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Identification and Characterization of a Membrane Protein (y^+L Amino Acid Transporter-1) That Associates with 4F2hc to Encode the Amino Acid Transport Activity y^+L

A CANDIDATE GENE FOR LYSINURIC PROTEIN INTOLERANCE*

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We have identified a new human cDNA (y^+L amino acid transporter-1 (y^+LAT-1)) that induces system y^+L transport activity with 4F2hc (the surface antigen 4F2 heavy chain) in oocytes. Human y^+LAT-1 is a new member of a family of polytopic transmembrane proteins that are homologous to the yeast high affinity methionine permease MUP1. Other members of this family, the *Xenopus laevis* IU12 and the human KIAA0245 cDNAs, also co-express amino acid transport activity with 4F2hc in oocytes, with characteristics that are compatible with those of systems L and y^+L, respectively. y^+LAT-1 protein forms a ≈135-kDa, disulfide bond-dependent heterodimer with 4F2hc in oocytes, which upon reduction results in two protein bands of ≈85 kDa (*i.e.* 4F2hc) and ≈40 kDa (y^+LAT-1). Mutation of the human 4F2hc residue cysteine 109 (Cys-109) to serine abolishes the formation of this heterodimer and drastically reduces the co-expressed transport activity. These data suggest that y^+LAT-1 and other members of this family are different 4F2 light chain subunits, which associated with 4F2hc, constitute different amino acid transporters. Human y^+LAT-1 mRNA is expressed in kidney ≫ peripheral blood leukocytes ≫ lung > placenta = spleen > small intestine. The human *y^+LAT-1* gene localizes at chromosome 14q11.2 (17cR ≈ 374 kb from D14S1350), within the lysinuric protein intolerance (LPI) locus (Lauteala, T., Sistonen, P., Savontaus, M. L., Mykkanen, J., Simell, J., Lukkarinen, M., Simmell, O., and Aula, P. (1997) *Am. J. Hum. Genet.* 60, 1479–1486). LPI is an inherited autosomal disease characterized by a defective dibasic amino acid transport in kidney, intestine, and other tissues. The pattern of expression of human y^+LAT-1, its co-expressed transport activity with 4F2hc, and its chromosomal location within the LPI locus, suggest *y^+LAT-1* as a candidate gene for LPI.

rBAT and 4F2hc are homologous proteins that induce amino acid transport in *Xenopus* oocytes (1, 2). These two proteins are slightly hydrophobic, which prompted the hypothesis that rBAT and 4F2hc are subunits or modulators of the corresponding amino acid transporter. This has been supported by several indirect observations: (i) rBAT and 4F2hc are involved in the induction of several activities in *Xenopus* oocytes (3–6); (ii) these two proteins can be immunodetected or immunoprecipitated as complexes of ≈125 kDa in the absence of reducing agents and as two proteins of ≈85 kDa (4F2hc or rBAT) and ≈40 kDa in the presence of reducing agents (7–9); and (iii) in oocytes, there is a dissociation between the expression of 4F2hc and rBAT at the plasma membrane and the induction of system y^+L and $b^{0,+}$ activity, respectively, indicating that this expression is limited by an endogenous factor (10, 11). We have recently provided new evidence that the amino acid transport system y^+L has a heterodimeric structure (11). Thus, we have shown that the y^+L activity induced in oocytes by a cysteine-less mutant of human 4F2hc is also inactivated by membrane-impermeant thiol-specific reagents, implying that another protein is required for this function, which would have external cysteine(s) that are targets of these reagents. Moreover, the sensitivity to inactivation is increased by reducing conditions and in 4F2hc mutants in which cysteine 109 has been mutated. These results indicate that Cys-109 may be linked by a disulfide bond to the cysteine target of these agents of the associated protein.

ASUR4 (Y12716), an adrenal steroid up-regulated cDNA from *Xenopus laevis* A6 cells (12) induces an L-type amino acid transport activity when co-expressed with 4F2hc in oocytes.[1] When comparing the amino acid sequence of ASUR4 to protein data bases, many highly homologous eukaryotic and prokaryotic amino acid transporter-related proteins are listed within the amino acids, polyamines, and choline (APC) family of transporters. Among these, and with highest degree of identity (between 38 and 82% when corresponding protein regions are compared) to ASUR4, are found its counterpart in human, E16 (Q01650) (13), and in rat, TA1 (Q63016) (14); a human cDNA, termed KIAA0245 (D87432) (15); five different *Caenorhabditis elegans* open reading frames deduced from genomic DNA sequence (Z68216, U50308, Z74042, U56963, and U70850) (16); and a *Schistosoma mansoni* cDNA, SPRM1 (L25068). In the same list appeared a yeast high affinity methionine permease, MUP1 (17) (U40316), and many other prokaryotic amino acid permeases. ASUR4 showed low, although significant, identity

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF092032.

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[1] F. Verrey, personal communication.

(between 26 and 31%) with the mammalian transporters for cationic amino acids CAT1[2] and CAT2 (18, 19). Human E16 was first identified from peripheral blood leukocytes and related to lymphocyte activation (13). Rat TA1 was cloned later on the basis of its differential expression between hepatoma cells and normal liver (14). E16, TA1, and ASUR4 cDNA were first described as proteins 241 amino acids long. The presence in the data base of a thyroid hormone regulated *X. laevis* cDNA, termed IU12 (AF019906) (20), which was 507 amino acid long and practically identical to ASUR4 (only one amino acid was different in the corresponding protein region), suggested that the former three cDNAs were indeed longer. Very recently, F. Verrey has submitted a new ASUR4 cDNA GenBank entry (accession number Y12716), which also has 507 amino acids. Although IU12 and the new entry of ASUR4 still differ in four disperse amino acids, we can consider that both sequences correspond to the same gene in *Xenopus*. We can now assume that E16 and TA1 are actually longer proteins.

In this study, we have identified a new human member of this group of amino acid permease-related proteins. This protein, which we have named y⁺L amino acid transporter-1 (y⁺LAT-1) does not induce transport of amino acids in oocytes when injected alone, but y⁺L activity is co-expressed when it is injected with 4F2hc. We demonstrate here that it forms an heterodimer with 4F2hc linked by disulfide bridges with residue cysteine 109 of human 4F2hc. Its pattern of expression and its chromosomal localization indicate that this gene could be responsible for lysinuric protein intolerance (21), an inherited disorder of cationic amino acid transport.

