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ORIGINAL ARTICLE

Molecular cloning, distribution and ontogenetic expression of the oligopeptide transporter PepT1 mRNA in Tibetan suckling piglets

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Abstract The gene encoding the oligopeptide transporter PepT1 (HGMW-approved gene symbol SLC15A1) from Tibetan porcine intestine was cloned. The open reading frame of this cDNA encodes 708 deduced amino acid residues that show high sequence similarity with its ovine and bovine counterparts. The putative protein has 12 putative transmembrane domains, including many structural features that are highly conserved among the vertebrate orthologs. PepT1 mRNA expression can be detected in duodenum, jejunum and ileum from Tibetan pigs at 28 days by RT-PCR. Real-time PCR analysis indicated that the jejunum had the highest expression of PepT1 when compared with the duodenum and ileum. PepT1 mRNA expression in the duodenum and proximal jejunum increases continuously from day 1 to day 14: expression was highest at day14 ($P < 0.01$) and then decreased gradually from day 21 to day 35. Our findings show that PepT1 mRNA expression in the distal jejunum increased gradually with age in suckling Tibetan piglet, and this may have important implications for amino acid and protein nutrition in young animals.

Keywords Amino acid transporter · PepT1 · Tibetan pig · Expression

Abbreviations

AA	Amino acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PBS	Phosphate-buffered saline

Introduction

A significant fraction of dietary protein digestion products are absorbed as oligopeptides rather than free amino acids (AA) (Fei et al. 1994). The cellular uptake of oligopeptides occurs through membrane transporter proteins in the Solute Carrier 15 (SLC15) family (Daniel and Kottra 2004). Members of this family have been characterized in bacteria, fungi, plants, fruit fly, *Caenorhabditis elegans*, birds and mammals. In higher vertebrates, two members of this family, PepT1 and PepT2, have been characterized extensively. Both proteins translocate di- and tri-peptides across the plasma membrane, even against a concentration gradient, using an inwardly directed electrochemical H+ gradient (Daniel 2004). They are also responsible for the transport of a variety of peptidomimetics, such as β-lactam antibiotics, aminopeptidase and angiotensin-converting enzyme inhibitors, δ-aminolevulinic acid, and many selected prodrugs (Rubio-Aliaga and Daniel 2002).

W. Wang and C. Shi have made equal contributions to the study.

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The PepT1 proteins range from 707 to 729 AA in size, and are present in various mammalian species, with approximately 50–80% homology. The proteins contain 12 membrane-spanning domains and a large extracellular loop between transmembrane domains 9 and 10. They contain several potential *N*-glycosylation as well as protein kinase recognition sites, which suggests that the transporters may be regulated by reversible phosphorylation (Brandsch and Leibach 2004). Very little research has been conducted on the developmental expression of PepT1 protein in the small intestine. Chen et al. (2005) observed a linear increase in cPepT1 with age, which suggested that it may be important in peptide transport in the post-hatch chick. However, the level of intestinal PepT1 mRNA in 4-day-old rats was 3.6-fold than that in adult rats. Thus, the intestinal PepT1 transporter may play a more important role in mammalian neonate (Shen et al. 2001). The ontogenetic development of PepT1 along the length of the rat small intestine was also evaluated from postnatal day 4 to day 50. The PepT1 mRNA levels on day 50 were less than those on day 21 in the proximal and median parts of the small intestine ($P < 0.05$), while there was no change in the distal part (Rome et al. 2002). Few studies have been conducted on the developmental changes in peptide transport in domestic animals. The objectives of this study were to clone the sequence of PepT1 from Tibetan suckling piglets, and then determine the tissue distribution and changes in PepT1 mRNA during their early development. These findings should increase our understanding of the relationships among developmental influences on PepT1 gene expression and peptide absorption.

Materials and methods

Tissue sample collection

The pigs used in this study were the offspring of purebred Tibetan sows and boars, which were maintained at the Laboratory Animal Center of Southern Medical University, P. R. China. Forty-two healthy newborn purebred Tibetan piglets were randomly obtained from eight litters for this study. All piglets were freely nursed by sows and killed during lactation (day 1, 4, 7, 14, 21, 28 and 35). Pigs were euthanized with an overdose injection of 10% sodium pentobarbital before sampling. The liver and the entire intestine were then rapidly removed from the animals. The duodenum, jejunum and ileum were separated and cleaned several times in ice-cold phosphate-buffered saline (PBS). The jejunum was divided into the distal and proximal jejunum. The isolated intestinal segments and other tissues were immediately frozen in liquid nitrogen and stored in a freezer at −70°C until molecular analysis. All procedures were approved by the Animal Care Committee at the Chinese Academy of Sciences.

