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Molecular Cloning, Tissue Distribution and Expression of Porcine y$^+$L Amino Acid Transporter-1*

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ABSTRACT : In this study, we cloned, sequenced and characterized porcine y+L Amino Acid Transporter-1 (y+LAT1). By screening a translated EST database with the protein sequence of the human y$^+$LAT1 and by using rapid amplification of cDNA ends (RACE), the full-length cDNA encoding porcine y$^+$LAT1 was isolated from porcine intestine RNA. It was 2,111 bp long, encoding a 511 amino acid trans-membrane glycoprotein composed of 12 transmembrane domains. The predicted amino acid sequence was found to be 91%, 90%, 87% and 87% identical to those of cattle, human, mouse and rat y$^+$LAT1 respectively. Real-time RT-PCR results indicated that the small intestine had the highest y$^+$LAT1 mRNA abundance and the lung had the lowest y$^+$LAT1 mRNA abundance. Baby hamster kidney (BHK) cells transfected with green fluorescent protein (GFP) tagged porcine y$^+$LAT1 cDNA indicated that the cellular localization of the gene product in BHK was on the plasma membrane. (**Key Words :** Cloning, Pig, Amino Acid Transporter, y$^+$LAT1, SLC7A7)

INTRODUCTION

Five transport systems that mediate the uptake of cationic amino acids (CAA) are known: the Na^+ dependent system $B^{0,+}$, Na^+ independent system b^+, system y^+, system y^+L and system $b^{0,+}$. cDNAs encoding the b0,+AT and CAT-2 of pigs have been reported by our groups (Zhi Ai-min et al., 2008; Zou Shi-geng et al., 2009). The SLC7A7 (HGMW-approved gene symbol SLC7A7, solute carrier family 7, member 7) gene encodes y$^+$LAT1 (y$^+$L amino acid transporter-1) and interacts with 4F2hc (4F2 heavy chain), responsible for the system y$^+$L amino acid transport activity at the membrane (Torrents et al., 1999). System y$^+$L is an antiporter, which exchanges cationic amino acids for large neutral amino acids, cotransported with Na^+, and plays a very important role in basic cellular functions such as cell volume regulation, the synthesis of glutathione (GSH), provision of amino acids for protein synthesis, and energy metabolism (Torrents et al., 1999).

System y$^+$L consists of two subunits, a polytopic membrane protein (light chain, SLC7 family) and an associated type II membrane protein (heavy chain, SLC3 family) subunit (Dall'Asta et al., 2000; Verrey et al., 2000; Chillaron et al., 2001). The heavy chain rBAT (i.e., related to $b^{0,+}$ amino acid transport) associates with the light chain $b^{0,+}$AT ($b^{0,+}$ amino acid transporter) to form the amino acid transport system $b^{0,+}$ isoforms (Verrey et al., 1999), whereas the homologous heavy chain 4F2hc (heavy chain of the surface antigen 4F2) interacts with several light chains (LSHATs; SLC7 family members) to form system L isoforms (with LAT1 and LAT2) (Gottesdiener et al., 1988), system y$^+$L isoforms (with y$^+$LAT1 and y$^+$LAT2) (Pfeiffer et al., 1999), system x_c^- isoforms (with xCT) (Bassi et al., 2001), or system asc isoforms (with asc1), and two (asc2 and AGT-1) seem to interact with as yet unknown heavy subunits (Verrey et al., 2004). System y$^+$L was first functionally described in erythrocytes (Deves et al., 1992). Further investigation revealed its presence in placenta (Novak et al., 1997), platelets (Mendes Ribeiro et al., 1999), skin fibroblasts (Dall'Asta et al., 2000), hepatocytes (Pineda et al., 1999), small intestine and kidney. The light subunit (y$^+$LAT1) has 12 transmembrane domains with the NH_2 and

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COOH termini located intracellularly (Mastroberardino et al., 1998; Sato et al., 1999) and is linked by a single disulfide bond at Cys109 of 4F2hc (Palacin et al., 1998; Chillaron et al., 2001). Functionally, y^+LAT1 obeys an obligatory exchange mechanism, transporting dibasic amino acids in the absence of Na^+ and neutral amino acids in the presence of Na^+ (Pfeiffer et al., 1999). It seems that y^+LAT1 preferentially mediates the efflux of arginine, which may be important in the kidney, where arginine is produced from citrulline and released into the blood to supply the rest of the body (Bröer et al., 2000).

