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Identification of LAT4, a Novel Amino Acid Transporter with System L Activity*

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System L amino acid transporters mediate the movement of bulky neutral amino acids across cell membranes. Until now three proteins that induce system L activity have been identified: LAT1, LAT2, and LAT3. The former two proteins belong to the solute carrier family 7 (SLC7), whereas the latter belongs to SLC43. In the present study we present a new cDNA, designated LAT4, which also mediates system L activity when expressed in *Xenopus laevis* oocytes. Human LAT4 exhibits 57% identity to human LAT3. Like LAT3, the amino acid transport activity induced by LAT4 is sodium-, chloride- and pH-independent, is not trans-stimulated, and shows two kinetic components. The low affinity component of LAT4 induced activity is sensitive to the sulfhydryl-specific reagent *N*-ethylmaleimide but not that with high affinity. Mutation in LAT4 of the SLC43 conserved serine 297 to alanine abolishes sensitivity to *N*-ethylmaleimide. LAT4 activity is detected at the basolateral membrane of PCT kidney cells. *In situ* hybridization experiments show that LAT4 mRNA is restricted to the epithelial cells of the distal tubule and the collecting duct in the kidney. In the intestine, LAT4 is mainly present in the cells of the crypt.

System L is a ubiquitous plasma membrane amino acid transport system that mediates sodium-independent transport of neutral amino acids (1). System L was first described in Ehrlich ascites tumor cells as a sodium-independent transport system for neutral amino acids that is specifically inhibited by a bicyclic amino acid, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH)[1] (1–5). Later, two subtypes for system L with distinct characteristics in substrate affinity, selectivity, and transport properties were reported in primary hepatocytes and named systems L1 and L2 (5).

To date several transporters with system L characteristics have been identified at the molecular level. The first cloned transporters with system L activity were LAT1 and LAT2, which are members of the SLC (solute carrier) 7 family of transporters (6–13). They mediate sodium-independent amino acid exchange and recognize a wide range of large neutral amino acids as substrates (14), expanding to small neutral amino acids in the case of LAT2 (8, 10, 11). These proteins form heteromeric complexes via a disulfide bond with the heavy chain of 4F2 antigen (4F2hc, SLC3A2), a single transmembrane domain protein essential for the functional expression of LAT1 and LAT2 at the plasma membrane (8, 11, 12).

Despite the identification of heteromeric amino acid transporters LAT1 and LAT2, some of the previously reported properties of system L, mainly those of system L2, remained to be explained. Recently, LAT3 was identified by expression cloning from the hepatocarcinoma-derived cell line FLC4 (15). LAT3 expressed in *Xenopus* oocytes induces system L2-like transport activity. Several features distinguish LAT3 from LAT1 and LAT2. (i) It is structurally distinct from the heteromeric transporters and does not require co-expression with 4F2hc to elicit transport activity at the plasma membrane; (ii) it exhibits a narrow substrate selectivity, preferring leucine, isoleucine, and phenylalanine as substrates; (iii) it is sensitive to pretreatment with NEM; (iv) it presents a two-component kinetics (high and low affinity); (v) it functions as a facilitative diffusion transporter, *i.e.* moves one substrate following its concentration gradient, whereas LAT1 and LAT2 are obligatory exchangers, *i.e.* move their substrates in exchange for other substrates (8, 16).

The mRNA of LAT3 was highly expressed in pancreas and liver (15). System L activities of low affinity have been described in other tissues, like placenta (17) or brain (18), that do not express LAT3 (15). We reasoned that other system L isoforms may exist in these tissues. In the present study, by homology to LAT3, we have cloned LAT4. LAT4-induced activity is very similar to LAT3, but LAT4 has a different tissue distribution.

EXPERIMENTAL PROCEDURES

Computer Analysis—The nucleotide sequence homology search was performed using basic local alignment search tool (BLAST) via an on-line connection to the National Center of Biotechnology Information. The BLASTn program was run using default parameters. Multiple amino acid sequence comparisons were performed with ClustalW via an on-line connection to the European Bioinformatics Institute (EMBL-

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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)BK005642 (human LAT4) and BK005643 (mouse LAT4)

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[1] The abbreviations used are: BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; LAT, L-type amino acid transporter; SLC, solute carrier family; HA, hemagglutinin; RT, reverse transcription; PBS, phosphate-buffered saline; NEM, *N*-ethylmaleimide.

