitol (DTT), dNTPs, 5 × reverse transcriptase buffer and 30 units of RNasin were added and the whole mixture (20 µl) was further incubated at 37 °C for 2 min. For cDNA synthesis 200 units of Superscript II-RT was added followed by incubation at 37 °C for 1 h. Before PCR, this mixture was inactivated by heating to 95 °C for 5 min. If mRNA was used for RT-PCR, a slightly different protocol was used. Polyadenylated RNA was isolated from total RNA by two passages over an oligo(dT)-cellulose column [43]. Random hexamers (0.2 µg) were added to 0.5 µg of mRNA in a total volume of 12 µl. The mixture was incubated for 10 min at 65 °C and then chilled on ice. DTT, dNTPs and 5 × RT buffer were added and the whole mixture (20 µl) was further incubated at 25 °C for 2 min. For cDNA synthesis 200 units of Superscript II-RT was added followed by incubation at 25 °C for 10 min and then at 37 °C for 1 h. Before PCR this mixture was inactivated by heating to 95 °C for 5 min.

A standard PCR protocol with 100 pmol of each primer and a 2 µl aliquot of the heat-inactivated RT reaction mixture was used for amplification of the fragments during 30 cycles (94 °C for 1 min; 50 °C for 1.5 min; 72 °C for 2 min) in a Trio-Thermoblock (Biometra, Göttingen, Germany). For quantification of PCR products, the RT reaction was started with identical amounts of RNA and only 25 cycles of PCR amplification were performed. Agarose gels were loaded with aliquots of the PCR mixture. Ethidium bromide-stained gels were analysed with a gel-documentation system (Cybertech CS1, Berlin, Germany). Amplified PCR fragments were detected and purified by agarose-gel electrophoresis. Purified fragments were cloned into the *Sma*I site of pUC18 by using the SureClone Ligation Kit (Pharmacia).

Sequencing of PCR fragments

The nucleotide sequences of the PCR fragments were determined by using the dideoxy chain-termination method [44] using the Sequenase DNA-sequencing kit. Both strands of the clone were sequenced using M13/pUC and T7 sequencing primers.

Calculations

Standard deviations are given for all values. In transport experiments the mean uptake activity from three culture dishes is given. Gauss' law of error propagation was applied when values had to be subtracted. Kinetic parameters of isoleucine transport were determined by non-linear regression using the following equation, in which K_{diff} is a first-order rate constant and all other parameters have their usual meaning:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} + K_{diff}[S]$$

Sequences were aligned using the clustalv multiple alignment algorithm [45]. Student's *t* test was used for the calculation of statistical significance. Each experiment presented was performed at least twice with similar results.

RESULTS

We have recently shown that injection of mRNA from C6-BU-1 glioma cells into *Xenopus laevis* oocytes induced Na⁺-independent isoleucine transport. This transport activity could be assigned to amino acid-transport system L [27]. To compare the transport activity induced in oocytes with that in C6-BU-1 glioma cells, basic transport parameters were also determined in this cell line. Na⁺-independent uptake of isoleucine was very fast and was proportional to time for only 30 s. Extrapolation to 0 s of determinations between 5 and 30 s revealed that no binding occurred. Basic kinetic parameters were determined in experiments with a standard uptake time of 15 s. The maximum velocity was 53 ± 11 nmol/min per mg of protein and the K_m value was 175 ± 57 µM. A non-saturable component was found with a K_{diff} of 0.025 ± 0.008 ml/min per mg of protein (results not shown). For competition experiments, a substrate concentration of 100 µM was chosen at which the non-saturable component amounted to only 12%.

