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Characterization of the transport of lysine-containing dipeptides by PepT1 orthologs expressed in *Xenopus laevis* oocytes

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ABSTRACT

During digestion, dietary proteins cleaved in di and tri-peptides are translocated from the intestinal lumen into the enterocytes via PepT1 (SLC15A1) using an inwardly directed proton electrochemical gradient. The kinetic properties in various PepT1 orthologs (*Dicentrarchus labrax*, *Oryctolagus cuniculus*, *Danio rerio*) have been explored to determine the transport efficiency of different combinations of lysine, methionine, and glycine. Species-specific differences were observed. Lys-Met resulted the best substrate at all tested potentials in sea bass and rabbit PepT1, whereas in the zebrafish transporter all tested dipeptides (except Gly-Lys) elicited similar currents independently on the charge position or amino acid composition. For the sea bass and rabbit PepT1, kinetic parameters, $K_{0.5}$ and I_{max} and their ratio, show the importance of the position of the charged lysine in the peptide. The PepT1 transporter of these species has very low affinity for Lys-Lys and Gly-Lys; this reduces the transport efficiency which is instead higher for Lys-Met and Lys-Gly. PepT1 from zebrafish showed relatively high affinity and excellent transport efficiency for Met-Lys and Lys-Met. These data led us to speculate about the structural determinants involved in substrate interaction according to the model proposed for this transporter.

1. Introduction

The cell membrane is a selective barrier for permeation of nutrient and xenobiotics and the transport across the plasma membrane is a crucial step of the translocation route. The solute enters the animal cell mainly through specialized proteins exploiting the Na^+ gradient or via uniport or antiport systems; other transporters energize the translocation by a transmembrane electrochemical proton gradient that, due to the negative potential inside the cell, supplies a force sufficient to accumulate nutrients above the extracellular concentration.

PepT1 (SLC15A1) is an electrogenic transporter that uses the inwardly directed proton electrochemical gradient to drive the transport of several di-tripeptides as well as peptido-mimetic molecules into a variety of cells. During digestion, dietary proteins are enzymatically cleaved and degradation products are translocated from the intestinal lumen into the enterocytes via PepT1. Lysine and methionine are two essential amino acids known to be growth-limiting in animals. On the other hand, fish meal (FM) supply has become a limiting factor for the further development of fish feed production, and diets for aquaculture species often have increasing proportions of plant-based ingredients. Although such feeds are effective for raising omnivorous and herbivorous species, the challenge in carnivorous fish is greater. Several studies have demonstrated that plant protein-based diets, often deficient in essential amino acids (EAA) such as lysine and/or methionine, caused poor growth performance of carnivorous European sea bass (*Dicentrarchus labrax*) reared in salt water. For this reason, it has been suggested that EAA supplementation will be suitable in solving the nutritional challenge with formulated purified diets for cultured fish. PepT1 is the major route of intake of small peptides and a better knowledge of its ability to transport lysine- and methionine-containing peptides will help in the formulation of the correct form of EAA to be supplemented in the fish diets.

The overall high degree sequence conservation of PepT1 through evolution (from prokaryotics to mammals) is, not only consistent with its essential role in growth and metabolism, but it also suggests that its biological action may be equally well conserved (Daniel et al., 2006; Solcan et al., 2012). Indeed, data presented in this last recent paper suggest that many functional aspects of the prokaryotic transporter are similar to the mammalian one, demonstrating high conservation of these mechanisms during evolution.

Different animal PepT1 orthologs have been analyzed in order to define a unified model of transport (Renna et al., 2011b), and the effects of temperature (Bossi et al., 2012), by comparing the presteady-state currents in the different species. In the present work, a comparative analysis of transport currents, substrate affinity and transport efficiency was conducted using an electrophysiological approach with the aim of exploring the characteristics of transport of some essential amino acids

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(lysine, methionine) and glycine in different combinations of peptides, in order to evaluate the uptake efficiency of these nutrients by various PepT1 proteins.