EXPERIMENTAL PROCEDURES

Oocytes, Injections, and Uptake Measurements—Oocyte origin, management, and injections were as described elsewhere (1, 2). Defolliculated stage VI *X. laevis* oocytes were injected with different amounts of human 4F2hc, human y⁺LAT-1, human y⁺LAT-2 (KIAA0245), or *X. laevis* IU12 cRNA. Amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those of uninjected oocytes (data not shown). Synthesis of human 4F2hc cRNA (22) was as described (11). IU12 was a gift from Shi and co-workers (20), and the cRNA was obtained by cutting the cDNA, cloned in pBluescript SK⁻ between the sites *Xho*I and *Eco*RI with *Apa*I and using T3 polymerase. The open reading frame of y⁺LAT-1 was obtained from the Integrated and Molecular Analysis of Genomes and their Expression (IMAGE) cDNA clone 727811 cloned in the vector pT7T3 between the restriction sites *Eco*RI and *Not*I. To obtain the y⁺LAT-2 cRNA, because it has a long 3′-untranslated region and is not expressed properly in *Xenopus* oocytes, we inserted the open reading frame of KIAA0245 (obtained from Takahiro Nagase from the Kasuza DNA Research Institute, (15) and cloned in pBluescript II SK⁺) in another vector with a shorter 3′ tail. Subcloning was done by cutting pBluescript-KIAA0245 with *Apa*I and filling with Klenow; the clone was then ethanol-precipitated, cut again with *Pst*I, and finally ligated into pSPORT1-human rBAT that had been cut with *Pst*I and *Bst*1107I. Influx rates of L-[³H]arginine, L-[³H]leucine were measured in 100 mM NaCl or 100 mM choline Cl medium at the indicated days after injection and under linear conditions. When presented, the induced uptake was calculated by subtracting uptake values in uninjected oocytes from those of the corresponding cRNA-injected oocytes.

PCR Amplification and Sequencing—For PCR amplification, first strand cDNA was synthesized from 5 μg of total RNA purified from opossum kidney (23) cells using the SuperScript II kit (Life Technologies, Inc.). Two degenerate forward and reverse primers were designed based on two highly conserved regions among KIAA0245, IU12, E16, TA1, and SPRM1 proteins. From region 1 sequence (A/S)REGHLP (corresponding to residues 347–353 of KIAA0245), a forward 5′-C(C/T)-(C/A)G(C/A/G)GA(G/A)GG(C/G)CA(C/T)CT(G/C/T/A)CC-3′ primer (1F) was synthesized, as well as a reverse (2R) 5′-A(T/G)G(A/C)(T/A)(A/G)-AA(C/G)A(C/A)(C/G)A(C/T)(T/A/G)GG-3′ primer deduced from region 2 sequence P(I/V)(V/F)F(I/C)(I/L) (corresponding to residues 429–434 of KIAA0245). Amplification was carried out in a Perkin-Elmer 9600 thermocycler, and conditions were as follows: hot start of 3 min at 94 °C; 15 cycles of denaturing (94 °C for 25 s), annealing (starting 65–50 °C lowering 1 °C each cycle for 30 s), and extension (74 °C for 70 s); 25 cycles of denaturing (94 °C for 25 s), annealing (50 °C for 30 s), and extension (74 °C for 70 s); and a final extension of 4 min at 72 °C. PCR-amplified DNA fragments with the expected length were subcloned into pGEM-T easy vector (Promega) and sequenced in one direction. The DNA sequence obtained and all frames of the deduced amino acid sequences were then compared with DNA and protein sequence data bases. All sequences carried out in this work were performed in one or both directions (in the case of clone 727811) with D-Rhodamine Dye Terminator Cycle Sequencing Ready Reaction (Perkin-Elmer). Analysis of the sequence reactions was done with an Abi Prism 377 DNA sequencer.

Computer Analysis—Amino acid or nucleotide sequence homology searching was performed using basic local alignment tool (BLAST) via on-line connection to the National Center of Biotechnology Information. The programs BLASTn, BLASTp, and BLASTx were run using default parameters. Data base searching was done against nonredundant or dbEST, when searching for nucleotide sequence homology, and *versus* nonredundant when comparing to peptide sequences. The clusters of Expressed Sequence Tag were identified and analyzed with the IMAGE data base and Telethon Institute of Genetics and Medicine EST assembly machine tool. Multiple nucleotide or amino acid sequence comparisons were done with CRUSTALW via on-line connection to the Genome World Wide Web server (University of Tokyo) and to the Baylor College of Medicine Search Launcher (University of Texas). Amino acid sequence deduction and other sequence analysis were done with Genetics Computer Group utilities.

The prediction of transmembrane segments of the proteins y⁺LAT-1, y⁺LAT-2, IU12, and SPRM1 was established on the basis of the combination of three criteria: (i) the prediction of transmembrane segments by the programs of Aloy *et al.* (23) and TopPred II (24) using the algorithms of G. von Heijne (25), Goldman, Engelman, and Steitz (26), and K_D (27) to determine the position of hydrophobicity peaks; (ii) the prediction of α-helix in the predicted secondary structure using a program that combines the algorithms of Chou-Fasman and Rose (28); and (iii) the surface probability and flexibility index plots, according to the algorithms of Boger (29) and Karplus and Schulz (30), respectively.

Northern Blot Analysis—A human adult poly(A⁺) membrane containing 12 different tissues, purchased from CLONTECH (Palo Alto, CA) was used. Insert of clone 727811 was separated from the pT7T3D-727811 vector with *Apa*I-*Not*I digestion. This 2250-bp-long DNA fragment was purified, labeled with [α-³²P]dCTP (Amersham) using a random oligonucleotide-priming labeling kit (Amersham), and used as a probe. Hybridization, carried out in Express HybTM Hybridization solution (CLONTECH), and wash conditions were as recommended by the manufacturer. To rule out differences in sample loading, the CLONTECH membrane was hybridized with human β-actin probe. A nonradioactive fluorescein and anti-fluorescein peroxidase-conjugated antibody detection kit was used (Amersham). Hybridization, washes, and detection conditions were as suggested by the supplier.

Chromosome Mapping—Chromosome mapping was done using the Stanford Human Genome Center G3 Radiation Hybrid panel (medium resolution). DNA samples of this panel, along with total genomic DNA and pT7T3-727811 (used as a positive controls), were PCR-screened for the presence of the genomic sequences flanked by the primers F7 (5′-GGAAGTTGAAAAGGAAAGC-3′) and R7 (5′-AAGGAGACAGGAAATTGG-3′), which are located at the 3′-untranslated region of the cDNA. PCR amplifications were carried out in a Perkin-Elmer 9600 thermocycler, using 200 μM dNTP, 3 pmol of each primer and DNA *Taq* polymerase (Boehringer Mannheim) in PCR buffer. Amplification conditions were as follows: 35 cycles of denaturing (94 °C for 30 s), annealing (56 °C for 40 s), and extension (74 °C for 30 s). PCR results were classified as 0 (for no amplification), 1 (for positive amplification), or r (for uncertain) and submitted to the Radiation Hybrid Mapping E-mail server at the Stanford Human Genome Center (SHGC). Resulting chromosomal location, referred to a SHGC marker, was obtained automatically via E-mail from this server.

Site-directed Mutagenesis—Construction of the mutants C109S and C330S of human 4F2hc was as described in Ref. 11.