Identification of the system-L amino acid transporter in different cell types

Inhibition of Na⁺-independent isoleucine transport by various amino acids was determined in C6-BU-1 glioma cells, COS-7 cells, primary cultures of mouse glial cells and activated lymphocytes (Table 1). CHO cells were not included in the analysis since their amino acid transport has been investigated in detail by Shotwell et al. [46]. Uptake activity was measured at an isoleucine concentration of 100 µM in the presence and absence of different amino acids at a concentration of 10 mM in transport buffer in which Na⁺ ions were replaced by choline ions. In all cell types,

Table 1 Inhibition of Na⁺-independent isoleucine transport in different cell types

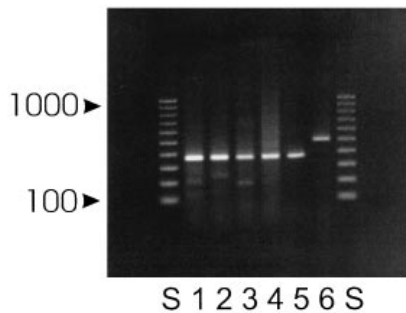
Isoleucine uptake was determined at a substrate concentration of 100 µM for 15 s. Competing amino acids were added at a concentration of 10 mM. Transport activities are given as nmol/min per mg of protein and are means ± S.D. from triplicate determinations. Resting lymphocytes were activated by the addition of PMA (5 ng/ml) and ionomycin (0.5 µg/ml). Student's *t* test was used for the calculation of statistical significance. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 compared with control.

Amino acid	Isoleucine uptake (nmol/min per mg of protein)			
	C6-BU-1 glioma cells	Primary mouse astroglial cells	COS-7 cells	Activated lymphocytes
None (control)	22.0 ± 1.6	16.3 ± 0.4	11.3 ± 0.4	6.8 ± 0.2
Taurine	21.2 ± 1.3	—	13.0 ± 0.6*	—
Proline	22.3 ± 1.3	—	11.9 ± 0.5	—
Glutamate	20.9 ± 1.5	15.9 ± 0.1	11.8 ± 0.4	—
MeAIB	22.6 ± 0.6	15.1 ± 0.1**	12.0 ± 0.3	6.1 ± 0.04**
Glycine	20.9 ± 0.9	—	11.1 ± 0.7	—
Alanine	20.7 ± 1.8	12.0 ± 1.1**	9.6 ± 0.3**	—
Lysine	19.5 ± 1.7	—	10.2 ± 0.3*	—
Arginine	19.2 ± 0.4*	15.4 ± 0.1*	11.3 ± 0.1	6.1 ± 0.09*
Serine	17.3 ± 1.4*	—	9.5 ± 0.4**	—
Threonine	17.0 ± 0.1**	—	7.0 ± 0.5***	—
Glutamine	15.1 ± 1.5**	10.0 ± 1.0***	8.0 ± 0.2***	2.4 ± 0.07***
Valine	4.0 ± 0.3***	—	1.7 ± 0.1***	—
Methionine	2.1 ± 0.1***	1.6 ± 0.1***	0.9 ± 0.1***	—
Histidine	1.9 ± 0.1***	—	1.0 ± 0.1***	—
Leucine	0.8 ± 0.1***	1.0 ± 0.1***	0.6 ± 0.1***	—
Tryptophan	0.8 ± 0.1***	—	0.5 ± 0.1***	—
Phenylalanine	0.7 ± 0.1***	0.8 ± 0.1***	0.4 ± 0.1***	0.3 ± 0.1***
BCH	1.2 ± 0.1***	1.6 ± 0.4***	1.0 ± 0.1***	1.2 ± 0.08***

Table 2 Absence of mutual inhibition of isoleucine and arginine transport in C6-BU-1 cells

Inhibition of isoleucine transport by arginine and of arginine transport by isoleucine was studied at substrate concentrations of 10 and 100 μ M, with inhibitor concentrations of 1 and 10 mM respectively. Competition was investigated in the presence and absence of Na⁺. Transport was determined as described for Table 1. Statistical significance was calculated for isoleucine uptake by comparison with data from line 1 and for arginine uptake by comparison with data from line 5. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

