Cloning and functional expression of a cDNA from rat jejunal epithelium encoding a protein (4F2hc) with system y^+L amino acid transport activity

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Two different protein families, designated CAT (cationic amino acid transporter) and BAT (broad-specificity amino acid transporter) mediate the plasma membrane transport of cationic amino acids in animal cells. CAT transporters have 12–14 transmembrane domains and are selective for cationic amino acids. BAT proteins, in contrast, have one to four transmembrane domains and induce the transport of both cationic and zwitterionic amino acids when expressed in *Xenopus* oocytes. Mutations in the human BAT gene cause type I cystinuria, a disease affecting the ability of intestinal and renal brush border membranes to transport cationic amino acids and cystine. We have used functional expression cloning in oocytes to isolate a BAT-related cDNA from rat jejunal epithelium. The cDNA encodes the rat 4F2 heavy chain (4F2hc) cell-surface antigen, a 527-residue (60 kDa) protein that is 26% identical in amino acid sequence with rat renal BAT (also known as NBAT/D2). Expression of rat jejunal 4F2hc in oocytes induced the lysine-inhibitable Na^+-dependent influx of leucine and the leucine-inhibitable Na^+-independent influx of lysine. Lysine efflux was stimulated by extracellular (Na^+ plus leucine). These characteristics identify the expressed amino acid transport activity as system y^+L, a transporter that has been implicated in basal membrane transport of cationic amino acids in intestine, kidney and placenta.

INTRODUCTION

Four plasma membrane transport systems for cationic amino acids have been identified functionally in animal cells. One (y^+), is specific for cationic amino acids [1]; the others ($B^{o,+}$, $b^{o,+}$ and y^+L) also transport zwitterionic amino acids [2–7]. $B^{o,+}$ is Na^+-dependent and y^+ and $b^{o,+}$ are Na^+-independent. System y^+L mediates the Na^+-independent transport of cationic amino acids but the Na^+-dependent transport of zwitterionic amino acids. cDNA species corresponding to three of these transport activities (y^+, $b^{o,+}$ and y^+L) have been cloned and belong to two different gene families, designated CAT (cationic amino acid transporter) and BAT (broad-specificity amino acid transporter) [8–11]. There are three different CAT isoforms (CAT1, CAT2A and CAT2B) and two BAT isoforms [BAT and 4F2 heavy chain (4F2hc)].

CAT1, CAT2A and CAT2B have 12–14 transmembrane domains and mediate system y^+ transport activity [8]. BAT and 4F2hc, in contrast, have one to four transmembrane domains and might not be complete transporters [9–11]. BAT, a 677–685-residue protein, is found in the brush border membranes of small intestine and renal proximal tubules [12,13] where it is disulphide-linked to an unidentified 50 kDa integral membrane protein [14]. When expressed in *Xenopus* oocytes, rat BAT (also known as NBAT/D2) and rabbit BAT induce system $b^{o,+}$ transport activity [15–17]. There is genetic linkage between the human BAT gene and type I cystinuria, a disease resulting from defective intestinal/renal brush border membrane transport of cationic amino acids and cystine [18–20]. Mis-sense mutations of human BAT have been demonstrated in cystinuria chromosomes [21–25], and expression of the most common mutation found in cystinuria patients [Met-467 changed to threonine (Met467Thr)] decreases transport activity in *Xenopus* oocytes by 90% [21]. Transfection of cultured kidney proximal-tubular (OK) cells with BAT antisense leads to depletion of system $b^{o,+}$ transport activity [26].

The BAT homologue 4F2hc is a 526–529-residue protein [27–29]. It is recognized by the monoclonal antibody mab4F2 [30], has a basolateral localization in renal epithelial cells of the proximal tubule [31] and is disulphide-linked to a 40 kDa light chain (4F2lc). Previous studies have suggested that the 4F2 molecule might be a growth factor receptor [32] or possibly involved in Na^+/Ca^{2+} exchange [33]. Recently, 4F2 has been implicated in virus-mediated cell fusion [34]. The amino acid transport activity associated with 4F2hc expression in oocytes has been variously described as system y^+L [human SV40-transformed fibroblast and human (JAR) choriocarcinoma cell 4F2hc] [35–37] or as system L [rat glioma (C6-BU-1) cell 4F2hc] [38], reflecting an apparent species difference in cation dependence for zwitterionic amino acid transport (Na^+-independent for the rat clone). The functional characteristics ascribed to the rat glial cDNA [38] correspond more closely to $b^{o,+}$, because cationic amino acids were also apparently transported and fluxes of isoleucine were inhibited by cationic amino acids.

We report here the isolation and functional expression of a

Abbreviations used: BAT, broad-specificity amino acid transporter; CAT, cationic amino acid transporter; 4F2hc, 4F2 heavy chain; 4F2lc, 4F2 light chain; MBM, modified Barth's medium.

Amino acid transport system nomenclature: $b^{o,+}$, a Na^+-independent system with a broad tolerance for cationic and zwitterionic amino acids; $B^{o,+}$, a Na^+-dependent system with a broad tolerance for cationic and zwitterionic amino acids; L, a Na^+-independent system selective for zwitterionic amino acids with branched and apolar side chains; y^+, a Na^+-independent system selective for cationic amino acids; y^+L, a broad-specificity system that exhibits Na^+-independent transport of cationic amino acids (e.g. lysine), but Na^+-dependent transport of zwitterionic amino acids (e.g. leucine).

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U59324.

