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Oligopeptide transporter PepT1 in Atlantic cod (*Gadus morhua* L.): cloning, tissue expression and comparative aspects

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Summary

A novel full-length cDNA that encodes for the Atlantic cod (*Gadus morhua* L.) PepT1-type oligopeptide transporter has been cloned. This cDNA (named codPepT1) was 2838 bp long, with an open reading frame of 2190 bp encoding a putative protein of 729 amino acids. Comparison of the predicted Atlantic cod PepT1 protein with zebrafish, bird and mammalian orthologs allowed detection of many structural features that are highly conserved among all the vertebrate proteins analysed, including (1) a larger than expected area of hydrophobic amino acids in close proximity to the N terminus; (2) a single highly conserved cAMP/cGMP-dependent protein kinase phosphorylation motif; (3) a large N-glycosylation-rich region within the large extracellular loop; and (4) a conserved and previously undescribed stretch of 8–12 amino acid residues within the large extracellular loop. Expression analysis at the mRNA level indicated that Atlantic cod PepT1 is mainly expressed at intestinal level, but that it is also present in kidney and spleen. Analysis of its regional distribution along the intestinal tract of the fish revealed that PepT1 is ubiquitously expressed in all segments beyond the stomach, including the pyloric caeca, and through the whole midgut. Only in the last segment, which included the hindgut, was there a lower expression. Atlantic cod PepT1, the second teleost fish PepT1-type transporter documented to date, will contribute to the elucidation of the evolutionary and functional relationships among vertebrate peptide transporters. Moreover, it can represent a useful tool for the study of gut functional regionalization, as well as a marker for the analysis of temporal and spatial expression during ontogeny.

Key words: oligopeptide transporter PepT1, comparative sequence analysis, tissue expression, peptide, teleost.

Introduction

Uptake of oligopeptides (di- and tripeptides) into cells is due to membrane transport proteins that belong to the so-called SoLute Carrier 15 (SLC15) family (Daniel and Kottra, 2004). One of the members of this family, namely PepT1 (or SLC15A1), is highly expressed in the intestine of vertebrates, where it is responsible for the transport of a significant fraction of dietary protein across the brush-border membrane of the small intestinal epithelium (for a review, see Daniel, 2004). PepT1 functions as a Na^+-independent, H^+-dependent, H^+-coupled transporter of a variety of di- and tripeptides. PepT1 is also responsible for the transport of orally active drugs, such as β-lactam antibiotics, aminopeptidase and angiotensin-converting enzyme inhibitors, δ-aminolevulinic acid, and many selected pro-drugs (for a review, see Rubio-Aliaga and Daniel, 2002).

PepT1 proteins have been characterized in great detail in higher vertebrates [mostly in mammals, but also in birds (Daniel et al., 2006)]. In contrast, information about these proteins in lower vertebrates is very limited, with the sole exception of the PepT1-type oligopeptide transporter of the teleost zebrafish *Danio rerio*, whose functional activity and pattern of expression in tissue has been assessed (Verri et al., 2003). In particular, zebrafish PepT1 is highly expressed in the proximal intestine, where it mediates the uptake of a large amount of di- and tripeptides derived from the protein digestion process.

We report here the cloning of a full-length cDNA that encodes for the PepT1-type oligopeptide transporter of the Atlantic cod (*Gadus morhua* L.). This is an important commercial species in many North Atlantic countries, and has recently been targeted for aquaculture, mainly due to depletion of natural stocks by overfishing (e.g. Brander, 2007). For this reason, Atlantic cod has become an important model fish species. The complete Atlantic cod PepT1 sequence, the second fish sequence resolved to date, has made a detailed comparison along the vertebrate series possible, allowing identification of highly conserved motifs/regions in all the vertebrate PepT1 transporters. It also became possible to study tissue expression as well as the regional distribution in the digestive tract of the transporter at the mRNA level.

Materials and methods

Animals and tissue sampling

Atlantic cods (*Gadus morhua* L.) used in this experimental work (45 cm in length) were reared in commercial systems, or at the Bergen High-Technology Centre (Bergen, Norway). Fish were routinely fed to satiety once daily, and fasted for 36–48 h prior to sampling. The fish were killed by a blow to the head and their organs were rapidly removed, transferred into RNA*later* (Ambion, Austin, TX, USA) and stored at –80°C until RNA extraction. In two fish, the gut was dissected into ten sections for regional analysis of the spatial distribution along the intestinal tract. The segments were stomach, pyloric area, pyloric caeca (inner and outer segments), and six segments in the remainder of the intestine based on divisions of the three loops present in the dissected gut. The last segment (segment 10) also comprised the hindgut.

Molecular cloning

Total RNA was isolated from cod intestine using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Total RNA (1 μg) was reverse transcribed at 37°C for 1 h using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT; Invitrogen, Carlsbad, CA, USA) and a reverse oligo(dT)-adapter [5′-ACGCGTCGAC-CTCGAGATCGATG$(T)_{18}$-3′]. Amplification of a partial Atlantic cod PepT1 (codPepT1) was performed by polymerase chain reaction (PCR) using *Taq* DNA polymerase and degenerate primers whose design was based on conserved regions of known vertebrate PepT1 genes. Among these, the sequences from icefish (*Chionodraco hamatus*; GenBank accession no. AY170828), European eel (*Anguilla anguilla*; GenBank accession no. AY167576) and zebrafish (*Danio rerio*; GenBank accession no. AY300011). In addition, by data-mining EST sequences and *via* manual and automated (http://genes.mit.edu/genomescan.html) predictions of locations and exon–intron boundaries, respectively, the partial cDNA for *Salmo salar* (TPA Database accession no. BK004882) and for *Takifugu rubripes* (TPA Database accession no. BK004883) were outlined and included in the design of the following degenerate primers: forward primer-1 (FP-1; 5′-GCDGCMTTYGGDGGAGAYCAGTT-3′) and reverse primer-1 (RP-1; 5′-CCAGTCCAKCCAGTGCKCYCTYTTKKGG-3′). The PCR reactions were conducted for 30 cycles (1 cycle: 30 s at 95°C, 45 s at 58°C and 1 min at 72°C), followed by a 10 min final extension at 72°C, with *Taq* DNA polymerase (Invitrogen). The PCR products were size-fractionated in a 2% agarose gel by electrophoresis and the fragment of the expected size was gel-purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Uppsala, Sweden), ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), and multiple clones were screened by sequencing. The identity of the resulting PCR product was confirmed by DNA sequence analysis at Macrogen Inc. (South Korea).

The full-length sequence of codPepT1 was obtained by 3′ and 5′ RACE using the Marathon cDNA Amplification Kit (Clontech, Mountain View, CA, USA) with Advantage Klen *Taq* polymerase (Clontech). PolyA(+) RNA was purified with the Oligotex mRNA Midi Kit (Qiagen GmbH, Hilden, Germany). 1 μg of this mRNA was used to construct one Marathon cDNA library (Clontech) and the 5′ and 3′ ends were obtained by rapid amplification of cDNA ends (RACE)-PCR using Advantage Klen *Taq* polymerase, the AP1 primer and gene-specific primers codPepT1-F1 (5′-CTCCATCTTCTAC-CTGTCCATCAACGCA-3′) and codPepT1-R1 (5′-GCTA-CGGTTCCTGAAGCGGTTTTTGACT-3′). Amplification conditions were those suggested by the supplier. PCR products were size-separated by agarose gel electrophoresis and visualized by ethidium bromide staining, extracted from the gel with GFX PCR DNA and the Gel Band Purification Kit, and cloned into the pGEM-T Easy vector. Final identification was made by DNA sequence analysis at Macrogen Inc.