Immunoprecipitation of Methionine-labeled Proteins from Xenopus Oocytes—Oocytes were injected with 10 ng of human 4F2hc or C109S human 4F2hc (CS1) or C330S human 4F2hc (CS2) alone or in combi-

[2] The abbreviations used are: CAT, cationic amino acid transporter; LAT, L amino acid transporter; LPI, lysinuric protein intolerance; IMAGE, Integrated and Molecular Analysis of Genomes and their Expression; SHGC, Stanford Human Genome Center; PCR, polymerase chain reaction; bp, base pair(s).

nation with 10 ng of y$^+$LAT-1 cRNA. After 24 h, [^{35}S]methionine (0.5 μCi in 50 nl of water; ICN) was injected, and the oocytes (usually 20 oocytes) were incubated for 48 h at 18 °C in 1 ml of modified Barth's solution. Oocytes were then harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethanesulfonyl fluoride. Extracts were centrifuged twice at 1000 $\times$ *g* in order to remove the yolk granules. Aliquots of 10^6 cpm were rotated overnight at 4 °C with 20 μl of human 4F2hc antibody (Immunotech, Marseille, France) previously bound to protein G-Sepharose (Sigma). The beads were washed five times in a buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 500 mM LiCl, and 0.5% Nonidet P-40 and five times in the same buffer without LiCl. The resulting immunoprecipitates were heated in sample buffer with or without 100 mM dithiothreitol for 5 min at 95 °C before gel loading. The labeled proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography after enhancement with 1 M sodium salicylate.

RESULTS

Our goal was to identify any new member of the amino acid transporter-related family expressed in kidney and potentially involved in reabsorption of amino acids. For this purpose reverse transcription PCR amplification of total RNA from opossum kidney cells (32) was performed. Degenerated primers were designed on the basis of two highly conserved protein regions (see under "Experimental Procedures") revealed after a multiple amino acid sequence comparison among KIAA0245, E16, TA1, IU12, and SPRM1 proteins. Electrophoretic analysis of the PCR showed one band of 228 bp, which was subcloned into pGEMT-easy vector and amplified in *Escherichia coli*. Several clones were then analyzed by sequencing. Nucleotide sequence of clone b4c2 showed a significant degree of identity to the amino acid transporter-related proteins when compared, using BLASTx program, to nonredundant peptide data bases. Deduced amino acid sequence comparison showed an identity of 75, 52, 49, 47, and 20% for b4c2 with KIAA0245, IU12, TA1, E16, and SPRM1, respectively. This high degree of identity with KIAA0245 suggested that we had cloned a fragment of the KIAA0245 ortholog cDNA in opossum. To rule out the presence of other human genes having high homology to KIAA0245, the coding region sequence of this gene was run as a query with BLASTn program against dbEST data bases. Flanking 3′- and 5′-untranslated regions were avoided to minimize the presence of KIAA0245 EST in the results. We identified an IMAGE EST cluster (46303) that corresponds to a new unidentified human gene with a high degree of identity (75%) to KIAA0245. EST AA393488 (located 5′ of this cluster) and EST AA400789 (located 3′ of this cluster and presenting a poly(A$^+$) tail) are flanking regions of IMAGE cDNA clone 727811 and comprised the whole cDNA. We named this clone y$^+$LAT-1 (y$^+$L amino acid transporter), and KIAA0245 tentatively as a y$^+$LAT-2 because they yielded system y$^+$L amino acid transport activity when co-injected with 4F2hc in oocytes (see below).

Two direction sequencing of clone 727811 (Fig. 1) showed a cDNA 2245 bp long. Sequence comparison of the corresponding region of y$^+$LAT-1 with the opossum b4c2 clone revealed 82 and 81% identity for DNA and protein, respectively. We then assumed that b4c2 clone is a fragment of the corresponding y$^+$LAT-1 in opossum. The size of the human y$^+$LAT-1 cDNA corresponds to the transcript seen in Northern blots (see Fig. 5). The first ATG codon lies within a good consensus initiation sequence (5′-CCACC) (33). The open reading frame continues to the first stop codon (TAA) at base 1820 and codes for a protein of 511 amino acid residues with a predicted molecular mass of 55,988 Da. The nucleotide sequence of y$^+$LAT-1 has been deposited in the GenBank data base (accession number: AF092032).

Hydrophobicity studies (see under "Experimental Procedures") show 12 transmembrane domains with both C- and N-terminal segments intracytoplasmatic, a typical protein structure similar to some previously reported organic solute transporters (34–36). There is only one putative N-glycosylation site underlined in Fig. 1 (Asn-Ala-Ser) between the putative transmembrane segments VIII and IX. In our prediction model, this segment is cytoplasmic and cannot be glycosylated. There are also two putative casein kinase II phosphorylation sites (threonine 8 and serine 11, located in the putative cytoplasmic N-terminal segment) and one putative protein kinase C phosphorylation site (threonine 96, located intracellularly between the putative transmembrane segments II and III). A multiple sequence alignment of the predicted amino acid sequence of y$^+$LAT-1, y$^+$LAT-2 (KIAA0245), IU12, E16, and SPRM1 is shown in Fig. 2. The percentages of identity between y$^+$LAT-1 and y$^+$LAT-2, E16, IU12, SPRM1, and the yeast permease MUP1 are 75, 51, 53, 39, and 31%, respectively. The predicted structural model of these proteins is also very similar. Only the consensus for the position of the transmembrane segment III can vary for the proteins presented in Fig. 2. For y$^+$LAT-1 and y$^+$LAT-2, this segment could be located in the position indicated in Fig. 2. However, in the case of IU12, the fragment is displaced 10 amino acids to the C-terminal end, and in the protein SPRM1, the fragment is moved 5 amino acids to the N-terminal end. Because 4F2hc is associated with y$^+$LAT-1 in a disulfide bond-dependent manner (see Fig. 7), we looked for cysteine residues conserved in these proteins. There are only two cysteines conserved in all these proteins: cysteine 151 of y$^+$LAT-1, located extracellularly in our structure model prediction, corresponding to residues 159, 164, and 137 of y$^+$LAT-2, IU12, and SPRM1, respectively; and cysteine 174, located in the transmembrane domain IV and corresponding to residues 182, 187, and 160 of y$^+$LAT-2, IU12, and SPRM1, respectively. These two cysteines are not conserved in the yeast permease MUP1.

The human *y$^+$LAT-1* gene was chromosome mapped by using a radiation hybrid panel (see under "Experimental Procedures") with primers corresponding to the 3′-untranslated region of the y$^+$LAT-1 cDNA. From this screening, we obtained 13 positive, 70 negative and 2 uncertain results. Chromosome mapping results, obtained from the SGHC server, linked *y$^+$LAT-1*, with a logarithm odds score of 10.4, to a distance of 17 cR (374 kb) from the marker SHGC-13532 (D14S1350) located at chromosome 14q11.2. When uncertain samples were submitted as positive, the localization was linked to the T-cell receptor α chain marker, which lies $\approx$150 kb telomeric of SHGC-13532.

