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Topical Review

Function and Molecular Structure of Brush Border Membrane Peptide/H^+ Symporters

H. Daniel
Biochemistry of Nutrition Unit, Institute of Nutritional Sciences, University of Giessen, Wilhelmstrasse 20, D-35392 Giessen, Germany

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Introduction

In both prokaryotic and eurkaryotic organisms, uptake of short chain peptides in intact form provides an efficient and economic route for cells to absorb amino acids which are needed for growth and development. Peptide carriers specialized in transport of di- and tripeptides have been cloned from bacteria, fungi, plants and mammalian cells. Whereas the importance of peptide transporters in bacteria and yeast could easily be demonstrated by growth experiments, establishing that mammalian cells also take up oligomers of amino acids was a difficult task due to rapid extracellular hydrolysis of peptides by membrane-bound hydrolases. However, by use of peptides and peptidomimetics resistant to hydrolysis it was demonstrated convincingly that epithelial cells of mammalian intestine and kidney also possess oligopeptide transporters in their apical membranes that are responsible for absorption of peptide-bound amino acids and peptide-derived drugs. Within the last two years a number of mammalian peptide transporters have been identified by different cloning techniques giving us a first insight into the molecular structure and function of this novel group of solute transporters This review focuses on the very recent advancements in peptide transport in mammalian cells.

Molecular Basis of Peptide Transport

The breakdown of dietary proteins in the gastrointestinal tract by the action of pancreatic proteases in concert with brush border membrane-bound peptide hydrolases generates a large amount and huge variety of oligopeptides as well as free amino acids. These products of luminal hydrolysis finally reach the apical membrane of enterocytes, are taken up into the cell and from there they are delivered into the circulation. Similarly in the kidney, proximal tubule peptides filtered in the glomerulum and short chain peptides released by hydrolysis of larger peptides by membrane-bound enzymes are efficiently reabsorbed into kidney tubular cells for conservation of amino acid nitrogen.

A large number of studies in various tissue preparations and species had provided indirect evidence that in addition to the transport systems for free amino acids there is a distinct electrogenic transport pathway for short-chain peptides in small intestinal epithelial cells [1, 8, 16, 23, 39, 40, 50, 51, 53, 57]. However, studies on the molecular mechanisms of peptide transport in vertebrate cells started only a decade ago with the demonstration of transport of intact dipeptides into isolated brush border membrane vesicles of rat and rabbit intestine and kidney [16–18]. One of the major findings was that peptide transport is electrogenic in nature and is energized by a transmembrane electrochemical H^+ gradient but not by a Na^+ gradient [16, 19]. At that time this was a novel finding, since a proton gradient as the driving force was thought to be of biological importance only in prokaryotes and yeast [20, 21]. A large number of studies using isolated membrane vesicles, tissue preparations and epithelial cells in culture have since confirmed the electrogenic proton-mediated nature of transport of di- and tripeptides in both intestine and kidney [3, 9, 10, 41–43, 61–63]. In addition it was also shown that not only di- and tripeptides but pharmacologically active compounds with a peptide backbone including β-lactam antibiotics and angiotensin converting enzyme (ACE) inhibitors

Correspondence to: H. Daniel

Key words: Peptide transport — Kidney — Intestine — Expression cloning — Molecular structure