In silico analysis

The codPepT1 amino acid sequence was deduced using the open reading frame (ORF) finder program at http://www.ncbi.nlm.nih.gov. Putative transmembrane domains were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), which is part of the Simple Modular Architecture Research Tool (SMART; at http://www.expasy.org/prosite/). Potential N-glycosylation, cAMP/cGMP-dependent protein kinase and protein kinase C recognition sequences were identified using the PROSITE 19.7 computational tools (http://www.expasy.org/prosite/).

Nucleotide sequences were routinely compared with the GenBank database using the BLAST algorithm (Altschul et al., 1997). Clustal W 1.82 was used to align amino acid sequences (www.ebi.ac.uk/clustalw). The phylogenetic reconstruction was generated using the neighbor-joining (NJ) method (Saitou and Nei, 1987), as implemented in MEGA 3.0 (Kumar et al., 2004). Phylogenetic trees were constructed with bootstrap confidence values based on 1000 replicates. GenBank accession numbers for amino acid sequence comparisons are: AAQ65244 [zebrafish PEPT1 (Verri et al., 2003)], AAA17721 [rabbit PEPT1 (Fei et al., 1994)], NP_001003036 (dog PEPT1), AAO43094 [pig PEPT1 (Klang et al., 2005)], XP_599441 (bovine PEPT1), AAB61693 [human PEPT1 (Liang et al., 1995)], NP_001028071 [macaque PEPT1 (Zhang et al., 2004a)], BAA09318 [rat PEPT1 (Miyamoto et al., 1996)], NP_444309 [mouse PEPT1 (Fei et al., 2000)], AAK14788 [sheep PEPT1 (Pan et al., 2001)], AAK39954 [chicken PEPT1 (Chen et al., 2002)], AAO16604 (turkey PEPT1).

Reverse transcriptase-polymerase chain reaction (RT-PCR) expression analysis

Total RNA was purified from heart, spleen, gill, eye, intestine, ovary, kidney and liver using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), and subjected to DNase treatment (Turbo DNase, Ambion). For regional analysis of the intestinal tract, total RNA was extracted from various gut segments, as depicted in Fig. 5 (see also *Animals and tissue sampling*). cDNA was synthesized starting from 1.5 μg total RNA, using SuperScript III (Invitrogen) and following the manufacturer's recommendations. The following Atlantic cod specific primers were designed on the basis of the nucleotide sequence GenBank accession no. CO541820 for *Gadus morhua* elongation factor 1α (EF1α) mRNA: forward primer CodEF1AF (5′-CCCCTCCAGGACGTCTACAAG-3′) and reverse primer CodEF1AR (5′-GGCAGAGCCACCGATCTTC-3′), while the forward primer CodPepT1F (5′-CCGCTTCAGGAACCGT-

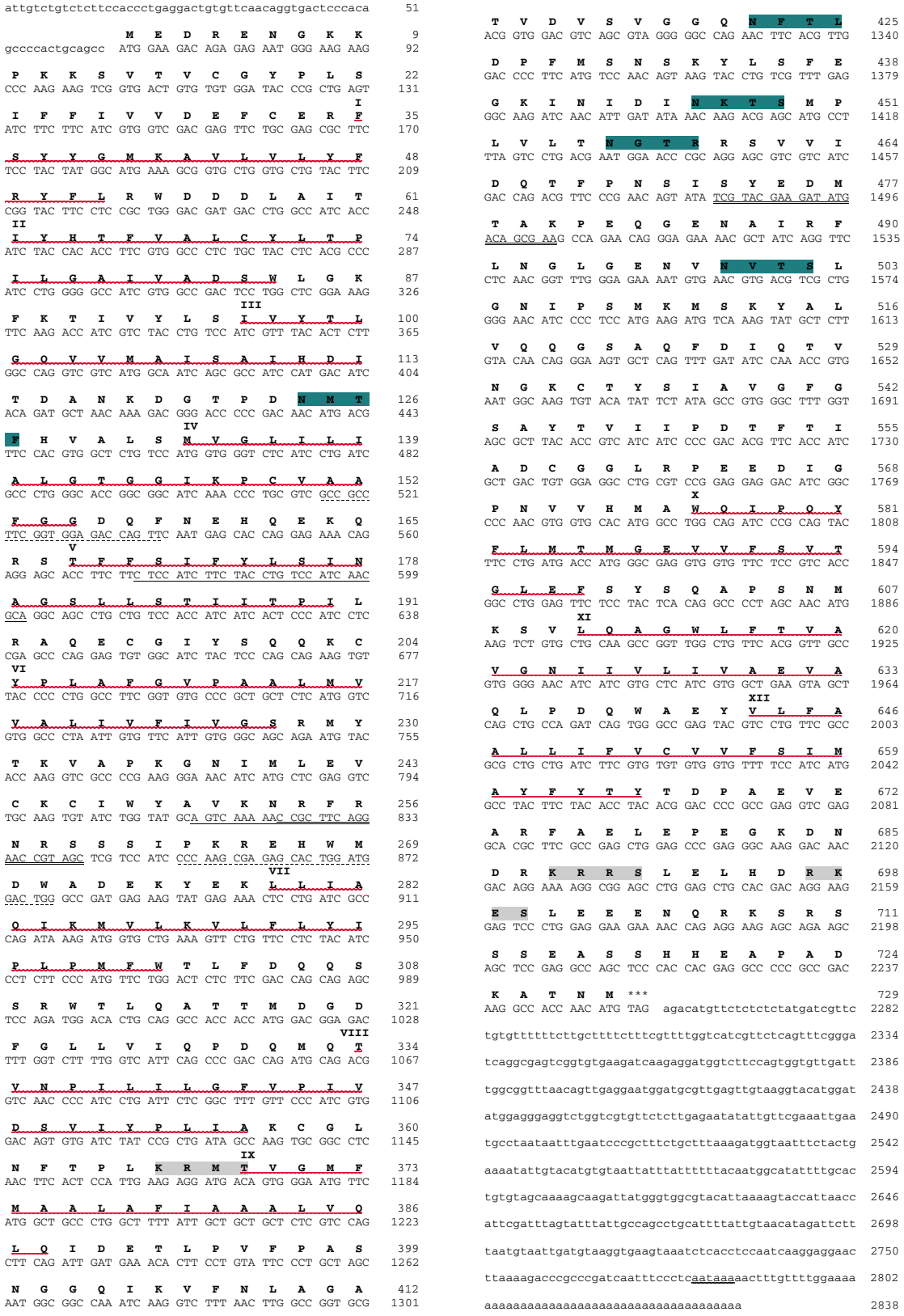
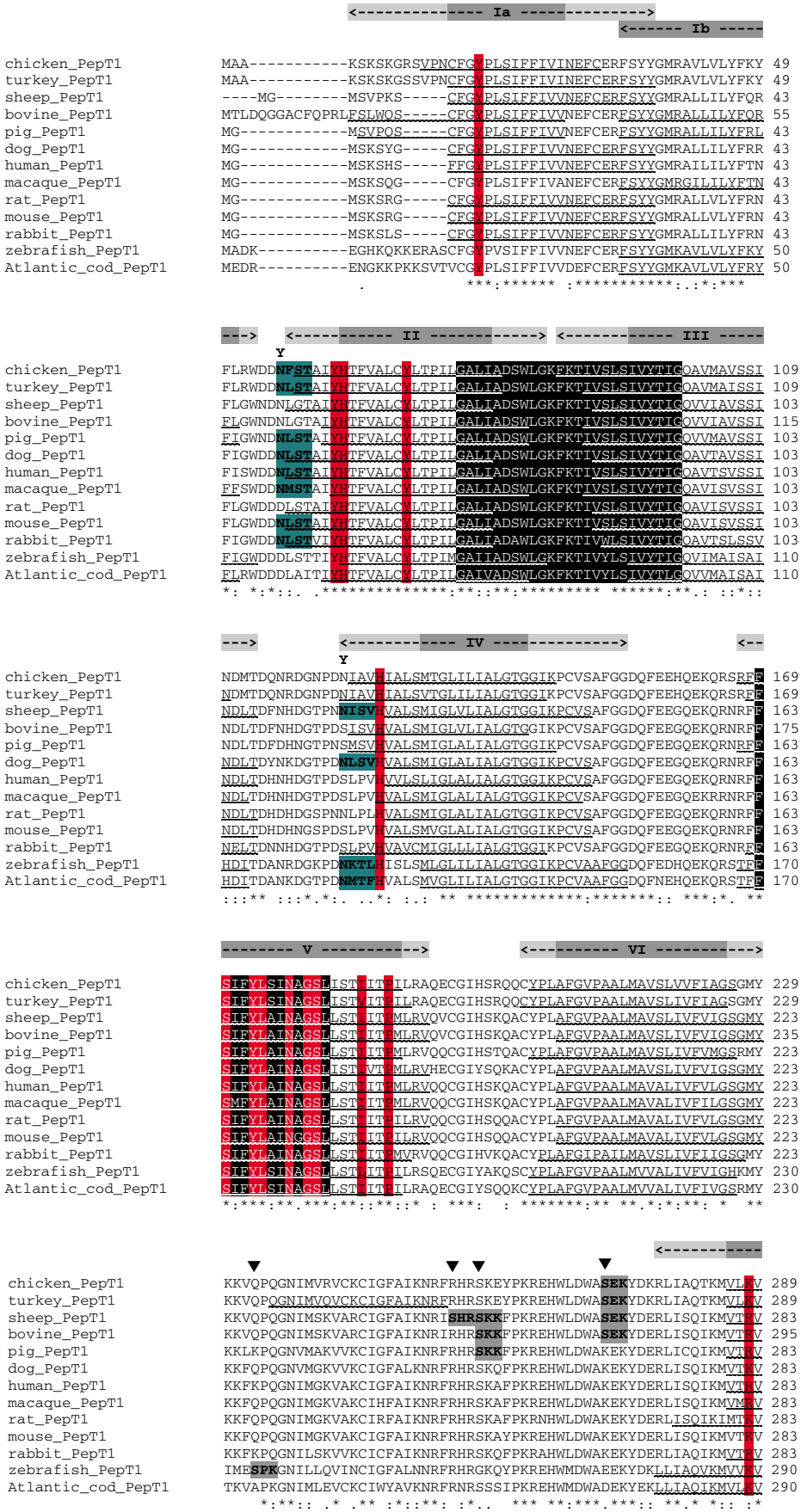


