Molecular Cloning and Functional Expression of Atlantic Salmon Peptide Transporter 1 in Xenopus Oocytes Reveals Efficient Intestinal Uptake of Lysine-Containing and Other Bioactive Di- and Tripeptides in Teleost Fish

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Abstract

Atlantic salmon (Salmo salar L.) is one of the most economically important cultured fish and also a key model species in fish nutrition. During digestion, dietary proteins are enzymatically cleaved and a fraction of degradation products in the form of di- and tripeptides translocates from the intestinal lumen into the enterocyte via the Peptide Transporter 1 (PepT1). With this in mind, a full-length cDNA encoding the Atlantic salmon PepT1 (asPepT1) was cloned and functionally characterized. When overexpressed in Xenopus laevis oocytes, asPepT1 operated as a low-affinity/high-capacity transport system, and its maximal transport activity slightly increased as external proton concentration decreased (varying extracellular pH from 6.5 to 8.5). A total of 19 tested di- and tripeptides, some with acknowledged bioactive properties, some containing lysine, which is conditionally growth limiting in fish, were identified as well transported substrates, with affinities ranging between ~0.5 and ~1.5 mmol/L. Analysis of body tissue distribution showed the highest levels of asPepT1 mRNA in the digestive tract. In particular, asPepT1 mRNA was present in all segments after the stomach, with higher levels in the pyloric caeca and midgut region and lower levels in the hindgut. Depriving salmon of food for 6 d resulted in a ~70% reduction of intestinal PepT1 mRNA levels. asPepT1 will allow systematic in vitro analysis of transport of selected di- and tripeptides that may be generated in Atlantic salmon intestine during gastrointestinal transit. Also, asPepT1 will be useful as a marker to estimate protein absorption function along the intestine under various physiological and pathological conditions. J. Nutr. 140: 893–900, 2010.

Introduction

The intestinal peptide transporter Peptide Transporter 1 (PepT1) plays an important role in protein nutrition by mediating the uptake of a fraction of dietary amino acids in di- and tripeptide form (1). Some of these peptides may have bioactive properties (2–11). The di- and tripeptides present in the gut derive from proteins of animal, plant, and microorganismal origin and may be released by digestive or microbial enzymes during gastrointestinal transit (4–11) or by microbial fermentation of foods during processing or ripening (2,3). Moreover, PepT1 is responsible for the absorption of orally active peptidomimetic drugs, including β-lactam antibiotics and selected pro-drugs (12).

PepT1 belongs to the Peptide Transporter family (13). Members of the this family have been well characterized in bacteria, fungi, plants, insects, nematodes, birds, and mammals. In vertebrates, PepT1 is also known as Solute carrier family member a1 (14). A detailed analysis of PepT1 function, as performed on mammalian and avian orthologs, has revealed that this protein operates as an Na⁺-independent, H⁺-coupled electrionic symporter (1,15). Coupling of substrate uptake to
the movement of protons down an inwardly directed electrochemical proton gradient allows vectorial transport of peptides across the plasma membrane, even against a substrate concentration gradient. The transport responds to both membrane potential and extracellular pH and exhibits a pH optimum varying between 4.5 and 6.5 depending on the net charge of the transported substrate (1). Although well characterized in mammals, information on PepT1 function in lower vertebrates, including teleosts, is very limited (16). Interestingly, the zebrafish (Danio rerio) transporter exhibits a unique pH dependence, with neutral to alkaline extracellular pH increasing its maximal transport rate (17).

Atlantic salmon (Salmo salar L.) is the most economically relevant cultured fish in cold-water regions. Preparation of feeds for carnivorous cultured fish, including salmon, has traditionally been based on cheap and readily available fish meal as the main protein source (18,19). However, a limited world supply combined with increased prices of fish meal has recently led to the replacement of fish meal with alternative protein sources, mainly of vegetable origin (20–22). The use of these new formulations in aquaculture has frequently led to both reduced growth and enteritis in carnivorous fish, and Atlantic salmon is particularly sensitive (21,22). Given its dual role in providing bulk quantities of dietary amino acids in peptide form and in mediating the transport of selected bioactive molecules, including antiinflammatory compounds such as Lys-Pro-Val (5), PepT1 may be a target in salmon aquaculture for improved delivery of more appropriate protein compositions and/or for antiinflammatory therapies.

Our aim in this study was to clone and functionally characterize PepT1 in the teleost Atlantic salmon. We report data of cloning, sequence analysis, phylogeny, functional characterization, transport kinetics, substrate specificity, and tissue expression. We also show that 6-d feed deprivation down-regulates intestinal PepT1 expression in salmon.