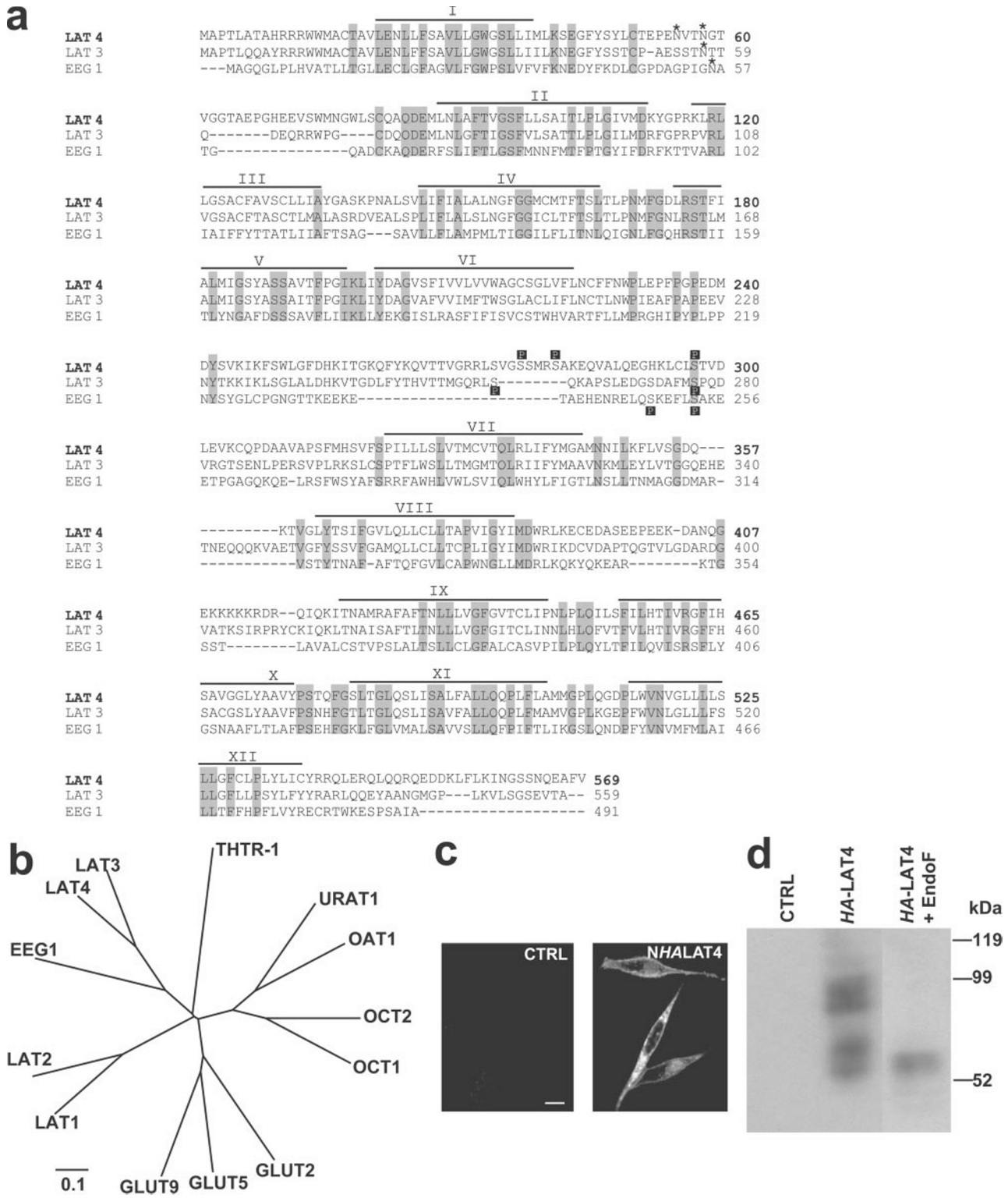

FIG. 1. **General characteristics of the SLC43 family of transporters.** *a*, multialignment of LAT4, LAT3, and EEG1 was done using the program ClustalW sequence alignment from the European Bioinformatics Institute (EMBL-EBI). The *thin horizontal lines* indicate the 12 putative transmembrane domains determined by computer analysis (see "Experimental Procedures"). The amino acid residues identical in the three proteins are marked with *gray boxes*. *Asterisks* note putative *N*-glycosylation sites, as determined by the NETGLYC program (www.cbs.dtu.dk/services/NetNGlyc). *P* indicates putative phosphorylation sites, determined by the NETPHOS program (www.cbs.dtu.dk/services/NetPhos); *b*, phylogenetic analyses. Protein sequences of some representative members of the SLC43, SLC2, SLC22, and the SLC7 L transporters together with the folate transporter THTR-1 (SLC19A2) were obtained. The tree was prepared from a multiple sequence alignment using Treetool (19) (Ribosomal Data Base Project, University of Illinois). The branch lengths are proportional to the identity of the amino acid sequences and to the phylogenetic distance. *c*, Localization of N-HA-LAT4 in HeLa cells. HeLa cells were transfected with N-HA-tagged LAT-4. 48 h after transfection, cells were processed for immunofluorescence with anti-HA as the primary antibody and Texas Red-conjugated goat anti-mouse as the secondary antibody (see "Experimental Procedures"). The HA-immunodetected signal (*white*) is visible at the plasma membrane. An intracellular staining is also visible, probably corresponding to the endoplasmic reticulum. The signal is absent in non-transfected cells (*CTRL*). *Bar*, 10 μm; *d*, LAT4 is *N*-glycosylated. HeLa cells were transfected or not (CTRL) with N-HA-tagged LAT-4, and after 48 h cells were harvested, solubilized, and incubated or not with endoglycosidase F (*EndoF*), which removes *N*-glycosylation. The HA signal corresponding to N-HA-LAT4 was detected in Western blot assays as a broad band between 66 and 99 kDa. Treatment with endoglycosidase F produces a mobility shift, indicating that LAT4 is *N*-glycosylated.

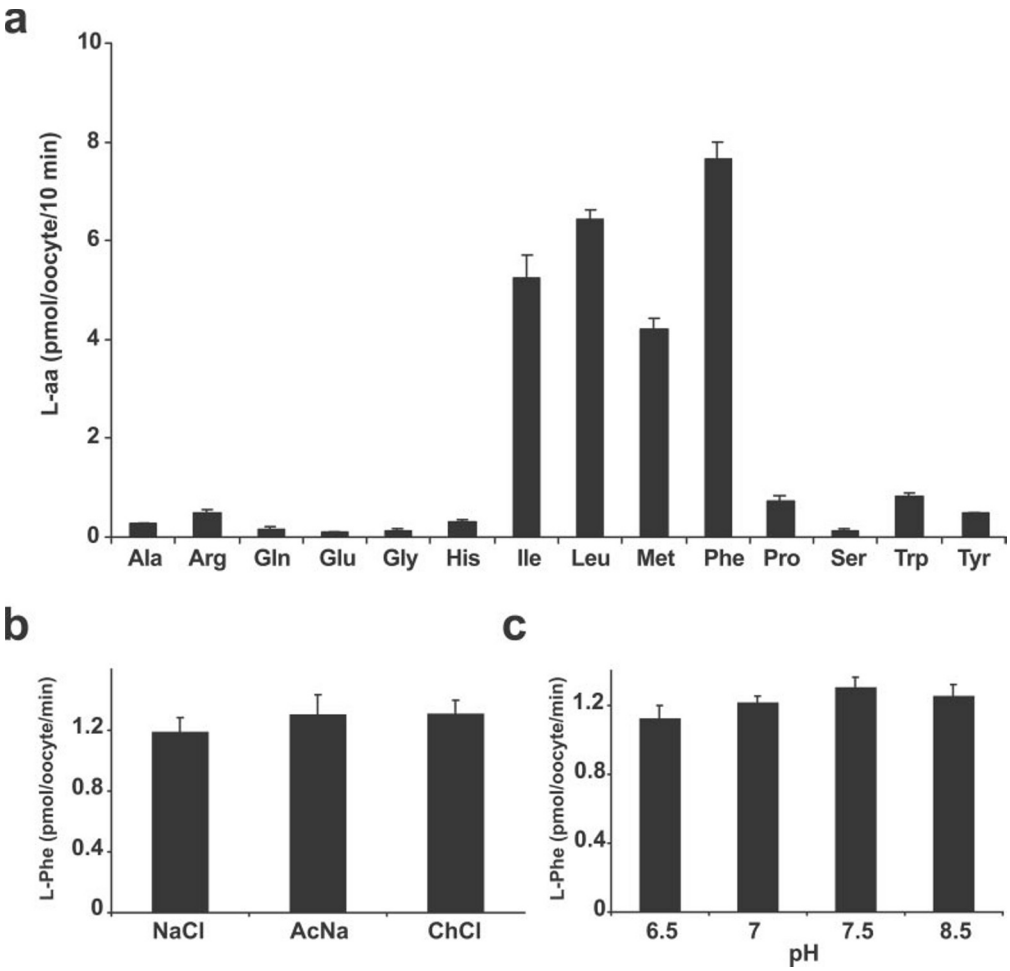

FIG. 2. **Functional expression of LAT4 in *X. laevis* oocytes.** *a*, oocytes were injected with LAT-4 cRNA (25 ng/oocyte). Two days after the injection, the uptake of 10 μM L-^{3}H amino acids (*L-aa*) was determined for 10 min. *b* and *c*, ion dependence of the L-phenylalanine transport induced by LAT4. Oocytes injected with LAT4 cRNA or uninjected controls were incubated in 10 μM L-phenylalanine uptake solution containing 100 mM sodium chloride, choline chloride (*ChCl*), or sodium acetate (*AcNa*) for 10 min (initial velocity conditions) (*b*) or at a range of pH in choline chloride (*c*). Amino acid uptake rates (pmol/10 min/oocyte) were calculated by subtracting the uptake of the non-injected group from that of the cRNA-injected groups. Data (mean ± S.E.) correspond to a representative experiment of three experiments with seven oocytes per group.

EBI). The prediction of transmembrane segments was performed with TopPred II. NETGLYC (www.cbs.dtu.dk/services/NetNGlyc) and NETPHOS programs (www.cbs.dtu.dk/services/NetPhos) were used for *N*-glycosylation and phosphorylation site prediction. The phylogenetic tree was prepared from a multiple sequence alignment using Treetool (19).

Molecular Biology—The open reading frames of mouse and human LAT4, embryonic epithelial gene 1 (EEG1) (20), and LAT3 were obtained from Deutches Ressourcenzentrum für Genomforschung GmbH. NcoI and XhoI restriction sites were introduced by PCR at the ATG site and directly after the stop codon, respectively. The PCR product obtained was cloned in the vector pTLN (21) and sequenced. A hemagglutinin epitope tag (HA) tag was added after the first methionine of mouse LAT4 using standard molecular biology techniques. We used the human LAT4 cDNA as a template for site-directed mutagenesis (QuikChange™; Stratagene) to replace serine 297 by alanine (LAT4SA). For HeLa cell studies, the cDNA was cloned into pCDNA3 (Invitrogen). All sequences in this study were performed in both directions with the Big Dye terminator V.3.1 cycle sequencing kit (Applied Biosystems). The reactions were analyzed with an Abi Prism 377 DNA sequencer.

Oocytes, Injections, and Transport Measurements—Oocyte origin, handling, and injections were as described elsewhere (22). Defolliculated stage VI *Xenopus laevis* oocytes were injected with 25 ng/oocyte human LAT4, human EEG1, or human LAT3 or with 10 ng each of 4F2hc and either *Xenopus* LAT1 or human LAT2 cRNA. cRNA was obtained using the mMessage mMachine™ kit (Ambion).