RNA extraction and cDNA synthesis

Approximately 100 mg of tissue from each sample was pulverized in liquid nitrogen. Total RNA was isolated using TRIZOL reagent (Invitrogen, USA) and treated with DNase I (Invitrogen, USA) according to the manufacturer's instructions. The RNA quality was checked by 1% agarose gel electrophoresis, after staining with 10 μg/mL ethidium bromide. The RNA had an OD260:OD280 ratio between 1.8 and 2.0. First-strand cDNA was synthesized with oligo(dT)20 and Superscript II reverse transcriptase (Invitrogen, USA).

Cloning of the PepT1 cDNA

Primers to recognize the Tibetan porcine PepT1 cDNA sequence were designed with Primer 5.0 (Biosoft International) based on the human and mouse PepT1 cDNA sequences. The polymerase chain reaction (PCR) primers used were 5′-ATGGGAATGTCCGTGCCACAGAGC-3′ (forward) and 5′-TCACATCTGCGTCTGTACGTCGGT-3′ (reverse). PCR conditions were as follows: denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 2% agarose gel in Tris–borate–EDTA buffer and visualized by staining with ethidium bromide. The purified PCR product was cloned into the pGEM-T easy Vector (Promega, USA) and sequenced by dideoxy-mediated chain termination sequencing at Sangon Biotechnology, Inc.

Intestinal distribution of PepT1 cDNA

cDNA samples from the duodenum, proximal jejunum, distal jejunum, ileum and liver at 28 days were chosen. The tissue distribution of PepT1 in different tissues of Tibetan pig was studied by PCR as described above using LA Taq polymerase (TaKaRa, Japan). DEPC-water was used to replace cDNA template to give a negative control.

Quantification of mRNA by real-time RT-PCR analysis

Primers for PepT1 and GAPDH were designed with Primer 5.0 based on the PepT1 cDNA sequence of the Tibetan pig to produce an amplification product (Table 1). GAPDH was used as an internal reference gene to normalize target gene transcript levels. Real-time PCR was performed using SYBR Green PCR Mix, containing $MgCl_2$, dNTP, and Hotstar Taq polymerase. Two microliters of cDNA template was added to a total volume of 25 μL containing 12.5 μL SYBR Green mix, and 1 μmol/L each of the forward and

Table 1 The primers used in real-time PCR

Gene	Acc. no	Primer sequence(5′-3′)	Amplicon size
PepT1	EU400159	Sense:5′- CATCGCCATACCCTTCTG-3′	144
		Antisense:5′-TTCCCATCCATCGTGACATT-3′	
GAPDH	X94251	Sense:5′-AAGGAGTAAGAGCCCCTGGA-3′	139
		Antisense:5′-TCTGGGATGGAAACTGGAA-3′	

reverse primers. We used the following protocol: (1) predenaturation program (10 s at 95°C); (2) amplification and quantification program, 40 cycles (5 s at 95°C, 20 s at 60°C); and (3) melting curve program (60–99°C with heating rate of 0.1°C/s and fluorescence measurement). The identity of each product was confirmed by dideoxy-mediated chain termination sequencing at Sangon Biotechnology, Inc. We calculated the relative expression ratio (R) of mRNA as $R = 2^{-\Delta\Delta Ct}$. The efficiency of real-time PCR was determined by the amplification of a dilution series of cDNA according to the equation $10^{(-1/\text{slope})}$ and was consistent between target mRNA and NADPH. Negative controls were created by replacing cDNA with water.

Bioinformatics analysis

The BLAST program was used to identify homologous sequences in the GenBank database. Sequences were aligned with the multiple alignment program CLUSTAL V. The neighbor-joining method was used to construct a phylogenetic tree. The transmembrane domain of the protein was predicted using the Transmembrane Hidden Markov Model (Version 2.0).

Statistical analysis

To determine the changes in PepT1 mRNA expression, data on the amount of mRNA were subjected to an analysis of unequally spaced orthogonal polynomial contrast (days 1, 4, 7, 14, 21, 28 and 35). Multiple comparisons of mRNA abundance in the duodenum, proximal jejunum, distal jejunum and ileum at days 7 and 21 were made using the Tukey test in the SAS software package. Data are presented as mean ± SEM. $P < 0.05$ was considered to be significant.