Fig. 1. Nucleotide and predicted amino acid sequence of Atlantic cod PepT1 (codPepT1). The numbers on the right refer to positions of the nucleotides (lower row) and amino acids (upper row). The stop codon is indicated by ***. A polyadenylation signal is underlined. Positions for degenerated (broken underline) and species specific (solid underline and double underline) primers are indicated in the nucleotide sequence. In the amino acid sequence, putative transmembrane domains are underlined in red and named I to XII. Potential extracellular N-glycosylation sites (dark green boxed areas) and potential cAMP/cGMP-dependent protein kinase phosphorylation sites at the cytoplasmic surface (light grey boxed areas) are indicated.



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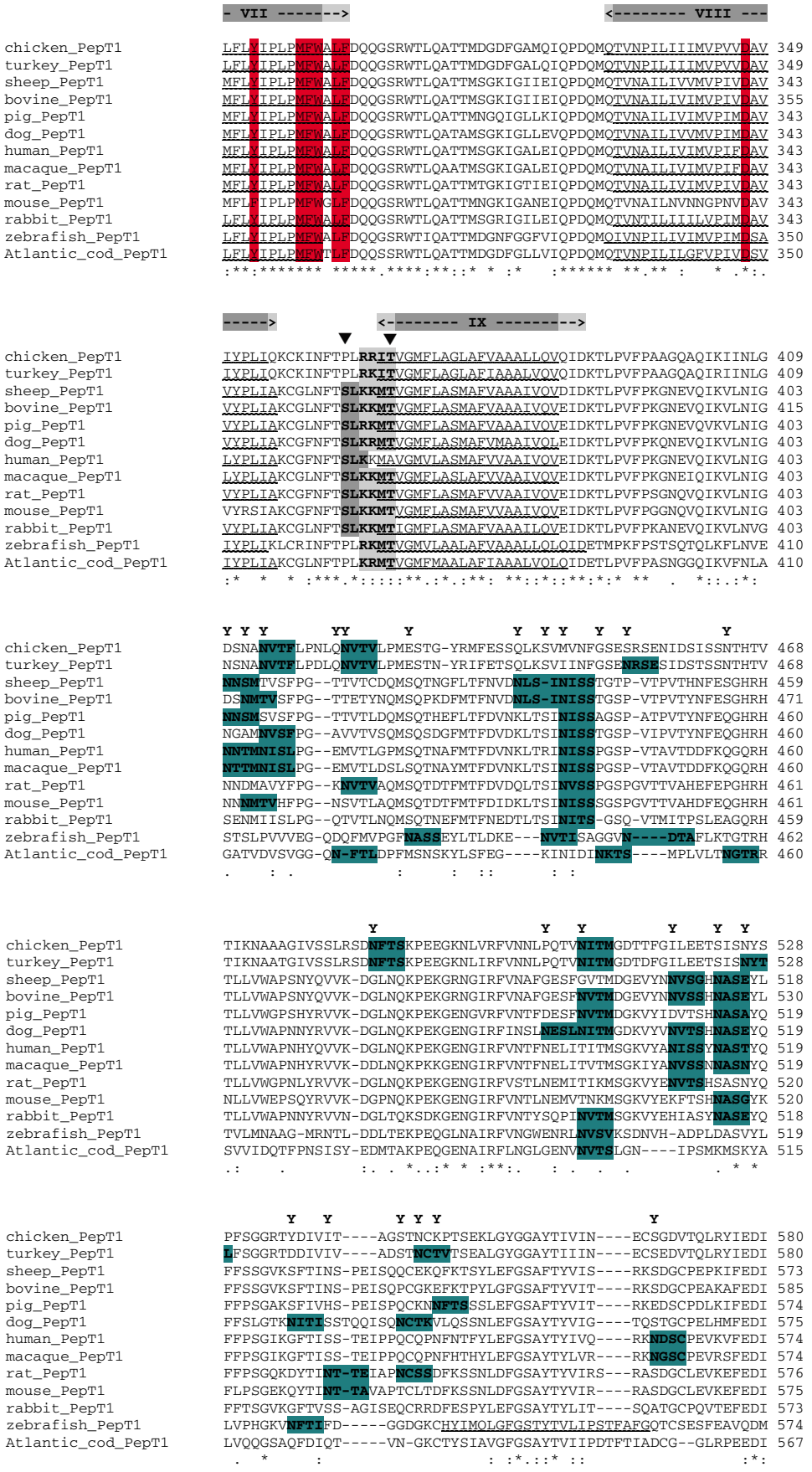

