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Intestinal amino acid absorption and control of transepithelial transport – Filling the gaps

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Outline

Abbreviations

ACE	angiotensin-converting enzyme
APA	aminopeptidase A
APN	aminopeptidase N
ASCT2	alanine, serine, cysteine transporter 2
BBMV	brush border membrane vesicles
BCAA	branched-chain amino acids
BMI	body mass index
Cas9	CRISPR associated protein 9
CELA	chymotrypsin-like elastases
CRISPR	clustered regularly interspaced short palindromic repeats
DPPIV	dipeptidyl peptidase IV
FOODBALL	food biomarkers alliance
GABA	gamma-aminobutyric acid
GFR	glomerular filtration rate
GGT	gamma-glutamyl transferase
LPI	lysinuric protein intolerance
MEP	meprin A
NEP	neutral endopeptidase
PEM	protein-energy malnutrition
PEPT1	peptide transporter 1
PITC	phenyl isothiocyanate
PSMA	prostate-specific membrane antigen
SLC	solute carrier
SPF	specific-pathogen-free
TEER	transepithelial electrical resistance
WHO	world health organization

Abstract

Essential physiological functions including metabolism, protein synthesis, and cell growth depend on the constant dietary supply of nitrogen in the form of protein. Dietary protein has to be hydrolyzed into free amino acids and di- and tripeptides before being absorbed in the intestine. Contrary to a multitude of different transport proteins for amino acids, intestinal mucosa only expresses a single transporter for the uptake of these oligopeptides: peptide transporter 1 (PEPT1, SLC15A1). Besides oligopeptide transport, PEPT1 facilitates the uptake of structurally similar drugs, and thus determines the oral bioavailability of peptidomimetics like β-lactam antibiotics, antivirals, and angiotensin-converting-enzyme (ACE) inhibitors. Numerous studies of intestinal peptide transport in the past 50 years, and particularly since PEPT1's discovery in 1994, have elicited many facets of peptide absorption, including PEPT1's structure, function, and control of expression. However, its contribution to intestinal amino acid availability from dietary protein remains unclear.

The aim of this dissertation was to establish an LC–MS/MS-based method for the quantification of di- and tripeptides and to study the oligopeptides' fate in different intestinal models, helping define the physiological role of PEPT1.

In a human study, we employed our method for peptide quantification to a panel of peptides representative for the 400 possible dipeptides from the 20 proteinogenic amino acids in order to determine postprandial peptide concentrations in circulation after the consumption of different quantities of chicken breast. When analyzing the kinetics of postprandial oligopeptide plasma levels, dose- and time-dependent increases were observed for a wide range of dipeptides of different charge, size, and polarity. These peptides' appearance did however not correlate with their affinity to PEPT1 as determined in in vitro studies, so their intrinsic resistance to hydrolysis can be assumed to be of great importance in this context.

In Caco-2 transwell cell culture, in in vitro and in vivo mouse studies with PEPT1 deficient animals, as well as in in vitro hydrolysis studies with human intestinal samples, we simultaneously assessed the panel peptides' rates of hydrolysis as well as their transport via

PEPT1. We observed strict structure dependent susceptibilities to hydrolysis at the brush border membrane and within enterocytes that varied greatly amongst the panel peptides, yet that were very similar across all tested models: a lower polarity of an oligopeptide's N-terminal residue is usually associated with hydrolysis predominating transport via PEPT1, while peptides that are distinctly stable against hydrolysis depend on PEPT1 for absorption.

In summary, the present studies were the first in using an LC-MS/MS method for the systematic quantification of dipeptides in biological samples that described the dose-dependent postprandial appearance of peptides in plasma after the consumption of a common source of dietary protein in humans. In addition, this newly developed quantification method enabled us to simultaneously study transport and hydrolysis of the panel peptides in different intestinal models. We discovered almost identical effects in all our models regarding individual peptides as well as the peptide panel, showing that Caco-2 cells and mouse intestines are valid models for human intestinal peptide transport and hydrolysis.

Zusammenfassung

Wesentliche physiologische Funktionen wie Stoffwechsel, Proteinsynthese und Zellwachstum sind auf eine kontinuierliche Zufuhr von Stickstoff in der Nahrung in Form von Protein angewiesen. Mit der Nahrung aufgenommene Proteine müssen vor ihrer Absorption im Darm in freie Aminosäuren und Di- und Tripeptide hydrolysiert werden. Im Gegensatz zur Vielzahl unterschiedlicher Transportproteine für Aminosäuren exprimiert die Darmmukosa nur einen einzigen Transporter für die Aufnahme dieser Oligopeptide, nämlich Peptidtransporter 1 (PEPT1, SLC15A1). Neben dem Transport von Di- und Tripeptiden ist PEPT1 ebenfalls für die Aufnahme strukturell ähnlicher Medikamente verantwortlich und bestimmt so die orale Bioverfügbarkeit von Peptidomimetika wie β-Lactam-Antibiotika, Virostatika und Angiotensin-Converting-Enzym (ACE)-Hemmern. Zahlreiche Studien zum intestinalen Peptidtransport der letzten 50 Jahre, insbesondere seit der Entdeckung von PEPT1 im Jahr 1994, konnten viele Aspekte der Peptidabsorption aufklären, einschließlich der Struktur, Funktion und Expressionskontrolle von PEPT1. Der Beitrag von PEPT1 hinsichtlich der intestinalen Aufnahme von Aminosäuren aus Nahrungsprotein ist jedoch weiterhin ungeklärt.

Das Ziel der vorliegenden Arbeit bestand darin, eine LC-MS/MS-basierte Methode zur Quantifizierung von Di- und Tripeptiden zu etablieren und damit das Schicksal von Oligopeptiden in verschiedenen Darmmodellen zu untersuchen, um so zur Aufklärung der physiologischen Rolle von PEPT1 beizutragen.

In einer Humanstudie setzten wir diese Methode zur Quantifizierung eines Peptidpanels ein, das für die 400 möglichen Dipeptide aus den 20 proteinogenen Aminosäuren repräsentativ war, um postprandiale Peptidplasmakonzentrationen nach dem Verzehr unterschiedlicher Mengen von Hähnchenbrust zu bestimmen. Bei der Analyse der Kinetik dieser postprandialen Oligopeptid-Plasmaspiegel konnten wir dosis- und zeitabhängige Anstiege für eine Vielzahl von Dipeptiden unterschiedlicher Ladung, Größe und Polarität beobachten. Das Erscheinen dieser Peptide im Plasma korrelierte jedoch nicht mit ihrer in in vitro-Studien

ermittelten Affinität zu PEPT1, so dass ihre intrinsische Hydrolyseresistenz in diesem Zusammenhang mitentscheidend sein dürfte.

In Caco-2-Transwell-Zellkulturen, in in vitro- und in vivo-Mausstudien mit PEPT1-defizienten Tieren sowie in in vitro-Hydrolysestudien mit menschlichem Darmgewebe untersuchten wir darüber hinaus gleichzeitig die Hydrolyseraten der Panel-Peptide sowie ihren Transport durch PEPT1. Wir beobachteten streng strukturabhängige Hydrolyseraten an der Bürstensaummembran und innerhalb von Enterozyten, die stark zwischen einzelnen Panel-Peptiden variierten, die sich jedoch insgesamt in allen getesteten Modellen sehr stark ähnelten: eine geringe Polarität des N-Terminus eines Oligopeptids ist in der Regel mit einer den PEPT1-Transport überwiegenden Hydrolyse verbunden, während Peptide, die ausgesprochen hydrolysestabil sind, auf die Absorption durch PEPT1 angewiesen sind.

Zusammenfassend waren die vorliegenden Studien die ersten, in denen mittels einer LC-MS/MS-Methode für die systematische Quantifizierung von Dipeptiden in biologischen Proben das dosisabhängige postprandiale Erscheinen von Peptiden im Plasma nach dem Verzehr einer gängigen Nahrungsproteinquelle im Menschen beschrieben wurde. Darüber hinaus ermöglichte uns diese neu etablierte Quantifizierungsmethode, simultan Transport und Hydrolyse eines Peptidpanels in verschiedenen Darmmodellen zu analysieren. In allen untersuchten Modellen konnten wir nahezu identische Effekte in Bezug auf einzelne Peptide sowie für das gesamte Peptidpanel beschreiben. Dies legt nahe, dass Caco-2-Zellen und Mausdarm valide Modelle für die Transportaktivität und die Hydrolyse von Peptiden im menschlichen Darm darstellen.

1 Introduction

1.1 Protein in human nutrition

Protein serves as the main dietary source of nitrogen and essential amino acids, which human metabolism cannot synthesize. In large parts of the developing world, adequate protein intake, or a lack thereof, still is a significant problem, while our average protein intake in Germany is well above daily requirements. Nevertheless, dietary protein plays an important role in everyday life in Western societies: Specific diets like Paleolithic, vegetarian, or vegan diets that feature increased or decreased amounts of dietary protein, respectively, become more and more popular, while protein malnutrition is not uncommon amongst elderly individuals. The role of dietary protein in human nutrition in the past and present, the consequences of protein deficiency and oversupply, its digestion and the absorption of its breakdown products, and diseases associated with impaired protein digestion and absorption will be detailed below.

1.1.1 Protein in human evolution

3 to 2.5 million years ago, our ancestors lived in a tropical forest environment, relying on a plant-based diet consisting of fruits, plants, and seeds [1]. Brain size of these early hominids, corrected for body size, was only slightly bigger than in living primates [2]. The current human brain is distinctly larger than that of other primates however, consuming 20% of our body’s total energy while representing only 2% of a person's total body weight [3]. Hominins had to expend a lot of energy on the digestion of raw plant-derived foods. Due to the higher effort to derive limited calories from these foods, their gut was longer and slower than it is in current humans. With a high amount of energy spent on digestion, our ancestors’ brains remained relatively small.

A worldwide climate shift approx. 2.8 million years ago led to a rise in temperature and a decrease in humidity [4]. This climate change, reducing the availability of their usual plant-

based foods in a now more arid and seasonal environment, is believed to have required our ancestors to include higher amounts of protein-richer animal-based foods into their diet as additional sources of energy. The start of the consumption of flesh between 2.6 and 2 million years ago is believed to be the trigger of a first period of encephalization, which correlates with the appearance of the genus *Homo* [2]. Prey was killed or scavenged from other predators and then processed with simple Lower Paleolithic stone tools available to our ancestors. Slicing, pounding, and flaking reduced the caloric expenditure required for chewing and digesting foods while at the same time providing a nutrition much richer in energy [5]. A second phase of encephalization marks the appearance of *H. sapiens* approx. 500.000 years ago, when human brain size evolved to its current level [2]. In this period, humans began cooking meat, which enabled an even faster and more efficient digestion, increasing the food's energy yield even further.

The changed diet including processed animal protein, being a source of energy with a higher quality and energy density than forage foods, reduced our ancestors' need for a long digestive tract designed for processing mainly plant matter. As a consequence, encephalization was associated with a compensatory reduction in mass of the human gut. Being a highly metabolically active organ as well, this freed up further energy available for brain development [2]. Regardless of which evolutionary factors were selecting for an increase in human brain size, encephalization could not be achieved to this extent without the adoption of a high-quality, protein-rich diet, which was achieved by an increased consumption of animal food [2].

1.1.2 Contemporary protein consumption

An approximation of the macronutrient composition of the hominin diet is difficult, but isotope analysis of Paleolithic skeletons suggests that the diet at that time was high in animal protein [6, 7]. Similarly, analysis of stomach contents of a Chalcolithic mummy revealed high

proportions of animal fat and protein in its diet [8]. Forms of nutrition similar to these early hominin diets can still be found in contemporary pre-agricultural societies: Data on nutrition collected from more than 200 hunter-gatherer populations worldwide show that their diet to date consists, to a large extent, of animal protein in the form of game and fish and uncultivated plant foods like roots, tubers, vegetables, berries, fruits, and nuts [9]. Analyses reveal an average macronutrient distribution of elevated protein (19–35% of energy) and fat (28-58% of energy) and reduced carbohydrate (22-40% of energy) [10, 11] when compared to the current dietary guidelines recommending 30% of energy fat and >50% of energy carbohydrates [12]. Over the course of 2 million years, human metabolism could genetically adapt to Paleolithic diet. After remaining fairly stable for this long period, starting with the agricultural revolution 10,000 years ago and particularly since the industrial revolution in the mid-18th century, nutrition changed drastically from the previous Paleolithic diet to the now common Western diet in many countries, a timespan that is acknowledged to not having been sufficient for our metabolism to adapt to the challenges of Western diet as well as the changes in lifestyle. For this reason, we are still genetically adapted to our ancestors' diet and lifestyle as hunters and gatherers [13, 14]. In contrast to Paleolithic diet, Western diet, characterized by overeating, frequent snacking, and the consumption of sucrose-containing soft drinks, features a large fraction of high-glycemic and high-fat foods like cereal grains, refined sugars and vegetable oils, domestic meats, milk and dairy products, cultivated legumes and plant food, and alcoholic beverages [15]. Together with a sedentary lifestyle and overall low physical activity, a Western style diet is associated with many chronic degenerative diseases in Western countries [16]. Particularly obesity, whose prevalence has doubled in more than 70 countries since 1980 [17] and, according to the World Health Organization (WHO), nearly tripled worldwide since 1975, results in 1.9 billion overweight adults, of which more than 650 million are obese.

As for the macronutrient composition of Western diet, for instance in Germany and in the US, based on data collected in the second National Nutrition Survey and the National Health and Nutrition Examination Survey from 2009–2014, the median carbohydrate intake is below recommendations, with a majority of the population not reaching the recommendations of >50% of energy from carbohydrates. Median fat uptake on the other hand exceeds recommendations, while the median protein uptake exceeds recommendations as well. At the same time, total energy intake of a large part of the population exceeds recommendations as well [12, 18, 19].

1.1.3 Recommended dietary allowance of protein

Protein, regardless if plant-based or animal-based, is vital to human nutrition as an important source of nitrogen and essential amino acids. The recommended dietary allowance of protein for healthy adults from sources like the WHO and DACH, based on nitrogen balance studies revealing an average requirement of 0.6 g/kg of body weight per day, is 0.8 g/kg of body weight per day, which corresponds to 60 grams per day for an average sedentary man with 75 kg body weight and roughly 50 grams per day for an average sedentary woman of 60 kg. Of course, individual requirements vary depending on many factors, including age, sex, activity level, and overall health. For instance, athletes may require protein of 1.4 to 2 g/kg of body weight per day [20], and during pregnancy, due to the deposition of body tissues, protein requirements are increased by an additional 7 g per day during the second and 21 g per day during the third trimester, resulting in a recommended dietary allowance of 0.9 and 1.0 g/kg/d, respectively [12]. During lactation, the DACH recommends women increase their daily protein intake by 23 g, i.e., increase their daily protein intake to 1.2 g/kg. The estimates for infants decrease from 2.5 g/kg/d after birth to 1.4 g/kg/d until the age of 4 months. Afterwards, the amount decreases further to 1.3 g/kg/d until the age of 12 months, to 1.0 g/kg/d until the age of 4 years, and slowly reach the 0.8 g/kg/d in adulthood as stated above.

Eventually, in adults aged 65 years and older, recommendations for protein intake are increased again to 1.0 g/kg/d to prevent sarcopenia and osteopenia [21, 22].

Protein deficiency

Diet continuously failing to meet protein requirements will eventually lead to malnutrition. Initially, the body will react by covering its nitrogen requirements by breakdown of skeletal muscle protein, which may not elicit any clinical symptoms at early stages [23]. In the longer term, the lack of dietary protein cannot be compensated by the breakdown of endogenous protein, resulting in a loss of muscle function, metabolic disorders, and impaired organ function [24]. Also, protein malnutrition is often accompanied by an impairment of the immune system, causing malnutrition to be associated with infectious comorbidities like malaria or diarrhea [25]. While primary forms of protein malnutrition are based on a reduced dietary protein intake, secondary forms may occur as a complication from cancer, chronic renal disease, inflammatory bowel disease, cystic fibrosis, etc. [26]. The blanket term protein-energy malnutrition (PEM) comprises different stages of malnutrition, including the severe forms kwashiorkor, marasmus, and mixed forms thereof. Kwashiorkor refers to a relative protein deficiency in the diet, i.e., a qualitatively inadequate protein supply, while marasmus refers to a general deficiency of energy. Kwashiorkor is characterized by oedema, believed to be caused mainly by hypoalbuminemia, and fatty liver [27], while the main symptom of marasmus is severe wasting in the absence of oedema [28]. Protein malnutrition nowadays is mostly found in children in developing countries, with more than 20% of all children worldwide being affected in 2001, and malnutrition being associated with more than half of child deaths in developing countries, amounting to the deaths of 6 million children each year [29, 30]. Adult malnutrition can also be frequently found in the developing world: For instance, up to 50% of all women of child-bearing age in Africa and south Asia are underweight [31]. In industrialized countries, PEM is mainly found in home-dwelling and hospitalized elderly. While total energy requirements of elderly are reduced associated with a

reduced basal metabolic rate, their protein requirements are equal to those of younger adults. Due to age-related physiological changes (e.g., reduced appetite, sense of taste, and sense of smell), psychological changes (e.g., cognitive impairment), and social changes (e.g., isolation), elderly often fail to meet their dietary protein needs [32]. In this population group, malnutrition is associated with increased morbidity, mortality, and an increased risk of complications in case of hospitalization [33], making PEM in elderly an important health concern, especially in the light of ageing Western populations, with PEM prevalence rates ranging between 2.5% and 16.5% in European community-dwelling adults [34].

Protein oversupply

Contrary to protein deficiency, an oversupply of dietary protein in the form of high-protein diets, i.e., diets with a protein content of more than 20% of energy, does not appear to have any serious adverse effects in humans. Due to a lack of data, there are no tolerable upper intake levels for protein available in humans. According to the National Academy of Medicine, dietary protein of up to 35% of energy of the diet may be acceptable, while this upper limit cannot be supported by data regarding its long-term safety [35]. High-protein intake is known to increase glomerular filtration rate (GFR) leading to renal hyperfiltration [36], yet even long-term high-protein diets have not been demonstrated to impair kidney function in healthy individuals [37, 38]. However, in individuals with impaired renal function, e.g., due to chronic kidney disease, increased amounts of dietary protein may accelerate the deterioration of renal function [39]. While an association of the consumption of high amounts of protein in general with health risks in healthy individuals has not been shown, specific sources of protein, like red and processed meat, may have adverse effects, e.g., by increasing the risk for cancer compared to other protein sources like plant protein [40]. In pregnant women, many studies have shown that high-protein diets do have adverse effects, leading to increased risk for increased blood pressure and overweight in offspring [41-43]. Increased protein levels in infant diet are also associated with an increased risk for

overweight in later life [44]. On the contrary, while not displaying any adverse effects in healthy individuals, literature describes even positive effects of increased protein consumption, with numerous studies demonstrating positive effects in various metabolic disorders like obesity, dyslipidemia, and hypertension [10, 15, 45-48].

1.2 Gastrointestinal protein digestion

Dietary protein is digested by a wide variety of proteases and peptidases upon oral intake, before its degradation end products - amino acids, di-, and tripeptides - can be absorbed and enter circulation (Figure 1). This range of hydrolytic enzymes includes gastric endopeptidases as well as pancreatic endopeptidases and exopeptidases. As this cascade of peptidases is not sufficient for completely breaking down dietary protein into free amino acids and absorbable di- and tripeptides, luminal protein digestion is completed by small intestinal brush border membrane peptidases. Once dietary protein is digested to the level of free amino acids and di- and tripeptides, it can be absorbed via intestinal amino acid transporters and PEPT1 (SLC15A1) located in the apical membrane of enterocytes. Part of the absorbed oligopeptides will be exported basolaterally into circulation via a so far unidentified basolateral peptide transporter [49]. The other part will be the target of cytosolic peptidases with different mechanisms and amino acid specificities.

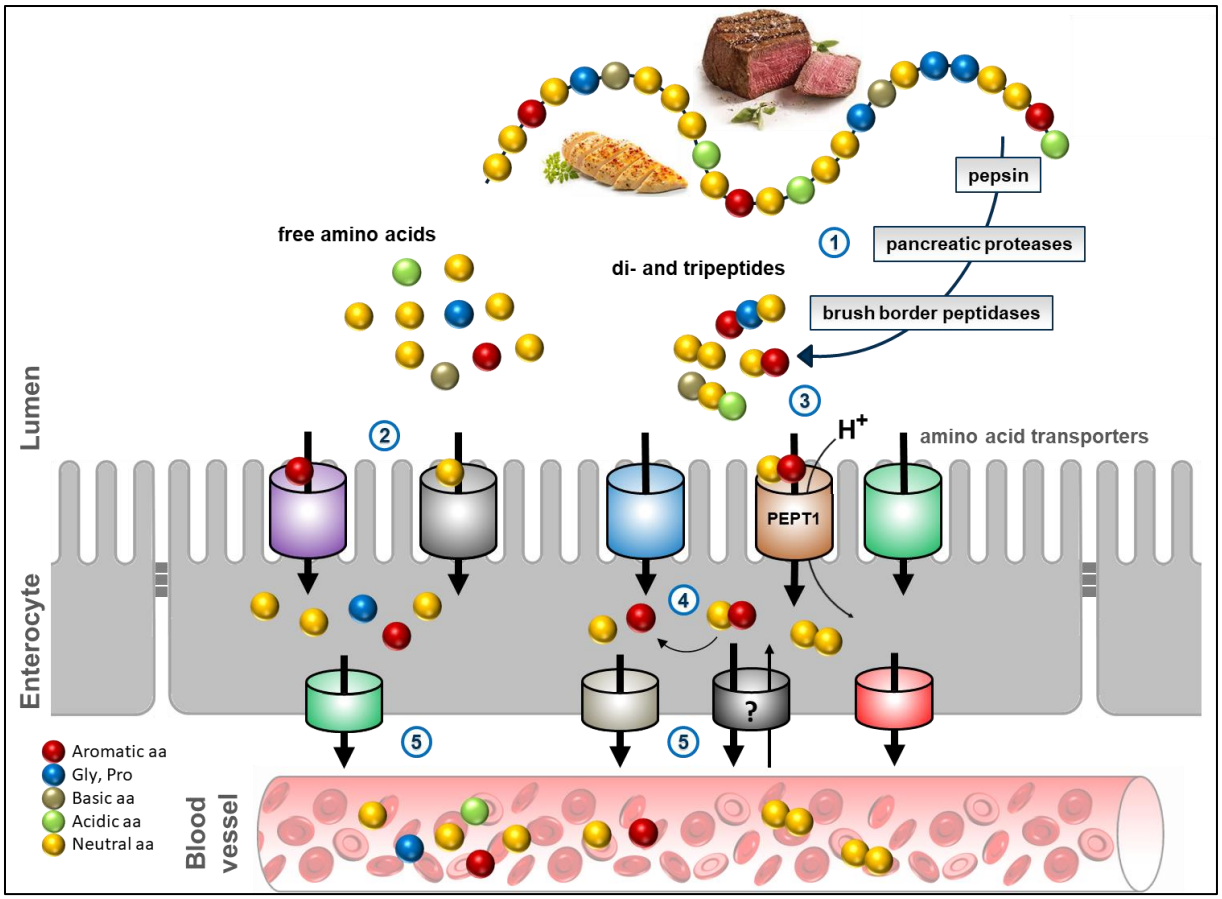

Figure 1: Intestinal fate of dietary protein.

Dietary protein is digested by pepsin, pancreatic proteases, and small intestinal brush border peptidases into free amino acids (aa) and di- and tripeptides (1). Free amino acids are transported across the apical plasma membrane of enterocytes by amino acid transporters (2). Di- and tripeptides cross the enterocytes' apical membrane via Peptide Transporter 1 (PEPT1) (3). In the enterocytes' cytosol, the absorbed di- and tripeptides are partially hydrolysed into free amino acids by cytosolic peptidases (4). The free amino acids absorbed from the intestinal lumen and generated during the intracellular hydrolysis of di- and tripeptides, as well as the di- and tripeptides that escaped intracellular hydrolysis, are exported across the basolateral plasma membrane into circulation by amino acid transporters and a so far unidentified peptide transporter (5).

1.2.1 Gastric protein digestion

Dietary protein digestion is initiated in the stomach. Upon stimulation by gastrin and acetylcholine, gastric chief cells release pepsinogens. These zymogens are inactive precursors of gastric peptidases. By secreting the peptidases in their inactive form, chief cells prevent proteolytic damages to the gastric mucosa. Parietal cells, another type of cells found

in gastric mucosa, release hydrochloric acid into the gastric lumen via a hydrogen potassium ATPase (H^+/K^+ ATPase). In combination with gastric peptidases and lipase, a high gastric acidity inactivates ingested microorganisms, including bacteria and viruses, as well as prions [50]. In addition, the acidic environment denatures dietary protein, thus making it more easily digestible, and activates the zymogens produced by the chief cells [51]. When both pepsinogen and hydrochloric acid are present in the gastric juice, pepsin is activated at a low pH of 1.5 to 2. Notably, the term “pepsin” does not refer to a single enzyme but to a group of proteolytic enzymes. These aspartate proteases are endopeptidases with a pH optimum of 1-4, which assume their inactive form at pH above 4. The major pepsins secreted in the human stomach are pepsin A (EC 3.4.23.1, 70%) and pepsin C (EC 3.4.23.3, 20%), also referred to as gastricin [52]. Pepsins preferentially hydrolyse peptide bonds with aromatic amino acids, e.g., tyrosine and phenylalanine, at positions P_1, defined as the residue N-terminal to the peptide bond [53], and P_1’, the C-terminal residue of the peptide bond (Figure 2), but also peptide bonds including acidic amino acids and leucine [54].

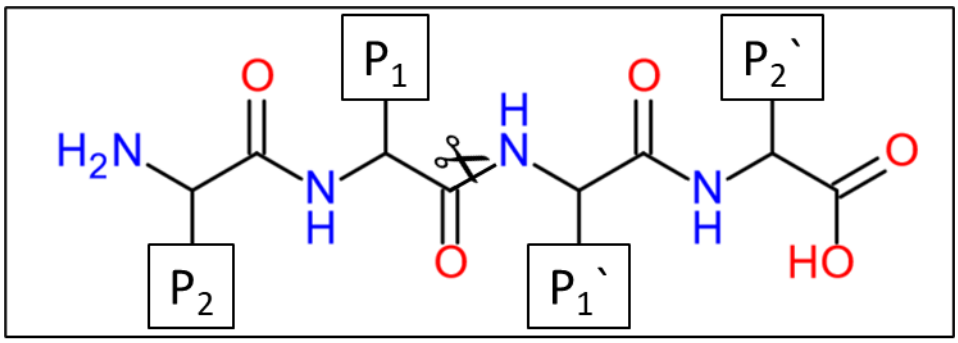

Figure 2: The Schechter and Berger notation for protease cleavage sites.
When the central peptide bond in this tetrapeptide is targeted by a peptidase, the N-terminal amino acid residue forming the peptide bond is considered in P_1 position, while the C-terminal amino acid residue with which it forms the peptide bond is considered in P_1’ position. Accordingly, the amino acid residues that are located one position farther towards the peptides N-terminus and C-terminus are referred to as in P_2 and P_2’ position, respectively.

1.2.2 Small intestinal protein digestion

Once dietary protein in the chyme leaves the stomach through the pylorus to the duodenum, small intestinal protein digestion completes the breakdown of dietary protein required for the absorption of di- and tripeptides and free amino acids. The major contributors at this point are pancreatic proteases and small intestinal brush border membrane peptidases.

Pancreatic proteases

The bulk of dietary protein digestion is accomplished by pancreatic proteases. A variety of these enzymes is synthesized in the acinar cells of the exocrine pancreas and released into the lumen of the duodenum via the ductus pancreaticus upon stimulation by cholecystokinin. Like gastric pepsins, all pancreatic proteases are released in the form of zymogens, protecting the tissue from damage due to inappropriate intrapancreatic activation of the enzymes. These zymogens are only activated once they reach the small intestinal lumen by an activation cascade: When trypsinogen reaches the duodenal lumen, a proteolytic enzyme expressed in the brush border membrane of enterocytes and goblet cells located in the mucosa of duodenum and proximal jejunum [55], namely enteropeptidase (see below), converts trypsinogen to the key protease trypsin. Once trypsin is formed, it activates additional molecules of trypsinogen, as well as the other pancreatic zymogens. Besides trypsin, the range of pancreatic hydrolytic enzymes includes endopeptidases and exopeptidases with different enzymatic mechanisms and different specificities.

Trypsin (EC 3.4.21.4), an endopeptidase and serine protease of which three isoforms, PRSS1, PRSS2, and PRSS3, are expressed, is most active in the pH range between 7 and 9. These trypsins preferentially hydrolyze peptide bonds with lysine or arginine at position P_1 [56]. Like trypsin, chymotrypsin (EC 3.4.21.1) is also an endopeptidase and serine protease, expressed in isoforms (CTRB1 and CTRB2), and excreted by the pancreas as chymotrypsinogens. Unlike trypsins, chymotrypsins preferentially cleave peptide bonds with large hydrophobic amino acids at the P_1 position, i.e., tyrosine, tryptophan, and

phenylalanine [57], but they also hydrolyze peptide bonds with other amino acids at slower rates, particularly those containing leucine and methionine at the P_1 position.

Pancreatic elastases, also referred to as chymotrypsin-like elastases (CELA), form another group of pancreatic serine proteases. Like the other pancreatic proteases, elastases are excreted as zymogens, referred to as proelastases, and are activated in the lumen of the duodenum by trypsin. CELA1 (EC 3.4.21.36) has been shown not to be expressed by human acinar cells [58]. However, two isoforms of CELA3 (EC 3.4.21.70) are expressed at similar mRNA and protein levels in human pancreas [59]. The specificities of human CELA3A and CELA3B are rather broad, with a preference for aliphatic amino-acid side chains, like branched-chain amino acids leucine, isoleucine, valine, and also alanine, at the P_1 position [60, 61].

In addition to these serine proteases, the pancreatic juice also contains metalloproteases. These pancreatic exopeptidases comprise carboxypeptidase A (EC 3.4.17.1) and carboxypeptidase B (EC 3.4.17.2). Both enzymes are zinc metalloproteases that are activated by trypsin from procarboxypeptidase A and B, respectively, and both enzymes hydrolyze peptide bonds at the carboxy-terminal end of dietary protein. While carboxypeptidase A preferentially releases C-terminal amino acid residues with aromatic or branched aliphatic side-chains, e.g., phenylalanine, tyrosine, tryptophan, leucine, and isoleucine, with only low affinity towards aspartate, glutamate, arginine, lysine, and proline [62, 63], carboxypeptidase B features a preferential release of C-terminal basic amino acids, i.e., arginine and lysine [64].

All these peptidases have in common that they display a reduced activity towards peptide bonds with proline at the P_1' site [65].

Brush border membrane peptidases

Up to this point, pepsin and the pancreatic endopeptidases have broken down dietary protein to smaller-sized oligopeptides, while the pancreatic carboxypeptidases have released absorbable free amino acids. More than two thirds of the amino acids in jejunal and ileal chyme are however still bound in peptides, while less than one third exists as free amino acids [66]. So, as a final step of protein digestion prior to intestinal absorption, the oligopeptides left after gastric and pancreatic proteolysis need to undergo further hydrolysis by peptidases located on the apical surface of small intestinal enterocytes, the brush border membrane. Due to the high share of peptides containing proline, particularly with proline in C-terminal position, brought about by the specificities of gastric and pancreatic proteases, different specificities compared to those hydrolases is required. This completion of protein digestion is accomplished by brush border membrane peptidases, comprising numerous hydrolases with different mechanisms and specificities, including aminopeptidases, carboxypeptidases, endopeptidases, and dipeptidases (Table 1) [67]. Notably, these peptidases are not expressed evenly throughout the entire small intestine: protein expression and activity of aminopeptidases and dipeptidyl peptidase IV in humans have been shown to increase along the length of the small intestine, reaching the highest levels in distal jejunum and ileum [68, 69], while the expression of enteropeptidase, the endopeptidase responsible for activating trypsin and thus the pancreatic protease cascade, is most abundant in the duodenum [70], where the zymogens in pancreatic juice enter the small intestinal lumen, i.e. where enteropeptidase activity is required the most.

Aminopeptidases

The most abundant type of peptidases expressed in the small intestinal brush border membrane are aminopeptidases, a group of exopeptidases that is not present in the pancreatic juice [71]. These peptidases play an important role in the degradation of dietary

peptides by stepwise release of N-terminal amino acids, which can be absorbed via intestinal amino acid transporters. They include, amongst others, aminopeptidase N, aminopeptidase A, aminopeptidase P, dipeptidylpeptidase IV, and gamma-glutamyl transferase.

One of the most abundant brush border aminopeptidases is aminopeptidase N (APN, EC 3.4.11.2), also referred to as membrane alanyl aminopeptidase. This zinc metalloprotease releases N-terminal amino acids from peptides with a preference for alanine at P_1 position. However, most other amino acids are also cleaved off as well, including proline, albeit at lower rates. In case of a hydrophobic amino acid at P_1 position and proline at P_1', the two amino acids may be released together as a dipeptide [67].

Another brush border membrane zinc metalloprotease is aminopeptidase A (APA) or glutamyl aminopeptidase (EC 3.4.11.7), which preferentially hydrolyzes N-terminal acidic amino acids, i.e., aspartic acid or glutamic acid [72]. Besides digesting dietary protein in the small intestine, aminopeptidase A is responsible for the proteolytic conversion of angiotensin II to angiotensin III and thus is involved in blood pressure regulation [73].

Aminopeptidase P (EC 3.4.11.9) is a manganese metalloprotease that hydrolyzes N-terminal amino acids that are linked to proline, including proline, also from di- and tripeptides [74].

Dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) is a serine protease that, contrary to the other brush border aminopeptidases above, releases N-terminal dipeptides exclusively. The exopeptidase is expressed on the surface of many cell types and in various body fluids [75]. As an exception to most proteases and peptidases concerning the ability to hydrolyze peptide bonds with proline, DPPIV is involved in the activation and inactivation of numerous biopeptides, including growth factors, vasoactive peptides, and chemokines that are protected by an N-terminal X-Pro, and thus participates in various processes including immune regulation and signal transduction [76-78]. It also plays an important role in glucose metabolism due to inactivation of the incretin GLP-1 [79] by cleaving His-Ala from its N-

terminus [80]. In the small intestinal brush border membrane, DPPIV activity interestingly complements the spectrum of aminopeptidase N. While the latter displays reduced activity in hydrolyzing peptide bonds with proline at P1' position of the N-terminal peptide bond, DPPIV has the highest preference towards proline, and, to a lesser extent, alanine, in penultimate N-terminal position, while its activity is reduced in peptide bonds involving other neutral amino acids in the penultimate position [81].

