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Expression of LAT1 and LAT2 amino acid transporters in human and rat intestinal epithelial cells

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Summary. The present study evaluated the presence of LAT1 and LAT2 amino acid transporters in human Caco-2 cells and rat IEC-6 cells along the mucosa of the rat digestive tract. The LAT1 cDNA was amplified by PCR using two sets of primers (one specific for rat LAT1 and another simultaneously specific for human, rat and mice). The LAT2 cDNA was amplified by PCR using one set of primers simultaneously specific for human, rat and mice LAT2. The presence of LAT1 and LAT2 protein was examined by means of immunoblotting using an antibody raised against the rat LAT1 and mouse LAT2. Caco-2 and IEC-6 cells, as well as the rat intestinal mucosa, are endowed with both LAT1 and LAT2 transporter transcripts and protein. LAT1 protein is most abundant in IEC-6 cells, which is in agreement with functional data previously reported. The findings in the rat intestinal mucosa indicate that LAT1 protein is most abundant in the colon and its abundance markedly decreases at the level of jejunum and ileum, which contrast with relative homogeneous presence of LAT2 across the digestive tract. In conclusion, Caco-2 and IEC-6 cells, as well as the rat intestinal mucosa, are endowed with both LAT1 and LAT2 amino acid transporter transcripts and protein.

Keywords: LAT1 – LAT2 – Caco-2 cells – IEC-6 cells – Intestinal epithelium

Introduction

Amino acid related drugs such as levodopa, melphalan, gabapentin, triiodothyronine, thyroxine and cysteine-conjugates have been described as substrates for amino acid transporters, namely system L because of its broad substrate selectivity (Kanai and Endou, 2001; Uchino et al., 2002). However, amino acid transporters fall into different families that are distinguished by their functional properties (specificity of amino acids transported, transport mechanism, coupling to ions) and molecular identity (Palacin et al., 1998; Verrey et al., 2000; Wagner et al., 2001). System L, for leucine preferring, conveys the Na^+-independent transport of large branched and aromatic neutral amino acids in almost all types of cells and can be differentiated against related transporters by its ability to transport the model substance 2-aminobicyclo-(2,2,1) heptane-2-carboxylic acid (BCH). On the basis of the affinity for its substrate, two subtypes have been described; LAT1, with a high affinity in the micromolar range, and LAT2, with a lower affinity in the millimolar range (Verrey, 2003; Wagner et al., 2001). Another difference between LAT1 and LAT2 is concerned with their sensitivity to extracellular pH for substrate uptake (Prasad et al., 1999).

LAT1 is expressed in brain, spleen, thymus, testis, skin, liver, placenta, skeletal muscle, and stomach (Kanai et al., 1998; Prasad et al., 1999). The second isoform for system L, LAT2 is highly expressed in polarized epithelia (Segawa et al., 1999), suggesting an important role in transepithelial amino acid transport. However, LAT1-specific mRNA is expressed in most human tissues with the notable exception of the intestine (Prasad et al., 1999). This conflicts with the view that their transport across the intestinal epithelium may correspond to LAT1. On the other hand, the mRNA corresponding to LAT2 examined by Northern blot analysis was strongly expressed in the small intestine (Rossier et al., 1999; Segawa et al., 1999). Though intestinal epithelial cell lines have been used quite often for the study of drug and nutrient absorption, the presence of LAT1 and LAT2 transporters has not been systematically addressed. Recently, we reported functional evidence for the presence of both LAT1 and LAT2 in intestinal epithelial Caco-2 and IEC-6 cells (Fraga et al., 2002a, b, c).

The present study was aimed at evaluate the presence of LAT1 and LAT2 transcripts and protein in human Caco-2 cells and rat IEC-6 cells by RT-PCR using specific

primers for either LAT1 or LAT2 rat cDNAs sequences and immunoblotting, respectively. In addition, the expression of LAT1 and LAT2 was also evaluated along the mucosa of the rat digestive tract. It is reported that Caco-2 and IEC-6 cells, as well as the rat intestinal mucosa, are endowed with the LAT1 and LAT2 transporter transcripts and protein.