Results

Identification and characterization of Tibetan porcine PepT1 cDNA

The PepT1 cDNA sequence of Tibetan piglets was obtained from small intestine RNA using RT-PCR (GenBank Accession number EU400159). The ORF of PepT1 cDNA of 2,127 bp encoded a 708-aa polypeptide (Fig. 1). Hydrophobicity analysis of the AA sequence suggested the presence of 12 putative transmembrane domains with a large extracellular loop between transmembrane domains IX and X (Fig. 2). Five putative extracellular *N*-glycosylation sites (Asn^{50}, Asn^{404}, Asn^{428}, Asn^{498} and Asn^{542}) and three putative protein kinase C phosphorylation sites (Ser^{252}, Ser^{357} and Thr^{362}) were identified (Fig. 1). A single intracellular cAMP/cGMP-dependent protein kinase phosphorylation site was close to the spanning domain IX (Fig. 1). A phylogenetic analysis of the AA sequence was performed, and the resulting neighbor-joining tree showed that the Tibetan pig has a closer genetic relationship with bovine, sheep and dog than with the other animals examined (Fig. 3).

Intestinal distribution of Tibetan porcine PepT1 mRNA

The intestinal distribution of PepT1 at day 28 is shown in Fig. 4. PepT1 transcript expression was not detected in the liver.

To investigate the segmental expression of PepT1 in the small intestine, we compared the PepT1 mRNA levels in four segments of Tibetan pig small intestine at day 28 by quantitative RT-PCR. A survey of PepT1 abundance in various segments is shown in Fig. 5. The distal jejunum had the most PepT1 mRNA and the ileum had the least. There was no difference in PepT1 transcript abundance among the duodenum, proximal jejunum and ileum ($P > 0.05$).

Relative abundance of PepT1 mRNA during ontogenesis

The developmental changes in the relative abundance of PepT1 mRNA are shown in Table 2. Significant differences in PepT1 mRNA expression were observed in all intestinal segments within the age-range examined. The amount of PepT1 mRNA in the duodenum and proximal jejunum was highest at day 14 ($P < 0.01$). However, the amount of PepT1 mRNA in the distal jejunum was highest at day 21 ($P < 0.05$) and no differences were observed between day 28 and day 35 ($P > 0.05$). The PepT1 mRNA level in the ileum was highest at day 7.

Fig. 1 Nucleotide and predicted amino acid sequences of Tibetan porcine PepT1**.** The *numbers* on the *right* refer to positions of the nucleotides. The stop codon is indicated by *. In the amino acid sequence, potential extracellular *N*-glycosylation sites (*light gray boxed* areas) and potential cAMP/cGMP-dependent protein kinase phosphorylation sites at the cytoplasmic surface (*empty boxed* areas) are indicated