Substrate	Inhibitor	Presence of Na ⁺	Transport (nmol/min per mg of protein)	
			10 μ M substrate	100 μ M Substrate
Isoleucine	—	—	7.1 \pm 0.7	40.3 \pm 3.2
	Arginine	—	6.8 \pm 0.3	44.9 \pm 6.5
	—	+	8.2 \pm 0.6	51.4 \pm 5.9*
	Arginine	+	8.0 \pm 0.3	43.4 \pm 1.1
Arginine	—	—	2.4 \pm 0.2	8.4 \pm 0.5
	Isoleucine	—	2.6 \pm 0.2	9.0 \pm 0.3
	—	+	3.7 \pm 0.4**	9.9 \pm 0.7*
	Isoleucine	+	3.3 \pm 0.2	8.5 \pm 0.5

**Figure 1 Detection of 4F2hc homologues in different cell types**

mRNA was prepared from different cell types and reverse-transcribed into cDNA. Aliquots of the cDNA were amplified by PCR using degenerate primers (lat7s and lat8a, see the Experimental section) corresponding to conserved regions of the rbAT/NBAT/D2 and 4F2hc cDNAs. Amplified fragments of rat 4F2hc and rabbit rbAT cDNA clones were used as references. Aliquots of the PCR mixture were separated on a 1.5% agarose gel and stained with ethidium bromide. A 100 bp ladder was used as size marker (S). The mRNA used for RT-PCR was isolated from rat astroglia-rich primary culture (lane 1), CHO cells (lane 2), COS cells (lane 3) or activated lymphocytes (lane 4). Lane 5, PCR performed with 4F2hc cDNA; lane 6, PCR performed with rbAT cDNA.

Na⁺-independent isoleucine transport was strongly inhibited by phenylalanine, leucine and the model substrate BCH. Glutamine and alanine competed weakly with Na⁺-independent isoleucine transport, whereas arginine, lysine, glutamate and MeAIB did not inhibit Na⁺-independent isoleucine transport (Table 1). The outcome of the competition experiments did not depend on the methods used for the transport experiments. When suspensions of C6-BU-1 cells were subjected to silicone oil centrifugation the same results were obtained as with adherent cells on culture dishes (results not shown).

In C6-BU-1 cells a possible involvement of system y^L in isoleucine transport was investigated (Table 2). For this, uptake of isoleucine and arginine was studied at concentrations of 10 and 100 μ M. Isoleucine transport could be divided into a Na⁺-dependent and a Na⁺-independent component. Less than 20% of isoleucine transport was Na⁺-dependent. Na⁺-independent

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NBAT  GVDGFSFDAVKFLEAKDLRNEIQVNTSQIPDVTVTRYSSELYHDFTTTQVG
*****
r4F2  GVDGF-----QVRDVGKLANASL--YLAEWQNITKNFSEDRL
CHO   GVDGF-----QFRDVEKLMASL--YLAEWQNITKNFSEDRL
m4F2  GVDGF-----QFRDVGKLMNAPL--YLAEWQNITKNLSEDRL
COS   GVDGF-----QVRDIENLKDASS--FLAEWENITKXFSEDRL
h4F2  GVDGF-----QVRDIENLKDASS--FLAEWQNITKGFSEDRL
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NBAT  MHDLVR--DFRQTMNQFSREPGRYRFMGTEVSAESTERTMVYGLSFIQEAD
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r4F2  IAGTASSDLQQIVNILESTSDLLTSSYLSQP-----VFTG----EHA
CHO   IAGTDSSDLQQIVTILESTSDLLTSSYLSNS-----SFTG----EHTN
m4F2  IAGTESSDLQQIVNILESTSDLLTSSYLSNS-----TFTG----ERTE
COS   IAGTSSDLQQIVSLLSNKDLLTSSYLSDS-----SFTG----EHTK
h4F2  IAGTSSDLQQILSLLSNKDLLTSSYLSDS-----GSTG----EHTK
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Figure 2 Comparison of 4F2hc sequences from different cell types