Methods

Cell culture

Caco-2 cells (ATCC 37-HTB; passages 39–49) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. Cells were grown in Minimal Essential Medium (Sigma Chemical Company, St. Louis, Mo, USA) supplemented with 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (Sigma), 20% foetal bovine serum (Sigma) and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanosulfonic acid (HEPES; Sigma). IEC-6 cells were obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (ACC-111; Passages 3–14) and maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. Cells were grown in Dulbecco's Modified Eagle's Medium (45%) and RPMI 1640 (45%) supplemented with 10% foetal bovine serum, 0.1 U/ml insulin, 100 U/ml penicillin G, 0.25 μg/ml amphotericin B and 100 μg/ml streptomycin. For subculturing, the cells were dissociated with 0.05% trypsin-EDTA, split 1:3 and subcultured in Costar Petri dishes with 21 cm^2 growth area (Costar, Badhoevedorp, The Netherlands). The cell medium was changed every 2 days, and the cells reached confluence after 4 (IEC-6) or 7 (Caco-2) days of initial seeding. For 24 hours prior to each experiment, the cell medium was free of foetal bovine serum. Experiments with IEC-6 and Caco-2 cells were generally performed 2 and 5 days after cells reached confluence, usually 6 and 12 days after the initial seeding, respectively; each cm^2 contained about 20 and 100 μg of cell protein.

Isolation of rat intestinal epithelial cells

Male Wistar rats (Harlan-Inferfauna, Barcelona, Spain) aged 12-weeks were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 22 ± 1°C) with food and water allowed *ad libitum*. The solid diet (rat maintenance diet, catalogue number 9609) was obtained from Harlan-Teklad (Oxon, UK). All animals interventions were performed in accordance with the European Directive number 86/609, and the rules of the "Guide for the Care and Use of Laboratory Animals", 7th edition, 1996, Institute for Laboratory Animal Research (ILAR), Washington, DC. Rats were sacrificed by decapitation and the intestine was removed.

The jejunal, ileal and colonic mucosa were dissected out and immediately processed. For the purpose of isolation of epithelial cells from the jejunum, ileum and colon, the selected intestinal segments were incubated with Ca^{2+}-free Hanks' medium with 1 mM EDTA at room temperature for 3 × 10-min periods (Ogawa et al., 2000). The modified Hanks' medium had the following composition (mM): NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $MgCl_2$ 1.0, Tris HCl 0.15 and sodium butyrate 1.0, pH = 7.4. At the end of each period, they were shaken rapidly with a glass rod. The supernatants were collected by centrifugation, and the resulting pellets were washed twice.

RNA extraction

Cells and the rat intestinal mucosa were homogenised (Diax, Heidolph) in Trizol Reagent (75 mg/ml; Invitrogen) and total RNA was extracted according to manufacturer's instructions. The RNA obtained was dissolved in diethylpyrocarbonate (DEPC)-treated water and quantified by spectrophotometry at 260 nm.

Reverse transcription (RT)-PCR

One microgram total RNA was reverse transcribed to cDNA with SuperScript First Strand Synthesis System for RT-PCR (Invitrogene) according to manufacturer's instructions. The reverse transcription was performed at 50°C and using 5 μg/μl random hexamers.