In contrast with intensive studies on the structure and function of human and mouse y^+LAT1, there have been few studies on other animals, including pigs. Therefore, the goal of the present study was to clone the y^+LAT1 gene of pigs and characterize it. Identification of porcine y^+LAT1 will aid in understanding cationic amino acid metabolism and may help in discovering new functions of SLC7A7 in porcine nutrition and physiology.

MATERIALS AND METHODS

Cell culture

BHK were maintained in Dulbecco's modified Eagle's minimal essential medium (Gebico) supplemented with 10% fetal bovine serum and antibiotics (100 μ/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin, and 2 μg/ml Fungizone).

Tissue sample collection

Six 60-day-old, crossbred pigs were purchased from a commercial farm. They were euthanized with an overdose injection of 10% sodium pentobarbital before sampling. The heart, liver, lung, kidney, brain, muscle, and intestines were separated. The isolated tissue samples were immediately put into liquid nitrogen for deep-freezing after flushing with ice-cold saline (154 mM NaCl, 0.1 mM PMSF, pH 7.4). Then, each tube, which contained approximately 10 g of tissue, was tightly capped and stored at -80°C.

RNA extraction and cDNA synthesis

Total tissue (intestines) RNA was isolated from 100 mg of intestine using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and digested by DNase I according to the manufacturer's protocol (TAKARA, Dalian, China). The concentration of RNA solution was determined spectrophotometrically and the quality was verified by visualization of 2:1 intensity ratio of 28S vs. 18S rRNA bands over UV light after electrophoresis through a 1% ethidium bromide stain agarose gel. The RNA had an OD260:OD280 ratio between 1.8-2.0. Synthesis of the first strand cDNA was performed with oligo (dt) 20 and Superscript II reverse transcriptase (Invitrogen).

Cloning strategy

A translated Expressed Sequence Tag (EST) was screened from the translated Expressed Sequence Tags database (which is composed of sequences from species other than humans or mice) at the National Center for Biotechnological Information (NCBI) using the human SLC7A7 protein sequence. This identified one EST sequence (GenBank accession no: DB794205) that showed high homology to the human SLC7A7. PCR primers (ZA1, ZA2) were designed based on this sequence. PCR was performed as described below: 94°C for 5 min, followed by 35 cycles of amplification (94°C for 30 s, 68°C for 30 s, 72°C for 40 s). After the PCR product was sequenced and homologically compared to certify that it was porcine SLC7A7 on the basis of sequence, porcine SLC7A7 gene-specific primers were synthesized and 3′/5′ RACE were carried out according to the manufacturer's instructions (BD Biosciences Clontech). Briefly, the first strand cDNA was generated from 1 μg total RNA using 3' RACE CDS primer (3' CDS) and 5'-CDS/SMART II (Clontech) for 3' RACE and 5' RACE, respectively. For 3' RACE, the amplification reaction was performed by first touch down PCR for 40 cycles (94°C for 5 min, 94°C for 30 s, 70°C, 65°C, 61°C for 30 s respectively, and 72°C for 10 min) using the GSP2 and the UPM (universal primer mix). After the first PCR, the second (nest) PCR was performed under similar conditions using nest primer NGSP2 and the NUP (nest universal primer). For 5' RACE, a similar amplification reaction but with a 3-min elongation time was carried out using the forward primer (UPM and NUP) and reverse primer GSP1 and NGSP1. The obtained fragment was subsequently cloned and sequenced. Based on the newly obtained sequence for the full-length cDNA, a pair of PCR primers, forward primer ZY1 and reverse primer ZY2, were designed to amplify the sequence covering the ORF (open reading frame) of porcine SLC7A7. All primers except the primers provided by Clontech RACE kit are shown in Table 1.