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atgggaatgtccgtgccacagagctgcttcggttatcccttgagcatcttcttcatcgtg      60
M  G  M  S  V  P  Q  S  C  F  G  Y  P  L  S  I  F  F  I  V
gtcaacgagttctgtgaaaggttttcctactatggaatgagagcactcctgatcctgtac     120
V  N  E  F  C  E  R  F  S  Y  Y  G  M  R  A  L  L  I  L  Y
ttccggcttttcatcggctggaatgacaatctgtccactgccatctaccacacctttgtg     180
F  R  L  F  I  G  W  N  D  N  L  S  T  A  I  Y  H  T  F  V
gctctgtgctacctgacgcccattctaggagccctcatcgccgactcctggctgggggag     240
A  L  C  Y  L  T  P  I  L  G  A  L  I  A  D  S  W  L  G  E
ttcaagacaattgtgtcgttgtccatcgtctacaccattggacaggtggtcatggccgtg     300
F  K  T  I  V  S  L  S  I  V  Y  T  I  G  Q  V  V  M  A  V
agctccatcaatgacctcacagacttcgaccacaacggaacccccaacagcatgtctgtg     360
S  S  I  N  D  L  T  D  F  D  H  N  G  T  P  N  S  M  S  V
cacgtggcgctgtccatgatcggcctggccctgattgctctgggtactggcgggataaag     420
H  V  A  L  S  M  I  G  L  A  L  I  A  L  G  T  G  G  I  K
ccctgtgtgtcggcctttgggggcgatcagtttgaagagggccaggaaaagcaaagaaac     480
P  C  V  S  A  F  G  G  D  Q  F  E  E  G  Q  E  K  Q  R  N
cgatttttttccgtctttttatttggccattaatgctggaagtttgctttctacgatcatc     540
R  F  F  S  V  F  Y  L  A  I  N  A  G  S  L  L  S  T  I  I
actcccatgctcagagttcaacaatgtggaattcacagtacccaggcttgctacccactg     600
T  P  M  L  R  V  Q  Q  C  G  I  H  S  T  Q  A  C  Y  P  L
gcatttggggttcctgctgctctcatggctgtatctctgattgtgtttgtcatgggcagc     660
A  F  G  V  P  A  A  L  M  A  V  S  L  I  V  F  V  M  G  S
agaatgtacaagaagctcaagccccagggtaatgtcatggccaaagtcgtcaagtgcatc     720
R  M  Y  K  K  L  K  P  Q  G  N  V  M  A  K  V  V  K  C  I
gggtttgccatcaaaaataggtttaggcatcggagtaagaagtttcccaagagggagcac     780
G  F  A  I  K  N  R  F  R  H  R  S  K  K  F  P  K  R  E  H
tggctggactgggccaaggagaaatatgacgagcggctcatctgtcaaatcaagatggtc     840
W  L  D  W  A  K  E  K  Y  D  E  R  L  I  C  Q  I  K  M  V
acgcgcgtgatgttcctgtatatcccgctccccatgttctgggctttgtttgaccagcag     900
T  R  V  M  F  L  Y  I  P  L  P  M  F  W  A  L  F  D  Q  Q
ggcttcaggtggacactgcaagcaacgaccatgaatgggcaaattgggttgcttaaaatc     960
G  F  R  W  T  L  Q  A  T  T  M  N  G  Q  I  G  L  L  K  I
cagccggatcagatgcagaccgtcaacgccatcctgatcgttattatggtccccatcatg    1020
Q  P  D  Q  M  Q  T  V  N  A  I  L  I  V  I  M  V  P  I  M
gatgctgtggtgtatcctctgatcgcgaagtgtggtttgaatttcacctccctgaggaag    1080
D  A  V  V  Y  P  L  I  A  K  C  G  L  N  F  T  S  L  R  K
atgacagttgggatgttcctggcttccatggctttcgtggcagctgccatcgtgcaggtg    1140
M  T  V  G  M  F  L  A  S  M  A  F  V  A  A  A  I  V  Q  V
gagattgacaaaactcttccagtcttccccaaaggaaatgaagtccaagttaaagtactg    1200
E  I  D  K  T  L  P  V  F  P  K  G  N  E  V  Q  V  K  V  L
aacataggaaataacagcatgtccgtatcttttcctggaacgacggtgacccttgaccag    1260
N  I  G  N  N  S  M  S  V  S  F  P  G  T  T  V  T  L  D  Q
```

Fig. 1 continued

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atgtctcaaacacacgaatttctgactttcgacgtcaacaaactgacaagtataaacatt   1320