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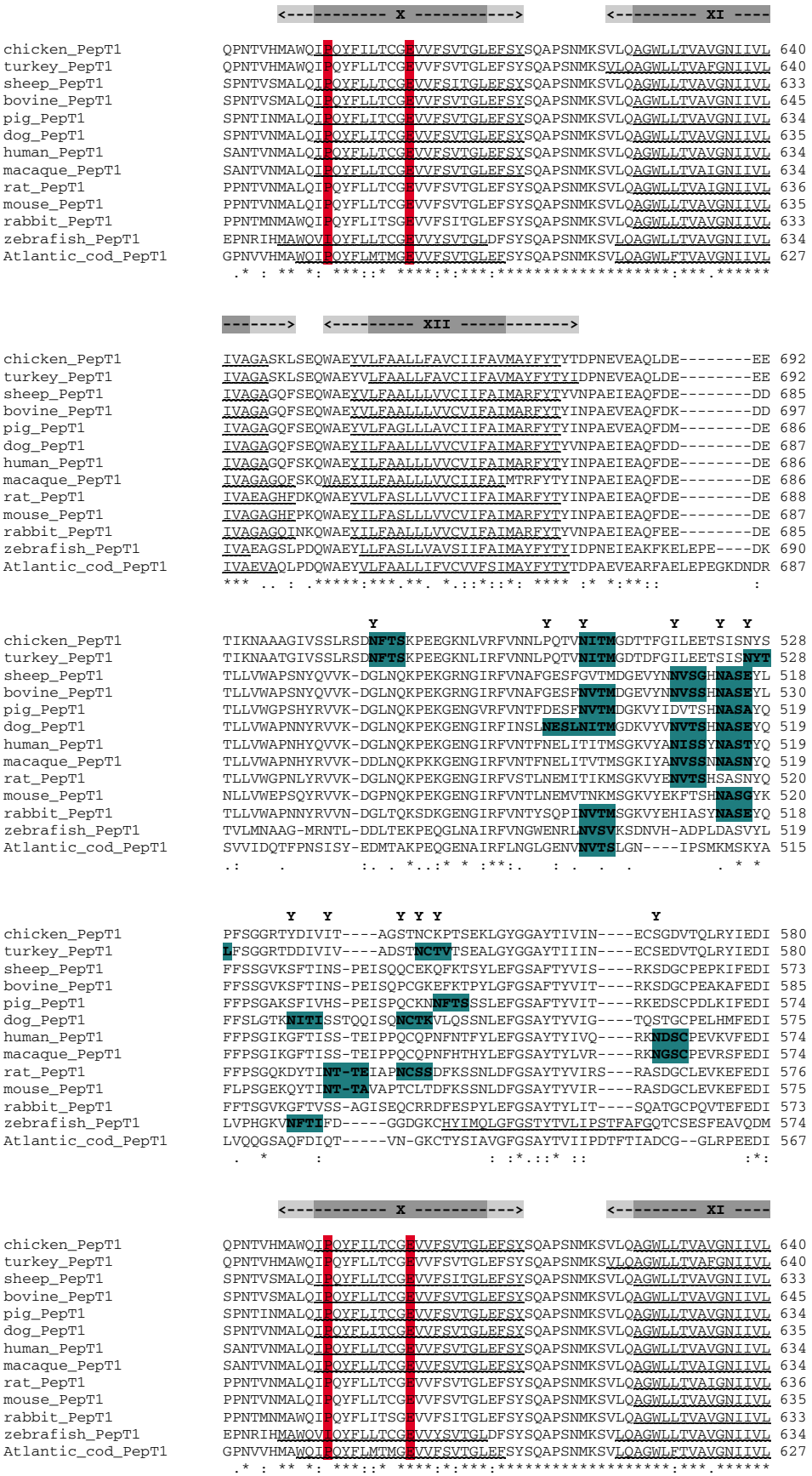

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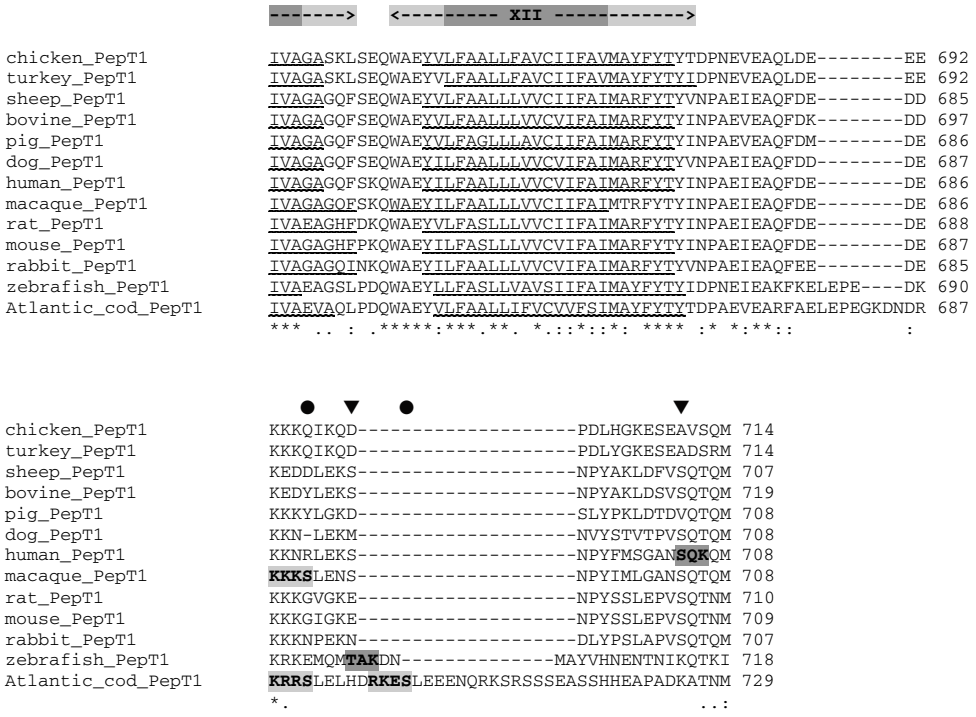

Fig. 2. Amino acid alignment of human, macaque, rat, mouse, dog, rabbit, sheep, cow, pig, chicken, turkey, zebrafish and Atlantic cod PepT1. Multiple sequence alignment was generated using Clustal W 1.82 using the tool at the www.ebi.ac.uk/clustalw web site with the following (default) parameters: Matrix=Gonnet 250, Gap Open=10, End Gaps=–1, Gap Extension=0.2, Gap Distances=4. Putative transmembrane segments (Ia to XII) were predicted using the TMHMM 2.0 program (TMHMM Server v. 2.0 at http://www.cbs.dtu.dk/services/TMHMM-2.0/), which is part of the Simple Modular Architecture Research Tool (SMART; at http://www.expasy.org/prosite/), and are indicated by grey-boxed double-headed broken arrows (with darker grey designating the core stretch of amino acids within each putative TMHMM-predicted transmembrane segment that is shared by at least two of the aligned sequences). Within each sequence, the topological location of each TMHMM2-predicted transmembrane helix is specifically underlined. Potential phosphorylation (solid triangle, protein kinase C; solid circle, protein kinase A) and *N*-glycosylation (**Y**) sites are indicated and correspondently highlighted in dark grey, light grey and dark green, respectively, along the sequences where found. The proposed 'PTR2 family proton/oligopeptide symporters signature 1' motif (PROSITE pattern: PS01022; amino acid residues 77–101 in Atlantic cod PepT1) and 'PTR2 family proton/oligopeptide symporters signature 2' motif (PROSITE pattern: PS01023; amino acid residues 170–182 in Atlantic cod PepT1) are highlighted in black. Individual amino acid residues identified by site-directed mutagenesis in PepT1 proteins from various mammalian species and found to be either involved in transport activity or responsible for incorrect synthesis and/or transport of the protein to the plasma membrane are indicated in red (for details, see Table 1).