Sequence and structural analysis

The RACE products were gel-purified and cloned into the pGMT vector (Invitrogen). After transformation into *Escherichia coli*, the plasmid purifications from the overnight-grown colonies were done and the cloned cDNA was sequenced. Nucleotide and amino acid sequence alignment was analyzed with the DNAMAN software package. Homology searches were performed using BLAST and FASTA at the National Center for Biotechnological Information (NCBI) and DNA Data Bank of Japan (DDBJ).

Detection of porcine SLC7A7 tissue distribution by

Table 1. Primers for Smart RACE cDNA and ORF Amplification

Primer	Application	Sequence
ZA1	EST	5' CCTTTGTTATGCGGAACTGGGCACC 3'
ZA2	EST	5' CCACAAAGAAAAGCCTAGAAGCAGCCAC 3'
GSP1	First PCR	5' CGAGGCTCCCTGACCAAGTCTAACAAT 3'
NGSP1	Second PCR	5' CCAGAGTCGGATGAAGGCAAGGAGTC 3'
GSP2	First PCR	5' ACAGGTGACATCGCTCTGGCACTCTACT 3'
NGSP2	Second PCR	5' CCAGTGATGCTGTTGCTGTGACTTTTGC 3'
y^+LAT1 forward	Real-time PCR	5'-GAGCCCACAAAGAAAAGC-3'
y^+LAT1 reverse	Real-time PCR	5'-GCCCATTGTCACCATCATC-3'
18S forward	Real-time PCR	5'- GGACATCTAAGGGCATCACAG -3'
18S reverse	Real-time PCR	5'- AATTCCGATAACGAA CGAGACT -3'
ZY1	ORF clone	5' CATGGTTGACGGCATGAAG 3'
ZY2	ORF clone	5' GTTTAGACTTGGGATCTTGTTGC 3'
ZM1	ORF clone	5' CCCAAGCTTATGGTTGACGGCATGA 3'
ZM2	ORF clone	5' GGCTGAATTCGTTTAGACTTGGGATCTTGTTGC 3'

Real-time RT-PCR analysis

Real-time PCR was performed using one-step SYBR Green PCR Mix (Takara, Dalian, China), containing $MgCl_2$, dNTP, and Hotstar *Taq* polymerase. Two microlitres of cDNA template was added to a total volume of 25 μl containing 12.5 μl SYBR Green mix, 0.25 μl RT mix and 1 μM each of forward and reverse primers shown in Table 1. Primers for 18S were designed with Primer 5 based on porcine sequence (Accession No. AY390526). The following protocol was used: i) denaturation program (15 min at 95°C); ii) amplification and quantification program, repeated 40 cycles (15 s at 95°C, 15 s at 58°C, 15 s at 72°C); iii) melting curve program (60-99°C with heating rate of 0.1°C s-1 and fluorescence measurement). An abundantly expressed gene, 18S, was used as the internal control to normalize the amount of starting RNA used for RT-PCR for all samples. Amplification and melt curve analysis were performed in an ABI 7500 (Applied BioSystems). Melt curve analysis was conducted to confirm the specificity of each product, and the size of products was verified on ethidium bromide-stained 2% agarose gels in Tris acetate-EDTA buffer. The identity of each product was confirmed by dideoxy-mediated chain termination sequencing at Takara Biotechnology, Inc. The relative expression ratio (R) of mRNA was calculated by $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001). Real-time PCR efficiencies were acquired by amplification of dilution series of RNA according to the equation 10 (-1/slope) and were consistent between target mRNA and 18S. Negative controls were performed in which water was substituted for RNA.

Transient expression of porcine SLC7A7 in BHK cells

For subcellular localization studies, the plasmid encoding the porcine y^+LAT1-GFP fusion protein was constructed as described below. Briefly, a porcine SLC7A7 cDNA containing the full-length CDS of porcine SLC7A7 was obtained using the following PCR primers (ZM1, ZM2) shown in Table 1. The reaction conditions of PCR were 94°C for 5 min, followed by 35 cycles of amplification (94°C for 30 s, 63°C for 30 s, 72°C for 2 min). After HindIII/EcoRI digestion and gel purification, the PCR product was inserted in the HindIII/EcoRI sites of pEGFP-N1. The resulting plasmid was termed py^+LAT1-EGFP. After transformation and amplification in *E. coli*, plasmids purified from the *E. coli* colonies using a TIANpure Mini plasmid kit (TianGen, BeiJing, China) were sequenced. The clone without mutations by PCR was further amplified in *E. coli* and then transfected into the BHK cells using Lipfectin 2000 (Invitrogen) following the manufacturer's protocol for the expressional localization of porcine y^+LAT1 by inverted fluorescent microscopy.