4F2hc cDNA from rat jejunal epithelium. The cDNA encoded a protein with an identical amino acid sequence to that cloned from rat glioma (C6-BU-1) cells [38]. In contrast with the rat glioma study, however, the cDNA induced system y^+L transport activity when expressed in *Xenopus* oocytes. The functional characteristics of the recombinant 4F2hc protein, including an ability to mediate the heteroexchange of cationic amino acid and (Na^+ plus zwitterionic amino acid), were consistent with a potential role in the basal membrane transport of cationic amino acids in intestine, kidney and placenta.

MATERIALS AND METHODS

Library screening

A size-selected rat jejunal pGEM-3Z cDNA library of 6800 primary recombinant clones from which we had previously isolated a cDNA encoding the Na^+-dependent nucleoside transporter protein rCNT1 was screened for [^{14}C]lysine and leucine transport activity (0.2 mM, 10 min, 20 °C) by functional expression in *Xenopus* oocytes [39]. RNA transcribed *in vitro* from the total library was injected into oocytes and induced a 2–3-fold increase in lysine influx, which was Na^+-independent. There was a parallel increase in leucine uptake, which was Na^+-dependent. The library was then subdivided into 20 pools, each of 700–800 clones. Four pools exhibited increased lysine/leucine transport and tested positive for 4F2hc by diagnostic PCR. All library pools were negative for BAT. Colonies from the master plate of one of the transport-positive pools were seeded individually into the wells of 96-well flat-bottomed microtitre plates to produce a grid system. Combined testing of rows and columns for lysine/leucine transport activity and 4F2hc identified a single positive colony from which a plasmid (pSY1) with a 1.8 kb insert was isolated.

PCR amplification of DNA

Primers corresponding to mouse pre-B cell/macrophage 4F2hc [28] and rat kidney BAT [15] were synthesized as follows: (a) sense 4F2hc oligonucleotide, corresponding to nt 795–811 of mouse 4F2hc cDNA (5′-GGTGTGGATGGTTTCCA-3′); (b) anti-sense 4F2hc oligonucleotide, complementary to nt 1146–1162 of mouse 4F2hc cDNA (5′-GTCCCTGGCAGAGTGAA-3′); (c) sense BAT oligonucleotide, corresponding to nt 76–94 of rat BAT cDNA (5′-CAAAGACAAGAGAGACTC-3′); and (d) anti-sense BAT oligonucleotide, complementary to nt 286–301 of rat BAT cDNA (5′-GTAGCGAGCCTGGCCA-3′). Reaction mixtures (100 μl) containing 10 mM Tris/HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin, 2 mM deoxyribonucleotides, 500 ng of template plasmid DNA, 100 pmol of each primer and 2.5 units of *Taq* polymerase were pipetted into 1.5 ml microcentrifuge tubes and layered with 100 μl of mineral oil to prevent evaporation. Amplification was accomplished by incubation at 94 °C for 1 min, 50 °C for 1.5 min and 72 °C for 1.5 min (Robocycler®40 Temperature Cycler; Stratagene). After 30 cycles, 15 μl of each reaction mixture was separated on a 2 % (w/v) agarose gel containing 0.25 μg/ml ethidium bromide. Negative controls were performed in which one or other of the primers or the template DNA was omitted from the reaction mixture. Single-stranded cDNA that had been reverse-transcribed from rat jejunal poly(A)$^+$ RNA [40,41] with oligo(dT)$_{12–18}$ as primer was used as a positive control. Positive PCR reactions were confirmed by the presence of approx. 370 bp (4F2hc) or approx. 230 bp [BAT] PCR products [40].

cDNA sequencing and sequence analyses

The pSY1 cDNA insert was subcloned into the vectors pBluescript II KS$^-$ or SK$^-$ (Strategene). Overlapping deletions were generated by exonuclease III digestion (Erase-a-base System; Promega) in accordance with the manufacturer's protocol. Sequencing by the dideoxynucleotide chain termination method was performed by the University of Alberta DNA Sequencing Laboratory (Department of Biochemistry) by using *Taq* Dye-Deoxy terminator cycle sequencing with an automated Model 373A (Applied Biosystems) DNA Sequencer. Database searches and sequence alignments were performed with programs of the Sequence Analysis Software GCG Package (Genetics Computer Group).

Expression in *Xenopus* oocytes of RNA transcribed *in vitro*

Plasmid DNA of pSY1 was digested with the restriction enzyme *Xba*I. The linearized DNA was then transcribed *in vitro* with T7 RNA polymerase in the presence of the m^{7}GpppG cap, with the MEGAscript® *in vitro* transcription system (Ambion). Healthy stage VI oocytes of *Xenopus laevis*, prepared as described previously [40,41], were injected (Inject + Matic System) with 10 nl of water containing pSY1 RNA (1 ng/nl) or 10 nl of water alone, and incubated at 18 °C in modified Barth's medium (MBM) for 5 days with a daily change of medium before the assay of transport activity.

Transport assays

The uptakes of lysine and leucine were traced with L-[4,5-^{3}H]lysine (5 μCi/ml; Amersham) and L-[U-^{14}C]leucine (2.5 μCi/ml; Amersham) respectively. Flux measurements were performed at 20 °C in a 48-well tissue culture plate positioned on a New Brunswick Scientific Model G2 Gyratory shaker (150 rev./min) on groups of 10–12 oocytes in transport buffer (0.2 ml) containing 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.5, and either 100 mM NaCl or 100 mM choline chloride. For competition experiments, oocytes were exposed to non-radioactive amino acids simultaneously with radiolabelled permeant. At the end of the incubation (5–60 min), extracellular label was removed by six rapid washes in 1 ml of the appropriate ice-cold transport buffer. The washing step was completed within 1 min. Individual oocytes were dissolved in 0.5 ml of 5 % (w/v) SDS for quantification of oocyte-associated radioactivity by liquid-scintillation counting (LS 6000 IC; Beckman). Results for flux studies are shown as means ± S.E.M. for 10–12 individual oocytes. Kinetic constants for lysine and leucine uptake were determined by non-linear regression analysis (ENZFITTER; Elsevier-Biosoft).