AGC-3′) and the reverse primer CodPepT1R (5′-TTCGC-TGTCATATCTTCGTACGA-3′) were used for amplification of Atlantic cod PepT1. The PCR reactions were conducted for 35 cycles (1 cycle: 45 s at 95°C, 30 s at 60°C and 50 s at 72°C), followed by a 10 min final extension at 72°C, with *Taq* DNA polymerase. The PCR products were size-fractionated by agarose gel electrophoresis.

In situ hybridization

Tracts of cod intestine were dissected out, cut into smaller pieces, and fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (PB: 0.028 mol l^{-1} NaH_2PO_4, 0.071 mol l^{-1} Na_2HPO_4, pH 7.2) for 16 h at 4°C. Buffer was changed twice to 25% sucrose in PB for 30 min and 15 min, and samples were kept in 25% sucrose in PB with 10% Tissue Tek (Miles Inc., Elkhart, IN, USA) overnight at 4°C. The tissues were embedded in Tissue Tek and stored at –80°C. Cryostat sections (10 μm thick) were collected on frozen Superfrost Plus glass slides (Merck, Darmstadt, Germany), air-dried and stored at –20°C until use.

The nucleotide fragment covering amino acids 254–480 of Atlantic cod PepT1 sequence was amplified by PCR using the forward primer CodPepT1F (starting at nucleotide 824) and the reverse primer CodPepT1R (starting at nucleotide 1504; see Fig. 1). The amplification product was subcloned into the pCR4-TOPO vector (Invitrogen) and the recombinant clone sequenced to confirm the identity of the insert. *In situ* analysis was performed with digoxigenin (DIG)-labeled cRNA probes (sense and antisense), using a DIG RNA labeling Kit (Roche, Mannheim, Germany). The DIG-labelled antisense riboprobe was synthesized *in vitro* with T7 RNA polymerase using the *Spe*I-cleaved recombinant pCR4-TOPO clone. The corresponding sense riboprobe was synthesized with T3 RNA polymerase using the *Not*I-cleaved recombinant pCR4-TOPO clone. DIG incorporation and the concentration of the probes were analyzed by spot tests (Roche).

In situ hybridization procedures on tissue sections were carried out according to published methods (Ebbesson et al., 2005).

Results

Sequence analysis

The Atlantic cod PepT1 cDNA (GenBank accession no. AY921634) was 2838 bp long, with an open reading frame of 2190 bp encoding a putative protein of 729 amino acids (Fig. 1). Hydropathy analysis predicted at least 12 potential membrane-spanning domains with a large extracellular loop between transmembrane domains IX and X (Fig. 1). Five putative extracellular N-glycosylation sites (Asn^{124}, Asn^{422}, Asn^{446}, Asn^{456} and Asn^{499}) and three putative intracellular cAMP/cGMP-dependent protein kinase phosphorylation sites (Thr^{369}, Ser^{691} and Ser^{700}) were identified (Fig. 1). No putative protein kinase C phosphorylation sites were found. When compared with PepT1-type members of the SLC15 family already characterized from other vertebrates (Fig. 2), the predicted Atlantic cod PepT1 amino acid sequence exhibited a higher percentage of identity with the zebrafish PepT1 (63%) than with any other PepT1-type transporter characterized so far in higher vertebrates (58–61%). The phylogenetic reconstruction of vertebrate PepT1-type proteins clustered codPepT1 to the 'fish' branch of the phylogenetic tree (together with zebrafish PepT1) and indicated early divergence both of the Atlantic cod sequence with respect to that of the zebrafish and of the two fish protein sequences with respect to those of the tetrapod group (Fig. 3).

Fish vs tetrapod PepT1 proteins: a comparison

To date, codPepT1 is the second full-length cDNA encoding for a PepT1-type transporter that has been cloned from a teleost fish and made available *via* public databases. Like zebrafish PepT1 (Verri et al., 2003), it is evolutionarily distant from the sequences already characterized from higher vertebrates, i.e. birds and mammals (for a review, see Daniel et al., 2006), and thus represents a novel reference sequence to support comparative analysis along the vertebrate series. With this in mind, alignment and comparison of the deduced amino acid sequence of Atlantic cod PepT1 to those of the other PepT1-type members of the SLC15 family already characterized in vertebrates were performed in order to recognize and recapitulate major structural/functional features common to vertebrate PepT1-type proteins (Fig. 2).

The first examination included analysis of putative membrane-spanning domains, N-glycosylation sites, cAMP/cGMP-dependent protein kinase phosphorylation sites and protein kinase C phosphorylation sites. Examination of the predicted membrane-spanning domains revealed 11 (out of 12) highly conserved regions of hydropathy among the proteins (membrane-spanning domains II–XII). Only in one case, i.e. transmembrane domain I, did the predicted membrane-spanning regions of the different proteins not (fully) overlap, but resulted in either of two adjacent sub-regions of hydropathic amino acids (Fig. 2; compare chicken, turkey, sheep, dog, human, rat, mouse and rabbit to macaque, zebrafish and Atlantic cod PepT1 sequences) or in the two sub-regions of hydropathic amino acids (Fig. 2; see bovine and pig PepT1 sequences). Such sub-regions were indicated as membrane-spanning domain Ia and membrane-spanning domain Ib, and may be indicative of a larger than expected area of hydrophobic amino acids in close proximity to the N terminus of the PepT1-type proteins. Analysis of single N-glycosylation, cAMP/cGMP-dependent protein kinase phosphorylation and protein kinase C phosphorylation predicted sites revealed (1) a large N-glycosylation-rich region within the large extracellular loop between membrane-spanning domains IX and X; (2) a single highly conserved cAMP/cGMP-dependent protein kinase phosphorylation motif close to the putative spanning domain IX (see Thr^{369} in the Atlantic cod sequence as a reference) that is present in every PepT1 sequence except the human (in which Ala^{357} is present instead of threonine); and (3) an adjacent protein kinase C phosphorylation (Ser-Leu-Lys) motif that is present in mammalian but not in avian and fish sequences. In the end, comparisons of tetrapod and fish sequences revealed at least one interesting conserved and previously undescribed stretch (of 8–12 amino acid residues) within the large extracellular loop, roughly centered around amino acid residues 480–491 in the Atlantic cod sequence. Further studies are necessary to assess the structural/functional significance of this stretch. Other regions/residues that are recognized as structurally or functionally

