Molecular Cloning and Functional Characterization of a Turkey Intestinal Peptide Transporter (tPepT1), and Developmental Regulation of PepT1 Expression in Turkey and Broiler Embryos

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(ABSTRACT)

A cDNA clone encoding a turkey intestinal peptide transporter, tPepT1, was isolated from a turkey small intestinal cDNA library by screening with our chicken PepT1 (cPepT1) cDNA probe. The tPepT1 cDNA is 2,921-bp long and encodes a 79.4 kDa protein of 714 amino acids (AA) with 12 predicted transmembrane domains. The isoelectric point (pI) of tPepT1 is 5.9, which is much lower than that of PepT1 cloned from chicken (pI = 7.5) and other species. The AA sequence of tPepT1 is 94.3% identical to cPepT1 and ~ 60% identical to PepT1 from rat, sheep, rabbit, and human. Using a two-electrode voltage-clamp technique in *Xenopus* oocytes expressing tPepT1, Gly-Sar transport was pH dependent, but independent of Na^+ and K^+. For the dipeptides Gly-Sar and Met-Met, the evoked inward currents indicated that the transporter was saturable and had a high affinity for these substrates. However, transport of the tetrapeptide, Met-Gly-Met-Met, exhibited a possible substrate inhibition. To study developmental regulation of PepT1 in broiler and turkey embryos, 12 Nicholas turkey or Cobb × Cobb broiler embryos (six males and six females) were sampled daily from 5 d before hatch to the day of hatch (d 0). The abundance of PepT1 mRNA in the small intestine was quantified densitometrically from northern blots after hybridization with full-length cPepT1 and tPepT1 cDNA as probes. There was a quadratic increase ($P < 0.001$) in PepT1 mRNA abundance with age in turkey and broiler embryos. The relative increase in abundance of PepT1 mRNA in intestinal tissue from 5 d before hatch to d 0 was much less in the turkey than in the broiler (3.2-fold vs 14-fold). The dramatic increase in PepT1 mRNA abundance indicates a developmental regulation of the PepT1 gene and that there may be a crucial role for PepT1 in the neonatal chick and poult.

Keyword: Peptide transporter, Developmental regulation, Broiler, Turkey, Embryo

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Chapter I

INTRODUCTION

In the lumen, peptides rather than free amino acids, had been shown to be the major digestion products after a protein meal (Matthews, 1991). The absorption of di- and tripeptides across the enterocyte brush border membrane is mediated by H^+/peptide cotransporter (PepT1; Ganapathy et al., 1994). PepT1 is unique among the solute transporters in that H^+, instead of Na^+, is the cotransported ion and PepT1 is able to bind and translocate a large number of substrates, including a wide range of di- and tripeptides, and several peptidomimetic drugs (Ganapathy et al., 1994). The molecular nature of peptide transporters has been identified by cloning of PepT1 from different species (Fei et al., 1994; 2000; Liang et al., 1995; Saito et al., 1995). The recent cloning and characterization of ovine (Pan et al., 2001) and chicken (Chen et al., 2002) peptide transporters in our laboratory improved our understanding of the molecular structure and basic functions of PepT1 in food-producing animals. PepT1 was mainly expressed in the small intestine of human and nonruminant animals (Liang et al., 1995; Saito et al., 1995; Chen et al., 1999). In ruminant animals sheep and Holstein cows, PepT1 was also expressed in the omasum and rumen (Chen et al., 1999).

Molecular identification of PepT1 provides an opportunity to elucidate the mechanisms of its regulation. PepT1 expression and activity can be regulated by several factors including development, diet, hormonal or neuronal signals in the small intestine (Meredith and Boyd, 2000). It was reported that the expression of PepT1 changed markedly during development in rats (Miyamoto et al, 1996; Shen et al.,

2000). In broilers, PepT1 mRNA increased dramatically from embryonic d 18 to the day of hatch (Chen, 2001). However, the natural pattern of PepT1 expression before hatch in broiler embryonic small intestine is not known. The purpose of this study was to clone and characterize the function of peptide transporter from turkey, another major domestic fowl. The developmental regulation pattern of PepT1 expression in turkey and broiler embryonic small intestine was also studied.

Chapter II

REVIEW OF LITERATURE

Protein is an important nutrient to animals. The intestinal oligopeptide transporter (PepT1) provides a major mechanism for protein assimilation in the small intestine. By taking advantage of molecular technology, specifically the cloning of PepT1 from the sheep and chicken, the understanding of nutritional and physiological impacts of PepT1 in domestic animals has been greatly improved. Compared with our knowledge of structures and mechanisms of PepT1, studies on the regulation of PepT1 have lagged behind. This review will focus on a summary of structural and functional characteristics and distribution of PepT1 cloned so far, developmental and dietary regulation of PepT1, and new findings on hormonal regulation and the diurnal rhythm of PepT1.

Structure and Functional Characteristics of Intestinal Peptide Transporters

Dietary protein entering the small intestine is digested by gastric and pancreatic proteases, resulting in a mixture of amino acids and small peptides. Numerous studies have shown that absorption of the products of protein digestion in the small intestine occurs primarily in the form of small peptides rather than amino acids (Ganapathy et al., 1994). Intestinal uptake of small peptides, primarily those consisting of two or three amino acids, is mediated by the H^+-coupled peptide transporter (PepT1) localized at the brush-border membranes of intestinal epithelial cells (Leibach and Ganapathy, 1996).

In 1994, a new era of peptide transport studies started with the cloning of the rabbit intestinal peptide transporter rPepT1 as the first mammalian peptide transporter

(Fei et al., 1994). Since then, PepT1 from human (Liang et al., 1995), rat (Saito et al., 1995), mouse (Fei et al., 2000), sheep (Pan et al., 2001), and chicken (Chen et al., 2002) have been cloned. Different from the earlier work on PepT1 from human or experimental animals, the recent cloning of ovine and chicken PepT1 in our lab revealed the structure and function of PepT1 in domestic animals.

Belonging to the POT (proton oligopeptide transporter) superfamily (Meredith and Boyd, 2000), PepT1 cloned from different species possess similar molecular structures. The molecular weight of PepT1 is 79 kDa although the number of encoded amino acids is different among species, with the maximum (714 amino acids) for chicken PepT1 (Chen et al., 2002) and the minimum (707 amino acids) for rabbit and ovine PepT1 (Fei et al., 1994; Pan et al., 2001). The predicted PepT1 protein has 12 transmembrane domains and intracellular amino and carboxyl groups (Fei et al., 1994; Liang et al., 1995; Saito et al., 1995; Fei et al., 2000; Pan et al., 2001; Chen et al., 2002). A long hydrophilic intracellular loop exists between transmembrane domains 9 and 10. Analysis of amino acid sequences also indicates that there are some potential N-linked glycosylation sites on the large extracellular loop and the number of the N-linked glycosylation sites differ from three (rabbit PepT1; Fei et al., 1994) to seven (human PepT1; Liang et al., 1995). PepT1 from most of species have one protein kinase C (PKC) and one protein kinase A (PKA) phosphorylation site intracellular, except for human PepT1 (Liang et al., 1995), which has two PKC phosphorylations sites but no PKA phosphorylation sites, and ovine PepT1 (Pan et al., 2001), which has four PKC and three PKA phosphorylation sites. The amino acid similarity of PepT1 among species is illustrated in Table 1 in Chapter III.