Materials and Methods

Animals and tissue sampling. Juvenile Atlantic salmon (mean body weight: 44.7 ± 2.1 g) were reared at the Bergen High-Technology Centre (Bergen, Norway) in indoor tanks supplied with a continuous flow of fresh water at 8°C. The fish were fed continuously a commercial pellet diet (EWSO; for diet composition, see Supplemental Table 1). At sampling, fish were killed with an overdose of 3-aminobenzoic acid ethyl ester (Sigma) and tissues were collected and stored in RNAlater (Ambion) at −20°C.

Molecular cloning. Total RNA was isolated from pyloric caeca of juvenile salmon using TRI Reagent (Sigma). Genomic DNA was removed using Ambion Turbo DNase (Applied Biosystems). First-strand cDNA was synthesized from total RNA using oligo(dT) primer with the SuperScript III First-Strand Synthesis system for RT-PCR reverse transcription kit (Invitrogen). To obtain the open reading frame of the Atlantic salmon PepT1 (asPepT1) sequence, single RT-PCR was performed. Primers for cloning were designed based on the sequence of salmon expressed sequence tags. In particular, the forward primer was designed to the sequence of slaspha501053, GenBank accession no. CA043920 (asPepT1 Fw1: 5′-GAC TGT CTT GGA CCG TGA CTC TCT TG-3′; see Supplemental Fig. 1, nucleotides 82 to 53 for primer reference), and the reverse primer was designed to the sequence of slasphn501122, GenBank accession no. CA044974 (asPepT1 Rv1: 5′-ATT GCA CAT GAC TCA ACT TGG TGT GGG-3′; see Supplemental Fig. 1, nucleotides 2519–2547 for primer reference). The PCR product was purified from agarose gel using a QIAquick Gel Extraction kit (Qiagen) and cloned into the pCR4-TOPO vector (TOPO TA Cloning kit; Invitrogen). The insert was sequenced at the University of Bergen Sequencing Facility (Bergen, Norway) using BigDye Terminator v3.1 chemistry and ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Computer analysis. Putative transmembrane domains were predicted using TMHMM 2.0 (23), which is part of the Simple Modular Architecture Research Tool (24,25). Potential N-glycosylation, cAMP/cGMP-dependent protein kinase, and protein kinase C recognition sequences were identified using the PROSITE 20.37 computational tools (26) (Supplemental Fig. 1).

Nucleotide sequences were routinely compared with the GenBank database using the BLAST algorithm. ClustalW2 (version 2.0.12) was used to align amino acid sequences (27) (Supplemental Fig. 2). The phylogenetic reconstruction was generated by using the neighbor-joining method as implemented in MEGA 4.0 (28) (Supplemental Fig. 3).

Expression in Xenopus laevis oocytes and electrophysiology. To obtain a poly(A)-tailed recombinant clone, amplification of the PepT1 cDNA insert was performed using salmon PepT1-specific primers (asPepT1 Fw2: 5′-TTG GAC CGT GAC TCC TCT TGT G-3′; Supplemental Fig. 1, nucleotides 75 to 54 for primer reference; asPepT1 Rv2: 5′-CCA GAG CCT CTC TCT TGG TAA CAC CCT(18-3); Supplemental Fig. 1, nucleotides 2264–2286 for primer reference limited to the PepT1-specific portion). PCR products were visualized on a gel, purified using the E.Z.N.A. Gel Extraction kit (Omega Bio-Tek), and subcloned into the pCR4-TOPO vector. To detect putative point mutations and to verify the insertion of the poly(A) tail, both strands of the cDNA insert were sequenced. A recombinant clone (named asPepT1-TOPOCloning vector) containing no mutations and the poly(A) tail was used for further functional analyses.

Female clawed frogs (Xenopus laevis) were purchased from African Xenopus Facility (Knysna, Republic of South Africa). To collect oocytes, the animals were anesthetized by immersion in 0.7 g/L 3-aminobenzoic acid ethyl ester. After the final collection, the frogs were killed with an anesthetic overdose. Oocytes were treated with 2.5 g/L collagenase for 90 min, separated manually, and incubated in Barth’s solution containing (in mmol/L): 88 NaCl, 1 KCl, 0.8 MgSO4, 0.4 CaCl2, 0.3 Ca(NO3)2, 2.4 NaHCO3, and 10 HEPEs (pH 7.5) at 17°C overnight. Stage V/VI oocytes were injected with ~50 ng (in 46 nL) of in vitro synthesized salmon PepT1-specific cRNA (Ambion) in Message Machine T7 kit; AMS), as obtained by using asPepT1-TOPOCloning vector as template, and incubated for 3–6 d at 17°C.