The LAT1 cDNA was amplified by PCR using two sets primers: one specific for rat LAT1 (forward: 5′-CAT CAT CGG TTC GGG CAT CTT-3′, and reverse: 5′-CAG GGT GAC AAT GGG CAA GGA-3′, corresponding to nucleotides 252–919 of rat cDNA) and another simultaneously specific for human, rat and mice LAT1 (forward: 5′-GG(C/T) TCG (G/T)GC ATC TTC GT-3′ and reverse: 5′-(G/A)CA (G/C)AG CCA GTT GAA GAA GC-3′) corresponding to nucleotides 285 and 1267 of the human cDNA (GenBank accession AF104032). The LAT2 cDNA was amplified by PCR using one set of primers simultaneously specific for human, rat and mice LAT2 (forward: 5′-CA(C/G) CCG (A/G)AC AAC ACC G(A/C)(A/C/G) AAG-3′ and reverse: 5′-TGC CAG TA(A/G) ACA CCC AGG AA(A/G)-3′) corresponding to nucleotides 242 and 1612 of the human cDNA (GenBank accession AF171669).

PCR was performed with Platinum TaqPCRx DNA Polymerase (Invitrogene) with 1× enhancer (LAT1) or without enhancer (LAT2). Amplification conditions were as follows: hot start of 2 min at 95°C; 30 cycles of denaturing (95°C for 30 s), annealing (58°C for 30 s), and extension (68°C for 45 s); and a final extension of 7 min at 68°C.

The PCR products were separated by electrophoresis in a 2% agarose gel and visualized under UV light in the presence of ethidium bromide.

Immunoblotting

Membrane fractions of Caco-2, IEC-6 cells and from rat intestinal epithelial cells were isolated and proteins extracted for LAT1 and LAT2 detection. Briefly, cell monolayers (Caco-2 and IEC-6 cells) and suspensions of epithelial cells from the intestinal mucosa (jejunum, ileum and colon) were washed twice with phosphate buffered saline (PBS) and centrifuged (900 *g*, 10 min, 4°C) in an Eppendorf tabletop refrigerated centrifuge. The pellets were resuspended in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM PMSF, aprotinin and leupeptin 5 μg/ml), homogenized and centrifuged (9,000 *g*, 20 min, 4°C). Thereafter, the supernatant was collected and centrifuged at 100,000 *g* (60 min, 4°C). The pellet was resuspended in lysis buffer with protease inhibitors (150 mM NaCl, 50 mM Tris.HCl pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/mL PMSF aprotinin and leupeptin 2 μg/mL each), briefly sonicated and incubated on ice for 1 h. After centrifugation (16,000 *g*, 30 min, 4°C), the supernatant was collected and protein concentration determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. Samples were combined with 6× sample buffer (0.35 M Tris-HCl, 4% SDS, 30% glycerol, 9.3% DTT, pH 6.8, 0.01% bromphenol blue) and boiled for 5 min, 95°C. The samples, which contained 40 μg of protein, were separated by SDS-PAGE with 10% polyacrylamide gel and then electroblotted onto nitrocellulose membranes (Bio-Rad). Blots were blocked overnight with 5% non-fat dry milk in PBS-T (0.05% Tween 20 in phosphate-buffered saline) at 4°C with constant shaking. Blots were then incubated with rabbit anti-LAT1 polyclonal antibody (1:500; Serotec, Oxford, UK) and rabbit anti-mouse LAT2 polyclonal antibody (Rossier et al., 1999) (1:2000; kindly provided by Prof. François Verrey) in 5% non-fat dry milk in PBS-T for 1.5 h at room temperature. Membranes were washed three times with PBS-T and then incubated with peroxidase-labeled goat anti-rabbit IgG (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1.5 h at room temperature and developed for detection of the specific protein using enhanced chemiluminescence reagents (Amersham).

Data analysis

Arithmetic means are given with S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference.