The peptide transport system in the intestinal brush border membrane is called a "tertiary active" transport system. In this system, H^+ are cotransported with small peptides across the apical membranes of enterocytes and released into the cytoplasm by PepT1. The extra- to intracellular H^+ gradient, which is the driving force of the peptide transport, is reestablished by the apical membrane-bound Na^+/H^+ exchanger which pumps H^+ out of cell in exchange for Na^+. The extra- to intracellular Na+ gradient is maintained by the basolateral membrane-bound Na^+/K^+ ATPase which pumps Na^+ actively out of the cell (Ganapathy et al., 1994). Utilization of two different driving forces, H^+ for the peptide transporter and Na^+ for amino acid transporters is advantageous to the organism to maintain optimal protein nutrition because this removes competition between amino acids and peptides for the translocating energy source and allows these absorptive processes to operate in parallel (Ganapathy et al., 1994).

Peptide transport activity of PepT1 is H^+ dependent but Na^+, K^+, Cl^- and Ca^+ independent (Fei et al., 1994; Pan et al., 2001; Chen et al., 2002). The pre-steady-state current study in oocytes expressing human PepT1 (hPepT1) indicated that H^+-coupled peptide transport for hPepT1 was an ordered, simultaneous transport model in which H^+ binds first to PepT1 (Mackenzie et al., 1996). Results from a recent study with PepT1 mediated dipeptide transport using giant patch clamp indicated that some dipeptides (Lys-Gly and Gly-Asp, but not Gly-Lys and Asp-Gly) can bind to PepT1 on the cytosolic site and be transported in the outward direction and, unexpectedly, enhance inward transport currents probably by increasing the turnover rate (Kottra et al., 2002).

PepT1 has rather broad substrate specificity. Wide range of di- or tripeptides, regardless of their molecular weight, electrical charge and hydrophobicity could be transported by ovine and chicken PepT1, demonstrating the importance of peptide transport in domestic animals (Pan et al., 2001; Chen et al., 2002). Moreover, the intestinal peptide transport system mediates the absorption of a broad range of peptidomimetic drugs, such as β-lactam antibiotics, the anti-cancer agent bestatin, angiotensin converting enzyme inhibitors, renin inhibitors, thrombin inhibitors, and thyrotropin-releasing hormone and its analogues, thereby playing an important pharmacological role for oral drug delivery (Yang et al., 1999).

The importance of PepT1 serving as a carrier for oral delivery of drugs has led to intensive studies on substrate specificity and the structural requirements for substrate recognition by PepT1. Transmembrane domains 7, 8, and 9 of PepT1 were reported to be responsible for substrate recognition (Fei et al., 1997). In addition, studies on mutations generated for histidine residues on rabbit PepT1 indicated that the H^+- binding site of PepT1 is located in the N-terminal half of the transporters and is associated with histidine residues at amino acid positions 57 and 121 (Terada et al., 1996). By employing computational analysis of possible substrate conformations in combination with transport assays in transgenic yeast cells and *Xenopus* oocytes expressing PepT1, Döring et al. (1998) suggested that electrogenic transport by PepT1 requires, as a minimum, the two ionized head groups separated by at lease four methylene groups. Consequently, a > 500 pm < 630 pm distance between the two charged centers (carboxylic carbon and amino nitrogen) is sufficient for substrate recognition and transport. Several studies on transport of charged or neutral dipeptides or peptidomimetics by PepT1 showed that the zwitterionic form of the

substrate is preferred by the peptide transporters under physiological conditions (Amasheh et al., 1997; Lister et al., 1997). Affinity of the anionic dipeptide increased dramatically by lowering the pH. A cationic substrate showed only a weak affinity for PepT1 at all pH values (Amasheh et al., 1997). However, the inward currents of cationic peptide (Lys-Lys) transport in oocytes expressing oPepT1 (ovine PepT1) was much higher than that of zwitterionic and anionic substrates (Gly-Sar, Met-Met, and Glu-Glu) at a neutral pH (7.0) (Pan et al., 2001).

Distribution of the Intestinal Peptide Transporters

Molecular cloning and expression studies have resulted in the identification of two distinct proton-coupled oligopeptide transporters, PepT1 and PepT2 in rabbit, rat and human. PepT1 and PepT2 both have 12 transmembrane domains, but have a low similarity (30 to 50%) between the two groups (Pan, 2000). PepT1 is mainly expressed in the small intestine and is responsible for intestinal absorption of protein digestion products, whereas PepT2 is mainly expressed in kidney, serving to reabsorb filtered peptides, peptide-derived antibiotics, and peptides produced as a result of the action of luminal peptidase (Miyamoto et al., 1996). In addition to its presence in the digestive tract, trace amounts of PepT1 mRNA were observed in the kidney of the rabbit, rat and human, the liver of the rabbit and human, the brain of the rabbit, and the placenta and the pancreas of human (Fei et al., 1994; Liang et al., 1995; Saito et al., 1995; Meredith and Boyd, 2000). In rat kidney, PepT1 mRNA was expressed in the early regions of proximal tubules, whereas PepT2 mRNA was predominantly expressed in the later regions of the proximal tubule (Meredith and Boyd, 2000). Thus, the reabsorption of peptides from the nephron appears to be a sequential, two-stage process, with the low-affinity, high-capacity PepT1 accounting for the majority

of uptake, and the high-affinity, low-capacity PepT2 scavenging remaining peptides from the ultrafiltrate.

Although it has been reported that the absorptive capability for small peptides is greater in the proximal small intestine than in the distal small intestine (Ganapathy et al., 1994), the PepT1 mRNA distribution along the small intestine varies among species. An even distribution profile of PepT1 mRNA was observed along the longitudinal axis of rat and mouse small intestine (Erickson et al., 1995; Miyamoto et al., 1996). In rabbit and pig, PepT1 mRNA abundance was higher in duodenum and jejunum than in ileum (Fei et al.1994; Chen et al., 1999). In White Leghorns and broilers, the strongest hybridization was found in the duodenum, and the jejunum and ileum showed only faint bands, whereas, in ruminant animals sheep and lactating Holstein cows, the abundance of PepT1 was observed mostly in the jejunum and ileum of the small intestine (Chen et al., 1999).