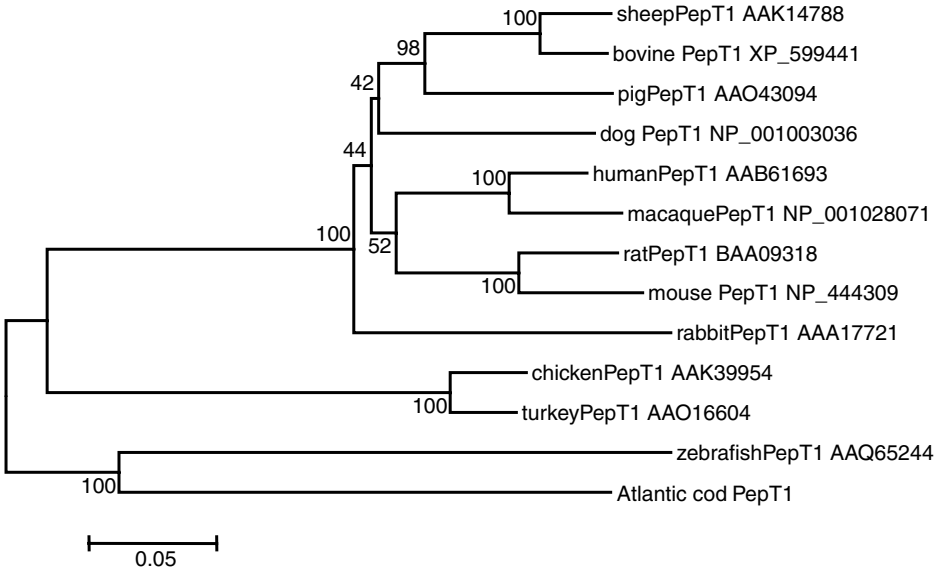

Fig. 3. Unrooted phylogenetic tree depicting the evolutionary relationship of vertebrate PepT1 transporters. The unrooted tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) based on the alignment of the complete amino acid sequences of known vertebrate PepT1 transporters. Bootstrap values (1000 replicates) indicating the occurrence of nodes are reported above each branch in the figure.

Table 1. *Effect of single amino acid substitutions (as obtained by site-directed mutagenesis) on the function of mammalian (human, rabbit and rat) PepT1 transport proteins*

Amino acid	Mutation	Effect of mutation	Proposed function of the residue	Experimental system	Reference
Human PepT1					
Y12	Y12A	Mutation has a modest effect on Gly-Sar uptake and primarily affects the V_{max} value of the translocation process		Transfection of HEK293 cells and radioactive peptide uptake	(Bolger et al., 1998)
H57	H57Q H57N	Mutants have no detectable peptide transport activity	H57 is involved in binding and translocation of H^+ cotransported with the peptide and is a principal proton-binding site	Expression in *Xenopus* oocytes and electrophysiology; transfection of HeLa cells and radioactive peptide uptake; transfection of HEK293 cells and radioactive peptide uptake	(Fei et al., 1997; Uchiyama et al., 2003)
	H57A H57R H57K	Mutants have no detectable peptide transport activity; moreover, mutant H57R evokes increasing steady-state currents with gradual increase of the pH (5.0–8.5)			
H121	H121A H121R H121K	Mutants show reduced uptake of Gly-Sar by 43% (H121A), 45% (H121R), 75% (H121K); moreover, mutants H121R and H121K show a significant decrease of Gly–Sar uptake at pH 7.4 and 8.5 compared to pH 6.0	H121 is probably involved in substrate recognition and is not involved in H^+ binding	Expression in *Xenopus* oocytes and electrophysiology; transfection of HEK293 cells and radioactive peptide uptake	(Uchiyama et al., 2003)
S164	S164C	Mutant is not expressed on the plasma membrane	This amino acid position is responsible for incorrect packaging and/or transport of the protein to the plasma membrane	Transfection of HEK293 cells and immunofluorescence experiments	(Kulkarni et al., 2003a)
Y167	Y167A Y167F Y167H Y167S Y167C	Uptake of Gly–Sar is abolished	This residue has an essential role in dipeptide uptake (due to the unique chemistry of its phenolic side chain)	Transfection of HEK293 cells and radioactive peptide uptake	(Bolger et al., 1998; Kulkarni et al., 2003a; Yeung et al., 1998)
L168	L168C	Mutant is not expressed on the plasma membrane	This amino acid position is responsible for incorrect packaging and/or transport of the protein to the plasma membrane	Transfection of HEK293 cells and immunofluorescence experiments	(Kulkarni et al., 2003a)
N171	N171C	Uptake of Gly–Sar is abolished	This amino acid plays a critical role in substrate binding	Transfection of HEK293 cells and radioactive peptide uptake	(Kulkarni et al., 2003a)
G173	G173C	Mutant is not expressed on the plasma membrane	This amino acid position is responsible for incorrect packaging and/or transport of the protein to the plasma membrane	Transfection of HEK293 cells and immunofluorescence experiments	(Kulkarni et al., 2003a)

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Table 1. *Continued*

Amino acid	Mutation	Effect of mutation	Proposed function of the residue	Experimental system	Reference
Human PepT1					
S174	S174C	Uptake of Gly–Sar is abolished	This amino acid plays a critical role in substrate binding	Transfection of HEK293 cells and radioactive peptide uptake	(Kulkarni et al., 2003a)
I179	I179C	Mutant is not expressed on the plasma membrane	This amino acid position is responsible for incorrect packaging and/or transport of the protein to the plasma membrane	Transfection of HEK293 cells and immunofluorescence experiments	(Kulkarni et al., 2003a)
P182	P182C	Mutant shows ~40% of Gly-Sar uptake		Transfection of HEK293 cells and radioactive peptide uptake	(Kulkarni et al., 2003a)
R282	R282A R282C R282K	Mutations have a modest effect on Gly–Sar uptake	The positive charge is important at amino acid position 282. A salt-bridge between R282–D341 may play a role in maximizing the efficiency of substrate translocation.	Transfection of HEK293 cells and radioactive peptide uptake	(Bolger et al., 1998; Kulkarni et al., 2007; Kulkarni et al., 2003b)
	R282E R282D	Mutants show significantly reduced uptake of Gly–Sar			
Y287	Y287C	Mutant is not expressed on the plasma membrane	Single cysteine mutation at this position is responsible for incorrect synthesis and/or misfolding of the mutated protein	Transfection of HEK293 cells and immunofluorescence experiments	(Kulkarni et al., 2003b)
M292	M292C	Mutant is not expressed on the plasma membrane	Single cysteine mutation at this position is responsible for incorrect synthesis and/or misfolding of the mutated protein	Transfection of HEK293 cells and immunofluorescence experiments	(Kulkarni et al., 2003b)
F293	F293C	Mutant displays negligible uptake of Gly–Sar	This residue probably plays a structural role in the transporter function	Transfection of HEK293 cells and radioactive peptide uptake	(Kulkarni et al., 2003b)
W294	W294A	Mutant shows reduced uptake of Gly–Sar (~8%); in particular, mutation has a significant effect on the Michaelis–Menten K_m value	This residue plays a role in maintaining the structural integrity of the protein. The larger size of the cysteine side chain, compared with that of alanine, sufficiently reflects the steric bulk of the tryptophan side chain and better maintains the correct helical packing	Transfection of HEK293 cells and radioactive peptide uptake	(Bolger et al., 1998; Kulkarni et al., 2003b)
	W294C	Mutant does not show reduced uptake of Gly–Sar			
L296	L296C	Mutant displays negligible uptake of Gly–Sar	This residue probably plays a structural role in the transporter function	Transfection of HEK293 cells and radioactive peptide uptake	(Kulkarni et al., 2003b)
F297	F297C	Mutant displays negligible uptake of Gly–Sar	This residue probably plays a structural role in the transporter function	Transfection of HEK293 cells and radioactive peptide uptake	(Kulkarni et al., 2003b)
D341	D341A D341E	Mutations do not show significantly reduced uptake of Gly–Sar	The negative charge is important at amino acid position 341. A salt-bridge between R282–D341 may play a role in maximizing the efficiency of substrate translocation	Transfection of HEK293 cells and radioactive peptide uptake	(Kulkarni et al., 2007)
	D341K D341R	Mutants show significantly reduced uptake of Gly–Sar			