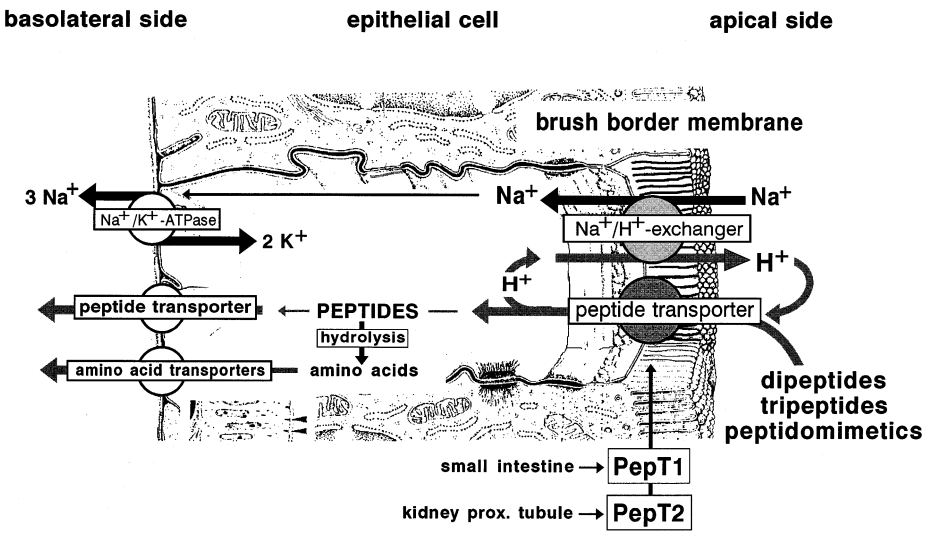

Fig. 1. Transport of peptides and peptidomimetics at the cellular level. This model of cellular coupling of epithelial peptide transport and Na^+/H^+ antiporter activity is currently used to explain the pH dependency of peptide uptake into intestinal and renal epithelial cells and its secondary dependence on the transmembrane Na^+ gradient.

[14, 15] serve as substrates for the intestinal and renal peptide transporters [11, 12, 24–28, 47, 48, 52, 54, 55, 61–63, 67, 68]. Figure 1 shows the current model used to explain oligopeptide transport at the cellular level in both intestine and kidney proximal tubule. Although peptide transport across the apical membranes appears to be phenomenologically similar in intestine and kidney, the renal transporter clearly shows a much higher substrate affinity (for the same substrates) and a different substrate specificity [6, 7, 9, 41].

Attempts to identify and isolate the apical peptide transporters by conventional techniques of protein chemistry have been undertaken for both intestine and kidney. In a series of papers, Kramer et al. [30–35] described the identification of a 127 kDa protein in brush border membranes of intestinal and renal epithelial cells that binds di- and tripeptides and β-lactam antibiotics as well as a number of other peptide mimetics including renin inhibitors. The 127 kDa protein was isolated and reconstituted into liposomes [31] and shown to bind cephalexin, a prototypical peptidomimetic of the aminocephalosporin class of β lactams. Similarly Boll and Daniel [5] identified a 105 kDa protein in kidney brush border membranes by photoaffinity labeling techniques employing cefadroxil as a high affinity type substrate of the renal peptide transporter [10, 52]. The 105 kDa protein could only be labeled by the photoreactive substrate in the presence but not in the absence of a transmembrane pH gradient, suggesting this protein could be involved in pH gradient dependent peptide transport. By use of the radiation inactivation size analysis, the functional molar mass of this putative peptide transporter protein was found to be 416 ± 15 kDa. Together with the labeling of a 105 kDa membrane protein it was speculated that the transporter may operate in an oligomeric arrangement consisting of four 105 kDa subunits [5].

Cloned Mammalian Peptide Transporters

In 1994, a new era of peptide transport studies started with the cloning of the rabbit intestinal peptide transporter rPepT1 as the first mammalian peptide transporter [6, 14]. Cloning of PepT1 was achieved by functional expression of the carrier protein in *Xenopus laevis* oocytes. Starting from poly(A)$^+$ mRNA isolated from the rabbit small intestinal mucosa and injected into oocytes the transport activity expressed in the oocytes was assessed by uptake of radiolabeled peptides. After size fractionation of the mRNA, a RNA-pool containing the message was used for constructing a cDNA library in a bacterial vector. Individual cDNA clones of the library were then transcribed into the corresponding cRNAs which were injected individually into oocytes. Oocytes containing different cRNAs were then assayed for the induced transport activity. By this procedure, the library was screened for a single clone encoding the transport activity of interest. Sequencing of the clone-cDNA finally allows the amino acid sequence of the transport protein to be determined and hydropathy analysis allows some first predictions of the transporters arrangement within the cell membrane. Using this approach Fei at al. [14] and Boll et al. [6] identified 2.7 and 2.9 kb cDNAs that, after injecting the corresponding cRNAs into oocytes, induced a transport activity that resembled the characteristics of peptide transport obtained in intestinal