In monogastric animals, PepT1 mRNA was only detected in the small intestine, whereas in ruminant animals such as sheep and lactating Holstein cows, PepT1 mRNA was also observed from the omasum and rumen, although the expression was lower compared with the small intestine (Chen et al., 1999). The results confirm the peptide transport capabilities of omasal and ruminal epithelial cells in ruminant animals, which had been demonstrated before by using in vitro studies (Matthews and Webb, 1995; Matthews et al., 1996). So far, no PepT1 expression in colon, cecum, skeletal muscle, heart, spleen, lung, or mammary gland has been found for all the animals studied (Fei et al, 1994, Chen et al, 1999; Meredith and Boyd, 2000).

PepT1 is only located on the apical microvillous plasma membrane of the absorptive epithelial cells of the small intestine (Origara et al., 1996, 1999). PepT1 mRNA became detectable at the villus/crypt junction and reached a maximum 100 to 200 μm from this point (Meredith and Boyd, 2000). Immunolocalization of PepT1 confirmed this, showing that expression of the PepT1 protein is regulated, and that only as cells leave the crypt and migrate towards the tip of the villus do they express the peptide transport activity (Meredith and Boyd, 2000). Results from a recent study of expression and cellular distribution of PepT1 during development in rat small intestinal epithelium indicated different PepT1 cellular distribution patterns dependent upon age (Hussain et al., 2002). The results of the immunocytochemical studies showed that, although the distribution of PepT1 protein was exclusively in the brush border membrane of the intestinal absorptive epithelial cells from both prenatal and mature animals, immediately after birth PepT1 was found in the subapical cytoplasm, in basal cytoplasm, and in the basolateral membrane.

The above study is the first direct evidence of the existence of an intestinal basolateral peptide transporter, although PepT1 expression was only observed on the day of birth. The existence of a basolateral peptide transporter is of considerable interest. The postulate of the existence of a basolateral peptide transporter is based on the evidence for the presence of circulating plasma peptides and that the membranes are impermeable to orally delivered peptidomimetic drugs (Terada et al., 1999). The apical and basolateral peptide transporters in human intestinal Caco-2 cells were functionally compared by the characterization of [^{14}C]glycylsarcosine transport. Differences in transport kinetics of apical and basolateral [^{14}C]glycylsarcosine transport indicated that a facilitative (not H^+-coupled) peptide transport system was

localized in the basolateral membranes of Caco-2 cells. Basal transport of β-lactam antibiotics and an orally active ACE inhibitor, fosinopril, were also found to be different from apical transport (Meredith and Boyd, 2000; Shu et al, 2001). The apical peptide transporter had a significantly greater affinity for fosinopril than did the basolateral peptide transporter in Caco-2 cells (K_m, 154 versus 458 μM, respectively), indicating that basolateral peptide transport was rate-limiting for transcellular peptide transport.

Regulation of Intestinal Peptide Transporter and Transport Activity

Luminal nutrient transport activity varies in response to many factors, such as development, dietary nutrients, and luminal hormonal or neuronal signals (Shiraga et al., 1999; Meredith and Boyd, 2000). The cloning of the intestinal peptide transporters has led to a surge of studies on cellular mechanisms regulating intestinal peptide transport. This review will focus on developmental and dietary regulation of peptide transporters. New findings on hormonal regulation and diurnal rhythm of PepT1 will also be discussed. Regulation of amino acids and sugar transport will also be discussed to provide a comparison to enhance understanding of transport regulation in general.

Developmental Regulation. The gut faces two abrupt shifts in the functional demands placed on it (Toloza and Miamond, 1992). First, at birth or hatching, the gut suddenly takes over the entire burden of nutrient acquisition from the placenta or yolk sac. Second, mammals make a major qualitative change in diet as they grow, such as the shift from milk to the adult diet at the time of weaning in mammals. Thus, the digestive system of animals undergoes dramatic structural and functional change

during development. However, ontogeny and developmental regulation of intestinal nutrient transport has received little attention.

It has been reported that intestinal glucose and amino acid transporters were present prenatally in humans, guinea pigs, sheep, rabbits, and rats (Pácha, 2000). The intestinal peptide transporter was also observed to be present prenatally. In rat, PepT1 mRNA and protein were present as early as d 20 of fetal life in intestinal tissue (Shen et al., 2001). In broilers, PepT1 mRNA was detected at embryonic d18, although in very low abundance (Chen, 2001).

In the rabbit, Gly-Pro influx in both jejunum and ileum increased from an early fetal stage to d 6 of postnatal life (Guandalini and Rubino, 1982) and then declined continually, reaching minimum values as an adult (> 3 mo). Further, the uptake of Gly-Gly was substantially higher than that of glycine in fetal (25 to 30 d gestational age), newborn (1 to 6 d old), and suckling or weaned (10 to 50 d old) rabbits, indicating the preferential uptake of small peptides over their free amino acids in newborns and infants (Guandalini and Rubino, 1982). It was also observed that Gly-Gly and Gly-Leu influx in isolated everted intestinal segments of guinea pig were significantly greater in sucklings (3 to 4 d) than in weanlings (10 to 14 d), which, in turn, were greater than in adults (Himukai et al., 1980). Kinetically, the developmental change in jejunal Gly-Gly influx was related to a decrease in maximal transport capacity.

Intestinal PepT1 mRNA levels were highest in 4-d-old rats, and then decreased reaching the adult level by d 28 after birth (Miyamoto et al., 1996). Results from another recent study in rats confirmed this result and indicated that expression levels of PepT1 mRNA and protein were maximal 3 to 5 d after birth in the

duodenum, jejunum, and ileum, and then declined rapidly (Shen et al., 2001). Expression of PepT1 was also observed to increase transiently at d 24, most notably in the ileum, but to a moderate extent (70% of that observed on d 3 to 5). Interestingly, significant PepT1 expression was observed in the colon during the first week of life, but levels were undetectable shortly thereafter through adulthood. Lactose absorption (Pácha, 2000) has also been found in the proximal colon of newborn sucklings. The transport ability of the colon to absorb peptides and amino acids was suggested as compensation for the temporary low capacity of the small intestine to absorb nutrients.

Toloza and Miamond (1992) reported that amino acid absorption in rat small intestine declined around two-fold from birth to adolescence. The postnatal decline is typically greater for essential amino acids than for nonessential amino acids. In rats, a high-protein milk diet is often replaced with an adult diet typically containing more carbohydrate than protein. The up-regulation of peptide and amino acid absorption early in development suggests an adaptation by the animal to a high-protein diet.