M  S  Q  T  H  E  F  L  T  F  D  V  N  K  L  T  S  I  N  I
agttctgctggatcaccagccactccagtaacttacaactttgagcagggccatcgccat   1380
S  S  A  G  S  P  A  T  P  V  T  Y  N  F  E  Q  G  H  R  H
acccttctggtgtggggccccagtcactaccgagtggtaaaggacggccttaaccagaag   1440
T  L  L  V  W  G  P  S  H  Y  R  V  V  K  D  G  L  N  Q  K
cctgaaaaaggagaaaacggagtcagatttgtaaatactttgacgagagcttcaatgtc   1500
P  E  K  G  E  N  G  V  R  F  V  N  T  F  D  E  S  F  N  V
acgatggatgggaaagtctacatagatgtcaccagtcacaacgccagcgcctatcagttt   1560
T  M  D  G  K  V  Y  I  D  V  T  S  H  N  A  S  A  Y  Q  F
tttctttcaggcgcaaaaagcttcatcgtgcactcaccggagatttcaccgcagtgtaaa   1620
F  L  S  G  A  K  S  F  I  V  H  S  P  E  I  S  P  Q  C  K
aataatttcacgtcctccagccttgaatttggcagcgcgtttacctatgtgatcacgagg   1680
N  N  F  T  S  S  S  L  E  F  G  S  A  F  T  Y  V  I  T  R
aaggaggacagctgccccgatctgaagatttttgaggatatttcccccaatacgattaac   1740
K  E  D  S  C  P  D  L  K  I  F  E  D  I  S  P  N  T  I  N
atggctctgcagatcccgcagtatttcctcatcacctgcggcgaggtggtcttctctgtc   1800
M  A  L  Q  I  P  Q  Y  F  L  I  T  C  G  E  V  V  F  S  V
acgggactggagttctcctattctcaggctccttccaacatgaagtcggtgcttcaagca   1860
T  G  L  E  F  S  Y  S  Q  A  P  S  N  M  K  S  V  L  Q  A
ggatggctgttgaccgtggctgttggcaacatcatcgtgcttatcgtggcaggagcaggc   1920
G  W  L  L  T  V  A  V  G  N  I  I  V  L  I  V  A  G  A  G
cagttcagtgaacagtgggccgagtacgttctgtttgcggggttgctcctcgccgtctgc   1980
Q  F  S  E  Q  W  A  E  Y  V  L  F  A  G  L  L  L  A  V  C
ataatatttgccatcatggctcgattccacacgtacatcaacccagcagaggttgaagct   2040
I  I  F  A  I  M  A  R  F  H  T  Y  I  N  P  A  E  V  E  A
cagtttgatatggacgaaaagaaaaagtacctgggaaaggatagcctgtaccccaagctg   2100
Q  F  D  M  D  E  K  K  K  Y  L  G  K  D  S  L  Y  P  K  L
gacaccgacgtacagacgcagatgtga                                    2127
D  T  D  V  Q  T  Q  M  *
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Discussion

The Tibetan pig PepT1 gene cloned in the present study is similar in size (2,127 bp) to the PepT1 genes reported in other animals. Tibetan porcine PepT1 shows high overall identity with PepT1-type transporters (58–98%) when compared to other known PepT1 proteins in vertebrates (Fig. 2), and clusters in the 'livestock' branch of the reconstructed phylogenetic tree, together with sheep, bovine and dog PepT1 (Fig. 3).

PepT1 usually has a conserved 12-transmembrane domain structure with a large extracellular loop located between transmembrane domains 9 and 10 (Covitz et al. 1998; Fei et al. 2000). However, analysis of Tibetan pig PepT1 using the Transmembrane Hidden Markov Model program revealed 13 putative transmembrane domains rather than 12. The extra predicted transmembrane domain of this porcine PepT1 is located at the amino terminus of the protein, whereas the locations of the other 12 transmembrane domains are conserved. In the 13-transmembrane domain model, a large hydrophilic loop of