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Transport Versus Hydrolysis: Reassessing Intestinal Assimilation of Di- and Tripeptides by LC–MS/MS Analysis

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Scope: The role of PEPT1 in the uptake of intact peptides as compared to hydrolysis prior to uptake of their constituents is unknown. Here, dipeptides, tripeptides, and amino acids are quantified to study the fate of selected peptides in different intestinal models.

Methods and results: An LC–MS/MS-based method is applied for the simultaneous assessment of rates of hydrolysis and transport of a peptide panel in Caco-2 transwell cell culture, in vitro and in vivo in mice expressing or lacking PEPT1, and in hydrolysis studies in vitro using human intestinal samples. It is shown that susceptibility to hydrolysis of peptides at the brush border membrane or within epithelial cells is practically identical in all tested models and strictly structure-dependent. Peptides with high luminal disappearance show substantial hydrolysis and low basolateral appearance, while peptides with low disappearance show strong PEPT1 dependency and high basolateral appearance in intact form in Caco-2 transwell culture.

Conclusion: Hydrolysis and transport of intact peptides are highly variable and structure-dependent. For peptides possessing less polar N-terminal residues, hydrolysis usually dominates over transport via PEPT1. For other peptides with high intrinsic hydrolysis resistance, including anserine, carnosine, γ-glutamyl-dipeptides, and aminocephalosporins, PEPT1 is the main determinant for appearance in peripheral blood.

1. Introduction

The composition of intestinal contents during digestion is in essence unknown. Studies by Adibi et al. from the 1970s suggested the majority of protein degradation products to comprise short-chain peptides and free amino acids.[1,2] However, the peptide fraction would be highly diverse in chain length and composition depending on the nature of the ingested protein, its sequence, and the accessibility of the peptide bonds to cleavage by a multitude of intestinal peptidases and proteases. Although intestinal amino acid transport processes had received considerable interest early on, the existence of a transport pathway for short-chain peptides was the subject of controversy over decades. Convincing evidence for its existence finally arose from studies in humans with inherited diseases of amino acid absorption, such as Hartnup disease and cystinuria, by demonstrating efficient absorption of the critical amino acids when provided in dipeptide form.[3–6] With the cloning of peptide transporter 1 (PEPT1) in 1992 and the ability of heterologous expression, a large number of studies could demonstrate the unique features of this protein for electrogenic transport of all possible 400 different dipeptides and 8000 tripeptides derived from the 20 proteinogenic amino acids.[7] However, almost all studies on the mechanism and the kinetic characteristics of the transport of peptides of highly diverse mass, polarity, and charge either employed electrophysiology or tracer flux studies using the very few radiolabeled substrates available, such as ^{14}C-labeled Gly-Pro and Gly-Sar or ^{3}H-labeled Gly-Gln, in combination with hundreds of competitors. The choice of dipeptides with N-terminal glycine was based on the demonstration of appearance of intact glycyl-peptides in human plasma after ingestion and their higher resistance to hydrolysis when compared to dipeptides with, e.g., leucine or other bulky residues in N-terminal position.[1,8,9] In this respect, almost all studies published in the last decades have used indirect methods to assess the transport features of PEPT1, including studies in mice lacking PEPT1, which did not reveal a distinct phenotype when these mice were fed diets with normal protein levels.[10] It is therefore currently not possible to define the role of PEPT1 in the uptake of intact peptides into epithelial cells as compared to hydrolysis of peptides prior to uptake of their constituents via the different brush border membrane amino acid transporters.

For this reason, we used an LC–MS/MS-based method to quantify dipeptides, tripeptides, and amino acids to study the fate of selected peptides in different intestinal models. The peptide panel comprised around 20 entities that differ in size, polarity, charge, and the arrangement of N- and C-terminal amino acid residues. We assessed rates of hydrolysis and

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transport of the selected peptides in transwell monolayers of Caco-2 cells, and in vitro and in vivo in mice expressing or lacking PEPT1, and complemented the mouse studies with in vitro hydrolysis studies using human intestinal samples.

2. Experimental Section

2.1. Chemicals, Reference Substances, and Internal Standards

Acetonitrile (LC–MS grade), ammonium acetate, formic acid (LC–MS grade), phenyl isothiocyanate (PITC), and pyridine were purchased from Sigma–Aldrich (Taufkirchen, Germany). LC–MS grade water was purchased from J. T. Baker Chemicals (Center Valley, PA). Ethanol and methanol (both LC–MS grade) were obtained from Merck (Darmstadt, Germany). Masschrom internal standard from ChromSystems (München, Germany) was used and expanded by glutamine-D5 and asparagine-15N2 (20.0 µmol L^{-1} each) from Cambridge Isotope Laboratories, Inc. (Andover, USA), and tryptophan-D5 (2.0 µmol L^{-1}) from Santa Cruz Biotechnology, Inc. (Dallas, USA). Analytes for the external standard solution comprised glycine, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamic acid, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine, purchased from Sigma–Aldrich (Taufkirchen, Germany). The di- and tripeptides for the external standard Ala-Gly, Ala-His, Ala-Phe, Arg-Gly, γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Leu, Gly-Asn, Gly-Asp, Gly-Gln, Gly-Gly-Ile, Gly-Pro, Gly-Sar, Gly-Val, Lys-Glu, Phe-Ala, Pro-Gly, Trp-Glu, and Val-Pro-Pro were purchased from Bachem (Bubendorf, Schweiz), Phe-Gly and Trp-Leu from Serva (Heidelberg, Germany), and anserine, carnosine, cefadroxil, cefalexin, cefradine, and Pen-Strep 100× from Sigma–Aldrich (Taufkirchen, Germany).

2.2. Animals

Mice lacking PEPT1 were created by targeted disruption of the PEPT1 gene and obtained from Deltagen (San Mateo, California, USA). Animals were backcrossed for 10 generations to C57BL/6N background and maintained at 22 ± 2 °C and a 12 : 12 h light/dark cycle in specific-pathogen-free (SPF) animal facility. These mouse strains were characterized in detail before.[10–12] Except for two female mice in the $PEPT1^{+/+}$ group, only male mice younger than 19 weeks were analyzed. All procedures were conducted according to the German guidelines for animal care and approved by the state of Bavaria (Regierung von Oberbayern) ethics committee (Reference number: 55.2-1-54-2532-234-2013).