Two-electrode voltage clamp experiments were performed as described (29). Briefly, the oocyte was placed in an open chamber (~0.5 mL total volume) and continuously superfused (~1 mL/min) with the Barth’s solution or with solutions containing the substrate peptide. Electrodes with resistance between 0.5 and 2 mol/L were connected to a TEC-05 amplifier (npi electronic). Oocytes were voltage-clamped at ~60 mV and current-voltage (I–V) relations were measured using short (100 ms) pulses separated by 200-ms pauses in the potential range of −160 to +80 mV. I–V measurements were made immediately before and 30–40 s after substrate application when current flow reached steady state. The asPepT1-evoked current at a given membrane potential was calculated as the difference between the currents measured in the presence and absence of substrate. I–V relations were calculated with a Visual Basic routine written in Microsoft Excel. Positive currents denoted positive charges flowing out of the oocyte.

Peptides (Sigma), with all amino acids of the α-type except in β-Ala-l-His (carnosine) and Gly and Sarc, which do not have l- or d-forms, were added to the solutions in concentrations as indicated in the text. After addition of the peptide, the pH was adjusted if necessary.

Transport parameters, i.e. apparent substrate affinity (K0.5; mmol/L) and maximal transport current (Imax,nA) were calculated by best-fitting of the Michaelis-Menten equation to at least 3 data points using the Solver function of Microsoft Excel. For the reference peptide (Gly-Sar), pH dependence of Imax was determined in paired experiments, i.e. each oocyte was perfused with solutions of pH values of 8.5, 7.5, and 6.5. At each pH, in addition to the substrate-free control solution, 3 substrate concentrations were applied, 1 high concentration (up to 20 mmol/L) evoking maximal current and 2 concentrations below and above the
respective pH-dependent $K_0.5$ value, which was estimated from preceding experiments. The sequence of pH values was varied to avoid systematic errors.

Analysis of gene expression in fish tissues. Total RNA was isolated from whole brain, pituitary, eye, gill, liver, stomach, pyloric caeca, midgut, heart, kidney, intraperitoneal adipose tissue, belly flap, skin, white muscle, red muscle, testis, and ovary of juvenile Atlantic salmon ($n = 8$). The first-strand cDNA was prepared as described above (see “Molecular cloning”). The tissue distribution of the mRNA for asPePT1 was analyzed by real-time quantitative RT-PCR (qPCR) using SYBR Green assays (Chromos 4 System; Bio-Rad Laboratories). Primer sets for the qPCR for asPePT1 were designed in the nucleotide sequence obtained (asPePT1 Fw3: 5'-CGA TGG GAC TAT CGA CGT CTG CTC TGT CCG C-3'; Supplemental Fig. 1, nucleotides 1251–1277 for primer reference; asPePT1 Rv3: 5'-AGG GCC GTA CGT GTG TGC GAC A-3'; Supplemental Fig. 1, nucleotides 1376–1400 for primer reference; product size: 150 bp). The PCR was performed as follows: 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Atlantic salmon elongation factor 1α (asEF1α; GenBank accession no. AF321836) was also amplified as an internal standard (asEF1α Fw1: 5'-GAG AAC CAT TGA GAA GTT CGA GAA G-3'; asEF1α Rv1: 5'-GCA CCC AGG CAT ACT TGA AAG-3'; product size: 71 bp).

Additional quantitative validation of the asPePT1 gene expression levels in posterior visceral organs of the intestinal tract was obtained by qPCR based on analysis of dissected intestinal segments (namely, pyloric caeca, midgut, and hindgut) isolated from additionally sampled juvenile fish ($n = 6$).

Effect of feed deprivation. Prior to evaluating the effect of feed deprivation, juvenile Atlantic salmon had been acclimatized to the rearing conditions for several weeks, with continuous feeding using a feeder machine. During sampling, a total of 10 fish (44.3 ± 1.1 g) were collected randomly from the tanks. The fed fish (fed group; $n = 5$) were sampled with filled guts. Subsequently, feeding was stopped for 6 d and then 5 fish (feed-deprived group; $n = 5$) were sampled at the same time as those of the fed group (1400 h). After the fish were killed, pyloric caeca were collected and stored in RNA later at −20°C until RNA isolations were performed. The first-strand cDNA was prepared from fed and feed-deprived fish as described above (see “Molecular cloning”). The asPePT1 gene expression levels were analyzed by qPCR as described in the previous section (see “Analysis of gene expression in fish tissues”).