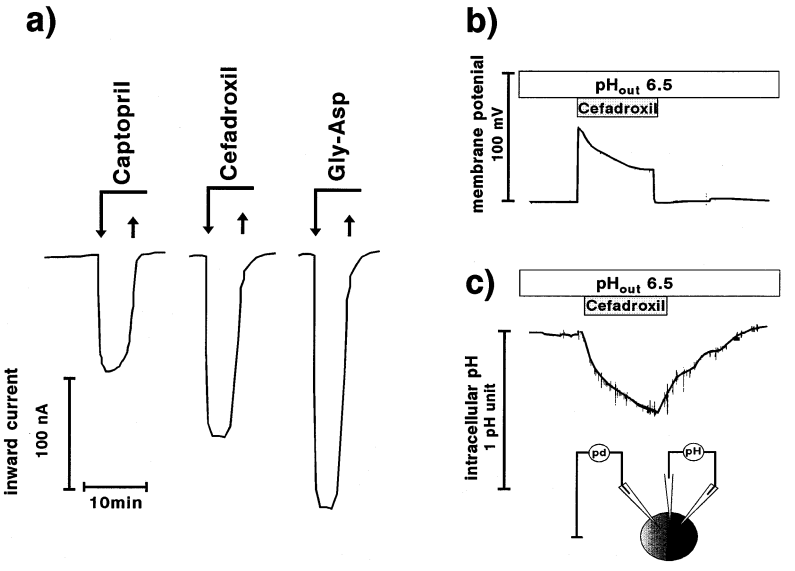

Fig. 2. Selected functional features of the intestinal peptide transporter PepT1 when expressed in *Xenopus laevis* oocytes. (*a*) Rheogenic character of peptide transport activity in *Xenopus* oocytes expressing the rabbit intestinal peptide transporter rPepT1. PepT1-cRNA was injected into individual oocytes and the expressed transport activity was assessed by the two electrode voltage clamp technique as described [6]. Perfusion of voltage-clamped oocytes (holding potential −60 mV) with 1 mM of captopril, cefadroxil or Gly-Asp at pH 6.5 causes substrate specific increases in inward currents. The arrows indicate the start of perfusion of a substrate containing medium or its removal. (*b* and *c*) Simultaneous recordings in membrane potential (V_m) and intracellular pH in *Xenopus* oocytes expressing PepT1 in response to addition of cefadroxil (2.5 mM). Membrane potential is recorded in the open circuit mode and intracellular pH is measured by use of a pH sensitive microelectrode. Addition of the dipeptide at pH_{out} 6.5 causes a membrane depolarization that is followed by a time-dependent decrease in intracellular pH with both parameters returning to initial values when the substrate is washed out.

membrane vesicles and tissue preparations in all aspects. The transporter (PepT1) is capable of translocating di- and tripeptides, aminocephalosporin antibiotics and angiotensin-converting enzyme inhibitors [6, 14]. PepT1 activity depends on the membrane potential and is rheogenic as a consequence of peptide flux coupled to proton cotransport [6, 14, 38]. Figure 2*a* shows the electrogenic influx of selected substrates into *Xenopus* oocytes expressing PepT1 as assessed by the two electrode voltage clamp technique. Uptake of peptides (glycyl-aspartate) or peptidomimetics (cefadroxil, captopril) causes substrate-dependent changes in inward currents in oocytes clamped to a membrane potential of −60 mV. As shown in Fig. 2*b* and 2*c*, the electrogenic transport of the zwitterionic compound cefadroxil is a consequence of a simultaneous H^+ influx into the oocytes expressing PepT1. Here we determined the intracellular pH by use of pH-sensitive microelectrodes and simultaneously registered the changes in membrane potential under open circuit conditions. Perfusion of the oocytes at pH 6.5 with 2.5 mM cefadroxil caused a significant depolarization of the membrane potential by almost 40 mV (Fig. 2*b*) followed by a decline in pH_{in} by 0.5 units (Fig. 2*c*). When oocytes were then perfused with a substrate-free medium, pH_{in} and membrane potential returned to their initial values. Fei et al. [14] calculated a 1:1 flux coupling ratio for glycyl-sarcosine:H^+ cotransport mediated by PepT1 in contrast to earlier predictions of >2, as derived from studies in isolated tissue preparations [1] and the human intestinal cell line Caco-2 [62]. We determined Hill coefficients as 1.15 ± 0.18 for activation of dipeptide uptake into oocytes expressing PepT1 at membrane potentials varying between ±0 and −100 mV. However, if PepT1 has multiple proton binding sites with identical affinity constants then Hill-coefficients of 1 do not necessarily rule out the possibility that more protons are translocated during one cycle of substrate translocation. With respect to the operational mode of PepT1, Mackenzie et al. [38] recently proposed an ordered simultaneous transport model for the human intestinal peptide transporter hPepT1. According to this model, H^+ ions bind first to the carrier. Reorientation of the empty carrier as well as in part H^+ binding and/or dissociation are crucial steps in the transporter performance.