2.3. Mouse Study Design and Plasma Sample Collection

For the gavage experiments, mice were fasted for 6 h. $PEPT1^{+/+}$ and $PEPT1^{-/-}$ animals (n = 3 per group) were gavaged with 200 µL of Gly-Pro/Pro-Gly solution (290 mM each in water, pH 6.0). Wild-type animals were gavaged with either 200 µL of water or Gly-Pro/Pro-Gly solution (n = 3 per group). Thirty minutes after gavage, the animals were anesthetized with isoflurane, followed by retro-orbital blood collection into EDTA-coated tubes (Sarstedt, Nürnbrecht, Germany).

2.4. Peptide Panel for Transport and Ex Vivo Digestion Assays

For oligopeptide transport in cell culture and digestion assays with mouse and human intestinal samples, a peptide panel representative for the entire range of 400 possible dipeptides from 20 proteinogenic amino acids was used, as previously described.[13] The panel comprised Ala-Gly, Ala-His, Ala-Phe, Ans, Arg-Gly, Car, Gly-Asn, Gly-Asp, Gly-Gln, Gly-Pro, Gly-Sar, Gly-Val, Lys-Glu, Phe-Ala, Phe-Gly, Pro-Gly, Trp-Glu, Trp-Leu, as well as Gly-Gly-Ile, Val-Pro-Pro, and γ-Glu-Leu as representatives for tripeptides and γ-glutamyl-dipeptides.

2.5. Cell Culture and Transport Assays

Transport and hydrolysis of a panel of di- and tripeptides, selected γ-glutamyl-dipeptides, and selected cephalosporins were determined in human colon carcinoma Caco-2 cells in transwell culture. PEPT1 knockout clones and corresponding control cells were generated with CRISPR/Cas9 technology and cultured as previously described.[13] Effective PEPT1 knockout was confirmed by DNA sequencing and Western blot analysis (Supporting Information Figure S7). At least 21-days post-confluent cells from passages 30 to 45 and a transepithelial electrical resistance (TEER) of at least 500 Ω cm^2 were rinsed with PBS. For the oligopeptide transport assay, 0.5 mL of MES-buffered transport medium (pH 6.0; Table S6, Supporting Information) containing 500 µM of each panel peptide were used as apical solution, while 1.5 mL of HEPES-buffered transport medium (pH 7.4; Table S6, Supporting Information) served as receiver in the basolateral compartment. For the γ-glutamyl-dipeptide transport assay, cells were incubated apically with 0.5 mL of MES-buffered transport medium (pH 6.0) containing 500 µM of either γ-Glu-Glu, γ-Glu-Gly, or γ-Glu-Leu. For the cephalosporin transport assay, cells were incubated apically with 0.5 mL of MES-buffered transport medium (pH 6.0) containing 500 µM of either cefadroxil, cefalexin, or cefradine. Medium samples were collected after 0 and 360 min of incubation at 37 °C and 5% CO_2.

2.6. Dosage Information

Mice were gavaged after a 6-h fasting period with a single dose of 200 µL of a Gly-Pro/Pro-Gly solution, containing 290 mM of each dipeptide in water at pH 6.0. Dipeptide concentrations were chosen in accordance with early peptide uptake studies in humans[14,15] and correspond to the maximal solubility of both dipeptides in water. This dose of 345 mg kg^{-1} in mice is not achievable through regular diet, and corresponds to a human equivalent dose (HED) of 28 mg kg^{-1}. Caco-2 cells were incubated for 6 h in 0.5 mL of MES-buffered transport medium (pH 6.0; Table S6, Supporting Information) containing 500 µM of each panel peptide as apical solution. Peptide concentrations were chosen to avoid potential slight cytotoxic effects described for certain peptides on Caco-2 cells during 24-h incubation.[16,17] The incubation time of 6 h was chosen to insure that changes in peptide concentrations were above detection limits.

2.7. Intestinal Oligopeptide Digestion by Mouse Tissue Ex Vivo

The intestines of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice ($n = 3$ per group) were removed and rinsed with PBS. Four 1 cm sections were removed 10 cm distally of the stomach, representing jejunal samples, and four sections of 1 cm were removed from the middle of the colon from each animal. The intestinal sections were split open longitudinally and three sections from each segment were incubated in 0.5 mL MES-buffered transport medium (pH 6.0) containing 250 µM of each panel peptide and 1:100 Pen-Strep, while one section each was incubated in 0.5 mL of MES-buffered transport medium (pH 6.0) containing 1:100 Pen-Strep at 37 °C as negative control. Medium samples were taken after 0, 30, 60, and 120 min.

2.8. Intestinal Oligopeptide Digestion by Human Tissue Ex Vivo

Healthy human intestinal samples from duodenum, ileum, and colon were provided by the Chirurgische Klinik at the Klinikum rechts der Isar in Munich (Prof. Dr. Ceyhan and PD Dr. Dr. Demir). The tissue was stored in ice-cold DMEM. Sample analysis was performed within 24 h after surgery. The sampling was approved by the ethical committee of the Technical University of Munich (project number: 51/17S). Intestinal samples were rinsed in PBS before further processing. From each gut section, four individual 0.25 cm^2 mucosal samples were prepared, of which three samples were incubated at 37 °C in 0.5 mL of MES-buffered transport medium (pH 6.0) containing 250 µM of each panel peptide and 1:100 Pen-Strep, while one sample each was incubated in 0.5 mL MES-buffered transport medium (pH 6.0) containing 1:100 Pen-Strep as negative control. Medium samples were taken after 0, 30, 60, 120, 180, and 240 min.

2.9. Sample Preparation, Derivatization, LC–MS/MS analysis, Calibration, Quantification

Sample preparation and derivatization, their analysis and quantification via LC–MS/MS, and calibration of the LC–MS/MS method were performed as previously described.[13] For details, see also the Materials and Methods in the Supporting Information.

2.10. Statistical Analysis

Comparison of concentrations and molar quantities of peptides and amino acids within groups was performed by one-way ANOVA with post hoc Bonferroni's Multiple Comparison Test, and comparisons between groups were performed by two-way ANOVA with post hoc Bonferroni's Multiple Comparison Test. Linear regression analysis of molar peptide quantities was performed with GraphPad PRISM 5.00 (GraphPad Software, Inc.), and outliers were identified based on Cook's distance, with $D_i > 4/n$. p-Values < 0.05 were considered statistically significant.