mRNA was prepared from different cell types and reverse-transcribed into cDNA. Aliquots of the cDNA were amplified by PCR using degenerate primers (lat7s and lat8a, see the Experimental section) corresponding to conserved regions of the rbAT/NBAT/D2 and 4F2hc cDNA. Amplified fragments were cloned into pUC18 and sequenced. Sequences of rat 4F2hc [17], human 4F2hc [47] and NBAT [14] are shown for comparison. Sequences were aligned using the clustalv program [45]. Rat, mouse and human 4F2hc are denoted by prefixes r, m and h respectively. Identical amino acids in the 4F2hc homologues are marked by an asterisk below the 4F2hc sequences, and amino acids that are also identical in the NBAT sequence are marked by an asterisk above the 4F2hc sequences. Similar amino acids are indicated by a dot.

isoleucine transport could not be inhibited by a 100-fold excess of arginine, at either substrate concentration (10 or 100 μ M). Only the Na⁺-dependent component of isoleucine transport was inhibited by arginine but only at a concentration of 100 μ M. Vice versa, arginine transport in the absence of Na⁺ could not be inhibited by a 100-fold excess of isoleucine, whereas the Na⁺-dependent component of arginine transport could be inhibited by isoleucine (Table 2), but also only at a substrate concentration of 100 μ M.

Identification of 4F2hc in different cell types

mRNA was isolated from the same cell types as used for the competition experiments. After cDNA synthesis, degenerate primers were used that not only recognized possible isoforms of 4F2hc but also homologues of rbAT/NBAT/D2 cDNAs. In all cell types only homologues of the 4F2hc cDNA could be identified (Figure 1). These were cloned and their sequences determined (Figure 2). The fragment amplified from mouse lymphocyte cDNA had the same size as the control fragment derived from rat 4F2hc cDNA. The sequence of this fragment was not determined because this cell type has already been shown to contain the 4F2hc cDNA [36]. COS cell 4F2hc showed a higher homology to human 4F2hc, whereas CHO 4F2hc showed a higher homology to the rat and mouse sequence. The NBAT sequence is shown for comparison.

Induction of 4F2hc mRNA and system-L-like neutral-amino acid transport after mitogenic stimulation of lymphocytes

Resting lymphocytes isolated from mouse spleen were stimulated by incubation with PMA (5 ng/ml) plus ionomycin (0.5 μ g/ml). Samples were taken from unstimulated control cells and stimulated cells at 0, 24 and 48 h. The rate of Na⁺-independent

Table 3 Increase in system L-like neutral-amino acid-transport activity and 4F2hc mRNA in activated lymphocytes

Lymphocytes were isolated from mouse spleen and purified by centrifugation through a Ficoll step gradient. Cells were stimulated for 24 or 48 h by the addition of PMA (final concentration 5 ng/ml) and ionomycin (final concentration 0.5 μ g/ml). Na⁺-independent isoleucine transport was determined after silicone oil centrifugation. The mean \pm S.D. activity from three samples is given. The levels of mRNA (presented in arbitrary units) were determined by RT-PCR followed by densitometric analysis of the gels. An aliquot of the RNA samples, used for the RT reaction, was subjected to agarose-gel electrophoresis to control the integrity and uniformity. Scanning of the 28 S rRNA was used to quantify the samples. ** $P < 0.01$, *** $P < 0.001$ compared with control. n.d., not determined.