By employing the *Xenopus* oocytes expression system, we recently succeeded in cloning the renal peptide transporter rPepT2 from the rabbit kidney cortex [7]. At the same time Liu et al. [36] identified the human renal peptide transporter PepT2 by homology screening procedures. Expression of rPepT2 in oocytes induces a transport activity with all the characteristics identified in brush border membrane vesicles from rabbit or rat kidney cortex [3, 9–12, 42, 43]. Similarly PepT2, when transfected into HeLa cells induces a high affinity transport activity for a variety of di- and tripeptides [36]. In contrast to PepT1 from the intestine, both renal transporters (human and rabbit) have a substrate affinity that is approximately 20-fold higher for the same substrates and under identical experimental conditions [6, 7, 14, 37]. Similar to the intestine, the renal peptide transporter rPepT2 when expressed in *Xenopus* oocytes shows Hill coefficients of 1.12 and 1.20 for activation of cefadroxil and D-Phe-L-Ala uptake, confirming earlier observations of a Hill-coefficient of 1 for coupling of Gly-Gln and H^+ cotransport in kidney brush border membrane vesicles [9]. With respect to substrate specificity, the renal trans-

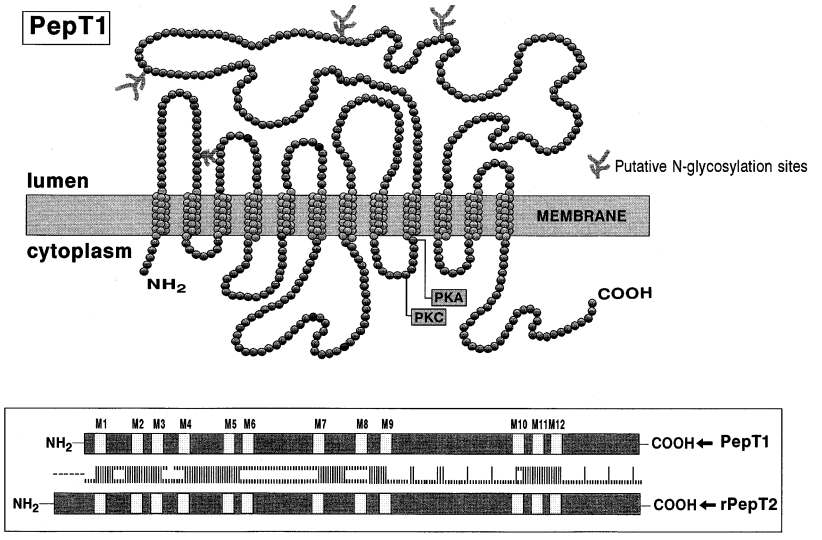

Fig. 3. Structural model and proposed membrane topography of the rabbit intestinal peptide/H^+ symporter PepT1. The amino acid sequence predicts twelve transmembrane domains for PepT1. Predicted N-glycosylation sites and potential phosphorylation sites for protein kinase C (PKC) and protein kinase A (PKA) are marked. Comparative analysis of the amino acid sequence of rabbit intestinal PepT1 and the renal transporter rPepT2 is demonstrated in the lower panel with vertical lines showing regions of identity or high homology and short lines indicating regions with low homology.

porters like the intestinal ones accept di- and tripeptides and selected β-lactam antibiotics as substrates but not ACE inhibitors [7]. Preliminary data on the structure affinity relationship of the cloned renal transporter support previous findings (10–12) on the importance of a free amino- and carboxyterminus as well as a bulky hydrophobic side chain as important determinants for a high affinity of a substrate.