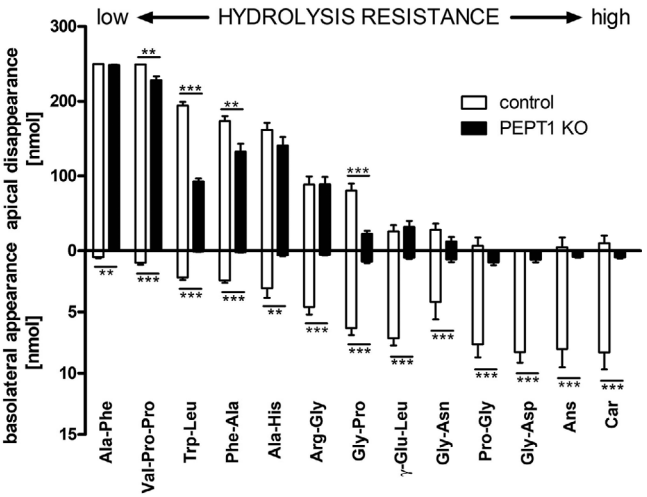

Figure 1. Transport and hydrolysis of oligopeptides in Caco-2 transwell cell culture, provided as mean ± SEM ($n = 6$). Apical disappearance and basolateral appearance of selected oligopeptides in transwell culture after 6 h of apical incubation in control and PEPT1 knockout cells. p-Values represent differences in molar quantity between control and PEPT1 knockout cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3. Results

3.1. Oligopeptide Transport and Hydrolysis by Caco-2 Cells

In order to assess the roles in intestinal protein assimilation of PEPT1 on the one hand and brush border peptidases on the other, PEPT1 knockout and control Caco-2 cells were incubated with a panel of 20 peptides in the apical compartment of transwell cell culture (V = 0.5 mL, 500 µM each, corresponding to 250 nmol per peptide per apical compartment). After a 6-h incubation, we discovered significant differences regarding changes in apical and basolateral concentrations between the panel peptides, reflected by different apical disappearance and basolateral appearance rates (**Figure 1**). Certain peptides, like Ala-Phe and Val-Pro-Pro, disappeared almost entirely from the apical compartment in both cell lines, indicating a low resistance to hydrolysis. For others, we observed marked apical disappearance rates, reducing their molar quantity by a third or more (Ala-His, Arg-Gly, Gly-Pro, Phe-Ala, Trp-Leu), while yet another group did not display any detectable, or only very low apical concentration changes after 6 h, reflected by low apical disappearance rates (Ans, Car, Gly-Asn, Gly-Asp, Pro-Gly, ɣ-Glu-Leu). Apical disappearance of half the panel peptides was less pronounced in PEPT1 knockout cells than in control Caco-2 cells (Ala-Gly, Gly-Gln, Gly-Pro, Gly-Val, Lys-Glu, Phe-Ala, Phe-Gly, Trp-Glu, Trp-Leu, Val-Pro-Pro; Figure S1 and Table S1, Supporting Information). For the other half, we did not observe any differences between the two cell lines, suggesting that apical disappearance was largely caused by brush border peptidase activity. In basolateral compartments of PEPT1 knockout cells, we detected only low increases in peptide concentrations of well below 1 µM, corresponding to basolateral appearance rates of 1 nmol or less, with the highest increase for Pro-Gly of 1 nmol. At the same time, basolateral appearance of all peptides in control cells was significantly higher than in PEPT1 knockout cells, varying between 0.5 nmol (Ala-Phe) and 8.3 nmol

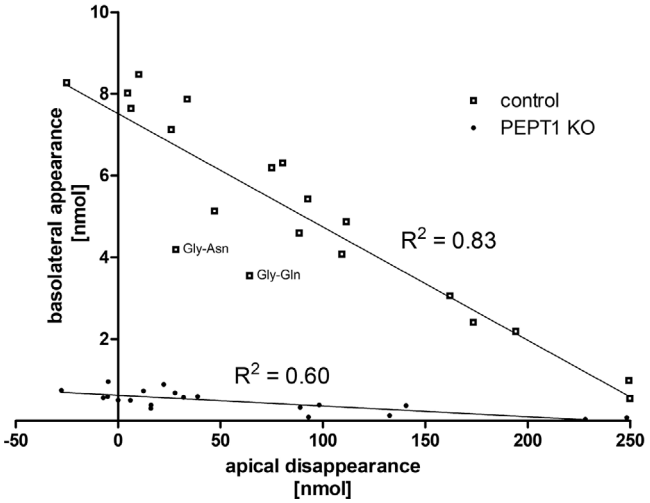

Figure 2. Linear regression of apical disappearance and basolateral appearance of panel peptides after 6 h of incubation with control and PEPT1 knockout Caco-2 cells in transwell cell culture. Gly-Asn and Gly-Gln are outliers in control cells.

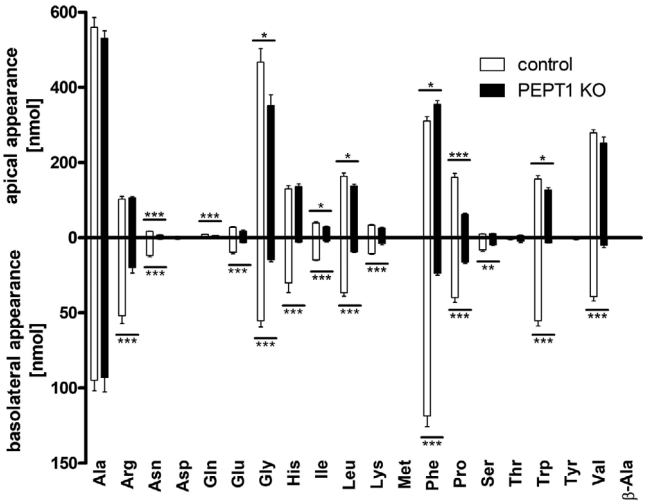

Figure 3. Free amino acids from oligopeptide hydrolysis in Caco-2 transwell cell culture provided as mean ± SEM ($n = 6$). Apical and basolateral appearance of amino acids in transwell culture after 6 h of apical incubation with peptide solution in control and PEPT1 knockout cells. p Values represent differences in molar quantity between control and PEPT1 knockout cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(Car), generally with peptides that featured the greatest apical disappearance showing the lowest basolateral appearance and vice versa. Linear regression analysis of apical disappearance rates and basolateral appearances rates revealed extremely significant differences in control and PEPT1 knockout cells ($p < 0.0001$), with $R^2 = 0.83$ in control cells and $R^2 = 0.60$ in PEPT1 knockout cells, X-intercepts of 271.0 nmol in control and 237.6 nmol in PEPT1 knockout cells, and Y-intercepts of 7.5 nmol in control and 0.6 nmol in PEPT1 knockout cells (**Figure 2**).

Apical amino acid appearance rates were higher in control cells for Asn, Gln, Gly, Ile, Leu, Pro, Trp, and Val, while apical appearance of Phe was higher in PEPT1 knockout cells (**Figure 3**). In basolateral compartments, amino acid appearance was increased for Arg, Asn, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Trp, and Val in control cells. Cumulative amino acid appearance in both apical and basolateral compartments was higher in control cells than in PEPT1 knockout cells (Table S2, Supporting Information).