Statistical analysis. GraphPad Prism v. 5.02 was used for statistical analysis. Values in the text are means ± SEM. To test $K_0.5$ and $I_{max}$ differences at different extracellular pH values, a paired $t$ test was conducted between data at a given membrane potential, followed by Bonferroni’s post hoc multiple comparison test (differences were considered significant if $P < 0.01$). For tissue distribution analysis, differences comparison of mRNA levels were done using 1-way ANOVA followed by Tukey’s post hoc multiple comparison test (differences were considered significant if $P < 0.01$). To test the effects of feed deprivation, an unpaired $t$ test was used after checking for homogeneity of variances and normality of distributions (differences were considered significant if $P < 0.05$).

Ethical treatment of animals. The research was conducted in accordance with regulations by National Animal Research Authority in Norway.

Results

Sequence analysis. The full-length cDNA for asPePT1 was 2629 bp long, with an open reading frame of 2205 bp encoding a putative protein of 734 amino acids (Supplemental Fig. 1). Hydropathy analysis predicted at least 12 potential membrane-spanning domains with a large extracellular loop between transmembrane domains IX and X (Supplemental Fig. 1). Four putative extracellular N-glycosylation sites (Asn123, Asn449, Asn502, and Asn517), 3 putative intracellular cAMP/GMP-dependent protein kinase phosphorylation sites (Thr168, Ser704, and Ser220), and 3 putative protein kinase C phosphorylation sites (Ser9, Ser258, and Ser709) were identified (Supplemental Fig. 1). As expected, the predicted asPePT1 amino acid sequence revealed a higher percentage of identity with PePT1 from other teleost fish, such as Atlantic cod (Gadus morhua) (68%), zebrafish (65%), and China rockfish (Sebastes nebulosus) (63%), than with PePT1 amino acid sequences characterized so far from birds (60%) and mammals (56–59%) (Supplemental Fig. 2). Overall, the phylogenetic reconstruction of the vertebrate PePT1 tree suggested substantially higher sequence divergence among the proteins of the fish group than among those of the mammalian group (Supplemental Fig. 3).

Function. The zwitterionic and hydrolysis-resistant dipeptide Gly-Sar was used as a test compound in 2-electrode voltage clamp experiments to establish the basic kinetic properties of the salmon transporter (Fig. 1). $I$-$V$ relations were initially measured in oocytes clamped at −60 mV and incubated with 0.1, 0.5, and 10 mmol/L Gly-Sar at the extracellular pH value of 6.5 (Fig. 1A). At pH 6.5, the transport current was inwardly directed at negative membrane potentials. However, the direction of current slightly reversed (i.e. it became outwardly directed) at more positive membrane potentials (at ~+34 and ~+58 mV when 0.5 and 10 mmol/L Gly-Sar were applied, respectively). Then, $K_0.5$ and $I_{max}$ were determined using extracellular Gly-Sar concentrations between 0.1 and 20 mmol/L at pH 6.5, 7.5, and 8.5 (Fig. 1B; Table 1). The dependencies of $K_0.5$ and $I_{max}$ on membrane potential and extracellular pH were also analyzed (Fig. 1C; Table 1). $K_0.5$ values were strongly affected by both membrane potential and pH. At each membrane potential less negative than −140 mV, apparent affinity for Gly-Sar at pH 6.5 was always higher than that at pH 7.5 and both were higher than that measured at pH 8.5 (Fig. 1C). However, these differences in affinities may result from a preference for transport of the zwitterionic form of Gly-Sar, which account for ~99% at pH 6.5, ~90% at pH 7.5, but only ~47% at a pH of 8.5 (Table 1). The measured $K_0.5$ values are therefore overestimating the true values for transport of the neutral form by ~1%, ~12%, and ~11% at pH 6.5, 7.5, and 8.5, respectively. Assuming that only the neutral form is transported, the corrected $K_0.5$ values correspond to 0.49 ± 0.08, 1.29 ± 0.11, and 4.41 ± 0.31 mmol/L at pH 6.5, 7.5, and 8.5, respectively. At each external pH, $K_0.5$ displayed a membrane potential-dependent minimum, which was shifted toward depolarizing membrane potentials with decreasing pH, i.e., $< -160$ mV at pH 8.5, $< -160$ mV at pH 7.5, and $-100$ mV at pH 6.5. A further depolarization below these potentials induced a sharp reduction in apparent substrate affinity (Fig. 1C). As expected from an electrogenic transporter, $I_{max}$ also exhibited a clear dependence on membrane potential and its value steadily decreased, passing from ~112% at pH 6.5, 7.5 and 8.5, respectively. Although less pronounced, this increase in $I_{max}$ as external proton concentration decreases is reminiscent of what has been observed with the zebrafish PePT1 transporter (17). On the contrary, the asPePT1 differs from mammalian PePT1, in which maximal transport activity is virtually independent of extracellular pH (29). Thus, despite general similarities, some differences exist between the kinetic behavior of fish and that of known mammalian PePT1 transmitters.