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Table 1. *Continued*

Amino acid	Mutation	Effect of mutation	Proposed function of the residue	Experimental system	Reference
Human PepT1					
P586	P586L	Mutant shows reduced transport capacity, lower protein level and lower plasma membrane expression	P586 may have profound effects on translation, degradation, and/or membrane insertion	Transfection of HeLa cells, radioactive peptide uptake and immunocytochemical and Western blot analyses	(Zhang et al., 2004b)
E595	E595A	Mutant shows reduced uptake of Gly–Sar (~95%)		Transfection of HEK293 cells and radioactive peptide uptake	(Bolger et al., 1998)
Rabbit PepT1					
Y56	Y56A Y56F	Mutant Y56F exhibits slightly decreased functional activities, while Y56A exhibits no significant activities	This aromatic residue stabilizes the charge on H^+ when interacting with H57	Expression in *Xenopus* oocytes and radioactive peptide uptake or electrophysiology	(Chen et al., 2000)
H57	H57R	Mutant has no detectable peptide transport activity	H57 residue is involved in the binding of H^+ to the transporter	Expression in *Xenopus* oocytes and radioactive peptide uptake or electrophysiology	(Chen et al., 2000)
Y64	Y64A Y64F	Mutant Y64F exhibits slightly decreased functional activities, while Y64A exhibits no significant activities	This aromatic residue stabilizes the charge on H^+ when interacting with H57	Expression in *Xenopus* oocytes and radioactive peptide uptake or electrophysiology	(Chen et al., 2000)
H121	H121R H121C	Affinity of H121 mutants for peptide substrates decreases depending on the charge of the substrate	H121 is involved in substrate recognition	Expression in *Xenopus* oocytes and radioactive peptide uptake or electrophysiology	(Chen et al., 2000)
R282	R282E	Mutation uncouples the H^+-peptide cotransport and creates a peptide-gated cation channel in the protein		Expression in *Xenopus* oocytes and radioactive peptide uptake or electrophysiology	(Meredith, 2004)
W294	W294F	Mutant does not show any peptide uptake and does not produce any depolarization of membrane potential		Expression in *Xenopus* oocytes and radioactive peptide uptake or electrophysiology	(Panitsas et al., 2006)
Rat PepT1					
H57	H57Q	Uptake of Gly–Sar is abolished	H57 is involved in substrate binding and/or is responsible for intrinsic activity of the transporter	Expression in *Xenopus* oocytes and radioactive peptide uptake	(Terada et al., 1996)
H121	H121Q	Uptake of Gly–Sar is abolished	H121 is involved in substrate binding and/or is responsible for intrinsic activity of the transporter	Expression in *Xenopus* oocytes and radioactive peptide uptake	(Terada et al., 1996)

Amino acid notation follows the single letter code.

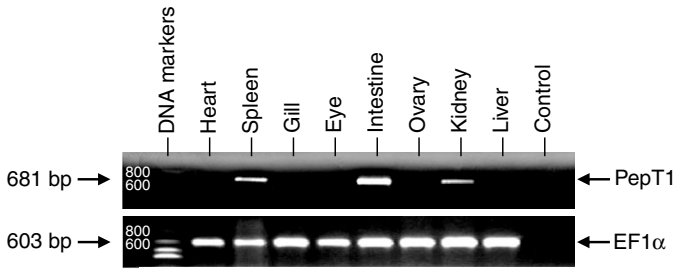

Fig. 4. Tissue distribution of Atlantic cod PepT1 mRNA in adult fish. RT-PCR performed on equal amounts of total RNA (1.5 μg) isolated from adult fish tissues using Atlantic cod PepT1- (top row) and EF1α-specific (bottom row) primers. Control, no RNA (H_2O instead) in RT (negative control). DNA markers indicate 10 kb SmartLadder (Eurogentec, Seraing, Belgium).

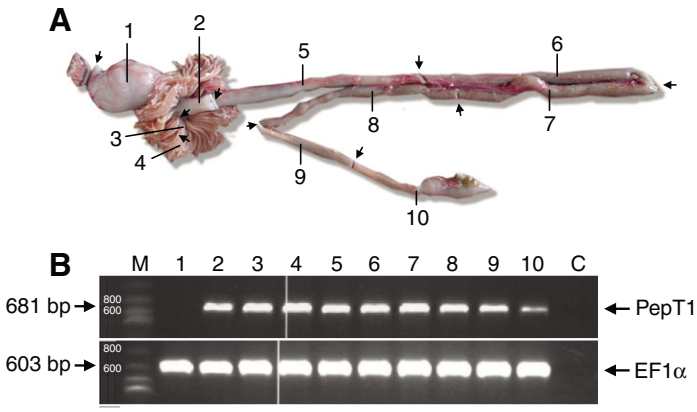

Fig. 5. Spatial distribution of Atlantic cod PepT1 mRNA in the digestive tract of adult fish. (A) A representative picture of Atlantic cod digestive tract. 1, stomach; 2, pyloric area; 3, pyloric caeca (inner segments); 4, pyloric caeca (outer segments); 5–10, six adjacent intestinal segments in the remainder of the digestive tract, starting after the pyloric area (segment 5) and ending with the anus (segment 10), and based on divisions of the three loops present in the dissected gut. The last segment (e.g. segment 10) also comprises the hindgut. Arrows indicate the incisions between the segments. (B) RT-PCR performed on equal amounts of total RNA (1.5 μg) isolated from the different segments of the digestive tract of Atlantic cod using PepT1- (top row) and EF1α-specific (bottom row) primers. Progressive numbers (1 to 10) indicate the segments of the digestive tract indicated in A. Control (C), no RNA (H_2O instead) in RT (negative control); M, DNA markers (10 kb SmartLadder; Eurogentec).

important within the mammalian PepT1 primary structure (for example, see Daniel, 2004) were invariably conserved in Atlantic cod PepT1, as well as the PepT1 sequence of the zebrafish. These include the 'PTR2 family proton/oligopeptide symporters signature 1' motif (PROSITE pattern: PS01022; amino acid residues 77–101 in Atlantic cod PepT1) and 'PTR2 family proton/oligopeptide symporters signature 2' motif (PROSITE pattern: PS01023; amino acid residues 170–182 in Atlantic cod PepT1).

The next examination involved comparative analysis to confirm identity along the vertebrate series of those amino acids for which mutational studies in mammalian systems had previously assessed a significant effect on function. Amino acids along the sequences for which mutational analysis was performed are highlighted in Fig. 2. All of them fall within putative membrane-spanning domains (namely membrane-spanning domains I, II, IV, V, VII, VIII and X) and are invariably conserved (identities or conservative substitutions) among the species. A list of the effects of a single amino acid substitution, as obtained by site-directed mutagenesis, on the function of human, rabbit and rat PepT1 transporters, is reported in Table 1.

Expression of Atlantic cod PepT1 in tissues

Expression of Atlantic cod PepT1 was analysed in various tissues/organs of adult fish. Using Atlantic cod PepT1-specific primers, a 681 bp RT-PCR product was amplified from total RNA isolated from intestine, kidney and spleen. No signal was obtained from samples of heart, gill, eye or liver, while a slight signal was obtained from ovary. As a control to assess RNA quality, EF1α RNA amplification was performed using Atlantic cod EF1α-specific primers, which invariably gave comparable 603 bp amplification products for all tissues tested (Fig. 4).