3.2. Gly-Pro, Pro-Gly, and Amino Acid Plasma Concentrations in Mice With and Without PEPT1 Expression after Dipeptide Gavage

Following up on the striking differences regarding transport and hydrolysis between the structural dipeptide isomers Gly-Pro and Pro-Gly in Caco-2 cell culture, C57BL/6N wild-type mice were gavaged with either 200 µL of a Gly-Pro/Pro-Gly solution (290 mM each in water) or 200 µL water as control. After 30 min, significant differences between Gly-Pro and Pro-Gly plasma concentrations were detected (**Figure 4**A). While plasma concentrations were similarly low for both dipeptides in the water-gavaged control group (Gly-Pro 0.27 µM, Pro-Gly 0.17 µM), Pro-Gly plasma concentration increased to 30 µM after dipeptide supplementation, whereas Gly-Pro increased to only 6.2 µM, which is ≈80% lower than Pro-Gly. At the same time, we did not observe any differences in amino acid plasma levels between the two groups except for the constituent amino acids of both dipeptides in the gavage solution. For glycine, we observed an almost fourfold increase from 200 µM in the control group to 760 µM in the dipeptide group, and for proline, there was a fivefold increase from 70 to 360 µM (Figure 4B). In PEPT1$^{-/-}$ and PEPT1$^{+/+}$ mice gavaged with 200 µL of the Gly-Pro/Pro-Gly solution, Gly-Pro levels in both groups as well as Pro-Gly plasma concentration in PEPT1$^{-/-}$ animals remained similarly low between 5.6 and 7.7 µM, while plasma Pro-Gly in the PEPT1$^{+/+}$ group increased to 45 µM, comparable to WT or even higher (Figure 4C). Regarding plasma amino acids, differences were observed only for glycine (750 µM in PEPT1$^{+/+}$ vs 175 µM in PEPT1$^{-/-}$) and proline (325 µM vs 85 µM) (Figure 4D), corresponding to approximately fourfold increases in PEPT1$^{+/+}$ compared to PEPT1$^{-/-}$ animals.

3.3. Intestinal Oligopeptide Digestion by Mouse Tissue Ex Vivo

In order to assess the role of brush border peptidase activity concerning the differences between Gly-Pro and Pro-Gly plasma concentrations observed in the previous experiments, jejunal and colonic sections from PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice were incubated in a solution of 21 di- and tripeptides (250 µM each). Changes in peptide concentration in the incubation solution were assessed after 30, 60, and 120 min, revealing distinct differences between samples from PEPT1$^{+/+}$ and PEPT1$^{-/-}$ animals (**Figure 5**). Certain panel analytes did not display any, or only small changes in concentration over time across all groups, like Ans, Car, Gly-Asp, Gly-Sar, Pro-Gly, and ɣ-Glu-Leu (Figure S2, Supporting Information), while others disappeared largely from the peptide solution in all groups. For assessing differences in peptide concentration changes between groups, we calculated the sum of concentrations of each panel peptide at all time points for

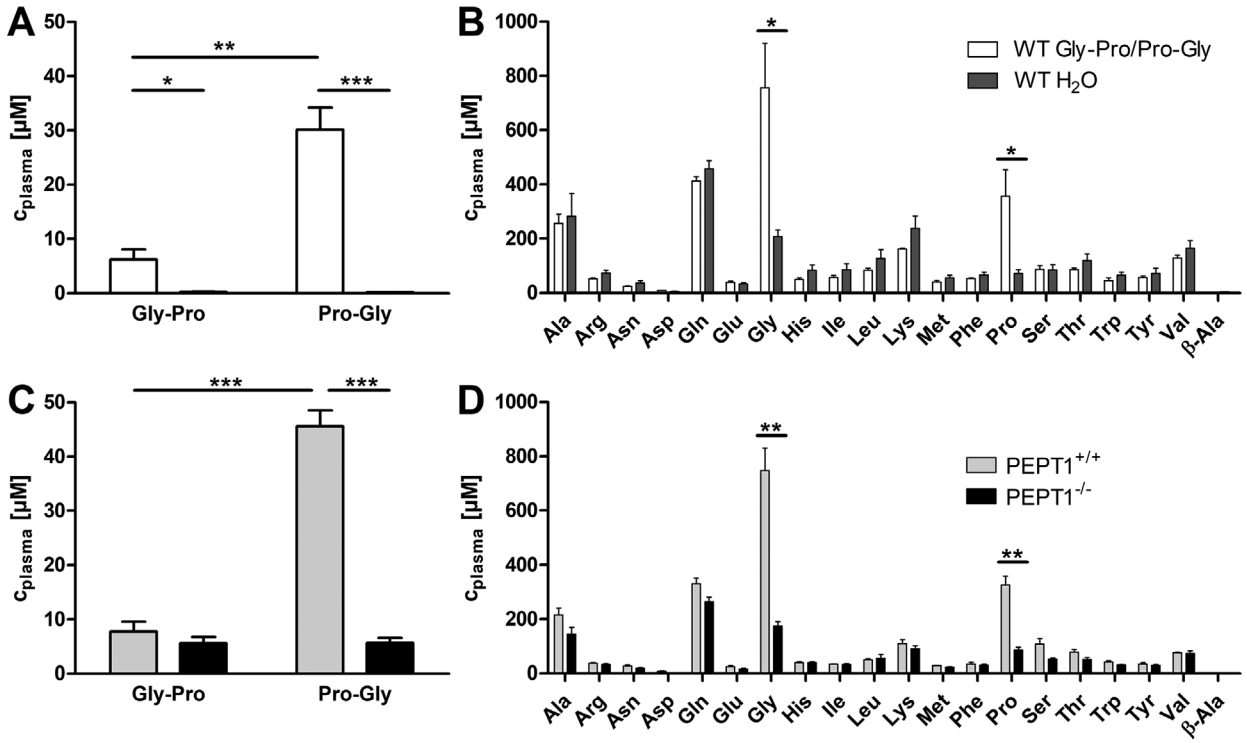

Figure 4. Gly-Pro, Pro-Gly, and amino acid plasma concentrations in wild-type (WT), PEPT1 knockout (PEPT1$^{-/-}$), and control mice (PEPT1$^{+/+}$) after gavage with peptide solution or water, provided as mean ± SEM ($n = 3$). A) Gly-Pro and Pro-Gly plasma concentrations 30 min after gavage with peptide solution or water in wild-type animals; B) amino acid concentrations in plasma 30 min after gavage with peptide solution or water in wild-type animals; C) Gly-Pro and Pro-Gly concentrations in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ plasma 30 min after gavage with peptide solution; D) amino acid concentrations in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ plasma 30 min after gavage with peptide solution. *p*-Values represent differences in plasma concentrations. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