Influx rates of L-radiolabeled amino acids (Amersham Biosciences) were measured under linear conditions (10 min incubation) 2 days after cRNA injection. The uptake medium was choline chloride uptake solution (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes/Tris, pH 7.5) containing the desired amino acid concentration and radioactive amino acid (10 μCi/ml for ^{3}H and 2 μCi/ml for ^{14}C). For L-phenylalanine efflux measurements, groups of three cRNA-injected or non-injected oocytes were loaded with the labeled amino acid by incubation in 50 μM L-[^{3}H]phenylalanine (1 μCi/100 μl) for 60 min. The loading ranged from 3,000 to 10,000 cpm/oocyte in non-injected or LAT4 injected oocytes, respectively. After loading, oocytes were washed 4 times in choline chloride uptake solution containing no amino acids. Efflux was then measured as tritium appearance in the incubation medium (500 μl) in the absence of amino acids or in the presence of phenylalanine (5 mM). To this end aliquots (100 μl) were removed from the medium at distinct times, and radioactivity was counted.

Northern Blot Analysis—For human samples, a commercial membrane containing adult poly(A) RNA was used (BD Biosciences). For the mouse LAT4 expression data, 25 μg of total RNA isolated using the Trizol^R reagent (Invitrogen) was loaded in each lane, separated by size in a 1% agarose gel, transferred to a positively charged nylon membrane (Hybond N$^+$, Amersham Biosciences) by capillarity in 10× SSC (standard saline citrate 1× = 150 mM NaCl, 15 mM sodium citrate, pH 7), and cross-linked with UV irradiation. Membranes were prehybridized in hybridization buffer (0.25 M sodium phosphate, pH 7.2, 1 mM EDTA, and 20% SDS) at 65 °C for 1–2 h and then hybridized in fresh buffer containing 25 ng/10 ml of the labeled DNA fragment used as a probe at a specific activity of 10^9 dpm/μg (nucleotides 1–984 for the human cDNA and 1–776 for the mouse cDNA) at 65 °C overnight. Probes were labeled with the Rediprime II random prime labeling system (Amersham Biosciences). Washes were performed at 65 °C for 30 min, the first in 2× SSC, 0.5% SDS and the second in 0.1× SSC, 0.5% SDS. After washing, membranes were sealed in a plastic bag and exposed to a

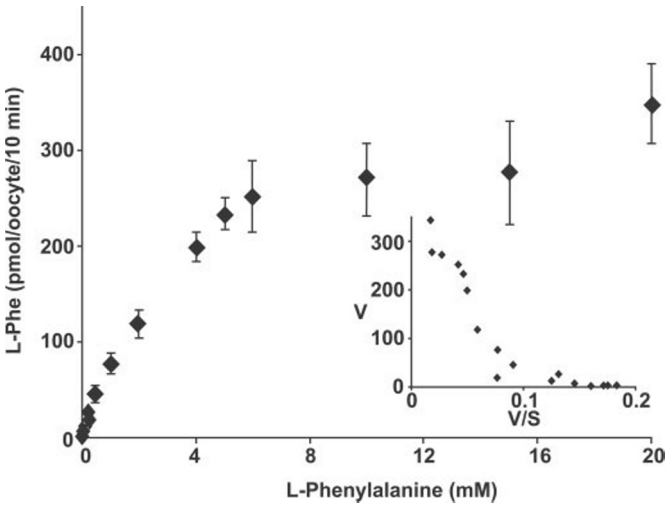

FIG. 3. **Kinetic analyses of LAT4-induced transport activity.** Oocytes were injected with LAT4 cRNA. Two days after the injection the uptake of L-phenylalanine at different concentrations was measured in the absence of sodium for 10 min. The transport activity in non-injected oocytes was subtracted from that of cRNA-injected oocytes. Data (mean ± S.E.) correspond to a representative experiment with seven oocytes per group. Another four independent experiments showed similar results. The substrate concentrations used were 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 4000, 5000, 6000, 10000, 15000, and 20000 μM. The *inset* shows the Eadie-Hofstee transformation of the data.

PhosphorImager screen (Molecular Dynamics).

RT-PCR Analysis—RNA from mouse PCT cells was isolated using the Trizol^R reagent (Invitrogen). RNA was retrotranscribed to cDNA using SuperScript II RNase H reverse transcriptase (Invitrogen). cDNA was then used to amplify mouse LAT1, LAT2, LAT3, and LAT4 using specific primer pairs for each transporter. PCR conditions were 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for 35 cycles. Products were visualized after size separation in a 1% agarose gel followed by ethidium bromide staining.

In Situ Hybridization—cRNA probes were labeled with digoxigenin-11-UTP (Roche Applied Science) by transcription of a mouse LAT4 fragment (nucleotides 1–322) or a human LAT4 fragment (nucleotides 1–345), linearized at either the 3′ (sense) or 5′ (antisense) end. Mouse tissues were embedded in paraffin after whole animal fixation with paraformaldehyde. 5-μm sections were cut on a Leica RM 2135 microtome and mounted on silanized slides (PerkinElmer Life Sciences). Sections were deparaffined with xylene, hydrated, and permeabilized with proteinase K (Roche Applied Science) (1 μg/ml) in Tris-EDTA buffer, pH 8 (37 °C, 3 min). Human kidney sections were processed in a similar manner.

Hybridization was done in 50% formamide, 10% dextran sulfate, 300 mM NaCl, 10 mM Tris, pH 8, 5 mM EDTA, 1× Denhardt's solution, 10 mM sodium phosphate, 200 μg/ml salmon sperm DNA, and 5 ng/μl labeled RNA probe at 42 °C overnight. Slides were washed in 2× SSC, treated with RNase A, and further washed to the final stringency achieved by 0.1× SSC at 37 °C. Probes were detected with an antibody to digoxigenin conjugated to alkaline phosphatase and incubation with nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (Promega). Slides were examined on an Olympus microscope, and images were captured with the Leica IM50 image manager at the Serveis Científico-Tècnics, University of Barcelona.

Cell Culture—PCT cells (23, 24) were grown in a mix of Dulbecco's modified Eagle's medium and Ham's F-12 medium (50% each) supplemented with 2% fetal calf serum, 5 mg/liter insulin, 50 nM dexamethasone, 60 nM selenium, 5 mg/liter transferrin, 50 nM triiodothyronine, 10 ng/ml epidermal growth factor, 20 mM Hepes, 2 mM glutamine, and 2.24 g/liter D-glucose. Subcultures were prepared by trypsinization and reseeding at high density (~1×10^6 cells/ml). For transport polarity studies, PCT cells were seeded (4.2×10^5 cells/filter) on polycarbonate filters (12-mm diameter, 3-mm pore size; Costar). Before seeding the cells, each polycarbonate filter was pretreated with Dulbecco's modified Eagle's medium containing 10 μg/ml collagen type I from rat-tail (Upstate Biotechnology). After incubation at 37 °C for 3 h, the collagen solution was removed. Cells were cultured in standard conditions. The formation of polarized monolayers was assessed by measuring transepithelial resistance with Millicell-Electrical resistance System (Millipore). Monolayers were considered optimal for transport polarity studies when resistance exceeded 300 ohm/cm^2 after about 15 days in culture.

TABLE I
Kinetic parameters for LAT4

K_m values correspond to either wild type- or LAT4SA-induced transport activities treated or not with NEM as described under "Results." Changes in V_m after NEM treatment are shown in the percentage of paired experiments. In five independent experiments, V_m values for LAT4 were V_m1 = 586 ± 86 pmol/oocyte·10 min and V_m2 = 34 ± 6 pmol/oocyte·10 min. In three independent experiments the V_m1 values for LAT4SA ranged from 37 to 60% of LAT4 values, and the V_m2 values ranged from 41 to 88% of LAT4 values. Values were estimated using KaleidaGraph 3.6.

	K_m1	% Activity V_m1	K_m2	% Activity V_m2
	μM		μM	
LAT4	4694 ± 510	100	178 ± 29	100
LAT4 NEM	3103 ± 442	36 ± 4	177 ± 30	90 ± 18
LAT4 SA	3323 ± 596	100	158 ± 32	100
LAT4 SA NEM	3742 ± 327	96 ± 7	106 ± 32	78 ± 28

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Transfections were performed by standard calcium phosphate precipitation.