cRNA from y$^+$LAT-1, y$^+$LAT-2, and IU12 was prepared and injected into oocytes alone or in combination with an equimolar quantity of human 4F2hc cRNA and tested for transport of arginine and leucine (50 μM) in the presence or in the absence (choline) of sodium (100 mM) (Fig. 3). These three proteins do not induce any amino acid transport activity when injected alone, but interestingly, they induce different activities when co-injected with 4F2hc. In the case of y$^+$LAT-1 and y$^+$LAT-2 (KIAA0245), the pattern of induced activity resembles that described as system y$^+$L (37) (*i.e.* sodium-independent uptake of dibasic amino acids and sodium-dependent uptake of some neutral amino acids). IU12, by contrast, induced an activity above that of 4F2hc alone, which is compatible with the activity described as system L (*i.e.* sodium-independent uptake of neutral amino acids). For y$^+$LAT-1, the induced activity is very similar to the component of y$^+$L activity induced by 4F2hc alone, but the level of induction is higher. From 10 independent experiments, the average fold induction relative to the induction of 4F2hc alone was 3.8 $\pm$ 0.9 (range, 2–14-fold). To explain this increase, we performed kinetic analysis, and from an in-

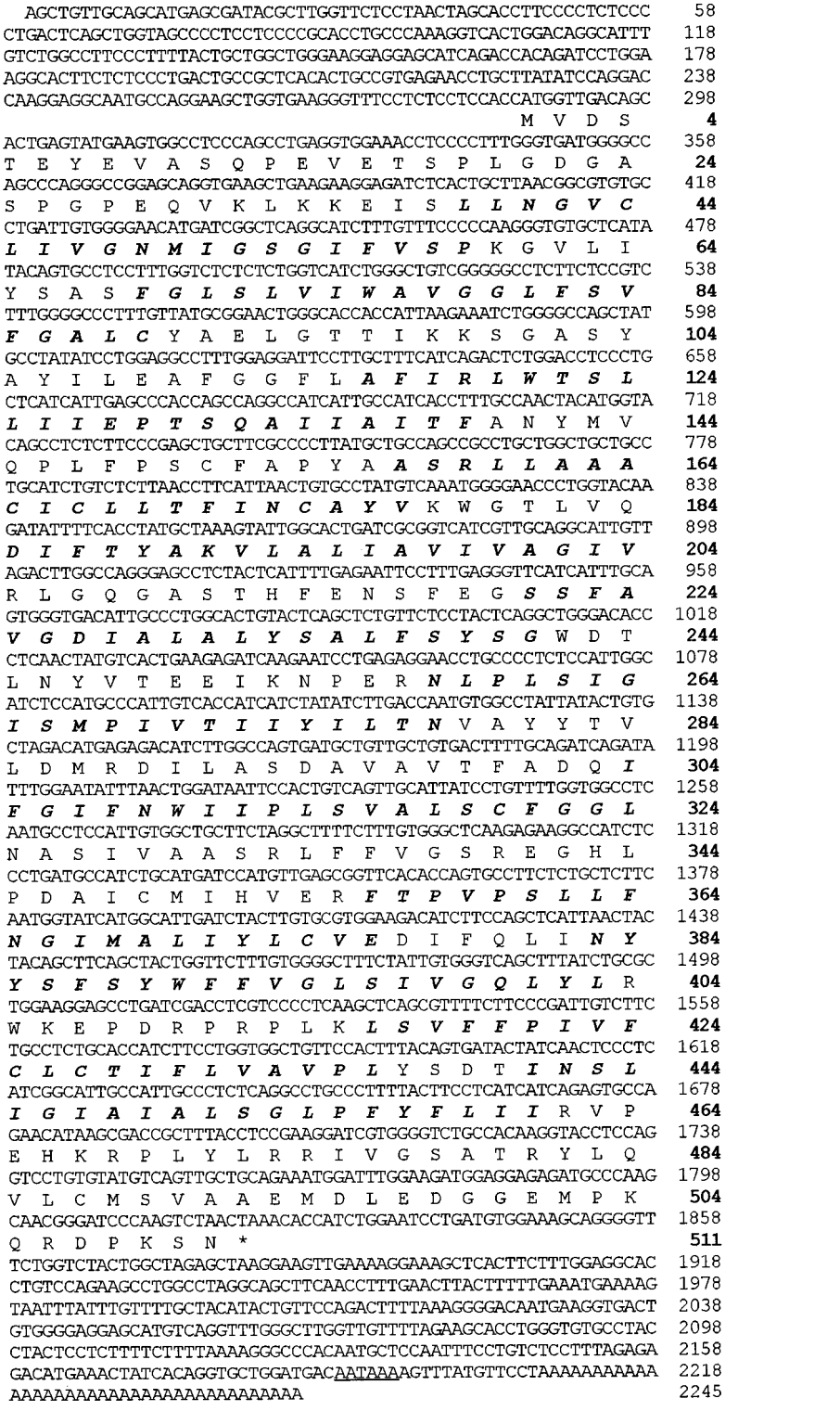

FIG. 1. **Nucleotide and deduced amino acid sequence of the y$^+$LAT-1 cDNA.** The size of the clone is 2245 bp and it contains a 5′-untranslated region of 268 bp, followed by an open reading frame of 511 amino acids and a 3′-untranslated region of 423 bp that contains a poly(A) tail of 38 bp. The stop codon (TAA) is indicated by one *star*. The possible polyadenylation signal is *underlined*. The putative transmembrane domains, deduced by hydrophobicity analysis (see under "Experimental Procedures"), are shown in *boldface*.

dividual experiment, the kinetic parameters showed an increase in V_{max} without apparent change in the $K_{0.5}$ parameter (4F2hc-induced uptake: $K_{0.5}$, 55 ± 15 μM; V_{max}, 18 ± 4 pmol of arginine (choline medium)/15 min per oocyte; 4F2hc plus y$^+$LAT-1-induced uptake: $K_{0.5}$, 45 ± 18 μM; V_{max}, 36 ± 5 pmol of arginine (choline medium)/15 min per oocyte). A further characterization of this transport activity co-expressed by y$^+$LAT-1 and y$^+$LAT-2 is in progress.[3]

To further characterize the uptake activity co-expressed by y$^+$LAT-1 and 4F2hc, we measured the inhibition of arginine uptake by different amino acids at a 100-fold excess concentration (5 mM). As shown in Table I, dibasic amino acids inhibit 50 μM arginine uptake in a sodium-independent manner, but in contrast, neutral amino acids inhibit more in the presence of sodium. In order to define better the effect of sodium on the inhibition by neutral amino acids, the uptake of L-arginine (50 μM) was measured in the presence or absence of sodium and in the presence of different concentrations of L-leucine (Fig. 4). These results showed clearly that sodium increased the affinity of L-leucine. This effect is indistinguishable in 4F2hc alone or

[3] M. Pineda, R. Estévez, and M. Palacín, manuscript in preparation.