In chickens, the critical period in development of intestinal functions occurs around the age of hatching, especially during the first 1 to 2 wks (Gonzalez and Vinardell, 1996). Villus height and width increased 25 to 100% in all segments of the small intestine between 4 to 10 d of age in chicks (Uni et al., 1995). It was found in chickens the uptake capacity for proline, like that for glucose, showed a peak when yolk reserves were exhausted, i.e. 2 wks post-hatching. The postnatal declines in proline transport would be at least partly caused by reductions in the density of transporters per square centimeter of intestinal segment (Gonzalez and Vinardell, 1996). Digestibility of amino acids in broilers increased with age and reached a

plateau at 10 d of age (Batal and Parsons, 2002). In broilers, intestinal PepT1 mRNA abundance increased dramatically from embryonic d 18 to the day of hatch (d 0; Chen, 2001). In broilers fed with 18 or 24% CP diets, PepT1 expression increased mostly during the first 2 wks after hatch.

The ontogenic development of PepT1 along the vertical and horizontal axes of the rat small intestine was evaluated using semiquantitative reverse transcriptase - polymerase chain reaction (RT-PCR) and immunohistochemistry. The results indicated that the regionalization of PepT1in the small intestine of rats was unchanged from birth (postnatal d 4) to adulthood (d 50) (Rome et al., 2002). On the cellular level, distribution of PepT1 along the enterocyte differed markedly on the day of birth in rat small intestine as shown by an immunocytochemistry (Hussain et al., 2002). In both prenatal and mature animals, distribution of PepT1 is exclusively in the apical brush border of enterocytes. Immediately after birth PepT1 immunoreactivity is increased and is no longer confined to the brush-border membrane, but also is present in the subapical cytoplasm, basal cytoplasm and the basolateral membrane. Although specific transport activity decreases with age, and development, the total activities of the transporters actually increased because of the dramatic increase in intestinal length and surface area (Iji et al., 2001).

Internal (i.e., genetically determined mechanisms) and external signals (i.e., nutrients) may directly regulate postnatal intestinal nutrient transport activities (Thiesen et al., 2000). Age-related changes in nutrient transporters are genetically programmed. Galactose absorption declines during early postnatal life, whereas fructose absorption is turned on at the end of weaning. Postponing the completion of weaning does not delay the appearance of the fructose transporter GLUT5, suggesting

that GLUT5 is a transporter that possesses a hard-wired ontogenic timing mechanism (Toloza and Miamond, 1992). Although it was assumed that induction of intestinal GLUT5 is independent of dietary signals, it is now generally accepted that diet consumed at an early age modifies the ability of the intestine to up- or down-regulate transport at a later age (Thiesen et al., 2000). Usually GLUT5 is expressed at significant levels in rat intestine only after completion of weaning at d 28. Recent studies demonstrated that this time course could be reprogrammed so that marked increases in GLUT5 activity and mRNA abundance could be observed in 14- to 26-d old pups, several days ahead of the natural schedule (Jiang and Ferraris, 2001; Jiang et al, 2001). In addition, dietary lipids fed to pregnant or to nursing dams are important in the development of the intestine of their offspring (Thiesen et al, 2000). Our laboratory demonstrated that, in broilers fed a 12% protein diet, PepT1 mRNA abundance decreased from hatch to d 35 and was at a lower level from d 7 after hatch to d 35 (Chen, 2001). In contrast, in broilers fed an 18 or 24% protein diet with isocaloric energy, PepT1 mRNA abundance increased with age, and the response was greater when 24% protein was fed. These results indicated that dietary protein can regulate PepT1 expression in development.

Neural-hormonal factors play major roles in the ontogeny of the gut. Aldosterone, thyroid hormones, glucocorticoid, and insulin are able to modulate the postnatal changes in intestinal transport (Pácha, 2000). The glucocorticoids may have possible regulatory effects on the developmental regulation of intestinal transport not only postnatally but also prenatally. Other hormones that are potentially important in regulating gastrointestinal development include cholecystokinin, gastrin, and secretin, which have trophic effects on the gastrointestinal tract, and insulin, insulin-like

growth factors, and epidermial growth factor (Lebenthal and Lebenthal, 1999). However, almost nothing is known about the neural-hormonal modulation of peptide transport in young animals.

Dietary Regulation. Transport rates of most nutrients in the small intestine are regulated by substrate levels in the intestinal luman. Ferraris and Diamond (1989) offered a general scenario for nutrient regulation of transport. They suggested that metabolizable nontoxic nutrients, such as sugars, nonessential amino acids, and short-chain fatty acids should be up regulated with increasing dietary substrate levels, and transporters for essential nutrients, which are toxic in large quantities, such as water-soluble vitamins and minerals, should be down regulated by their substrates. Further, for essential amino acids that are potentially toxic but also yield calories, there are various responses.

Substrates can regulate nutrient transport specifically and nonspecifically. Nonspecific regulation involves mechanisms that are not specific for a single nutrient and includes changes in mucosal surface area, transcellular electrochemical gradient, paracellular permeability, and plasma membrane lipid composition and fluidity (Ferraris, 1994, 2000). Specific regulation includes a change in site density of transporters in enterocytes as a result of changes in protein synthesis or degradation rate or an increased insertion of preformed cytoplasmic transporters into the brush border membrane (Ferraris and Diamond, 1989). Dietary regulation of nutrient transport can be independent and/or in concert with neural and hormonal control (Bates et al., 1998; Matosin-Matekalo et al., 1999).

The pattern of dietary control of amino acid uptake is complex. Karasov et al. (1987) reported that high-protein diets up regulated the absorption of nonessential

amino acids. Diet-induced increases in brush border uptake of essential amino acids tend to be smaller (5 to 76%) compared to those of nonessential ones (26 to 102%). In some cases, high-protein diets have no effect on uptake of essential amino acids (Ferraris, 1994). Low dietary protein levels tend to stimulate brush border uptake of essential amino acids and depress the uptake of nonessential ones. Thus, animals may compensate for a deficiency in protein by increasing the uptake of essential amino acids (Diamond and Kavasov, 1986). Switching from a 15% to a 70% casein diet stimulated proline uptake in mice (Karasov et al., 1983). Both $b^{0,+}$ - and y^{+}-like transport systems were up regulated in chicken jejunum by a lysine-enriched (4.8%) diet (Torras-Llort et al., 1998). However, a L-methionine-enriched (0.4%) diet reduced L-methione uptake in chicken jejunum (Soriano-Garcia et al., 1999) through reducing the V_{max} (30%) of system L, the V_{max} (51%) and K_m (30%) of system B; and the V_{max} (26%) of y^{+} with no effect on $b^{0,+}$.

Amino acids may be absorbed by more than one carrier; hence, it is possible for one dietary amino acid to regulate the transport of another. Stein et al. (1987) found that, in mice, dietary lysine stimulates basic amino acid transporters while aspartate can induce its own transport as well as that of lysine. Dietary arginine stimulates transporters for neutral and acidic amino acids. Segawa et al. (1997) reported that a high aspartate diet increased the amount of NBAT (neutral and basic amino acid transporter), a neutral and basic amino acid transporter, and NBAT mRNA in mouse ileum and also induced a marked increase in cystine transport activity.