Transport Measurements in Transwell Chambers—For basolateral membrane uptake experiments, apical and basal compartments were washed 3 times in preheated (37 °C) washing solution (10 mM HEPES, 5.4 mM KCl, 1.2 mM $MgSO_4 \cdot 7\ H_2O$, 2.8 mM $CaCl_2 \cdot 2H_2O$, 1 mM KH_2PO_4, and 137 mM *N*-methylglucamine, pH 7.4). Approximately, 0.5 ml of washing solution was left on the apical side of the filter during the experiment. We then added to the basal side different uptake solutions (washing solution supplemented with substrate at the desired concentration, radioactive amino acids, and inhibitors when indicated). Uptake was arrested by removing the uptake solution and washing three times with cold stop solution (washing solution at 4 °C supplemented with 10 mM substrate). Filters were then left to dry, cut, and placed in an Eppendorf tube with 200 μl of 0.1% SDS and 100 mM NaOH at 37 °C for 30 min. From these, 100 μl were used to count the radioactivity associated to the filter in a β scintillation counter, and 20 μl were used to determine the protein concentration in duplicate.

Immunofluorescence on Transfected HeLa Cells—HeLa cells grown on cover slips were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, then washed three times in PBS, incubated in PBS containing 50 mM NH_4Cl for 10 min then in PBS containing 20 mM glycine for 10 min, and blocked with 10% fetal bovine serum in PBS for 30 min. Subsequently, cover slips were incubated with primary antibody (anti-HA 1:100, Berkeley Antibody Co., Inc.) diluted in PBS containing 10% fetal bovine serum at RT for 1 h. After washing in PBS, the cover slips were incubated with 7.5 mg/ml Texas red-conjugated goat anti-mouse (Molecular Probes, Leiden, The Netherlands) at RT for 45 min. The cells were washed three times in PBS before mounting in Immunofluore medium (ICN Biomedicals Inc., Aurora, OH). The confocal images were obtained using a Leica TCS 4D laser confocal fluorescence microscope with a 63× objective at the Serveis Científico-Tècnics, University of Barcelona.

Cell Extraction and Western Blot Analysis—48 h after transfection cells were harvested, and membrane proteins were extracted using PBS, 1% Triton X-100 containing protease inhibitors. Proteins were separated on 7.5% SDS-polyacrylamide gels and electrotransferred (Bio-Rad) to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk powder in PBS and probed with the primary antibody 3F10 (Roche Applied Science) at 4 °C by overnight incubation. Three washes in PBS, 0.1% Tween 20 at room temperature for 10 min each were performed before incubation with horseradish peroxidase-conjugated anti-rat guinea pig (Jackson ImmunoResearch) that was used as the secondary antibody. After 3 washes in PBS, 0.1% Tween 20 at RT for 10 min, antigen-antibody complexes were detected with enhanced chemiluminescence (ECL, Amersham Biosciences) and exposure to x-ray films. Endoglycosidase F treatment was done following the manufacturer's instructions (New England Biolabs).

RESULTS

Identification of LAT4—LAT3 has recently been identified as a new protein, previously known as POV1 (25), that medi-

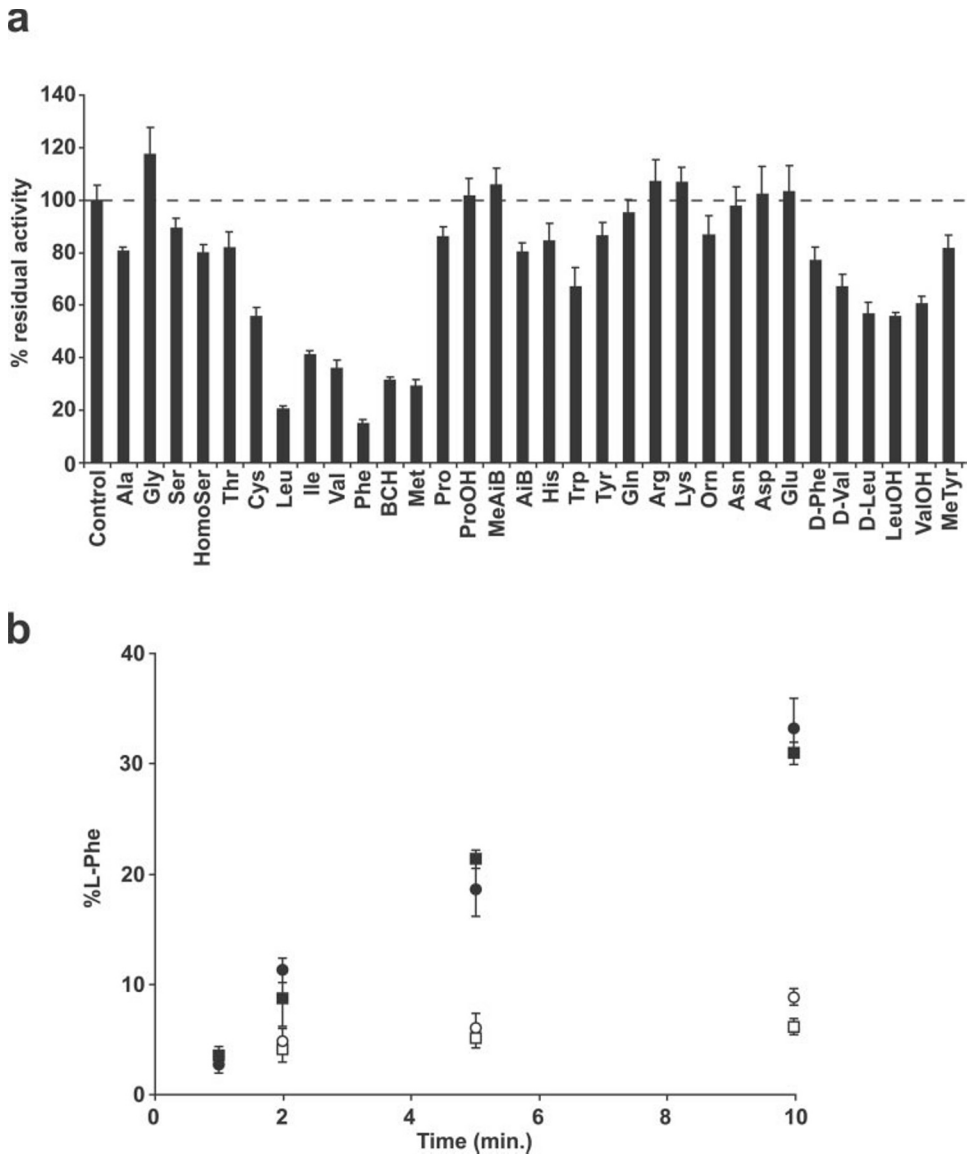

FIG. 4. **L-phenylalanine transport characteristics of LAT4-injected oocytes.** *a*, substrate selectivity. Two days after injection of LAT4 cRNA, the uptake of 100 μM L-phenylalanine in the absence of sodium was measured in the absence (control) or in the presence of the amino acids indicated or amino acid analogs at 20 mM for 10 min. Due to its poor solubility in water, methyltyrosine was used at 2 mM to inhibit L-phenylalanine transport at 20 μM. The uptake values in the control group at 100 μM were 11.22 ± 0.6 pmol/10 min per oocyte and 1.48 ± 0.07 pmol/10 min per oocyte for LAT4 and non-injected oocytes, respectively. At 20 μM values were 2.4 ± 0.2 pmol/10 min per oocyte and 0.3 ± 0.02 pmol/10 min per oocyte for LAT4 and non-injected oocytes, respectively. Data (mean ± S.E.) are the percentages of the amino acid transport in the presence of inhibitors and were calculated from the induced transport (*i.e.* subtracting transport of non-injected oocytes to that of LAT4 injected oocytes). Data correspond to two-four independent experiments, in which seven oocytes per group were used; for clarity, a *horizontal dashed line* is shown corresponding to 100% activity. *MeAiB*, methyl amino isobutyric acid. *b*, efflux of L-phenylalanine via LAT4. Oocytes were injected with LAT4 cRNA (*filled symbols*) or left uninjected (*open symbols*). Two days after the injection, oocytes were loaded with 50 μM L-[^{3}H]phenylalanine for 60 min (the radioactivity accumulated in this representative experiment was 8750 ± 315 and 3269 ± 276 cpm per oocyte in LAT4-injected and non-injected oocytes, respectively). The efflux of radioactivity was then measured at the indicated periods of time, with the oocytes in media (washing solution; see "Experimental Procedures") containing either no amino acid (*circles*) or 5 mM L-phenylalanine (*squares*). For this purpose, after loading oocytes were incubated in 500 μl of the corresponding medium, and aliquots of 100 μl were removed at the indicated times for radioactivity counting. After removing all the aliquots, oocytes were lysed to assess the total amount of radioactivity loaded. Data (mean ± S.E.) correspond to a representative experiment with three groups of three oocytes per data point and are expressed as % of the total radioactivity loaded.