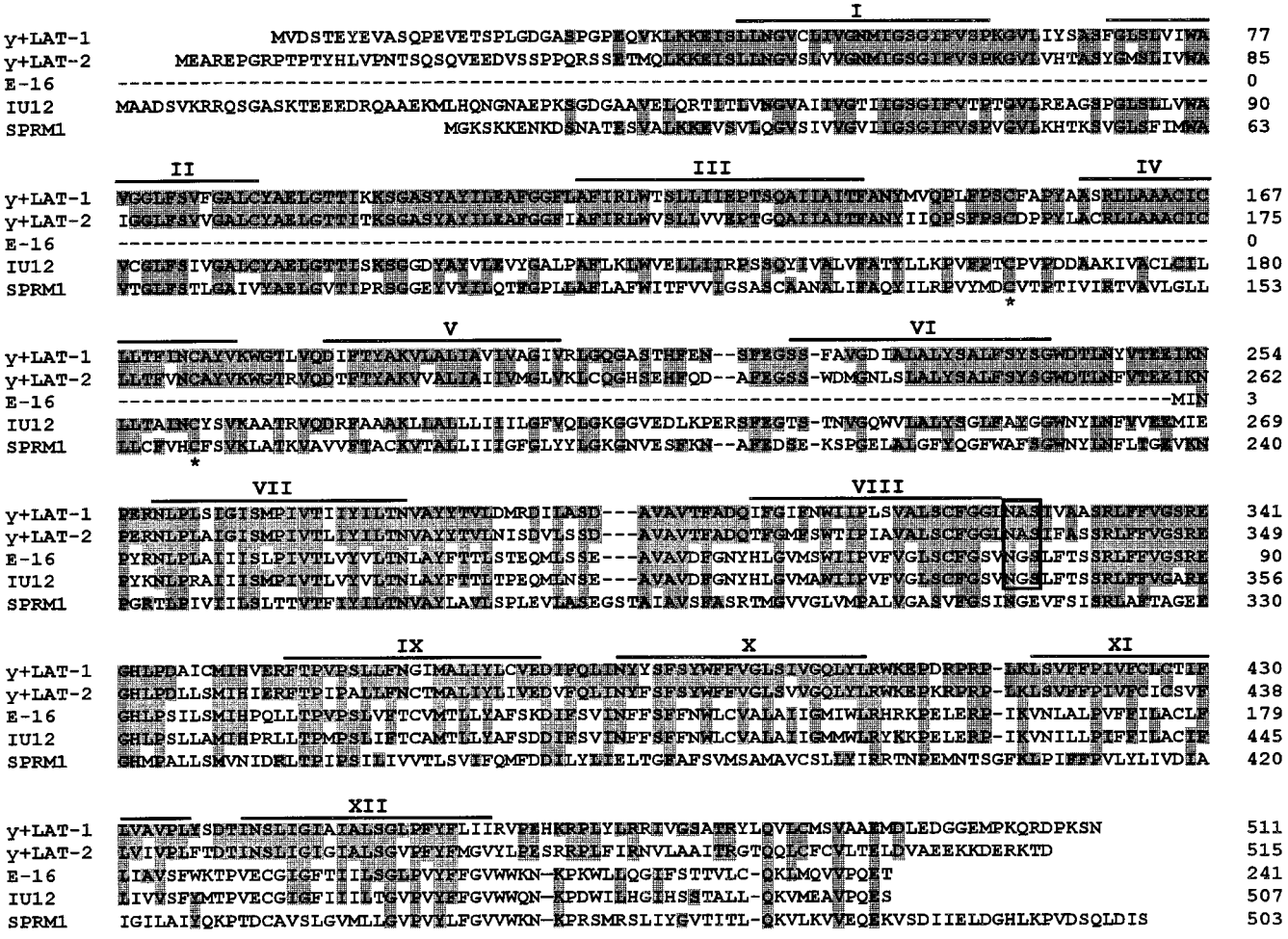

FIG. 2. **Amino acid sequence comparison of five members of the family of amino acid transporter-related proteins.** Multialignment was done using the program CLUSTALW Sequence Alignment from Baylor College of Medicine. The *thin horizontal lines* indicate the putative 12 transmembrane domains determined by computer analysis (see under "Experimental Procedures"). The amino acid residues identical to y⁺LAT-1 sequence are indicated by *gray boxes*. The *solid frame box* indicates a potential N-glycosylation site, but according to our membrane topology prediction, this site is intracellular and cannot be glycosylated. Two cysteine residues conserved in all the proteins presented here are indicated by a *star*.

4F2hc plus y⁺LAT-1-injected oocytes. All of this is consistent with the expression of y⁺L transport activity (38).

The tissue expression of the mRNA corresponding to y⁺LAT-1 was examined by Northern blot analysis at high stringency conditions (Fig. 5). The mRNA species of ≈2.4 kb hybridizes with the y⁺LAT-1 cDNA. Transcript expression is as follows: kidney ≫ peripheral blood leukocytes ≫ lung > placenta = spleen > small intestine.

Recently (11), we have postulated that residue cysteine 109 of human 4F2hc could be involved in the formation of a disulfide bond with a putative membrane protein already present in the *Xenopus* oocyte to express system y⁺L transport activity. To test whether this is also the case with human y⁺LAT-1 protein, we performed co-injection experiments with C109S (CS1) or C330S (CS2) human 4F2hc mutants (Fig. 6). The CS1 mutant injected alone led to a decrease of 56% in the induced activity compared with the wild type. This agrees with previous results (11) that showed a V_{max} decrease of 50% without changes in the $K_{0.5}$ parameter for this mutant. Moreover, CS1 co-injected with y⁺LAT-1 showed a 74% decrease in transport expression compared with wild type 4F2hc co-injected with y⁺LAT-1. In contrast, the CS2 4F2hc mutant showed no decrease in the induced activity when injected alone (similar to previous results; Ref. 11) or co-injected with y⁺LAT-1.

In the batch of oocytes used in the experiment shown in Fig. 6, we checked whether y⁺LAT-1 and 4F2hc proteins could form a heterodimeric structure via a disulfide bond. This was done by [^{35}S]methionine labeling and immunoprecipitation using a monoclonal antibody directed to human 4F2hc (Fig. 7). Under nonreducing conditions, two 4F2hc-specific protein bands were detected in 4F2hc-injected oocytes with ≈85- and ≈169-kDa electrophoretic mobilities. A band of ≈110 kDa was also visible, but it did not correspond to 4F2hc because it was also detected after immunoprecipitation of extracts from oocytes co-expressing 4F2hc and y⁺LAT-1 with protein G-Sepharose without 4F2hc antibody. The 85-kDa band corresponds to 4F2hc, as detected in activated lymphocytes (9). This band is also detected in oocytes not injected with 4F2hc cRNA, suggesting that *Xenopus* oocytes express a homologous 4F2hc protein. The 169-kDa band is not visible in reducing conditions or in oocytes expressing CS1 4F2hc, suggesting that this band might represent 4F2hc homodimers linked by a disulfide bridge involving cysteine residue 109. In oocytes co-injected with wild type or CS2 4F2hc plus y⁺LAT-1, a new 4F2hc-specific band of ≈135 kDa appears. Under reducing conditions, this band is drastically reduced and a new y⁺LAT-1-specific ≈40-kDa band appears (Fig. 7). In contrast, neither the 135- nor the 40-kDa band is visible, even after very long film exposures, in oocytes co-injected with CS1 4F2hc and y⁺LAT-1. This indicates that the 135-kDa band corresponds to a heterodimer of 4F2hc and