An aminopeptidase with an entirely different mechanism than the previous aminopeptidases, is gamma-glutamyl transferase (GGT, EC 2.3.2.2). Instead of releasing free amino acids or dipeptides from the N-terminus of peptides, GGT transfers an N-terminal gamma-glutamyl moiety to a wide range of acceptor molecules including water, certain L-amino acids, and peptides. Besides small intestinal mucosa, GGT is expressed in various tissues, including the kidneys, heart, and brain, where it plays an essential role the gamma-glutamyl cycle for the synthesis and degradation of glutathione [82, 83].

Carboxypeptidases

Another, yet smaller, group of exopeptidases expressed in the small intestinal brush border membrane are carboxypeptidases, including angiotensin-converting enzyme (ACE), glutamate carboxypeptidase II, and carboxypeptidase P.

ACE (EC 3.4.15.1) is a zinc metalloprotease, whose name reveals its main role in the organism, which is the proteolytic conversion of angiotensin I to the vasoconstrictor angiotensin II. In addition, ACE proteolytically inactivates the vasodilator bradykinin, emphasizing its importance for the regulation of blood pressure [84]. The general function of ACE consists in the release of a wide range of C-terminal dipeptides, unless the penultimate amino acid is proline and the C-terminal residue is an acidic amino acid, i.e., aspartate or glutamate [85].

Glutamate carboxypeptidase II (EC 3.4.17.21) is a zinc metalloprotease that preferably releases glutamate, but also aspartate from the C-terminus of peptides [86]. Besides intestinal mucosa and other tissues like neural ganglia and the proximal renal tubules, this exopeptidase is highly expressed in the prostate epithelium, which is why it is also referred to as prostate-specific membrane antigen (PSMA). It therefore also serves as a biomarker for the diagnosis and therapy of prostate cancer [87, 88].

The exopeptidase carboxypeptidase P (EC 3.4.17.16), also known as membrane Pro-X carboxypeptidase, is mainly expressed in the renal brush border membrane. In both brush border membranes, it releases C-terminal amino acid residues other than proline, with a preference for proline in the penultimate position [89]. Studies have shown that carboxypeptidase P, in conjunction with ACE, is an important factor in the digestion of proline-rich peptides [90].

Endopeptidases

In addition to the exopeptidases above, the brush border membrane of small intestinal enterocytes also expresses a set of endopeptidases, including the aforementioned enteropeptidase, meprin A (MEP), and neprilysin (NEP).

As detailed above, the serine protease enteropeptidase (EC 3.4.21.9), also referred to as enterokinase, is expressed in duodenal mucosa, where it is responsible for the proteolytic activation of trypsinogen to trypsin, triggering the pancreatic protease cascade. The importance of this process is highlighted by the congenital deficiency of this enzyme leading to protein malabsorption associated with nutrient deficiency and failure to thrive [91]. Enteropeptidase specifically catalyzes the hydrolysis of peptide bonds following a lysine residue after a tetra-aspartate motive, i.e., after Asp-Asp-Asp-Asp-Lys [92]. In this manner, enteropeptidase releases the N-terminal activation peptide from trypsinogen by selective

cleavage of a Lys-Ile bond following a tetra-Asp sequence in the zymogen and thus activates trypsin [93].

Neprilysin, also referred to as neutral endopeptidase (NEP, EC 3.4.24.11), is a zinc-dependent metalloprotease. NEP preferentially hydrolyzes peptide bonds between hydrophobic amino acids, primarily with phenylalanine or tyrosine at P_1' position [94]. Besides small intestinal brush border membrane, NEP is expressed in many different tissues, particularly in kidney. It is involved in the proteolytic inactivation of peptide hormones such as glucagon-like peptide 1 (GLP-1), oxytocin, and bradykinin [94, 95]. It is also expressed in leukemic cells and therefore serves in the diagnosis of acute lymphocytic leukemia in clinical pathology, under the name common acute lymphoblastic leukemia antigen (CALLA) or cluster of differentiation 10 (CD10) [96].

Meprin A (MEP, EC 3.4.24.63) shares many commonalities with NEP. It is a zinc metalloprotease that hydrolyzes peptide bonds preferentially between hydrophobic amino acid residues [97]. MEP, also referred to as endopeptidase-2, is also expressed in renal brush border membranes [98] and is involved in the degradation of biologically active peptides like bradykinin, oxytocin, and substance P [99].

Dipeptidases

Contrary to the previous groups of brush border peptidases, for all of which various members have been identified in human small intestinal mucosa, only a single dipeptidase has been identified so far expressed in the brush border membrane. Membrane dipeptidase, also known as renal dipeptidase or dehydropeptidase 1 (DPEP1, EC 3.4.13.19), is a zinc metalloprotease with a broad substrate specificity that hydrolyzes dipeptides to their component amino acids [100]. Besides dipeptides, membrane dipeptidase is able to hydrolyze beta-lactam structures and thus inactivate beta-lactam antibiotics [101]. In addition to small intestinal and renal brush border membranes, membrane dipeptidase is

overexpressed in colon tumor cells, making the enzyme a viable marker for colon cancer [102].

Brush border membrane vesicles

The variety of peptidases expressed in the apical membrane of small intestinal enterocytes enables the final steps of protein digestion, releasing absorbable amino acids and di- and tripeptides in close vicinity of apical amino acid transporters and PEPT1, while the unstirred layer and the mucus layer help maintain a high concentration of these transport substrates to facilitate transcellular, and possibly also paracellular, transport [103].

Recent studies have demonstrated that plasma membrane is actively shed from microvillar tips into the periapical space of brush border membrane in the form of vesicles (Figure 3) [104, 105]. These brush border membrane vesicles (BBMV) have been shown to contain membrane proteins also found in brush border membrane, including alkaline phosphatase and brush border peptidases [104]. The former detoxifies bacterial products like lipopolysaccharide (LPS) and thus contributes to the prevention of intestinal inflammation and to host defense mechanisms in the gut lumen [106, 107]. Brush border peptidases in the membrane of BBMV feature quantitatively similar aminopeptidase activity as brush border membrane, as compared by equal total protein, suggesting that BBMV may be a means of extending brush border peptidase activity beyond the periapical space into the lumen [104].

Besides being attached to brush border membrane and BBMV, brush border peptidases may also target oligopeptides as free peptidases, after being released from the enterocytes' apical membrane and BBMV by biliopancreatic secretions, i.e., bile acids and pancreatic proteases, particularly elastase, or BBMV disruption [108, 109]. However, the quantitative contribution to protein digestion of brush border peptidases bound to BBMV and liberated into the lumen is unknown so far.

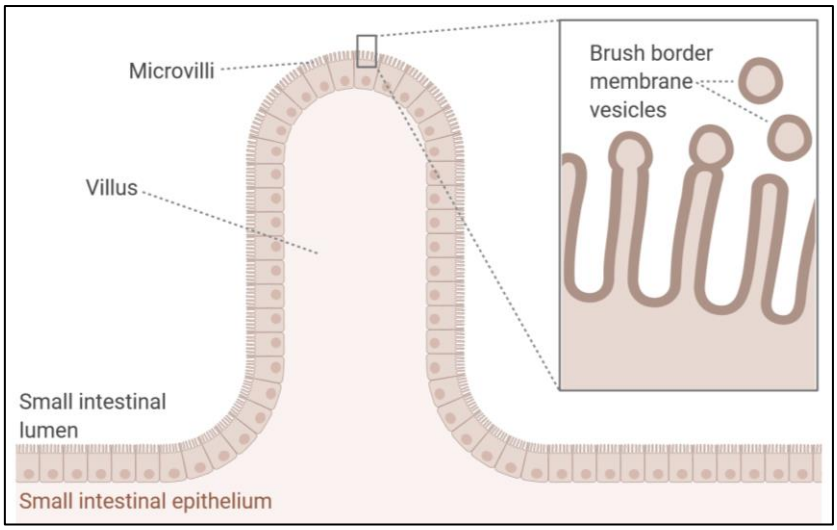

Figure 3: Brush border membrane vesicles

In the intestinal brush border membrane, plasma membrane vesicles are constantly shed from the tips of the microvilli into the intestinal lumen. These brush border membrane vesicles (BBMV) contain the same proteins as the brush border membrane that they were generated from, including brush border peptidases. Created with BioRender.com.

Table 1: Selected human intestinal brush border membrane peptidases

Peptidase	Catalytic mechanism	Substrate preference	References
Aminopeptidases			
Aminopeptidase N (APN)	Zinc metalloprotease	N-terminal Ala	[67]
Aminopeptidase A (APA)	Zinc metalloprotease	N-terminal Asp and Glu	[72, 73]
Aminopeptidase P (APP)	Manganese metalloprotease	N-terminal amino acids with Pro in P_1' position	[74]
Dipeptidylpeptidase IV (DPPIV)	Serine protease	N-terminal X-Pro dipeptides	[75-81]
Gamma-glutamyl transferase (GGT)	Acylation/ deacylation	Transfer of N-terminal ɣ-glutamyl moiety to water, L-amino acids, peptides	[82, 83, 110]
Carboxypeptidases			
Angiotensin-converting enzyme (ACE)	Zinc metalloprotease	C-terminal dipeptides except Pro-X, X-Asp, X-Glu	[85]
Glutamate carboxypeptidase II	Zinc metalloprotease	C-terminal Asp and Glu	[86-88]

Carboxypeptidase P	Zinc metalloprotease	C-terminal amino acids other than Pro with Pro in P_1 position	[89, 90]
Endopeptidases			
Enteropeptidase Enterokinase	Serine protease	After Asp-Asp-Asp-Asp-Lys	[91-93]
Meprin A (MEP) Endopeptidase-2	Zinc metalloprotease	Between hydrophobic amino acids	[97-99]
Neprilysin (NEP) Neutral endopeptidase	Zinc metalloprotease	Between hydrophobic amino acids, with Phe or Tyr at P_1' position	[94-96]
Dipeptidases			
Membrane dipeptidase Dehydropeptidase 1 (DPEP1)	Zinc metallo-protease	Dipeptides and β-lactam structures	[100-102]

Cytosolic peptidases

Once dietary protein is hydrolyzed to the level of free amino acids and di- and tripeptides, these products can be absorbed via intestinal amino acid transporters and PEPT1 located in the apical membrane of enterocytes (see **1.3**). Part of the absorbed oligopeptides will be exported into circulation via a so far unidentified basolateral oligopeptide transporter [49], whereas the other part will undergo intracellular digestion by cytosolic peptidases with different mechanisms and amino acid specificities [71, 111]. While small intestinal peptidase activity against peptides with more than 3 amino acids is predominantly located in the apical plasma membrane, general enzyme activity against di- and tripeptides is slightly higher in the cytosol fraction compared to the membrane fraction of mucosa of jejunum and ileum [112, 113]. Like brush border peptidases, the general expression of cytosolic peptidases appears to not be uniform along the small intestine: Studies have observed no or only low activities of cytosolic peptidases in proximal duodenum, gradually increasing to a maximum in distal jejunum and proximal ileum, before decreasing again towards the terminal ileum, with significant activity at the ileocecal valve [114]. These early findings in humans have been supported by later studies in different mammals, like sheep [115] and pigs [112].

Amongst these cytosolic peptidases, tripeptide aminopeptidase, also referred to as aminotripeptidase (EC 3.4.11.4) is distributed in a variety of tissues besides small intestinal mucosa, especially in liver [116]. This zinc metalloprotease releases N-terminal neutral amino acid residues exclusively from tripeptides. While tripeptides with charged N-terminal amino acids like lysine or glutamic acid are viable substrates as well, tripeptide aminopeptidase displays reduced activity towards these oligopeptides [117].

A cytoplasmatic peptidase that focuses on the breakdown of proline dipeptides in enterocytes is Xaa-Pro dipeptidase, also known as prolidase or X-Pro dipeptidase (EC 3.4.13.9). This manganese metalloprotease, expressed in most mammalian tissues, is a strict dipeptidase that is highly specific for dipeptides with proline at the C-terminus, except for Pro-Pro, which is not a substrate of Xaa-Pro dipeptidase [118].

The zinc metalloprotease leucyl aminopeptidase (LAP, EC 3.4.11.1) is an exopeptidase that preferentially hydrolyses peptide bonds of N-terminal leucine residues. Other N-terminal residues are released as well, yet with lower affinity [119]. LAP does not display a high specificity regarding the amino acid residue in P1’ position, including proline [120].

Cytosol alanyl aminopeptidase, also referred to as puromycin-sensitive aminopeptidase (PSA, EC 3.4.11.14) releases N-terminal amino acids, preferentially alanine, from a wide range of peptides. Multiple forms of this exopeptidase are widely distributed in mammalian tissues and body fluids [121, 122].

The exopeptidase aminopeptidase B (APB), also known as arginine aminopeptidase (EC 3.4.11.6) is expressed across many mammalian tissues, releasing N-terminal arginine and lysine residues from oligopeptides when the amino acid at P_1' position is not proline [123, 124].

1.2.3 Large intestinal protein digestion

Despite this wide array of gastrointestinal luminal, membrane-bound, and intracellular proteases and peptidases, protein digestion is not fully completed before the chyme leaves the small intestine via the ileocecal valve, so that a significant amount of dietary and endogenous protein - more than 10 g per day - enters the large intestine [125]. While the colon is known to be responsible, amongst others, for absorbing water, vitamins supplied by the gut microbiota, and bacterial fermentation products of undigested carbohydrates in the form of short fatty acids [126], a role in protein digestion and uptake has not been generally acknowledged [127, 128]. However, studies in the past decades have demonstrated colonic expression and activity of several peptidases in humans: Enzyme activity has been shown for the brush border membrane peptidases aminopeptidase N (APN), aminopeptidase A (APA), and dipeptidylpeptidase IV (DPPIV) [129, 130], as well as for cytosolic peptidases, e.g., cytosol alanyl aminopeptidase (PSA), aminopeptidase B (APB), and leucyl aminopeptidase (LAP) [129]. So, besides being the target of bacterial degradation, it is conceivable that peptides in the lumen of the colon are also the target of colonic membrane-bound peptidases and, upon uptake into colonocytes, of cytosolic peptidases.

1.2.4 Defects in protein digestion

There are only few rare primary defects in the digestive process that have been described in humans. Trypsin deficiency and enteropeptidase deficiency are associated with a similar phenotype as both disorders lead to an impaired activation of pancreatic zymogens essential for protein digestion [91, 131]. Both genetic defects in enteropeptidase and trypsinogen are linked to impaired growth and weight gain, as well as hypoproteinemia and edema. In addition, a primary defect in a single intracellular peptidase has been described in literature: Xaa-Pro dipeptidase or prolidase is expressed in many other tissues, so prolidase deficiency is associated with the disturbance of multiple functions, including impaired collagen turnover and skin abnormalities, mental retardation, and an increased susceptibility towards respiratory infections [132, 133]. It is not clear if these symptoms are associated with an

impaired hydrolysis of proline-containing peptides in the intestine, or if under normal dietary conditions the intestinal assimilation of proteins is unaffected.

1.3 Intestinal uptake of proteolysis products

As detailed above, the recommended dietary allowance of protein is, depending on age, sex, activity, etc., 0.8 g/kg of body weight per day, corresponding to approx. 50 to 60 g of dietary protein daily. The actual protein intake in developed countries is usually distinctly higher, up to 90 to 100 g per day [134]. In addition to dietary protein, large amounts of endogenous proteins are released in the gastrointestinal tract each day: Secretions of digestive glands, mainly mucins and digestive enzymes, contribute at least 20-30 g protein per day, desquamated cells from intestinal mucosa at least another 30 g per day, as well as smaller amounts of plasma proteins like albumin and gamma globulin [135]. These endogenous proteins are broken down and absorbed in the small intestine together with dietary protein, resulting in a total daily protein absorption of 170 g, while only 10 g thereof are estimated to reach the colon [136, 137]. Also, small intestinal and colonic microbiota synthesize and thus constantly contribute a considerable additional pool of proteins and amino acids, which is subject to digestion and absorption in addition to dietary and endogenous protein in the human gastrointestinal tract: for instance, almost 10% of plasma lysine is estimated to be of microbial origin in humans [138], as well as 20% of total body leucine [139]. The absorption of protein in the small intestine, regardless its source, after hydrolysis by gastric, pancreatic, and brush border membrane peptidases, is accomplished in the form of free amino acids and di- and tripeptides by a range of solute carriers (SLCs) expressed in the brush border membrane of small intestinal enterocytes. A small portion of dietary and endogenous protein, including microbial peptides and amino acids, is also absorbed in the colon. Upon uptake via PEPT1, di- and tripeptides are either released via the basolateral membrane by a so far unknown transport protein or rapidly hydrolyzed into amino acids by cytosolic peptidases. These breakdown products, together with the amino acids absorbed from the intestinal

lumen, may then also enter portal circulation via basolateral amino acid transporters. Overall, transport activity for amino acids and peptides is highest in the proximal jejunum, but the other parts of the intestine, including colon, also display significant transport of these breakdown products [140].

1.3.1 Small intestinal amino acid transport

Amino acids are transported across the intestinal epithelium by a battery of apical and basolateral amino acid transporters, with different specificities, mechanics, and structure: These SLCs can be uniporters, symporters, or antiporters, they can be monomeric or heterodimeric proteins, and they all have different specificities, ranging from a group of structurally related amino acids, such as neutral amino acids, to the transport of a single amino acid. Besides their classification in SLC families, based on similarities in their gene sequences, amino acid transporters are also grouped according to major transport activities: system B^0 (for broad neutral) transporting all neutral amino acids, system $b^{0,+}$ (for broad neutral and cationic), transporting cationic amino acids and cystine, a transport system for acidic amino acids aspartate and glutamate, referred to as X^-_{AG}, the iminoglycine system for proline, hydroxyproline, and glycine, a separate system transporting β-amino acids, etc. [135].

To demonstrate the versatility of amino acid transporters, the most important representatives of these families and systems of amino acid transporters identified in human are introduced below (Figure 4).

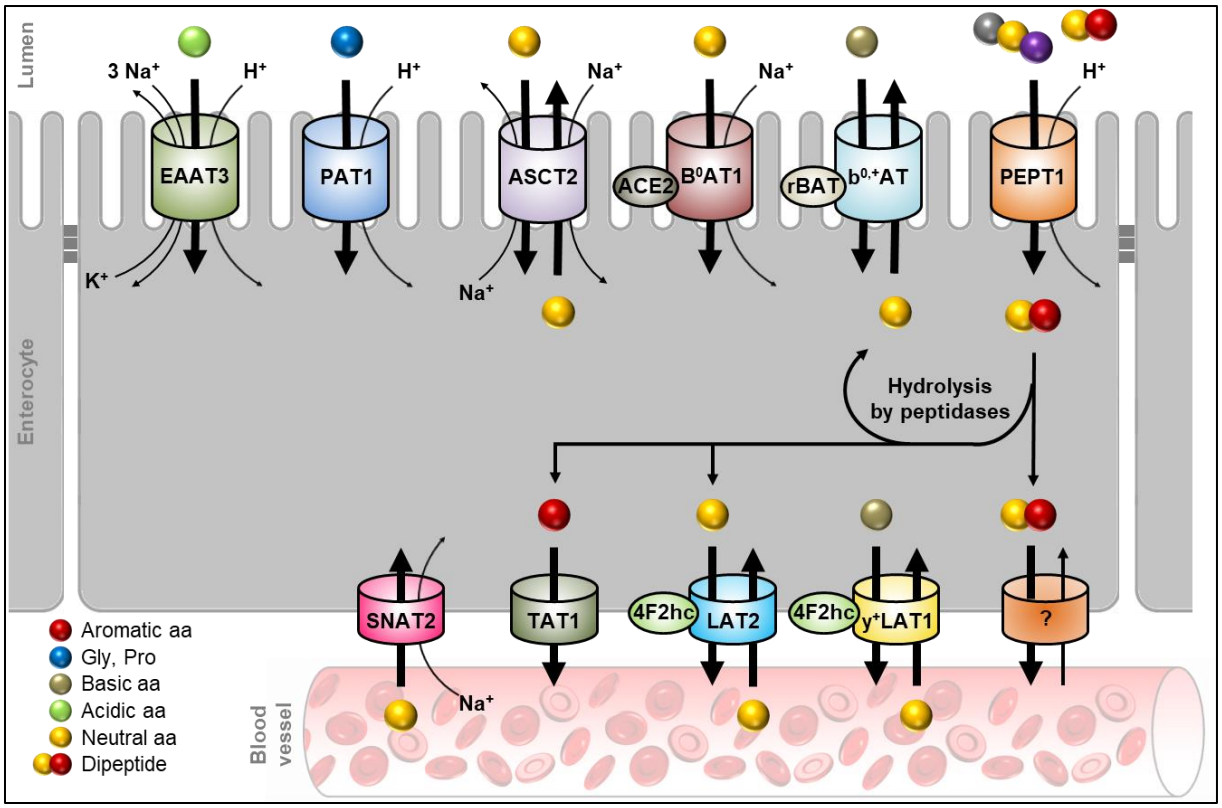

Figure 4: Plasma membrane transporters of amino acids and di- and tripeptides in human enterocytes.

The plasma membrane of enterocytes is equipped with SLC transporters that facilitate the absorption of proteinogenic amino acids (aa) as well as di- and tripeptides formed by these amino acids. The usually Na^+ and/or H^+-dependent electrogenic apical amino acid transporters include transporters specific for neutral amino acids, like the heterodimeric B^0AT1/ACE2 and the neutral amino acid exchanger ASCT2, transporters for glycine and proline, like PAT1, transporters for acidic amino acids, like EAAT3, and transporters for basic amino acids, like the heterodimeric amino acid exchanger $b^{0,+}AT$/rBAT). Contrary to the wide range of different amino acid transporters, all di- and tripeptides are transported only by a single SLC transporter, PEPT1. In the cytosol, di- and tripeptides are cleaved by cytosolic peptidases, releasing free amino acids that cross the basolateral membrane into circulation via basolateral amino acid transporters. Peptides that escape hydrolysis are released across the basolateral membrane by a so far unidentified peptide transporter. Free amino acids are exported into circulation by basolateral amino acid transporters, including the heterodimeric neutral amino acid exchanger LAT2/4F2hc, TAT1 for the transport of aromatic amino acids, and the heterodimeric amino acid exchanger y^+LAT1/4F2hc that releases basic amino acids and imports neutral amino acids from circulation in return. In addition, the basolateral plasma membrane also contains uniporters that transport amino acids from circulation into the enterocytes' cytosol, like the Na^+-dependent neutral amino acid transporter SNAT2 (modified from Spanier and Rohm 2018) [141].

Apical neutral amino acid transport

System B^0 features transport activity for all neutral amino acids, and its most important member is SLC6A19, also referred to as B^0AT1. SLC6A19 is also expressed in the kidney, transports all neutral amino acids, and is the most important apical transporter for almost all neutral amino acids [142]. Transport affinities vary however, decreasing the shorter and bulkier the amino acid substrate is [143], resulting in the highest affinities for methionine, leucine, isoleucine, and valine. SLC6A19 is a Na^+-dependent symporter that cotransports 1 Na^+ ion for every amino acid [144]. SLC6A19 requires the apical coexpression of angiotensin converting-enzyme 2 (ACE2), a zinc metalloprotease that otherwise also catalyzes the hydrolysis of angiotensin II to angiotensin (1-7) [145]. ACE2 regulates the transporter's membrane trafficking, so in the absence of ACE2, transport activity of SLC6A19 is distinctly reduced [146, 147]. Small intestinal expression of SLC6A19 increases from proximal to distal, while colonic expression has not been demonstrated [148]. SLC6A19 is essential for the uptake of neutral amino acids, particularly for tryptophan. The transporter's loss of function results in a serious metabolic disorder (Hartnup disease, see **1.3.5**).

Another system B^0 apical transporter of neutral amino acids is SLC1A5 or ASCT2 (for "Alanine, Serine, Cysteine Transporter 2"), which can also be found, amongst others, in renal epithelia. SLC1A5 generally features higher preference towards smaller neutral amino acids except glycine, with the highest affinity in alanine [149]. Despite its historical name, SLC1A5 has the highest affinity towards glutamine however [150]. At acidic pH values found at the intestinal brush border, the transporter has also been demonstrated to transport glutamate [151]. SLC1A5 is an amino acid antiporter, i.e., for every amino acid transported from the intestinal lumen into the enterocyte, another amino acid is exported in the opposite direction, so its transport activity does not contribute to a net transport of neutral amino acids across the apical enterocyte membrane but, due to different affinities towards its substrates, serves in the harmonization of amino acid pools [150]. Together with this amino acid antiport, a

Na^+/Na^+ exchange is performed [150]. The intestinal expression pattern of SLC1A5 differs from SLC6A19, being expressed mainly in jejunum and colon, and, only to a lesser extent, in duodenum and ileum [152].

Basolateral neutral amino acid transport

An important neutral amino acid transporter in the basolateral enterocyte membrane, also present in renal epithelia, is SLC7A8 or LAT2. SLC7A8 constitutes the subunit with a Na^+-independent transport activity of a heterodimeric transport protein that it forms with SLC3A2 or 4F2hc, with which it is connected by a disulfide bridge [153]. Like for other heterodimeric complexes, the 4F2 heavy chain is responsible for trafficking the amino acid transporter to the basolateral plasma membrane [154]. SLC3A2/SLC7A8 is expressed mainly in jejunum and ileum [155], where it transports a wide range of amino acid substrates, including all neutral amino acids except proline [156]. The heterodimer is an amino acid antiporter, which is assumed to facilitate the efflux of smaller amino acids and an associated influx of branched-chain amino acids (BCAA) and aromatic amino acids, which could then be re-exported by the corresponding basolateral transporters like SLC16A10 [157, 158].

SLC16A10 or TAT1 is another basolateral neutral amino transporter. Besides other tissues, it is expressed in kidney and features a high expression throughout all small intestinal segments [157, 159]. SLC16A10 is a Na^+-independent uniporter with generally low affinities that transports preferentially aromatic amino acids and their N-methylated derivatives like L-Dopa [157].

Besides transporters that are responsible for an exchange of amino acids or the efflux of amino acids from the enterocytes' cytosol across the basolateral membrane, there are also transporters that facilitate the basolateral uptake of amino acids from circulation: SLC38A1 and SLC38A2 (SNAT1 and SNAT2), are two Na^+-dependent uniporters expressed throughout the small intestinal and colonic mucosa [148], with a transport preference for

small hydrophilic amino acids [160]. In states of amino acid depletion, e.g., in case of low luminal amino acid levels, expression of these two transport proteins in upregulated, supplying the enterocytes with amino acids as energy substrates from circulation, including their prime energy substrate glutamine [161, 162].

Apical cationic amino acid transport

System $b^{0,+}$, the system facilitating the transport of neutral and cationic amino acids across the apical membranes of enterocytes, corresponds to the heterodimeric amino acid exchanger rBAT/$b^{0,+}$AT, which comprises the two subunits SLC3A1 and SLC7A9. SLC7A9 is the main apical intestinal transporter for cationic amino acids and cystine, with a lower affinity for neutral amino acids. The antiporter transports cationic amino acids and cystine from the intestinal lumen across the apical enterocyte plasma membrane while exporting neutral amino acids from the cytosol [163]. As in 4F2hc/LAT2 above, the two subunits in rBAT/$b^{0,+}$AT are connected by a disulfide bridge, and similar to 4F2hc, rBAT (SLC3A1) is responsible for trafficking the heterodimer to the apical enterocyte membrane [164]. SLC3A1/SLC7A9 is essential for the uptake of cationic amino acids in human. Mutations in either subunit may lead to a metabolic disorder called cystinuria, which is characterized by frequent cystine kidney stones (see **1.3.5**).

Basolateral cationic amino acid transport

The transporter mainly responsible for basolateral export of cationic amino acids is y^+LAT1 (SLC7A7), corresponding to system y^+L. The antiporter's direction of amino acid fluxes is determined by prevalent Na^+ concentrations: while the transport of cationic amino acids is independent of Na^+, neutral amino acids are preferred transport substrates in the presence of Na^+, like in extracellular space. So, due to naturally low intracellular Na^+ levels, cationic amino acids are preferentially exported from the cytosol in exchange for neutral amino acids from the extracellular space, where high Na^+ concentrations prevail [165]. Similar to

4F2hc/LAT2, y^+LAT1 is a subunit of the heterodimeric transporter 4F2hc/y^+LAT1 (SLC3A2/SLC7A7), in which 4F2hc is required for trafficking the transport molecule to the basolateral membrane [166]. Mutations of SLC7A7 that lead to a loss of function of the y^+LAT1 subunit cannot be compensated by other intestinal transporters and lead to lysinuric protein intolerance (see **1.3.5**).

Apical anionic amino acid transport

SLC1A1 or EAAT3 is the molecular correlate of system X^-_{AG}, which can, besides other tissues, also be found in kidney. The transporter is a Na^+-dependent uniporter with a high affinity for aspartate and glutamate [167], and with a lower affinity for cystine [168]. The transport of its transport substrates from the intestinal lumen across the apical plasma membrane requires several co-substrates: together with each amino acid molecule, SLC1A1 imports 3 Na^+ ions and 1 proton and exports 1 K^+ ion [169, 170]. Glutamate, together with glutamine and aspartate being an important energy substrate of enterocytes [171], is metabolized by enterocytes to 95% [172], which explains why very little of dietary aspartate and glutamate enter portal circulation and why so far, no transport proteins for the basolateral export of glutamate have been identified [135]. Rare mutations in SLC1A1 that impair the intestinal glutamate transport activity of EAAT3 are associated with dicarboxylic aminoaciduria (see **1.3.5**).

Apical imino acid and glycine transport

SLC36A1 or PAT1 is expressed in the brush border membrane throughout all intestinal sections, with the strongest expression in jejunum [173, 174]. In addition, the transporter is also expressed in lysosomes in various tissues, which accounts for the transporter's pH dependency with highest activity at low pH [175]. With each substrate molecule, PAT1 co-transports 1 proton across the brush border membrane into the cytosol, inducing an intracellular acidification [173]. PAT1 displays a fairly low affinity for all its substrates, which

include proline, glycine, and alanine, but also structurally related molecules like β-alanine, sarcosine, and GABA, yet with lower affinities [173].

1.3.2 Colonic amino acid transport

The vast majority of dietary and endogenous protein in the gastrointestinal tract is broken down and assimilated in the small intestine. Dietary proteins that escape small intestinal digestion, as well as colonic endogenous and bacterial protein are digested in the large intestine by the remaining pancreatic proteases, bacterial proteases [176], and colonic membrane-bound peptidases (see **1.2.3**). Degradation products of these proteins may then be utilized by gut microbiota for their metabolism, synthesizing, amongst others, amines and short-chain fatty acids [177], or they may be available for uptake by colonic mucosa. There is evidence for the transfer of amino acids released by gut microbiota and by brush border peptidases across the colonic epithelium under physiological conditions: Several studies on the resorptive capability of colonic mucosa for amino acids have been conducted in piglets [178-180], an established model for human nutrition studies [181], which could demonstrate colonic amino acid absorption *in vivo*. In rats, colonic amino acid transport activity under physiological conditions, alongside gene expression of 20 amino acid transporters along the colon, has been demonstrated systematically [182]. Clear evidence for colonic amino acid transport in humans however is scarce. In human tissue, expression of several amino acid transporters has been shown in the apical membrane of enterocytes in colon: $ATB^{0,+}$ (SLC6A14) has been demonstrated to transport L-enantiomers of neutral and cationic amino acids, but also D-serine produced by the gut microbiota [183-185]. Also, mRNA expression of several other plasma membrane amino acid transporters has been described in human colonic mucosa, e.g., ASCT2 (SLC1A5), 4F2hc/LAT1 (SLC3A2/ SLC7A5), y^+LAT2 (SLC7A6), and SNAT 1 (SLC38A1) [186]. Of course, mRNA expression cannot prove protein expression of the corresponding transporter, nor its localization in the plasma membrane or transport activity. These findings are supported by a study assessing colonic amino acid

absorption in infants, which demonstrated the absorption of ^{15}N-labeled yeast protein and its incorporation into body protein after colonic instillation [187]. However, it is not clear whether absorption occurred in the form of oligopeptides, amino acids, or ammonia, nor do the results allow conclusions about colonic amino acid transport in adult humans, nor if it contributes to a nutritionally relevant absorption under physiological conditions. So, overall, there is some evidence for the human colon's capacity to absorb degradation products of protein digestion, but the uptake of nutritionally relevant amounts of amino acids in colon requires further research.

1.3.3 Di- and tripeptide transport

Until the 1960s, scientists believed that dietary protein had to be completely broken down into free amino acids in order to be absorbed in the intestine. Around that time, the first studies demonstrated also di- and tripeptides, yet not peptides with more than 3 amino acid residues [188], to be absorbed in the gastrointestinal tract [189, 190], which eventually led to the identification of the peptide transporter as SLC15A1 or PEPT1 [191].

PEPT1 features high expression rates throughout all segments of the small intestine, with expression increasing along the course of the small intestine [192-194]. In addition, protein expression of SLC15A1 (PEPT1) has been demonstrated immunohistochemically in human colon, as well as functional PEPT1 transport activity [195]. Colonic PEPT1 expression under physiological conditions is however discussed controversially in literature [141]. Besides gastrointestinal mucosa, PEPT1 is also expressed, amongst others, in renal and bile duct epithelia [196, 197].

PEPT1 co-transports its peptide substrates with one or more protons per peptide across the enterocytes' apical membrane, following the proton gradient [198, 199]. Due to PEPT1's transport activity being dependent on the pH gradient, which is strongest in duodenum, PEPT1 activity is strongest in duodenum and decreases towards the distal small intestine

accordingly, despite inverse expression levels [200, 201]. Notably, contrary to the multitude of intestinal amino acid transporters as detailed above, PEPT1 is the only intestinal brush border membrane transporter for di- and tripeptides. PEPT1 features a very wide substrate specificity, covering the vast majority, yet not all [202], of the 400 dipeptides and 8,000 tripeptides that can be formed from proteinogenic amino acids [201, 203]. Preferred substrates are peptides consisting entirely of L-amino acids, while peptides containing a mix of L- and D-amino acids are accepted as transport substrates by PEPT1 as well, yet with lower affinity than peptides consisting entirely of L-amino acids; peptides consisting entirely of D-amino acids are not transported however [204, 205]. Overall, di- and tripeptide affinities vary by several orders of magnitude, with reported K_i values ranging from 8 µM for Ala-Ala to 22 mM for Pro-Gly [141]. As detailed above, di- and tripeptides are mostly degraded into free amino acids by cytosolic peptidases in the enterocytes' cytosol (see **1.2.2**). Free amino acids and the remaining peptides that escape hydrolysis can then enter portal circulation across the basolateral membrane. Interestingly, the transporter that facilitates the basolateral export of di- and tripeptides has not been identified yet [49]. The high intestinal expression of PEPT1 in addition to its high transport capacity [201, 206] is assumed to overall facilitate amino acid absorption rates via PEPT1 that are distinctly higher than those of free amino acids in the intestine [207].