Spatial distribution of Atlantic cod PepT1 expression in the digestive tract was studied in detail (Fig. 5). A 681 bp RT-PCR product was amplified from total RNA isolated from a total of ten segments (see Fig. 5A) along the intestine of adult fish. No signal was obtained from the stomach (segment 1; see Fig. 5B), while strong signals were obtained in the following segments. There appeared to be a weakening in the signal from segment 9 (the last 1/5 of the intestine), but in the last segment, which included the hindgut, the expression was very low (see Fig. 5B). The control for RNA quality (EF1α RNA amplification) invariably gave comparable 603 bp amplification products for all tested segments.

Expression of PepT1 mRNA at the intestinal level was further analyzed by *in situ* hybridization (Fig. 6). Using the DIG-labeled antisense probe, expression of the PepT1 mRNA was detected only in the epithelial layer of the intestine of Atlantic cod (see Fig. 6B), while the sense probe revealed no staining (see Fig. 6A).

Discussion

We have identified a full-length cDNA from Atlantic cod that encodes for a novel PepT1-type peptide transporter (Fig. 1). The predicted protein, designated as codPepT1, shares a high overall identity with PepT1-type transporters (58–63%) when compared to other known PepT1 proteins in vertebrates (Fig. 2), and clusters to the 'fish' branch of the reconstructed phylogenetic tree, together with zebrafish PepT1 (Fig. 3). To date, codPepT1 represents the second PepT1-type peptide transporter recognized from a teleost fish [after zebrafish PepT1 (Verri et al., 2003)], thus representing an additional molecular tool for the understanding of evolutionary and functional relationships among vertebrate peptide transporters.

In this paper, the comparative analysis of two fish (Atlantic cod and zebrafish) amino acid sequences *vs* a collection of sequences from various tetrapod (two avian and nine mammalian) species has allowed the recognition of a number of highly conserved motifs, regions and protein domains (and the exclusion of others) simply on the basis of the overall

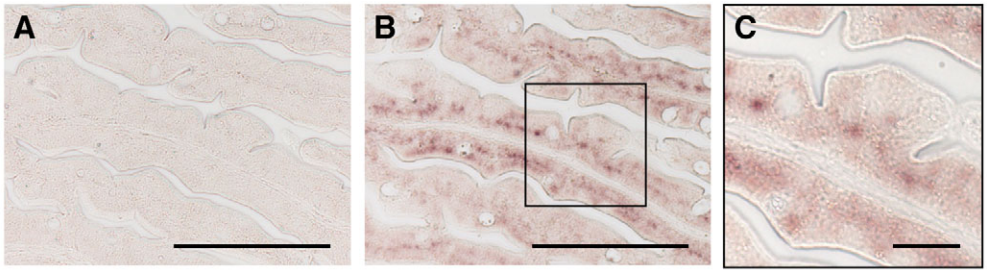

Fig. 6. PepT1 gene expression on neighbor sections of Atlantic cod intestine by *in situ* hybridization. (A) The sense probe showed no staining (negative control). (B) The antisense probe revealed mRNA expression in the epithelial layer of the intestine. (C) Epithelium from boxed area in B enlarged. Scale bars, 100 μm (A,B), 20 μm (C).

sequence alignment (see Fig. 2), as well as a better representation of some still unresolved areas of the protein. It is noteworthy that the individuation of two adjacent sub-regions of hydropathic amino acids instead of one single membrane-spanning domain I is suggestive of the existence of a very large hydrophobic area close to the N terminus of the PepT1 proteins. Moreover, the occurrence, beside the large N-glycosylation-rich region, of short, well-conserved stretches of amino acids within the big extracellular loop is novel structural evidence that deserves further study. The only highly conserved cAMP/cGMP-dependent protein kinase phosphorylation motif close to membrane-spanning domain IX (found in all vertebrate sequences with the exception of the human) and the adjacent protein kinase C phosphorylation motif (found in mammalian sequences only) would also merit further analysis, mostly in the light of the well-documented regulation of PepT1 protein activity in Caco-2 cells, mammalian models and human intestine, *via* agonists or antagonists of protein kinase A and C and hormones/extracellular signals (for a review, see Daniel, 2004). It is also noteworthy that none of the PepT1 protein kinase phosphorylation motifs have been analyzed so far with respect to their ability to functionally transmit the observed effects of second messengers at the protein level.

Functional analysis of mammalian PepT1 transporters has led to a general scheme of peptide transport and to the concept that such transport occurs in virtually all vertebrates according to the basic design (in terms of mode of transport, kinetics, proton- and membrane potential-dependence, pH dependence, electrogenicity, substrate specificity, protein sorting to the membrane, etc.) defined in the mammalian systems (for reviews, see Brandsch et al., 2004; Daniel, 2004; Daniel and Kottra, 2004; Daniel et al., 2006; Terada and Inui, 2004). Interestingly, the structural/functional characterization of zebrafish PepT1 partly contradicted this assumption, revealing that this transporter closely resembles mammalian systems in terms of low-affinity/high-capacity properties of the transport, but also exhibits some peculiarities, such as a unique pH dependence (Verri et al., 2003). It also suggested that fish PepT1 proteins might have allowed a useful comparative approach to focus on amino acid residues, motifs, conserved regions and protein domains that are relevant to the general function of the transporter or, conversely, to the determination of a species-specific phenotype. Since 1997, the function of mammalian PepT1 proteins has been progressively investigated with the support of single amino acid mutational analysis to define relevant structural/functional motifs or domains involved in the manifestation of the phenotype (see Table 1 and references therein). Such investigation allowed the identification of many amino acids that play a crucial role in the function of the transporter, although it was performed on an almost random basis and focused primarily on the functional role of selected transmembrane domains. In this respect, using our comparative analysis, we confirmed identity or conservative substitution along the vertebrate series of all those amino acids (23 amino acid residues distributed in membrane-spanning domains I, II, IV, V, VII, VIII and X; see Fig. 2) for which functional mutational analysis had established a significant effect on function. In perspective, such a comparative approach might help to rationalize the selection of the most suitable amino acid residues to target in site-directed mutagenesis experiments.

Atlantic cod PepT1 is highly expressed in the intestine, and a significant RT-PCR signal was also found in both kidney and spleen tissue (Fig. 4). Interestingly, this is the same pattern of expression as is found in zebrafish for PepT1 (Verri et al., 2003), which confirms a common theme among fish. A more detailed analysis of the regional distribution along the intestinal tract of cod, as performed herein for the first time in an adult fish to our knowledge, revealed that PepT1 is ubiquitously expressed in all segments after the stomach, including the pyloric caeca (Figs 5 and 6). This suggests that Atlantic cod may have a very high capacity to absorb small peptides from dietary protein digestion, with absorption occurring in most parts of the intestine. The low expression in the last segment that included the hindgut indicates that this segment is not, or only slightly, involved in peptide absorption. However, it may be involved in final adjustments of ion and water composition (for a review, see Marshall and Grosell, 2005).

In conclusion, the Atlantic cod intestinal PepT1-type oligopeptide transporter has been identified and characterized with respect to its expression in tissues. On the basis of the overall amino acid sequence alignment, conserved amino acids and novel motifs, regions and protein domains have been recognized in all vertebrates. In a perspective, Atlantic cod PepT1 can represent a useful tool for the study of gut regionalization, as well as a marker for the functional analysis of temporal and spatial expression during ontogeny, under the effects of various dietary sources, and in pathological states.

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