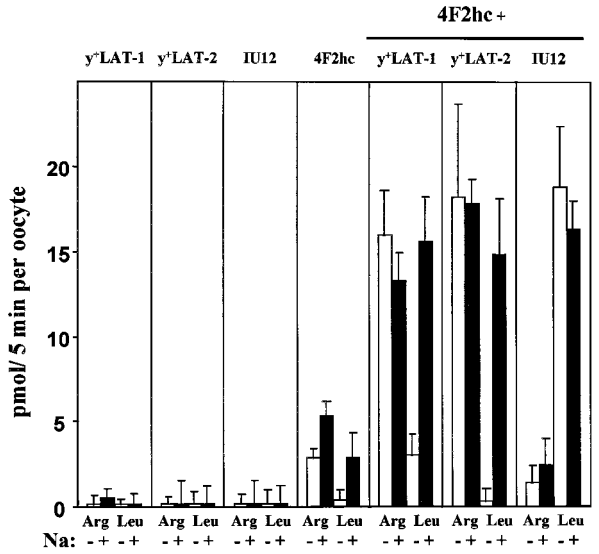

FIG. 3. **Different co-expressed transport activities with three members of the family of amino acid transporter-related proteins and 4F2hc.** Oocytes were injected with 10 ng of y⁺LAT-1 (IMAGE clone 727811), y⁺LAT-2 (KIAA0245), or IU12 alone or coinjected with 10 ng of human 4F2hc. Three days after the injection, the uptake of 50 μM L-[^{3}H]arginine (*Arg*) and 50 μM L-[^{3}H]leucine (*Leu*) in the presence (+, *closed bars*) or absence (−, *open bars*) of 100 mM NaCl was determined for 5 min. Amino acid uptake (pmol/oocyte × 5 min) was calculated by subtracting the uptake in uninjected from that of the cRNA injected groups. Amino acid uptake in uninjected oocytes was as follows: (i) L-[^{3}H]arginine uptake: 1.1 ± 0.2 (choline medium) and 3.5 ± 0.5 (sodium medium); (ii) L-[^{3}H]leucine uptake: 3.1 ± 0.6 (choline medium) and 3.8 ± 0.4 (sodium medium).

TABLE I
Inhibition of y⁺LAT-1/4F2hc-induced transport activity by different amino acids

Uptake was measured at 50 μM arginine concentration in either the absence (choline medium) or the presence (sodium medium) of 100 mM sodium and inhibited by different amino acids at a concentration of 5 mM. Each data point is the mean of values obtained in seven oocytes and expressed as the residual percentage of uptake. Basal values of uptake (mean ± S.E.), expressed in pmol/10 min per oocyte, were 32.7 ± 2.3 (choline medium) and 43.3 ± 3.3 (sodium medium) for y⁺LAT-1 plus 4F2hc-injected oocytes and 3.4 ± 0.2 (choline medium) and 7.8 ± 0.5 (sodium medium) for uninjected oocytes.

Inhibitor (5 mM)	Residual arginine (50 μM) uptake	
	Choline medium	Sodium medium
	%	
Arginine	2 ± 0.3	ND[a]
Lysine	2 ± 0.3	1 ± 0.2
Ornithine	0 ± 0.6	ND
Leucine	48 ± 5	3 ± 0.9
Isoleucine	43 ± 3	16 ± 2
Glutamine	73 ± 9	15 ± 2
Valine	69 ± 9	40 ± 9

[a] ND, not determined.

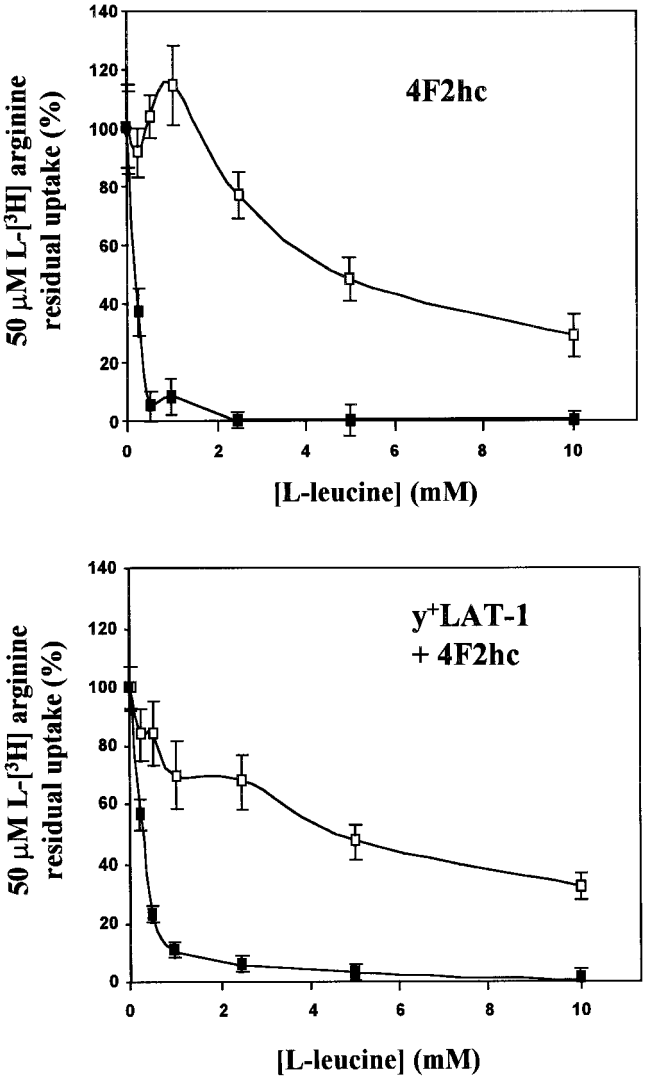

FIG. 4. **Inhibition by L-leucine of the y⁺LAT-1 and 4F2hc co-expressed transport activity.** Three days after injection of 10 ng of human 4F2hc alone or human 4F2hc plus 10 ng of y⁺LAT-1, the uptake of 50 μM L-[^{3}H]arginine in the absence (choline medium) (*open squares*) or in the presence (*closed squares*) of 100 mM sodium was measured for 10 min in the presence of different concentrations of leucine (0, 250 μM, 500 μM, 1 mM, 2.5 mM, 5 mM, and 10 mM). The percentage of the amino acid residual uptake was calculated by subtracting the uptake of uninjected oocytes and dividing by the uptake of 4F2hc alone or 4F2hc plus y⁺LAT-1-injected oocytes without leucine in the medium. The basal values of the uptake of 50 μM L-arginine were 3.4 ± 0.2 and 7.8 ± 0.5 pmol/10 min for uninjected oocytes in choline or in sodium medium, respectively, 19.5 ± 5.0 and 27.4 ± 3.0 pmol/10 min for 4F2hc-injected oocytes in choline or in sodium medium, respectively, and 32.7 ± 2.3 and 43.2 ± 3.3 pmol/10 min for 4F2hc plus y⁺LAT-1-injected oocytes in choline or in sodium medium, respectively.

y⁺LAT-1, linked by a disulfide bridge involving cysteine 109 of 4F2hc. The 135-kDa band is also visible after very long film exposures in 4F2hc-injected oocytes and might represent the association of 4F2hc with a *Xenopus* y⁺LAT-1 homologous protein (data not shown). It is worth mentioning that this band is the only one that correlates with the induced y⁺L transport activity (see Figs. 6 and 7).