Time (h)	Isoleucine transport (nmol/min per mg of protein)		4F2hc mRNA		28 S rRNA in RT reaction	
	Control	Activated	Control	Activated	Control	Activated
0	2.2 \pm 0.2	2.2 \pm 0.2	n.d.	n.d.	—	—
24	1.3 \pm 0.01	9.2 \pm 0.08**	40	328	1110	1222
48	0.8 \pm 0.06	10.8 \pm 0.07***	36	368	1076	1384

isoleucine transport was determined and RNA was isolated for RT-PCR assay. From 20×10^6 unstimulated cells, 14 μ g of RNA was isolated, whereas stimulated cells yielded 42 μ g of RNA and 91 μ g of RNA after 24 and 48 h respectively. To normalize the RT-PCR, 1 μ g of RNA was used for reverse transcription in all cases. The uniformity of the aliquots was controlled by gel electrophoresis and scanning of the rRNA (Table 3). Glyceraldehyde-3-phosphate dehydrogenase mRNA could not be used as an internal reference, because transcription of this mRNA also increased after stimulation. The number of cycles was chosen so that the amount of the PCR fragment was proportional to the amount of mRNA. After 48 h stimulation of lymphocytes with phorbol ester plus ionomycin, Na⁺-independent isoleucine transport was 13-fold higher than in unstimulated cells (Table 3); in two other experiments transport increased five- and eight-fold (not shown). In competition experiments, Na⁺-independent isoleucine transport was inhibited only by large neutral amino acids and was therefore assigned to system L (Table 1). In parallel to the elevation of transport activity, a tenfold increase in 4F2hc mRNA was detected in this experiment (Table 3); in two other experiments a four- and nine-fold increase was detected (not shown).

Antisense experiments and transient expression of 4F2hc in mammalian cell lines

Antisense oligonucleotides have been used for the selective suppression of mRNA expression in mammalian cells. C6-BU-1 glioma cells were treated with an antisense oligonucleotide complementary to 4F2hc mRNA for 72 h. Na⁺-independent isoleucine transport was inhibited between 17 and 20 %, whereas arginine transport was unaffected by this treatment (Table 4A). Primary cultures of astroglial cells were also susceptible to inhibition by antisense oligonucleotides. After 31 days incubation with antisense oligonucleotide, Na⁺-independent isoleucine transport was inhibited by 25 %, whereas arginine transport increased by 24 % (Table 4A).

Transient expression of 4F2hc cDNA in CHO cells was used as a further approach to the elucidation of the relationship between 4F2hc and amino acid transport. CHO cells were electroporated in the presence of 4F2hc cDNA that had been cloned into the eukaryotic expression vector pCS2+. Expression was determined between 40 and 72 h after electroporation.

Table 4 Antisense experiments and transient expression of 4F2hc in cell culture

In the antisense experiments, C6-BU-1 glioma cells or astroglia-rich primary cultures were incubated with 3 or 5 μ M antisense oligonucleotide for the times indicated. In the transient expression experiments, CHO cells were transfected by electroporation with either pB19/3, which is pCS2+ containing the 4F2hc cDNA, or with pCS2+ alone. Na⁺-independent isoleucine uptake was determined after the indicated times at an isoleucine concentration of 100 μ M for 15 s. Arginine uptake activity was determined in the presence of Na⁺ at a substrate concentration of 100 μ M. Transport activities are means \pm S.D. from triplicate determinations. *** $P < 0.001$ compared with control.

(A) Antisense experiments

Treatment	Incubation time	Cell type	Transport activity (nmol/min per mg of protein)	
			Isoleucine	Arginine
Control	72 h	C6-BU-1	36.1 \pm 1.5	5.6 \pm 0.1
Oligo (3 μ M)	72 h	C6-BU-1	28.6 \pm 0.2***	5.7 \pm 0.2
Control	31 days	Astroglia	25.1 \pm 1.1	5.1 \pm 0.1
Oligo (5 μ M)	31 days	Astroglia	19.0 \pm 1.6***	6.3 \pm 0.2***

(B) Transient expression

Plasmid	Expression time (h)	Cell type	Transport activity (nmol/min per mg of protein)	
			Isoleucine	Arginine
pCS2+ (control)	48	CHO	34.5 \pm 0.6	Not determined
pB19/3	48	CHO	41.4 \pm 1.4***	Not determined

Transient expression of 4F2hc caused a 20 % increase in Na⁺-independent isoleucine transport in CHO cells (Table 4B). Control cells, transfected with the vector alone or with a β -galactosidase control plasmid, did not exhibit increased transport activity. In experiments with a β -galactosidase expression vector the efficiency of electroporation was determined. Usually around 20 % of the surviving cells expressed β -galactosidase.