Intestinal dipeptide absorption in vivo and in vitro increases with dietary protein and peptide level. Jejunal uptake of carnosine increased by 30% in mice fed a high-protein (72%) compared with a low protein (18%) diet (Ferraris et al., 1988).

Changing of dietary protein from 4% casein to 50% gelatin resulted in a 1.5- to twofold increase in rPepT1 mRNA, a two- to threefold increase in high affinity glutamate transporter (EAAC1) mRNA but no change in the neutral/dibasic amino acid transporter mRNA abundance in rat intestine (Erickson et al., 1995). Addition of Gly-Sar in the Caco-2 cell culture medium for 24 h increased the V_{max} of Gly-Gln transport and PepT1 mRNA abundance and PepT1 protein by increasing de novo synthesis (Thamotharan et al., 1998). Walker et al. (1998) suggested that the dipeptide-induced increase in hPepT1 and hPepT1 mRNA abundance in Caco-2 cells was due to an increase in hPepT1 mRNA half-life as well as an increase in hPepT1 transcription. In this study, the magnitude of the increases in hPepT1 activity, mRNA and protein levels in the range 1.64 to 1.92 times basal values, demonstrating that control of mRNA accumulation, rather than translation or post-translational modification, is the primary mechanism of regulation. In broilers fed an isocaloric energy diet, cPepT1 mRNA abundance decreased in birds fed a 12% protein diet from hatch to d 35. Broilers fed 18 or 24% protein diets showed an increase in cPepT1 mRNA abundance. However, in broilers fed 24% protein diet ad libitum, cPepT1 mRNA abundance decreased until d 14 then increased modestly (Chen, 2001). Although low protein diet seems to decrease PepT1 gene expression, starvation increased PepT1 expression and activity (Ogihara et al., 1999). Starvation markedly increased the amount of peptide transporter present, whereas dietary administration of amino acids reduced it, as determined by immunoblotting. The maximal Gly-Gln uptake (V_{max}) increased twofold without changing the K_m in brush-border membrane vesicles prepared from the jejunum of 1-d fasted rats (Thamotharan et al., 1999b). Both the amount of intestinal PepT1 protein in the brush-border membrane and PepT1

mRNA abundance in the intestinal mucosa increased by threefold, indicating starvation induced gene expression.

There is a direct interplay in amino acid and peptide transport in intestinal cells. The induction of PepT1 synthesis may occur by selective amino acids and peptides (Shiraga et al., 1999). Feeding casein, Gly-Phe, and Phe diets stimulated Gly-Sar transport activity and rPepT1 mRNA and protein abundance compared with feeding a protein-free diet. In contrast, Gly-Gln, Gly, and Gln diets did not increase intestinal dipeptide transport activity. Preincubation of Caco-2 cells with 10 *mM* of selected neutral, mono- or dicationic dipeptides increased the influx of L-Arg up to fourfold (Wenzel et al., 2001). Preloading with the corresponding free amino acids and dipeptides increased L-Arg influx but dipeptides always proved to be more efficient (Wenzel et al., 2001). Other studies, however, showed that protein, protein hydrolysates, and free amino acid diets are equally effective at stimulating brush border amino acid and dipeptide transporters (Karasov et al., 1987).

The peptide/H^+ co-transporter is under the regulatory control of protein kinase C and cAMP in Caco-2 cells. Down regulation of hPepT1 by both cAMP and PKC has been reported (Brandsch et al., 1994). Additionally, Shiraga et al. (1999) identified the 5'-flanking region of the rat PepT1 gene and characterized its function to further elucidate the mechanism by which dietary amino acids or dipeptides induce PepT1 synthesis. The proximal promoter region of the rat PepT1 gene has a TATA-like box and a GC box sequence. The luciferase activities of the clone -351 RPT-LUC responded to particular amino acids (phenylalanine, arginine, and lysine) and dipeptides (Gly-Sar, Gly-Phy, Lys-Phe, and Asp-Lys). An AP-1 binding site and an amino acid-responsive element (AARE) were present at -295 and -277 nucleotides

relative to the transcription start site in this region. AP-1 is known to be associated with gene expression by amino acid deprivation. Whether the AARE-like element is involved in PepT1 gene regulation by amino acids needs to be clarified.

Hormonal Regulation. Insulin regulation of peptide transport was reported earlier (Meredith and Boyd, 2000) indicating that insulin stimulates peptide uptake by increasing the number of transporters by the insertion of the transport protein from a cytoplasmic pool. More recently, leptin and thyroid hormone were also suggested to have regulatory effects on peptide transport (Buyse et al, 2001; Ashida et al., 2002). Leptin (from the Greek *leptos*, meaning thin) is a protein hormone produced predominantly by adipocytes (Tartaglia et al., 1995). It is released into the circulation and transported across the blood-brain barrier into the hypothalamus, where it activates specific leptin receptors that regulate energy homeostasis by altering energy intake and expenditure. Smaller amounts of leptin are also secreted by cells in the epithelium of the stomach (Sobhani et al, 2000). Stomach-derived leptin is rapidly secreted into the blood and the gastric lumen after exogenous CCK-8 (cholecystokinin) administration or feeding in rats, and after infusion of pentagastrin or secretin in humans. Since stomach-derived leptin was not fully degraded by proteolysis in the gastric juice, it can reach the intestine in an active form, and thus can initiate biological processes involved in controlling functions of the intestinal tract, such as absorption and secretion (Buyse et al, 2001).

Buyse et al. (2001) investigated the in vitro and in vivo effects of leptin on peptide transport by using Gly-Sar and the β-lactam antibiotic cephalexin (CFX) as substrates for PepT1. An in vitro study was conducted using Caco-2 cells, which express both PepT1 and leptin receptors. Apical (but not basolateral) leptin induced a

short-term two- to fourfold stimulation of Gly-Sar and CFX transport via PepT1 in Caco-2 cells, which was associated with an increase in V_{max} without change in K_m, increased membrane PepT1 protein, decreased intracellular PepT1 content, and no change in PepT1 mRNA levels. In the rat jejunum, intrajejunal leptin also induced a rapid twofold increase in plasma CFX after jejunal perfusion with CFX, indicating enhanced intestinal absorption of CFX (Buyse et al., 2001). The regulation of peptide transport by leptin was specific since intrajejunal leptin did not alter the flux of glucose, water, or electrolytes. Leptin stimulation of peptide transport was suppressed by colchicine, a chemical disrupting cellular transloction of proteins to plasma membranes, indicating that leptin enhanced peptide transport involved increased translocation of the cytoplasmic pool of PepT1 to the apical membrane (Buyse et al., 2001). A similar mechanism was also reported for insulin-induced increase in the uptake of dipeptides by Caco-2 cells (Thamotharan et al, 1999a).