2. Materials and methods

2.1. Oocyte expression

Xenopus laevis oocytes and RNAs were prepared as previously described in detail (Bossi et al., 2007a). To prepare the mRNA, the cDNAs encoding the different orthologs of PepT1 transporters cloned into the pSPORT-1 vector (Invitrogen, Milan, Italy, www.lifetechnologies.com) were linearized with NotI for the rabbit (*Oryctolagus cuniculus*) PepT1 (rbPepT1) and with HindIII for the seabass (*D.labrax*) (sbPepT1) and the zebrafish (*Danio rerio*) (zfPepT1) PepT1. Subsequently cRNAs were synthesized in vitro in the presence of Cap Analog and 200 units of T7 RNA polymerase. All enzymes were supplied by Promega Italia (www.promega.com, Milan, Italy). Oocytes were obtained from adult female *X. laevis* (Xenopus express, France, www.xenopus.com), the frogs were anesthetized in MS222 (tricaine methanesulfonate salt) (Sigma, Milan, Italy, www.sigmaaldrich.com) 0.10% w/v solution in tap water and portions of the ovary were removed through an incision on the abdomen. The oocytes were treated with collagenase Type IA (Sigma) 1 mg/mL in calcium-free ND96 for at least 1 h at 16 °C. After 24 h at 16 °C in modified Barth's saline solution (MBS), selected oocytes were injected with 12.5 ng of cRNA in 50 nL of water, using a manual microinjection system (Drummond Scientific Company, Broomall, PA, www.drummondsci.com). The oocytes were then incubated at 16 °C for 3–4 days in MBS before electrophysiological studies. The experiments were carried out according to the institutional and national ethical guidelines (permit no. 05/12).

2.2. Point mutations

Mutations in rabbit and zebrafish PepT1 were obtained by site-directed mutagenesis. Briefly, 20 ng of the plasmids containing the FLAG-wild-type rbPepT1 and the wild-type-zfPepT1 cDNAs was amplified with 2.5 units of *Pfu* DNA polymerase in the presence of overlapping primers containing in their sequences the mutated codons:

rbPepT1_T327I 5′-CAGCCGGATCAGATGCAG**ATC**GTGAACACCATCTTG ATTATTATCC-3′
zfPepT1_I334T 5′-GCCAGATCAGATGCAG**ACC**GTGAACCCTATACTG-3′.

PCR amplification was performed with 25 thermal cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 14 min. Then, 10 units of *Dpn*I was added directly to the amplification reaction, and the samples were incubated for 1 h at 37 °C to digest the parental, methylated DNA. JM109 competent cells were finally transformed with 1 μL of the reaction mixture and plated onto LB-ampicillin plates (Liu and Naismith, 2008; Edelheit et al., 2009; Bossi et al., 2011). Plasmids were purified and sequenced (Eurofin MWG Operon, Ebersberg, Germany, www.eurofinsdna.com/) to confirm the nucleotide substitutions.

2.3. Single-oocyte chemiluminescence

To evaluate the expression at the oocyte plasma membrane, we used single-oocyte chemiluminescence (SOC) to quantify a tagged protein expressed at the cell surface (Bossi et al., 2011). Briefly, oocytes expressing FLAG-wild-type rbPepT1 and FLAG-rbPepT1_T327I, as well as non-injected oocytes, were fixed with 4% paraformaldehyde in ND96, then rinsed for three times in cold ND96 for 5 min and, after 1 h of incubation in blocking solution (BSA 1% + ND96 pH 7.6) they were incubated for 1 h in primary mouse anti-FLAG M2 (Sigma, Milan, Italy, www.sigmaaldrich.com) monoclonal antibody (1 μg/mL in 1% BSA-ND96) (all steps at 4 °C). At this point the oocytes were transferred at room temperature and kept for 1 h in peroxidase-conjugated goat anti-mouse IgG (HRP-IgG) 1 μg/mL (www.jacksonimmuno.com). For chemiluminescence readings, each oocyte was transferred into a well of a 96-well plate (Assay Plate White not treated flat bottom, Corning Costar, www.corning.com) filled with 50 μL SuperSignal Femto (Pierce, Euroclone, Milan, Italy, www.euroclonegroup.it). Chemiluminescence was quantified with a Tecan Infinity 200 microplate reader. The plates were read not later than 5 min after the transfer of the first oocyte. The data were then acquired at least three times in 10 min. Results were normalized to the mean value of wild-type FLAG-PepT1 for each batch and are given in arbitrary units (AU).