DISCUSSION

Transport processes induced by the 4F2hc surface antigen have so far only been studied in the *Xenopus* oocyte expression system. Several problems arise in the case of the 4F2hc surface antigen. First, the hydropathy plot indicates an unusual structure for a transport protein with only one typical transmembrane helix [47]. Secondly, a 4F2 light chain has been identified which is linked to the 4F2 heavy chain by a disulphide bridge [26]. Thirdly, competition experiments performed with the 4F2hc cRNA in comparison with the whole pool of C6-BU-1 mRNA indicated a switch in the substrate specificity of the expressed transport protein(s) [17,27]. Na⁺-independent isoleucine transport in oocytes expressing mRNA from C6-BU-1 glioma cells was inhibited by large neutral amino acids only and was therefore assigned to system L. Na⁺-independent isoleucine transport in oocytes expressing 4F2hc cRNA alone was not only inhibited by large neutral amino acids but also by small neutral and cationic amino acids. The reason for this switch in substrate specificity was not the cloning of a minor transport component, because it could be demonstrated in hybrid depletion experiments that the 4F2hc mRNA was responsible for the isoleucine-uptake activity

in the mRNA pool of the glioma cells [17]. We assumed the 4F2hc surface antigen to be an activator of the system-L amino acid transporter. A second (or more) component(s) must therefore be present in the mRNA pool from C6-BU-1 glioma cells, which on co-expression with 4F2hc give(s) rise to the typical system-L-like transport behaviour. When 4F2hc alone was expressed in oocytes, an endogenous oocyte amino acid transporter was activated, resulting in the observed switch of substrate specificity. Endogenous oocyte proteins have been shown in many cases to interfere with expressed proteins [48,49]. This has also been proposed for expression of the rbAT/NBAT/D2 protein [18,24]. It was therefore necessary to correlate the expression of the 4F2hc surface antigen with amino acid transport in mammalian cells. Three different approaches were used to investigate the possible relationship between 4F2hc expression and system-L amino acid transport. (i) The 4F2hc surface antigen should be present in all cell types that have been shown to possess amino acid-transport system L. (ii) 4F2hc expression should be induced in parallel with induction of system L. (iii) Antisense oligonucleotides directed against 4F2hc and transient expression of 4F2hc should alter the expression of system-L-like neutral amino acid transport. The 4F2hc surface antigen was detected in all cell types that display system L. We have tested C6-BU-1 glioma cells, primary cultures of astroglial cells, CHO cells and COS cells. The transport data obtained with primary cultures of astroglial cells are in agreement with data on transport of kynurenine and gabapentine, both of which are substrates of transport system L in astrocytes [50,51]. 3T3 cells have been shown to express system L [52] and 4F2hc [53]. The ubiquitous tissue distribution of 4F2hc, as detected by Northern blot [53], is likely to apply also to system L. Furthermore the presence of 4F2hc has been demonstrated immunocytochemically on the basolateral side of the proximal tubule [54], the side of neutral amino acid release into the blood [30]. The presence of 4F2hc in CHO cells gives a rationale for the puzzling oocyte expression data obtained by Su et al. [55]. Expression of size-fractionated mRNA from CHO cells in oocytes resulted in the induction of a transport process with similar characteristics, as described recently for 4F2hc [17].