RESULTS AND DISCUSSION

We searched the translated EST database (which is composed of sequences from species other than humans or mice) with the human y^+LAT1 protein sequence at the National Center for Biotechnological Information (NCBI) and found a length of 771-bp EST (Accession no. DB794205) with high homology to y^+LAT1. Using a RACE approach, the full-length cDNA clone was obtained. Based on this sequence, four gene-specific primers were synthesized and 3'/5' rapid amplifications of cDNA ends (RACE) were performed. 3' RACE (~1 kb) and 5' RACE (~0.5 kb) products were cloned into the pGMT vector and sequenced. Finally, a total of 2,111-bp long cDNA was assembled from the overlapping 3' (1,002 bp), EST and 5' RACE (498 bp).

Sequence analysis of the porcine SLC7A7 cDNA revealed i) an ORF of 1,536 bp that would encode a protein

of 510 amino acid residues, ii) 134 bp of 5' untranslated region (UTR), and iii) 441 bp of 3' UTR with a consensus AATAAA polyadenylation signal at 15-20 nt upstream of a poly(A) stretch. BLASTn or BLASTp analysis demonstrated that the porcine sequence shared a high degree of sequence identity, both in the nucleotide sequences, especially in coding sequence (CDS) regions (91, 90, 87 and 87%), and in the deduced amino acid sequences (93.2, 92.4, 88.5 and 88.9%), with those of cattle (Accession no. NM_001075151), human (Accession no. NM_003982), mouse (Accession no. NM_011405), and rat (Accession no. NM_031341) SLC7A7, respectively. The nucleotide sequence alignments among pig, human and mouse are shown in Figure 1. Furthermore, unlike rat and mouse y^+LAT1 which are shorter or longer than cattle and human y^+LAT1 by 3 amino acids, the porcine y^+LAT1 was of the same length as those of cattle and human. Alignment of amino acid sequence is shown in Figure 2. Hydrophobicity prediction (Hofmann and Stoffel, 1993) suggested 12 putative membrane-spanning domains within