Characterization of Peptide Transporter 1 in salmon
Kinetic parameters ($K_{0.5}$ and $I_{\text{max}}$) were also measured for a selected number of other di- and tripeptides, some of them with acknowledged bioactive properties (namely Ala-Gln, carnosine, Val-Pro-Pro, Ile-Pro-Pro, Lys-Pro-Val) and others containing Lys, an indispensable amino acid that conditionally limits fish growth (namely Lys-Pro, Lys-Val, Ala-Lys, Glu-Lys, Lys-Glu, Arg-Lys, Lys-Pro-Val; Table 2). asPepT1 displayed broad substrate specificity for transport of both neutral and charged di- and tripeptides (Table 2). All di- and tripeptides examined evoked inward currents according to Michaelis-Menten-type kinetics, with $K_{0.5}$ values ranging from 0.462 mmol/L (Phe-Tyr) to 11.32 mmol/L (Pro-Gly) and percent $I_{\text{max}}$ values (relative to Gly-Sar $I_{\text{max}}$) ranging from 50% (Val-Pro-Pro) to 207% (Lys-Pro). To compare and rank the different peptide compounds, the ratio between $I_{\text{max}}$ and $K_{0.5}$ ($I_{\text{max}}$: $K_{0.5}$) was calculated as an index of the ability of each peptide for active transport by asPepT1 (Table 2). This index designates the efficiency of a transporter for a given substrate and, under our experimental conditions, it is indicative of the ability of asPepT1 to move net charges from the outside to the inside of the oocyte.

Tissue distribution. Expression of asPepT1 was analyzed in a variety of tissues/organs of juvenile fish by qPCR (Fig. 2). Expression analysis indicated that asPepT1 mRNA is highly expressed in the intestinal tract, whereas lower levels of expression were detected in brain. Very low, but still detectable, levels of expression were measured in belly flap, skin, and heart. Expression levels in other tissues, including the stomach, were very low (Fig. 2A).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>pH dependence of the kinetic parameters of inwardly directed Gly-Sar transport via asPepT1 by 2-electrode voltage clamp experiments$^{1-3}$</th>
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<tr>
<td>pH</td>
<td>Neutral form $^4$</td>
</tr>
<tr>
<td>6.5</td>
<td>98.9</td>
</tr>
<tr>
<td>7.5</td>
<td>89.9</td>
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<td>8.5</td>
<td>47.1</td>
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$^1$ Values are means ± SEM, $n = 8$ (oocytes; each oocyte represents an independent observation). **Different from control (data at pH 7.5), $P < 0.01$.

$^2$ X. laevis oocytes voltaged clamped at -60 mV and -120 mV and perfused with solutions at pH 6.5, 7.5, and 8.5.

$^3$ Kinetic parameters ($K_{0.5}$ and $I_{\text{max}}$) calculated by least-square fit to the Michaelis-Menten equation (see Fig. 1B).

$^4$ The percentage of Gly-Sar present in its neutral (zwitterionic) form at a given pH is calculated using the Henderson-Hasselbach equation and the following pK$\alpha$ values: pK$\alpha_1$ = 2.83; pK$\alpha_2$ = 8.45 (57).

$^5$ $I_{\text{max}}$ values are expressed as the percentage of $I_{\text{max}}$ calculated at pH 7.5 in the same experiment.
FIGURE 2 Tissue distribution of asPepT1 mRNA, as assessed by qPCR performed on total RNA from juvenile Atlantic salmon tissues using PepT1- and EF1α-specific primers. Data are reported as PepT1 copy:EF1α copy ratio. (A) Body tissue distribution. Values are means ± SEM, n = 8 for all tissues except testis and ovary, n = 4. (B) Validation of postgastric PepT1 mRNA expression (pc, pyloric caeca; mg, midgut; hg, hindgut) in additional sampled fish. Values are means ± SEM, n = 6. Bars not sharing a common letter differ, P < 0.05.