approximately 200 AA is located between transmembrane domains 10 and 11, rather than between domains 9 and 10. We believe that membrane-spanning domain I was divided into two adjacent sub-regions of hydrophobic AA, and a very large hydrophobic area may be close to the N terminus of the PepT1 protein. Moreover, the presence, beside the large *N*-glycosylation-rich region, of short, well-conserved stretches of AA within the large

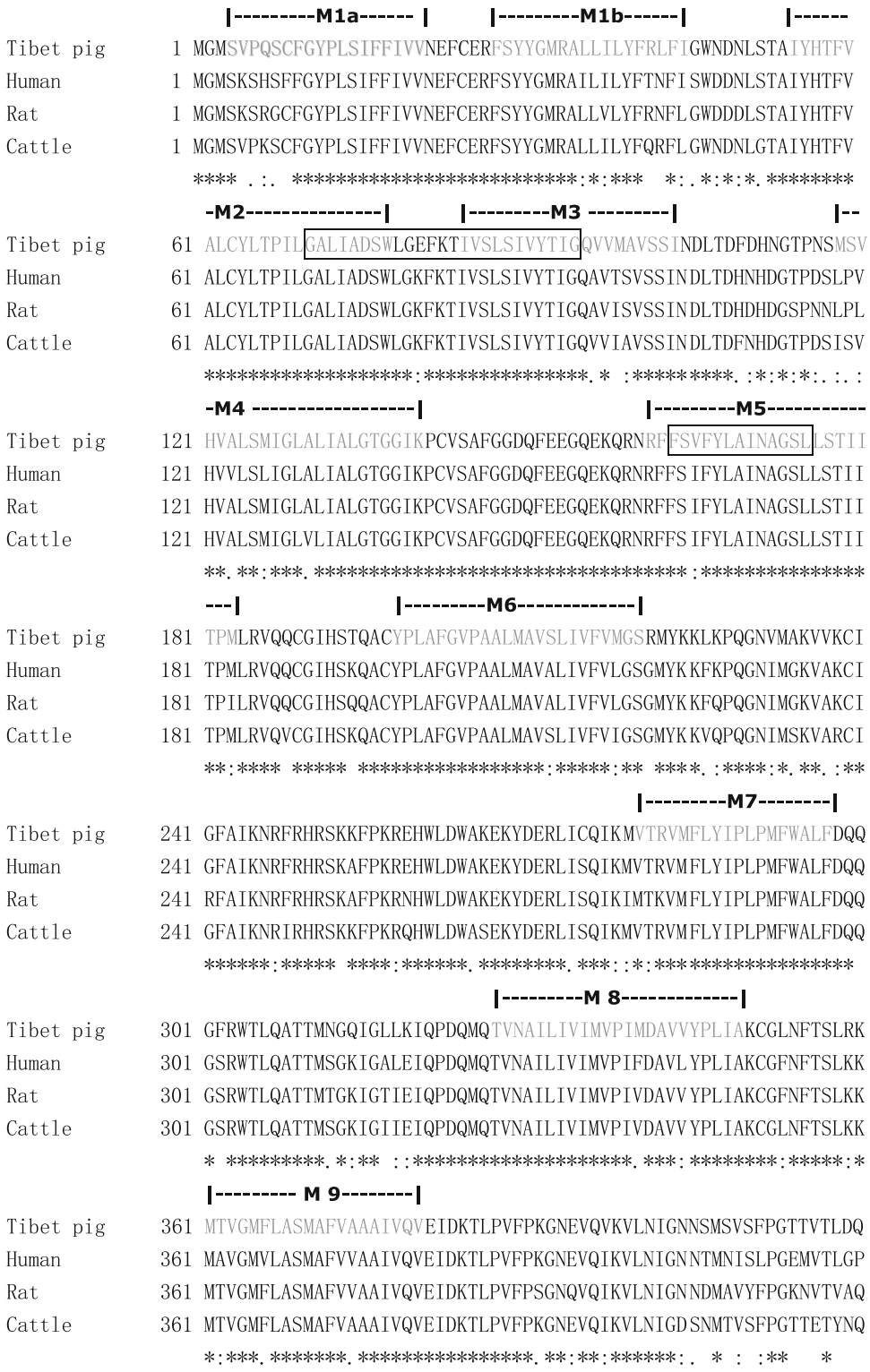

Fig. 2 Amino acid alignment of human, rat, cattle and Tibetan porcine PepT1. Multiple sequence alignment was generated using Clustal W 1.82. Putative transmembrane segments (Ia–XII) were predicted using the TMHMM 2.0 program. The beginnings and ends of the transmembrane segments, as established for mammalian PepT1, are indicated by (+) and (−), respectively. The proposed 'PTR2 family proton/ oligopeptide symporters signature 1' motif and 'PTR2 family proton/oligopeptide symporters signature 2' motif are highlighted in *box*

extracellular loop is novel structural evidence that deserves further study. Since PepT1 protein activity can be regulated by agonists or antagonists of protein kinase A and C as well as by hormones/extracellular signals, the highly conserved cAMP/cGMP-dependent protein kinase phosphorylation motif close to membrane-spanning domain IX and the adjacent protein kinase C phosphorylation motif should also merit further analysis (Daniel 2004).

PepT1 mRNA is expressed in the small intestine of rabbits, rats, sheep, chickens and bear, with little

Fig. 2 continued

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Tibet pig   421 MSQTHEFLTFDVNKLTSINISSAGSP-ATPVTYNFEQGHRHTLLVWGPSHYRVVKDGLNQ
Human       421 MSQTNAFMTFDVNKLTRINISSPGSP-VTAVTDDFKQGQRHTLLVWAPNHYQVVKDGLNQ
Rat         421 MSQTDTFMTFDVDQLTSINVSSPGSPGVTTVAHEFEPGHRHTLLVWGPNLYRVVKDGLNQ
Cattle      421 MSQPKDFMTFNVDNLS-INISSTGSP-VTPVTHNFESGHRHTLLVWAPSNYQVVKDGLNQ
                ***.. *:**:*::*: **:**.*** .*.*: :*: *:*******.*. *:********

Tibet pig   480 KPEKGENGVRFVNTFDESFNVTMDGKVYIDVTSHNASAYQFFLSGAKSFIVHSPEISPQC
Human       480 KPEKGENGIRFVNTFNELITITMSGKVYANISSYNASTYQFFPSGIKGFTISSTEIPPQC
Rat         481 KPEKGENGIRFVSTLNEMITIKMSGKVYENVTSHSASNYQFFPSGQKDYTINTTEIAPNC
Cattle      479 KPEKGRNGIRFVNAFGESFNVTMDGEVYNNVSSHNASEYLFFSSGVKSFTINSPEISQPC
                *****.**:***.::.* :.::.*.*:** :::*:.** * ** ** *.: : :.**.  *
                                                                |--------M10-
Tibet pig   540 KNNFTSSSLEFGSAFTYVIT-RKEDSCPDLKIFEDISPNTINMALQIPQYFLITCGEVVF
Human       540 QPNFNTFYLEFGSAYTYIVQ-RKNDSCPEVKVFEDISANTVNMALQIPQYFLLTCGEVVF
Rat         541 SSDFKSSNLDFGSAYTYVIRSRASDGCLEVKEFEDIPPNTVNMALQIPQYFLLTCGEVVF
Cattle      539 GKEFKTPYLGFGSAFTYVIT-RKSDGCPEAKAFEDISPNTVSMALQIPQYFLLTCGEVVF
                  :*.:  * ****:**::  * .*.* : * ****..**:.**********:********