Sequencing the open reading frames of the cDNAs of PepT1 and rPepT2 (both rabbit) predict gene products of 707 (PepT1) and 729 (rPepT2) amino acids with only approximately 50% overall sequence identity. The human clones hPepT1 and PepT2 similarly have 708 and 729 amino acids with more than 80% identity to the rabbit transporters [36, 37]. Meanwhile, the clones from the rat small intestine [44] and kidney [55] have been isolated and they are virtually identical to the corresponding rabbit and human isoforms. Hydropathy analysis predicts all gene products to contain 12 membrane-spanning domains with a large hydrophilic extracellular loop between membrane domains 9 and 10 (Fig. 3). Whereas the amino acid identity between the intestinal and renal transporters is 61% within the transmembrane regions, the large extracellular loop possesses only a 21% sequence identity. All proteins have a number of potential N-glycosylation sites and protein kinase recognition regions which indicate the transporters to be regulated by reversible phosphorylation. In vitro translation of the cRNA in the presence of microsomes (for core glycosylation) reveals gene products with apparent molecular weights of 71 kDa (PepT1) and 107 kDa (rPepT2) [7, 14]. The molecular mass of the glycosylated renal transporter rPepT2 is therefore almost identical with the 105 kDa protein detected by photoaffinity labeling in membrane vesicles, which was predicted to operate in an oligomeric arrangement [5]. Northern blot analysis with probes derived from PepT1 revealed messages of 2.9 kB predominantly in the rabbit intestine with weaker signals in liver, brain and kidney [14]. *In situ* hybridization [15] and immunolocalization with a peptide antibody against a synthetic peptide corresponding to the 15 carboxyterminal amino acids of PepT1 [46] identified the gene as well as the gene product throughout the small intestine and predominantly in mature epithelial cells lining the upper part of the villi. In contrast to the restricted expression of PepT1, *in situ* hybridization and northern blot analysis performed with a 1.2 kB probe derived from the open reading frame of the rPepT2 ascertained mRNA of 4.8 kB size in a number of other tissues including rabbit brain, lung, muscle and liver but not in the small intestine [7].

In 1994, Dantzig et al. [13] reported the cloning of a gene from the human intestinal cell line Caco-2 that after transfection into CHO cells induced a pH-dependent transport/binding activity for cephalexin and peptides in the transfectant cells. This protein (HPT-1) was found to have a high homology to the cadherin family of cell adhesion proteins but showed no homology to any of the prokaryotic or eukaryotic peptide transporters of the PTR-(peptide transporter)-family [58]. It is therefore currently unclear whether HPT-1 resembles the first member of a new class of peptide transporters or whether

it is a protein associated with the classical peptide carriers.

Sequence comparisons of HPT-1 with other members of the cadherin family reveal that HPT-1 is identical with the human LI-cadherin recently cloned (GenBank database). Immunolocalization studies of the rat intestinal LI-cadherin [4] suggest that LI-cadherin expression in the small intestine is restricted to the basolateral membrane of enterocytes [4]. It is therefore likely that HPT-1 is also localized in the contraluminal membrane of epithelial cells and is not responsible for or directly associated with peptide transport across the apical membrane.

Within the PTR group of peptide transporters from prokaryotes and eukaryotes the mammalian carriers build a separate branch in the proposed evolutionary tree [58]. A unique PTR signature motif is found in the mammalian transporters, the peptide permease from *Saccharomyces cerevisiae* (Ptr2p), the dipeptide transporter of *Lactococcus lactis* (DtpT) and the root di-/tripeptide transporters (AtPTR2-A,B) of the plant *Arabidopsis thaliana* [22, 49, 59]. Additionally a nitrate transporter (AtCHL1) and a histidine transporter from *Arabidopsis thaliana* belong to the PTR family based on significant sequence homologies [58, 65].