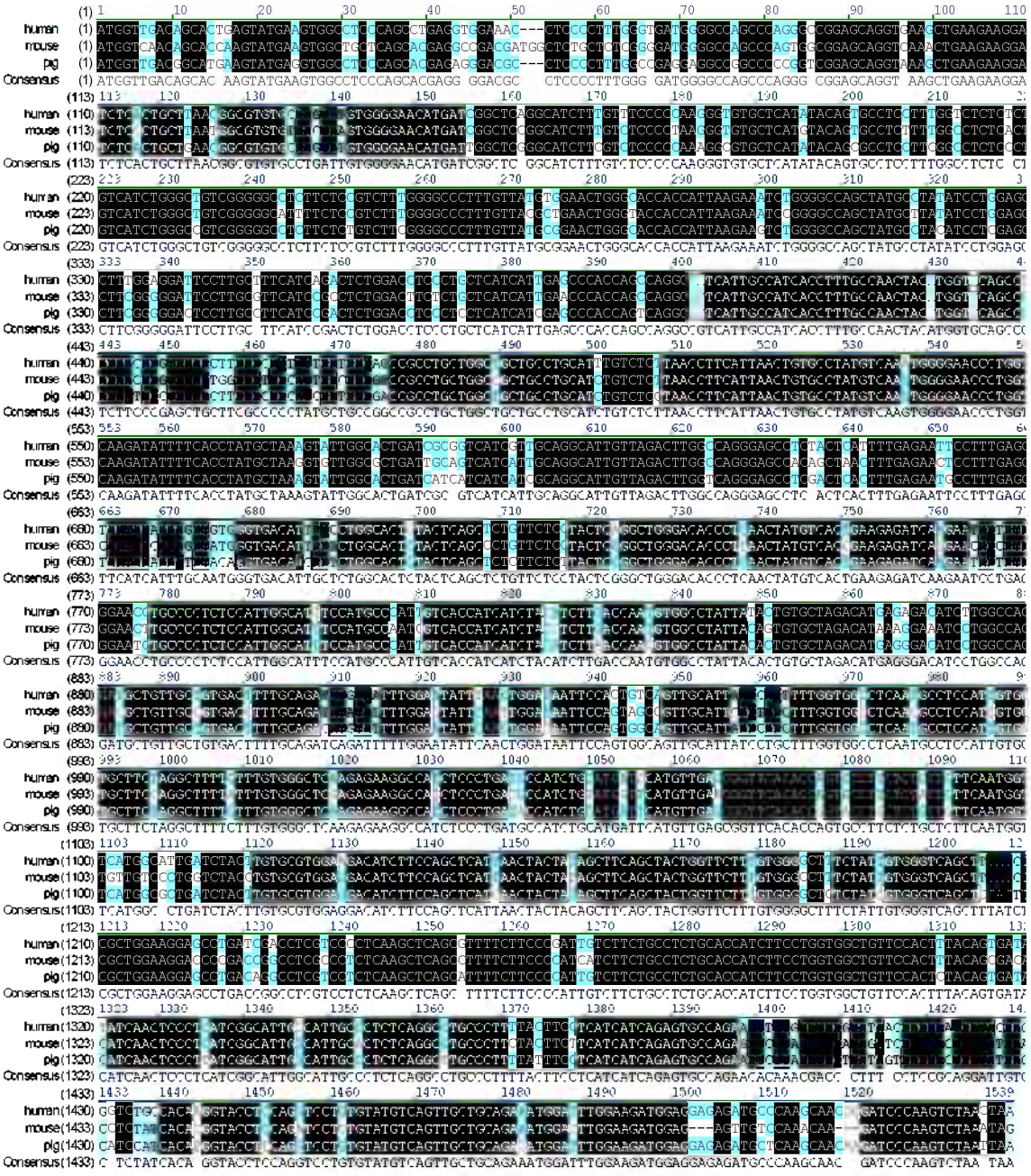

Figure 1. Comparison of code sequence of y^+LAT1 from pig, human and mouse. Identical nucleic acids are shown in black background. The porcine code sequence of y^+LAT1 (Accession No. EU047705) shows 90 and 87% homology with the human (Accession No. NM_003982) and mouse (Accession No. NM_011405) y^+LAT1, respectively.

porcine y^+LAT1, similar to other mammalian y^+LATs and consistent with the results of homologous comparison. Analysis of the amino acid sequence by ScanProsite (Gasteiger et al., 2003) revealed several consensus sites for post-translational modification (Figure 2). There was one glycosylation site on the fourth putative extracellular loop. Two consensus sites for protein kinase C phosphorylation were located at 58-60 and 96-96. The two sites were also present in the amino acid transporter of human y^+LAT1.

In order to examine the tissue distribution of the y^+LAT1 and ascertain whether pig y^+LAT1 was expressed in tissues other than intestine, Real-time PCR results were performed on mRNA from various tissues. The tissue distribution of y^+LAT1 mRNA at day 60 is presented in Figure 3. The small intestine had the highest y^+LAT1 mRNA abundance, while the lung had the lowest ($p<0.05$).