To measure *trans*-stimulation of lysine efflux by extracellular leucine, batches of 25 oocytes that had been injected with rat jejunal 4F2hc RNA transcript or water were preloaded with 0.2 mM [^{3}H]lysine (28 μCi/ml) in NaCl transport buffer for 3 h at 20 °C. After loading, the oocytes were washed free of extracellular radioactivity with ice-cold choline chloride transport buffer and kept on ice. Efflux was initiated by aspiration of the ice-cold wash buffer and the addition of 1.5 ml of either NaCl or choline chloride transport buffer at 20 °C. Duplicate 5 μl aliquots of transport buffer were removed at 10 or 15 min intervals and counted for radioactivity to determine baseline efflux. After 75 min, incubations were supplemented with 75 μl of 100 mM non-radioactive leucine in NaCl or choline chloride transport buffer (to give a final extracellular leucine concentration of 5 mM) and continued for a further 125 min. As was the case for

influx, the oocytes were continually mixed on a Gyratory shaker to prevent the formation of unstirred layers and to ensure uniform mixing.

RESULTS

Nucleotide and deduced amino acid sequences of rat jejunal 4F2hc

Screening of the rat jejunal pGEM-3Z cDNA library by a combination of lysine/leucine transport expression in *Xenopus* oocytes and diagnostic PCR with primers for 4F2hc resulted in the isolation of a 1797-bp cDNA named pSY1 (see nomenclature footnote). The open reading frame of pSY1 was flanked by 19 bp of 5′-untranslated sequence and 194 bp of 3′-untranslated sequence and encoded a 527-residue protein with a predicted molecular mass of 60 kDa (Figure 1). Hydropathy analysis of the deduced amino acid sequence of pSY1 by the Kyte–Doolittle algorithm [42] revealed the presence of only one potential transmembrane domain between residues 76 and 98. This region of the pSY1 coding sequence was flanked by cationic residues (Arg-75 and Arg-99). There was no 5′-hydrophobic signal sequence, suggesting that the pSY1-encoded protein is orientated with its N-terminus in the cytoplasm and its C-terminus outside the cell. The deduced amino acid sequence contained two cysteine residues (positions 103 and 325) and eight potential *N*-linked glycosylation sites (Asn-166, Asn-249, Asn-259, Asn-263, Asn-318, Asn-386, Asn-400 and Asn-510), all of which were predicted to be extracellular. The pSY1-encoded protein exhibited 76% amino acid sequence identity (85% similarity) with human SV40-transformed fibroblast 4F2hc [27,29], 89% identity (93% similarity) with mouse pre-B cell/macrophage 4F2hc [28] and 100% identity with rat glioma 4F2hc [38]. Small differences in nucleotide sequence between pSY1 and the rat glioma 4F2hc cDNA [38] were limited to the 5′- and 3′-untranslated regions. Structural features shared by rat, human and mouse 4F2hc included the single potential transmembrane domain and both cysteine residues. A -Ser-Ser-Ser-Ser- motif present at residues 439–442 of rat 4F2hc was absent from both human and mouse 4F2hc. Other membrane proteins that have been reported to contain serine clusters include the rat jejunal Na^+-dependent nucleoside transporter rCNT1 [39], the rat brain and rabbit intestinal GLAST1 and EAAC1 Na^+/K^+-dependent glutamate transporters [43] and the human α-2A-adrenergic receptor [44].

In Figure 1 we compare the amino acid sequence of rat jejunal 4F2hc with that of rat BAT [15–17]. The alignment gave 26% amino acid identity (51% similarity). Structural relationships between the two proteins are reviewed in the Discussion section.