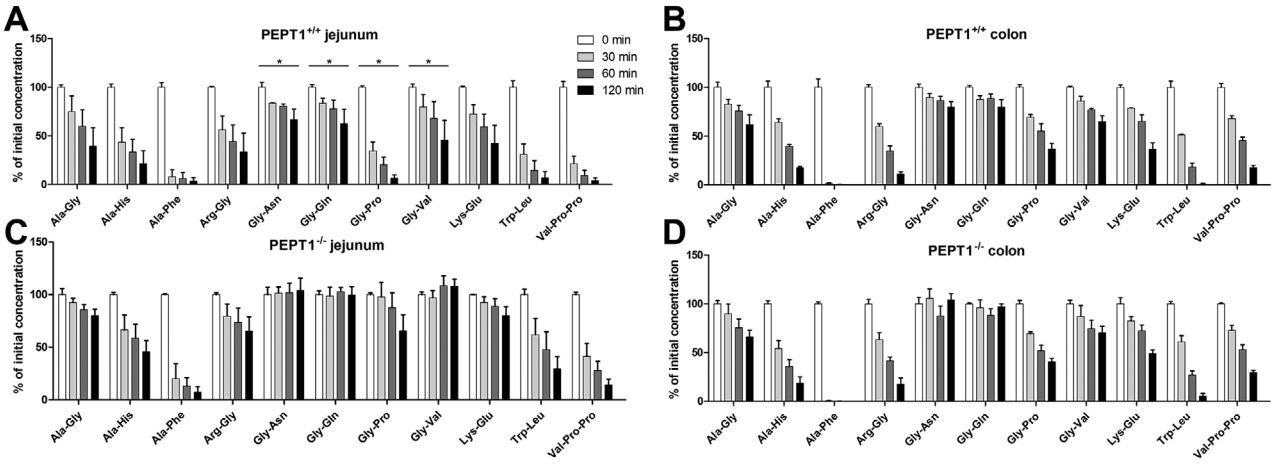

Figure 5. Changes in selected oligopeptide concentrations during incubation with sections of mouse intestines from PEPT1$^{+/+}$ and PEPT1$^{-/-}$ animals, provided as mean ± SEM ($n = 3$). Relative concentration in oligopeptide solution (compared to 250 µM per peptide at t_0) during incubation with A) PEPT1$^{+/+}$ mouse jejunum, B) PEPT1$^{+/+}$ mouse colon, C) PEPT1$^{-/-}$ mouse jejunum, and D) PEPT1$^{-/-}$ mouse colon. *p*-Values represent differences in cumulative peptide concentration between PEPT1$^{+/+}$ and PEPT1$^{-/-}$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

each sample, and used these cumulative concentrations for statistical analysis. We did not observe any differences in peptide concentrations and therefore peptidase activity between PEPT1$^{+/+}$ colon and PEPT1$^{-/-}$ colon (Table S3, Supporting Information). In jejunal samples however, peptidase activity was significantly reduced in PEPT1$^{-/-}$, indicated by a lower hydrolysis rate both of certain oligopeptides (Gly-Asn, Gly-Gln, Gly-Pro, Gly-Val, Phe-Gly, and Trp-Glu) as well as of the entire peptide panel. Analysis of amino acid concentrations in the incubation solution revealed identical amino acid patterns in all groups, with the strongest

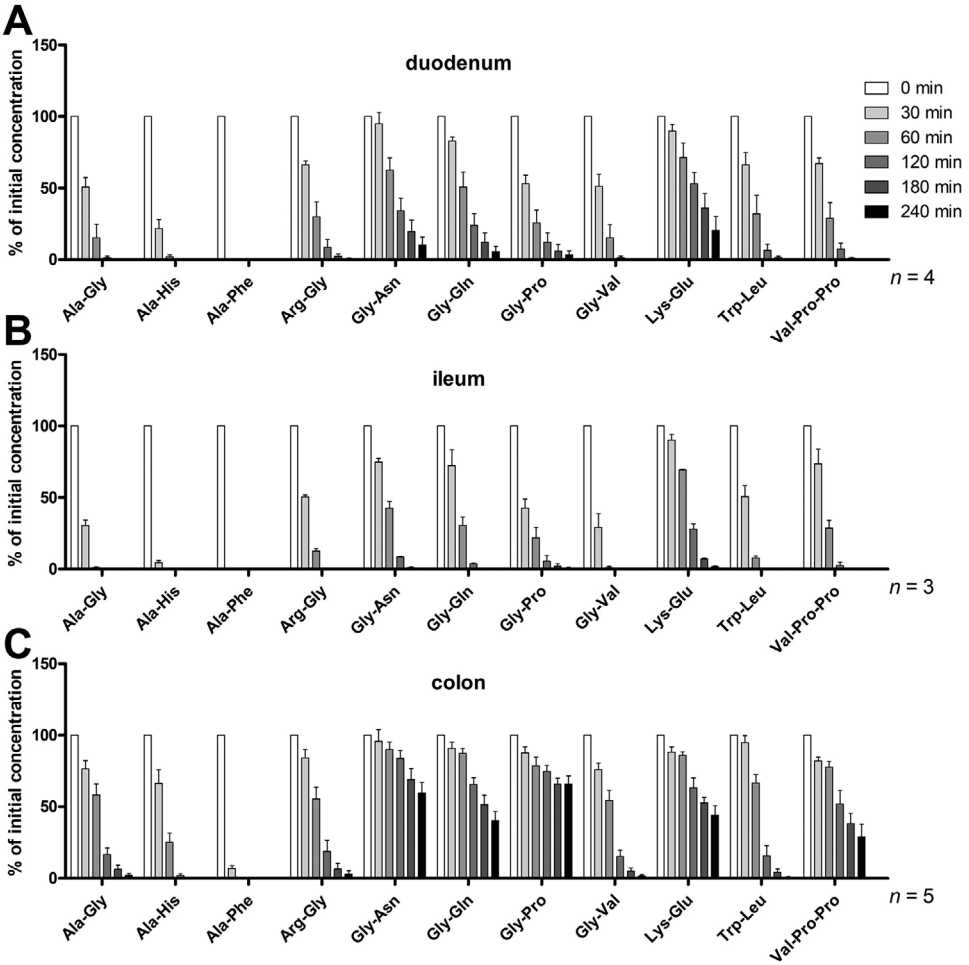

Figure 6. Selected changes in oligopeptide concentrations during incubation with human intestinal mucosa samples, provided as mean ± SEM (n = 3–5). Relative concentration in oligopeptide solution (compared to 250 μM per peptide at t_0) during incubation with human mucosa samples from A) duodenum, B) ileum, and C) colon.

increases for Ala, Gly, Phe, and Pro (Figure S3, Supporting Information), without any significant differences concerning cumulative concentrations.