In addition to peptides, a wide range of substrates that are structurally related to di- and tripeptides are readily transported by PEPT1, including peptidomimetics like aminocephalosporins and other β-lactam antibiotics, antivirals, protease inhibitors, and inhibitors of angiotensin-converting enzyme (ACE) [208, 209].

PEPT1 transports its substrates mainly from the intestinal lumen across the brush border membrane, but, depending on substrate concentrations, membrane potential, and proton gradient, peptide export from the cytosol across the brush border membrane is possible as well, making PEPT1 a bidirectional transporter [201].

1.3.4 Tetrapeptides and larger peptides

As stated above, PEPT1 (SLC15A1) does not accept peptides comprising more than three amino acid residues as transport substrates. Transporters for larger peptides do exist in nature, they are however restricted to prokaryotes [210]. While the uptake of intact peptides across the intestinal barrier is being discussed controversially in literature, particularly in the field of dietary bioactive peptides, no transporters for peptides with more than 3 amino acids have been identified in humans [211]. Under certain circumstances, peptides are known to be able to cross the intestinal mucosa intact and enter portal circulation: γ-globulins from colostrum cross the small intestinal wall of newborns intact, transferring passive immunity from mother to child [212]. Also in healthy adults, small amounts of larger peptides and proteins can cross the intestinal wall: via paracellular diffusion (mostly cationic and inert molecules) [213, 214], via passive diffusion through enterocytes (apolar peptides) [213], and via transcytosis [215, 216]. During their passage across the intestinal mucosa, these peptides may be further hydrolysed, allowing only very low amounts of peptides to reach portal circulation [217], where they rapidly become the target of endothelial and plasma peptidases [218]. Contrary to di- and tripeptide uptake via PEPT1, these non-transporter mediated processes do however not allow the uptake of peptides in physiologically and nutritionally relevant concentrations, but are believed to represent pathways of antigen sampling [217].

1.3.5 Primary defects in absorptive processes

Whenever an amino acid transporter is impaired whose transport activity cannot be compensated by other transporters, this may lead to a metabolic disorder or disease. Several rare amino acid transporter-associated disorders have been described in the past, amongst them Hartnup disease, cystinuria, lysinuric protein intolerance, and dicarboxylic aminoaciduria, which have helped identify the underlying genes and transport proteins [219].

Contrary to these primary defects an amino acid transport, no cases of PEPT1 deficiency are known in humans.

Hartnup disease

Mutations in SLC6A19, the neutral amino acid transporter B^0AT1 (see **1.3.1**), lead to renal aminoaciduria and impaired intestinal amino acid absorption of neutral amino acids responsible for Hartnup disease [220]. The symptoms of this rare autosomal recessive disorder with a frequency of 1:30,000 [221] are caused by a deficiency of the essential amino acid tryptophan [222], which, together with other neutral amino acids, is lost as a consequence of amino acid malabsorption in feces and urine. Tryptophan being a precursor of the niacin (vitamin B_3) in human metabolism, a lack of intestinal tryptophan absorption leads to niacin deficiency. For this reason, the wide spectrum of symptoms of Hartnup disease is similar to niacin deficiency, including photosensitive pellagra-like skin rash, cerebellar ataxia, dementia, and other psychiatric symptoms such as depression and psychosis [223]. Symptoms usually present themselves in childhood, at times of increased demand of amino acids due to growth. However, many individuals suffering from mutations in SLC6A19, particularly adults, do not develop any symptoms, especially when consuming a diet sufficient in protein [224]. In these cases, other intestinal amino acid transporters, as well as peptide transporter SLC15A1 (PEPT1) are assumed to achieve a sufficient supply with neutral amino acids including tryptophan [225]. The treatment of Hartnup disease comprises high-protein diet and nicotinamide supplements [221].

Cystinuria

Cystinuria is a rare inherited autosomal recessive disease, with a worldwide prevalence of 1:7000 [226]. It's underlying cause are mutations in either or both subunits of the rBAT/$b^{0,+}$AT heteromeric amino acid transporter (see **1.3.1**). Depending on which gene is affected, cystinuria is classified as type A (mutations in rBAT), type B (mutations in $b^{0,+}$AT), or type AB

(in case of mutations in both genes) [227]. Due to the transporter's inability to transport its substrates, cationic amino acids and cysteine, across the brush border membrane of intestinal and renal mucosa, cystinuria is characterized by high urinary concentrations of cystine, lysine, arginine, and ornithine, while in feces, increased levels of dibasic amino acids lysine, arginine, and ornithine can be detected as well [228]. Both in urine and in feces, the amino acids' metabolic breakdown products produced by the gut microbiota, e.g., cadaverine, putrescine, and citrulline, can be detected. Due to the high cysteine concentrations in urine, cysteine precipitates and frequently forms kidney stones that are composed mostly of cystine in the urinary tract, i.e., in kidney, ureters, and bladder. The treatment of cystinuria entails the prevention of kidney stones by adequate hydration, a reduction in protein intake, urine alkalinization, and chelation therapy, and the therapy of kidney stones by extracorporeal shock wave lithotripsy or surgery, e.g., percutaneous nephrolithotripsy [229]. If untreated, kidney stones cause pain, urinary tract infections, and eventually lead to kidney damage [230].

Lysinuric protein intolerance

Lysinuric protein intolerance (LPI) is a rare autosomal recessive disorder mainly reported in Finnish population with a frequency of 1 in 60,000 births [231]. Symptoms presented by affected individuals include vomiting and diarrhea, growth retardation, hepatosplenomegaly, osteoporosis and osteopenia, pulmonary alveolar proteinosis, and chronic kidney disease [232, 233]. Urinary concentrations of cationic amino acids (arginine, lysine, ornithine) are elevated, while their plasma concentrations are reduced due to impaired intestinal absorption [234]. The impaired uptake and consequent lack of arginine and ornithine leads to an impairment of the patients' urea cycle, causing hyperammonemia after protein uptake [235]. LPI is caused by defective cationic amino acid transport due to mutations of SLC7A7 (y^+LAT1) (see **1.3.1**), i.e., by an impaired export of cationic amino acids across the basolateral membrane of epithelial renal and intestinal cells [236]. Therefore, LPI, contrary to

diseases associated with defective apical amino acid transporters, cannot be treated with peptides containing these amino acids as they would be hydrolyzed intracellularly upon apical uptake via PEPT1, leading to an accumulation of cationic amino acids in the enterocytes. Consequently, the treatment of LPI consists of dietary protein-restriction and oral supplementation of citrulline [237].

Dicarboxylic aminoaciduria

Mutations in SLC1A1 (EAAT3, see **1.3.1**) cause dicarboxylic aminoaciduria with an estimated incidence of 1:35,000 births [238]. This rare autosomal recessive disorder, clinically asymptomatic in most affected individuals [239], is characterized by highly elevated urinary aspartate and glutamate concentrations. Reports on impaired intestinal uptake of these amino acids in dicarboxylic aminoaciduria are controversial however [240, 241]. This, together with the fact that dicarboxylic aminoaciduria is usually asymptomatic, supports the notion that there are additional intestinal glutamate transporters, e.g., SLC1A5 (ASCT2), for which glutamate transport activity has been demonstrated [150].

1.4 Research questions

The fate of the breakdown products that arise during the intestinal digestion of dietary protein has not yet been deciphered. It is not known to what extent intact di- and tripeptides reach the circulation postprandially or to what extent dietary protein is absorbed in the form of free amino acids. The aim of this dissertation is to describe processes in the context of intestinal protein digestion with regard to hydrolysis and transport of peptides.

To this purpose, the main objectives of this dissertation are:

1. Development of a method for the quantification of peptides in biological samples.
2. Assessment of human peptide plasma concentrations in fasting state.

3. Assessment of the kinetics of peptide plasma concentrations after a protein-rich meal in humans.
4. Generation of a PEPT1 deficient Caco-2 cell clone using CRISPR/Cas9 technology.
5. Simultaneous assessment of peptide transport and hydrolysis in different models.

2 Methods

2.1 Human study design and plasma sample collection

The study included six healthy male and six healthy female volunteers. The inclusion criteria were an age of 18 to 40 years, a BMI below 30 kg/m^2, and the participants had to be non-smokers. Exclusion criteria consisted of gastro-intestinal diseases that could affect nutrient uptake, digestion, metabolism, and excretion, chronic diseases, acute illnesses, antibiotic therapy in the 6 months prior to the study, a regular medication intake (except oral contraceptives), pregnancy and lactation, as well as allergies and intolerances to the foods used in the study. The volunteers' baseline characteristics were an age of 28 ± 5 years and 25 ± 3 years for the males and females, respectively. The participants' BMI was 25.2 ± 2.8 kg/m^2 (males) and 21.5 ± 2.4 kg/m^2 (females).

The intervention study was part of the EU Joint Programming Initiative (JPI) within the Food Biomarkers Alliance (FOODBALL), performed at the ZIEL-Institute for Food and Health, Core Facility for Human Studies, Technical University of Munich, Freising, Germany, and registered in the German Clinical Trials Register (DRKS #00010133). The ethical committee of the Technical University of Munich approved the study protocol (project number: 51/16S). Written informed consent was obtained from all participants prior to the study. The study was a randomized controlled intervention study with a crossover design. It comprised three treatment periods of 48 h each that were separated by an at least 1-week wash-out period and a total maximum duration of 6 weeks. The volunteers were randomized and allocated into the study using sequentially numbered, opaque, sealed envelopes (SNOSE). The principal study design can be found elsewhere [242]. The volunteers were randomly allocated either to the control group that received rice without meat (125 g, cooked, 30 g margarine, 1.5 g salt), rice with 100 g chicken breast that was prepared sous vide (dose 1), or with 200 g chicken breast (dose 2). During the 48-hour run-in period, the subjects followed a strict vegetarian diet. The day prior to the intervention, the subjects were served the control

rice meal as dinner. The control and test foods were given for breakfast in a randomized order. In addition, the standardized meals were served for lunch and dinner, together with 250 ml water per hour on the intervention day. The participants' blood samples were taken at time point 0 and after 1, 2, 4, 6, 12, and 24 h and plasma was immediately separated (3000×g, 10 min, 4 °C) and stored at −80 °C until analysis.

2.2 Mouse study design and plasma sample collection

The PEPT1 deficient mice used in the studies were characterized in detail before [243-245]. They were generated by targeted disruption of the PEPT1 gene and obtained from Deltagen (San Mateo, California, USA). The animals were backcrossed for 10 generations to a C57BL/6N background. They were maintained in a specific-pathogen-free (SPF) animal facility at 22 ± 2 °C and a 12:12 h light/dark cycle. All but two female mice in the $PEPT1^{+/+}$ group were male and younger than 19 weeks. 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).

For the gavage experiments, the animals were fasted for 6 h. $PEPT1^{+/+}$ and $PEPT1^{-/-}$ mice (n = 3 per group) were each gavaged with a single dose of 200 µl of Gly-Pro/Pro-Gly solution that contained 290 mM of each peptide in water at pH 6.0. We chose these dipeptide concentrations in accordance with early peptide uptake studies that were performed in humans [188, 246], which represent the peptides' maximal solubility in water. Corresponding to a dose of 345 mg/kg in mice, these amounts are not achievable through a regular diet, and corresponds to a human equivalent dose (HED) of 28 mg/kg. 3 wild-type animals per group were gavaged with either 200 µl of water or Gly-Pro/Pro-Gly solution. The animals were anesthetized with isoflurane 30 minutes after the gavage, at which point retro-orbital blood was collected into EDTA-coated tubes.

2.3 Cell culture and transport assays

We assessed the rates of transport and hydrolysis of selected di- and tripeptides, ɣ-glutamyl-dipeptides, and cephalosporins in human colon carcinoma Caco-2 cells in transwell culture. We generated PEPT1 knockout clones and control cells using CRISPR/Cas9 technology. To this purpose, we targeted exon 5 of *pept1* with a double nickase approach. We transfected Caco-2 cells using the Cell Line Nucleofector Kit T (Lonza, Basel, Switzerland), the nickase vector pMB-Casv2-D10A containing the gRNAs 5'-TTGTCCAATTGTGTAGACAA-3' and 5'-CTCACAGACCACAACCATGA-3', as well as the homology-directed repair vector HR110PA-1 (System Biosciences, Mountain View, USA). The latter contained homology arms consisting of 800 bp 5' and 3' of the single strand breaks that were introduced by the nickases at positions 98713044 and 98713114 of chromosome 13. At these positions, puromycin resistance and red fluorescent protein genes were inserted as selections markers in exon 5 of the human PEPT1 gene. We verified an effective PEPT1 knockout by DNA sequencing and Western blot analysis. The Caco-2 cells were cultured in uncoated culture flasks in minimal essential medium (MEM) containing 10% fetal calf serum, 1% MEM non-essential amino acids solution (Sigma-Aldrich, Taufkirchen, Germany), and either 50 µg/ml gentamicin or 5 µg/ml puromycin. Cells were trypsinized at confluence and seeded in 12-well culture plates with transwell inserts at 180 000 cells per well. The cells were incubated at 37 °C and 5% CO_2. At least 21-days post-confluent cells from passages 30 to 45 and a transepithelial electrical resistance (TEER) of at least 500 $\Omega * cm^2$ were rinsed with PBS before performing any transport assays.

0.5 ml of MES-buffered transport medium containing 500 µM of each panel peptide were used as apical solution in the peptide transport assays, and 1.5 ml of HEPES-buffered transport medium served as receiver in the transwell culture basolateral compartment. Accordingly, for the ɣ-glutamyl-dipeptide transport assay, the Caco-2 cells were incubated in the apical compartment with 0.5 ml of MES-buffered transport medium at pH 6 containing 500 µM of either ɣ-Glu-Glu, ɣ-Glu- Gly, or ɣ-Glu-Leu. We chose these peptide concentrations

to avoid potential cytotoxic effects described in literature for some peptides in Caco-2 cells during long-term incubation [247, 248]. For the transport assay on cephalosporins, the cells were incubated in the apical compartment with 0.5 ml of MES-buffered transport medium at pH 6.0 that contained 500 µM of either cephalosporin cefadroxil, cefalexin, or cefradine. In all transport assays, medium samples were collected after 0 and 360 min of incubation at 37 °C and 5% CO_2. We chose the incubation time of 360 min in order to ensure changes in peptide and cephalosporin concentrations above detection limits.

2.4 Representative peptide panel for transport and *ex vivo* peptide digestion assays

We employed a di- and tripeptide panel representative for the 400 possible dipeptides from 20 proteinogenic amino acids regarding their constituents' size, charge, side-chain polarity, and side-chain class for the cell culture oligopeptide transport assays as well as the peptide digestion assays using mouse and human intestinal samples. This panel contained the dipeptides 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, and Trp-Leu, and Gly-Gly-Ile, Val-Pro-Pro, and ɣ-Glu-Leu as representatives for tripeptides and ɣ-glutamyl-dipeptides, respectively.

2.5 Intestinal di- and tripeptide digestion by intestinal mouse mucosa *ex vivo*

We removed the intestines of $PEPT1^{+/+}$ and $PEPT1^{-/-}$ mice (n = 3 per group). After rinsing them with PBS, we removed four sections of 1 cm 10 cm distally of the stomach as jejunal samples, and four additional 1 cm sections from the center of the colon of each animal. We split these intestinal sections open longitudinally and incubated three sections from each segment in 0.5 ml MES-buffered transport medium (pH 6.0) that contained 250 µM of each panel peptide and 1:100 Pen-Strep. As negative control, one additional jejunal and colonic section each was incubated in 0.5 ml of MES-buffered transport medium (pH 6.0) containing 1:100 Pen-Strep at 37 °C. Medium samples were collected after 0, 30, 60, and 120 min.

2.6 Intestinal di- and tripeptide digestion by intestinal human mucosa *ex vivo*

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

2.7 Sample preparation and derivatization for LC-MS/MS analysis

Samples of 20 µl of plasma or 10 µl of transport medium were evaporated with 25 µl internal standard under a nitrogen stream before being derivatized with phenyl isothiocyanate (PITC). 10 µl of PITC was added following an incubation with 190 µl of derivatization mix at room temperature while shaking at 750 rpm for 5 min. Afterwards, the samples were incubated for 20 min at room temperature and 750 rpm shaking. Samples were then again dried under a nitrogen stream and reconstituted in 300 µl 5 mM ammonium acetate in methanol. 70 µl of this sample solution was diluted 7:10 with water and then transferred to Eppendorf microplates for LC-MS/MS analysis.

2.8 LC-MS/MS analysis

We performed all MC-MS/MS analyses on a triple quadrupole 3200 Q Trap LCMS/ MS system (AB Sciex, Framingham, MA) with a 1200 Series binary pump, a degasser, and a

column oven (Agilent, Santa Clara, CA). Samples were injected by an HTC pal autosampler (CTC Analytics, Zwingen, Switzerland). The parameters of the ion spray source operating in positive ESI mode were: curtain gas, 20 psi; collision gas, medium; ion spray voltage, 5500 V; temperature, 500 °C; ion source gas 1, –40 psi; ion source gas 2, 50 psi. Chromatographic separation was performed using a VDSpher 100 PUR C18-SE column (length 150 mm, internal diameter 3.0 mm, particle size 3.5 µm; VDS optilab), at a column temperature of 50 °C. Eluent A was 0.2% formic acid in water, and eluent B was 0.2% formic acid in acetonitrile. Gradient elution comprised the following steps: a linear decrease from 98% to 60% A at 500 µl/min over 6 min, hold for 2 min, followed by a linear decrease to 0% A at 500 µl/min over 7 min. Re-equilibration was achieved by a linear increase to 98% A at 500 µl/min over 1 min, increase of flow rate to 800 µl/min over 1 min, hold for 2 min, and decrease to 500 µl/min over 1 min. Accordingly, the total running time was 20 min. Analytes were quantified in scheduled multiple reaction monitoring (MRM) with a target scan time of 1 s and a detection window of 60 s, and quadrupoles were set to unit resolution.

2.9 Calibration and quantification of LC-MS/MS analysis

Calibration was performed by spiking control plasma with certain concentrations of amino acids and di- and tripeptides. We conducted a seven-point calibration by adding increasing amounts of each standard, as well as internal standard. The calibration curves were drawn and fitted by linear regression through zero with 1/x weighting. Analyst 1.5 software (AB Sciex) was used for data analysis.

2.10 Statistical analysis

Areas under curve (AUC) of plasma concentrations were determined with GraphPad PRISM 5.00 (GraphPad Software, Inc.). One-way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was applied for the comparison of AUC and maximum values regarding plasma concentrations, as well as oligopeptide concentrations in cell culture experiments. p-

values <0.05 were considered statistically significant. The comparison of concentrations and molar quantities of di- and tripeptides and amino acids within groups was performed by one-way ANOVA with post hoc Bonferroni's Multiple Comparison Test, while comparisons between groups were performed by two-way ANOVA with post hoc Bonferroni's Multiple Comparison Test. GraphPad PRISM 5.00 (GraphPad Software, Inc.) was used for the linear regression analysis of molar peptide quantities. Outliers were identified based on Cook's distance, with Di > 4/n, and p-Values < 0.05 were considered statistically significant.

3 Short description of publications[1]

3.1 Publication 1: Proton Coupled Oligopeptide Transporter 1 (PepT1) Function, Regulation, and Influence on the Intestinal Homeostasis

SLC15A1, the peptide transporter 1 (PEPT1) is the key transporter for the small intestinal uptake of di- and tripeptides as well as structurally similar drugs like β-lactam antibiotics and angiotensin-converting enzyme (ACE) inhibitors. Since its molecular identification in 1994, hundreds of studies have focused on different aspects of PEPT1. This review on PEPT1 focuses primarily on recent findings regarding the regulation of PEPT1 gene and protein expression as well as PEPT1's role in health and disease, and it outlines the transporter's expression pattern, substrate specificity, 3D structure, as well as its interaction with other proteins.

Personal contribution: Florian Rohm prepared the chapters "Role of PepT1 in Intestinal Homeostasis in Health and Disease" and "PepT1 Deficiency Models" of this review article.

Spanier B, Rohm F. Proton Coupled Oligopeptide Transporter 1 (PepT1) Function, Regulation, and Influence on the Intestinal Homeostasis. Compr Physiol. 2018;8(2):843-869.

3.2 Publication 2: Appearance of Di- and Tripeptides in Human Plasma after a Protein Meal Does Not Correlate with PEPT1 Substrate Selectivity

Many aspects of the peptide transporter SLC15A1 (PEPT1) have been revealed in numerous studies, including the transporter's expression pattern and its gene expression regulation, its substrate specificity and the affinities of various substrates. However, little is known about the physiological role of PEPT1 in the assimilation of dietary protein by absorbing di- and tripeptides generated during intestinal protein breakdown. In order to enable us to assess concentrations of these PEPT1 transport substrates in biological samples, we established an

[1] See 6.1 for the original publications and 6.2 for the corresponding letters of approval

LC-MS/MS-based method for the quantification of di- and tripeptides. Based on a peptide panel that is representative for the 400 possible dipeptides from the 20 proteinogenic amino acids in terms of their constituents' size, charge, side-chain polarity, and side-chain class, we applied this LC-MS/MS method in a human study, assessing peptide plasma levels in fasting state and after the intake of different amounts of chicken breast. We could demonstrate significant amounts of di- and tripeptides in human peripheral blood in fasting state. In addition, we found dose- and time-dependent postprandial increases for the majority of the panel peptides in plasma, whose spectrum does however not match their affinity to PEPT1. This suggests that the type and quantity of peptides reaching circulation is influenced by their resistance to hydrolysis by pancreatic, brush border, and cytosolic peptidases. Furthermore, many of the dipeptides whose plasma concentrations increased postprandially featured N-terminal glycine, which is generally associated with a higher resistance to hydrolysis. The contribution of the peptides' affinity to PEPT1 and their stability against hydrolysis, respectively, to their appearance in peripheral blood remains unclear, however, and warrants further research.

Personal contribution: Florian Rohm developed the LC-MS/MS method, performed the LC-MS/MS plasma analysis, and contributed substantially to the interpretation of the data and the writing and revising of the manuscript.

Rohm F, Skurk T, Daniel H, Spanier B. Appearance of Di- and Tripeptides in Human Plasma after a Protein Meal Does Not Correlate with PEPT1 Substrate Selectivity. Mol Nutr Food Res. 2019;63(5):e1801094. Copyright Wiley-VCH GmbH. Reproduced with permission.

3.3 Publication 3: Transport Versus Hydrolysis: Reassessing Intestinal Assimilation of Di- and Tripeptides by LC-MS/MS Analysis

The respective contributions of absorption of peptides from dietary protein via PEPT1 and their hydrolysis in dietary protein assimilation have not been identified. In order to shed

further light on the fate of di- and tripeptides released from dietary protein during intestinal digestion, we applied a previously developed LC-MS/MS-based method and a representative peptide panel for the quantification of peptides in several intestinal models *in vitro* and *in vivo*. These models ranged from Caco-2 cells to PEPT1 deficient mice to human mucosa from different intestinal segments and allowed us to simultaneously assess peptide transport by PEPT1 and hydrolysis. Across all models, we observed a wide range of susceptibility to hydrolysis amongst the panel peptides. Certain peptides were highly susceptible to hydrolysis and featured low transport rates in intact form, while others were stable against hydrolysis and featured higher PEPT1-dependent transport rates in intact form. Overall, the patterns of panel peptides with a high susceptibility and resistance to hydrolysis, respectively, were highly similar across all tested models. Susceptibility to hydrolysis appears to be structure-dependent. Peptides with less polar amino acid residues in N-terminal position feature reduced resistance to hydrolysis and low rates of transport. Other panel peptides with a high stability to hydrolysis on the other hand are dependent on PEPT1 for crossing the small intestinal mucosa. Besides these regular peptides, we could also demonstrate hydrolysis resistance associated with PEPT1 dependency for several ɣ-glutamyl-dipeptides and aminocephalosporins. Interestingly, translocation rates of members of either group of PEPT transport substrates varied greatly despite similar structure and PEPT1 affinity.

Personal contribution: Florian Rohm contributed substantially to the design of the study, performed all experiments and LC-MS/MS analyses, and contributed substantially to the interpretation of the data and the writing and revising of the manuscript.

Rohm F, Daniel H, Spanier B. Transport Versus Hydrolysis: Reassessing Intestinal Assimilation of Di- and Tripeptides by LC-MS/MS Analysis. Mol Nutr Food Res. 2019;63(21):e1900263. https://doi.org/10.1002/mnfr.201900263

4 Discussion

4.1 Major findings

We aimed to better define the role of PEPT1 in the assimilation of dietary protein in the form of the absorption of dietary amino acids as di- and tripeptides. Based on a newly developed LC-MS/MS-based method for the quantitative analysis of di- and tripeptides we assessed the fate of a panel of 20 dipeptides defined as representative for the possible 400 dipeptides from proteinogenic amino acids in terms of size, charge, and polarity, as well as two tripeptides. In a human study, we showed that di- and tripeptides, including ɣ-glutamyl-peptides, are present in considerable concentrations in systemic circulation in fasting states [249]. In addition, we observed dose-dependent postprandial increases in plasma for half the peptide panel after the intake of a common source of dietary protein, chicken breast. Plasma levels in general however did not reflect the affinity of these peptides for interaction with PEPT1, leading to the conclusion that the rate of hydrolysis and thus the liberation of the peptides from protein during digestion and the uptake into the tissue vary greatly and are highly structure-dependent.

Applying the developed method for peptide quantification in several different models, we assessed intestinal transport and hydrolysis of di- and tripeptides *in vitro*, *ex vivo*, and *in vivo* [250]. Results confirmed the other findings regarding transport and hydrolysis of peptides as depending on structure and composition. Peptides with less polar amino acid residues in N-terminal position are usually hydrolysed to a greater extent rather than transported and are thus mostly not absorbed in intact form, while in case of peptides with higher stability against hydrolysis, like certain glycyl dipeptides, Pro-Gly, and ɣ-glutamyl-dipeptides, PEPT1-dependent transport dominates over hydrolysis. Also, intestinal transepithelial transport of xenobiotic substrates like aminocephalosporins is dependent on PEPT1, while translocation rates of structurally nearly identical substrates may vary greatly despite comparable affinities for PEPT1.

4.2 Role of PEPT1 in nutrition

Together with intestinal amino acid transporters, the peptide transporter SLC15A1 (PEPT1) holds a critical position in dietary protein assimilation. Many aspects of PEPT1 structure and transport mechanism are well characterized and its expression pattern in the small intestine is well established, yet expression in colon is still discussed controversially in literature. PEPT1's role in nutrient sensing acting as a transceptor has been described, as well as its regulation and interaction with other proteins [141]. Its substrate specificity and affinities of various substrates have been assessed in numerous studies, while ongoing research is focused on identifying PEPT1's crystal structure, which has already succeeded for several bacterial peptide transporter isoforms [251]. The structural elucidation of bacterial PEPT1 homologues in complex with transport substrates has enabled a good understanding of the interaction between PEPT1 type proteins and substrates [252, 253].

Despite the ever-growing knowledge regarding PEPT1 accumulated from research of almost half a century, little is known to date about the transporter's physiological role, i.e., the extent of dietary amino acids absorbed in the form of di- and tripeptides as compared to free amino acids absorbed by amino acid transporters across the intestinal brush border membrane. The luminal absorption of amino acids bound in di- and tripeptides via PEPT1 is believed to distinctly exceed the intestinal absorption of free amino acids by 75%, presumably due to PEPT1's high capacity paired with its high expression in the small intestine [201, 207]. On the other hand, based on the increases we observed postprandially for plasma amino acids and plasma di- and tripeptides, our human study suggests that most dietary protein is hydrolyzed into free amino acids before reaching circulation [249]. Little is also known about the total quantity and physiological role of di- and tripeptides in circulation, as well as the types of di- and tripeptides that escape intestinal hydrolysis and reach circulation. It is unknown if the intestinal uptake of peptides via PEPT1 and their presence in circulation is a) an alternative uptake pathway when the quantity of amino acids after a protein-rich meal

exceeds the capacity of intestinal amino acid transporters, or b) if plasma peptides from dietary protein actually have an additional function beyond the nutritional value of their constituent amino acids, e.g., in the form of bioactivity, which has been reported for numerous di- and tripeptides as discussed above. The fact that PEPT1 deficient mice are phenotypically normal on a standard diet and only develop a phenotype different from wild-type animals on high-protein diets [244] indicates that plasma peptides from dietary protein are not essential for metabolism. Moreover, di- and tripeptides escaping hydrolysis from cytosolic peptidases are usually broken down rapidly by endothelial peptidases and plasma peptidases and often only have a very short half-life after reaching circulation [213, 254]. On the other hand, while plasma peptides are assimilated predominantly by kidney and liver [255, 256], cytosolic peptidases as well as PEPT1 and other peptide transporting members of the SLC15 family, namely PEPT2 (SLC15A2), PhT1 (SLC15A4), and PhT2 (SLC15A3), are expressed in many tissues, mainly the intestine, kidney, and brain, but also lung, spleen, pancreas, liver, and bile duct [257]. An organism's efforts of this extent to facilitate the uptake of small peptides into various tissues system-wide and to equip cells with the tools to break down these peptides suggests that intestinal di- and tripeptide absorption may have a role that goes beyond a mere overflow mechanism during excess amino acid supply. In addition, certain pathophysiological states illustrate PEPT1's importance for dietary protein assimilation, e.g., diseases like Hartnup disease that are associated with a loss of function of essential apical amino acid transporters. Here, PEPT1 can completely cover the defective transporter's function by facilitating the corresponding amino acids' uptake in the form of di- and tripeptides, a fact that contributed significantly to the identification of PEPT1 (see **1.3.5**).

Plasma di- and tripeptides do not originate exclusively from the assimilation of dietary protein and uptake via PEPT1: In fasting states, we observed stable plasma levels of di- and tripeptides that result in an estimated cumulative plasma concentration of 20 µM for dipeptides and 10 µM for tripeptides [249]. Small amounts of larger intestinal oligopeptides

may reach circulation via alternative pathways, e.g., paracellular diffusion or transcytosis (see **1.3.4**), after which they may become subject to hydrolysis by endothelial and soluble plasma peptidases, and contribute to plasma peptide concentrations. Also, endogenous intracellular protein is constantly degraded by cytosolic peptidases, and breakdown products may cross the plasma membrane into circulation, just like proteins and peptides that are continuously being hydrolyzed in the blood by plasma and endothelial peptidases.

While transporter affinity has been determined for a considerable number of PEPT1 transport substrates *in vitro* and in model systems [141], the pattern of peptides we observed postprandially in systemic circulation cannot be explained by their affinity to PEPT1 [249]. The appearance of a given peptide in plasma after a meal depends on several factors: the presence of the corresponding amino acid sequence in the dietary protein, and the resilience of the involved peptide bonds towards hydrolysis in the intestinal lumen, the enterocytes' cytoplasm, as well as in blood vessels. Due to the wide range of pancreatic and brush border membrane peptidases, it is currently impossible to predict a pattern of distribution of protein breakdown products in the chyme, i.e., which amounts and types of di- and tripeptides and amino acids will result from hydrolysis of a given protein and thus be available for absorption by peptide and amino acid transporters. Moreover, cytoplasmatic peptidases, plasma and endothelial peptidases, as well as the first pass effect in liver render the pattern of postprandial plasma peptides in systemic circulation even more unpredictable. Early uptake studies that demonstrated for the first time dipeptides to be able to cross the intestinal mucosa utilized mainly glycyl peptides administered directly into the intestine [189]. These glycyl peptides were chosen due to their established generally higher resistance towards peptidases to ascertain their detection in circulation [250, 258]. This is also reflected by the increases in postprandial peptide plasma levels we observed after a protein meal in our human study [249]. Accordingly, glycyl peptides in our studies also showed higher resistance towards hydrolysis, while dipeptides with hydrophobic constituents overall featured rapid

hydrolysis. Nevertheless, this high intrinsic hydrolysis resistance does not appear to apply to all glycyl dipeptides as we unexpectedly did not observe any increases in postprandial Gly-Pro plasma concentration. In addition, we demonstrated that this glycyl dipeptide is susceptible to hydrolysis in all our models [249, 250] despite its peptide bond involving proline, which usually is assumed to render a dipeptide more resistant to hydrolysis [81]. These observations question the use of the radiolabeled tracer substrates commonly used in peptide transport studies like [14C]Gly-Pro [259, 260]. To a certain extent, this also applies to [3H]Gly-Gln [261], as we did observe postprandial plasma increases of Gly-Gln, yet it is subject hydrolysis [249, 250].

Although frequently used in uptake studies, glycyl peptides are not representative for the entire range of dietary PEPT1 substrates that may arise from the hydrolysis of dietary protein. In theory, 400 different dipeptides and 8000 different tripeptides constituted of the 20 proteinogenic amino acids can be released from dietary protein, almost all of which are transport substrates of PEPT1. Their transport affinity depends on several factors, including side-chain size, stereospecificity, polarity, and charge [204]. Tracer flux studies using radiolabeled dipeptides (competition assays) as well as electrophysiological transport studies are indirect methods for assessing transport of intact peptides via PEPT1. and peptides subject to hydrolysis and the subsequent uptake of resulting free amino acids by intestinal amino acid transporters. In addition, studies determining affinities are often performed using heterologous expression systems, e.g., *Xenopus laevis* oocytes. These experiments may reveal interactions between transporter and substrate and allow the assessment of affinities, yet the absence of membrane peptidases in these systems does not allow conclusions to be drawn on whether such a peptide may ever interact with PEPT1 in the intestine [262, 263]. We showed that a high affinity for PEPT1 as observed in such studies is not necessarily associated with high transepithelial flux rates and high postprandial plasma concentrations *in vivo*, while a low PEPT1 affinity does not automatically mean that a substrate will show low

transepithelial flux [250]. Transepithelial transport of intact peptides under physiological conditions (as after a protein meal) appears to be determined to a great extent by peptide composition: An unpolar N-terminal amino acid residue is often associated with a higher susceptibility towards hydrolysis and therefor lower rates of uptake via PEPT1.

Despite the physiological role of PEPT1 being not entirely clear, its importance in clinical nutrition is well recognized for enteral and parenteral nutrition. The human organism's ability to efficiently metabolize di- and tripeptides, mainly in liver and kidney but also many other tissues (see above), allows the patients' efficient nutrition via peptide solutions, which are superior to amino acid solutions due to their higher stability and solubility compared to free amino acids [264, 265].