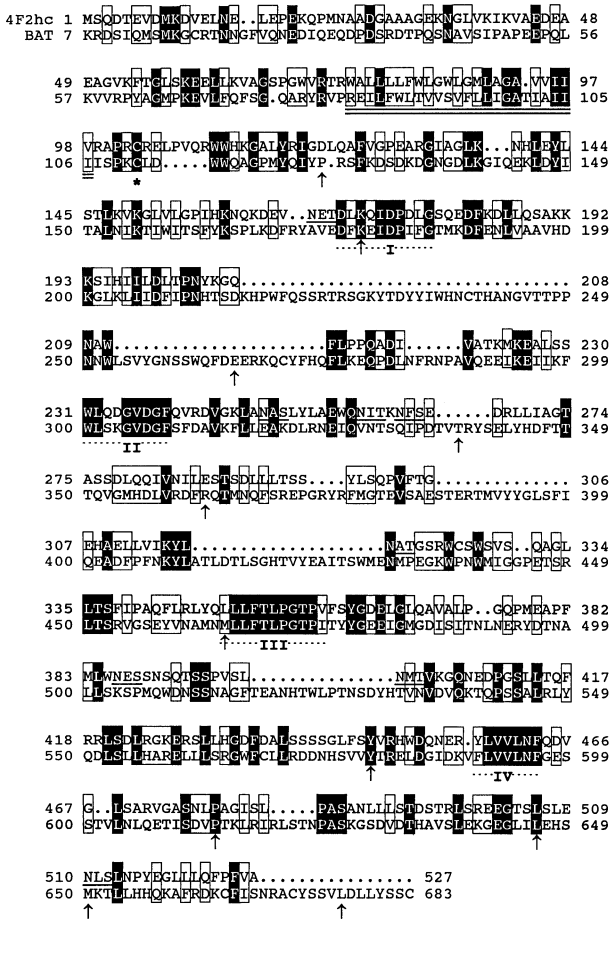

Figure 1 Alignment of rat jejunal 4F2hc with rat BAT

Rat jejunal 4F2hc has eight potential N-linked glycosylation sites (underlined) and a single putative transmembrane domain (doubly underlined). The corresponding putative transmembrane region of BAT is between residues 86 and 106. A cysteine residue common to 4F2hc and BAT is indicated by an asterisk. Four conserved regions of amino acid sequence (I–IV) are highlighted by dots. Amino acid residues that are mutated in human BAT and result in cystinuria are indicated by arrows. The alignment was performed with the GCG BESTFIT program. Gaps were introduced to optimize homology. Identical amino acid residues in the two proteins are indicated in black: similar amino acid residues are boxed.

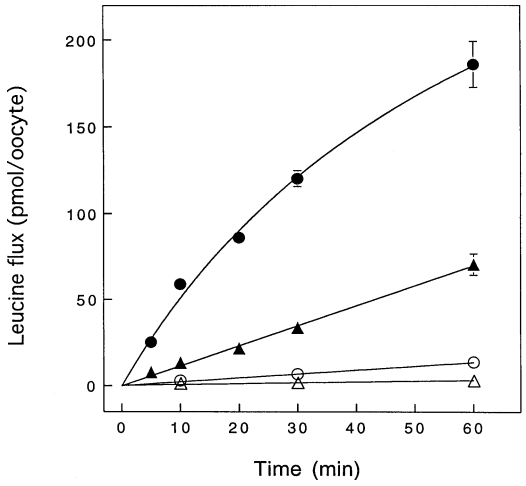

Figure 2 Time course of leucine uptake

Oocytes injected with either 10 nl of rat jejunal 4F2hc RNA transcript (1 ng/nl) (○,●) or 10 nl of water (△,▲) were incubated for 5 days at 18 °C in MBM. Fluxes (0.2 mM, 20 °C) were determined in transport buffer containing 100 mM NaCl (●,▲) or 100 mM choline chloride (○,△). Each value is the mean ± S.E.M. for 10–12 oocytes.

Expression of amino acid transport activity

Leucine transport

Representative time courses of [^{14}C]leucine uptake (0.2 mM, 20 °C) in oocytes injected with rat jejunal 4F2hc RNA transcribed *in vitro* or with water are shown in Figure 2. Uptake was approximately linear with time for the first 10 min. With the use of this time interval to determine initial rates of transport, leucine influx in RNA-injected oocytes in NaCl transport buffer was

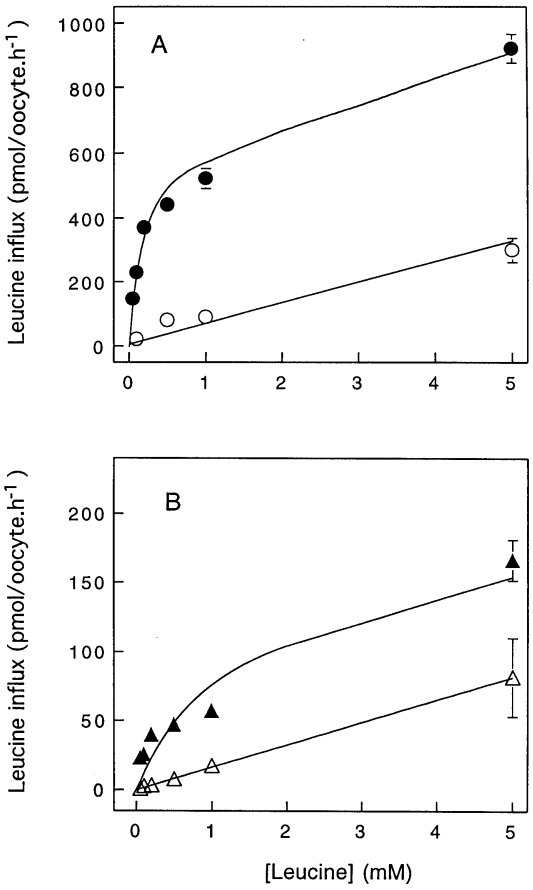

Figure 3 Concentration dependence of rat jejunal 4F2hc-induced leucine influx

Oocytes injected with either 10 nl of RNA transcript (1 ng/nl) or 10 nl of water were incubated for 5 days at 18 °C in MBM. Influx of leucine (50 μM to 5 mM, 20 °C, 10 min flux) was determined in transport buffer containing 100 mM NaCl (●,▲) or 100 mM choline chloride (○,△). Each value is the mean ± S.E.M. for 10–12 oocytes. (**A**) Influx in RNA-injected oocytes. (**B**) Influx in water-injected oocytes. Kinetic constants were determined by non-linear regression analysis (ENZFITTER, Elsevier-Biosoft) and are reported in Table 1.

Table 1 Kinetic parameters of leucine and lysine influx

Kinetic parameters (means ± S.E.M.) for the data presented in Figures 3 and 4 were determined by non-linear regression analysis (ENZFITTER, Elsevier-Biosoft). Abbreviation: n.d., not detected.