3.4. Intestinal Oligopeptide Digestion by Human Tissue Ex Vivo

In order to confirm our findings in mouse tissue, human mucosa samples from duodenum, ileum, and colon were incubated in a solution of 21 di- and tripeptides (250 μM each). Changes in peptide concentration in the incubation solution were assessed after 30, 60, 120, 180, and 240 min (**Figure 6**). Similar effects were observed in the small intestinal segments: the concentration of certain peptides did not decrease at all or only slightly, like Ans, Car, Gly-Sar, and ɣ-Glu-Leu (Figure S4, Supporting Information), while others rapidly disappeared from the solution, like Ala-Gly, Ala-His, Ala-Phe, Arg-Gly, Gly-Val, Phe-Ala, Trp-Leu, and Val-Pro-Pro. Considering that both groups contained peptides with similar affinities for PEPT1 uptake (Gly-Sar K_m = 0.86 mM, Arg-Gly K_m = 1.06 mM),[18,19] and based on the apical peptide disappearance rates in PEPT1 knockout clones in Caco-2 transwell cell culture described above, we believe that the disappearance of peptides from the incubation solution is caused mainly by peptidase activity rather than uptake into intestinal tissue by PEPT1.

Overall, there appears to be a slight, yet not significant, increase in small intestinal peptidase activity from proximal to distal, as indicated by increased disappearance rates of Ala-Gly, Gly-Gln, Gly-Val, Phe-Ala, Phe-Gly, Pro-Gly, and Trp-Leu in ileal compared to duodenal samples. Colonic samples displayed a peptidase activity pattern similar to small intestinal samples. Peptide-specific peptidase activity for more than half the panel peptides (Ala-Gly, Ala-His, Ala-Phe, Gly-Asn, Gly-Gln, Gly-Gly-Ile, Gly-Pro, Gly-Val, Phe-Ala, Phe-Gly, Trp-Glu, Val-Pro-Pro) as well as overall peptidase activity were reduced in colonic mucosa compared to duodenal and ileal mucosa (Table S4, Supporting Information). Accordingly, total amino acid concentrations did not differ between duodenum and ileum, while they were significantly elevated both in duodenum and ileum compared to colon (Figure S5 and Table S5, Supporting Information).

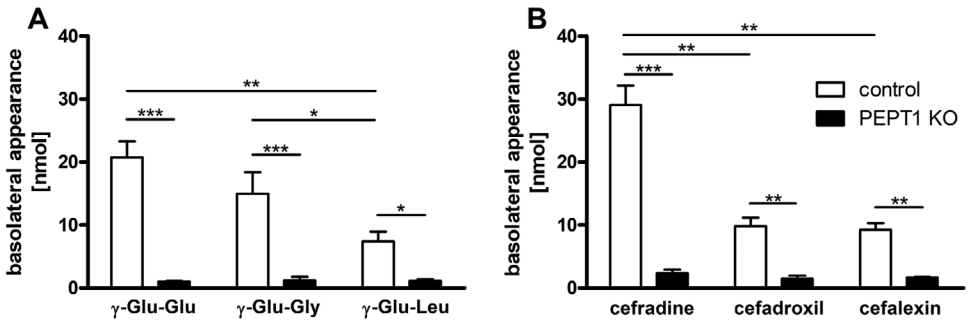

Figure 7. PEPT1-dependent transport of γ-glutamyl-dipeptides and aminocephalosporins in Caco-2 transwell cell culture, provided as mean ± SEM (n = 5). Basolateral appearance in transwell culture of control and PEPT1 knockout cells after 6 h of apical incubation with A) γ-glutamyl-peptide solutions and B) cephalosporin solutions. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.5. PEPT1-dependent Transport of γ-Glutamyl-Dipeptides by Caco-2 Cells

As we had observed transepithelial flux of γ-Glu-Leu in Caco-2 transwell cell culture that appeared to be PEPT1-dependent, additional transport studies with γ-glutamyl-dipeptides were performed. After a 6-h incubation of Caco-2 cells with 500 μM of γ-Glu-Glu, γ-Glu-Gly, or γ-Glu-Leu, PEPT1 knockout and control cells displayed significant differences regarding basolateral γ-glutamyl-dipeptide appearance rates (**Figure 7**A). In PEPT1 knockout cells, basolateral appearance ranged between 1 and 2 nmol. Control cells displayed the greatest transport activity for γ-Glu-Glu (20 nmol) and γ-Glu-Gly (15 nmol), while γ-Glu-Leu featured the lowest concentration at 7.5 nmol.

3.6. PEPT1-dependent Transport of Aminocephalosporins by Caco-2 Cells

After applying our novel LC–MS/MS method for oligopeptide quantification to different sample matrices from different experimental setups, quantifying physiological PEPT1 transport substrates in the form of regular di- and tripeptides as well as special dipeptides in the form of γ-glutamyl-dipeptides, we assessed the possibility of also quantifying non-physiological PEPT1 transport substrates. To this purpose, PEPT1 knockout and control Caco-2 cells were incubated with solutions containing 500 μM of a single cephalosporin in the apical compartment of transwell cell culture for 6 h. The assessment of basolateral appearance revealed significant differences between the transport of the tested cephalosporins, as well as between the transport activity of the two cell lines (Figure 7B). Basolateral appearance in PEPT1 knockout cells did not exceed 2.5 nmol after 6 h, while in control cells, cefradine increased to almost 30 nmol and cefadroxil and cefalexin to almost 10 nmol each.