Results

Detection of LAT1 and LAT2 transcripts

The expression of LAT1 and LAT2 in human Caco-2 cells and rat IEC-6 cells was examined by RT-PCR using specific primers for both LAT1 or LAT2 rat and human cDNAs. The expected 1029 bp fragment corresponding to human LAT1 was identified in Caco-2 cells, whereas the 1391 bp fragment corresponded to human LAT2 (Fig. 1A). The products obtained for rat IEC-6 cells had the expected size: a 688 bp fragment corresponding to LAT1 and a 1391 bp fragment corresponding to LAT2 (Fig. 1B). The identity of Caco-2 amplified fragments was further confirmed by restriction analysis with *Rsa* I. The expected banding patterns are shown in Fig. 2: LAT1 (244 bp, 356 bp and 429 bp) and LAT2 (25 bp, 256 bp, 510 bp and 600 bp). The presence of LAT1 and LAT2 transcripts in rat jejunal mucosa was examined by RT-PCR using specific primers for either LAT1 or LAT2 rat cDNA sequences. The expected 1391 bp fragment corresponding to LAT2 was identified in total RNA from rat jejunal mucosa (Fig. 3). The 688 bp product was also obtained when the same RNA samples were reverse transcribed and amplified by PCR with the LAT1 rat specific primers, as shown in Fig. 3.

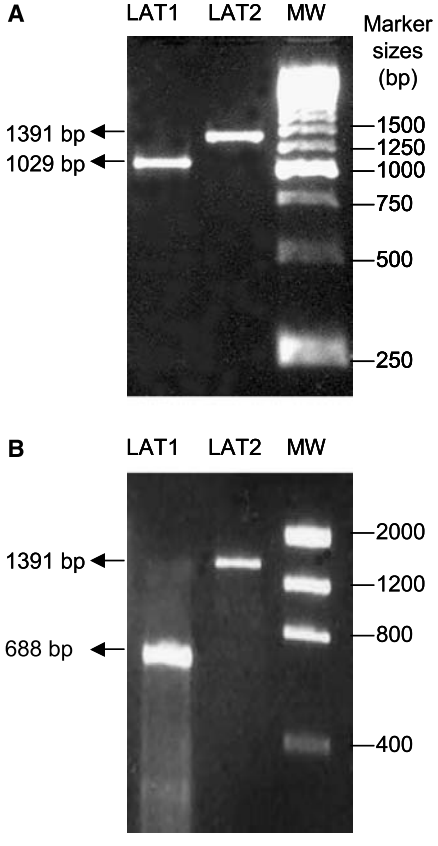

Fig. 1. A Detection of LAT1 and LAT2 in human intestinal epithelial Caco-2 cells by RT-PCR, using both LAT1 and LAT2 set of primers simultaneously specific for rat, human and mice (corresponding to nucleotides 285–1267 and nucleotides 242–1612 of human cDNA, respectively). MW – 250 bp DNA ladder (Invitrogen). **B** Detection of LAT1 and LAT2 in rat intestinal epithelial IEC-6 cells by RT-PCR, using rat LAT1 specific primers (nucleotides 252–919 of rat cDNA) and LAT2 set of primers simultaneously specific for rat, human and mice (nucleotides 242–1612 of human cDNA). MW – 250 bp DNA ladder (Invitrogen)

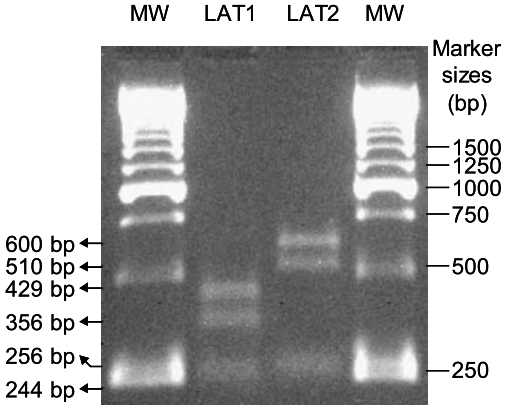

Fig. 2. Restricition analysis of LAT1 and LAT2 fragments with Rsa I. LAT1 – 244 bp, 356 bp and 429 bp. LAT2 – 25 bp, 256 bp, 510 bp and 600 bp. MW – 250 bp DNA ladder (Invitrogen)