			Values of parameters	
Amino acid	Na^+	Parameter	4F2hc	Water
Leucine	+	Apparent K_m (μM)	110 ± 20	32 ± 20
		V_{max} (pmol/h per oocyte)	491 ± 36	34 ± 4
		K_d (h^{-1})	88 ± 9	26 ± 2
	−	Apparent K_m (μM)	n.d.	n.d.
		V_{max} (pmol/h per oocyte)	n.d.	n.d.
		K_d (h^{-1})	54 ± 5	17 ± 1
Lysine	+	Apparent K_m (μM)	55 ± 10	77 ± 30
		V_{max} (pmol/h per oocyte)	440 ± 27	142 ± 16
		K_d (h^{-1})	n.d.	n.d.
	−	Apparent K_m (μM)	59 ± 10	120 ± 20
		V_{max} (pmol/h per oocyte)	345 ± 12	113 ± 5
		K_d (h^{-1})	n.d.	n.d.

Table 2 Amino acid inhibition of leucine influx

Leucine influx (0.2 mM, 20 °C, 10 min flux) in RNA transcript-injected oocytes was measured in NaCl transport buffer in the absence (control) or in the presence of 1 mM competing non-radioactive amino acids (leucine, lysine, alanine or phenylalanine) and compared with influx in water-injected oocytes. Non-radioactive amino acids were added to incubations at the same time as [^{14}C]leucine. Each value is the mean ± S.E.M. for 10–12 oocytes.

	Leucine influx (pmol/h per oocyte)	
Addition	4F2hc	Water
None (control)	322 ± 18	99 ± 4
Leucine	129 ± 15	52 ± 6
Lysine	165 ± 7	50 ± 3
Alanine	298 ± 19	75 ± 6
Phenylalanine	302 ± 21	85 ± 7

353 ± 22 pmol/h per oocyte, compared with 80 ± 4 pmol/h per oocyte in control water-injected oocytes. These values give a net 4F2hc-induced transport activity (influx in RNA-injected oocytes *minus* influx in water-injected oocytes) of 273 ± 22 pmol/h per oocyte and a ratio of 4F2hc-induced flux to endogenous flux of 3.4:1. In the absence of Na^+, leucine influx was 18 ± 1 and 9 ± 1 pmol/h per oocyte, respectively. Similar patterns of Na^+ dependence were found in eight additional independent experiments, demonstrating that both endogenous and 4F2hc-induced transport activities were largely Na^+-dependent. In the nine experiments, leucine flux ratios (0.2 mM) in NaCl transport buffer ranged between 2.2 and 11.1 (mean 5.4 ± 1.2).

These results contrast markedly with results obtained previously with rat glioma 4F2hc, where induced fluxes of isoleucine were reported to be Na^+-independent [38]. In that study, the basal rate of 0.1 mM isoleucine uptake by water-injected oocytes was less than 1 pmol/h per oocyte, compared with 80 pmol/h per oocyte for 0.2 mM leucine in Figure 2; 4F2hc-induced isoleucine transport activities (0.1 mM) were in the range 50–100 pmol/h per oocyte [38].

Figure 3 presents representative data for the concentration dependence of leucine influx in oocytes injected with either rat jejunal 4F2hc RNA or water, measured in the presence and in the absence of Na^+ over the concentration range 0.1–5.0 mM. Transport was resolved into saturable and non-saturable components, the former being Na^+-dependent. Non-saturable leucine influx was largely Na^+-independent. 4F2hc expression was associated with an increase in apparent K_m and V_{max} for saturable leucine influx, and an increase in K_d for non-saturable leucine influx (Table 1). The saturable component of uptake was the major route of 0.2 mM leucine transport in both RNA-injected and water-injected oocytes. Induction of more than one transport activity has also been reported for BAT [45,46].

The net leucine flux (0.2 mM) induced by rat jejunal 4F2hc was inhibited by 1 mM non-radioactive lysine (and leucine), but not by alanine or phenylalanine (Table 2). This inhibition pattern is consistent with previous studies on human 4F2hc [35–37], but differs from that of rat glial 4F2hc [38], where high concentrations of alanine and phenylalanine were inhibitory. Endogenous leucine transport activity in water-injected oocytes was inhibited by each of the amino acids tested (lysine, leucine, alanine, phenylalanine) (Table 2).

Table 3 Rat jejunal 4F2hc-induced lysine influx

Oocytes injected with either 10 nl of RNA transcript (1 ng/nl) or 10 nl of water were incubated for 5 days at 18 °C in MBM. Influx of lysine (0.2 mM, 20 °C, 30 min flux) was determined in transport buffer containing either 100 mM NaCl or 100 mM choline chloride. Each value is the mean ± S.E.M. for 10–12 oocytes.