4. Discussion

To the best of our knowledge, this is the first study that simultaneously assesses transport and hydrolysis of a panel of di- and tripeptides selected based on different physicochemical characteristics by quantification of substrates and products via LC–MS/MS in different intestinal models. The study reveals that susceptibility to hydrolysis of peptide substrates at the brush border membrane or within epithelial cells is strictly structure-dependent and can vary by orders of magnitude. Whereas peptides with the highest luminal disappearance rates almost all showed substantial if not complete hydrolysis, peptides with the lowest disappearance rates generally showed highest basolateral appearance in intact form in Caco-2 transwell culture. For stable peptides, for example, anserine, carnosine, Gly-Asn, Gly-Asp, Pro-Gly, or γ-Glu-Leu, uptake via the apical peptide transporter was obviously the key determinant for transepithelial flux since the lack of PEPT1 drastically reduced their basolateral appearance. In contrast, for peptides with marked disappearance rates from apical solution, such as Ala-His, Ala-Phe, Arg-Gly, or Val-Pro-Pro, only low basolateral appearance rates were observed. Intermediate substrates like Gly-Pro and Trp-Leu revealed a PEPT1-dependency for both luminal disappearance and basolateral appearance, but also a substantial release of their constituent amino acids into apical and basolateral compartments. Linear regression analysis revealed a striking relationship of apical disappearance and basolateral appearance rates, particularly in the presence of PEPT1. Interestingly, we detected two outliers in control cells, Gly-Gln and Gly-Asn, without which R^2 increases even to 0.95. These dipeptides are notably the only panel peptides containing the prime energy substrate for enterocytes, glutamine,[20] and its structurally related amino acid asparagine, which has been shown to be an important energy substrate for enterocytes as well.[21,22] Their reduced basolateral appearance (in relation to their apical disappearance) could be interpreted as an intracellular retention of the cells' preferred energy substrates, potentially caused by, e.g., increased peptidase activity against dipeptides containing these amino acids or a low affinity for the basolateral peptide transporter.

The observed rate of degradation of Gly-Pro in Caco-2 transwell culture seems to contradict its wide use as a radiolabeled tracer peptide in studies on peptide transport.[23,24] We here show that the dipeptide has a low intrinsic hydrolysis resistance. However, what makes Gly-Pro particularly interesting is the comparison of its features to its mirror-peptide Pro-Gly, which shows absolute resistance to hydrolysis and a strict PEPT1-dependence for its transepithelial flux. This remarkable difference in handling of Gly-Pro and Pro-Gly could also be observed in almost

identical manner in mice in vivo when the pair of dipeptides was provided by intragastric application. The appearance rate of Pro-Gly in peripheral blood was several-fold higher than that of Gly-Pro, for which in contrast a considerable increase in plasma levels of the constituent amino acids was observed. In animals lacking PEPT1, Pro-Gly plasma levels hardly exceeded those of animals receiving water, demonstrating the key role of the intestinal peptide transporter, and recommending Pro-Gly as a possible "lead" substrate for testing PEPT1 functionality in animals and humans. This seems contradictory to its rather low affinity for interaction with PEPT1 as derived from competition assays in vitro, with an affinity 100-fold lower than that of Gly-Pro, which was determined around 0.2 mM.[25] Even more surprising is that we recently observed the same striking differences in postprandial plasma appearance of Gly-Pro and Pro-Gly in humans after consumption of 100 or 200 g of chicken meat.[13] Although not proven experimentally, it is conceivable that the consumed meat comprised in a random fashion roughly the same amount of Gly-Pro and Pro-Gly sequences that could be liberated during digestion. Nevertheless, just like in the mouse study at hand, Pro-Gly levels exceeded Gly-Pro levels approximately fivefold while Gly-Pro levels remained at fasting state. Among other dipeptides that revealed a rise in postprandial levels in human plasma after meat consumption, we detected anserine, carnosine, Gly-Asn, Gly-Asp, Phe-Gly, Trp-Leu, but also γ-Glu-Leu.[13] These peptides, when tested here in the Caco-2 cell system, most surprisingly all revealed a high intrinsic stability against hydrolysis but also a strict PEPT1-dependence for basolateral appearance. Besides Gly-Pro and Pro-Gly, another pair of structural isomers in the panel, Ala-His and carnosine (β-Ala-His), emphasizes the peptides' structure dependence for PEPT1 transport and hydrolysis. Despite the N-terminal amino group in carnosine in β-position greatly diminishing affinity for PEPT1,[26] its low susceptibility for intestinal hydrolysis[27] elicited a postprandial plasma concentration exceeding Ala-His fourfold, an effect we also observed in Caco-2 transwell culture. Similar observations regarding other pairs of structural peptide isomers, e.g., for Trp-His and His-Trp, have been reported in literature.[28]

Which contribution the basolateral efflux has in overall transepithelial fluxes is still unknown. All attempts to identify the basolateral efflux system for peptides and peptidomimetics like aminocephalosporins that share apical PEPT1 for uptake have failed so far. With the means of a Caco-2 cell line lacking PEPT1 at our disposal, we were at least able to test basolateral-to-apical uptake and permeation across the monolayer in the absence of a reuptake from the apical facing into the cells (Supporting Information Materials and Methods and Figure S6). When test peptides were administered from the basolateral compartment, measurements of basolateral disappearance and apical appearance showed only very low disappearance rates for all peptides in both cell lines. While overall apical appearance of peptides and amino acids did not differ between cell lines, we observed significant apical appearance rates for Pro-Gly and γ-Glu-Leu in control cells. Most strikingly, the basolateral-to-apical transfer of intact Pro-Gly and γ-Glu-Leu revealed a significant reduction in apical appearance in cells lacking PEPT1. The main characteristics of the basolateral transport system as revealed by studies in membrane vesicles of Caco-2 cells resembled many of the features of PEPT1.[29–31] However, all attempts to identify the PEPT1-like basolateral efflux protein—either by classical protein isolation techniques or by genetic approaches—have so far failed. We have previously demonstrated that PEPT1 can transport substrates bidirectionally, and that directionality is determined by membrane potential but also asymmetric affinities for substrates.[19,32] It thus seems possible that peptides reach the Caco-2 cells' cytosol via the basolateral peptide transporter. From there, PEPT1 enables outward transport of peptides to the apical compartment in control cells, and subsequently reuptake of panel peptides with higher affinities for PEPT1, leading to increased apical concentrations of peptides with the lowest affinities, such as Pro-Gly and possibly γ-Glu-Leu. PEPT1 affinity of dipeptides with isopeptide bonds has never been determined, but considering their transport could not be demonstrated before, it seems plausible to assume that their affinity is rather low.[26]