DISCUSSION

In this study, we have identified a new member (y⁺LAT-1) of a family of amino acid transporter-related proteins also composed in humans by y⁺LAT-2 (KIAA0245) and E16. We have characterized the human y⁺LAT-1 cDNA sequence, chromosomal location, and pattern of expression of its mRNA and demonstrated that when co-expressed with 4F2hc, it yields y⁺L amino acid transport activity and forms a disulfide bond-dependent complex with 4F2hc through residue Cys-109 in oocytes. Therefore, y⁺LAT-1 is a putative light subunit of the surface antigen 4F2hc. Moreover, we also present human *y⁺LAT-1* as a strong candidate for the lysinuric protein intolerance (LPI) gene.

The surface antigen 4F2 from lymphocytes has been previously immunoprecipitated as a complex of 125 kDa, which upon reduction resulted in two protein bands of 85 kDa (the heavy chain of 4F2 surface antigen, or 4F2hc) and an unidentified light chain with an electrophoretic mobility of 40 kDa; this light chain is known to be nonglycosylated and very hydrophobic (9,

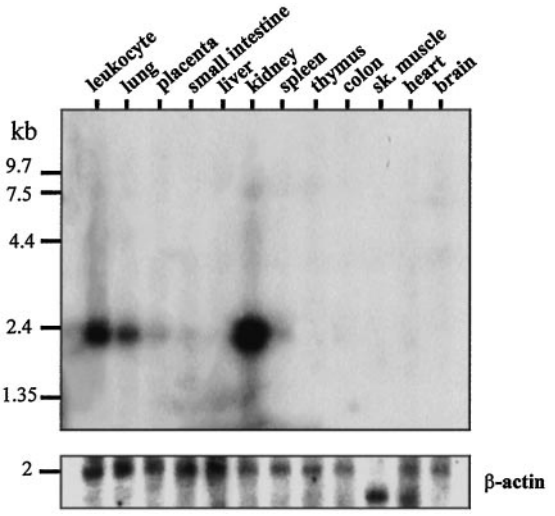

FIG. 5. **Northern blot analysis for y⁺LAT-1 mRNA in human tissues.** A poly(A) RNA (2 μg per lane) membrane containing 12 different human adult tissues was purchased from CLONTECH. Blots were probed with ^{32}P-labeled human IMAGE clone 727811 and washed at high stringency conditions (see under "Experimental Procedures"). Human y⁺LAT-1 cDNA hybridizes to a transcript of ≈2.4 kb and is expressed predominantly in kidney, leukocytes (from peripheral blood), lung, placenta, spleen, and total small intestine. Proper quality and control of loading was substantiated by hybridization with human β-actin cDNA (CLONTECH), used as a control probe.

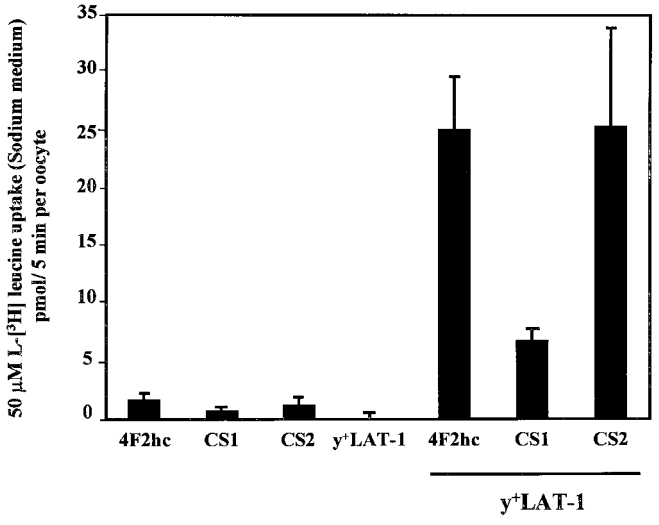

FIG. 6. **Co-expression defect of mutant C109S (CS1) 4F2 but not C330S (CS2) 4F2 with y⁺LAT-1 protein.** Oocytes were injected with 10 ng of human 4F2hc, C109S-human 4F2hc (CS1), C330S-human 4F2hc (CS2), and 10 ng of y⁺LAT-1 cRNA alone or in combination. Three days after the injection, the uptake of 50 μM L-[^{3}H]leucine in the presence of 100 mM sodium was measured in the linear region of the time course. Amino acid uptake was calculated by subtracting the uptake of uninjected oocytes. Data are the mean ± S.E. obtained from the uptake of seven oocytes per group of a representative experiment. Another experiment gave similar results. The rate of 50 μM L-[^{3}H]leucine uptake in the presence of sodium in uninjected oocytes was 2.0 ± 0.1 pmol/5 min.

39). We have recently demonstrated that system y⁺L transport activity induced by 4F2hc in oocytes requires association, most probably by disulfide bridges, with a plasma membrane endogenous protein (11). Here we demonstrated that human y⁺LAT-1 and 4F2hc combine to generate system y⁺L amino acid transport activity in oocytes and form a heterodimeric complex of ≈135 kDa. Moreover, this complex correlates with the induction of y⁺L transport activity by 4F2hc and y⁺LAT-1 co-expression in oocytes. Interestingly, immunoprecipitation of

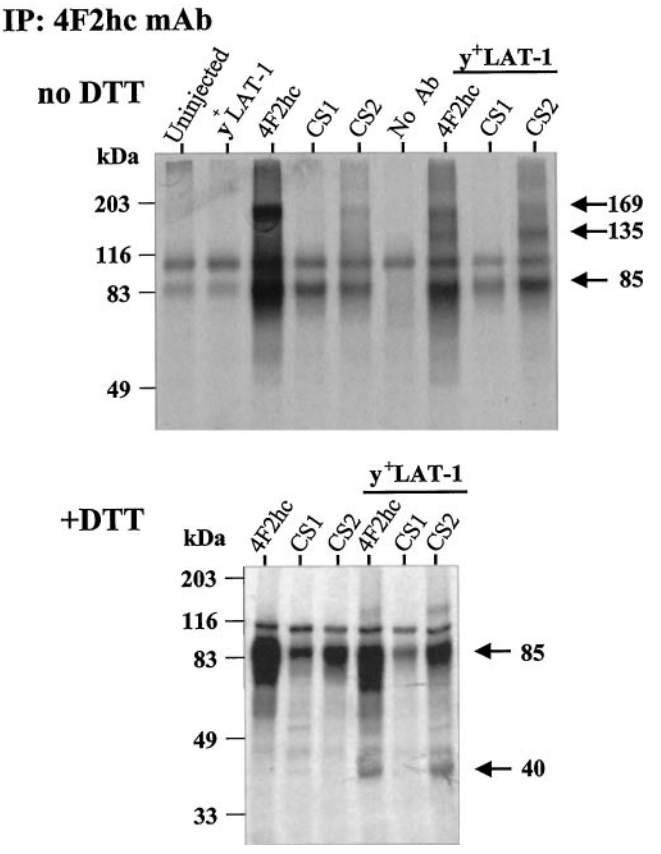

FIG. 7. **4F2hc protein forms a heterodimeric disulfide bond-dependent structure with y⁺LAT-1 through the Cys-109 residue.** Oocytes from the experiment shown in Fig. 6 were injected with 10 ng of each different cRNA as indicated. [^{35}S]Methionine labeling and immunoprecipitation with a monoclonal h4F2hc antibody (*4F2hc mAb*) was performed as described under "Experimental Procedures." Two autoradiographs (under nonreducing conditions (*no DTT*) and under reducing conditions (*+DTT*)) from a representative experiment are shown. Another independent experiment, with higher CS1 expression, gave similar results.