	Lysine influx (pmol/h per oocyte)	
Transport medium plus	4F2hc	Water
NaCl	289 ± 18	46 ± 6
Choline chloride	255 ± 9	27 ± 6

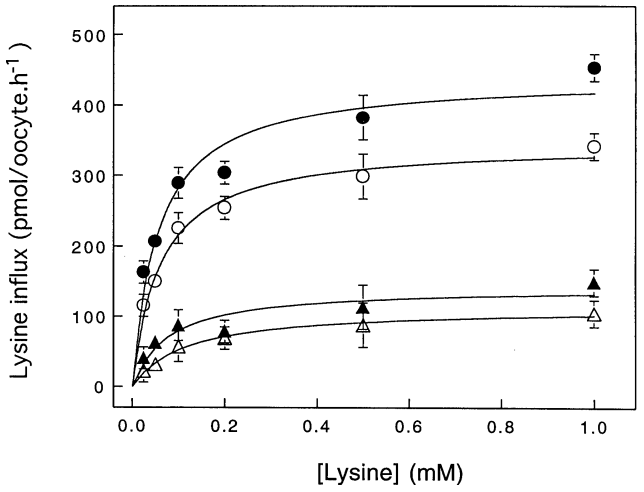

Figure 4 Concentration-dependence of rat jejunal 4F2hc-induced lysine influx

Oocytes injected with either 10 nl of RNA transcript (1 ng/nl) (○,●) or 10 nl of water (△,▲) were incubated for 5 days at 18 °C in MBM. Influx of lysine (25 μM to 1 mM, 20 °C, 30 min flux) was determined in transport buffer containing 100 mM NaCl (●,▲) or 100 mM choline chloride (○,△). Each value is the mean ± S.E.M. for 10–12 oocytes. Kinetic constants were determined by non-linear regression analysis (ENZFITTER, Elsevier-Biosoft) and are reported in Table 1.

Lysine transport

Uptake of [^{3}H]lysine (0.2 mM) by oocytes injected with rat jejunal 4F2hc RNA transcript or water was linear for 30 min at 20 °C, so this incubation period was used to determine initial rates of lysine transport. In the representative experiment shown in Table 3, influx of lysine (0.2 mM) in RNA-injected oocytes measured in NaCl transport buffer was 289 ± 18 pmol/h per oocyte, compared with 46 ± 6 pmol/h per oocyte for control water-injected oocytes, giving a net 4F2hc-induced lysine transport activity of 243 ± 19 pmol/h per oocyte and a ratio of 4F2hc-induced to endogenous flux of 5.3:1. Corresponding values for lysine influx measured in choline chloride transport buffer were 255 ± 9 and 27 ± 6 pmol/h per oocyte respectively, demonstrating that, in contrast with that for leucine, the expressed lysine transport activity was largely Na^+-independent. This was confirmed in concentration dependence studies (0.025–1 mM). Transport was saturable and largely Na^+-independent (Figure 4). 4F2hc expression was associated with an increase in V_{max}, with little change in apparent K_m (Table 1). Competition of net lysine influx (0.2 mM) with other amino acids (10 mM) in the presence of Na^+ gave almost complete inhibition by leucine and arginine, and smaller amounts of inhibition by alanine, valine and phenyl-

Table 4 Inhibition of lysine influx by amino acids

Lysine influx (0.2 mM, 20 °C, 30 min flux) was measured in RNA transcript-injected oocytes in the absence (control) or presence of 10 mM competing non-radioactive amino acids (arginine, leucine, alanine, valine or phenylalanine) and compared with influx in water-injected oocytes. Non-radioactive amino acids were added to incubations at the same time as [^{3}H]lysine. Each value is the mean ± S.E.M. for 10–12 oocytes.

		Lysine influx (pmol/h per oocyte)	
Transport medium plus	Addition	4F2hc	Water
NaCl	None (control)	224 ± 18	63 ± 4
	Arginine	12 ± 2	8 ± 4
	Leucine	16 ± 2	9 ± 1
	Alanine	129 ± 14	27 ± 3
	Phenylalanine	103 ± 7	20 ± 1
	Valine	139 ± 13	30 ± 3
Choline chloride	None (control)	155 ± 15	33 ± 2
	Arginine	11 ± 2	9 ± 1
	Leucine	123 ± 11	27 ± 2
	Alanine	125 ± 11	35 ± 3
	Phenylalanine	125 ± 7	23 ± 2
	Valine	161 ± 16	33 ± 3

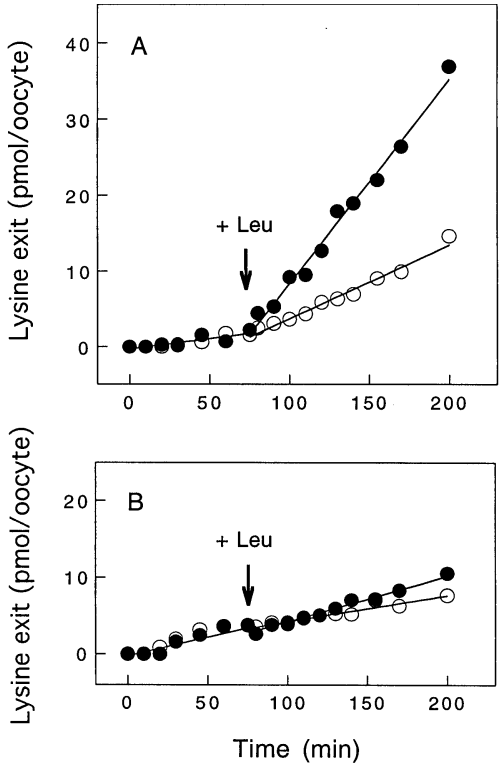

Figure 5 Stimulation by leucine of lysine efflux from *Xenopus* oocytes

Batches of 25 oocytes that had been injected with 10 nl of rat jejunal 4F2hc RNA transcript (1 ng/nl) (**A**) or 10 nl of water (**B**) and incubated for 5 days at 18 °C in MBM were preloaded with 0.2 mM [^{3}H]lysine as described in the text. Efflux at 20 °C was measured in NaCl transport buffer (●) or in choline chloride transport buffer (○). At the time indicated by the arrows, non-radioactive leucine was added to give an extracellular concentration of 5 mM. Values are means of duplicate determinations.

alanine (Table 4). Replacement of Na^+ with choline had no effect on arginine inhibition, but markedly decreased the extent of inhibition by leucine. Na^+ replacement also abolished valine inhibition and decreased the extent of inhibition by alanine and phenylalanine. Endogenous lysine transport activity in water-injected oocytes showed broadly similar characteristics (Table 4).