TABLE 2 Kinetic parameters of the inwardly directed transport of selected di- and tripeptides via asPepT1 by 2-electrode voltage clamp experiments 1−3

<table>
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<tr>
<th>Bioactive 4</th>
<th>Lysine-containing Substrate 5</th>
<th>$K_{i5}$</th>
<th>$I_{max}$ 6</th>
<th>$I_{max}/K_{i5}$</th>
<th>Oocytes</th>
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<td>—</td>
<td>Yes</td>
<td>Lys-Pro</td>
<td>0.607 ± 0.083</td>
<td>207 ± 16</td>
<td>341</td>
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<td>—</td>
<td>Yes</td>
<td>Lys-Val</td>
<td>0.607 ± 0.083</td>
<td>188 ± 11</td>
<td>310</td>
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<td>—</td>
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<td>Phe-Tyr</td>
<td>0.482 ± 0.079</td>
<td>115 ± 5</td>
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<td>Ala-Gln</td>
<td>0.528 ± 0.061</td>
<td>123 ± 6</td>
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<td>Yes</td>
<td>Yes</td>
<td>Lys-Pro-Val</td>
<td>0.658 ± 0.023</td>
<td>145 ± 10</td>
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<td>Tyr-Ala</td>
<td>0.620 ± 0.029</td>
<td>129 ± 7</td>
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<td>Gly-Leu</td>
<td>0.651 ± 0.087</td>
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<td>Yes</td>
<td>—</td>
<td>Ala-Lys</td>
<td>0.634 ± 0.100</td>
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<td>Phe-Phe</td>
<td>0.486 ± 0.140</td>
<td>84 ± 16</td>
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<td>Carnosine</td>
<td>0.972 ± 0.128</td>
<td>171 ± 10</td>
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<td>Gly-Gln</td>
<td>0.648 ± 0.176</td>
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<td>—</td>
<td>Gly-Sar</td>
<td>0.601 ± 0.060</td>
<td>100 (73.4 ± 6.5 nA)</td>
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<td>Glu-Lys</td>
<td>0.697 ± 0.100</td>
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<td>Yes</td>
<td>Lys-Glu</td>
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<td>Val-Pro-Pro</td>
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<td>Pro-Leu</td>
<td>1.028 ± 0.163</td>
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<td>Ile-Pro-Pro</td>
<td>0.975 ± 0.058</td>
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<td>Arg-Lys</td>
<td>1.538 ± 0.551</td>
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<td>Pro-Gly</td>
<td>11.32 ± 4.032</td>
<td>98 ± 14</td>
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</table>

1 Values are means ± SEM of n (ocytes; see last column; each oocyte represents an independent observation).
2 X. laevis oocytes voltage clamped at –60 mV and perfused with solutions at pH 6.5.
3 $K_{i5}$ and $I_{max}$ calculated by least-square fit to the Michaelis-Menten equation (see Fig. 1B).
4 For documented biological effects of tested di- and tripeptides that are referred to as "bioactive," see Supplemental Table 2.
5 All amino acids of the L-type, except in carnosine and Gly and Sar, which do not have L- or D-form.
6 $I_{max}$ values are expressed as the percentage of Gly-Sar $I_{max}$ in the same experiment.

Effect of feed deprivation. As assessed by qPCR performed on pyloric caeca samples, feed depriving juvenile Atlantic salmon for 6 d resulted in a ~70% reduction of PepT1 mRNA levels. In particular, PepT1 relative expression (calculated as the PepT1 copy:EF1α copy ratio) was 0.220 ± 0.045 (means ± SEM) in the fed group and 0.069 ± 0.025 (means ± SEM) in the feed-deprived group (P < 0.05; unpaired t test).

Discussion

We have cloned a full-length cDNA from Atlantic salmon that encodes a novel PepT1-type transporter. The predicted protein exhibits a high overall identity (56–68%) with other known vertebrate PepT1 proteins and clusters to the fish branch of the reconstructed phylogenetic tree, showing highest similarity with Atlantic cod PepT1.