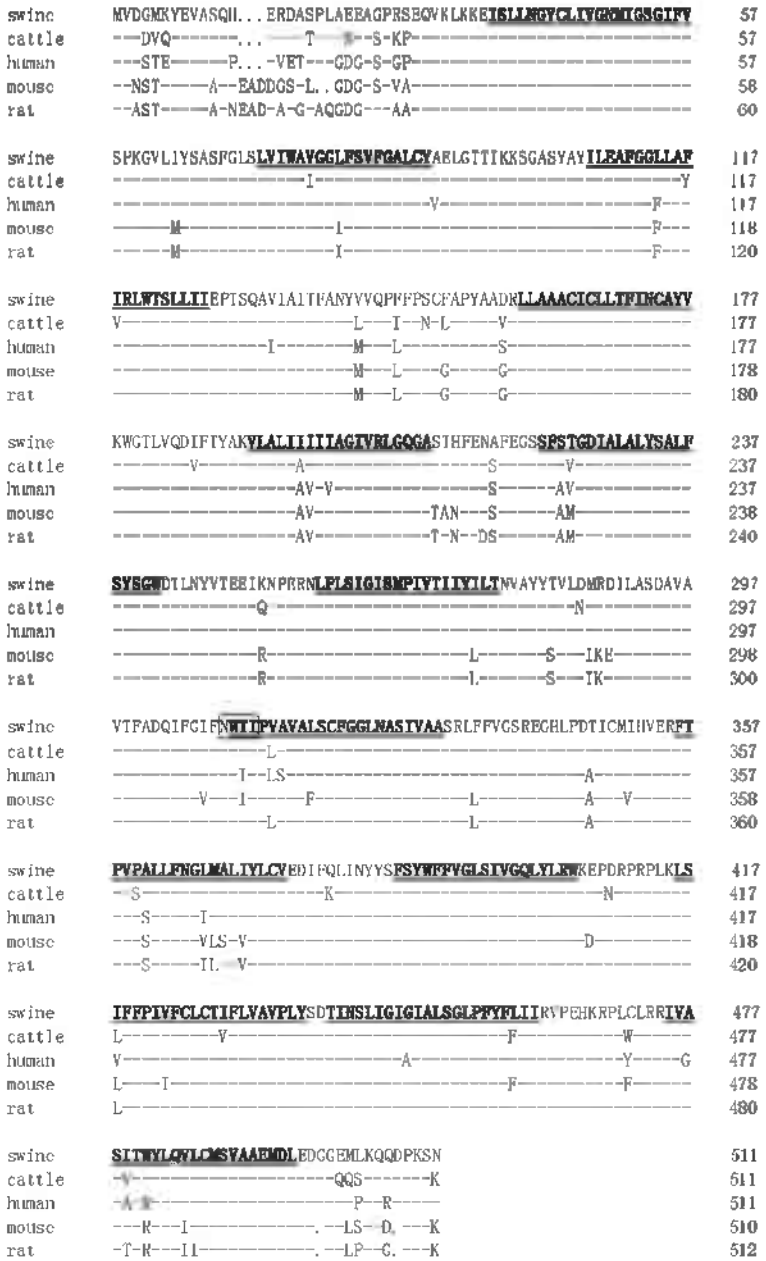

Figure 2. Comparison of amino acid sequence of y^+LAT1 from cattle, pig, human, mouse, and rat. Amino acid sequence determined from the porcine y^+LAT1 cDNA (accession no. EU047705) is shown on the top line. Amino acid sequence for cattle y^+LAT1 (accession no. NM_001075151), human y^+LAT1 (accession no. NM_003982), mouse y^+LAT1 (accession no. NM_011405), and rat y^+LAT1 (accession No. NM_031341) are shown below. Putative membrane-spanning domains are underlined and in bold. Potential N-glycosylation site is indicated by an open box. Dashes indicate identity of amino acid to pig y^+LAT1. Dots indicate the gaps inserted to maintain alignment.