Representative time courses for [^{3}H]lysine efflux are shown in Figure 5. Exit from oocytes expressing recombinant 4F2hc was initially slow, Na^+-independent and similar in rate to that found in water-injected ooyctes. The addition of extracellular leucine resulted in a 12-fold *trans*-stimulation of lysine efflux. This increase in lysine exit was markedly decreased in choline chloride transport buffer, consistent with heteroexchange of intracellular lysine for extracellular (Na^+ plus leucine). Extracellular leucine had a much smaller effect on lysine exit from water-injected oocytes.

DISCUSSION

Both Na^+-dependent and Na^+-independent transport of cationic amino acids in the intestinal brush border membrane has been described, although the existence of both activities in all parts of the small intestine and in all species is not a consistent finding. This transport, attributed to systems $B^{o,+}$, $b^{o,+}$ and y^+ [47–51], is subject to regulation during ontogeny [52] and in response to changes in dietary protein load [53]. Subsequent efflux across the basolateral membrane against an outside-positive membrane potential represents the rate-limiting step of cationic amino acid absorption [54]. In the rat and other species, this process is stimulated (not inhibited) by zwitterionic amino acids [55–58] and is consistent with electroneutral heteroexchange of cationic amino acid with extracellular (Na^+ plus zwitterionic amino acid), mediated by system y^+L. A similar apical/basal disposition of cationic amino acid transport systems has been proposed for kidney [31] and placenta [37,59–61]. The latter tissue lacks $b^{o,+}$ but also functions as a polarized epithelium. Gestational changes in system y^+L transport activity in rat placenta are correlated with changes in the relative abundance of 4F2hc transcript [61], and the induction of system y^+L transport activity has been demonstrated in *Xenopus* oocytes injected with mRNA from a human placental (JAR) cell line [37]. Hybrid-depletion of JAR mRNA with a 4F2hc anti-sense oligonucleotide abolished the flux. In kidney, 4F2hc has been localized to basolateral membranes of the proximal tubule [31].

We have studied lysine transport in oocytes injected with mRNA extracted from mucosal scrapings of rat jejunum [41]. Three distinct transport activities were induced, corresponding to systems y^+L, $b^{o,+}$ and $B^{o,+}$. Multiple transport activities were also observed with leucine as permeant [40]. In the experiments described here, a rat jejunal cDNA library [39] was screened for expression of lysine/leucine transport activity. Pools of clones were also tested by diagnostic PCR for 4F2hc and BAT, both of which have been shown to be present in rat jejunum [40]. All pools were negative for BAT. Induction of transport activity coincided with expression of 4F2hc, resulting in the isolation of a full-length cDNA (pSY1) of 1797 bp encoding a rat jejunal 4F2hc protein with 527 residues.

Rat jejunal 4F2hc and its human and mouse isoforms [27–29] are homologous with BAT [15–17]. The alignment shown in Figure 1 between rat jejunal 4F2hc and rat BAT identifies four C-terminal (extracellular) regions of amino acid sequence that are particularly conserved, corresponding to rat jejunal 4F2hc residues (1) 169–178, (2) 231–239, (3) 349–359 and (4) 457–463. One cysteine residue (Cys-103) is common to rat 4F2hc and BAT, and to their homologues in other species. This residue, which is predicted to be extracellular and adjacent to the 4F2hc transmembrane domain, might represent the site of disulphide linkage between 4F2hc and 4F2lc and, correspondingly, between the two subunits of the BAT heterodimer. BAT has been suggested to contain between one and four transmembrane domains [9–11]. Our analysis of the deduced amino acid sequence of rat BAT by various algorithms, including that of Kyte and Doolittle [42], identified a single potential transmembrane domain located in a position (rat BAT residues 86–106) corresponding closely to that occupied by the rat jejunal 4F2hc transmembrane region (Figure 1).

Twelve different mis-sense mutations of the human BAT gene have been demonstrated in cystinuria [21–24]. Five of the eleven affected residues are common to BAT and 4F2hc, suggesting parallel functions in the two proteins. For example, the amino acid residue most commonly mutated in cystinuria (Met-467 of human BAT, residue 464 of rat BAT) is replaced conservatively by a leucine residue (Leu-349) in rat jejunal 4F2hc (Figure 1). Similarly, Arg-181 of human BAT (residue 178 of rat BAT) corresponds to Lys-171 of 4F2hc. Both these residues are within conserved regions of the 4F2hc/BAT amino acid sequence. Human BAT mutation sites Tyr-582, Pro-615 and Phe-648 correspond to residues 579, 613 and 646 of rat BAT, and to Tyr-447, Pro-478 and Leu-506 of rat jejunal 4F2hc. A C-terminal leucine zipper motif (Leu-548, Leu-555, Leu-562 and Leu-569 of rat BAT) is absent from rat jejunal 4F2hc.