4.3 Pharmacological aspects of PEPT1

Besides peptides from dietary protein, PEPT1 is important for the oral bioavailability of dietary peptide supplements as well as a wide range of drugs that are structurally related to peptides, including angiotensin converting enzyme (ACE) inhibitors, a broad spectrum of beta-lactam antibiotics, and certain cancer therapeutics [205, 266]. Due to the improved understanding of PETP1's prodrug recognition, PEPT1 has come further into the focus of pharmaceutical research. Scientists are now able to specifically modify substances to become better targets for PEPT1 transport, e.g., by joining an amino acid residue to a drug molecule in order to greatly enhance the resulting prodrug's bioavailability compared to the drug [267]. For instance, in case of the antiviral valacyclovir, a prodrug derivative of acyclovir used in the treatment of e.g., herpes zoster, joining the original drug to the amino acid valine and thus enabling transport by PEPT1 improved its oral bioavailability from 15% to more than 50% [268]. Our aminocephalosporin transport studies suggest that, despite similar affinities to PEPT1 predicted *in silico* or determined *in vitro* based on highly similar chemical structures [269-271], actual PEPT1 transport rates of these structurally similar compounds

may vary distinctly [250]. This demonstrates the importance of assessing the bioavailability of PEPT1 transport substrates in *in vivo* studies.

Besides transport of xenobiotics via PEPT1, there is also a growing interest in transport of food-derived PETP1 substrates. In many dietary proteins, sequences of peptides have been discovered that are believed to elicit biological effects in the host. Over the past decades, numerous such effects have been attributed to these bioactive peptides once released from their protein precursors during digestion, expanding the role of dietary protein beyond its mere nutritional value as a source of nitrogen and essential amino acids [272-277]. For example, a review in 2012 listed more than 130 antihypertensive peptides from numerous protein sources with different proposed mechanisms of action [278], most of which involve ACE inhibition or the interaction with opioid receptors as agonists or antagonists [279]. Of these dietary bioactive peptides, the tripeptides Val-Pro-Pro and Ile-Pro-Pro derived from casein are amongst the most frequently discussed peptides for which antihypertensive properties have been described [211], the former of which we included in the peptide panel that we applied in our studies. These lactotripeptides are often claimed to exert their effect via ACE inhibition, e.g., after reaching systemic circulation and subsequent uptake into vascular endothelial cells [280, 281]. However, most studies postulating antihypertensive effects of peptides derived from milk proteins are performed *in vitro*, applying analyte concentrations in the high micromolar, sometimes even millimolar ranges for long incubation times of up to 24 hours, or in animal models, which makes the transfer of the observed effects to humans *in vivo* appear questionable [211, 262]. Plasma concentrations required for ACE inhibition of these lactotripeptides are estimated to be in the micromolar range [282]. The question remains if this bioactivity described in *in vitro* assays can also be achieved in humans by reaching physiologically relevant levels in plasma. *In vivo* studies assessing bioavailability and plasma concentrations of these proline-rich tripeptides demonstrated only a very low bioavailability and a short half-life: In pigs, bioavailability of Val-Pro-Pro and Ile-

Pro-Pro was 0.1%, and the peptides' half-lives in plasma were no longer than approx. 10 minutes [283]. Peak plasma concentrations only reached nanomolar ranges, attributed to the hydrolytic activity of pancreatic, brush border membrane, and cytosolic peptidases. For Val-Pro-Pro, we observed a high susceptibility towards hydrolysis in all our employed models, including human intestinal mucosa, which appears to confirm this assumption. A human study reported even lower postprandial plasma concentrations for Val-Pro-Pro and Ile-Pro-Pro of below 1 pmol/ml [284]. Accordingly, in our human study using meat as a protein source, we did not observe any plasma concentration increases for Val-Pro-Pro, which may of course be due to a lower abundance of the tripeptide sequence in chicken meat than in milk protein [249]. Consequently, the observed maximum plasma concentrations in the above-mentioned studies are several orders of magnitude lower than those needed for ACE inhibition *in vivo*. For reference, the bioavailability of the common drug ACE inhibitors captopril and enalapril ranges at 60-70% and their elimination half-lives are 1.5 hours for captopril and several hours for enalapril [285]. The inhibitory constants (IC_{50}) of these ACE inhibitors are in the nanomolar range for captopril and the micromolar range for enalapril, respectively [286]. Considering the minimal oral bioavailability, the very short plasma half-life, and the low plasma concentrations of oral Val-Pro-Pro and Ile-Pro-Pro under physiological conditions, it seems improbable that any antihypertensive effects of lactotripeptides via ACE inhibition observed *in vitro* and in model systems can be achieved in humans *in vivo*. Nevertheless, there are few *in vivo* studies in humans on antihypertensive effects of orally provided lactotripeptides: Hypertensive participants who received different dosages of oral Val-Pro-Pro and Ile-Pro-Pro displayed dose-dependent systolic blood pressure decreases, although no reduction in diastolic blood pressure was observed [287]. In another, albeit not placebo-controlled, clinical study, hypertensive participants showed reduced systolic and diastolic blood pressure after Val-Pro-Pro and Ile-Pro-Pro ingestion [281]. A possible alternative explanation for the observed blood pressure lowering effects of dietary bioactive

peptides is their effects being mediated by intestinal receptors [288, 289]. This interaction of bioactive peptides with intestinal receptors seems conceivable because this mode of action has been demonstrated for other types of dietary bioactive peptides: Besides antihypertensive proline-rich tripeptides, milk proteins contain a wide range of sequences of peptides for which bioactivity has been shown, including around 50 peptides with blood pressure lowering effects [278]. Many of these milk protein fragments are small peptides that interact with opioid receptors in the intestinal wall. These opioid receptor ligands, also referred to as exorphins, which are also found in other sources of dietary protein like gluten and meat, may act as receptor agonists or antagonists [290, 291]. Exorphins are peptides with more than 3 amino acid residues, so intact absorption via PEPT1 is not required for exerting their biological effects [211], which mainly involve effects on intestinal function and blood pressure [278, 290].

Our studies clearly demonstrate the importance of hydrolysis over transport of di- and tripeptides in overall bioavailability and that is relevant also in the context of potential effects of bioactive peptides derived from food. Our findings regarding the limited stability of Val-Pro-Pro to intestinal hydrolysis that we observed across all models – which supports the low bioavailability of proline-rich lactotripeptides demonstrated in *in vivo* studies – ask for alternative explanations and mechanisms of action of the observed antihypertensive effects in humans.

4.4 Impact of intestinal brush border peptidases on amino acid homeostasis

A multitude of intestinal brush border peptidases has been identified, and for many of them, small intestinal expression in humans is well described in literature, showing an overall differential expression along the small intestine: Enteropeptidase for instance is expressed mainly in the duodenum, where it activates the pancreatic protease cascade [70], while the expression of aminopeptidases and DPPIV is strongest in distal parts of the small intestine

[68, 69]. Interestingly, these individual differences in expression of small intestinal peptidases described in literature were not reflected by differences in the hydrolytic capacity of duodenum and ileum in our *ex vivo* studies, where hydrolysis patterns were very similar in human samples from both small intestinal segments, but also in mouse samples and Caco-2 cells.

In these assays, we also observed a remarkable digestive capacity for peptides in mucosa from mouse and human colon. As detailed above, a considerable amount of dietary protein, together with endogenous protein, escapes small intestinal digestion [125], so it is not surprising that the organism provides the capacity to assimilate these proteins, together with endogenous and bacterial protein released in the colon, by expressing brush border membrane peptidases to assist the remaining intraluminal pancreatic proteases in the digestion of these proteins [176]. While colonic peptidase expression and activity in humans has not been systematically assessed to our knowledge, few studies have demonstrated expression and activity of certain brush border membrane peptidases like APN, APA, and DPPIV as well as cytosolic peptidases in human colonocytes [129, 130]. Nevertheless, a significant contribution of human colon to overall protein assimilation is not generally assumed [127]. As expected, overall peptidase activity as well as peptidase activity against the provided individual peptides was reduced in colonic as compared to small intestinal mucosa samples both in mice and in humans [250]. However, we unexpectedly observed a spectrum of peptidase activity in colon samples that was very similar to small intestinal peptidase activity observed across all our models. This capacity for peptide hydrolysis of colon is even more remarkable when considering the smaller absolute surface area of colonic mucosa samples when compared to small intestinal mucosa samples of the same size: In the small intestine, villi, which are absent in colonic mucosa, increase the mucosal surface by a factor of ~6.5, in addition to microvilli that increase the surface area to a greater extent in the small intestine than the fewer and shorter microvilli in colonic mucosa [292].

Overall, our studies suggest that protein reaching the human colon is targeted by a wide range of colonic brush border membrane peptidases that have the capacity to break it down into di- and tripeptides and free amino acids, which may then be further processed by colonocytes upon uptake via PEPT1 and amino acid transporters, as well as utilized by gut microbiota.

4.5 Strengths and limitations

To our knowledge, the present studies were the first in using an LC-MS/MS method for the systematic quantification of dipeptides in biological samples based on a representative peptide panel. For the first time, the dose-dependent postprandial appearance of peptides in plasma after the consumption of a common source of dietary protein could be observed and quantified in humans. At the same time, the presence of considerable amounts of plasma di- and tripeptides could be demonstrated in fasting states in humans. In addition, this newly developed quantification method enabled us to simultaneously study transport and hydrolysis of the panel peptides in different intestinal models. We are confident that the approx. 20 dipeptides in our panel are well representative for the possible 400 dipeptides from proteinogenic amino acids, yet with the exception of cysteine-containing dipeptides. Due to the easily oxidized sulfhydryl group in cysteine, e.g., by the formation of disulfide bonds, cysteine-containing peptides were not included in the panel. Also, individual dipeptides may not act in accord with expectations, as was the case with Gly-Pro and Pro-Gly, so further studies using a panel expanded by additional dipeptides could consolidate our findings. Furthermore, the two panel tripeptides out of the 8,000 possible tripeptides from proteinogenic amino acids do not allow any solid assertions regarding intestinal tripeptide assimilation in general, for which an expansion of the peptide panel by a representative set of tripeptides is required.

Some of our studies featured conditions that were not physiological in some degree. In the mouse gavage experiments, Gly-Pro and Pro-Gly were applied in unphysiologically high concentrations close to the dipeptides' maximal solubility in water that are not achievable through regular diet. Also, incubation times of the transport studies in Caco-2 cells were rather long with up to 6 hours. Both parameters were chosen in order to ascertain that changes in peptide concentrations were above detection limits. While our models allowed to quantify changes in extracellular peptide and amino acid concentrations, intracellular concentrations and hydrolytic processes, e.g., in enterocytes in our *ex vivo* experiments or in the cytoplasm of Caco-2 cells, eluded our assessment. However, despite these occasionally artificial conditions, the effects we observed in all our models regarding individual peptides as well as the peptide panel were almost identical, suggesting that Caco-2 cells and mouse intestines are valid models for human intestinal peptide transport and hydrolysis.

Overall, the expansion of our LC-MS/MS analysis to a larger peptide panel and its application in further human nutrition studies could greatly enhance our knowledge regarding intestinal protein assimilation, help researchers evaluate claims regarding biological effects of bioactive peptides, and possibly develop peptide supplements tailored for states of protein or amino acid deficiency.

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6 Appendix

6.1 Original publications

Proton Coupled Oligopeptide Transporter 1 (PepT1) Function, Regulation, and Influence on the Intestinal Homeostasis

Britta Spanier[*1] *and Florian Rohm*[1]

ABSTRACT

As the organ with one of the largest surface areas facing the environment and responsible for nutrient uptake, the small intestine expresses numerous transport proteins in its brush-border membrane for efficient absorption and supply of dietary macro- and micronutrients. The understanding of regulation and functional interplay of these nutrient transporters is of emerging interest in nutrition and medical physiology research in respect to development of diabetes, obesity, and inflammatory bowel disease worldwide. The peptide transporter 1 (PepT1, SLC15A1) is abundantly expressed particularly in the intestinal tract and provides highly effective transport of amino acids in the form of di- and tripeptides and features a substantial acceptance for structurally related compounds and drugs. These characteristics bring PepT1 into focus for nutritional and medical/pharmaceutical approaches, as it is the essential hub responsible for oral bioavailability of dietary protein/peptide supplements and peptide-like drugs in eukaryotic organisms. Detailed analysis of molecular processes regulating PepT1 expression and function achieved in the last two decades has helped to define and use adjusting tools and to better integrate the transporter's role in cell and organ physiology. In this article, we provide an overview of the current knowledge on PepT1 function in health and disease, and on regulatory factors modulating its gene and protein expression as well as transport activity. © 2018 American Physiological Society. *Compr Physiol* 8:843-869, 2018.

Didactic Synopsis

Major teaching points

1. Peptide transporter PepT1 is responsible for absorption of di- and tripeptides and of numerous structurally related peptidomimetic drugs into intestinal epithelia cells and regulates their bioavailability.

2. The di- and tripeptides uptake is driven by the membrane potential in form of an inward-directed proton gradient which is maintained by sodium-proton exchanger NHE3.

3. PepT1 expression and function is controlled at the transcriptional, translational, and post-translational level.

4. Systemic changes and compensatory processes after the loss of PepT1 are studied in various models including *Caenorhabditis elegans*, *Mus musculus*, and the human colon carcinoma cell line Caco-2.

5. PepT1 is involved in intestinal homeostasis regarding metabolite profiles and tissue physiology, both in health and disease (inflammatory bowel disease, obesity, diabetes, and celiac disease).

Introduction

Metabolism, protein synthesis, and cell growth are dependent on the supply of nitrogen. The plasma membrane functions as a barrier for amino acids, peptides, and other hydrophilic nutrients. Consequently, a large spectrum of membrane transporter proteins for these molecules has emerged during evolution. For efficient transport of amino acids and small peptides, various specialized amino acid transporters in parallel to a single oligopeptide transporter (Pept1) are expressed in the mucosa of the small intestine, the organ with one of the largest contact areas with the environment (79). PepT1 belongs to the solute carrier (SLC) family SLC15A, also referred to as the proton-coupled oligopeptide transporter (POT) family, which includes four members in eukaryotes: the well-characterized proton-dependent oligopeptide transporters PepT1 (SLC15A1) and PepT2 (SLC15A2) (for review

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see (47, 160)), and the peptide/histidine transporters PhT1 (SLC15A4) and PhT2 (SLC15A3). The latter enable the efflux of histidine and selected di- and tripeptides from lysosomes into the cytoplasm and are involved in pathogen sensing (129). PepT1, the predominant intestinal isoform, and PepT2, the predominant renal isoform, transport almost all di- and tripeptides and various peptidomimetics like β-lactam antibiotics, specific angiotensin-converting-enzyme (ACE) inhibitors, protease inhibitors, and antivirals (148), making both peptide transporters considerably important for pharmaceutical and medical approaches. However, free amino acids as well as peptides consisting of more than three amino acids do not constitute accepted substrates for either peptide transporter. The export of selected, metabolically stable small peptides and drugs into the blood stream has been demonstrated (14), but the transporter for these molecules in the basolateral cell membrane has not been characterized yet. On background of the rising incidence of metabolic disorders including obesity, type 2 diabetes, and inflammatory bowel disease (IBD) in almost all industrial countries worldwide, there is growing interest in a better understanding of the regulation and function of oligopeptide transporter PepT1.

Since the last overview article about the transcriptional and functional regulation of PepT1 in eukaryotes published in 2014, more than 50 new studies about oligopeptide transporters have shed light on various aspects regarding structure, function, and control of expression. In the present more detailed review, we will focus on the (new) roles of PepT1 in the intestinal epithelium, and summarize the current knowledge about regulatory processes that modulate gene and protein expression as well as function in respect to intestinal homeostasis.

Function of PepT1

For many decades it was believed that the gastrointestinal proteinases and peptidases hydrolyze dietary proteins completely into free amino acids, which are then absorbed into the cells, serving as substrates in central metabolism and as building blocks for protein *de novo* synthesis. In studies performed in the 1960s and 1970s, it became more and more obvious that not only free amino acids as monomeric breakdown products are released into the intestinal lumen during the digestion of dietary proteins but also remarkably high amounts of oligopeptides (3). Most of these oligopeptides are then further hydrolyzed by pancreatic proteases and membrane-associated endo-, amino-, and carboxypeptidases into di- and tripeptides and free amino acids, the latter serving as substrates for a variety of amino acid transporters.

The proton-coupled absorption of di- and tripeptides by specific peptide transporters in the plasma membrane of renal and intestinal epithelia was proven in brush-border membrane vesicles (BBMVs) prepared from kidney and small intestinal tissues (66, 125). In the 1990s, the intestinal oligopeptide transporter PepT1 from various eukaryotes was cloned and characterized (20, 62, 63, 110, 151). With that, the discussion about the existence of parallel transport routes for free and peptide-bound amino acids was practically completed. The ability to absorb free and peptide-bound amino acids via multiple transport proteins ensures an organism's survival also under restricted living conditions by efficient absorption of amino nitrogen from digested protein. Figure 1 shows an overview of the transport proteins for amino acids and di- and tripeptides in the apical and basolateral plasma membrane of intestinal epithelial cells. Various amino acid transporters, including the Na^+-dependent B^0-system for neutral amino acids, the pH-driven PAT1 for glycine and proline, and the $b^{0,+}$ system for the influx of basic in exchange for neutral amino acids, plus the peptide transporter PepT1 facilitate the uptake of dietary amino acids and small peptides across the brush-border membrane of enterocytes. The complex interplay of these transporters in respect to cellular and systemic amino acid homeostasis was recently summarized by Bröer and Bröer (29). However, until today the ratio between absorbed di- and tripeptides on the one side and free amino acids on the other, that is, the transport rate of the peptide transporter compared to the transport rate of all amino acid transporters in the intestinal brush-border membrane is still not fully clarified.

Expression pattern

The task sharing between amino acid transporters on the one hand and peptide transporters on the other has developed early in evolution. In bacteria, except for archeal species, various classes of transporters for di- and tripeptides (PTR) but also for larger oligopeptides (Opp and Dpp) are present (78, 166). These oligopeptide transporters are specific for prokaryotes and cannot be found in eukaryotic cells. All eukaryotic organisms, from yeasts to mammals, express only transport proteins specific for di- and tripeptides. In organisms with a defined tissue distribution, the two peptide transporter isoforms PepT1 and PepT2 can be well discriminated by their expression pattern which is evolutionarily conserved since millions of years (49). PepT2 has a broad expression pattern and can be detected mainly in kidney, but also in various other tissues including brain, neurons, lung, and mammary gland (47). On the other hand, in agreement among many mammalian species PepT1 is abundantly expressed in intestinal cells of duodenum, jejunum, and ileum and in low to very low concentrations in healthy colon (140, 191). PepT1 is also active in the renal proximal tubulus, in the epithelium of the bile duct and can be found in nuclei and lysosomes of pancreas and placenta (17, 68, 100, 161) (Fig. 2). Human PepT1, a 78 kDa membrane protein, consists of 708 amino acids and has around 50% identity and 70% similarity to the sequence of human PepT2. Its amino acid sequence predicts 12 transmembrane domains, with N- and C-terminus facing toward the cytosol. Functional analysis of both peptide transporters in cell culture and in *Xenopus laevis* oocytes revealed isoform-specific transport characteristics: PepT1 is

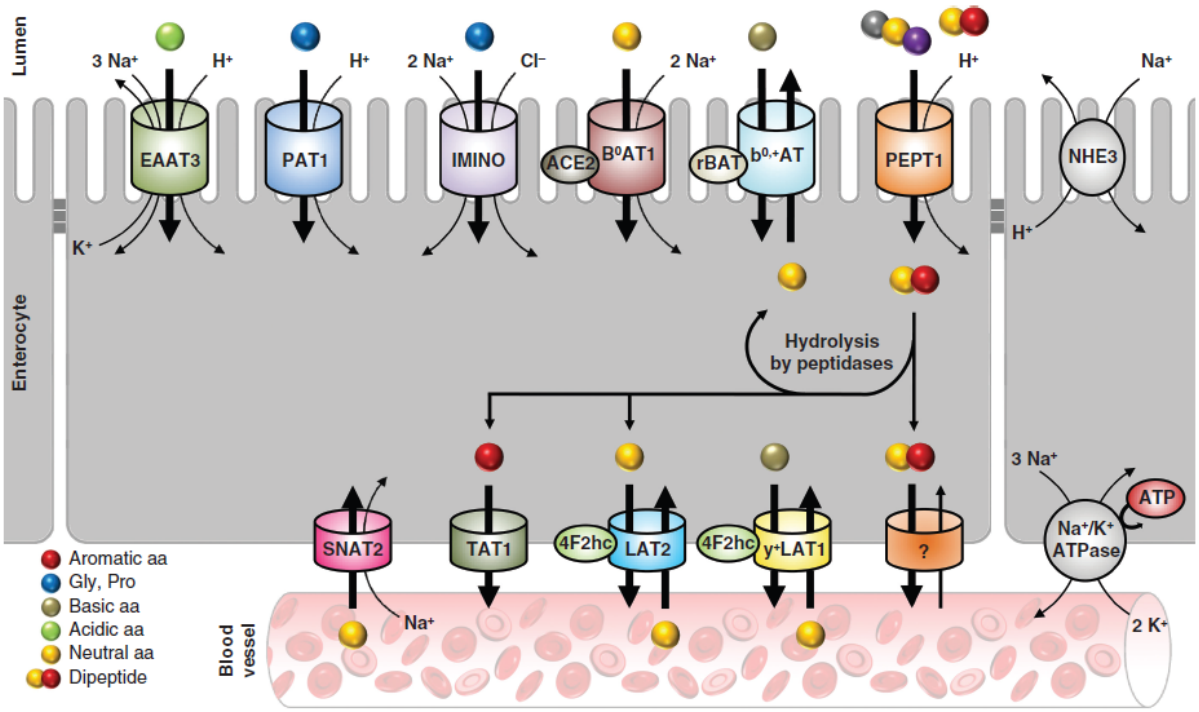

Figure 1 Amino acid and di- and tripeptide transporters in the apical and basolateral membrane of mammalian enterocytes in small intestine. The brush-border membrane contains transport proteins for acidic amino acids (EAAT3), for glycine/proline (PAT1, IMINO), for neutral amino acids (B0AT1/ACE2), for basic in exchange with neutral amino acids (b0,+AT/rBAT), and for di- and tripeptides (PepT1). All heterodimeric amino acid transporters only function in the presence of their heavy chain (ACE2, rBAT, or 4F2hc). Most transporters are electrogenic and couple substrate uptake with ion movement. The function of PAT1 and PepT1 is dependent on the pH gradient between gut lumen and cytoplasm, which is maintained by the sodium-proton exchanger NHE3. The Na^+ gradient on the other hand is stabilized by the Na^+/K^+-ATPase. In the cytoplasm, most peptides are hydrolyzed by numerous cytosolic peptidases and the released amino acids will be exported via basolateral amino acid transporters. TAT1 exports aromatic amino acids, SNAT2 imports neutral amino acids, while LAT2/4F2hc and y+LAT1/4F2hc are responsible for exchange of neutral or basic amino acids against neutral ones. The postulated basolateral peptide transporter that allows release of small peptides and structurally related drugs into the blood stream has not been identified yet.

a low-affinity/high-capacity transporter, while PepT2 represents a high-affinity/low-capacity transporter (62, 147). Both transporters however essentially accept the same substrates. With respect to the tissue distribution of both transport proteins, these characteristics explain the extensive absorption of large amounts of di- and tripeptides from the intestinal lumen into the enterocytes and the highly selective reabsorption of small peptides from the primary urine in the kidney.

Within the intestinal tube, the presence of PepT1 protein follows two gradients: its expression increases from the proximal to the distal part of the small intestine (74, 90). However, the protein expression cannot be directly translated into function, because the pH gradient across the plasma membrane is stronger in the proximal part than in the distal part, leading to a PepT1 transport activity that follows the gradient duodenum > jejunum > ileum. Moreover, on the vertical axis from villus to crypt, the highest transporter expression is detectable at the villus tip, while no expression is detectable in goblet cells and lower crypt cells (140). From an anatomical and physiological point of view, this is a very plausible expression pattern for PepT1 in the intestinal tube, as it allows: (a) a rapid absorption of di- and tripeptides in the proximal gut region, as well as (b) an effective uptake of dietary di- and tripeptides passing the villus tips, which protrude into the intestinal lumen.

The presence of PepT1 in the colon however has been controversially discussed over the past years. While in many studies no or a very low expression of PepT1 protein was observed in healthy colons of mice, rats, and humans (2, 41, 124, 138, 190, 193), the proof for transporter activity in the colon is scarce (74, 191). Although Groneberg and coworkers reported about uptake of the labelled dipeptide D-Ala-Lys-AMCA in mouse gut (74), we demonstrated 12 years later that none of the tested vertebrate PepT1 transporters accept β- or D-Ala-Lys-AMCA as a substrate (104). However, studies in our group detected PepT1 protein in the apical membrane of colonocytes of various healthy wild-type mouse strains, with higher abundance in distal than in proximal colon (167, 191). Yet its expression is significantly lower than in the small intestine. These data were confirmed in colonic samples from C57BL/6 N wild-type mice in comparison to samples from various parts of the small intestine (Fig. 3). The observed differences in colonic PepT1 protein expression might be

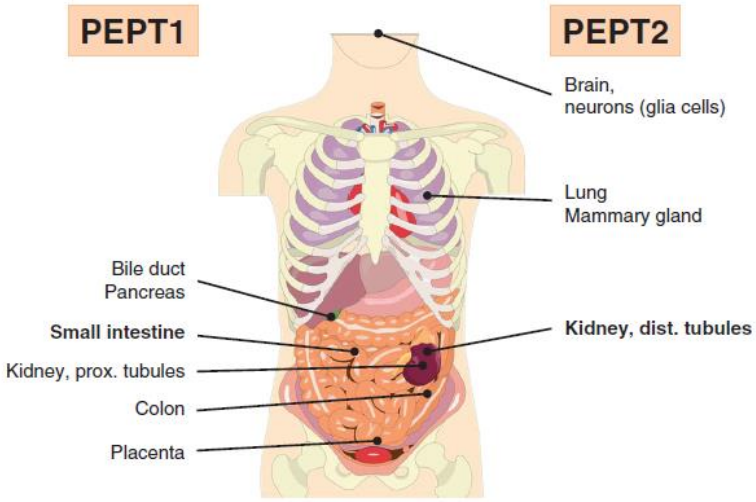

Figure 2 Tissue distribution of peptide transporters PepT1 and PepT2 in humans. The PepT1 protein is mainly expressed in the small intestine, with increasing expression from duodenum to ileum. It can also be found in low concentrations in the distal colon, in the proximal tubulus of the kidney and in bile duct, pancreas, and placenta. PepT2 is the renal isoform, predominantly expressed in the distal tubulus, but also present in brain, glia cells, lung, and mammary gland. The scheme of the human corpus is from an open source (https://pixabay.com).

result of variations in sample preparation, antibody source and specificity, and species differences as discussed in detail by the Smith group (86). The role of PepT1 in the colon in health and disease will be discussed later in this review in the paragraph "PepT1 in inflammatory bowel disease."

Substrate specificity

Oligopeptide transporter PepT1 transports almost all known forms of di- and tripeptides in symport with protons following the inward-directed electrochemical gradient and inside-negative membrane potential, which allows substrate transport against a concentration gradient. Regarding substrate specificity, PepT1's broad acceptance of peptides is noteworthy, nearly independent of their size, net charge, and side-chain anatomy, but with substantial differences in affinity to the transporter's pore, including dietary peptides, peptidomimetic drugs as well as prokaryotic peptides such as fMLF and Tri-DAP (see paragraph "PepT1 in Inflammatory Bowel Disease"). Peptides containing solely L-amino acids

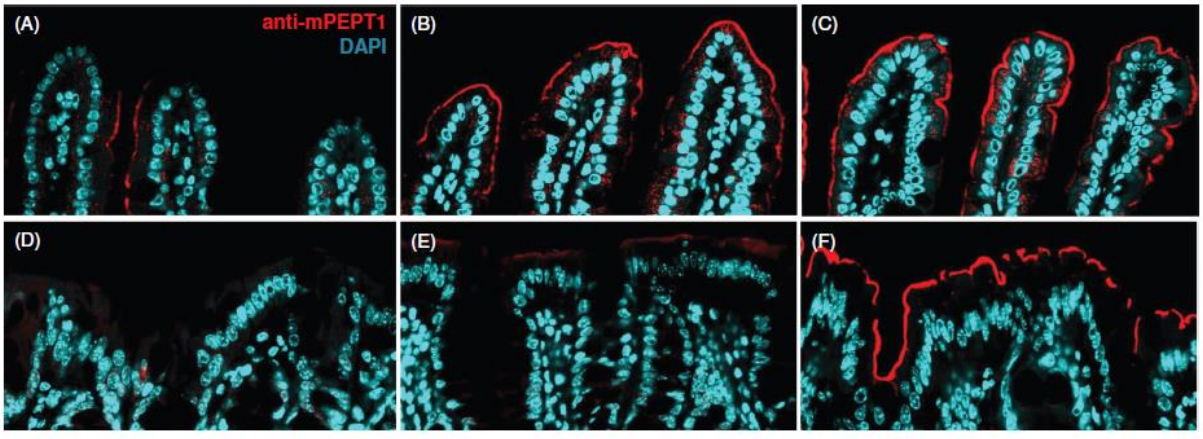

Figure 3 Expression of PepT1 protein along the mouse small intestine and colon. Representative images of duodenum (A), jejunum (B), ileum (C), and proximal (D), middle (E), and distal (F) colon tissue. Nuclei are stained with DAPI (blue), and mouse PepT1 protein expression was detected by custom-made anti-mPepT1 antibody, which was used before (130). PepT1 expression increases from proximal to distal colon.

have the highest affinity to the transporter, while those containing D-amino acids are poorly accepted or not transported at all (48). Moreover, a typical PepT1 and PepT2 substrate should fulfill the following structural features, as summarized by Rubio-Aliaga and Daniel: "... an acidic or hydrophobic C-terminus, a weakly basic group in alpha-position at the N-terminus, a ketomethylene or acid amine bond, and a *trans*-formation of peptide bonds ... " (148). Typical peptides with high transport rates are Gly-Gly, Gly-Gln, Ala-Ala, Ala-Lys, Ala-Asp, Glu-Pro, Ala-Ala-Ala, Ala-Phe-Leu, Leu-Ala-Arg, Val-Ala-Leu, and the model peptide Glycyl-Sarcosine (Gly-Sar) (Table 1), while peptides containing proline at the N-terminus have low affinity and low transport rates due to the bulky side group (23). Contrary to overviews found in literature usually focusing on a single group of PepT1 substrates, Table 1 offers—to our knowledge for the first time—a comprehensive list of different substrate groups of PepT1, including physiological, that is, dietary oligopeptides, synthetic oligopeptides and drugs, as well as competitive transport inhibitors. Note that for a large part of these potential transport substrates, measurements of actual transport activity have not been performed. In these cases, data are based on competition experiments, assessing a compound's affinity for PepT1, which however does not necessarily prove correlating transport activity.

The stoichiometry of the substrate-to-proton ratio is dependent on the charge of the peptide. Mammalian PepT1 is predicted to transport neutral and cationic di- and tripeptides in symport with one proton. On the other hand, anionic peptides are transported with one proton in their neutral form, and with two protons in their charged form. In that case, the second proton neutralizes the charge of the side chain, so that substrate transport via PepT1 always produces a positive inward current, independent of the charge of the peptide (89, 115). These observations were updated by Newstead and colleagues who reported a proton-substrate ratio of 3:1 for tripeptides and an even higher ratio (5-6:1) for dipeptides for the stoichiometry of the bacterial peptide transporter isoform $PepT_{St}$ (143). These data were generated with a reconstituted liposome-based transporter assay. The authors found that at a given pH value more dipeptides are concentrated inside the liposomes than tripeptides, which points to a thermodynamic selection of dipeptides over tripeptides in *Streptococcus thermophilus*.

The substrate transport via PepT1 is strictly dependent on the activity of sodium-proton exchangers of the SLC9 family in the intestinal epithelium to prevent acidification of the cells. While the basolaterally expressed NHE1 and the apical isoform NHE2 both have only minor effects on PepT1, the brush-border membrane Na^+/H^+ exchanger NHE3 is a main regulator of PepT1 function (55). NHE3 is responsible for the majority of intestinal and renal sodium uptake and mediates the electroneutral efflux of protons in exchange with sodium ions which are pumped out basolaterally of the cell via the Na^+/K^+-ATPase (194), which makes the peptide transporter secondary sodium-dependent and tertiary ATP-dependent. A loss of function of PepT1 in mammalian cells in the presence of NHE3 inhibitors and in NHE3 knockout (KO) mice was reported before (37, 144, 184), and can also be observed in the nematode *Caenorhabditis elegans* (12). These data underline the close functional interplay of PepT1 with NHE3.

3D structure

More than 15 years after the first cloning of mammalian peptide transporters passed without detailed information about its three-dimensional (3D) protein structure. However, in the last 5 years knowledge regarding the 3D structure and architecture of peptide transporters and especially of their substrate binding pocket was expanded enormously. RNA or protein isolates from various bacterial species provided a safe basis for preparation of sufficient amounts of recombinant or native peptide transport protein. In 2011, the crystal structure of the bacterial PepT homologue from *Shewanella oniedensis* was published (136), followed by detailed structural analysis of POT family proteins from *Streptococcus thermophilus* (162), *Geobacillus kaustophilus* (54), a second isoform from *Shewanella oniedensis* (75), one from *Escherichia coli* (197), and from *Yersinia enterocolitica* (19). These bacterial peptide transporter isoforms all are organized in 12 transmembrane domains, arranged equally in one N- and one C-terminal bundle and connected via the helical hairpins HA and HB. The current advances regarding PEPT architecture and transporter-substrate interaction were recently reviewed by Newstead (135), and will thus not be discussed in detail in this review.

However, with respect to the eukaryotic and especially mammalian form of PepT1 the large extracellular domain between transmembrane domain 9 and 10 of PepT1 is worth mentioning. This roughly 200 amino acid long segment is not present in bacterial POT proteins. Beale and colleagues recently performed a crystallographic analysis of the extracellular domain from human PepT1 and PepT2, and they found two tandem-oriented immunoglobulin-like domains that form a binding site for the intestinal peptidase trypsin (11). The coupling of PepT1 with one of the most abundant serine proteases in the intestinal tract is plausible, especially considering the release of arginine- and lysine-containing di- and tripeptides in direct vicinity of the transporter's pore, which might improve their bioavailability. The pancreatic acinar cells secret a mixture of zymogen precursors into the intestine where active trypsin is cleaved off from its precursor trypsinogen. From a physiological point of view, linkage of trypsin to PepT2, which is expressed in many tissues but not in the intestine, is less reasonable. However, some selected studies found active trypsin or trypsin-like proteins also in murine and human kidney, lung, and brain (103). How peptide transporter and protease work together at the brush-border membrane and how it is that PepT1 is well protected against tryptic cleavage will be later explained in the text (see paragraph "Direct and indirect interaction of PepT1 with proteins").