                ----------|          |--------M11----------|    |--------M12-
Tibet pig   599 SVTGLEFSYSQAPSNMKSVLQAGWLLTVAVGNIIVLIVAGAGQFSEQWAEYVLFAGLLLA
Human       599 SVTGLEFSYSQAPSNMKSVLQAGWLLTVAVGNIIVLIVAGAGQFSKQWAEYILFAALLLV
Rat         601 SVTGLEFSYSQAPSNMKSVLQAGWLLTVAIGNIIVLIVAEAGHFDKQWAEYVLFASLLLV
Cattle      598 SVTGLEFSYSQAPSNMKSVLQAGWLLTVAVGNIIVLIVAGAGQFSEQWAEYVLFAALLLV
                ********************************:********* **:*.:*****:***.***.
                -------|
Tibet pig   659 VCIIFAIMARFHTYINPAEVEAQFDMDEKKKYLGKDSLYPKLDTDVQTQM
Human       659 VCVIFAIMARFYTYINPAEIEAQFDEDEKKNRLEKSNPYFMSGANSQKQM
Rat         661 VCIIFAIMARFYTYINPAEIEAQFDEDEKKKGVGKENPYSSLEPVSQTNM
Cattle      658 VCVIFAIMARFYTYINPAEVEAQFDKDDKEDYLEKSNPYAKLDSVSQTQM
                **:********:*******:***** *:*:. : *.. *    .   *.:*
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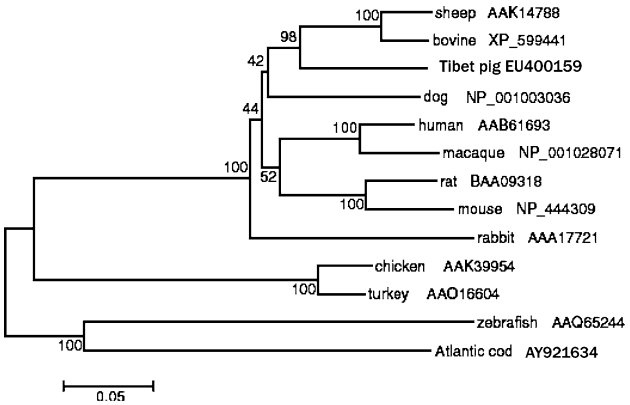

Fig. 3 Unrooted phylogenetic tree depicting the evolutionary relationship of vertebrate PepT1 transporters. The unrooted tree was constructed using the neighbor-joining (NJ) method based on the alignment of the complete amino acid sequences of known vertebrate PepT1 transporters. Bootstrap values (1,000 replicates) indicating the occurrence of nodes are reported above each branch in the figure

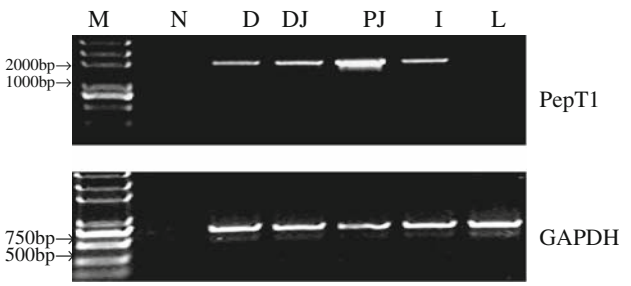

Fig. 4 Tissue distribution of Tibetan *PepT1* mRNA at 28 ages using RT-PCR. PCR were performed using primers specific for PepT1(30 cycles) and *GAPDH* (20cycles). *GAPDH* was amplified as internal control. *M*, Marker; *N*, negative control; *D*, duodenum; *DJ*, distal jejunum; *PJ*, proximal jejunum; *I*, ileum. *L*, liver

expression in the liver and kidney (Fei et al. 1994; Saito et al. 1995; Pan et al. 2001; Chen et al. 2005; Van et al. 2005). In the present study, RT-PCR signals of Tibetan

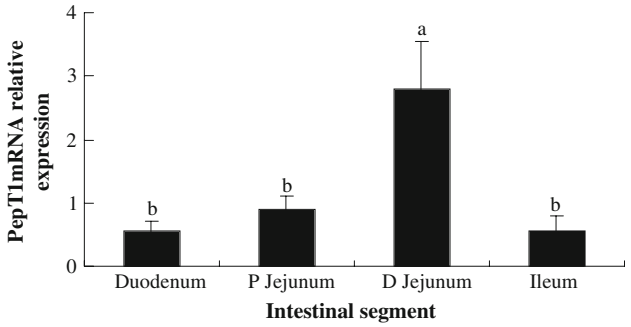

Fig. 5 Relative expression of Tibetan pig PepT1 mRNA along longitudinal axis of intestine at day 28. All samples were normalized using GAPDH expression as an internal control in each real-time PCR. Relative levels of PepT1 mRNA were analyzed by the $2^{-\Delta\Delta Ct}$ method. *Bars* that share a common superscript do not differ ($P > 0.05$). Data are presented as mean ± SE ($n = 6$), in arbitrary units

Table 2 PepT1 mRNA abundance of Tibetan piglets in intestinal distribution during sucking development from Day 1 to Day 35

Age (days)	Segment			
	Duodenum	Proximal jejunum	Distal jejunum	Ileum
1	1.009 bc	1.244 b	0.785 b	0.573 c
7	1.294 b	0.730 b	1.729 ab	1.093 ab
14	3.575 a	2.257 a	2.588 a	0.177 d
21	1.709 b	1.090 b	3.072 a	0.819 bc
28	0.531 c	0.885 b	2.784 a	0.527 c
35	0.424 c	1.023 b	2.850 a	1.411 a
SEM[1]	0.239	0.186	0.416	0.104

All samples were normalized using GAPDH expression as an internal control in each real-time PCR. Relative levels of PepT1 mRNA were analyzed by the $2^{-\Delta\Delta Ct}$ method