ates low affinity system L amino acid transport activity (15). LAT3 is expressed mainly in pancreas and liver, being nearly absent from kidney, brain, or placenta. We hypothesized the existence of LAT3 homologue proteins that mediate this activity in these tissues. LAT3 shows a significant degree of identity (BLASTn) with two human cDNAs, the embryonic epithelial gene 1 (EEG1) (20) and the predicted cDNA MGC34680, encoding LAT4. Human LAT4 cDNA (3024 bp) contains a single open reading frame encoding a putative 569-amino acid protein (GenBank™ TM/EMBL/DDBJ accession number BC027923) with a predicted molecular weight of 62.7. The first ATG codon (position 90) lies within a good consensus initiation sequence (5′-ACCATGG) (26), and the open reading frame continues to the first stop codon (TAG) at base 1797. The cDNA contains a poly(A) tail of 25 adenines that starts 17 bases downstream from a typical polyadenylation signal (AATAAA) at nucleotide 2977. LAT4 presents a 57% identity to LAT3 and 30% identity to EGG1 (Fig. 1*a*). In addition we have cloned mouse LAT4 (NM_173388). Mouse LAT4 is 91% identical to human LAT4. Phylogenetic analyses indicated that LAT3, LAT4, and EEG1 belong to the same family (Fig. 1*b*). LAT4 presents only about 10% homology to other proteins with system L transport activ-

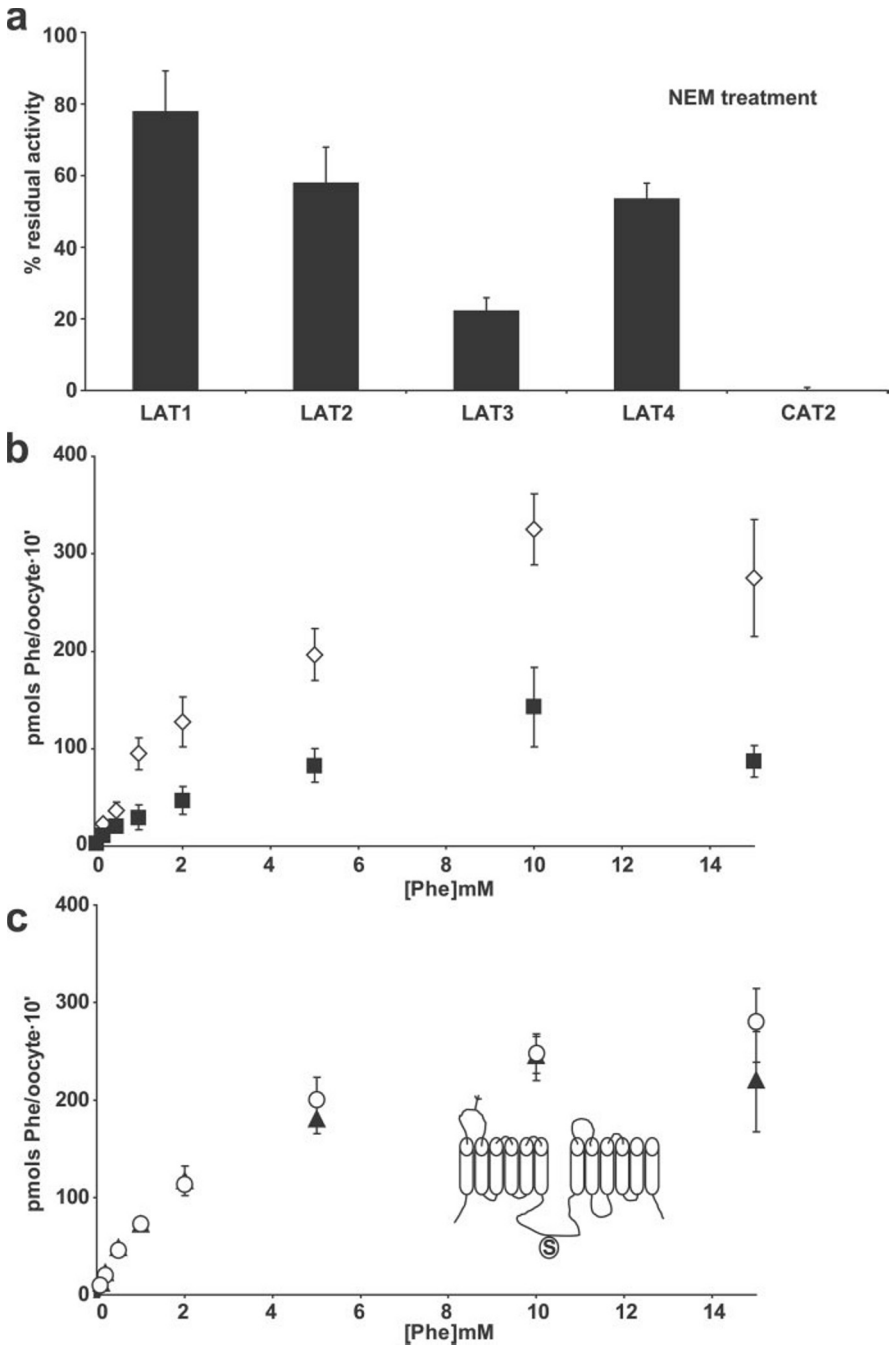

FIG. 5. **Analysis of NEM effect on LAT4-induced transport activity.** *a*, NEM inhibition of LAT-induced system L activity. Two days after injection of cRNA, the uptake of 100 μM L-phenylalanine was measured in oocytes after preincubation with 5 mM NEM for 15 min. Data correspond to a representative experiment with seven oocytes per group and are percentages of uptake values without NEM preincubation. *b*, oocytes were injected with 25 ng of LAT4 cRNA or left uninjected. Two days after the injection, the uptake of radiolabeled phenylalanine at different concentrations was measured in oocytes treated (*filled symbols*) or not (*open symbols*) with NEM (5 mM, 15 min). The transport activity in non-injected oocytes was subtracted from that in cRNA injected oocytes. Data (mean ± S.E.) correspond to a representative experiment with seven oocytes per group. *c*, same as in *b* using oocytes expressing a mutant form of LAT4 in which serine 297 has been substituted by alanine as described under "Experimental Procedures" (see "Results" for details). *Open symbols* correspond to non-treated oocytes, and *filled symbols* correspond to NEM-treated oocytes. The *inset* in *c* represents the LAT4 protein at the plasma membrane with its 12 predicted transmembrane domains. Serine 297 in the putative intracellular loop between transmembrane domains 6 and 7 is indicated.

ity (LAT1 and LAT2) or to other members of the SLC7, SLC2, and SLC22 families of transporters.

The predicted topology (see under "Experimental Procedures") of these proteins indicates 12 transmembrane domains with both the N and C termini located intracellularly. The PSORT2 program (bioweb.pasteur.fr/seqanal/interfaces/psort2.html) predicted a possible signal peptide in the N terminus, and the NETGLYC program (www.cbs.dtu.dk/services/NetNGlyc) predicted that the extracellular loop between the first two transmembrane domains contains *N*-glycosylation sites (*asterisks* in Fig. 1*a*) conserved in mouse LAT4. We tested whether LAT4 contains a signal peptide and whether the protein is glycosylated. To this end, we introduced an HA tag just after the first ATG (N-HA-LAT4), transfected HeLa cells, and assayed its subcellular localization by immunofluorescence (Fig. 1*c*). No signal was observed in non-transfected cells, whereas a clear plasma membrane staining was detected in N-HA-LAT4-transfected cells, indicating that epitope-tagged LAT4 reaches the plasma membrane and that the N-terminal part of LAT4 is not cleaved. In addition, N-HA-LAT4 was as functional as the wild-type LAT4 in amino acid uptake assays (data not shown). Next, we performed Western blot assays with transfected cells incubated or not with endoglycosidase F, which removes *N*-glycosylation. N-HA-LAT4 is detected as a broad band between 66 and 99 kDa. This broad mobility is indicative of glycosylation. In fact, treatment with endoglycosidase F reduces the molecular weight to ~66 (Fig. 1*d*).