Single or Multiple Transport Systems for Peptide Uptake into Epithelial Cells?

Enzymatic hydrolysis of dietary proteins formed by 20 amino acids could cause the release of 400 different dipeptides and 8,000 different tripeptides. Not only will these peptide substrates vary with respect to net charge and solubility, they also cover a wide range of molecular weights from 96.2 Da (di-Gly) to 522.6 Da (tri-Trp). Taking this diversity of structures into account, it is hard to imagine that a single peptide transport system might be capable of transporting all these substrates. For this reason, a number of studies in search of different intestinal peptide transport systems have been conducted. However, for most of the peptides and peptidomimetics and in a variety of model systems only single Michaelis-Menten type kinetics have been obtained, indicating the presence of only one transport pathway for the different compounds (di- tripeptides, β-lactams) investigated. Only a few studies provided indirect evidence for more than one peptide transporter in apical membranes of intestinal epithelial cells. For example, it had been suggested that differently charged β-lactam antibiotics might utilize different peptide transporters for uptake into Caco-2 cells and rabbit intestinal brush border membrane vesicles [26, 34, 60]. This suggestion was mainly based on differences in the pH dependence of uptake of zwitterionic and anionic substrates. Moreover, by comparing the inhibitory effects of selected dipeptides on uptake of anionic or zwitterionic β-lactams, distinctly different patterns were observed [26, 34, 60] supporting the notion that these substrates might utilize different transport pathways. However, we recently demonstrated in Caco-2 cells and in *Xenopus* oocytes expressing PepT1 that anionic (cefixime) and zwitterionic (cefadroxil) substrates share the same transporter [67, 68]. PepT1 is capable of transporting both groups of compounds although with a different pH dependence. Whereas neutral compounds have a pH optimum for uptake at pH 6.0 to 6.5, uptake of the anionic cefixime similar to the anionic dipeptide glycyl-aspartate, is most pronounced at pH 5.0. In addition, competition experiments with selected peptides and β-lactams in addition showed that all substrates depending on their net charge at a particular pH do interact with the substrate binding site of PepT1. Together with the hybrid depletion experiments reported by Fei et al. [14] in which blocking PepT1 expression almost abolished peptide transport in oocytes injected with intestinal mRNA, it may be concluded that PepT1 is predominantly (or entirely) responsible for apical transport of a large variety—if not all—di- and tripeptides and related peptide mimetics.

With respect to the kidney tubule, microperfusion experiments [56] and studies in tubular brush border membrane vesicles [9] provided evidence that in addition to the described and cloned high affinity type peptide transporter, a low affinity transport pathway for peptides and peptide mimetics might exist. Furthermore, northern blot analysis with probes derived from PepT1 and immunoblot analysis suggest a low expression of PepT1 also in the kidney cortex [14, 46]. In contrast to the high affinity system this second renal transport pathway has not been characterized in detail in tissue or membrane preparations and its importance remains to be determined.

Post Cloning Perspectives

Although the cloning and selective expression of peptide transporters provide basic information on molecular structure and functions of this novel family of membrane transporters, the operational mode of substrate translocation is not yet understood. Site-directed mutagenesis and the construction of chimeric gene products will be important tools for gaining information on substrate binding and transport function. The mapping of the substrate binding site will be of particular importance not only for understanding how the transporters are able to accept such a huge variety of substrates but also for the rational design of peptidomimetics for drug delivery. Knowledge of the substrate recognition sites and its size limitations will enable the synthesis of peptide analogues with potent pharmacological and therapeutic activity and a high oral availability.

The isolation of peptide transporters from tissues

such as liver, lung or brain identified by northern analysis and RT-PCR as well as their morphological mapping will soon advance our understanding of the nutritional role of peptide transporters in peripheral tissues. Investigating the regulation of expression of peptide transporters by Northern and Western analysis in intestine and kidney, but possibly also in other tissues, will shed light on the importance of peptide transporters in nutritional and metabolic adaptation. And lastly, knocking out the genes for the peptide transporters will finally help to answer the open question of how important the transporters are in overall metabolism of amino acid nitrogen.

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