Table 1 Selected Substrates and Inhibitors of PepT1

Substrate	Ki or Kt [mmol/L]	Reference	Substrate/Inhibitors	Ki or Kt [mmol/L]	Reference
Physiological substrates			Leu-Pro-Arg	0.10	(15)
Ala-Ala	0.08	(96)	Leu-Thr-Leu	0.11	(15)
Ala-Asp	0.26	(96)	Lys-Pro-Val	0.16	(40)
Ala-Glu	0.32	(96)	Met-Met-Met	0.10	(15)
Ala-Gly	0.14	(96)	Phe-Leu-Leu	0.14	(15)
Ala-His	0.33	(96)	Pro-Gly-Gly	16	(15)
Ala-Lys	0.21	(25)	Pro-Phe-Lys	2	(15)
Ala-Pro	0.15	(96)	Ser-Pro-Ile	0.17	(15)
Ala-Ser	0.14	(96)	Thr-Lys-Tyr	1.1	(15)
Ala-Tyr	0.15	(96)	Trp-Gly-Tyr	0.24	(15)
Arg-Pro	0.39	(26)	Trp-Trp-Trp	0.17	(15)
Asp-Ala	0.32	(96)	Tyr-Gly-Gly	0.35	(15)
Asp-Asp	1.2	(127)	Tyr-Pro-Ile	0.25	(15)
Asp-Gly	0.56	(96)	Val-Ala-Leu	0.14	(15)
Asp-Lys	0.86	(96)	Val-Pro-Pro	0.10	(15)
Glu-Ala	0.25	(96)	**Exotic substrates**		
Glu-Lys	0.51	(96)	D-Ala-Ala	2.1	(96)
Glu-Pro	0.26	(26)	Ala-D-Ala	4.2	(96)
Gly-Ala	0.38	(96)	D-Ala-D-Ala	>30	(96)
Gly-Asp	0.35	(96)	D-Ala-Lys	6.9	(21)
Gly-Gln	0.26	(96)	Ala-D-Lys	>30	(21)
Gly-Gly	1	(96)	D-Ala-D-Lys	>30	(21)
Gly-His	1	(96)	Ala-Orn	0.97	(96)
Gly-Lys	2.5	(96)	D-Ala-Pro	4.9	(96)
Gly-Pro	0.3	(26)	Ala-D-Pro	15	(21)
His-Ala	0.48	(96)	D-Ala-D-Pro	>30	(96)
Leu-Pro	0.18	(26)	Gly-Sar	0.86	(83)
Lys-Pro	0.35	(96)	Orn-Ala	0.48	(96)
Lys-Ala	0.34	(96)	Sar-Pro	2.5	(26)
Lys-Asp	0.33	(96)	Val-Pro-D-Pro	4.3	(15)
Lys-Glu	1.3	(96)	D-Ala-Ala-Ala	7.9	(15)
Lys-Gly	0.62	(96)	Ala-Ala-D-Ala	8.3	(15)
Lys-Lys	3.4	(58)	D-Tyr-Val-Gly	14	(15)
Pro-Ala	9.5	(26)	Tyr-D-Ala-Gly	14	(15)
Pro-Arg	2.5	(26)	Ala-D-Ala-Ala	17	(15)
Pro-Glu	20	(26)	Ala-D-Phe-Ala	19	(15)
Pro-Gly	22	(26)	D-Leu-Gly-Gly	25	(15)
Pro-Leu	0.47	(26)	D-Met-Met-Met	0.52	(15)
Pro-Phe	1.9	(26)	Tri-DAP	4.78	(41)
Pro-Pro	1.2	(26)	**Inhibitors**		
Pro-Ser	14	(96)	Lys[Z] Pro	0.033	(96)
Pro-Tyr	0.73	(26)	Lys[Z(NO_2)] Pro	0.01	(96)
Ser-Ala	0.14	(96)	Lys[Z(NO_2)]-Pro	0.007	(96)
Trp-Pro	0.54	(26)	Lys[(Z)NO_2]-Val	0.002	(97)
Tyr-Ala	0.09	(96)	**Drugs**		
Tyr-Pro	0.53	(26)	Benazepril	0.96	(101)
Ala-Ala-Ala	0.2	(101)	Bestatin	0.34	(150)
Ala-Phe-Leu	0.10	(15)	Captopril	46	(101)
Ala-Phe-Pro	0.11	(15)	Cefadroxil	2.69	(83)
Ala-Pro-Leu	0.15	(15)	Cyclacillin	0.5	(28)
Glu-Phe-Tyr	0.20	(15)	Enalapril	14	(101)
Gly-His-Lys	4.1	(15)	Flucloxacillin	3.2	(15)
Ile-Pro-Pro	0.28	(15)	Losartan	0.024	(99)
Ile-Val-Tyr	0.20	(15)	Piperacillin	>30	(15)
Leu-Ala-Arg	0.11	(15)	Valacyclovir	0.49	(67)
Leu-Arg-Pro	0.30	(15)	Valsartan	0.39	(99)
Leu-Gly-Gly	0.39	(15)	δ-Aminolevulinic acid	0.23	(98)

Note: List of selected physiological substrates of PepT1 consisting of proteinogenic L-amino acids, exotic substrates containing D- and nonproteinogenic amino acids, as well as PepT1 inhibitors and drugs that serve as PepT1 substrates. Unless noted otherwise, amino acids refer to the corresponding L-isomer.

Drugs and pharmacy

The intestinal surface is the largest interface for orally administered nutrients and pharmaceuticals which needs to be passed, before the nutrients and drug molecules can enter the blood stream and become bioavailable. Most of these transfer processes are highly dependent on the presence and function of membrane transport proteins. PepT1 is important for the oral bioavailability of numerous drugs and pharmaceuticals. In the last two decades, a broad collection of xenobiotics and synthetic prodrug molecules that are transported by PepT1 has been designed (for review see (24, 148). The extreme flexibility of the transporter's pore that accepts, based on the 20 proteinogenic L-α-amino acids, more than 8000 di- and tripeptides with significant differences in size, charge, and polarity, is the fundamental feature of PepT1 that shifted it into the focus of pharmaceutical industry. Many established drugs on the market, including beta-lactam antibiotics, selected ACE inhibitors and renin inhibitors, as well as antitumor agents are substrates of PepT1. As predicted by Rubio-Aliaga and Daniel already in 2008, the spectrum of ester prodrugs accepted by PepT1 as substrates was further developed and significantly increased over the last years. 5′-amino acid ester modification of Cytarabine (170), and of the antiviral agent guanidine oseltamivir carboxylate (76) increased their affinity to the peptide transporter, and thus led to a significantly increased oral bioavailability. Similar effects were achieved by 5′dipeptidyl ester modification of the mGlu2/3 agonist LY354740 (61), of the dopamine precursor L-dopa, a drug for Parkinson's disease patients (178), and of the antiarthrosis agent glucosamine (73). In general, ester modifications of otherwise poorly absorbed pharmaceuticals are a potent tool to increase their acceptance by PepT1 and improve their membrane permeability and systemic distribution.

In 2014 the Ogihara group reported about the use of L-Phenylalanyl-Ψ[CS-N]-l-alanine (Phe-Ψ-Ala), a metabolically stable thiourea dipeptide, as a nonradioactive probe for PepT1 (6). In the same year, Smith and colleagues reported about the development of a humanized PepT1 mouse model (huPepT1), which allows a better prediction of pharmacokinetics and bioavailability of peptidomimetic drugs in humans (86). The mouse model is predicted to mimic the human PepT1 expression pattern and specific transporter characteristics under physiological conditions. As previously reported species differences exist between mouse and human PepT1 in respect to location and substrate affinity (83, 104), so that translation of drug screening data from cell-culture models or mice to humans is difficult. For that purpose, the authors transfected the human peptide transporter 1 gene huPepT1 into mice nulled for the murine PepT1 gene. The uptake kinetic of the model peptide Gly-Sar was comparable between wild-type mice and the huPepT1 mice indicating a full rescue of PepT1 function (86). In a first pharmacokinetic test assay, the affinity for the aminocephalosporin cefadroxil for PepT1 was twofold higher in huPepT1 mice, and its permeability in the small intestine was 50% to 60% lower in huPepT1 mice when compared to wild-type mice (84). These data support the interspecies differences in PepT1 function *in vivo* and underline the importance of humanized mouse models as screening tools for peptidomimetic drugs.

Nutrient sensing: Transceptor functions of PepT1

In the last years, it became more and more obvious that membrane proteins that act in first line as nutrient transporters also function as sensing systems. Mace and colleagues reported about a macronutrient-driven PKC betaII-dependent regulation of the presence of PepT1 and the glucose transporter GLUT2 in the brush-border membrane of rat jejunum (114). L-glutamate or sucralose supplementation sensed by amino acid or sweet-taste receptors activated PKC beta II. Consequently, PepT1 protein was internalized from the apical membrane to vesicles, while the glucose transporter GLUT2 was recruited to the apical membrane. The apical localization of GLUT2 allows absorption of large quantities of glucose because of its low-affinity/high-capacity transport characteristics. Based on these findings, an energy-supply network was proposed in which membrane transporters and receptors regulate the nutrient absorption in enterocytes. However, the coregulation of PepT1 and GLUT2 by a high intestinal glucose load could not be shown *ex vivo* in gut rings from C57BL/6 N wild-type mice (145), which might be explained by species or methodic differences. Four years after the study by Mace, PepT1 was reported to be a functional sensing system for short oligopeptides in the gut epithelium in concert with the calcium-sensing receptor (53). The model peptide Gly-Sar evoked an influx of Ca^{2+} into murine wild-type enteroendocrine L-cells that induced secretion of the glucagon-like peptide 1 (GLP-1), while this effect was significantly reduced in cells of PepT1 KO mice. These findings were confirmed by studies in intestinal organoids prepared from mouse small intestine. Zietek and colleagues showed PepT1 protein expression at the apical side (in case of organoids the inner side) of these "mini guts" and detected absorption of the hydrolysis-resistant dipeptide Gly-Sar into organoid cells suggesting PepT1 function (200). However, in organoids derived from gut tissue of PepT1 KO mice, still 40% of Gly-Sar uptake remained, indicating peptide influx via predicted basolateral peptide transporter(s) (14). Nevertheless, GLP-1 secretion in the presence of Gly-Sar was blunted in PepT1 KO organoids, which clearly points to PepT1-dependent uptake of small peptides from the apical side to induce GLP-1 release. An overview on the transceptor function of PepT1 and glucose transporter SGLT1 can be found in the review of Daniel and Zietek (50).

Regulation of PepT1

Nutrient uptake is essential for survival of eukaryotic cells. Given the importance of the presence of functional PepT1 in the plasma membrane for an adequate supply of peptide-bound amino acids, the molecular regulation of PepT1 mRNA

and protein expression under various conditions needs to be understood.

Regulation by fasting/refeeding and dietary amino acid supply

The quantity, but also the quality and composition of food, not only have a tremendous impact on the architecture of the gut tissue but also dictate the expression and function of various nutrient transporters and digestive enzymes in the gut epithelium. These modifications in gastrointestinal physiology are highly obvious under food deprivation, meaning situations where the organism is short- or long-term fasted or even starved. For example, it is well known that fasting reduces villus length and density in the small intestine, decreases cell proliferation and increases apoptotic processes, leading to gut mucosal atrophy (35). On the other hand, fasting for 24 h induced PepT1 mRNA and protein expression in rats approximately twofold when compared to *ad libitum* fed littermates (173) (Table 2). The same effect was observed by Ogihara and colleagues, who fasted rats for a longer time period of 4 days, which increased PepT1 protein expression 1.8-fold (141), and by Ma and colleagues, who found a twofold upregulation of PepT1 protein concentration and function in mice after overnight fasting (113). These effects are not rodent-specific, because a 24-h food deprivation in broiler chickens led to a nearly twofold increase in PepT1 protein expression and therefore confirmed the data found in rodents (117). Fasting-induced PepT1 expression is a plausible physiological compensation for the general shrinkage of the intestinal surface area during food deprivation. The increased number of PepT1 transporters per membrane space allows for di- and tripeptide uptake also under those restricted living conditions, and intensely argues for the peptide transporter's crucial role in amino acid supply. Interestingly, not all animal groups show such changes in the PepT1 expression pattern. In teleost fish, exactly the opposite effect was observed: under food deprivation, a significant reduction of PepT1 homologous gene and protein expression was reported for sea bream (172), Atlantic salmon (146), Nile tilapia (87), and for zebrafish (106, 111). Furthermore, refeeding significantly increased PepT1 protein expression in fish. This argues for an essential involvement of the intestinal peptide transporter in compensatory growth during periods with high food accessibility. A comprehensive overview on fish peptide transporters can be found in the overview article of Verri et al. (176) and will not be further discussed in the present review.

In mammals however, refeeding and a sufficient nutrient supply suppress PepT1 expression. Supplementation with amino acids lowered PepT1 protein expression in the jejunal mucosa by 30% compared to samples from *ad libitum* fed rats (141). Newer studies further assessed that not only amino acids influence peptide transporter expression. Feeding a high fat diet (HFD) containing 35 energy percent fat reduced the protein expression of PepT1 in mice, besides other nutrient transporters including glucose transporters SGLT1, GLUT2, and Na^+/K^+-ATPase (188). Furthermore, Arakawa and colleagues analyzed whether excessive intake of D-glucose and L-glutamic acid as donors of sweet and umami taste impair intestinal absorption of orally supplied PepT1 substrates (5). The authors found a reduced PepT1 protein expression in D-glucose-administered rats that led to a significantly lower uptake of the peptide transporter substrate cephalexin. The same effect was induced by L-glutamic acid supplementation, which suggests a taste receptor-dependent mechanism that modulates PepT1 expression in the brush-border membrane.

Table 2 Selected Nutrients and Dietary Interventions that Modulate the Gene and/or Protein Expression and Function of the Intestinal Oligopeptide Transporter PepT1

Dietary intervention, nutrient	Effect on PepT1	Modulation type	References
Fasting	Stimulates the mRNA and protein expression and function of PepT1 in rats, mice, and broiler chicken	Transcriptional	(113, 117, 141, 173)
Long-term fasting	Reduces the mRNA and protein expression in teleost fish	Transcriptional	(87, 106, 111, 146, 172)
Refeeding, supplementation with amino acids	Reduces the mRNA and protein expression and function of PepT1 in mammals	Transcriptional	(141)
Dipeptides Gly-Sar, Gly-Phe, Phe-Val, Lys-Phe, Asp-Lys	Stimulate the mRNA and protein expression of PepT1 in human colon carcinoma cells Caco-2	Transcriptional	(158)
Amino acids leucine, lysine, arginine and phenylalanine	Induces PepT1 promoter activity via the AARE in rats	Transcriptional	(158)
High protein diet (50%), supplementation with a dipeptide	Induces PepT1 promoter activity *in vivo* via the AARE in rats	Transcriptional	(60, 158)
High protein diet	Increases colonic PepT1 protein expression in neonate piglets with low birth weight	Transcriptional	(22)
High fat diet	Reduces PepT1 protein expression in rats	Transcriptional	(188)
D-glucose, L-glutamic acid	Impair PepT1 function in rats		(5)

AARE, amino acid regulatory element

Nevertheless, not only food composition but also the model organisms' age at the time of nutrient administration seems to be an important factor. In most of the studies mentioned, young adult individuals were used. When neonate piglets with a low birth weight were supplemented with a high-protein diet to support development and weight gain, colonic PepT1 expression was increased and influenced colonic homeostasis in later life. When these animals reached adulthood, their colonic mucosa showed higher susceptibility for oxidative stress and inflammatory mediators (22). The authors concluded that neonate infants, who are routinely fed a high-protein diet, may also have a higher colonic PepT1 expression in early life. This may not only increase the absorption of dietary but also of bacterial-derived peptides and may influence immunological processes and stress response in adulthood.

Regulation by oligopeptide breakdown and related signaling pathways

The previous paragraph indicates that changes in the intracellular and systemic amino acid homeostasis have a significant impact on the expression of related nutrient transporters. From the metabolical point of view these interactions are plausible and essential for cell survival, as the cells must be loaded with a pool of all 20 proteinogenic amino acids to ensure tRNA loading for protein *de novo* synthesis and to maintain cell metabolism. Therefore, involvement of digestive enzymes, receptor molecules, and underlying signaling pathways allows a fine adjustment of transporter function.

The close interplay between cytosolic and brush-border membrane peptidases and peptide transport has been known for some time and was already discussed in detail earlier (164). Low peptidase activity, induced by gene silencing or by peptidase inhibitors (amastatin, bestatin), leads to a shift in cellular amino acid homeostasis, due to an accumulation of small peptides in the cytosol, which induces low intracellular amino acid concentrations and a decline in PepT1 protein expression and function (12). The intracellular free amino acid concentration is the main driving force in this process, as supplementation with amino acids brought the peptide transporter activity back to wild-type levels. Interestingly, the positively associated regulation of PepT1 expression and function with peptidase expression is conserved in humans, as shown in colon carcinoma Caco-2 cells (12). The major regulator linking cellular amino acids and protein breakdown and *de novo* synthesis is the target of rapamycin (TOR) signaling pathway, which brought this pathway into focus for further analysis. The role of TOR signaling in intestinal epithelial cell morphogenesis and in enterocyte function has been studied in mammals (118, 153) and in *C. elegans* (112). In this context, TOR complex 1 (TORC1) acts as an intracellular amino acid sensor with high sensitivity to leucine, arginine, and glutamine (198). PepT1 KO *C. elegans* were observed to suffer from a severe amino acid deficiency, with at least 50% lower concentrations of the mentioned amino acids compared to wild type, suppressing TOR signaling and culminating in a low protein *de novo* synthesis rate (71). To further explore the systemic and molecular alterations caused by a loss of PepT1, a quantitative proteomics approach coupled to transcriptomics analysis was applied to PepT1 KO *C. elegans* in comparison to wild-type nematodes. At mRNA and protein level the expression of various enzymes and factors involved in "response to endoplasmic reticulum stress" and "protein folding" was decreased in PepT1 KO worms, indicating a direct correlation between PepT1 function and the activity of the cellular protein folding machinery. The molecular link between these two processes is the transcription factor (TF) XBP-1, a central regulator of the unfolded protein response. Loss of PepT1 leads to reduced splicing of the *xbp-1* mRNA by IRE-1, and therefore to a reduced amount of active XBP-1 protein. In addition, the low protein *de novo* synthesis rate further suppresses the XBP-1 signaling axis. These data allow a closer view on the complex molecular machinery that is coupled to PepT1 in eukaryotic cells. A more detailed overview on various modulator molecules and their impact at the transcriptional, post-transcriptional, and translational level plus protein-protein interactions to PepT1 is presented in the following paragraphs.

Regulation by transcription factors

TFs are the main regulators of gene expression. Detailed analysis of the promoter regions of oligopeptide transporter genes in the genomes of *C. elegans*, mouse, rat, and human revealed only a relatively small number of predictive TF binding sites. Until today the direct impact on PepT1 gene expression has been demonstrated for only a handful of candidates. Fei and colleagues reported about three GC-rich motifs for the binding of SP1, the zinc finger TF "specificity protein 1" at the mouse PepT1 promoter (63). Five years later, control of the basal PepT1 gene expression by SP1 was demonstrated in human cells (155) (Table 3). CDX2 binds to the PepT1 promoter in the presence of SP1 (157) and butyrate (45), its binding site is still unknown though. Although PPAR-alpha, the peroxisome proliferator-activated receptor alpha, is a central regulator for lipid metabolism in mammals, it is also known to regulate the expression of several hundred genes involved in other metabolic pathways. The Inui group identified it as mediator for fasting-induced stimulation of PepT1 expression (156). Two years later, they could exclude the influence of PPAR-alpha on the diurnal rhythm of PepT1 expression using PPAR-alpha KO mice (152). However, Okamura and colleagues reported about a feeding- and bile acid-dependent mediation of PepT1 expression via PPAR-alpha (142). Bile acids released during feeding periods suppress PPARα, causing a low PepT1 expression in murine small intestine during the night phase and a higher expression during the day phase, therefore leading to circadian changes in the intestinal absorption of small peptides. In 2008, a circadian regulation of PepT1 by the clock-controlled TF DBP was discovered (152). Noteworthy, the DBP binding site is located

Table 3 Transcription Factors that Modulate the Gene and/or Protein Expression of the Intestinal Oligopeptide Transporter PepT1

Transcription factor	Effect on PepT1	Modulation type	References
SP1	Binds to the PepT1 promoter and modulates the basal PepT1 transcription	Transcriptional	(63, 155)
CDX2	Modulates Pept1 transcription in concert with SP1 and with butyrate in mammals	Transcriptional	(45, 157)
DAF-16	Represses PepT1 mRNA transcription in *C. elegans*	Transcriptional	(123)
PPAR-alpha	Fasting-induced and bile acid-suppressed transcription of PepT1	Transcriptional	(142, 156)
DBP	Circadian regulation of PepT1 in mouse	Transcriptional	(152)
NRF2	Increases PepT1 expression in presence of sulfuraphane and resveratrol	Transcriptional	(70)

in the distal promoter of rat PepT1, 6305 bp ahead of the start codon. Other genes possess the DBP binding site in the proximal promoter less than 300 bp ahead of ATG (108). In *C. elegans*, the FOXO transcription factor DAF-16 represses the expression of the PepT1 gene (123). This effect seems to be nematode-specific, as it was not found for the DAF-16 homologue FOXO3 in mammalian cells. The *C. elegans* PepT1 promoter contains several TF binding sites for SKN1, a homologue of the mammalian nuclear factor erythroid 2-related factor 2 (NRF2), which orchestrates the expression of various cytoprotective enzymes. Recently, the regulation of human PepT1 gene expression by NRF2 in human colon carcinoma cells Caco-2 was demonstrated (70). The basal PepT1 gene expression is independent of NRF2. NRF2 is however responsible for higher PepT1 expression and activity in the presence of NRF2-activating stimuli like the cancer-chemoprotective plant products resveratrol and sulfuraphane from red wine and broccoli, respectively. NRF2 is known to modulate the expression of proteins involved in glutathione synthesis, and therefore modulates the concentration of this essential cellular antioxidant (122). As PepT1 transports precursors of glutathione, for example, in form of the dipeptide cystinylglycine, its expression seems to be coupled to cellular glutathione metabolism via the NRF2 pathway.

Regulation by microRNA

In the last decade, microRNAs (miRNA) were found to act as potent post-transcriptional regulators of gene expression. They are 21 to 23 nucleotide short noncoding RNAs, encoded in genomic DNA from plants to mammals, that silence genes by binding to complementary sequences within mRNA molecules. miRNAs play numerous roles in differentiation, developmental timing, cell migration, immune, and barrier function in mammalian small and large intestine (121, 149). In humans, roughly 30% of all genes might be influenced by miRNAs (13), the PepT1 gene being one of them (Table 4).

Dalmasso and colleagues found a negative impact of miRNA-92b (miR-92b) on mRNA and protein expression and on transporter function of PepT1 in human Caco2-BBE cells (46). miR-92b targets to the 3′ untranslated region of the PepT1 gene. Furthermore, in fractions from mouse intestinal epithelium, the mature mmu-miR-20a, a potential repressor of mouse PepT1, was less abundant in the villus fraction than in the crypt fraction, and, thereby, supports the typical expression pattern of the peptide transporter in intestinal epithelium. In humans, Dai and colleagues described a direct regulation of PepT1 gene expression by miRNAs in inflamed gut tissue (41). In IBD, microRNA miR-193a-3p was down-regulated, leading to a higher expression of its target gene

Table 4 MicroRNAs that Modulate the Gene Expression of the Intestinal Oligopeptide Transporter PepT1

MicroRNA	MiRNA sequence	Species	Characteristics	Binding site	References
hsa-miRNA-92b	CCUCCGGCCCUGCUCACGUUAU	human	Concentration decreases during Caco-2 cell differentiation, inhibits PepT1 gene expression	3′ untranslated region of the Pep1 gene	(46)
mmu-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	mouse	Lower concentration in villus than in crypt		(46)
hsa-miR-193a-3p	ACUGGCCUACAAAGUCCCAGU	human	miRNA is downregulated during inflammatory bowel disease		(41)
mmu-miRNA-23b	GGGUUCCUGGCAUGCUGAUUU	mouse	Higher expressed in hPepT1-overexpressing colonocytes, activates Marcksl-1 gene in macrophages, increased macrophage activity and inflammation	No impact on PepT1, but PepT1 overexpression increases miRNA abundance	(9)

PepT1. This elevated PepT1 expression is postulated to be the main trigger for bacterial peptide-driven inflammatory processes in enterocytes. Further tests in murine colitis models showed a lower inflammatory response when miR-193a-3p concentration was high and PepT1 protein expression was low, indicating a negative correlation between inflammation and the peptide transporter. But not only the gene expression of PepT1 is influenced by miRNAs, it seems to be also *vice versa*: the presence of PepT1 protein in epithelial cells itself modulates the expression of miRNAs. Three years after their miR-92b study, the Merlin group analyzed the effects of PepT1 overexpression on the miRNA pattern in inflamed and noninflamed murine colon (9). For that purpose, they used genetically modified mice that overexpress hPepT1 in colonocytes as a model for IBD (44). They did not find any significant differences in the miRNA pattern of inflamed and noninflamed colon, but detected 10 miRNAs that were differentially expressed when colonic PepT1 expression is high. Out of these, miRNA-23b, known to be involved in human IBD, is more abundant in inflamed colonocytes of hPepT1-overexpressing mice than in inflamed wild-type colonocytes. In the transgenic animals, the macrophages displayed increased activity due to a reduced expression of the miRNA-23b target gene Marcksl-1, leading to a greater degree of inflammation. These findings are a first hint for a connection between colonic PepT1 expression and an increased susceptibility to inflammatory processes in the gastrointestinal tract. The same group analyzed changes in miRNA patterns in enterocytes of PepT1 KO mice (196). The loss of PepT1 significantly reduced (villus: 15 miRNAs; crypt: 11 miRNAs) and increased (villus: 11 miRNAs; crypt: 14 miRNAs) the expression of various miRNAs in the crypt and in the villus fraction, followed by an altered expression of predicted protein targets. These data indicate for the first time that PepT1 has an impact on the expression gradient of selected miRNAs and their protein targets and might thus be involved in homeostasis across the villus-crypt axis of the small intestine. Just recently, microRNAs miR-193b-3p and miR-27a-3p were found to interact with PepT1 3′-UTR (31). In human intestinal tissue samples, miRNAs were selected that showed a negative correlation to PepT1 protein expression, therefore suggesting an inverse functionality to transporter expression. As the sequence of miR-193b-3p is very similar to miR-193a-3p, which was active in murine tissue, the interaction of miR-193b-3p with the 3′UTR of human PepT1 is an additional confirmation of the conserved molecular regulatory circuits in the gut epithelium.

Direct and indirect interaction of PepT1 with proteins

In polarized epithelial cells, the localization of membrane proteins in the apical or basolateral compartment is precisely defined to enable their correct function. Therefore, it is noteworthy that although numerous studies describe the transporter characteristics of oligopeptide transporters, information on protein interaction partners for membrane trafficking, anchoring, and functional support is still limited. Since 2008, when Boehmer and colleagues described a direct protein-protein interaction between the sodium-proton exchanger regulation factor 2 (NHERF2) and human PepT2 (18), no additional studies were published in this field. NHERF2 is a typical scaffold protein that contains a PDZ (PSD-95/Discs-large/ZO-1) domain which binds to the PDZ binding motif S/T-X-# (S/T: serine/threonine, X: any residue, and #: hydrophobic residue) at the C-terminus of membrane proteins. The PDZ binding motif is conserved in both isoforms of the mammalian oligopeptide transporter, and a direct interaction of mouse PepT1 to scaffold protein PDZK1 proves the molecular utilization of that specific site (169) (Table 5). However, no evidence for the direct interaction of human PepT1 with a scaffold protein has been published yet.

By functional coexpression of peptide transporters PepT1 and PepT2 with the tyrosine kinase Janus kinase 2 or 3 (JAK2 or JAK3) in *Xenopus* oocytes, a positive regulation of PepT1 and PepT2 protein expression and function by JAK2 (82) and JAK3 (182) was observed. Both kinases are involved in the JAK/STAT (signal transducers and activators of transcription) signaling pathway that is a central regulator of cell proliferation and apoptotic processes. A gain-of-function mutation in the JAK3 gene supports tumor cell proliferation and growth by upregulation of nutrient transporter expression in the cell membrane, leading to excessive nutrient uptake. Data in *Xenopus* oocytes suggest a role of peptide transporters in the cellular amino acid supply, especially in situations involving high cell proliferation.

Ubiquitination is a central regulator of cellular protein stability and turnover, which has also been reported for transport proteins. It is a highly dynamic and reversible process that is achieved via the balance of enzyme-driven ubiquitination and deubiquitination. In 2014, the Lang group reported three enzymes that are involved in the ubiquitination of PepT1 and PepT2: E3 ligase NEDD4 for ubiquitination, and the deubiquitin enzymes USP18 and USP30 (183). Coexpression of rabbit PepT1 or PepT2 with human USP18 or USP30, respectively, resulted in increased Gly-Gly uptake into *Xenopus* oocytes, which was explained by an increased PEPT protein stability due to deubiquitination. Ubiquitination of oligopeptide transporters by NEDD4 on the other hand led to a decreased dipeptide uptake. Whether these processes are relevant in intestinal and renal epithelia needs to be further analyzed. However, the *in vivo* interplay of NEDD4 and another deubiquitin enzyme USP46 for the ubiquitination of the neuronal AMPA receptor was reported recently (88).

Beta-Klotho, the soluble part of the membrane protein Klotho with beta-glucuronidase activity that was previously shown to regulate the function of ion channels and vitamin transporters (163), is a negative modulator of PepT1 and PepT2 function (1). When rabbit PepT1 or PepT2 expressing *Xenopus* oocytes were treated with beta-Klotho protein in the incubation solution, Gly-Gly-induced currents were significantly reduced compared to nontreated controls. *Ex vivo*

Table 5 Selected Proteins, Factors, and Hormones that Modulate the Gene and/or Protein Expression and Function of the Intestinal Oligopeptide Transporter PepT1

Protein, factor, hormone	Effect on PepT1	Modulation type	References
Scaffold protein PDZK1	Interacts with mouse PepT1, trafficking to and anchoring in the plasma membrane	Direct protein-protein interaction	(169)
Janus kinase JAK2		Translational, functional	(82)
Janus kinase JAK3		Translational, functional	(182)
AMP-activated protein kinase AMPK	Recruitment and stabilization of the PepT1 protein in the apical membrane	Protein stability, functional	(144)
Deubiquitin enzymes USP18, USP30	Deubiquitination, stabilization of the PepT1 protein in the apical membrane	Protein stability, functional	(183)
E3 ligase NEDD4	Ubiquitination, initiation of PepT1 protein degradation	Protein stability, functional	(183)
beta-Klotho	Negative modulator of PepT1 and PepT2 expression		(1)
STE20-related proline/alanine-rich kinase SPAK	Suppressed PepT1 and PepT2 protein expression in *Xenopus* oocytes	Translational, functional	(180)
Oxidative stress-responsive kinase OSR1	Suppressed PepT1 and PepT2 protein expression in *Xenopus* oocytes	Translational, functional	(181)
Protease trypsin	Interacts with the extracellular loop of mammalian PepT1 protein, cleavage of dietary protein next to the transporters pore	Direct protein-protein interaction	(11)
RNAi of intracellular peptidases	Reduced protein expression and function of PepT1 in Caco-2 cells and *C. elegans*	Translational, functional	(12)
Sodium-proton exchanger NHE3/NHX-2	Proton export is necessary for proper function of PepT1 in mammalian cells and in *C. elegans*	Functional interaction via proton gradient, protein-protein interaction not yet proven	(12, 95, 184)
Insulin	Increased recruitment of PepT1 to the apical membrane of enterocytes, increased function (rat and human)	Translational, functional	(16, 139, 185, 186)
Glucose-dependent insulinotropic polypeptide (GIP)	Increased PepT1 transport function	Functional	(39, 40)
Leptin	Increased PepT1 protein expression, dependent on transcription factors CDX2 and CREB	Translational, functional	(80, 81, 132, 171)

analysis of Gly-Gly transport in intestinal epithelia of control and beta-Klotho KO mice supported these findings by a twofold increased transport rate of the dipeptide in beta-Klotho deficient mice.

Two kinases, the STE20-related proline/alanine-rich kinase SPAK and the oxidative stress-responsive kinase OSR1 suppress PepT1 and PepT2 protein expression, as shown by coexpression in *Xenopus* oocytes (180,181). Both kinases are known to modulate ion transport, cell volume, and blood pressure in the kidney, but are also expressed in the mammalian intestine, where SPAK is a suppressor of glucose transporter SGLT1 (59). Therefore, the regulation of peptide transporters both in kidney and small intestine is plausible.

The direct interaction of human PepT1 and PepT2 with the protease trypsin via specific motives in the large extracellular loop was recently described (11), as mentioned above in the paragraph about the 3D structure of PepT1. The direct coupling of the digestive enzyme and the peptide transporter represents a highly efficient functional unit. Oligopeptides released by trypsin are immediately recognized by their transporter and are taken up into epithelial cells. The mechanism with which PepT1 protects itself against proteolytic cleavage by trypsin will be explained in the following paragraph.

Post-transcriptional modifications (glycosylation)

Numerous proteins in eukaryotic cells undergo post-transcriptional modifications. The dominant alteration during protein processing is the N-glycosylation of asparagine residues, which was recently reported for murine and human PepT1 (167). Murine PepT1 contains six asparagine molecules at positions N50, N406, N439, N510, N515, and N532. Interestingly, only N50 is located at the N-terminus of the transport protein between transmembrane domains (TMD)

1 and 2, while the other five are located in the large extracellular loop between TMD 9 and 10. The asparagines are differently glycosylated in the small and large intestine, leading to a protein mass higher by 10 kDa in the colon than in the small intestine. The different glycosylation patterns of PepT1 in different parts of the intestinal tube seem to be unique, as this has not been reported for other membrane proteins yet. Nevertheless, almost all proteins in the intestinal epithelium carry glycan groups as protective shield against proteolytic cleavage by pancreatic proteases like trypsin or by brush-border membrane peptidases. However, the proteolytic cleavage of PepT1 by trypsin, a direct interactor with PepT1 (11), was generally slow and independent of the glycosylation status of the peptide transport protein (168). Stelzl et al. suggested that this high resistance against the tryptic cleavage is due to structural properties of the transport protein. Loss of the glycans by single-site mutations or treatment with tunicamycine, an inhibitor of N-glycosylation, leads to distinct changes in PepT1 substrate affinity and transport rate. Especially the loss of glycosylation at position N50 reduced the affinity for the model peptide Gly-Sar by more than 50%, while the transport rate was doubled both inward and outward (168). A replacement of glycans at position N50 by biotin derivatives of comparable mass was able to reverse the gain-of-function phenotype and significantly reduced Gly-Sar transport rate. These changes can be explained by the localization of N50 at the extracellular side of the PepT1 binding cavity. When the corresponding glycans are missing, the rotation of the protein from the "outward open" to the "inward open" position and back to "outward open" is faster, resulting in a higher uptake rate of di- and tripeptides into the cell. These data demonstrate a functional role of N-glycans on the transport characteristics of a nutrient transport protein for the first time.