With the methods employed here, we could identify peptides that are completely resistant to hydrolysis, which included γ-Glu-Leu. To ascertain that the high transepithelial flux observed for γ-Glu-Leu in transwell culture was not an artifact, we used two additional γ-glutamyl-dipeptides as a reference. And, indeed, all three peptides showed high transepithelial flux rates as well as a strict PEPT1-dependence. An interesting finding is that we observed distinctly different basolateral appearance rates for the γ-glutamyl-peptides despite similar physicochemical characteristics. To extend this observation to another class of compounds known to be PEPT1 substrates that are resistant to hydrolysis, we used aminocephalosporins and quantified their basolateral appearance in Caco-2 transwell culture. From the three compounds of very similar chemical structure, cefradine showed the highest appearance with almost 30 nmol in basolateral compartments, while the appearance rates of cefadroxil and cefalexin amounted to only around one-third of that. It thus is a consistent finding that different appearance rates in the basolateral compartment exist in the Caco-2 model for peptide substrates and peptidomimetics that have a strict PEPT1-dependence (by comparison of wild-type and PEPT1-deficient cells). This, however, was unexpected, taking into account that most of these substrates do not reveal major differences in chemical structure and physical characteristics. The three aminocephalosporins have been reported to feature very similar affinities for interaction with peptide transporters,[33–35] yet they show almost threefold differences in basolateral appearance. Curiously, other sources conveyed dissimilar affinities of aminocephalosporins for PEPT1 in Caco-2 cells, with K_m values of 1.5 mM for cefradine and 6 resp. 7 mM for cefadroxil and cefalexin,[36] which would explain the different rates of appearance we observed in Caco-2 cells. Very similarly, the three γ-glutamyl-dipeptides as well show threefold differences in basolateral appearance despite only minor differences in composition in one terminal residue, which may be the consequence of different affinities for apical uptake via PEPT1 or different affinities for efflux via the so far unidentified basolateral peptide transporter.

Taken together, we show impressive differences in hydrolysis resistance of selected di- and tripeptides, affinity for uptake, and translocation across the epithelium in intact form. The susceptibility to cleavage by a wide array of peptidases found at the brush border membrane[28,37] or in the cytosol[36,38] was predictable to some extent, yet other observations turned out contrary to expectations. It is sensible to verify whether certain structural or

chemical features associate with susceptibility to cleavage. Dipeptides with more hydrophobic residues such as Ala, Trp, Phe, Leu, or Val mostly showed rapid cleavage, whereas dipeptides containing Gly residues featured above average hydrolysis resistance. Surprising is the almost complete resistance of Gly-Asp and Gly-Asn as compared to Gly-Pro. Although the latter would have been predicted to have a higher resistance due to peptide bonds formed with Pro having a different length and existing in *cis* and *trans* configuration, which renders the products more hydrolysis resistant,[39] Gly-Pro showed a rather high degradation rate consistent across all models (Caco-2, mouse in vivo and in vitro, human in vivo and in vitro). In Pro-Gly, a dipeptide with proline and its imino group in N-terminal position, but also in anserine and carnosine, which feature N-terminal ß-alanine with its amino group in ß-position, hardly any hydrolysis and the highest transepithelial flux rates were observed. This is surprising since analysis of the structural determinants in PEPT1-substrates revealed that exactly features like the above markedly reduced affinity for binding to PEPT1 by up to one order of magnitude.[25,26] Consequently, studies determining affinity in heterologous expression systems in the absence of surface peptidases, like in *Xenopus laevis* oocytes, may reveal features for substrates that are not necessarily applicable for in vivo conditions in epithelial cells or intact tissue, as discussed previously.[28,40] Therefore, they do not allow any predictions about transepithelial flux rates: high PEPT1 affinity is not necessarily associated with high transepithelial flux rates, as shown for Val-Pro-Pro (K_i = 0.1 mM),[41] while low PEPT1 affinity is not necessarily associated with low transepithelial flux rates, as shown for Pro-Gly (K_i = 22 mM).[25]

Whether Caco-2 cells are indeed representative for the small intestine per se is of course a critical question. For this reason, we additionally used intestinal segments isolated from wild-type mice and mice lacking PEPT1, and determined hydrolysis rates of the peptide panel with samples derived from jejunum and colon. We also used human mucosa samples from duodenum, ileum, and colon from surgical resections to assess hydrolysis in a similar fashion. With the data obtained on time-dependent disappearance of intact peptides and the release of constituent amino acids, we can conclude that there are no major differences in hydrolysis patterns found in the different models including human tissue samples. Although some interesting differences in the velocity of hydrolysis became apparent in mouse and human samples, the overall classification of the test peptides regarding their susceptibility to hydrolysis was fairly consistent across all models. A remarkable finding is a rather high hydrolysis capacity in mouse and human colonic samples and the lack of major longitudinal differences along the human small intestine. We are not aware of any similar studies demonstrating this intestinal hydrolysis capacity for such a wide range of different peptide substrates. Another interesting finding from the mouse studies is that in animals lacking PEPT1, hydrolysis rates appeared to be reduced compared to wild-type samples. This could mean that expression of certain peptidases—either at the brush border membrane or in the cytosol—is reduced in the absence of PEPT1. These findings warrant further studies.

In summary, with a new analytical approach that allows the simultaneous quantification of transport and hydrolysis of substrates, we reassessed intestinal assimilation of di- and tripeptides in different intestinal models. We show that hydrolysis and transport of intact peptides is highly variable and structure-dependent. For di- and tripeptides possessing less polar N-terminal residues, hydrolysis usually dominates over transport, with either no or only a minimal contribution of PEPT1 and thus uptake in intact form. For other peptides with high intrinsic resistance such as Pro-Gly and some Gly-X substrates, but also free dipeptides such as anserine and carnosine (found in meat), PEPT1 is the main determinant for appearance in peripheral blood. This also holds true for ɣ-glutamyl-dipeptides, generated from the hydrolysis of glutathione—either provided by diet or derived from secretions such as bile—and released by ɣ-glutamyl-transpeptidase. Moreover, for transepithelial delivery of peptidomimetics such as aminocephalosporins, PEPT1 is the limiting factor. However, PEPT1-substrates revealed different translocation rates despite almost identical chemical structure and similar affinity for PEPT1.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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F.R., H.D., and B.S. designed the study; F.R. undertook data collection and performed LC–MS/MS analysis of all samples; F.R. and B.S. interpreted results of experiments; H.D. provided essential reagents and materials; F.R., H.D., and B.S. wrote the manuscript; and all authors critically read the manuscript and approved the final version. The authors thank Katrin Petzold and Margot Siebler for technical assistance with cell culture, Beate Rauscher for human sample preparation, Ronny Scheundel for mouse sample preparation, and Barbara Gelhaus for LC–MS/MS analysis assistance. We thank our colleagues, especially Pieter Giesbertz and Kurt Gedrich, for fruitful discussions and statistical support. F.R. was financially supported by the DFG Graduate School GRK 1482 "Interface functions of the intestine between luminal factors and host signals."

Conflict of Interest

The authors declare no conflict of interest.

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