A correlation between the level of 4F2hc mRNA and Na⁺-independent isoleucine transport activity is difficult to demonstrate, since system L appears to be constitutive. In earlier work it was found that system L is regulated by the availability of leucine for protein biosynthesis [31]; however, this regulation occurs at the level of translation. Lymphocytes are the only known cell type with an inducible system L. In resting lymphocytes, the system is nearly undetectable but increases greatly after activation of lymphocytes by phorbol esters [32]. Transport activity in activated lymphocytes reached the same level as in other cell types (Table 1). Concomitant with activation of Na⁺-independent isoleucine transport, 4F2hc mRNA levels also increased greatly, which is in agreement with earlier data [34,36]. Isoleucine is not only transported by system L but also by the recently identified systems b^{0,+} and y⁺L [28]. However, Na⁺-independent isoleucine transport in activated lymphocytes was mediated only by system L, similar to the situation in the other cell types (Table 1). rbAT mRNA could not be detected in lymphocytes or other cell types.

To elucidate further the relationship between amino acid transport and 4F2hc, antisense oligonucleotides directed against 4F2hc were used. In cell culture experiments, a modest but significant inhibition of system-L transport was observed, which was not found for arginine transport. Arginine transport was used as a control for non-specific effects of antisense oligonucleotides instead of sense oligonucleotides. The same modest

influence was also observed in transfection experiments which resulted in an increase in system-L-like transport activity. CHO cells were chosen for the transfection experiments because of the presence of system L. If the 4F2 light chain or another protein participates in Na⁺-independent neutral amino acid transport, it must be present in this cell type. Owing to the very high endogenous level of Na⁺-independent isoleucine transport in this cell line, the increment was quite small. In addition, only about 20% of all cells were transfected by electroporation, causing a further dilution of the expression signal.

Other groups have implicated the 4F2hc surface antigen in system-y⁺L-like transport activity [23,56]. System y⁺L is defined as a system that transports neutral amino acids in the presence of Na⁺, and cationic amino acids in the absence of Na⁺. Therefore competition between neutral and cationic amino acids occurs only in the presence of Na⁺ [29]. Several arguments indicate that this system is not activated by 4F2hc in C6-BU-1 cells. Most of the data supporting a role for 4F2hc in y⁺L-like transport have been obtained using oocytes that express human 4F2hc. Rat 4F2hc exhibited different behaviour from the human homologue in oocytes. Rat 4F2hc mediates Na⁺-independent isoleucine transport in oocytes [17], whereas human 4F2hc mainly mediates Na⁺-dependent isoleucine transport [13]. We therefore investigated whether y⁺L activity was present in C6-BU-1 cells. We could not detect y⁺L activity at a substrate concentration of 100 or 10 μM, which is around the K_m determined for the y⁺L system [29]. Na⁺-dependent isoleucine transport was only about 10% of the total transport activity in C6-BU-1 cells. At 10 μM isoleucine, transport could not be inhibited by arginine, no matter whether Na⁺ was present or not. At 100 μM, only Na⁺-dependent isoleucine transport was inhibited by arginine. This excludes the presence of the y⁺L system in glioma cells. The possibility still remains that, in human cells, system y⁺L is activated by 4F2hc, whereas, in the rat, system L is activated. In agreement with activation of system L in rat cells is a publication by Yao et al. [28], in which involvement of 4F2hc in y⁺L activity in rat intestine was excluded. A more general role for 4F2hc in exocytosis and membrane fusion has been proposed by Ohgimoto et al. [57].

The data presented here from antisense and transient expression studies support the hypothesis that the 4F2hc protein is not the transport itself; however, they indicate that expression of 4F2hc affects the activity of amino acid-transport system L. Overexpression of the related NBAT/rbAT/D2 cDNA in mammalian cells did not result in any increase in amino acid-transport activity [14,18]. Therefore, a cell line was used that endogenously expresses the system-L transporter and could supply the 4F2 light chain or other additional proteins during expression experiments. In this expression system we achieved a 20% increase during transient expression of the 4F2hc cDNA.

In conclusion, the present investigation provides evidence supporting the notion that the 4F2hc surface antigen is linked to amino acid-transport system L, but is probably not the transporter itself.

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