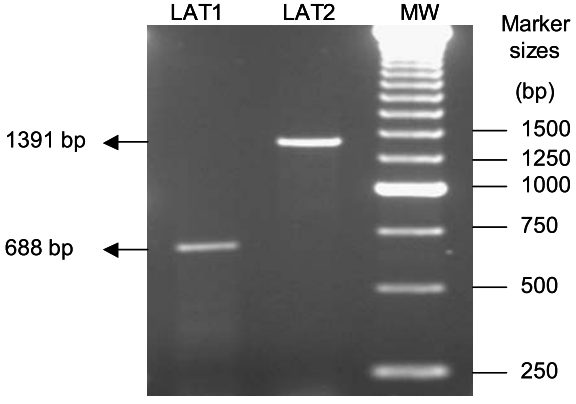

Fig. 3. Detection of LAT1 and LAT2 in total RNA extracted from rat jejunal mucosa, using rat LAT1 specific primers (nucleotides 252–919 of rat cDNA) and LAT2 set of primers simultaneously specific for rat, human and mice (nucleotides 242–1612 of human cDNA). MW – 250 bp DNA ladder (Invitrogen)

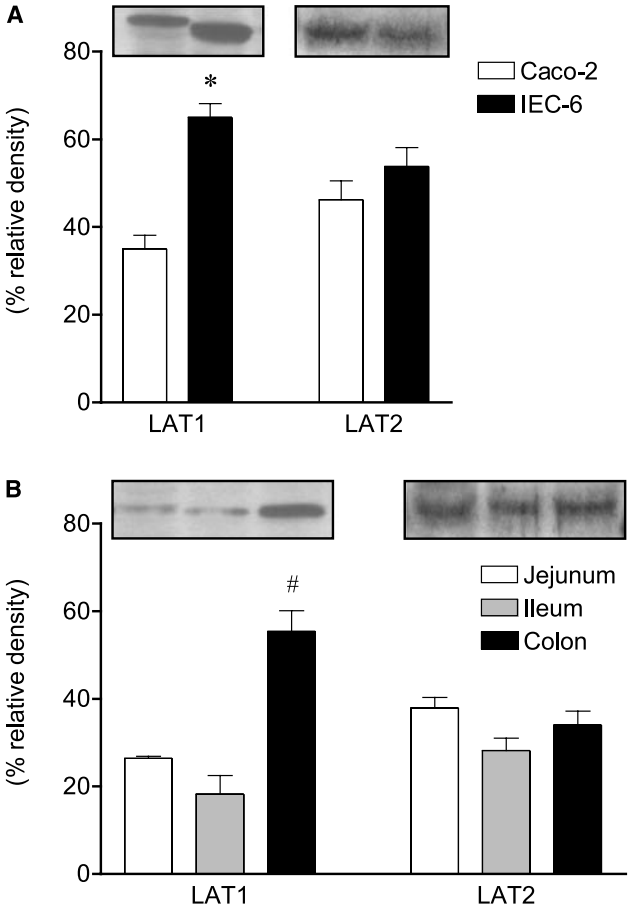

Fig. 4. Abundance of LAT1 and LAT2 in membrane fractions of (**A**) Caco-2 and IEC-6 cells and (**B**) rat jejunal, ileal and colonic epithelial cells. Each lane contains equal amount of protein (40 μg). Western blots were repeated 4–6 times. Columns indicate relative density and represent the mean of 4–6 separate experiments; vertical lines indicate SEM. Significantly different from values for Caco-2 cells ($^{*}P < 0.05$) and jejunal or ileal mucosa ($^{\#}P < 0.05$)

LAT1 and LAT2 immunoblotting

The presence of LAT1 and LAT2 protein was examined in Caco-2 and IEC-6 cells by means of immunoblotting using an antibody raised against the rat LAT1 and mouse LAT2. As shown in Fig. 4A, this antibody recognized the presence of LAT1 and LAT2 in cell membranes from both Caco-2 and IEC-6 cells. In agreement with the functional data (Fraga et al., 2002c), the abundance of LAT1 in cell membranes was greater in IEC-6 than in Caco-2 cells. On the other hand (Fig. 4B), it is interesting to underline that the level of expression of LAT1 in the colon was 2-fold that in the jejunum and 3-fold that in the ileum. In contrast, the level of expression of LAT2 was identical in all three segments of the rat digestive tract (jejunum, ileum and colon).