Values are mean for six piglets. Means in the same column without a common letter (a, b, c) differ ($P < 0.05$)

[1] SEM, pooled standard error of means for the age effects

porcine PepT1 were detected in the duodenum, jejunum and ileum, but not in the liver. In this research, a more detailed real-time-PCR analysis of its distribution along the small intestinal tract of Tibetan pig revealed that PepT1 is ubiquitously expressed in all segments. This suggests that Tibetan pig may have a very high capacity to absorb small peptides from dietary protein, with peptide absorption occurring in most parts of the small intestine. PepT1 mRNA was evenly distributed in the small intestine of rat. However, Tibetan porcine PepT1 mRNA levels increased from the duodenum to the distal jejunum of the small intestine ($P < 0.05$) and were dramatically reduced in the ileum ($P < 0.05$). Such high expression in the distal jejunum suggests that this segment is predominantly involved in peptide absorption.

The suckling period is a critical time in mammalian life. The intestinal peptide transport system is more important for animals in the suckling period (Himukai et al. 1980; Guandalini and Rubino 1982; Freeman 1995; Miyamoto et al. 1996; Rome et al. 2002; Xiao et al. 2004). The PepT1 mRNA levels in the small intestine of rat increased rapidly from the early days to the middle of the suckling period, and then decreased from the middle to the end of the suckling period (Miyamoto et al. 1996; Rome et al. 2002; Shen et al. 2001). In the present study, PepT1 mRNA expression in the duodenum, proximal jejunum and distal jejunum of Tibetan piglets increased gradually from day 1 to the middle of the suckling period, and then gradually decreased from the middle to the end of the suckling period. We concluded that PepT1 gene expression was unregulated by the onset of suckling colostrum or milk, which is rich in nutrients and growth factors, and thus the transport activity of the entire small intestine increases due to a dramatic increase in intestinal mucosal mass. However, from the middle to the end of the suckling period, the nutrients obtained by piglets were limited by the milk yield of sows and the transport activity of the entire small intestine decreased.

In summary, we have cloned Tibetan pig PepT1, which encodes a 708-AA protein with 12 transmembrane domains. It has a high degree of sequence and structure similarity with bovine PepT1. Among the segments of the small intestine, the distal jejunum is the predominant expression site and probably the most active in peptide absorption. The expression of PepT1 mRNA along the small intestine is regulated according to age in early development, which may have important implications for protein nutrition in young animals.

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