It was suggested that leptin secreted in the gut lumen may speed up the absorption of dietary proteins, which is consistent with the suggested role of leptin in reducing fat accumulation (Buyse et al, 2001). Under physiological conditions, gastric leptin might balance the intestinal absorption of dietary proteins and fats, and deregulation of this function may contribute to obesity. More research needs to be done to clarify the effects of luminal leptin on peptide transport.

Thyroid hormone, secreted from the thyroid, is a multipotent hormone that exerts effects on development, cell growth and metabolism in vivo (Matosin-Matekalo et al., 1998). 3,3',5-L-triiodothyronine (T_3) has been reported to stimulate SGLT1 and GLUT5 mRNA abundance and sugar transport activities in T_3 treated Caco-2/TC7 cells (Matosin-Matekalo et al., 1998; 1999). However, treatment of

Caco-2 cells with T_3 inhibited [^{14}C]glycylsarcosine uptake in a time- and dose-dependent manner (Ashida et al., 2002). There was also a significant decrease in the amount of PepT1 mRNA (25% of the control) and protein (70% of the control), indicating a decrease in the transcription and/or stability of PepT1 mRNA by T_3 administration. There was an increase in [^{3}H]threonine uptake, but no changes in methyl-α-D-[U-^{14}C]glucopyronoside uptake was observed in the same study. These results, however, are inconsistent with an earlier study which indicated that glucose transport was stimulated by T_3 (Matosin-Matekalo et al., 1998). The reasons for the discrepancy might be the different T_3 pretreatment time on Caco-2 cells in the two studies (4 d in Ashida's study compared to 20 d in Matosin-Matekalo's study).

A region (-308/-290) of the GLUT5 gene promoter containing a thyroid-hormone-responsive element (TRE) might be involved in the response of the GLUT5 gene to T_3 (Matosin-Matekalo et al., 1999). However, thyroid hormone-responsive elements were not found in the promoter regions of rat and mouse PepT1 genes, although there might be differences among rat, mouse, and human homologues (Ashida et al., 2002). In T3-treated Caco-2/TC7 cells, mRNA and protein abundance of Na^+/K^+ATPase increased significantly (Matosin-Matekalo et al., 1998). PepT1 is a tertiary transporter where first small peptides and H^+ are cotransported and this is driven by the extra- to intracellular H^+ gradient. Seconds, this H^+ gradient is maintained by a Na^+/H^+ exchanger which is driven by an extra- to intracellular Na^+ gradient. Thirds, this gradient is established by the Na^+/K^+ATPase (Gardner, 1994). Thus, it might be expected that T_3 can regulate peptide transport at least in part through exerting its effects on Na^+/K^+ATPase. Further studies are required to investigate the

precise mechanism underlying the regulation of thyroid hormone on expression and function of PepT1 in the small intestine.

Diurnal Rhythm of PepT1. In mammals, most physiological processes, including nutrient transport and absorption, display a circadian rhythm that may be regulated by complicated neuroendocrine mechanisms. In rats with free access to chow, daily increases in SGLT1 and GLUT5 expression did not occur at the same time as increases in food intake. The maximal SGLT1 and GLUT5 transport activity was just before darkness (just before maximum food intake) and then gradually decreased towards early morning hours (Ferraris, 2001). Highest activities of intestinal absorption of L-histidine were also observed in dark phases in nocturnal rodents feeding mainly at night (Furuya and Yugari, 1974).

To investigate the diurnal rhythms of peptide transport, diurnal changes of [^{14}C]Gly-Sar transport and PepT1 mRNA and protein expression in the small intestine and kidney were examined in rats maintained in a 12-h photoperiod with free access to chow (Pan et al., 2002). The results indicated that abundance of intestinal PepT1 mRNA and protein was much greater in the small intestine from 1600 to 2400. The intestinal [^{14}C]Gly-Sar uptake was significnatly faster at 2400 than at 1200. However, renal PepT1 showed little diurnal rhythmicity in protein and mRNA expression.

Since rats have a nocturnal feeding behavior, the diurnal rhythms of PepT1 function and expression present an intestinal anticipatory regulating pathway for the preparation of nocturnal dietary load. Studies have been done to delineate the mechanisms regulating the diurnal rhythms of SGLT1 (Rhoads et al., 1998; Tavakkolizadeh et al., 2001). The SGLT1 transcription rate in rats was found to be seven fold higher in the morning (1000 to 1100) than in the afternoon (1600 to 1700).

The periodicity change of the hepatocyte nuclear factor 1 (HNF-1) isoform complement at the HNF-1 site contributed to the circadian rhythm of SGLT1 transcription (Rhoads et al., 1998). Because there is a potential site for HNF-1 in the rat PepT1 promoter region, this factor may be involved in the daily anticipatory mechanism of intestinal PepT1 expression (Pan et al., 2002). A neuroendocrine mechanism was proposed to regulate genes involved in the circadian rhythm of nutrient transport (Tavakkolizadeh et al., 2001). Insulin level in rats undergoes circadian variation with maximal plasma concentrations late in the afternoon, just before the peak period of food intake. Truncal vagotomy abolishes rhythmicity in food intake, insulin secretion, and glucose tolerance in normal rats. The specific molecular events and neuroendocrine mechanisms underlying the circadian rhythm of PepT1 remain to be elucidated.

Chapter III

Molecular Cloning and Functional Characterization of a Turkey Intestinal Peptide Transporter and Developmental Regulation of Peptide Transporter Expression in Turkey and Broiler Embryos

ABSTRACT

A cDNA clone encoding a turkey intestinal peptide transporter, tPepT1, was isolated from a turkey small intestinal cDNA library by screening with our chicken PepT1 (cPepT1) cDNA probe. The tPepT1 cDNA is 2,921-bp long and encodes a 79.4 kDa protein of 714 amino acids (AA) with 12 predicted transmembrane domains. The isoelectric point (pI) of tPepT1 is 5.9, which is much lower than that of PepT1 cloned from chicken (pI = 7.5) and other species. The AA sequence of tPepT1 is 94.3% identical to cPepT1 and ~ 60% identical to PepT1 from rat, sheep, rabbit, and human. Using a two-electrode voltage-clamp technique in *Xenopus* oocytes expressing tPepT1, Gly-Sar transport was pH dependent, but independent of Na^+ and K^+. For the dipeptides Gly-Sar and Met-Met, the evoked inward currents indicated that the transporter was saturable and had a high affinity for these substrates. However, transport of the tetrapeptide, Met-Gly-Met-Met, exhibited a possible substrate inhibition. To study developmental regulation of PepT1 in broiler and turkey embryos, 12 Nicholas turkey or Cobb × Cobb broiler embryos (six males and six females) were sampled daily from 5 d before hatch to the day of hatch (d 0). The abundance of PepT1 mRNA in the small intestine was quantified densitometrically from northern blots after hybridization with full-length cPepT1 and tPepT1 cDNA as probes. There was a quadratic increase ($P < 0.001$) in PepT1 mRNA abundance with

age in turkey and broiler embryos. The relative increase in abundance of PepT1 mRNA in intestinal tissue from 5 d before hatch to d 0 was much less in the turkey than in the broiler (3.2-fold vs 14-fold). The dramatic increase in PepT1 mRNA abundance indicates a developmental regulation of the PepT1 gene and that there may be a crucial role for PepT1 in the neonatal chick and poult.
Keyword: Peptide transporter, Developmental regulation, Broiler, Turkey, Embryo