Discussion

The results presented here show that Caco-2 and IEC-6 cells, as well as the rat intestinal mucosa, are endowed with the LAT1 and LAT2 transporter transcripts and protein. LAT1 protein is most abundant in IEC-6 cells, which is in agreement with the functional data previously reported (Fraga et al., 2002c). The findings in the rat intestinal mucosa indicated that LAT1 protein is most abundant in the colon and its abundance markedly decreases at the level of jejunum and ileum, which contrast with relative homogeneous presence of LAT2 across the digestive tract. LAT2 is highly expressed in polarized epithelia (Segawa et al., 1999), suggesting an important role in transepithelial amino acid transport.

The difference in LAT1 abundance along the intestinal tract is likely to negatively correlate with the levels of amino acids that reach each segment. Epithelial colonic cells probably express more units of LAT1 to overcome the lower concentration of amino acids that reach the colon, in contrast with that occurring at the level of the jejunum and ileum. Concerning the cell lines used in this study, the human Caco-2 and the rat IEC-6 cells, they also differently express LAT1, but not LAT2. Although Caco-2 cells derived from human colonic adenocarcinoma, they undergo enterocyte differentiation in culture (Pinto et al., 1983) and possess attributes that make it a suitable *in vitro* model for the study of the small intestine (Hidalgo et al., 1989; Meunier et al., 1995). The IEC-6 cell line, on the other hand, derives from rat intestine epithelial cells that in culture have features of small intestinal crypt cells (Quaroni et al., 1979). This may explain the high expression level of LAT1 in this cell line, as this protein plays a role in cell growth and proliferation (Kanai et al., 1998) and crypt cells are characterized by rapid proliferation.

Apparently, this conflicts with the view that the Na^+- and pH-independent L-type amino acid transporter in Caco-2 and IEC-6 cells may correspond to LAT1. However, the finding that both Caco-2 and IEC-6 cells are endowed with both LAT1 transporter transcripts and express LAT1 protein gives support to the observation that both cell lines transport L-Leucine through the Na^+-independent and pH-insensitive L system (LAT1) (Fraga et al., 2002c). On the other hand, the finding that these cell lines are also endowed with the LAT2 transporter transcript supports the observation that both Caco-2 and IEC-6 cells transport L-DOPA through the Na^+-independent and pH-sensitive L system (LAT2) (Fraga et al., 2002a; 2002b). In fact, LAT1 induces Na^+-independent transport of large neutral amino acids with K_m values in the micro-

molar range. LAT2 also transports small neutral amino acids with a lower affinity to substrate amino acids than that of LAT1 (Segawa et al., 1999; Wagner et al., 2001). A major difference between LAT1 and LAT2 is concerned with their sensitivity to extracellular pH for amino acid uptake (Prasad et al., 1999). These findings are consistent with K_m values for L-DOPA and L-leucine in IEC-6 and Caco-2 cells (Fraga et al., 2002a, b, c).

In conclusion, Caco-2 and IEC-6 cells, as well as the rat intestinal mucosa, are endowed with both LAT1 and LAT2 transporter transcripts and protein. The functional studies indicate that L-leucine transport in both Caco-2 and IEC-6 cells is performed by the LAT1 isoform that is characterized for being Na^+-independent and pH-insensitive. LAT1 protein is most abundant in IEC-6 cells, which is in agreement with the functional data previously reported (Fraga et al., 2002c). The findings in the rat intestinal mucosa indicate that LAT1 protein is most abundant in the colon and its abundance markedly decreases at the level of jejunum and ileum, which contrast with relative homogeneous presence of LAT2 across the digestive tract.

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