Functional Characterization—To test the hypothesis that LAT4 is an amino acid transporter, we measured the uptake of isotopically labeled amino acids in oocytes injected with human LAT4 cRNA. An increased uptake of L-phenylalanine, L-leucine, L-isoleucine, and L-methionine was observed when compared with non-injected oocytes (Fig. 2*a*). Mouse LAT4 and HA-tagged mouse LAT4 also induced L-phenylalanine trans-

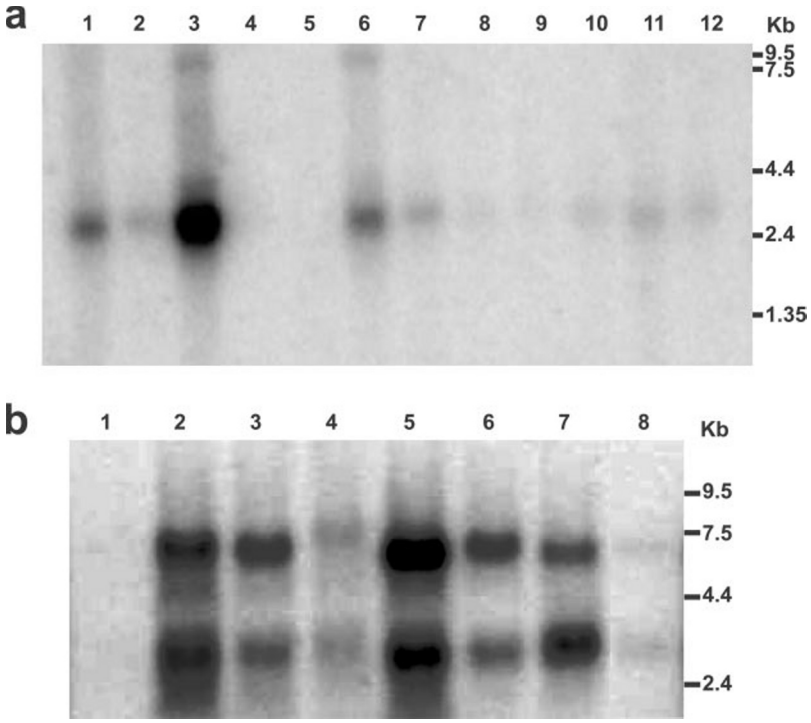

FIG. 6. **Northern blot analysis for LAT-4 mRNA in human and murine tissues.** *a*, a poly(A) RNA membrane (2 μg/lane) containing 12 different human adult tissues was purchased from BD Biosciences. The blot was probed with a ^{32}P-labeled fragment of the human cDNA (nucleotides 1–984; MGC34680) and washed in high stringency conditions (0.1× SSC, 0.5% SDS, 65 °C). Human LAT-4 cDNA hybridizes to transcripts of about 8–9 and ~3.1 kilobases (*kb*). *Lanes*: *1*, peripheral blood leukocytes; *2*, lung; *3*, placenta; *4*, small intestine; *5*, liver; *6*, kidney; *7*, spleen; *8*, thymus; *9*, colon; *10*, skeletal muscle; *11*, heart; *12*, brain. *b*, Total RNA (25 μg/lane) was extracted from several mouse tissues and established cell lines, separated by size in an agarose gel, transferred to a Hybond N$^+$ nylon membrane (Amersham Biosciences), cross-linked, and hybridized to a mouse LAT4 cDNA fragment (nucleotides 1–776) labeled with ^{32}P. After washing in high stringency conditions, blots were exposed to a PhosphorImager screen (Molecular Dynamics). Two transcripts of ~8 and ~3.1 kilobases are visualized. *Lanes*: *1*, liver; *2*, kidney; *3*, brain; *4*, heart; *5*, small intestine; *6*, white adipose tissue; *7*, testis; *8*, PCT cells. The mouse blot was overexposed to visualize the low abundance transcripts in PCT cells.

port when expressed in oocytes (data not shown). LAT4-mediated L-phenylalanine transport is not chloride-, sodium-, nor pH-dependent (Fig. 2, *b* and *c*).

We next performed kinetic analysis for the induced transport activity of L-phenylalanine (Fig. 3 and Table I) and L-leucine. Interestingly, and similar to LAT3 (15), the expression of LAT4 in oocytes leads to the presence of a transport activity with two kinetic components. The low affinity component has a K_m of 4694 ± 510 μM for L-phenylalanine and 3733 ± 1019 μM for L-leucine, and the high affinity component has a K_m of 178 ± 29 μM for L-phenylalanine and 103 ± 62 μM for L-leucine.

To further characterize the activity induced by LAT4, we measured the inhibition of sodium-independent L-phenylalanine uptake by distinct amino acids at a 200-fold excess concentration (100 μM *versus* 20 mM) (Fig. 4*a*). Acidic and basic amino acids did not inhibit the transport. Small neutral amino acids were poor inhibitors for LAT4, except for L-cysteine, which inhibited 45% of the entry of L-phenylalanine. In contrast, L-isoleucine, L-leucine, L-methionine, and L-valine were strong inhibitors (60–85% inhibition) of LAT4. The system L-specific amino acid analog BCH also inhibited (70%) the entry of L-phenylalanine through LAT4. These data indicate that the activity induced by LAT4 corresponds to system L.

In addition, we analyzed the substrate selectivity using amino acid analogs (Fig. 4*a*). LAT4-induced activity is stereospecific since D-isomers of leucine, phenylalanine, and valine are by far less efficient than the L-isomers in inhibiting L-phenylalanine transport. Similar to LAT3 (15), L-leucinol and L-valinol, which have a hydroxymethyl group instead of a α-carboxyl group, also inhibit the uptake of L-phenylalanine. Methyltyrosine, a LAT1-specific inhibitor (27), is a poor inhibitor of LAT4 (Fig. 4*a*).

To further characterize the mechanism of transport, oocytes were loaded with L-phenylalanine (50 μM, 1 μCi/100 μl) for 1 h, and the efflux of the labeled amino acid was measured by exposing the oocytes to the uptake solution with or without unlabeled substrate amino acid. The rate of efflux of the labeled amino acid was identical in the presence or absence of transamino acid (Fig. 4*b*). This, together with the ion dependence data presented in Fig. 2, indicates that the transporter moves the substrate following its concentration gradient; therefore, the transporter works through facilitated diffusion.

NEM Sensitivity of LAT4 Kinetic Components—LAT3-induced transport activity has been shown to be sensitive to NEM (15). We studied the sensitivity to NEM for the different LAT transporters (Fig. 5*a*). CAT2, a system y+ transporter known to be very sensitive to NEM (28), was included as a positive control. LAT1 is almost insensitive to NEM, LAT2 and LAT4 show an intermediate sensitivity, and LAT3 is the most severely affected system L transporter. To further characterize the effect of NEM on LAT4, the inhibition of the activity by NEM was monitored at different substrate concentrations (Fig. 5*b*). Interestingly, only the low affinity component was sensitive to NEM pretreatment, resulting in a reduction of the V_m of transport (Table I).

Babu *et al.* (15) suggested that phosphorylation may be involved in the kinetic behavior of LAT3. Serine 297 in LAT4 is conserved in the three proteins of the family (Fig. 1*a*). This residue may be phosphorylated *in vivo* (NETPHOS program, (www.cbs.dtu.dk/services/NetPhos)). We reasoned that mutation of this residue might have an impact on the activity of the protein. Mutation of serine 297 to alanine resulted in a partial inhibition of the transport activity (see the legend to Table I). To further characterize this mutant, we evaluated the impact of NEM treatment on the activity of LAT4SA. Surprisingly, the activity of LAT4SA was insensitive to NEM at any substrate concentration (Fig. 5*c*, Table I). Therefore, replacement of LAT4 Ser-297 for alanine renders the transporter resistant to NEM treatment.