Regulation by hormones

Hormones are signaling molecules that act as systemic regulators of cell physiology and metabolism. They are produced by the endocrine system (glands, tissue-specific endocrine cells), which secretes the hormones in the circulatory system to allow communication between tissues and organs. Specialized cells in the gut epithelium, called enteroendocrine cells, are active in direct neighborhood to enterocytes. Significant effects of selected hormones on peptide transporter PepT1 have been shown in the last decade. Leptin, an adipokine secreted by white adipose tissue, promotes PepT1 protein expression in mice *in vivo* (80), dependent on the transcription factors CREB and CDX2 (132). Intestine-specific depletion of leptin signaling substantially reduced di- and tripeptides uptake in the gut of leptin receptor B deficient mice (171). In a previous study, mice with diet-induced obesity showed a 50% reduced PepT1 expression and function, which was explained by a mild leptin signaling deficiency due to a 50% reduced leptin receptor expression in enterocytes (81). These findings are first hints toward an adaptation of oligopeptide transporter expression and function connected to leptin sensitivity in an individual. However, these data from rodents have not been confirmed in humans yet, as described in more details in the paragraph "PepT1 in obesity." Coon and colleagues found a threefold increase in PepT1 transporter function in the presence of glucose-dependent insulinotropic polypeptide (GIP) in the intestinal epithelial cells IEC-6 with CDX2 expression, while the same amount of glucagon-like peptide 1 (GLP-1) had no effect on PepT1 expression and function (39,40). The influence of insulin on the expression and transport activity of PepT1 will be discussed in the paragraph "PepT1 in diabetes mellitus."

Role of PepT1 in Intestinal Homeostasis in Health and Disease

PepT1 in inflammatory bowel disease

IBD is classified as a group of inflammatory intestinal disorders, the main types of IBD being ulcerative colitis (UC) and Crohn's disease (CD) (51). While in UC exclusively the mucosa of colon and rectum is affected, CD features transmural lesions in any segment of the gastrointestinal tract. Both entities have in common that their exact etiology has not been identified yet. Several contributing factors to the pathogenesis of CD and UC leading to intestinal inflammation have been characterized so far in genetically predisposed individuals, showing a disturbed interplay of external, that is, environmental and dietary, as well as internal factors, like gut microbiota and the host immune system. Over time, both CD and UC bear the potential to induce the development of malignancies in the form of colorectal cancer (CRC) caused by chronic intestinal inflammation (57,91,174). Since the turn of the millennium, the involvement of PepT1 in the pathogenesis of IBD has been studied by different groups, coming to conflicting conclusions however.

Merlin and colleagues (2001) could not detect any mRNA nor protein expression of PepT1 in large intestinal tissue of healthy human individuals, contrary to human small intestinal samples (124). Concomitantly, the small intestine-like cell line Caco-2 BBE was reported to feature hPepT1 expression, which is not the case in human colonic HT29-Cl.19A cells, again neither on mRNA nor on protein level. However, both PepT1 mRNA and protein expression were documented in colonic mucosa of CD and UD patients, suggesting an induction of aberrant PepT1 expression in a state of chronic inflammation. These findings were confirmed by Wojtal et al. (2009), who assessed changes in intestinal mRNA expression of members of the SLC gene superfamily in IBD patients (189). They observed a dysregulation of SLC mRNA expression in inflamed human colonic biopsy specimens. Besides several other SLCs, PepT1 mRNA expression was significantly increased in inflamed colonic tissue of both UC and CD patients as compared to specimens from healthy control subjects. As stated above, IBD is associated with the development of CRC, in this context also referred to as colitis-associated cancer (CAC). In mice overexpressing intestinal PepT1,

Viennois and coworkers reported enhanced tumor growth, associated with stronger increases in proliferation and concurrently less-pronounced increases in apoptosis rate in colonic intestinal epithelia cells (IECs) compared to wild-type animals following azoxymethane (AOM)/dextran sodium sulfate (DSS) treatment (177). At the same time, PepT1 KO mice displayed reduced tumor growth compared to wild-type controls, possibly due to reduced proinflammatory cytokine mRNA expression and thus less-pronounced intestinal inflammation, together with an inhibition of colonic proliferation and a weaker increase in apoptosis after AOM/DSS treatment. When PepT1 expression was determined in the AOM/DSS-treated wild-type mice, an increase both in mRNA as well as in protein expression was observed. In human tissue samples of both benign and malign colonic tumors, an increase in PepT1 protein expression was documented.

Wuensch and colleagues however could not reproduce these findings. Contrary to the above mentioned studies, PepT1 expression had been observed—in addition to small intestinal epithelium—also in healthy distal colon and rectum of rodents and humans, both on mRNA and protein level (191). In proximal human colon however, PepT1 could not be detected, neither in health nor during inflammation. Biopsy specimens from the distal colon of IBD patients during acute inflammation showed reduced PepT1 expression compared to healthy controls, which recovered however in tissue of IBD patients in remission. These findings were supported by mouse studies. Interleukin-10 (IL-10) deficient mice, which spontaneously developed colitis when mono-associated with *Enterococcus faecalis* OG1RF, displayed reduced colonic PepT1 expression compared to both wild-type and germfree IL-10 KO mice, both in acute and chronic inflammation. These observations however are not in line with a previous study, in which upregulated PepT1 protein and mRNA expression as well as transport activity were reported in IL-10 KO mice compared to wild-type animals (36). In addition to the decrease in PepT1 expression in inflamed colonic tissue described above, reduced PepT1 protein levels were also observed in the terminal ileum of TnfΔARE/wild-type mice, which serve as a model for CD-like ileitis. There is no apparent explanation for the conflicting results of these studies. While the ones showing increased colonic PepT1 expression during inflammation do not state if human samples were taken from the proximal or distal colon, Wuensch et al. examined samples from distal colonic segments. So there may be region-specific effects of inflammation on PepT1 expression, leading to a downregulation in colonic segments in which PepT1 is expressed physiologically, while PepT1 expression is possibly induced in segments which normally do not express PepT1.

Contradictory results were also reported in studies investigating possible mechanisms leading to a change in PepT1 expression in a state of inflammation as observed in IBD. In rats treated with endotoxin, that is, lipopolysaccharides (LPS) found in the membrane of Gram-negative bacteria, a decrease in PepT1 expression in jejunum and ileum was observed, both on mRNA and protein level, together with an increase in TNF-α and IL-1ß levels in plasma and intestinal mucosa (159). Both effects could be reduced by glucocorticoid coadministration, suggesting a connection between the downregulation of PepT1 expression through the upregulation of proinflammatory cytokine secretion. In Caco-2 BBE cells however, the administration of proinflammatory cytokines TNF-α and IFN-γ was associated with a concentration- and time-dependent increase in PepT1 protein expression and transport activity, while PepT1 mRNA expression was not affected, indicating post-transcriptional upregulation (32, 175). Similar effects were observed in mouse experiments *in vivo*. While IL-1β increased PepT1 mRNA levels in proximal and distal colon, at the same time PepT1 mRNA expression in jejunum and ileum was reduced, confirming the findings of Shu and coworkers. However, TNF-α and IFN-γ injection in mice did not elicit any changes in PepT1 expression in the small intestine, while PepT1 protein expression and function was increased in proximal and distal colon, with no increase in PepT1 mRNA levels, again illustrating significant region-specific differences of how PepT1 expression is affected by different stimuli. Furthermore, an upregulation of protein expression and function of PepT1 in primary human enterocytes by IFN-gamma was reported (65).

As a possible mechanism of involvement of PepT1 in the pathogenesis if IBD, the group of Merlin described that the transport of fMLF, a bacterial chemotactic oligopeptide activating the innate immune system, induced an increase in antigen presenting MHC class I molecule surface expression in HT29-Cl.19A cells stably transfected with hPepT1 (124). In this regard, PepT1 would be involved in the amplification of intestinal inflammation by an enhanced transport of proinflammatory oligopeptides from the intestinal lumen across the intestinal epithelium. L-Ala-D-Glu-meso-DAP (Tri-DAP), a proinflammatory tripeptide originating from the breakdown of bacterial peptidoglycan, has been demonstrated to be a transport substrate of PepT1 as well (43). Upon the uptake of Tri-DAP, activation of NF-κB and MAP kinases and, as a result, an increase in proinflammatory cytokine interleukin-8 (IL-8) expression was observed. Like with fMLF before, PepT1-mediated transport of bacterial products may thus aggravate intestinal inflammation in states of chronic inflammation as described in IBD (Fig. 4). An additional proinflammatory interleukin that has been reported to be associated with IBD is IL-16 (179). In a DSS-induced intestinal inflammation pufferfish model (Tetraodon), a significant increase in IL-16 serum concentration comparable to that of IBD patients was observed. In the colon-like inflamed Tetraodon intestine, PepT1 mRNA was increased. When the effect of IL-16 was reversed via anti-IL-16 antibodies, inflammation and concurrently PepT1 mRNA levels were reduced to healthy control levels. Intestinal inflammation comparable to that of the DSS-induced model was also detected upon IL-16 administration. Besides an increase in other proinflammatory cytokine concentrations including TNF-α, IFN-γ, and IL-1β, again increases in PepT1 mRNA and protein expression were observed, paralleling the degree of intestinal inflammation.

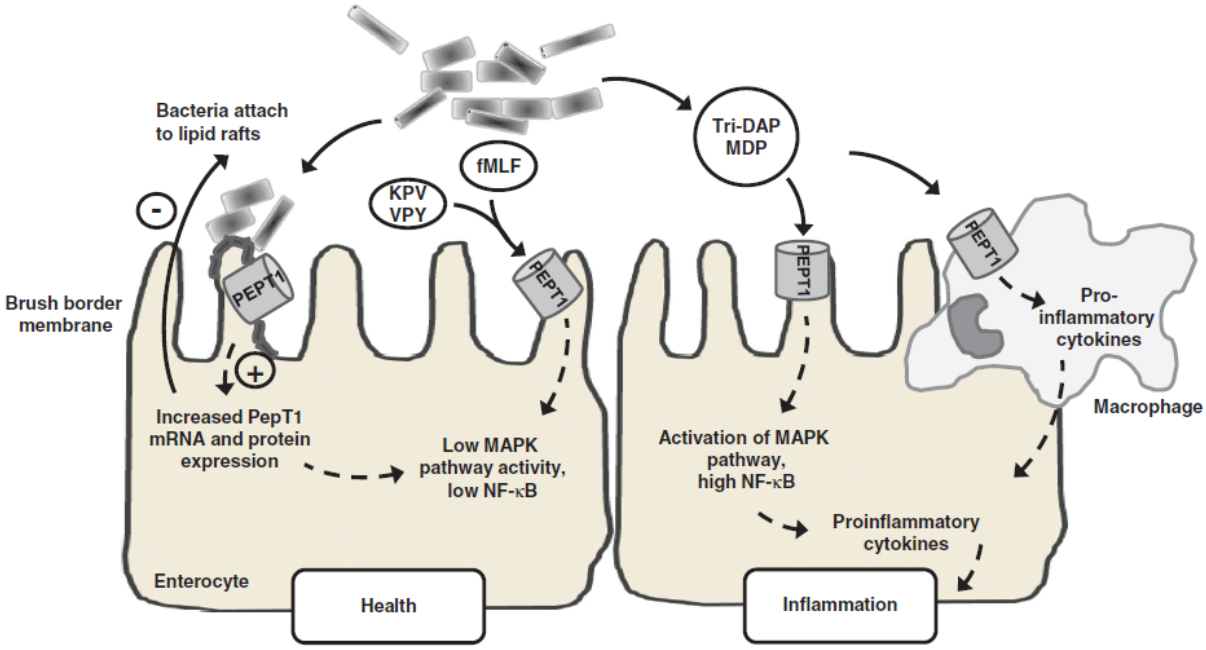

Figure 4 The role of PepT1 in intestinal homeostasis. The wide variety of PepT1 substrates includes exogenous oligopeptides like the C-terminal sequence of α-melanocyte stimulating hormone (α-MSH) Lys-Pro-Val (KPV) and the dietary soy tripeptide Val-Pro-Tyr (VPY), as well as endogenous bacterial oligopeptides. Uptake via PepT1 of bacterial products like L-Ala-γ-D-Glu-meso-diaminopimelic acid (Tri-DAP) and muramyl dipeptide (MDP), a constituent of bacterial cell walls, is associated with the activation of mitogen-activated protein (MAP) kinase pathway and NF-κB, and subsequently an increase in proinflammatory cytokine expression in enterocytes. In intestinal macrophages, PepT1-mediated uptake of bacterial products induces an increase in proinflammatory cytokine secretion that, together with the increased cytokine levels in enterocytes, may contribute to the pathogenesis or promotion of intestinal inflammation. PepT1-mediated uptake of other bacterial peptides like N-formyl-Met-Leu-Phe (fMLF) as well as of aforementioned KPV and VPY on the other hand conveys anti-inflammatory properties by inhibition of proinflammatory cytokine secretion due to reduced activation of NF-κB and MAP kinase inflammatory signaling pathways. Besides these anti-inflammatory effects associated with PepT1 transport activity, PepT1 may also directly affect bacterial-epithelial interactions. Certain enteropathogenic bacteria attaching to enterocytes specifically via lipid rafts induce PepT1 expression in said lipid rafts. Increased intestinal PepT1 expression in turn reduces bacterial lipid raft attachment, while at the same time reducing activity of NF-κB, MAP kinase and secretion of proinflammatory cytokines, implying an anti-inflammatory role of PepT1 in intestinal host defense against pathogenic enterobacteria.

The subsequent increase in PepT1 fMLF transport activity, shown in an *in vitro* transport study, may accentuate intestinal inflammation. In PepT1 knockdown pufferfish, all signs of intestinal inflammation, like mucosal damage and leukocyte infiltration, were distinctly reduced in IL-16-induced colitis, suggesting a role of PepT1 in the propagation of intestinal inflammation. Similar effects could be expected from muramyl dipeptide (MDP), a further bacterial oligopeptide known to be a PepT1 transport substrate and moreover a ligand of the intracellular pattern recognition receptor NOD2. However, when treating distal colonic tissue cultures from wild-type and PepT1 KO mice with MDP, no differences in terms of immune response could be detected (192). Increases in proinflammatory cytokine levels, like IL-1ß, IL-6, and keratinocyte-derived chemokine, the murine IL-8 homolog, were found in both mouse strains, showing that MDP-induced inflammation seems to be independent of the presence of PepT1. In addition, PepT1 protein expression was compared in distal colon of NOD2 KO and wild-type mice. No differences were observed between the two strains, suggesting that NOD2, or a lack thereof, is not involved in the regulation of colonic PepT1 expression.

Effects described in PepT1 deficiency in intestinal inflammation may however not be caused exclusively by the lack of PepT1 in the brush-border membrane. Besides intestinal epithelial cells, PepT1 is also expressed in cells of the immune system like macrophages, which may be involved in the immune response during intestinal inflammation (8). In PepT1 KO mice with DSS-induced colitis, body weight loss, rectal bleeding, and diarrhea were less marked compared to corresponding control animals. In addition, several signs of intestinal inflammation were less pronounced in PepT1 KO compared to wild-type mice. There were reduced impairments of colonic architecture, mucosal and submucosal immune cell infiltration, tissue myeloperoxidase (MPO) activity, as well as inflammatory cytokine mRNA levels. At the same time, proinflammatory cytokine levels in colonic immune cells were higher in control animals compared to PepT1 KO mice. These differences between the two mouse strains suggest that the absorption of bacterial products like fMLF, MDP,

and Tri-DAP by macrophage PepT1 may modulate the secretion of proinflammatory cytokines and this way contribute to the development of intestinal inflammation (Fig. 4).

In addition to the interaction via these bacterial peptides, pathogenic bacteria may also have an effect on intestinal PepT1 expression by interacting directly with the intestinal mucosa. In HT29-Cl.19A cells, which under normal conditions do not express detectable amounts of PepT1, attaching and effacing (A/E) enteropathogenic *E. coli* (EPEC) were reported to induce PepT1 and Cdx2 mRNA and protein expression (138). As already described in the paragraph about the PepT1-regulating transcription factors, the homeobox protein CDX2 had been previously shown to be involved in the regulation of PepT1 expression (132). An induction in PepT1 mRNA and protein expression was observed in HT29-Cl.19A cells overexpressing Cdx2. At the same time, HT29-Cl.19A cells in which Cdx2 was silenced by Cdx2 siRNA displayed reduced EPEC-induced PepT1 expression, indicating an involvement of Cdx2 in EPEC-induced PepT1 expression. Nduati et al. could show that EPEC specifically attach to the HT29-Cl.19A cell membrane via lipid rafts, leading to an increased PepT1 expression in lipid rafts, which the group had already observed in IECs (137). Overexpression of PepT1 in HT29-Cl.19A cells distinctly reduced EPEC lipid raft attachment as well as activity of NF-κB, MAP kinase, and IL-8. These findings were confirmed *in vivo* and *ex vivo*. *Citrobacter rodentium*, a murine A/E pathogen related to EPEC was associated with increased colonic PepT1 mRNA and protein expression in wild-type mice. When infected with EPEC, PepT1 transgenic mice ubiquitously expressing hPepT1 however exhibited reduced bacterial colonization, production of proinflammatory cytokines, and signs of colonic inflammation. These findings suggest a role of PepT1 in intestinal host defense against pathogenic enterobacteria by affecting bacterial-epithelial interactions, attenuating intestinal inflammation.

Besides a potential contribution to the pathogenesis or promotion of IBD, PepT1 may also be considered for IBD therapy. Recently, therapeutic approaches have been developed in IBD employing probiotics (120). Evidence for the efficacy of probiotic therapy of IBD however is still sparse (56). When incubated with a probiotic *Lactobacillus casei* strain, Caco-2 cells displayed markedly increased PepT1 transport activity by 100% after 48 h of coincubation, while no changes in PepT1 mRNA expression were detected (134). These effects could not be reproduced with conditioned *L. casei* culture medium, suggesting that the induction of PepT1 transport activity may be caused by a direct interaction between probiotics and Caco-2 cells.

Bacterial products of probiotics may also convey positive health effects on their host. In a study on the effects of the chemotactic peptide fMLF, which is released by bacteria and is present in the healthy human colon, protective properties of fMLF were discovered (34). Upon PepT1-dependent uptake, physiological concentrations of fMLF induced the expression of cytoprotective heat-shock protein 27 (Hsp27) in Caco-2 BBE cells, which in turn protected the cells from oxidative stress. At the same time, fMLF reduced the activation of NF-κB by TNF-α in Caco-2 BBE cells and accordingly mRNA expression of NF-κB target genes TNF-α and IL-8. *Ex vivo*, in primary tissue culture of human ileal and cecal biopsy specimens from healthy individuals, the concentration of proinflammatory cytokines following the stimulation with TNF-α was reduced when pretreated with fMLF.

While PepT1 has been demonstrated to transport bacterial peptides that may be harmful or protective to the host, it may as well transport other bioactive oligopeptides that have the potential to be beneficial in a state of intestinal inflammation, like the tripeptide Lys-Pro-Val (KPV). KPV is the C-terminal sequence of α-melanocyte stimulating hormone (α-MSH) and has been demonstrated previously to have anti-inflammatory properties (119). Upon the incubation of the human intestinal cell line Caco-2 BBE and the human T cell line Jurkat with KPV, the tripeptide was shown to be absorbed via PepT1 in both cell lines and effectively led to an inhibition of proinflammatory cytokine secretion due to a reduced activation of NF-κB and MAP kinase inflammatory signaling pathways, which are typically involved in intestinal inflammation (42). These findings were confirmed *in vivo*. Both in mice with DSS- and trinitrobenzene sulfonic acid (TNBS)-induced colitis, oral coadministration of KPV together with DSS or TNBS managed to reduce symptoms of intestinal inflammation compared to the control animals that received DSS or TNBS without KPV. In both experimental colitis models, weight loss and MPO activity were reduced. Histological analysis revealed reduced levels of colonic wall damage, interstitial edema, and immune cell infiltration, as well as reduced mRNA expression of proinflammatory cytokines. In AOM/DSS-induced CAC model mice, coadministration of the KPV peptide was associated with a significant reduction in colonic tumorigenesis in a PepT1-dependent manner (177), revealing KPV also as a potential therapeutic approach in the therapy of CAC. Similar properties were described for the soy-derived tripeptide VPY upon uptake via PepT1. In Caco-2 cells, VPY reduced IL-8 secretion after TNF-α stimulation, while in the human monocytic cell line THP-1, TNF-α secretion following stimulation with LPS was reduced by VPY (105). These observations could be confirmed *in vivo* in mice with DSS-induced colitis. As with KPV, the coadministration of the VPY peptide with DSS reduced the severity of clinical signs of intestinal inflammation and histopathological parameters, while also intestinal expression of proinflammatory cytokines TNF-α, IL-1β, and IFN-γ was reduced in VPY-treated animals compared to mice treated with DSS alone. Overall, these anti-inflammatory properties of selected bioactive tripeptides like KPV and VPY may be interesting for the development of new approaches in IBD therapy.

As stated before, genetics play an important role in the etiology of IBD, displayed by familial aggregation and higher incidence in monozygotic compared to dizygotic twins. Human single nucleotide polymorphisms (SNPs) related to intestinal barrier function and bacterial-epithelial interactions

predispose to both CD and UC (94). A protein commonly affected by SNPs in CD is the intracellular pattern recognition receptor NOD2. As mentioned before, several bacterial peptides serve as transport substrates for PepT1. Upon uptake, these bacterial products can bind to NOD2 and subsequently induce NF-κB activation and intestinal inflammation.

Studies have been conducted to identify SNPs in SLC15A1 in general and also with respect to the susceptibility toward IBD. Two studies in ethnically diverse cohorts aimed to identify SNPs in the PepT1 gene (4,195) could only detect one SNP each associated with altered PepT1 activity. The low-frequency SNP PepT1-F28Y was connected with reduced cephalexin uptake due to an increased Km value and modified pH dependence, while for SNP PepT1-P586L an association with impaired transport capacity and decreased PepT1 protein expression was observed. Overall, PepT1 exhibits a remarkably low genetic variability, indicating the importance of unimpaired PepT1 functionality for the organism. Zucchelli and colleagues analyzed two cohorts of Swedish and Finnish IBD patients and controls with regard to coding SLC15A1 SNPs associated with IBD (202). They discovered one SNP that met the criteria, namely rs2297322, which exchanges serine 117 to asparagine. Interestingly, the common allele of this SNP was risk-associated with CD in both cohorts, however with opposite effects. In the Swedish cohort, rs2297322 was associated with increased CD predisposition, while in the Finnish cohort, a reduction in CD susceptibility was associated with the SNP. These contrasting effects may be explained by possible different genetic backgrounds of the two cohorts affecting SLC15A1. In a German cohort of IBD patients and healthy controls however, PepT1 expression was identical in IBD patients with and without the SNP rs2297322 (192). Furthermore, no association between rs2297322 and the susceptibility for IBD could be detected in individuals carrying the SNP as compared to individuals without the SNP. So far, no single PepT1 SNP has been identified that is conclusively associated with IBD. PepT1 expression in intestinal immune cells may have an inflammation-promoting role by sensing the presence of bacterial products. For PepT1 expression in IECs on the other hand, some studies demonstrated proinflammatory, yet others anti-inflammatory properties of intestinal PepT1, while one study showed that PepT1 is not required for the induction of intestinal inflammation by bacterial peptides. Therefore, based on the numerous studies in this field, the role of PepT1 in IBD still remains unclear.

PepT1 in diabetes mellitus

Diabetes mellitus (DM) is an endocrine metabolic disease characterized by the organism's inability to control blood glucose levels, manifesting itself as hyperglycemia which can lead—if untreated—to serious complications in the form of macro- (e.g., coronary atherosclerosis) and microangiopathy (e.g., diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy). In type 1 DM, pancreatic beta cells perish due to autoimmune processes, leading to an absolute lack of insulin, contrary to type 2 DM. Here, a relative lack of insulin is observed as a result of insulin resistance. PepT1 is relevant in DM in two respects: On the one hand, peptidomimetic drugs used in the therapy of DM complications, like ACE or renin inhibitors are PepT1 transport substrates; on the other hand insulin is associated with the regulation of PepT1 expression. In streptozotocin-induced diabetes (which serves as a model for type 1 DM) in male rats, increased small intestinal and also renal PepT1 mRNA and protein concentrations as well as increased transport activity were observed 96 h after streptozotocin treatment (69). These effects appeared to be induced by increased PepT1 mRNA stability rather than its rate of transcription. However, long-term treatment with streptozotozin reversed the PepT1 expression profile. After 1 month, jejunal PepT1 protein expression and activity were reduced in female streptozotocin-induced diabetic rats (16). At the same time point, PepT1 expression and activity reached near-normal levels in insulin-treated streptozotocin-induced diabetic animals, showing that PepT1 expression may be associated with systemic insulin levels. These previous findings were mostly confirmed by Der-Boghossian et al. in a study analyzing both male and female streptozotocin-induced diabetic and nondiabetic rats after a duration of 1 month (52). In noniabetic rats, insulin treatment did not alter PepT1 protein expression and activity. However, a decrease in PepT1 mRNA expression was observed in male animals, while PepT1 mRNA expression was increased in healthy insulin-treated female animals. In male diabetic rats, PepT1 mRNA expression was increased; however, PepT1 protein expression and activity were reduced. Female diabetic rats on the other hand displayed reduced PepT1 mRNA and activity levels, however accompanied with increased protein levels. In female diabetic rats, insulin treatment led to the complete restoration of PepT1 mRNA and protein expression as well as activity to levels of nondiabetic rats, while in male rats all three parameters were only partially reversed (52). These findings imply sex-dependent differences in PepT1 regulation by insulin, probably driven by estrogen, which may be relevant for the establishment of gender-specific treatments of DM with peptidomimetic or related alternative drugs.

PepT1 in obesity

Obesity, generally caused by a hypercaloric diet in association with a sedentary lifestyle and—to some extent—a genetic predisposition, is commonly classified as a body mass index of at least 30 kg/m^2 (27). Obesity is a risk factor for a plethora of diseases like cardiovascular disease, DM, osteoarthritis, and certain types of cancer. One of several pathophysiological mechanisms involved in the development of obesity is leptin resistance. Leptin is an adipokine, that is, a cytokine secreted by adipocytes that binds to receptors in hypothalamic neurons and regulates appetite by inducing satiety. In obesity, increased serum leptin levels as well as leptin resistance are observed, possibly due to impaired leptin receptor signaling (128).

In cell culture, human colon carcinoma Caco-2 cells treated with leptin levels corresponding to normoleptinemia for 7 days exhibited an increase in PepT1 protein expression and transport activity, while hyperleptinemic concentrations did not induce any changes in PepT1 expression (81). At both leptin concentrations, PepT1 mRNA expression was only transiently elevated, with no differences between control cells and leptin-treated cells after 7 days. At the same time, also leptin receptor (Ob-R) mRNA and protein expression were transiently elevated at both concentrations, but decreased to below control levels after 7 days, showing a desensitization of leptin by leptin downregulating its own receptor. Feeding mice a hypercaloric diet over the course of 4 weeks resulted in diet-induced obesity, associated with a decrease in jejunal PepT1 mRNA and protein expression as well as transport activity. The diet-induced obesity was also accompanied by hyperleptinemia, while, as previously observed *in vitro*, jejunal Ob-R mRNA expression was downregulated leading to leptin resistance. These results show that intestinal PepT1 activity in mice may be impaired by leptin resistance in obesity, possibly affecting the uptake of PepT1 substrates. Reduced peptide uptake could possibly prevent protein-induced satiety, while at the same time reduced PepT1 transport activity may negatively affect the bioavailability of peptidomimetics. These findings could however not be reproduced in humans yet. A proteomics approach in jejunal tissue samples from morbidly obese individuals and healthy control subjects, in which expression levels of transport and drug-metabolizing enzymes in human jejunum were assessed, did not show any differences in jejunal PepT1 protein expression in morbidly obese compared with nonobese subjects (126). Summarizing these cell culture and *in vivo* data we might conclude that the body mass of an individual, which is translated in the systemic leptin concentration, has no or only a minor impact on the protein expression of PepT1.

PepT1 in short bowel syndrome

Whenever surgical resection of substantial small intestinal segments or small intestinal together with colonic segments is required, for example, in CD, a malabsorption disorder characterized by diarrhea and ensuing dehydration and malnutrition known as short bowel syndrome (SBS) may be the consequence. Over time, the human gastrointestinal tract manages to adapt to the loss of intestinal surface area, marked by a decrease in severity of diarrhea and increase in nutrient uptake. In animal tests, adaptive mechanisms have been observed leading to an upregulation in proliferation of intestinal mucosa with a concomitantly increased protein expression of intestinal nutrient transporters (72).

In a study on rats submitted to 70% mid-small intestine (SI) resection and control rats which were subject to intestinal transection and reanastomosis, PepT1 expression and activity as well as intestinal histomorphology remained unchanged in the control animals 4 weeks postoperation (116). In the remnant proximal jejunum, the rats submitted to SI resection displayed an increase in villus height and peptide uptake, while no changes were detected regarding PepT1 mRNA and protein expression. In distal ileum, PepT1 protein expression was reduced, while mRNA levels were decreased only transiently at 1 week postoperation and returned to normal levels after 4 weeks. PepT1 transport activity and villus height remained unchanged throughout the 4 weeks compared to the control animals. The colon on the other hand did not exhibit any changes regarding histomorphology, PepT1 expression, and transport activity.

In a different study, colonic changes were observed in rats during 4 weeks following 80% SI resection (154). In the transection control group, colonic PepT1 mRNA was not detectable, while in the resection group, PepT1 mRNA peaked at 2 weeks postoperation and decreased over the course of the following 2 weeks. Regarding PepT1 transport activity, a transient increase compared to the control group was observed after 2 weeks, correlating to the increase in PepT1 mRNA expression, but was no longer detectable after 4 weeks. Besides the beta-lactam antibiotic cephalexin, which was used as transport substrate for the assessment of PepT1 transport activity, rat colons were also perfused with the PepT1 substrate fMLF at 2 and 4 weeks postoperation. MPO activity was measured as a marker for inflammation. The control group did not display any changes in MPO activity at 2 weeks postoperation, while a distinct increase was observed in the resection group at that time point, accompanied by colon wall damage and inflammation, as assessed by histological analysis. Colonic perfusion with fMLF at 4 week postoperation did not induce any increase in MPO activity or signs of colonic inflammation in neither group. When the colonic perfusion of resection group animals with fMLF was performed in the presence of high concentrations of the dipeptide Gly-Gly, the increase in MPO activity and colon wall damage observed with fMLF alone could be inhibited entirely. These findings support the conclusion that there is no relevant fMLF uptake in a healthy state associated with physiological colonic PepT1 expression. Increased colonic PepT1 expression after SI resection however may enable an increase in the uptake of bacterial peptides, which may further contribute to intestinal inflammation, reflecting a mechanism possibly involved also in other inflammatory intestinal diseases like CD and UD.

Ziegler et al. examined the effects of SBS on intestinal morphology and PepT1 expression (199). Analysis was based on biopsy samples from individuals suffering from chronic malabsorption following intestinal resection of various lengths and from subjects with an intact intestine. No differences between the two groups were observed regarding intestinal mucosal growth, assessed by SI and colonic crypt depth as well as villus height and width. Both SI PepT1 mRNA and protein expression did not differ between the two groups either, while in the colon, PepT1 mRNA and protein expression were increased in the SBS group compared to the control group. Judging from these results, there seems to be no adaptive processes to compensate for the reduction in surface area accompanying small bowel resection in terms of mucosal

hyperplasia. There appear however to be mechanisms leading to the upregulation of colonic PepT1 expression, possibly in order to compensate for the SI malabsorption of dietary peptides.

A prospective study with patients that had undergone jejunocolonic anastomosis assessed the parameters colonic morphology and colonic PepT1 expression (93). Colonic crypt depth was increased in patients compared to healthy controls. The total number of epithelial cells per crypt was elevated in patients as well, while the rate of proliferating and apoptotic cells remained unchanged in both groups. No changes were detected in terms of PepT1 mRNA and protein expression.

The results from both human studies appear to directly contradict one another, with Ziegler et al. observing signs of adaptation after partial intestinal resection in terms of increased colonic PepT1 expression, in the absence of any changes detectable regarding gut morphology (199), while Joly and colleagues reported unchanged PepT1 expression but signs of mucosal growths (93). In both studies, colonic biopsies were taken from the mid-portion of the remaining colon. There are however differences in the design of the two studies that may account for the conflicting results: While the prospective study assessed long-term effects, with at least 2 years between resection and study, the group of Ziegler studied shorter-termed effects. In addition, Joly et al. excluded patients with intestinal inflammation, which has been associated with increased intestinal PepT1 expression, as discussed before.

PepT1 in celiac disease

Celiac disease is an autoimmune disorder that affects primarily the small intestine. Upon the ingestion of gluten, which comprises different proteins found in cereals like wheat and barley, genetically susceptible individuals experience gastrointestinal symptoms like chronic diarrhea, malabsorption, and abdominal distention (109). These symptoms are caused by an inappropriate immune response, leading to the production of autoantibodies and eventually, if untreated, to a loss of villus surface area due to small intestinal villous atrophy.