INTRODUCTION

A significant fraction of dietary amino nitrogen is absorbed as intact oligopeptides rather than free amino acids (Ganapathy, 1994). In fowls, uptake of lysine in vivo was more rapid in the form of the dipeptide glycylleucine rather than as a mixture of glycine and leucine (Boorman, 1975). The intestinal H^+-dependent oligopeptide transporter (PepT1) plays an important role in absorbing small peptides arising from digestion of dietary proteins in the small intestine (Matthews, 1991). PepT1 from rabbit (Fei et al., 1994), human (Liang et al., 1995), rat (Saito et al., 1995), and mouse (Fei et al., 2000) have been cloned. PepT2, another member of the H^+/peptide transporter family which is specifically expressed in the kidney, serving to reabsorb filtered peptides, peptide-derived antibiotics, and peptides produced as a result of the action of luminal peptidase, has also been cloned (Boll et al., 1996). The recent cloning and characterization of ovine (Pan et al., 2001) and chicken (Chen et al., 2002) peptide transporters in our laboratory improved our understanding of the molecular structure and basic functions of PepT1 in food-producing animals. PepT1 mRNA is present in the small intestine of sheep, cows, pigs, and chickens and the omasal and ruminal epithelium of sheep and dairy cows with little expression in liver and kidney (Chen et al., 1999). Ultrastructural localization of PepT1 in the rat small intestine indicated that it was abundant in the absorptive epithelial cells of the villi in the small intestine (Ogihara et al., 1999).

PepT1 expression can be regulated markedly by diet and development at the level of gene transcription (Meredith and Boyd, 2000). In the rat, intestinal PepT1 mRNA was most abundant in 4-d-old rats, and levels decreased to the adult level by d 28 of age (Miyamoto et al, 1996). Although some studies have investigated the

developmental regulation of nutrient transport in mammals, little has been done in poultry. In a recent study conducted with broilers in our lab, PepT1 mRNA was observed to increase dramatically from embryonic d 18 to the day of hatch (Chen, 2001). However, the natural pattern of PepT1 expression before hatch in broiler embryonic small intestine is not known. The purpose of this study was to clone and characterize the function of the peptide transporter from turkey, another major domestic fowl. The developmental regulation pattern of PepT1 expression in turkey and broiler embryonic small intestine was also studied.

MATERIALS AND METHODS

Materials

All chemicals, substrates, and reagents were of either molecular biology or cell culture tested chemical grades and purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise stated. The ZAP Express cDNA synthesis system and Gigapack III were purchased from Stratagene (La Jolla, CA). Restriction enzymes were from New England BioLabs (Beverly, MA). The RNA transcription kit, mMESSAGE mMACHINE was obtained from Ambion (Austin, TX). *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). Collagenase A was purchased from Boehringer Mannheim Corporation (Indianapolis, IN). Streptomycin, penicillin, diethyl pyrocarbonate, and dipeptides were purchased from Sigma Chemical (St. Louis, MO). TriReagent was purchased from Molecular Research Center (Cincinnati, OH). RNA ladder (0.24 to 9.5 kb) was purchased from Invitrogen Life Technologies (Carlsbad, California). Magna nylon transfer membranes were purchased from Osmonics (Westborough, MA). [α-^{32}P]dATP was purchased from ICN

Pharmaceutical (Costa Mesa, CA). DNA polymerase I/DNase I was purchased from Invitrogen Life Technologies (Carlsbad, California).

Construction of a Turkey Intestinal cDNA Library

The turkey intestinal cDNA library was constructed and screened by Y. X. Pan. Total RNA was extracted from turkey intestinal tissue and poly(A)$^+$ RNA was purified on oligo(dT) cellulose following established procedures (Sambrook et al. 1989). A cDNA library was constructed using the ZAP Express cDNA synthesis system. Only cDNA of a size > 400 bp were used for library construction. Phage DNA containing cDNA were then packaged with Gigapack III Gold packaging extract and introduced into the XL1-Blue MRF' *Escherichia coli* cell line. The phage library was plated out immediately on a series of large, 150-mm NZY agar plates (50,000 plaques/plate) for screening.

Screening of the cDNA Library

Positive clones were identified by plaque hybridization of the cDNA library transferred to Magna nylon transfer membranes. The cDNA probe used for screening was our cloned cPepT1. The probe was labeled with [α-^{32}P]dATP (ICN Pharmaceutical, Costa Mesa, CA) by nick translation using DNA polymerase I/DNase I (Invitrogen Life Technologies, Carlsbad, California). Hybridization was carried out for 16 h at 42°C in a solution containing 50% formamide, 5X Denhardt's solution, 6X SSPE (1 x SSPE = 0.15 mmol/L NaCl, 10 mmol/L NaH_2PO_4, and 1 mmol/L EDTA), 0.5% SDS and 10 mg/L yeast tRNA. Posthybridization washing was done under medium stringency conditions, which involved washing twice with 5X SSPE, 0.5% SDS at room temperature for 15 min and twice with 1X SSPE, 0.5% SDS at 42°C for 15 min. Positive clones were subjected to three more rounds of screening using the

same conditions. After the quaternary screening, 100% of the plaques showed positive hybridization by autoradiography.

Sequencing of the Full-length cDNA Insert

The positive plaques identified after screening of the cDNA library were used to generate the excised pBK-CMV phagemid containing the cDNA insert. Automated DNA sequencing was performed using standard methods on an ABI 377 automated DNA Sequencer and using Applied Biosystems BigDye (version 2.0) Terminator ReadyReaction kit (Applied Biosystems, Foster City, CA). Analysis of nucleotide and amino acid sequence was performed using the sequence analysis software Lasergene (DNAStar, Madison, WI). Database searches were done using the GenBank Program BLAST.