Injection of oocytes with rat jejunal 4F2hc RNA transcript induced an up to 11-fold increase in leucine influx, compared with approx. 2-fold reported previously for human fibroblast 4F2hc [35,36]. The major component of expressed leucine transport activity was Na^+-dependent, saturable and inhibited by lysine. Rat jejunal 4F2hc also induced saturable, Na^+-independent uptake of lysine. This lysine flux was inhibited by both zwitterionic (leucine $\gg$ phenylalanine, valine, alanine) and cationic (arginine) amino acids, but the interaction with zwitterionic amino acids was abolished in the absence of Na^+. Therefore rat jejunal 4F2hc induced an amino acid transport process that was both Na^+-dependent (zwitterionic amino acid transport) and Na^+-independent (cationic amino acid transport). Apparent K_m values in sodium-containing medium were approx. 100 and 50 μM for leucine and lysine respectively, and the system exhibited selectivity for leucine over other zwitterionic amino acids. These characteristics identify the expressed transport activity as system y^+L, a process first reported in human erythrocytes and also described in placenta [5,62]. Like rat jejunal 4F2hc, the erythrocyte and placental y^+L transporters mediate the Na^+-dependent transport of zwitterionic amino acids and the Na^+-independent transport of cationic amino acids [5,62]. Inhibition of cationic amino acid transport by zwitterionic amino acids was Na^+-dependent, with leucine showing greatest inhibition. Apparent substrate affinities for zwitterionic and cationic amino acids were similar (10–30 μM) to those reported here for 4F2hc.

The functional properties of the rat jejunal 4F2hc-induced transport activity were similar to, but not identical with, those exhibited by endogenous mechanism(s) of leucine and lysine transport in control water-injected oocytes. This was most noticeable in exit experiments, which demonstrated Na^+-dependent, 4F2hc-induced *trans*-stimulation of lysine efflux by extracellular leucine. Similar findings have been reported recently for human 4F2hc [63] and suggest that 4F2hc and its associated protein(s) function as electroneutral tertiary active amino acid transport systems with obligatory exchange characteristics. Cationic heteroexchange of amino acid with (Na^+ plus zwitterionic

amino acid) provides a potential mechanism to allow the exit of cationic amino acids across intestinal, renal and placental basal membranes against an outside-positive membrane potential, and is consistent with the long-standing observation that efflux of cationic amino acids across the enterocyte basolateral membrane is stimulated by vascular leucine [55–58]. BAT (system $b^{o,+}$), in contrast, which has a brush border localization in intestinal and kidney epithelia, induces the Na^+-independent electrogenic exchange of cationic and zwitterionic amino acids [64–66].

Our results with rat jejunal 4F2hc, and those published previously for human 4F2hc [35,36,63] are at variance with oocyte expression experiments reported by Broër et al. [38] for rat glioma 4F2hc, which showed that induced isoleucine transport activity was Na^+-independent and inhibited by alanine and phenylalanine. It was proposed that the different transport characteristics of human and rat 4F2hc could be due either to the low expression of the human transcript or to the difference in amino acid sequence between human and rat 4F2hc [38]. Neither possibility can explain the discrepancy with our results because (1) our expressed amino acid transport activities were similar in magnitude to those found for rat glial 4F2hc, and (2) rat jejunal 4F2hc and rat glial 4F2hc have identical amino acid sequences.

Unlike conventional amino acid transporter proteins, such as those belonging to the CAT gene family, 4F2hc and BAT have single, or in BAT up to four, transmembrane domains. Both have a conserved extracellular cysteine residue that, in 4F2hc, is postulated to form a disulphide linkage with 4F2lc, a protein whose primary structure and function are unknown. Theoretical models have been proposed for the 4F2 transport assembly in which 4F2lc is the catalytic (transporting) subunit and 4F2hc functions as a regulatory/modulatory subunit [9–11]. A corresponding subunit model for BAT is supported by immunological studies that found disulphide linkage of native BAT to unidentified rat and rabbit intestinal and renal brush border membrane proteins [14]. Recombinant BAT formed an equivalent linkage with oocyte plasma membrane proteins [14]. The amino acid transport phenotype(s) induced by heterologous expression of recombinant 4F2hc/BAT in oocytes might therefore be influenced by their interaction with catalytic subunits endogenous to the oocyte plasma membrane. Such proteins might be subject to genetic, physiological and/or biochemical variability. Evidence that oocytes might have the potential for such variability is provided by the different endogenous isoleucine fluxes reported in two studies by Broër et al. [38,67] and by that group's description of 4F2hc-induced Na^+-independent zwitterionic amino acid transport activity [38], if substantiated. Our results were obtained with oocytes that exhibited endogenous amino acid transport capability. The experiments describing expression of the rat glial 4F2hc cDNA, in contrast, were performed on oocytes with very low background fluxes [38].

In summary, a cDNA encoding a 4F2hc-type membrane protein was isolated from a rat jejunal cDNA library. Rat jejunal 4F2hc is homologous with the BAT proteins that have a physiological role in the epithelial brush border transport of cationic amino acids and L-cystine. Expression of recombinant rat jejunal 4F2hc in *Xenopus* oocytes induced the Na^+-dependent influx of leucine, the Na^+-independent influx of lysine and the Na^+-dependent leucine stimulation of lysine efflux. These characteristics identified the expressed transport activity as amino acid transport system y^+L and are consistent with other evidence suggesting that 4F2hc might have a role in the basal membrane transport of cationic amino acids in intestine, kidney and placenta. Mutations in the human BAT gene cause the genetic disease cystinuria [18–25]. By analogy, 4F2hc is a plausible candidate for a second human amino acid transport disorder, lysinuric protein intolerance [68]. This autosomal recessive disease is associated with the decreased intestinal absorption (and increased renal excretion) of cationic amino acids and results from a specific defect in cationic amino acid transport at the basolateral membrane of both tissues.

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