The assessment of mRNA levels of aquaporins (AQPs) and selected transporter mRNAs, amongst others PepT1, in human duodenal biopsies from individuals suffering from Celiac disease revealed distinct differences between patients and healthy controls (107). In untreated celiac disease patients, a reduction in mRNA expression of PepT1 and other solute transporters like SGLT1 and NHE3, as well as certain AQPs was observed. These reduced mRNA levels were accompanied in all transporters, including PepT1, by reduced duodenal protein expression, which was however completely restored in samples from individuals with treated celiac disease, that is, in gluten withdrawal. A loss of function of these transporters associated with reduced expression may help explain the mechanisms behind the malabsorption of water and nutrients encountered in Celiac disease. The intestine's already impaired absorptive capacity due to the reduction in intestinal surface area in the wake of villous atrophy could this way be aggravated by the transporters' reduced capacity. Impaired PepT1 transport activity may lead to the retention of oligopeptides in the intestinal lumen. Laforenza and coworkers speculate that due to the luminal increase of harmful peptides derived from gluten in the context of Celiac disease, reduced PepT1 expression may this way contribute to the disease progression and promote villous atrophy. We think, that this thesis is vague because most of the luminal oligopeptides longer than tripeptides will be hydrolyzed rapidly by brush-border peptidases to amino acids and di- and tripeptides. These small molecules should not be recognized by the immune system and should not induce degradation processes in the epithelium. Recognized peptide fragments of the gluten protein on the other hand are peptidase-resistant 12-mers or longer (201), but their way across the epithelium to the Lamina propria is PepT1 independent.

PepT1 Deficiency Models

Caenorhabditis elegans

C. elegans is a free-living, nonparasitic nematode whose natural habitat can be found in the soil, feeding mainly on bacteria (64). For many years, it has been used extensively as model organism in research, being a simple organism sharing many similarities with mammals in terms of physiology and genetics. In 1998, it became the first multicellular eukaryotic organism whose complete genome was sequenced (38). *C. elegans* is one of the most important model organisms employed for studying PepT1 deficiency and has contributed a great deal to the understanding of regulatory mechanisms that PepT1 is involved in.

Gene inactivation of the Na^+/H^+ exchanger *nhx-2* in *C. elegans* was reported to induce a reduction of intestinal lipid stores and longevity compared to wild-type worms (7, 133). Gene KO of PepT1, completely preventing intestinal uptake of di- and tripeptides in *C. elegans*, also leads to a severe delay in development as well as reduced body and brood size (123). Compared to wild-type animals, retardation in postembryonic development as well as an increase in reproductive life span was described. Nevertheless, dietary amino acid supplementation could not entirely make up for the lack of intestinal peptide absorption. While amino acid supplementation did not alter brood size in wild-type *C. elegans*, a partial recovery of brood size and generation time was observed in individuals with PepT1 KO, indicating that PepT1 transport activity is crucial for amino acid homeostasis in *C. elegans* required for physiological growth and development, and that dietary free amino acids cannot compensate for the lack of di- and tripeptides caused by PepT1 loss of function compared to wild-type animals.

Contrary to the observations made in previous reports, a study linking PepT1 to lipid metabolism in *C. elegans* reported an obesity phenotype in PepT1 KO *C. elegans*, featuring an

increase in total body lipids by 100% (165). Further analysis revealed that lipid accumulation was not caused by endogenous but exogenous fatty acids. Long-chain fatty acid lipogenesis was impaired in PepT1 KO animals, marked by a reduction in mRNA levels of enzymes and transport proteins involved in fatty acid synthesis, elongation, and desaturation. Uptake of intestinal free fatty acids on the other hand was markedly increased in PepT1 KO animals, which accounts for the observed increase in body fat. This accelerated fatty acid uptake is proposed to be induced by an increase in intracellular pH due to the absence of PepT1-mediated proton transport, leading to the promotion of the fatty acid flip-flop mechanism (77). In parallel, Brooks and colleagues found out that the obesity phenotype in adult PepT1 deficient animals is independent of the macronutrient composition of their diet (30). Other than in selected *C. elegans* strains with KO of various genes involved in nutrition-related processes and signaling pathways, the PepT1 KO worms gained as much body fat on bacteria with high carbohydrate/low fatty acid content (*E. coli* HB101) as on their standard bacteria food (*E. coli* OP50). Furthermore, the reproductive rate of PepT1 KO *C. elegans* further dropped when fed with the carbohydrate-rich *E. coli* HB101, indicating a severe prevention of nutrient assimilation followed by impaired fertility.

To investigate the biomolecular mechanisms behind the phenotypic changes associated with PepT1 deficiency in *C. elegans*, including reduced body and brood size and increased stress resistance, quantitative proteome analysis was conducted (71). The assessment of cytosolic amino acid concentrations revealed decreased levels of the vast majority of free cytosolic amino acids in PepT1 KO compared to wild-type worms. This lack in intracellular amino acids in turn was shown to affect TOR signaling (see paragraph "Regulation by oligopeptide breakdown and related signaling pathways"), leading to reduced expression of several TOR target proteins. Proteome analysis revealed differences in protein expression for several proteins between PepT1 KO and wild-type animals, predominantly a downregulation of ribosomal proteins, leading to impaired ribosome biogenesis and thus to low protein *de novo* synthesis in PepT1 deficiency. This reduced protein synthesis rate may contribute to the phenotypical changes including reduced body and brood size as well as delayed development observed in PepT1 KO *C. elegans*. The regulation of *C. elegans* PepT1 by the unfolded protein response signaling pathway via transcription factor XBP-1 was described in detail in paragraph "Regulation by oligopeptide breakdown and related signaling pathways."

Mouse models

The laboratory mouse, *Mus musculus*, is the classic mammal model used in research. It fulfils many criteria required in model organisms, for example, small body size, short generation time, close genetic similarity to humans (the human and mouse genomes have more than 90% in common), while being easily genetically manipulable (187). This characteristic has been utilized to generate PepT1 deficiency mouse models for the assessment of the role of PepT1 (or lack thereof) in mammalian organisms.

PepT1 deficient C57BL/6 mice do not exhibit any phenotypical impairment in terms of viability, fertility, growth rate, and weight gain compared to wild-type mice (85). Histological analysis did not reveal any differences between the two mouse strains, while on a functional level, a distinct decrease in intestinal dipeptide transport and permeability were observed in PepT1 KO animals, both *in vitro* in everted small intestinal gut rings, *in situ* in jejunal perfusion, as well as *in vivo* after gastric gavage. The results support the conclusion that the loss of function of PepT1 is not compensated by upregulation of other peptide transporters, while judging from the absence of a pathological phenotype in PepT1 deficiency, other adaptive responses are conceivable, for example, in the form of increased uptake of free amino acids.

When fluid absorption rates were measured *in vivo* in murine jejunal segments perfused with Gly-Sar solution, a considerable reduction in intestinal fluid absorption was observed in PepT1 KO mice compared to wild-type animals (37). In addition, feces of PepT1 KO mice display increased water content compared to wild-type animals (191). Accordingly, PepT1 may facilitate a significant amount of small intestinal salt and fluid absorption via substrate transport in postprandial state as well as general colonic water absorption.

Following up on the hypothesis of a compensatory induction of intestinal amino acid transport in case of PepT1 deficiency, amino acid transporter expression as well as amino acid concentrations in different body fluids and organs were determined (130). PepT1 KO mice exhibited an increase in total free amino acids in plasma compared to wild-type animals, while there were no differences in total urinary free amino acids. In urine, a strong increase in proline was observed in PepT1 deficiency. No differences were detected regarding liver tissue amino acid profiles. Analysis of jejunal tissue with regard to gene expression and proteome profiling of intestinal amino acid transport proteins did not reveal any differences between PepT1 KO mice and related controls. Comparing amino acid transport activity, assessed by glycine and L-proline transport in mucosal preparations of small intestine in Ussing chamber experiments as well as ^{3}H-proline uptake in jejunal everted gut sacs, did not show any differences between the two mouse strains. In *in vivo* experiments using gastric gavage with low-dose protein, no differences in amino acid plasma appearance rates were observed after 30 min. When the amount of protein introduced by gastric gavage was increased however, distinct differences between amino acid plasma levels between the two mouse strains were observed. Already 10 min after gavage, some amino acid concentrations in control mice were elevated compared to PepT1 KO animals. This effect increased over time, until 60 min after gavage the levels of 15 amino acids, including the essential branched-chain amino acids valine and isoleucine, as well as total amino acids were increased in wild-type compared to PepT1-deficient animals. These results were reflected by

amino acid levels in liver tissue 1 h after protein load. Overall, no indication was found for a compensation of reduced PepT1 activity by an upregulation of intestinal amino acid transporter expression or function. In the presence of low amounts of protein in the intestinal lumen, PepT1 transport activity appears to play a minor role regarding nutrient absorption. After the intake of large amounts of protein however, in a situation in which intestinal amino acid carriers may be saturated, additional amino acids in the form of di- and tripeptides may be absorbed via PepT1.

These findings are supported by an animal study in which PepT1 KO mice were fed low- and high-protein diets (HPDs) (131). When fed a low-protein diet containing 8 energy percent or a control diet with 21 energy percent of protein, food intake, and weight gain of PepT1 KO mice corresponded to those observed in wild-type animals. When fed a HPD containing 45 energy percent protein, a stronger initial reduction in food intake was observed in PepT1 KO compared to wild-type mice. No weight gain was detected in PepT1 KO animals despite a subsequent increase in food uptake to wild-type levels, which was accompanied by a so far unexplained impaired intestinal energy assimilation leading to higher fecal energy losses. In addition, plasma leptin—which has been linked to intestinal PepT1 regulation—was distinctly decreased in PepT1 deficient mice during the initial period of reduction in food intake, which would actually be expected to have an orexigenic effect. At the same time, plasma arginine levels were increased in PepT1 KO compared to wild-type mice, both on control as well as high-protein diet, indicating an interaction between arginine and hypothalamic leptin signaling, with arginine antagonizing the effect of low leptin on food intake, as suggested in previous studies (33). Other serum amino acid concentrations did not differ between mouse strains however. Analysis of liver tissue amino acid levels on the other side did reveal differences: Some metabolites, methionine, for instance, were decreased in PepT1 KO mice, while others like leucine and tyrosine were increased. Gastric gavage of 15N-labeled protein did not reveal any substantial changes in terms of 15N-labeled amino acid serum levels in PepT1 deficient mice, suggesting unaltered intestinal amino acid uptake.

In addition to HPD, also the effects of high carbohydrate diet (HCD) and high fat diet (HFD) in PepT1 deficiency were examined. Like low-protein diet before, HCD did not induce any obvious phenotypical changes in PepT1 KO mice (102). In PepT1 KO mice on HFD however, both reduced weight gain and reduced body fat depots were observed. Compared to wild-type animals, energy expenditure and body temperature were unaltered in PepT1 KO mice, while a concomitant reduction in caloric intake and increase in fecal energy loss was reported, which were not observed in PepT1 deficient animals. PepT1 KO mice displayed a diet-independent increase in cecal biomass, containing high amounts of carbohydrates in the HCD group resp. free fatty acids in animals on HFD, indicating, together with an observed fecal energy loss, impairment in small intestinal nutrient assimilation. Regarding gut morphology, an elongation of the small intestine was detected in PepT1 KO mice compared to wild-type animals on both diets, while on HCD, a slight reduction in villus length with unchanged villus width was observed. A possible explanation for the absence of weight gain and reduced fat storage in PepT1 KO mice on HFD could be the accentuation of these changes in SI mucosal architecture. While in wild-type mice, HFD led to an increase in small intestinal surface area associated with an increase in villus length, this trophic effect could not be observed in PepT1 deficient animals. Plasma levels of IL-6, which was previously shown to promote intestinal growth (92), were reduced diet-independently in all animals with PepT1 deficiency, which may explain the missing morphological response in PepT1 KO mice to the type of diet observed in wild-type animals.

Under standard conditions, the previously stated decrease in villus length in PepT1 deficiency was not observed in a further mouse study (196). However, transmission electron microscopic analysis revealed a previously unreported reduction in microvillus length in jejunal samples from PepT1 KO mice, leading to a distinct decrease in small intestinal surface area. In addition, body weight was lower in PepT1 KO mice compared to wild-type controls, which may be a consequence of the reduced intestinal absorptive surface and consequently reduced intestinal energy assimilation capacity. The assessment of intestinal proliferation and apoptosis rates revealed an increase in cellular apoptosis in villus IECs of PepT1 KO mice and a concurrent increase in proliferation that was, in contrast to wild-type mice, not restricted to crypt IECs. This altered IEC homeostasis along the crypt villus axis may be related to changes in miRNA expression observed in both villus and crypt cells of PepT1 deficient animals. Changes in expression levels and profiles of miRNA, which are involved in RNA silencing and post-transcriptional regulation of gene expression (10), may explain altered expression of certain IEC proteins that were detected in PepT1 KO animals.

Overall, these results show that the lack of PepT1 may have extensive effects on the mouse organism. However, the observed changes are highly dependent on different conditions: It is conceivable, that the animals' composition of microbiota (specific-pathogen-free vs. conventional), housing conditions and also the mouse strain may influence results. As demonstrated before, different locations of intestinal samples for analysis, for example, jejunum versus ileum or proximal versus distal colon, may produce different results. But above all, the composition of the diet clearly has a great impact on the outcome of such mouse studies.

Conclusion

Extensive analysis of the effect of numerous factors and molecular regulators on the expression and function of peptide transporter PepT1 have provided essential knowledge about this nutrient/drug transport system (Fig. 5). Significant progress was made in the field of crystal structure analysis, regarding the bacterial and human PepT isoforms, which not only allowed the design of new peptidomimetic drugs but also

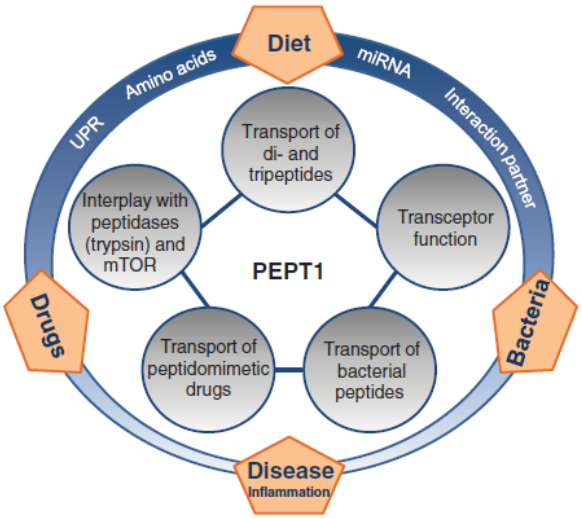

Figure 5 Functions of peptide transporter PepT1. In the last decade it became more and more obvious, that next to the transport of di- and tripeptides and peptidomimetic drugs, the activity pattern of PepT1 is much more complex. In distal ileum and in the colon the uptake of bacterial-derived peptides plays a role in inflammatory processes and in the development of diseases. For stabilization of the intestinal homeostasis PepT1 is embedded in a network consisting of protein hydrolysis (peptidases), amino acid supply, mTOR signaling, protein *de novo* synthesis, and unfolded protein response (UPR). The analysis of its transceptor function, its regulation by micro RNAs (miRNA), and its direct protein-protein interactions are relatively novel fields and they will improve the understanding of the peptide transporters.

revealed a direct interaction between mammalian PepTs and trypsin. The position of PepT1 in the cellular network regulating amino acid homeostasis, including dietary protein breakdown by proteases and peptidases, mTOR signaling, protein *de novo* synthesis, and the unfolded protein response, has been further defined. Without any doubt the existing *C. elegans* and mouse *in vivo* models will be complemented with the novel small intestine organoid cultures, both from mouse and human, which provide an excellent *in vitro* model to study transporters in their physiological environment. However, the picture of PepT1 is still incomplete, and over the next years the regulation of PepT1 gene expression by miRNAs, its protein-protein interactions, and its role as cellular transceptor and in IBD will require further study to understand its function in the complex network of gut physiology.

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RESEARCH ARTICLE

Peptide Homeostasis

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Appearance of Di- and Tripeptides in Human Plasma after a Protein Meal Does Not Correlate with PEPT1 Substrate Selectivity

*Florian Rohm, Thomas Skurk, Hannelore Daniel, and Britta Spanier**

Scope: Peptide transporter 1 (PEPT1) function is well understood, yet little is known about its contribution toward the absorption of dietary amino acids in the form of di- and tripeptides. In the present human study, changes in plasma concentrations of a representative oligopeptide panel are investigated after meat intake.

Methods and results: Based on a method for quantitative analysis of a panel of selected di- and tripeptides in biological samples, the kinetics of plasma changes of peptides derived from a widely accessible dietary protein source are described. The findings demonstrate postprandial changes of a whole spectrum of dipeptides of different size, charge, and polarity in peripheral blood in a dose-dependent manner after consumption of chicken breast in healthy human volunteers. Although the substrate specificity of PEPT1 is well known, the spectrum of peptides appearing in blood cannot be matched to the affinity to PEPT1. Stability against hydrolysis by exo- and endopeptidases appears to be another factor influencing their presence in blood. In addition, the study shows that dipeptides, including gamma-glutamyl-peptides, as well as tripeptides are common components present in human plasma.

Conclusion: Besides amino acids, human peripheral blood contains numerous di- and tripeptides. The dietary source determines their abundance and composition.

1. Introduction

Intestinal peptide transport has been studied for more than four decades, but it is still uncertain to which extent it contributes to overall amino acid availability from dietary proteins. The corresponding transporter PEPT1 was identified in 1994,[1,2] and since then its structure and many of its functional features have been elucidated, mainly ex vivo and in some in vivo systems such as *Caenorhabditis elegans* or mice.[3–6] PEPT1 mediates proton-dependent electrogenic uptake of di- and tripeptides but also of a variety of peptide-like drugs, defining their oral bioavailability.[7] Dating back to the 1970s, in vivo studies in humans revealed a significant absorption of di- and tripeptides into systemic circulation[8,9] when selected glycyl-peptides were provided via the intestine. Glycyl-peptides were chosen because of their higher stability against peptidases, allowing a higher chance for detection in the blood stream. However, these peptides are not representative for the entirety of all 400 possible dipeptides that can arise from 20 proteinogenic amino acids when dietary proteins undergo hydrolysis in the small intestine. Although the uptake of peptides into enterocytes via PEPT1 is fairly well understood, their mechanism of crossing the enterocytes' basolateral membrane remains unclear, as the corresponding transporter has eluded identification so far.[10]

To shed further light on the physiological role of PEPT1, we developed a method for quantitative analysis of postprandial plasma concentrations of peptides by Liquid chromatography (LC) coupled with double mass spectrometry (MS) (LC-MS/MS). We applied this method in a human study to profile appearing peptides in peripheral blood after serving two quantities of chicken breast or rice for comparison to assess also the dose-dependency of postprandial peptide plasma levels. Anserine and carnosine, two well-described markers for the consumption of poultry and red meat, respectively,[11] were included in the method for comparison. We generated a representative panel of peptides that best can reflect the entire range of 400 possible dipeptides from 20 proteinogenic amino acids to assess changes in plasma peptide concentrations after protein ingestion.

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2. Experimental Section

2.1. Chemicals, Reference Substances, 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). The internal standard Masschrom from ChromSystems (München, Germany) was used and expanded by glutamine-D5 and asparagine-15N2 (20.0 µmol L^{-1} each), both 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 the amino acids glycine, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-citrulline, L-glutamic acid, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, 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-Leu, Gly-Asn, Gly-Asp, Gly-Gln, Gly-Gly-Ile, Gly-Pro, 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 and carnosine from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Study Design and Plasma Sample Collection

Twelve healthy volunteers (six males, six females) were enrolled into the study. Inclusion criteria were: age between 18 and 40 years, BMI <30 kg m^{-2}, and non-smokers. Exclusion criteria were any gastro-intestinal diseases affecting nutrient uptake, digestion, metabolism, and excretion; chronic diseases (e.g., hepatitis B and C, diabetes mellitus); other acute illnesses; antibiotic therapy in the last 6 months; regular intake of medication (except oral contraceptives); pregnancy and lactation; known allergies or intolerances to the tested foods. The volunteers' baseline characteristics were an age of 28 ± 5 years (males) and 25 ± 3 years (females). The BMI was 25.2 ± 2.8 kg m^{-2} (males) and 21.5 ± 2.4 kg m^{-2} (females).

The intervention was part of the EU Joint Programming Initiative (JPI) within the Food Biomarkers Alliance (FOODBALL) and was carried out at the ZIEL-Institute for Food and Health, Core Facility for Human Studies, Technical University of Munich, Freising, Germany. It was registered in the German Clinical Trials Register (DRKS #00010133), and the study protocol was approved by the ethical committee of the Technical University of Munich (project number: 51/16S). Written informed consent was obtained from all subjects prior to the beginning of the study.

The study represented a randomized controlled intervention study using a crossover design with three treatment periods of 48 h separated by a wash-out period of at least 1 week and a maximum duration of 6 weeks. Randomization and allocation of volunteers into the study was done with SNOSE (sequentially numbered, opaque, sealed envelopes). The principle study design can be found elsewhere.[12] Volunteers were randomly assigned either to receive rice alone (125 g, cooked, 30 g margarine, 1.5 g salt) as control, or rice with 100 g chicken breast (dose 1; prepared sous vide) or with 200 g chicken breast (dose 2). During a 2-day run-in period, participants followed a strict vegetarian diet. The day before the intervention, participants received the standardized rice meal as dinner. Test foods and control were given as breakfast in a random order. Additionally, standardized meals were provided for lunch and dinner plus 250 mL water per hour during the intervention day. Blood samples were taken at time points 0, 1, 2, 4, 6, 12, and 24 h. Plasma was immediately separated (3000 × g, 10 min, 4 °C) and stored at −80 °C until analysis.

2.3. Cell Culture and Dipeptide Transport Assay

The human colon carcinoma cell line Caco-2 was used to assess transport and hydrolysis of selected dipeptides (Gly-Pro and Pro-Gly) in transwell culture. PEPT1 knockout clones and corresponding control cells were generated using CRISPR/Cas9 technology by targeting exon 5 of *pept1* with a double nickase approach: Caco-2 cells were transfected, using Cell Line Nucleofector Kit T (Lonza, Basel, Switzerland), with nickase vector pMB-Casv2-D10A (kindly provided by Oskar Ortiz Sanchez, Helmholtz Zentrum München, Munich, Germany) containing the gRNAs 5′-TTGTCCAATTGTGTAGACAA-3′ and 5′-CTCACAGACCACAACCATGA-3′, and the homology-directed repair vector HR110PA-1 (System Biosciences, Mountain View, USA). HR110PA-1 contained homology arms of ≈800 bp 5′ and 3′ of the single strand breaks introduced by the nickases at positions 98713044 and 98713114 of chromosome 13, inserting puromycin resistance and red fluorescent protein genes in exon 5 of the human *PEPT1* gene as selection markers. Cells were cultured in uncoated culture flasks in minimal essential medium (MEM) containing 10% fetal calf serum, 1% MEM non-essential amino acids solution (Sigma-Aldrich, Taufkirchen, Germany), and 50 µg mL^{-1} gentamicin, respectively 5 µg mL^{-1} puromycin. At confluence, cells were trypsinized and seeded in 12-well culture plates with transwell inserts at 180 000 cells per well. Cells were incubated at 37 °C and 5% CO_2 and differentiated for 21 days after reaching confluency. For the transport study, cells were rinsed with PBS, and 0.5 mL of MES-buffered transport medium (pH 6.0) containing 500 µM of each peptide were used as apical solution, while 1.5 mL of HEPES-buffered transport medium (pH 7.4) served as receiver in the basolateral compartment. Samples were collected after 0 and 360 min of incubation at 37 °C and 5% CO_2.

2.4. Sample Preparation and Derivatization

20 µL of plasma or 10 µL of transport medium were evaporated with 25 µL internal standard under a stream of nitrogen and then derivatized with PITC.[13] After incubation with 190 µL of derivatization mix at room temperature while shaking at 750 rpm for 5 min (Eppendorf Thermomixer Comfort; Eppendorf, Hamburg, Germany), 10 µL of PITC was added, followed by a 20-min incubation at room temperature and 750 rpm shaking. Samples were dried under a stream of nitrogen and then reconstituted in 300 µL 5 mM ammonium acetate in methanol.

Table 1. Dipeptide panel and properties of amino acid constituents.

Dipeptide	N-terminus	C-terminus
Ala-Gly	Neutral, aliphatic	Neutral, aliphatic
Ala-His	Neutral, aliphatic	Basic, polar, positive, essential
Ala-Phe	Neutral, aliphatic	Neutral, aromatic, non-polar, essential
Arg-Gly	Positive, basic, polar	Neutral, aliphatic
Gly-Asn	Neutral, aliphatic	Neutral, amide, polar
Gly-Asp	Neutral, aliphatic	Negative, acid, polar
Gly-Gln	Neutral, aliphatic	Neutral, amide, polar
Gly-Pro	Neutral, aliphatic	Neutral, cyclic, non-polar
Gly-Val	Neutral, aliphatic	Neutral, aliphatic, non-polar, essential
Lys-Glu	Positive, basic, polar, essential	Negative, acid, polar
Phe-Ala	Neutral, aromatic, non-polar, essential	Neutral, aliphatic
Phe-Gly	Neutral, aromatic, non-polar, essential	Neutral, aliphatic
Pro-Gly	Neutral, cyclic, non-polar	Neutral, aliphatic
Trp-Glu	Neutral, aromatic, non-polar, essential	Negative, acid, polar
Trp-Leu	Neutral, aromatic, non-polar, essential	Neutral, aliphatic, non-polar, essential

70 µL of this sample solution was diluted 7:10 with water and transferred to Eppendorf microplates for measurement.

2.5. LC-MS/MS Analysis

Analyses were performed on a triple quadrupole 3200 Q Trap LC-MS/MS system (AB Sciex, Framingham, MA) with a 1200 Series binary pump, a degasser, and a column oven (Agilent, Santa Clara, CA). Samples were injected by an HTC pal autosampler (CTC Analytics, Zwingen, Switzerland). Parameters of the ion spray source operating in positive ESI mode were set to: curtain gas, 20 psi; collision gas, medium; ion spray voltage, 5500 V; temperature, 500 °C; ion source gas 1, –40 psi; ion source gas 2, 50 psi. Chromatographic separation was accomplished by a VDSpher 100 PUR C18-SE column (length 150 mm, internal diameter 3.0 mm, particle size 3.5 µm; VDS optilab), at a column temperature of 50 °C. Eluent A consisted of 0.2% formic acid in water, and eluent B consisted of 0.2% formic acid in acetonitrile. Gradient elution comprised the following steps: a linear decrease from 98% to 60% A at 500 µL min^{-1} over 6 min, hold for 2 min, followed by a linear decrease to 0% A at 500 µL min^{-1} over 7 min. Re-equilibration was achieved by a linear increase to 98% A at 500 µL min^{-1} over 1 min, increase of flow rate to 800 µL min^{-1} over 1 min, hold for 2 min, and decrease to 500 µL min^{-1} over 1 min, leading to a total running time of 20 min. Analytes were quantified in scheduled multiple reaction monitoring (MRM) with a target scan time of 1 s and a detection window of 60 s. Quadrupoles were set to unit resolution.

2.6. Calibration and Quantification

Calibration was achieved by spiking control plasma with different concentrations of amino acids and peptides. A seven-point calibration was performed by addition of increasing amounts of each standard, as well as internal standard. Calibration curves were drawn and fitted by linear regression through zero with 1/x weighting. Data analysis was performed with Analyst 1.5 software (AB Sciex).

2.7. Statistical Analysis

Areas under curve (AUC) of plasma concentrations were calculated with GraphPad PRISM 5.00 (GraphPad Software, Inc.). For comparison of AUC and maximum values of plasma concentrations, and dipeptide concentrations from cell culture experiments, one-way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was applied. p-values <0.05 were considered statistically significant.

3. Results

3.1. Representative Panel for Peptide Quantification by LC-MS/MS

The representative dipeptide panel for LC-MS/MS analysis comprised peptides with amino acids differing in charge (positive/negative/neutral), in side-chain polarity (acidic/basic polar/non-polar), and in side-chain class (aromatic/aliphatic/cyclic/basic/acid/amide) in either N- or C-terminal position, when possible (Table 1). Anserine and carnosine were included as known markers for meat intake. Two tripeptides were also added, comprising Gly-Gly-Ile and Val-Pro-Pro, which have been studied for its predicted function as angiotensin-converting-enzyme (ACE) inhibitor.[14] Furthermore, γ-Glu-Leu, an "atypical" dipeptide containing a pseudo-peptide bond between the γ-carboxyl group in glutamate and the α-amino group in leucine was included as a representative lead of gamma-glutamyl dipeptides, which are found, among others, in dairy and soy products and meat (Table S1, Supporting Information).[15–17] We also included pairs of structural isomers (Gly-Pro/Pro-Gly and

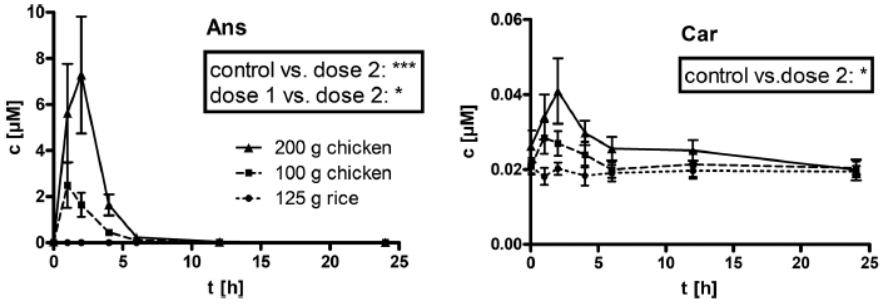

Figure 1. Changes in postprandial anserine (Ans) and carnosine (Car) plasma concentrations in healthy volunteers in response to 125 g rice (control), 100 g chicken breast (dose 1), and 200 g chicken breast (dose 2) provided as means ± SEM ($n = 12$). p values represent differences in AUC between the three test meals. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Ala-Phe/Phe-Ala) to assess if dipeptides made up of identical amino acids, yet in a different conformation, are handled any differently during protein digestion and peptide uptake.

3.2. Quantitative Analysis of Postprandial Peptide Plasma Concentrations

3.2.1. Anserine and Carnosine—Markers for Meat Consumption

Anserine, which is known to be present in high concentrations in skeletal muscle of birds, displayed strong increases in plasma when volunteers consumed chicken breast, both in a time- as well as in a dose-dependent manner. While no increase could be observed in the rice-fed group (control), plasma concentrations in the 200 g chicken breast fed group (dose 2) increased distinctly already after 1 h, reaching their maximum of more than 7 µM after 2 h, rapidly decreasing after 4 h, and reaching almost pre-meal levels after 6 h (**Figure 1**). A similar profile was observed after 100 g chicken (dose 1), where anserine concentrations reached a maximum of 2.5 µM after 2 h. For carnosine, a food marker peptide for the consumption of red meat, which is also present at low concentrations in poultry, a slight dose- and time-dependent increase in plasma concentration could be observed.

3.2.2. Peptides with Dose- and Time-Dependent Plasma Increases

In addition to anserine and carnosine, we detected plasma concentration increases in samples of dose 1 and dose 2 for the majority of the dipeptides in the panel (Gly-Asn, Gly-Asp, Gly-Gln, Gly-Val, Lys-Glu, Phe-Gly, Pro-Gly, Trp-Leu, and γ-Glu-Leu) as shown in **Figures 2** and 4. For these analytes, a pronounced plasma level increase could be observed in dose 2, and a less pronounced increase in dose 1, with no or hardly any increase in the rice-fed group. Dose-dependency was reflected by an average 2.5-fold increase in AUC of plasma concentrations of these 11 peptides in dose 2 compared to dose 1 (Table S2, Supporting Information). Maximum concentrations were usually reached after 4 h, and for Gly-Val, Phe-Gly, and Trp-Leu already after 2 h. Usually after 12 h, plasma concentrations reached levels equal to the fasting state. Peak concentrations varied greatly between the different dipeptides, ranging from 0.013 µM for Gly-Asn to a maximum of 1.2 µM for Pro-Gly.

3.2.3. Peptides without Changes in Postprandial Plasma

The other dipeptides of the panel, namely Ala-Gly, Ala-His, Ala-Phe, Arg-Gly, Gly-Pro, Phe-Ala, and Trp-Glu, as well as the two tripeptides Gly-Gly-Ile and Val-Pro-Pro did not display any significant increases in any test plasma. Concentrations showed great variability, ranging from as low as 0.01 µM for the two tripeptides and Ala-Phe and Arg-Gly to 0.2 µM for Gly-Pro (Figure 2).

3.2.4. Differences between Dipeptide Isomers

We detected distinct differences in plasma concentrations of structural dipeptide isomers in response to oral protein supply. While this was not the case for Ala-Phe and Phe-Ala, both of which did not appear in plasma (**Figure 3**), Gly-Pro and Pro-Gly behaved markedly differently. For Gly-Pro, we did not detect any notable increase in plasma concentration after consumption of rice or chicken, whereas Pro-Gly showed one of the strongest increases of all analytes, reaching a maximum level of 1.2 µM after 4 h, only second to anserine (**Figure 4**).

3.3. Postprandial Amino Acid Plasma Levels

In addition to peptides we also analyzed postprandial amino acid plasma concentrations (Figure S1, Supporting Information). For all proteinogenic amino acids except for cysteine (which was not analyzed), glycine, and glutamine, dose-dependent increases in postprandial plasma levels were observed, with only small changes after rice consumption and major changes after chicken intake, with highest concentrations in dose 2 samples (Table S3, Supporting Information). Dose-dependency was reflected by an average 1.9-fold increase in AUC of plasma concentrations of these 17 amino acids in dose 2 compared to dose 1 (Table S2, Supporting Information). For almost all amino acids, peak plasma concentrations were reached after 2 h, with the exception

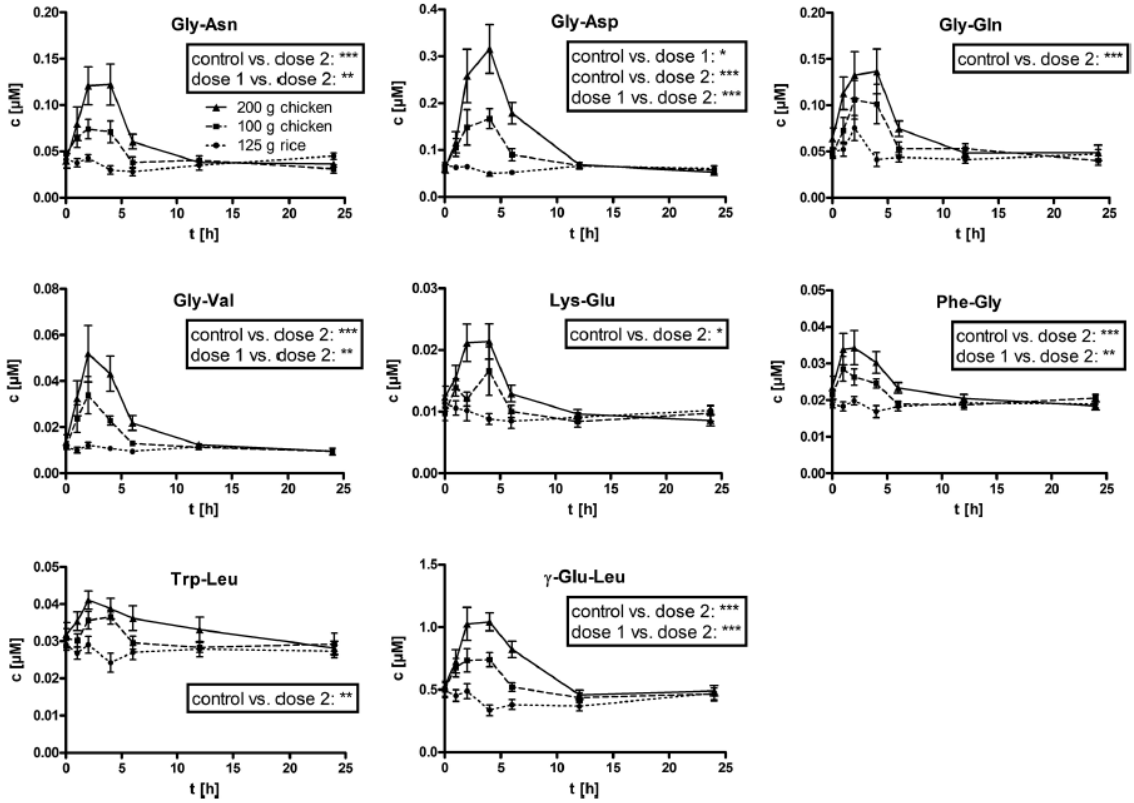

Figure 2. Selected dipeptides with significant increases in postprandial plasma concentrations in healthy volunteers in response to 125 g rice (control), 100 g chicken breast (dose 1), and 200 g chicken breast (dose 2) provided as means ± SEM ($n = 12$). p values represent differences in AUC between the three test meals. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

of proline and glycine at 1 h, and valine reaching maximum levels after 4 h.