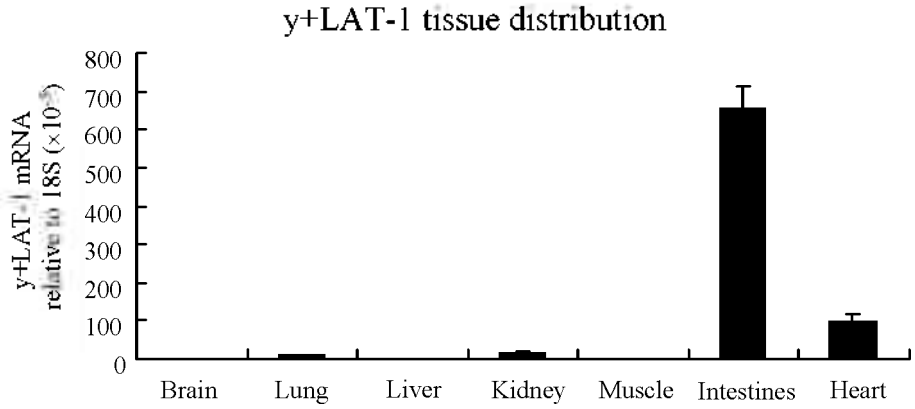

Figure 3. Tissue distribution of porcine y^+LAT1 in the heart, liver, lung, kidney, brain, muscle and intestine. The small intestine had the highest y^+LAT1 mRNA abundance, while the lung had the lowest ($p<0.05$, n = 6). However, undetectable levels of y^+LAT1 mRNA expression were observed in the brain, muscle and liver.

However, undetectable levels of y^+LAT1 mRNA expression were observed in the brain, muscle and liver. The number of transcripts that SLC7A7 genes could express seems to depend on species. For example, a Northern blot showed that high amounts of mouse y^+LAT1 RNA are expressed in the kidney and intestine. Lower amounts of mouse y^+LAT1 RNA appear to be expressed in the other tissues tested (Pfeiffer et al., 1999).

Having verified that the cloned cDNA behaved functionally like porcine SLC7A7, we next sought to identify the cellular localization of the gene product in BHK cells using green fluorescent protein (GFP) tagging. The expressed porcine y^+LAT1-GFP localized to the plasma membrane at 36 h after transfection. Peak expression time was at 60 h, as shown in Figure 4. We detected the signal using inverted fluorescent microscopy 48 h after the transfection. The mutation of SLC7A7 can cause the occurrence of LPI (Lysinuric Protein Intolerance) and affect the trafficking of y^+LAT1 to the plasma membrane in mammalian cells (Borsani et al., 1999; Toivonenb et al., 2002). The right orientation of GFP tagged y^+LAT1 protein in the plasma membrane of BHK cells indicated that the product of cloned cDNA had bioactivity.

In conclusion, we have cloned a cationic amino acid transporter y^+LAT1 from the pig. This cationic amino acid transporter showed significant homology with human and murine y^+LAT1. BHK cell expression showed that the transporter was located in the cell membrane. Therefore, we consider it to represent the porcine homologue of human y^+LAT1 (SLC7A7). Future studies will identify which cationic amino acid is transported by y^+LAT1 as well as its function in porcine nutrition and physiology.

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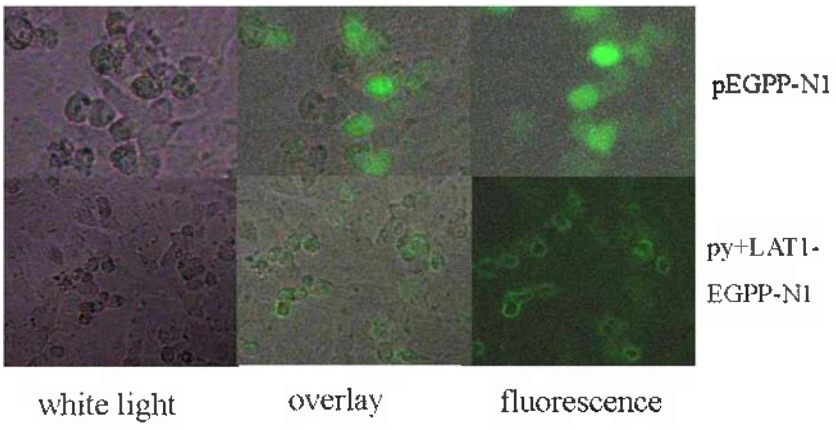

Figure 4. Cellular localization of porcine y^+LAT1-GFP fusion protein detected by invert fluorescent microscopy. BHK cells transfected with the porcine y^+LAT1-GFP or control pEGFP-N1 were observed using inverted fluorescent microscopy 48 h after the transfection. The fluorescence at the midsection of cells is demonstrated. Most of the porcine y^+LAT1-GFP fusion protein targets to the plasma membrane 48 h after transfection, whereas GFP distributes evenly in the cell (Original magnification 40×).

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