2.4. Electrophysiology and data analysis

Measurements of the currents generated by the transporters in controlled voltage conditions were performed using the two-electrode voltage clamp (TEVC) technique (GeneClamp, Molecular Devices, Sunnyvale, CA, USA) (Giovannardi et al., 2007). Intracellular glass microelectrodes, filled with KCl 3 M and with tip resistance between 0.5 and 4 MΩ were used. Agar bridges (3% agar in 3 M KCl) connected the bath electrodes to the experimental chamber. The holding potential (V_h) was −60 mV. Voltage pulses from −140 mV to +40 mV in 20 mV increments were applied for 200 ms. Data were analyzed using Clampfit 10.2 (Molecular Devices, www.moleculardevices.com), while statistics and figures were done with Origin 8.0 (originalLab Corp., Northampton, MA, USA, www.originlab.com).

2.5. Solutions

The oocyte culture and washing solutions had the following composition (in mM), calcium-free ND96: 96, KCl 2, $MgCl_2$ 1, Hepes 5, pH 7.6; ND96: NaCl 96, KCl 2, $MgCl_2$ 1,$CaCl_2$ 1.8, Hepes 5, pH 7.6; MBS: NaCl 88, KCl 1, $NaHCO_3$ 2.4, Hepes 15, $Ca(NO_3)_2$ 0.30, $CaCl_2$ 0.41, $MgSO_4$ 0.82, sodium penicillin 10 μg/mL, streptomycin sulfate 10 μg/mL, gentamicin sulfate 100 μg/mL, pH 7.6; PBS: NaCl 138, KCl 2.7, Na_2HPO_4 10, KH_2PO_4 2, pH 7.6. The external control solution during the electrophysiological recordings had the following composition (mM): NaCl, 98; $MgCl_2$, 1; $CaCl_2$, 1.8, Hepes 5 mM. The final pH values (6.5–7.5) were adjusted with NaOH. The dipeptide substrates were added at the indicated concentrations. Experiments were conducted at room temperature (20–25 °C).

The peptides tested were: Glycine-Glutamine, Lysine-Lysine, Methionine-Lysine, Lysine-Glycine (Sigma, Milan, Italy) Glycine-Lysine, Lysine-Methionine, Lysine-Lysine-Lysine (Genicbio, Shanghai, China, www.genicbio.com) at concentrations from 0.1 mM to 30 mM.

2.6. Sequence alignment

PepT1 sequences were obtained from NCBI database (GenBank accession no. AAQ65244.1 for *D. rerio*, AAK39954.1 for *Gallus gallus*, NP_999512.1 for *Sus scrofa*, AAK14788 for *Ovis aries*, NP_001075806 for *O. cuniculus*, NP_005064 for *Homo sapiens*, ACI49693.2 for *D.labrax*, 2XUT_B for *Shewanella oneidensis*, 4APS_B for *Streptococcus thermophilus*, and PepT1, respectively), aligned with ClustalW (www.ebi.ac.uk/clustalw2) and the consensus sequences were visualized with Jalview program (www.jalview.org).

3. Results

3.1. Transport current

Measurements of the transport associated currents generated by the transporters, in the presence of different substrates, were performed at constant (Vh = −60 mV) (Fig. 1A,B,C), or at different membrane

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