3.4. Gly-Pro and Pro-Gly Isomer Transport by Caco-2 Cells

After a 6-h incubation of Caco-2 cells with 500 µM each of Gly-Pro and Pro-Gly in the apical compartment of transwell cell culture, we observed distinct differences regarding changes in apical and basolateral dipeptide concentrations (**Figure 5**). In apical compartments of Caco-2 control cells, Gly-Pro concentration was reduced by one-third, while in PEPT1 knockout cells it was reduced by less than 10%. In case of Pro-Gly we did not observe a quantifiable decline in apical concentrations in either cell line. Concentrations in the basolateral compartment increased to 4 µM in case of Gly-Pro and to almost 6 µM for Pro-Gly in control cells. In PEPT1 knockout, basolateral concentrations never exceeded around 0.5 µM for either dipeptide after 6 h.

4. Discussion

Dietary proteins are digested by a multitude of proteases and peptidases in the small intestine, releasing short-chain peptides and free amino acids. Given the huge variety of protein sequences in a daily diet and the capacity of endo- and exoproteases and corresponding peptidases for cleavage of normal peptide bonds (with the exception of those formed with proline), in essence an undefined mixture of thousands of different short-chain peptides and amino acids is generated. Among these oligopeptides, only di- and tripeptides are known to be absorbed in intact form. This is exclusively achieved by PEPT1, which was shown not to transport free amino acids or peptides >3 amino acid residues.[7] Based on 20 proteinogenic amino acids, a theoretical number of 8400 different di- and tripeptides could be generated during digestion and serve as substrates. Extensive studies on the unique substrate specificity of PEPT1 have demonstrated that indeed all the different substrates could be transported—with few exceptions—but with pronounced differences in transport affinity, depending on polarity and charge, but also side-chain size and stereospecificity.[18] Peptides taken up by PEPT1 are subjected to hydrolysis by a variety of cytosolic peptidases with a preference for short-chain peptides, which cleave substrates intracellularly, releasing amino acids to the basolateral but also to the apical membrane. The latter can serve as counter-substrates in the uptake of free amino acids via exchange transporters.[19] However, a yet undefined fraction of di- and tripeptides escapes hydrolysis and is exported across the basolateral membrane in intact form

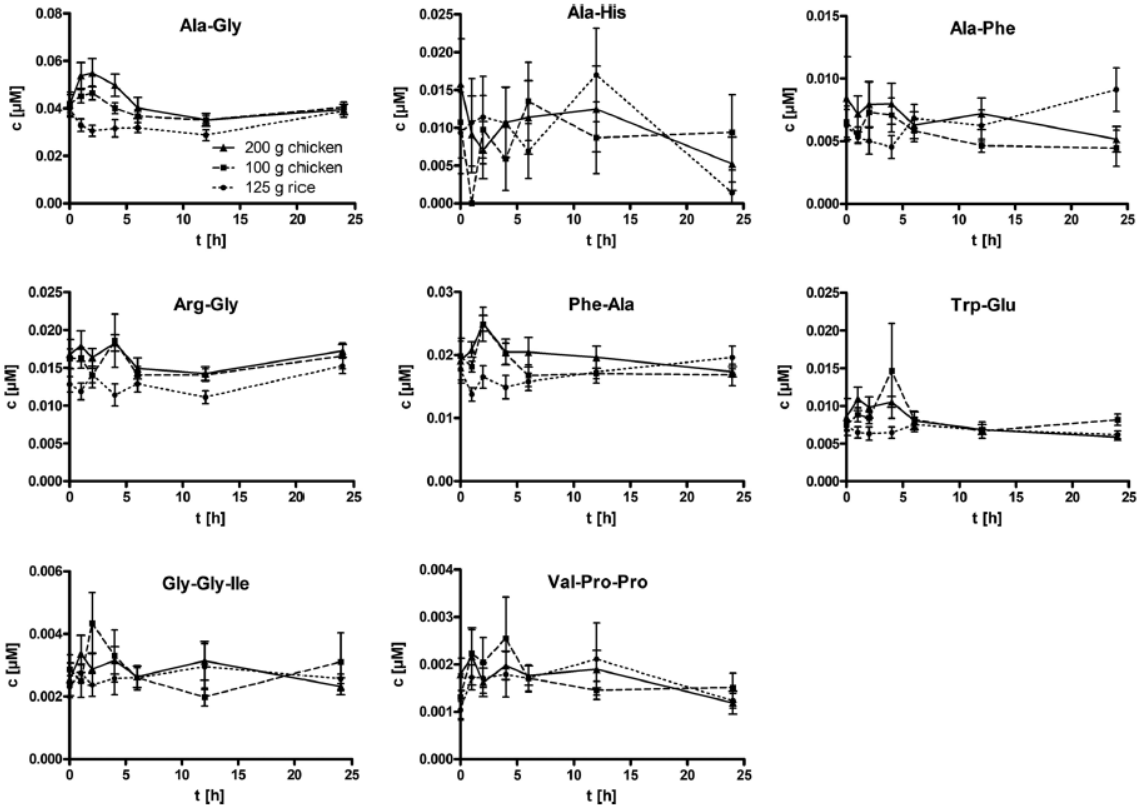

Figure 3. Dipeptides without significant increases in postprandial plasma concentrations in healthy volunteers in response to 125 g rice (control), 100 g chicken breast (dose 1), and 200 g chicken breast (dose 2) provided as means ± SEM ($n = 12$).

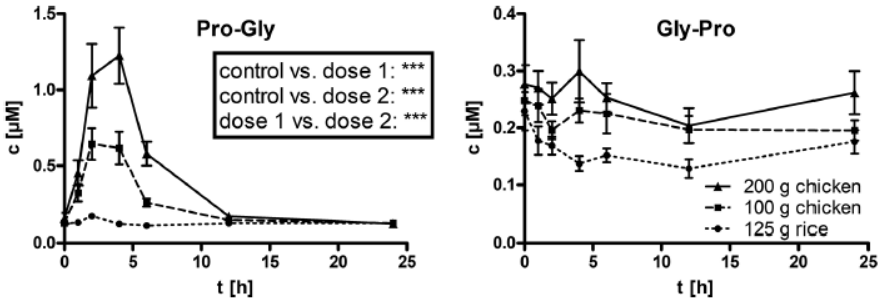

Figure 4. Changes in postprandial plasma concentrations of structural dipeptide isomers Pro-Gly and Gly-Pro in healthy volunteers in response to 125 g rice (control), 100 g chicken (dose 1), and 200 g chicken (dose 2) provided as means ± SEM ($n = 12$). p values represent differences in AUC between the three test meals. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

by a so far unidentified basolateral oligopeptide transporter.[10] That selected di- and tripeptides can cross the intestine in intact form to appear in peripheral blood has been demonstrated for a variety of glycine-containing peptides[8] and for some selected tripeptides such as Val-Pro-Pro and Ile-Pro-Pro,[14] which were identified as ACE-inhibitors and were incorporated into dairy products for lowering blood-pressure.[20] In those cases concentrations of intact peptides in the nanomolar range were detected.

To date it is unknown if the postprandial appearance of oligopeptides in circulation constitutes an overflow mechanism in states of excess supply, or if this has a physiological function, considering bioactivity has been reported for food-derived di- and tripeptides, like Val-Pro-Pro and Ile-Pro-Pro mentioned above.[21] Phenotypical differences between PEPT1 knockout and wild-type mice only manifesting under high-protein, but not standard or low-protein diets[22] suggests that exogenous peptides are not essential for the organism, and that the appearance of peptides in

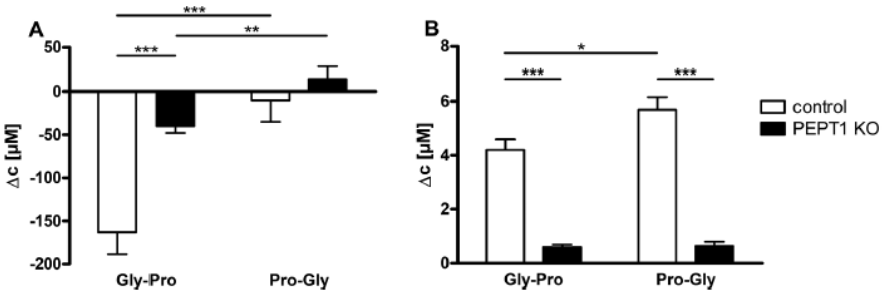

Figure 5. Transport of Gly-Pro and Pro-Gly in Caco-2 transwell cell culture (means ± SEM, $n = 6$). A) Concentration changes in apical compartments of transwell culture after 6 h of incubation in control and PEPT1 knockout cells; B) Concentration changes in basolateral compartments of transwell culture after 6 h of incubation in control and PEPT1 knockout cells. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

plasma is probably an overflow mechanism. On the other hand, many tissues express peptide transporters[23] and cytosolic peptidases, which enable them to absorb and metabolize peptides, while overall kidney and liver, the latter of which does not express plasma membrane peptide transporters, are the main organs for plasma oligopeptide assimilation.[24,25] This efficient peptide metabolism makes oligopeptide solutions fundamental in parenteral nutrition due to the increased stability and solubility of dipeptides compared to certain free amino acids.[26,27]

What has to our knowledge never been studied is the level and appearance of intact di- and tripeptides in systemic circulation after intake of ordinary dietary proteins. We therefore developed an LC-MS/MS method for the quantification of 20 different di- and tripeptides in biological samples and applied it to human plasma.

We used chicken breast as a test meal because of its high protein content of 20–25%,[28] with the myofibrillar proteins myosin and actin making up 40% of the total muscle, but also containing titin, tropomyosin, troponin, and nebulin, as well as the stromal proteins collagen and elastin.[29] Chicken muscle contains large quantities of anserine—a dipeptide composed of ß-alanine and 1-methylhistidine—for which appearance in plasma following chicken meat consumption is known to occur. In addition, anserine in urine is reported to serve as a marker for poultry consumption.[11] This is remarkable since anserine, together with carnosine (another ß-alanyl-dipeptide) undergoes hydrolysis by carnosinase, so that only a fraction of those dipeptides is cleared into urine. Anserine and carnosine in plasma here served as "benchmarking" dipeptides, contained in chicken meat and known as PEPT1 substrates.[30] Anserine and carnosine concentrations indeed increased in plasma in a dose-dependent manner for two servings of chicken meat of 100 or 200 g, with anserine reaching peak levels of around 7 µM whereas carnosine did not exceed 0.04 µM. In this respect it is surprising that some of the other dipeptides for which we developed quantification reached even higher plasma levels than carnosine.

A systematic search in the amino acid sequences of chicken muscle protein for dipeptide-motifs that showed strong plasma level increases such as Gly-Asn, Gly-Asp, Gly-Gln, or Pro-Gly, as compared to those that did not show any increase (Ala-Gly, Arg-Gly, Gly-Pro, Gly-Gly-Ile, Val-Pro-Pro) could not explain the differences found between these two groups of analytes (Table S4, Supporting Information). There are a number of reasons why different peptides may become detectable in peripheral blood or not. Among them are a) that the corresponding sequence might not occur in proteins of the test food, b) that the corresponding sequence is present in the protein but the peptide is further hydrolyzed to free amino acids, c) that the peptide released is taken up by PEPT1 into enterocytes followed by rapid hydrolysis by cytosolic peptidases, or d) that the peptide reaches circulation intact (portal and peripheral blood) but is rapidly hydrolyzed by endothelial or plasma peptidases.

Among the analyzed dipeptides we found the highest plasma peak concentrations for Gly-Asn, Gly-Asp, Gly-Gln, and Pro-Gly. Interestingly, the atypical dipeptide γ-Glu-Leu, a dipeptide with a pseudo-peptide bond between the γ-carboxyl group of L-glutamic acid and L-leucine, was also among the dipeptides displaying the strongest plasma concentration increases, with a peak level of more than 1 µM after 2 and 4 h. γ-glutamyl di- and tripeptides can be found after meat proteolysis, and contribute to the meat's sour, salty, or brothy taste.[16] It is however not clear to which extent γ-glutamyl dipeptides are suitable PEPT1 transport substrates. Yet, a variety of γ-glutamyl-peptides have been identified recently in biomarker discovery studies, and some of these peptides can be associated with diseases.[31] We here show that uptake from the intestine from dietary sources contributes to the plasma level of these dipeptides.

The reason why many of the dipeptides for which we observed significant plasma concentration increases are entities that contain glycine in N-terminal position may be explained by the fact that those are more stable toward hydrolysis.[32] But otherwise we cannot identify any particular features in the peptides appearing in plasma that are known to define PEPT1 substrate specificity.[18] A particularly striking finding was that Gly-Pro, while having a similar fasting plasma concentration, did not show any postprandial plasma level increase, while Pro-Gly showed one of the strongest effects, both in terms of AUC as well as peak plasma concentration for both servings of chicken. This is surprising since previous studies suggested that proline in N-terminal position of a dipeptide causes a substrate's very low affinity for transport, the nitrogen being part of the ring system.[33] Moreover, peptide bonds with proline as a constituent have special features such as *cis* and *trans* configuration, with only the *trans* form in a peptide serving as PEPT1 substrate.[34]

There are other factors besides PEPT1 substrate selectivity conceivable for contributing to the observed postprandial serum

peptide pattern. Peptides >3 amino acid residues may cross the intestinal mucosa via the paracellular pathway and may subsequently be hydrolyzed by endothelial or plasma peptidases, releasing di- and tripeptides in the blood. In addition, peptides generated during intracellular protein turnover may not be hydrolyzed entirely to free amino acids, but some of them might escape hydrolysis and reach circulation, just like dietary oligopeptides absorbed by PEPT1, which may explain or at least contribute to the fasting serum peptide concentrations we observed in our study. All these processes are entirely independent from PEPT1, and may contribute to the disparity between the observed postprandial plasma peptide pattern and PEPT1 substrate specificity.

Since we cannot conclude that the differences observed for Pro-Gly and Gly-Pro are indeed linked to their specific transport behavior by PEPT1, or that the presence of the Gly-Pro or Pro-Gly motif or the probability of being released by hydrolysis is different, we employed a Caco-2 cell system in transwell culture with exposure to the two isolated dipeptides. Although both dipeptides appeared at similar rates in the basolateral compartment, we only observed a distinct reduction in apical Gly-Pro concentration, while there was no detectable decline in apical Pro-Gly levels. Based on the difference in volume of both transwell compartments (apical 0.5 mL, basolateral 1.5 mL), a basolateral increase of ≈5 µM accounts for an apical decrease of around 15 µM. This corresponds to around 10% of what was observed as apical loss, which in turn suggests a major contribution of hydrolysis of Gly-Pro by brush border membrane or even cytosolic peptidases, but not of Pro-Gly. In the absence of PEPT1, basolateral levels were reduced significantly to a residual level of around 0.5 µM—which is likely attributable to paracellular permeation. Interestingly, we observed also a less pronounced reduction in apical Gly-Pro concentration, suggesting not only reduced absorption but also a reduced rate of hydrolysis of Gly-Pro in PEPT1 knockout cells. Further experiments will have to determine if this is due to a reduced expression of brush border membrane peptidases or reduced intracellular hydrolysis due to much lower dipeptide uptake into the cells. Based on our findings, we are not able to explain the differences in plasma appearance of Pro-Gly versus the lack of appearance of Gly-Pro. It is clearly not associated with differences in cellular uptake via PEPT1 and/or release into blood. Inspection of amino acid sequences of various muscle proteins revealed that Pro-Gly is a rare motif in myofibrillar muscle proteins like myosin and actin, but it is fairly abundant in collagen and elastin. The finding that Gly-Pro, for which we did not observe any postprandial increases, displays a distribution pattern similar to Pro-Gly, suggests that there may be either differences in the liberation of the two peptides during digestion or that Pro-Gly is far less prone to intestinal hydrolysis than Gly-Pro, for which the Caco-2 data provide some evidence.

The magnitude of postprandial increases in amino acid plasma concentrations as opposed to the concentrations reached for individual dipeptides suggests that the majority of dietary proteins from chicken breast are degraded to free amino acids. Interestingly, according to the data on amino acid composition of chicken breast (see Figure S2, Supporting Information and Table S5, Supporting Information), the most abundant amino acids aspartic acid/asparagine, glutamic acid/glutamine, and glycine, do not display the strongest postprandial increases in plasma levels. Despite its high abundance in many food sources, glutamic acid has one of the lowest plasma levels among all amino acids in peripheral blood.[35] This can be explained by its use, like aspartate and their corresponding amines, as prime energy sources of enterocytes[36] and by high rates of hepatic extraction in first pass. In contrast lysine, leucine, and alanine showed strong increases, but even for amino acids with low abundance in the protein sequences of chicken breast (Figure S1, Supporting Information) peak plasma levels of 20 µM for tryptophan, 40 µM for tyrosine, and 50 µM for methionine and histidine were reached. β-alanine displayed the lowest baseline plasma level but showed an ≈15-fold and thus the highest fold-increase in plasma concentrations among all amino acids when volunteers consumed 200 g chicken, being the product of hydrolysis of anserine and carnosine.[37] Although we identified only around ten dipeptides with pronounced changes in plasma levels following consumption of chicken breast, our study demonstrates that there is a certain probability that dipeptides escape intestinal hydrolysis and hepatic first pass and appear in intact form in peripheral blood. For most of these peptides, plasma levels even showed dose-dependent increases. Other protein sources and quantities of proteins would likely reveal other peptides to appear—possibly also in different quantities. We here show that most of the quantified peptides have plasma levels in fasting state in the range of 10–50 nM. This level remains fairly stable over time (for controls receiving rice), and fasting levels are also usually reached again after 6–12 h for the peptides that showed altered postprandial levels. Based on fasting levels of 10–50 nM with an estimated average of 25 nM, an estimated pool size of 10 µM of dipeptides composed of L-a-amino acids is circulating in peripheral blood. With all possible γ-glutamyl-dipeptides shown to exist in human plasma and with a surprisingly high concentration of around 500 nM found here for γ-glutamyl-leucine, another 10 µM of dipeptides can be estimated to circulate. As we only quantified two different tripeptides of possible 8000 possible entities (Gly-Gly-Ile and Val-Pro-Pro), based on concentrations of 1–2 nM, another peptide fraction of around 10 µM total can be postulated to circulate in peripheral blood. Besides biological activities proposed for a large variety of individual di- and tripeptides, this pool of amino acids in bound form also needs to be considered when whole body nitrogen metabolism is discussed. Given that metabolomics platforms now more often contain dipeptides in their panels, the future will likely bring much more knowledge to this largely "unknown" fraction of plasma constituents.

In conclusion, based on an LC-MS/MS method for the quantitative analysis of a panel of selected di- and tripeptides in biological samples, we describe the kinetics of plasma changes of peptides derived from an ordinary dietary protein source. Our findings demonstrate postprandial changes of a whole spectrum of dipeptides of different size, charge, and polarity in peripheral blood in a dose-dependent manner after consumption of chicken breast in healthy human volunteers. Although the substrate specificity of the intestinal transporter PEPT1 is well known, the spectrum of peptides appearing in blood while the plasma concentrations of others did not change cannot be matched to the specificity of PEPT1. However, our study also shows that dipeptides including γ-glutamyl-peptides as well as tripeptides are "normal constituents" of human plasma that together could represent up to 5% of the total plasma pool of amino acids.

Supporting Information

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

Acknowledgements

T.S., H.D. designed the study; F.R. undertook data collection, developed the new method, and performed LC-MS/MS analysis of human samples; F.R., B.S. interpreted results of experiments; H.D., T.S. provided essential reagents and materials; F.R., B.S. wrote the manuscript; and all authors critically read the manuscript and approved the final version. The authors thank Barbara Gelhaus and Ronny Scheundel for technical assistance. The authors thank their colleagues, especially Pieter Giesbertz and Kurt Gedrich, for fruitful discussions and statistical support. The authors are also grateful to the study team of the ZIEL Institute for Food and Health, namely Yu-Mi Lee and Beate Brandl for their engagement in the clinical study. F.R. was financially supported by the DFG Graduate School GRK 1482 "Interface functions of the intestine between luminal factors and host signals." The intervention study was part of the HDHL Joint Programming Initiative "The Food Biomarkers Alliance" (FOOBALL) and was financially supported by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2814ERA01E.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

amino acids, peptidases, peptide transporter 1, peptide transporter, protein digestion

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RESEARCH ARTICLE

Peptide Homeostasis

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

*Florian Rohm, Hannelore Daniel, and Britta Spanier**

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.

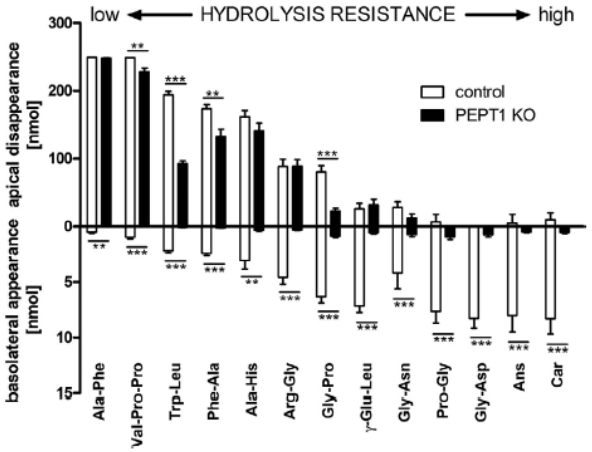

Figure 1. Transport and hydrolysis of oligopeptides in Caco-2 transwell cell culture, provided as mean ± SEM ($n = 6$). Apical disappearance and basolateral appearance of selected oligopeptides in transwell culture after 6 h of apical incubation in control and PEPT1 knockout cells. p-Values represent 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, γ-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

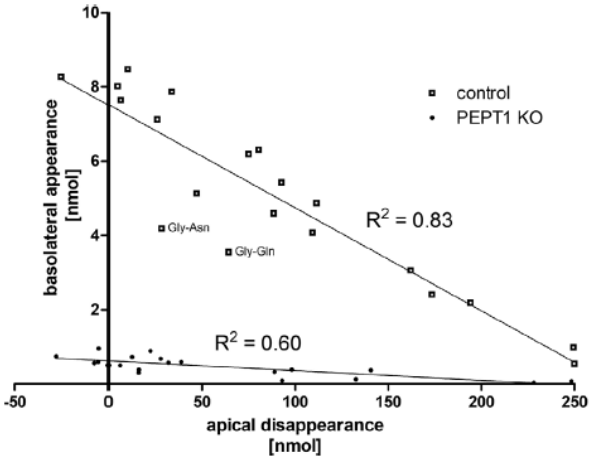

Figure 2. Linear regression of apical disappearance and basolateral appearance of panel peptides after 6 h of incubation with control and PEPT1 knockout Caco-2 cells in transwell cell culture. Gly-Asn and Gly-Gln are outliers in control cells.

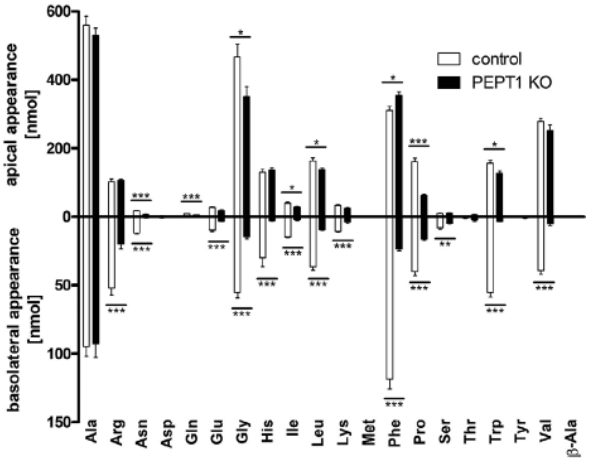

Figure 3. Free amino acids from oligopeptide hydrolysis in Caco-2 transwell cell culture provided as mean ± SEM ($n = 6$). Apical and basolateral appearance of amino acids in transwell culture after 6 h of apical incubation with peptide solution in control and PEPT1 knockout cells. p Values represent 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 4A**). 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

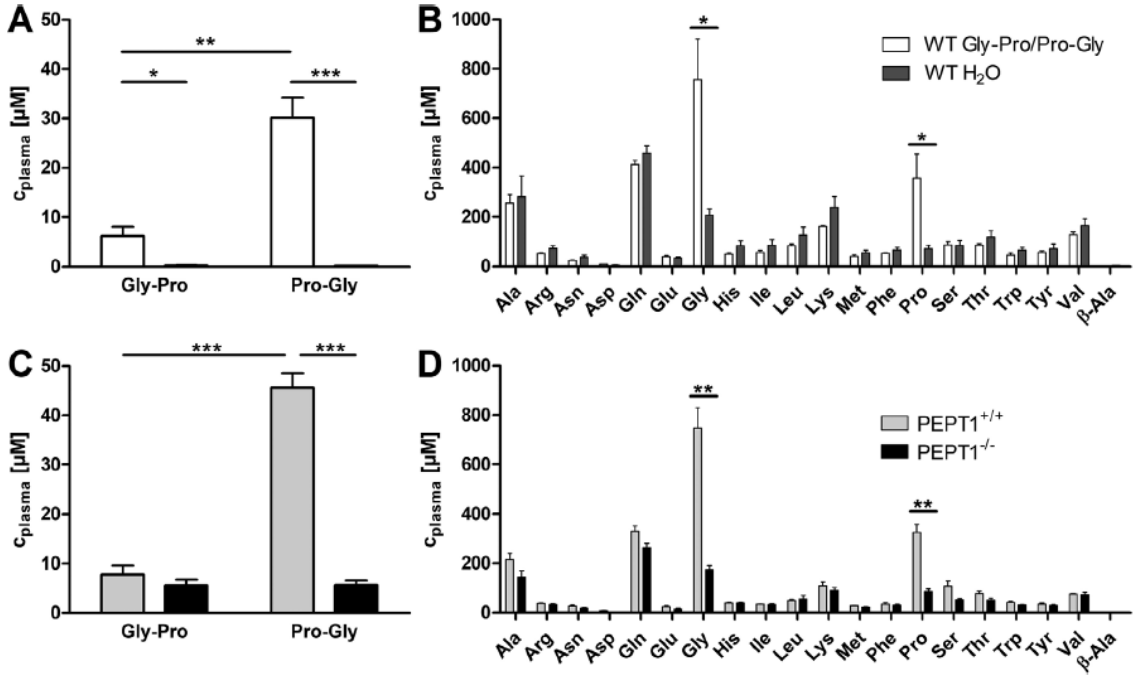

Figure 4. Gly-Pro, Pro-Gly, and amino acid plasma concentrations in wild-type (WT), PEPT1 knockout (PEPT1$^{-/-}$), and control mice (PEPT1$^{+/+}$) after gavage with peptide solution or water, provided as mean ± SEM ($n = 3$). A) Gly-Pro and Pro-Gly plasma concentrations 30 min after gavage with peptide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

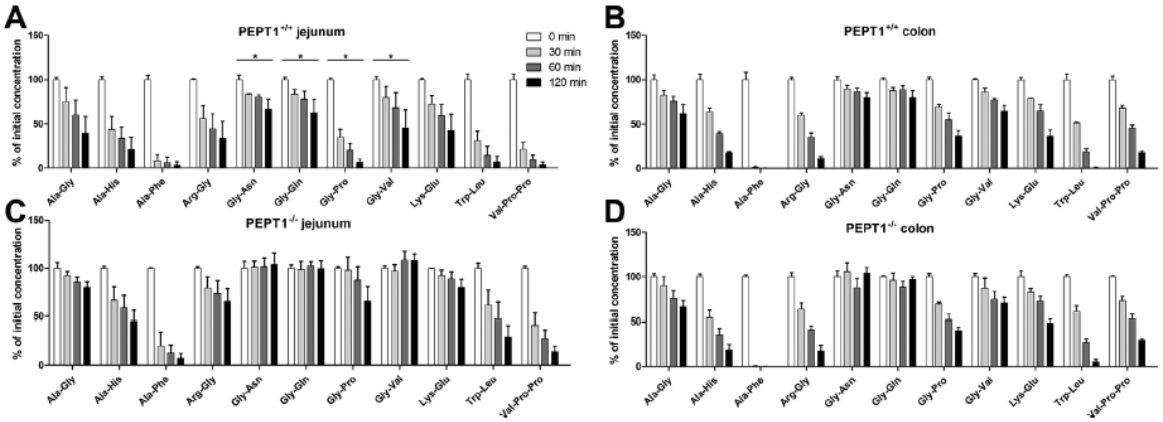

Figure 5. Changes in selected oligopeptide concentrations during incubation with sections of mouse intestines from PEPT1$^{+/+}$ and PEPT1$^{-/-}$ animals, provided as mean ± SEM ($n = 3$). Relative concentration in oligopeptide solution (compared to 250 µM per peptide at t_0) during incubation with 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

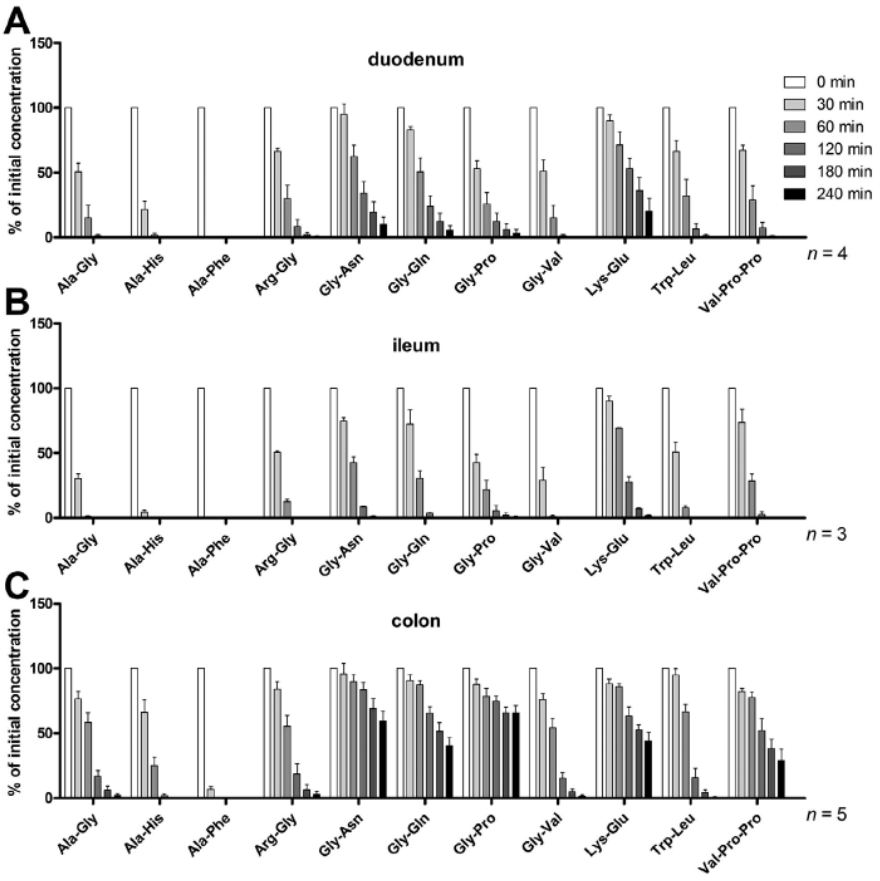

Figure 6. Selected changes in oligopeptide concentrations during incubation with human intestinal mucosa samples, provided as mean ± SEM (n = 3–5). Relative concentration in oligopeptide solution (compared to 250 µM per peptide at t_0) during incubation with human mucosa samples from A) duodenum, B)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

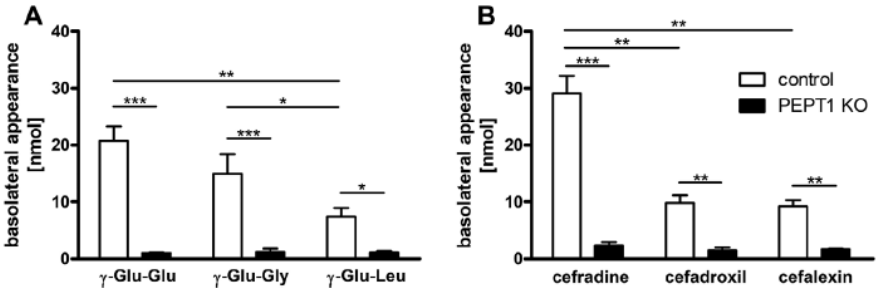

Figure 7. PEPT1-dependent transport of γ-glutamyl-dipeptides and aminocephalosporins in Caco-2 transwell cell culture, provided as mean ± SEM ($n = 5$). Basolateral appearance in transwell culture of control and PEPT1 knockout cells after 6 h of apical incubation with A) γ-glutamyl-peptide solutions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

Keywords

amino acids, PEPT1, peptidases, peptide transporters, protein digestion

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6.2 Letters of approval

6.2.1 Letter of approval – Comprehensive Physiology

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