Functional analysis of mammalian PepT1 transporters has led to the notion that, in all vertebrates, PepT1-mediated transport occurs by the same kinetic mechanism and with very similar features (1,14,30). However, the functional characterization of the zebrafish PepT1 revealed that this piscine transporter resembles the mammalian systems in terms of low-affinity/high-capacity properties of the transport but also exhibits peculiarities in terms of pH dependence (17). In particular, zebrafish PepT1 $I_{max}$ increases at alkaline extracellular pH values. With asPepT1, varying extracellular pH from 6.5 to 8.5 also results in an increase of $I_{max}$. This increase, although less pronounced, confirms the kinetic behavior of the zebrafish ortholog (17). In this context, it has to be emphasized that while zebrafish and Atlantic salmon represent agastric and gastric teleosts, respectively, both are carnivorous fish with a short intestinal tract. Moreover, like in zebrafish, Atlantic salmon exhibit a high overall identity (56–68%) with other known vertebrate PepT1 proteins and clusters to the fish branch of the reconstructed phylogenetic tree, showing highest similarity with Atlantic cod PepT1.
intestinal lumen is alkaline under normal physiological conditions, with luminal pH values of 7.6–8.0 in the pyloric caeca, 8.2–8.5 in the midgut, and 7.8–8.5 in the hindgut, whereas stomach exhibits pH values of 4.0–5.3 (Ashild Krogdahl, Norwegian School of Veterinary Science, Oslo, personal communication). Thus, a slightly acidic environment may only be found in a short segment of the upper small intestine. Then, like zebrafish (17,31), other cyprinids (32), and other salmonoids (33,34), Atlantic salmon possibly has no or only very low levels of mRNA of the Na+/H+ exchanger NHE3 in apical membranes of enterocytes, which, by functional coupling with PepT1, is essential for peptide uptake in mammals (35). Taken together, these observations suggest that PepT1 in salmon is adapted to work at alkaline pH under normal physiological conditions.

The broad substrate specificity of asPepT1 for transport of both neutral and charged di- and tripeptides is supported by the observation that all di- and tripeptides examined in this study evoked inward currents according to Michaelis-Menten-type kinetics. In particular, Lys-Pro was the most efficient peptide in activating charge transfer, immediately followed by Lys-Val, whereas Pro-Gly was by far the least efficient peptide. In Lys-Pro and Lys-Val, the good $I_{\text{max}}^{K_{0.5}}$ ratios were mainly due to the high $I_{\text{max}}$ values, whereas in case of Pro-Gly, the low $I_{\text{max}}^{K_{0.5}}$ ratio was exclusively due to the high $K_{0.5}$ value, which was at least 1 order of magnitude higher than those of the other peptides. All the other peptides were between these extremes and their position in the rank was due to the differential contribution to the ratio of $I_{\text{max}}$ and $K_{0.5}$. However, it has to be considered that under our experimental conditions, the high $I_{\text{max}}$ values of Lys-Pro ($I_{\text{max}} = 207\%$), Lys-Val ($I_{\text{max}} = 188\%$), and carnosine ($I_{\text{max}} = 171\%$) are probably caused by the positive charge of Lys and His at pH 6.5, so that 2 charges, 1 from the proton and 1 from the positively charged Lys (Lys+) or His (~50% His+), are transported within each transport cycle. This means that the “true” transport rate is nearly equal to that of Gly-Sar (and to those of the other neutral dipeptides tested, i.e. Gly-Gln, Gly-Leu, Tyr-Ala, Ala-Gln, Phe-Tyr, and Phe-Phe). The same should hold true for Lys-Glu ($I_{\text{max}} = 151\%$), but here the positive charge of Lys is most probably (partially) compensated by the negative charge of Glu. Theoretically, Arg-Lys ($I_{\text{max}} = 99\%$) should also generate higher currents, but it is known from studies in mammals [rabbit (36)] and, more recently, fish [zebrafish (16)] that PepT1 orthologs that Lys+ in the carboxy-terminal position reduces transport currents. This applies also to Ala-Lys ($I_{\text{max}} = 123\%$) and Glu-Lys ($I_{\text{max}} = 90\%$). It is well accepted that Pro-X dipeptides (among them Pro-Gly and Pro-Pro) have much lower apparent affinities for PepT1 than some other Pro-X peptides like Pro-Leu (37,38) and previous studies established that X-Pro dipeptides but not all Pro-X dipeptides are taken up efficiently into the cells via PepT1. The 3 Pro-containing dipeptides tested here (i.e. Lys-Pro, Pro-Leu, and Pro-Gly) fully confirm this that this is also the case in salmon. In fact, the $K_{0.5}$ value for Lys-Pro (0.607 mmol/L) was only slightly lower than that for Pro-Leu (1.028 mmol/L) and both were lower than that for Pro-Gly (11.319 mmol/L). In addition, the 3 Pro-containing tripeptides tested exhibited $K_{0.5}$ values comparable to those measured for Lys-Pro and therefore their rank order was largely determined by the different contribution to the $I_{\text{max}}$ value of the amino acid composition. Even though the peptides evaluated in this study constitute only a sample of the possible di- and tripeptides present as a result of protein hydrolysis in the gut, there appears to be considerable variability in the affinities of these substrates for asPepT1.