Tissue and Subcellular Distribution—We next evaluated the expression pattern of LAT4 in human and mouse tissues. The Northern blot analysis revealed two transcripts, one of ~3.1 kilobases and another of about 8–9 kilobases, in both human and mouse samples. Human LAT4 mRNA was detected in several tissues (Fig. 6*a*), with higher expression in placenta (*lane 3*), kidney (*lane 6*), and peripheral blood leukocytes (*lane 1*). Mouse LAT4 is expressed in intestine (*lane 5*) > kidney (*lane 2*) > brain (*lane 3*) = white adipose tissue (*lane 6*) = testis (*lane 7*) > heart (*lane 4*) (Fig. 6*b*). In contrast to LAT3 (15), no expression was observed in human or mouse liver (Fig. 6, *a*

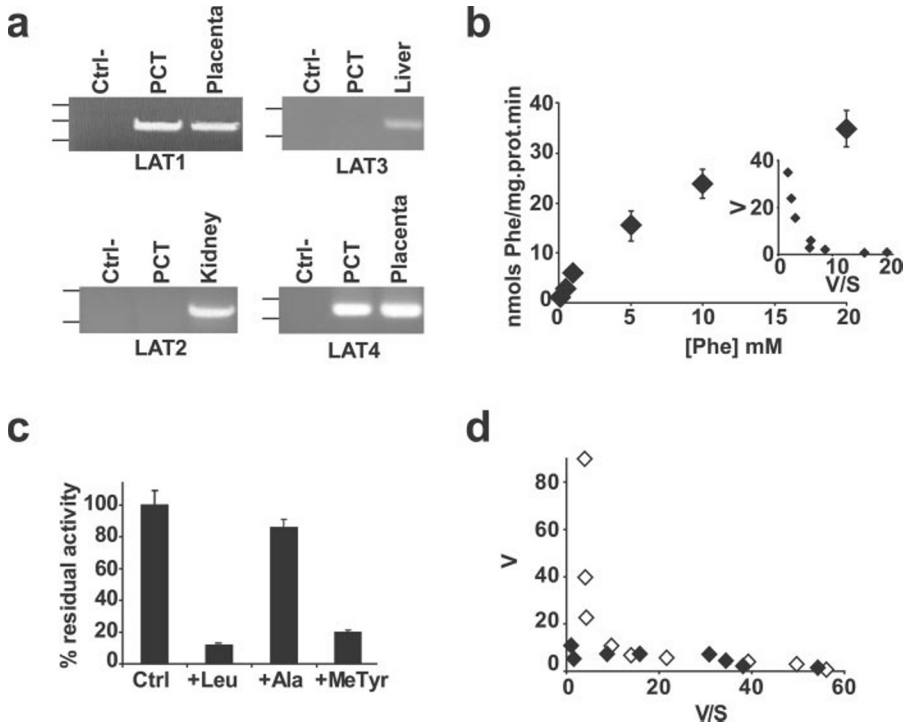

FIG. 7. **LAT mRNA expression and activity in PCT cells.** *a*, RT-PCR analyses for the different LAT transporters. RNA was retrotranscribed as described under "Experimental Procedures," and the resulting cDNA was used to amplify LAT1-, LAT2-, LAT3-, and LAT4-specific sequences. Bands of the expected size were detected in positive controls (placenta for LAT1 and LAT4, kidney for LAT2 and liver for LAT3). No bands were observed in negative control lanes (*Ctrl−*). Molecular weight markers migrated to the positions indicated with *horizontal lines* and correspond to 1000, 850, and 650 base pairs for the LAT1 gel, 850 and 1000 base pairs for the LAT2 gel, 650 and 850 base pairs for the LAT3 gel, and 200 and 300 base pairs for the LAT4 gel. *b–d*, PCT cells were grown in filters and used to perform transport studies in the basolateral side. *b*, kinetic analyses of L-phenylalanine transport. The L-phenylalanine concentrations used were 10, 50, 250, 500, 1,000, 5,000, 10,000, and 20,000 μM. The *inset* shows the Eadie-Hofstee transformation of the data. Each data point corresponds to mean ± S.E. of three independent filters of a representative experiment. *c*, inhibition pattern of L-phenylalanine transport. Uptake of L-phenylalanine (100 μM) was measured in the presence of 10 mM L-alanine, 10 mM L-Leu, or 2 mM methyltyrosine. When methyltyrosine was used as an inhibitor, the substrate concentration was 20 μM. The inhibition by L-alanine is not statistically significant. Data are mean ± S.E. of three independent filters of a representative experiment and are presented as % activity of controls with no inhibitor. *d*, Eadie-Hofstee plot of the effect of NEM on the kinetics of L-phenylalanine transport in PCT cells. Cells were treated with NEM 5 mM for 15 min (*filled symbols*) or left untreated (*open symbols*), and the transport of L-phenylalanine was measured at different substrate concentrations. Data correspond to mean values of triplicate measures for each individual point. S.E. of the mean were lower than 10% in all cases.

(*lane 5*) and *b* (*lane 1*) for human and mouse, respectively). A faint LAT4 signal was detected in PCT cells, derived from mouse kidney proximal convoluted tubules (23, 24) (*lane 8*). This expression pattern differs from that of other LAT transporters (6, 8, 10, 15).

RT-PCR analyses confirmed expression of LAT4 in PCT cells. LAT2 and LAT3 transcripts could not be detected, whereas the other known system L protein, LAT1, is also expressed in these cells (Fig. 7*a*). Next, we studied the presence of a LAT4-like activity in these cells. Cells were cultured in transwell chambers to perform uptake assays (see "Experimental Procedures"). Kinetic analysis of basolateral L-phenylalanine entry into these cells revealed two distinct components with different affinities, a high affinity component with a K_m of 217 ± 19 μM and a V_m of 4.1 ± 0.1 nmol/mg of protein·min and a low affinity component with a K_m of 13.2 ± 3.6 mM and a V_m of 57.2 ± 8 nmol/mg protein·min (Fig. 7*b*). To study the high affinity component, we performed inhibition studies (Fig. 7*c*). There is a basolateral L-phenylalanine transport activity that is not blocked by alanine, a typical LAT2 substrate, but it is almost completely inhibited by methyltyrosine, a LAT1-specific substrate, corroborating the RT-PCR data in Fig. 7*a*. In all, these data indicate that the high affinity component corresponds mainly to LAT1. As shown in Fig. 7*d*, NEM treatment results in inhibition of the low affinity component without affecting the high affinity component. This together with the data in Fig. 7*a* suggests that the low affinity component corresponds mainly to LAT4.

To study which cells in kidney and intestine express LAT4, we performed *in situ* hybridization analyses in paraffin-embedded sections of these tissues derived from mice and in human kidney sections (Fig. 8). In the kidney human (Fig. 8*a*) and mouse (data not shown) LAT4 mRNA was detected in epithelial cells of the distal tubule (*D*) and collecting duct (*CD*). No expression was seen in the glomerulus (*G*) or the proximal tubule (*PT*). In the intestine the transporter mRNA was mainly expressed in crypt cells (*C*) of the intestinal microvilli and epithelial cells in the base of the villus (*V*). No expression was seen in the muscularis mucosae (*MM*) (Fig. 8*c*). No signals were detected using sense probes for human LAT4 in kidney (Fig. 8*b*) or mouse LAT4 in intestine (Fig. 8*d*), confirming the specificity of the results.