the 135-kDa complex and subsequent reduction results in the appearance of a y⁺LAT-1-specific ≈40-kDa protein band. All of this strongly indicates that human y⁺LAT-1 is a light chain of the surface antigen 4F2hc.

Three proteins, y⁺LAT-1 and y⁺LAT-2 (present study) and IU12 (present study, and for the equivalent protein ASUR4 or the human ortholog E16)[2] induce with 4F2hc several amino acid transport activities in oocytes: system y⁺L activity for y⁺LAT-1 and y⁺LAT-2, or system L-type for IU12 or E16. This suggests that at least these three proteins (human y⁺LAT-1, y⁺LAT-2, and E16) might be light subunits of 4F2hc with associated amino acid transport activities. This is in full agreement with the fact that both y⁺L and L transport activities have been associated with the expression of 4F2hc cRNA or 4F2hc-containing mRNA (2, 40–42, 5–6, 43). Interestingly, y⁺LAT-1 is expressed in tissues where mRNA-induced y⁺L activity has been reported (small intestine, placenta and lung) (41, 42).[4] The final demonstration that y⁺LAT-1, y⁺LAT-2, and E16 are light subunits of the surface antigen 4F2 awaits co-immunoprecipitation studies from tissue or cell samples.

Our data strongly suggest that the 4F2hc and y⁺LAT-1 heterodimeric complex is linked by a disulfide bridge involving 4F2hc residue cysteine 109. Thus, both 4F2hc-induced (present study and Ref. 11) and 4F2hc/y⁺LAT-1-induced system y⁺L transport activity is drastically reduced when the 4F2hc resi-

[4] R. Estévez and M. Palacín, unpublished results.

due cysteine 109 is mutated to serine. In parallel to this, the formation of the 4F2hc/y$^+$LAT-1 heterodimer is abolished by this mutation. This suggests that C109S 4F2hc mutant is able to form an active transporter heterodimer with y$^+$LAT-1, albeit with lower efficiency than wild type 4F2hc. Most probably, weak protein-protein interactions between C109S 4F2hc and y$^+$LAT-1 are destabilized during detergent solubilization prior to immunoprecipitation. In favor of this, the 4F2hc-induced y$^+$L transport activity is not sensitive to β-mercaptoethanol treatment, even though this increases sensitivity to inactivation by cysteine-specific reagents (11). Two cysteine residues of y$^+$LAT-1 (residues 151 and 174) are conserved among the known full-length protein members of this family. Site-directed mutagenesis studies are currently in progress to identify the y$^+$LAT-1 residue involved in the disulfide bridge with the Cys-109 residue of 4F2hc.

One intriguing question is why y$^+$LAT-1 does not induce amino acid transport when injected alone in oocytes and why 4F2hc does. One possible explanation is that the exogenous 4F2hc may constitute a functional y$^+$L transporter with a homologous protein of the y$^+$LAT-1 family already present in the oocyte. The oocyte would synthesize more y$^+$LAT-1-like subunits than 4F2hc-like subunits. This would be similar to the activation of the oocyte catalytic α subunit of the Na^+/K^+ ATPase by expression of foreign β subunits (44). By analogy to Na^+/K^+ ATPase (45, 46), the oocyte y$^+$LAT-1-like subunits might be present in the endoplasmic reticulum and would be transported to the plasma membrane when exogenous 4F2hc is added. In this sense, the y$^+$L activity is already present in the *Xenopus* oocyte (2), and we can visualize an immunoprecipitated 4F2hc antibody protein with the same molecular weight as 4F2hc in uninjected oocytes (Fig. 7).

Structural and functional evidence suggested that rBAT also associates with an oocyte plasma membrane protein to express system b$^{o,+}$-like amino acid transport activity (see under "Introduction," and see Refs. 47 and 48 for recent reviews). Therefore, it will be interesting to determine whether some of the members of the transporter-related family can also interact with the rBAT protein. Preliminary results[5] indicate that y$^+$LAT-1, y$^+$LAT-2, and IU12 do not cause b$^{o,+}$-like amino acid transport activity with rBAT in oocytes.

LPI is an autosomal recessive disease in which transport of the cationic amino acids lysine, arginine, and ornithine is defective. This defect has been localized at the basolateral membranes of epithelial cells in small intestine (49, 50) and in the renal tubules (51). Simell and co-workers (52) reported that LPI fibroblast showed a reduced *trans*-stimulated efflux of cationic amino acids. Clinical signs of LPI include hyperammonemia and episodes of stupor, immunological abnormalities (53), growth retardation, and muscle hypotonia. Potentially fatal interstitial lung disease and progressive renal failure may occur at any age (54). Recently, Lauteala *et al.* (55) have assigned, through linkage analysis of 20 Finnish LPI families, the LPI gene locus to the proximal long arm of chromosome 14. In this work, recombination studies placed the locus between markers D14S72 and MYH7; the phenotype showed the highest linkage desequilibrium with marker T-cell receptor α chain within this locus. Although functional criteria pointed to the cationic amino acid transporters (hCAT-1 and hCAT-2) as candidate genes, linkage studies, using flanking microsatellite markers, excluded both as the mutated gene in LPI (56). The human *y$^+$LAT-1* gene is a good candidate for LPI: (i) 4F2hc is expressed at the basolateral membrane of proximal tubule epithelial cells in the kidney (57). (ii) The y$^+$L activity induced by 4F2hc in oocytes is an exchanger activity that mediates the efflux of cationic amino acids and the influx of neutral amino acids plus sodium (58). This would explain why the efflux and not the influx (because of the presence of a member of the CAT family of transporters) is affected. (iii) The expression pattern of this gene is consistent with the tissues in which some defect in LPI has been detected (lung, immune system cells, kidney, and intestine). (iv) Finally, its chromosomal localization is within the locus of the LPI gene (55). Mutational analysis to prove this hypothesis is currently in progress.

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Note Added in Proof—After this paper was accepted for publication the observations by Verrey and co-workers were published (Mastroberardino, L. Spindler, B., Pfeiffer, R., Skelly, P. J., Loffing, J., Shoemaker, C. B., and Verrey, F. (1998) *Nature* **395,** 288–291).

[5] M. Pineda, D. Torrents, and M. Palacín, unpublished observations.

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