PepT1 has recently received considerable attention as a target for delivery of critical amino acids by designing/formulating novel diets with more appropriate protein compositions and for antiinflammatory therapies. Particularly in salmon, enteritis is a well-known problem caused by diets high in plant-based protein. Fish, as other vertebrates, rely on a dietary supply of a well-balanced profile of indispensable and dispensable amino acids (39). Lys is generally one of the first limiting amino acids during preparation of fish feeds today (40–42) and this problem increases when plant-based protein sources, particularly gluten, are used to replace fish meal (43). Lys availability may therefore limit protein synthesis, protein accretion, and growth of fish (44,45), and impair metabolism (46). In Atlantic salmon, Lys requirements up to 5.0% of dietary protein have been reported (45). Based on this fundamental role in fish nutrition, Lys-containing peptides were particularly addressed in the functional characterization of asPepT1. The observed kinetic aspects, including how structural features like the Lys positioning in di- and tripeptides and net charge affect binding affinity and functional transport, will be beneficial in the formulation of new diets that may contain peptide hydrolysates or individual peptides rather than free amino acids (47–52). Pro-containing peptides that derive mostly from collagen (the most abundant protein in fish feeds of animal origin) are continuously generated by specific peptidases during proteolysis in the intestinal lumen. In particular, dipeptidylpeptidase IV, which is highly active at the intestinal epithelium, releases N-terminal dipeptides of the X-Pro or X-Ala type from larger polypeptides in a sequential manner. These peptides are taken up into intestinal epithelial cells via PepT1 (38). Our study also demonstrates that Pro-containing peptides are taken up by asPepT1, which complements previous but fragmented data obtained in a variety of fish by using other approaches (16). We also have shown that many di- and tripeptides with recognized bioactivity (Supplemental Table 2 and literature cited therein) serve as good substrates for asPepT1. In particular, Lys-Pro-Val, which has recently been recognized in mammals to exert potent antiinflammatory effects in the intestinal mucosa by uptake via PepT1 (5), is also a very good substrate for asPepT1. This finding opens the possibility to assess the effects of Lys-Pro-Val for treatment of fish enteritis and associated inflammation.

asPepT1 is highly expressed in the postgastric intestinal tract with a distinct proximal-to-distal gradient, which suggests that not only pyloric caeca and midgut but also hindgut are involved in peptide absorption. Interestingly, feed depriving fish for 6 d reduces intestinal PepT1 mRNA expression levels, which is in strong agreement with data recently reported for Asian weather-loach [Misgurnus anguillicaudatus (53)] and European sea bass [Dicentrarchus labrax (54)] PepT1. The downregulation of PepT1 mRNA expression thus seems to emerge as a hallmark of a state of malnutrition in fish and as an adaptive response that contrasts with all the experimental evidence obtained so far in higher vertebrates (mammals and birds), in which an upregulation of PepT1 mRNA levels is always observed in malnourished animals (1,15,55). With this in mind, one could speculate that PepT1 dynamically cooperates in a larger and more integrated network of adaptive programs/strategies with major differences between ectotherms and endotherms to adapt to food limitation/deprivation (56).

In conclusion, we have cloned and functionally characterized a novel PepT1-type transporter, which is highly expressed in Atlantic salmon intestine. Transport kinetics were obtained for 16 dipeptides and 3 tripeptides, some with nutritional relevance and/or proposed bioactive properties. We also demonstrated
that fasting downregulates PepT1 expression in salmon. Dietary proteins differ in their amino acid composition and thus also in the composition of peptides produced upon hydrolysis. To what extent the tested peptides and their subsequent absorption are related to the quality of different protein sources and to the composition of peptides produced when digested needs to be determined. Additional information will come from systematic and continued studies of various peptide substrates, which will eventually allow fish nutritionists to formulate diets that are more efficiently used and more nutritionally adequate. The finding that peptides with bioactive properties are good substrates of fish PepT1 allows their exploitation for use in treatment of fish diseases. In particular, those with antimicrobial properties in the intestine and at a systemic level might be relevant to alleviate enteritis in cultured Atlantic salmon by targeting PepT1.

Note Added in Proof. During the editorial process, a paper reporting the cloning and functional characterization of the sea bass (Dicentrarchus labrax) PepT1 was published [Sangaletti R, Terova G, Peres A, Bossi E, Corà S, Saroglia M. Functional expression of the oligopeptide transporter PepT1 from the sea bass (Dicentrarchus labrax). Pflugers Arch. 2009;459:47–54].

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