DISCUSSION

On the basis of significant homology (57% identity) to the recently described LAT3 transporter (15), we have identified a new amino acid transporter with system L transport activity (LAT4). LAT4 and LAT3 show significant identity (30%) to yet another protein, EEG1, of unknown function. Because of their structural similarity, we propose that LAT3 and LAT4 should denote a new family of organic solute transporters (SLC43) that will also include the orphan member EEG1. Phylogenetic analyses show that these proteins are weakly related to members of the organic cation/anion transporter family (SLC22), to members of the facilitated glucose transporter family (SLC2),

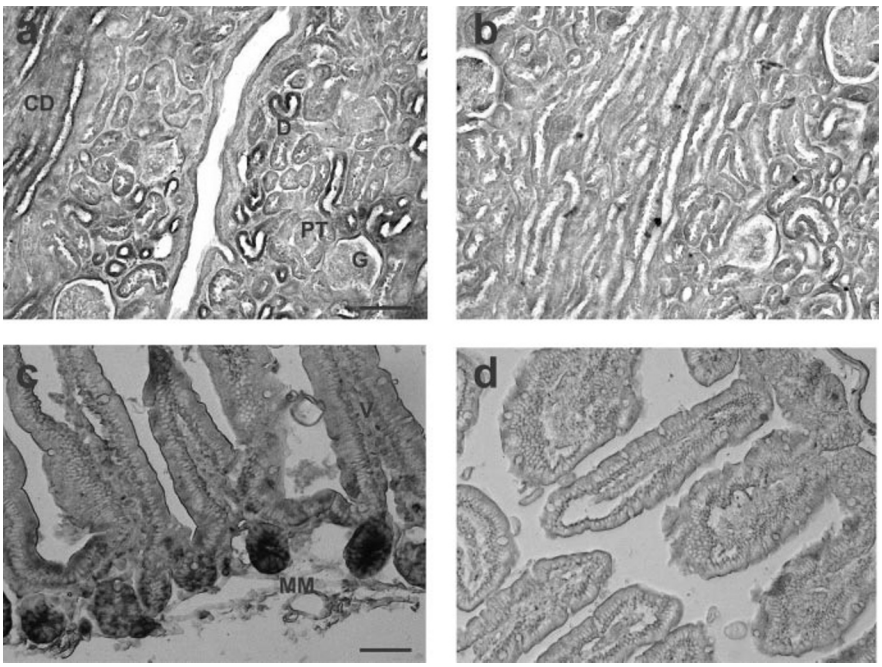

FIG. 8. ***In situ*** **hybridization of LAT-4 mRNA.** Serial paraffin-embedded sections of human kidney cortex (*a* and *b*) or mouse intestine (*c* and *d*) were incubated with antisense (*a* and *c*) or sense (*b* and *d*) LAT4 cRNA probes as described under "Experimental Procedures." Results are representative of two independent experiments. *a*, in the kidney LAT4 mRNA-specific detection is restricted to the distal tubule (*D*) and the collecting duct (*CD*). No signal is detected in the glomerulus (*G*) or the proximal tubule (*PT*). *c*, in the intestine the signal corresponds to crypt cells (*C*), and there is a faint labeling in the epithelial cells of the villus (*V*). No staining was seen in the muscularis mucosae (*MM*). *b* and *d*, no signal was seen with LAT4 sense probes in kidney (*b*) or intestine (*d*), confirming the specificity of the results. *Bars*: 0.1 mm (*a* and *b*); 0.04 mm (*c* and *d*).

and to members of the heteromeric amino acid transport family (SLC7A5 and SLC7A8, encoding LAT1 and LAT2, respectively) (Fig. 1*b*) (29, 30). LAT4 induces amino acid transport activity in oocytes; however, we could not detect induction of any amino acid transport activity in EEG1-injected oocytes. Additional experiments are required to demonstrate or rule out that EEG1 encodes a transporter for organic solutes.

LAT3 and LAT4 share many functional characteristics like sodium, chloride, and pH independence, narrow substrate specificity, a two-component kinetics, lack of trans-stimulation, stereospecificity, and sensitivity to the amino acid analog BCH, and they accept substitutions of the α-carboxyl group by an hydroxymethyl group in the transported substrates. However, the activities of these two transporters can be distinguished on the basis of NEM sensitivity. Although NEM strongly inhibits LAT3 activity (Ref. 15, Fig. 5*a*), it only partially blocks that of LAT4. An unusual finding in this new family of transporters is the two-component kinetic behavior. For LAT4, only the low affinity component is sensitive to NEM (Fig. 5*b,* Table I). It has been suggested that phosphorylation might regulate the kinetic performance of LAT3 (15). In the present study we show that mutation of a putative phosphorylation site conserved in the SLC43 family results in a transport activity resistant to NEM treatment (Fig. 5*c*). Serine 297 is located in a putative intracellular loop in the middle of the protein. This loop may contain binding sites for modulators of the activity and contains cysteines that may be targets for NEM action. Mutation of serine 297 might cause conformational changes that make the target cysteine inaccessible to NEM. Alternatively, such changes might cause NEM modification of cysteine residues in LAT4 not to have an effect on the transporter activity. Further research is needed to clarify this issue.

System L variants L1 (substrate affinity in the micromolar range and substantially inhibited by cysteine, valine, isoleucine, leucine, methionine, histidine, tryptophan, tyrosine, phenylalanine, and BCH) and L2 (substrate affinity in the millimolar range and narrower substrate selectivity) have been described previously (1). These subtypes were reported in hepatoma cell lines and hepatocytes, respectively (5). An L3 subtype with an affinity between that of the L1 and L2 subtypes has been described in fibroblasts (31). Uptake by system L2 is inhibited by isoleucine, leucine, phenylalanine, and BCH (5, 32, 33). System L2 is sensitive to inhibition by NEM, whereas system L1 is not (32). LAT1 fits the transport characteristics and the tissue distribution of subtype L1 (6), and LAT3 coincides with the traits of L2 (15). Due to the functional similarities to LAT3, LAT4 could contribute to L2 activity in some tissues.

The expression of LAT4 mRNA in PCT cells prompted the study of a LAT4-like activity in these cells. PCT cells can be grown on filters allowing the study of transporters at the basolateral and apical membranes separately. We have detected a LAT4-like activity at the basolateral membrane (Fig. 7) that suggests a basolateral location for LAT4. A final demonstration requires the availability of a specific antibody. Due to its facilitative diffusion transport mechanism and its basolateral location, it is tempting to speculate that LAT4 might have an important role in the net flux of substrates from inside the cell to the exterior of the cell contributing to the transcellular flux of amino acids.

In the kidney, the absence of expression in the proximal tubule, where more than 90% of the reabsorption of amino acids occurs, rules out a major role of LAT4 in renal reabsorption of amino acids. Recycling of amino acids has been reported between Henle's loop and vasa recta in the juxtamedullary nephrons of the rat kidney (34). This recycling could play a role in osmotic regulation at the initial stage of cellular adaptation (35). LAT4 is expressed in distal tubules and collecting ducts where it could contribute to the basolateral fluxes of neutral amino acids between tubule epithelial cells and vasa recta during recycling (34). In the intestine, LAT4 is mainly expressed in the crypts, where it may play a role in the homeostasis of enterocytes. Moreover, high K_m bulky neutral amino acid transport agencies have been detected in cerebral cortex (18) and astrocytes (36). LAT4 mRNA was also detected in the brain (Fig. 6). It remains to be established whether LAT4 is responsible for these transport activities.

Interestingly, human LAT4 is highly expressed in placenta. Kinetic analysis using a single circulation paired-tracer dilution technique revealed L-phenylalanine uptake kinetics on the fetal side (basolateral membrane of the trophoblast) with an apparent K_m of 17 mM (17). This value is within the range of the K_m for LAT4-like activity in PCT cells (13 mM). The detection of a LAT4-like activity at the basolateral membrane of PCT cells

and the kinetic properties of LAT4 suggest that LAT4 might be responsible for this activity. In maternal phenylketonuria (37, 38), the raised levels of L-phenylalanine could block the delivery of several amino acids including L-tyrosine and L-tryptophan to the fetus (38). This amino acid imbalance may lead to the disaggregation of polysomes in fetal heart and brain, as seen in pregnant rats loaded with L-phenylalanine (39). This fact may contribute to the congenital heart disease and mental retardation of maternal phenylketonuria (40). LAT4 transports substrates with high capacity; therefore, L-phenylalanine flux through the placenta will not be limited by the function of this transporter. Therefore, the activity of LAT4 at the fetal side could contribute to the pathogenesis of phenylketonuria.

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