In Vitro Transcription of cRNA.

cRNA was synthesized using the RNA transcription kit mMESSAGE mMACHINE (Ambion) according to the manufacturer's protocol. For sense cRNA synthesis, phagemid containing the cDNA insert was linearized using *Xba* I and transcribed in vitro by T3 RNA polymerase in the presence of an RNA cap analog. For antisense cRNA synthesis, phagemid containing the cDNA insert was linearized using *EcoR* I and transcribed in vitro by T7 RNA polymerase in the presence of an RNA cap analog. The resultant cRNA was purified with phenol/chloroform, precipitated with ethanol, and then resuspended in nuclease-free water and stored frozen at – 80°C. Concentration was determined by UV spectrophotometry and the cRNA was verified by denaturing 1% agarose-formaldehyde gel electrophoresis and visualization using ethidium bromide staining.

Electrophysiology

Healthy *Xeonopus* oocytes at stage V were dissected free and defolliculated. Membrane potential was measured on approximates 10 % of these defolliculated oocytes. Only batches of oocytes with a resting membrane potential more negative than – 30 mV after defolliculation were used for injection 1-d later. Using a microinjection system, either sense cRNA or antisense cRNA was injected into the vegetal pole of each oocyte near the polar interface. Antisense cRNA or water was used as a control. The injected oocytes were incubated in culture solution at 17°C for 1 to 7 d. The two-electrode voltage-clamp technique was used to characterize the induced peptide transport activity in oocytes injected with sense cRNA or antisense cRNA. All responses were monitored by a two-electrode voltage-clamp amplifier (TEV-200, Dagan, Minneapolis, MN), and analyzed by a MacLab (AD Instruments, Milford, MA). *Xenopus* oocytes were injected with different amounts of tPepT1 cRNA (4, 8, 16, 32, and 64 ng). The current in the oocytes in response to 1 *mM* Gly-Sar (pH 6.0) perfusion were examined from d 1 to d 7 after injection. For functional characterization, 64 ng of sense-cRNA- or antisense-cRNA was injected into oocytes and electrophysiologic measurements were carried out 3 to 6 d after injection. Only oocytes with a resting membrane potential (V_m) more negative than – 30 mV were used for recordings. An oocyte was perfused continuously with buffer with or without peptide at a rate of 1.2 mL/min using a gravity feed perfusion system (Model BPS4, Ala Scientific Instruments, Westburg, NY). All peptide substrate solutions were prepared by dissolving the peptides in buffer (96 mmol/L NaCl, 2 mmol/L KCl, 1mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 5 mmol/L HEPES). All experiments were performed at room temperature (~ 21°C).

Animals and Tissue Sampling

Cobb × Cobb eggs were obtained from a commercial hatchery. Turkey eggs were from Nicholas Turkey Breeding Farms (Sonoma, CA). The entire small intestine was removed from six males and six females each day from chicken embryonic d 16 (e16) to the day of hatch (d0) and from turkey embryonic d 23 (e23) to the day of hatch (d0). The birds were killed by cervical dislocation and sexed. The small intestine was removed and washed with ice-cold 1 X phosphate-buffered saline (PBS), and then chopped. Approximately ~ 0.2 g of tissue were immediately immersed in 2 mL ice-cold TriReagent for total RNA extraction. All animal procedures were approved by Viriginia Tech's Animal Care Committee.

Northern Blot Analysis

Total RNA from each sample was extracted using TriReagent following the procedures provided by the company with minor modification (RNA precipitation was at - 20°C for 2 hrs in stead of at room temperature for 10 min). Absorbance at 260 and 280 nm (Model U-2000 Spectrophotometer, Hitachi Instrument Inc, Japan) was used to quantify the RNA extracted and the isolated RNA was stored at – 80°C. For northern blot analysis, 20 μg of total RNA was denatured and size-fractionated on 1% agarose gels in 2.2 mol/L of formaldehyde and stained with ethidium bromide. The size of the mRNA bands was determined by an RNA ladder (0.24 to 9.5 kb). The size-fractionated RNA was then transferred onto a Nylon membrane overnight and cross-linked with UV light at 0.30 J/cm^2. Following prehybridization for 2 h in a solution containing 50% formamide, 5 × Denhardt's solution, 6 × SSPE (1 × SSPE = 0.15 mmol/L NaCl, 10 mmol/L NaH_2PO_4, and 1 mmol/L EDTA), 0.5% SDS and 10 mg/L yeast tRNA at 42 °C, RNA blots were hybridized for 16 to18 h under identical

conditions with the addition of tPepT1 or cPepT1 cDNA as the probe for turkey and chicken RNA, respectively. The probes were labeled with [α-^{32}P]dATP by nick translation using DNA polymerase I/DNase I and purified by Sephadex G-50 spin column chromatography.

Post-hybridization washing was under high-stringency conditions, which involved washing twice in 5 × SSPE. 0.5% SDS at room temperature for 15 min, twice in 1 × SSPE, 0.5% SDS at 42 °C for 15 min, and twice in 0.1 × SSPE, 1% SDS at 65 °C for 15 min. Washed filters were exposed to K-type imaging screens (Bio-Rad Laboratories, Hercules, CA) which were then scanned using a FX laser scanner (Bio-Rad Laboratories, Hercules, CA). Images of the signal from tPepT1 or cPepT1 were acquired by using Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA) for radioisotopes, K-screen by PDQUEST, version 6.2.1 (Bio-Rad Laboratories, Hercules, CA). To correct for differences in RNA loading onto gels or in RNA transfer to membranes, membranes were stripped of the tPepT1 or cPepT1 probe and rehybridized to a [α-^{32}P]-labeled 18S rRNA probe. The density of hybridiztion bands was quantified using volume tools of Quantity One Quantification Software (Bio-Rad Laboratories, Hercules, CA).

Computational Analysis

A hydrophobicity plot of tPepT1 was constructed according to the Kyte and Doolittle (1982) hydropathy analysis using a window of 21 amino acid residues via the Tmpred service program from the European Molecular Biology Network. Protein phosphorylation/dephosphorylation sites of tPepT1 were predicted on the basis of consensus amino acid sequences as substrate specificity determinants for protein kinases and phosphatases (Kennelly and Krebs, 1991). The consensus amino acid

sequences for cAMP-dependent protein kinase A (PKA) site prediction are R-R/K-X-S/T, R-X_2-S/T or R-X-S/T. The consensus amino acid sequences for protein kinase C (PKC) site prediction were (R/K_{1-3}, X_{2-0})-S/T-(X_{2-0}, R/K_{1-3}) or (R/K_{1-3}, X_{2-0})-S/T. The consensus amino acid sequence for N-linked glycosylation site prediction was N-X-T/S (X = any amino acid).

Calculations and Statistics.

The kinetic parameters, including the Michaelis-Menten constant (*Kt*), the maximal velocity (*I max*), and all other calculations (linear as well as nonlinear regression analysis) were performed using PRISM (GraphPad, San Diego, CA). Data from northern blots and data for ion and pH dependency of tPepT1 in cRNA-injected *Xenopus* oocytes were subjected to analysis of variance procedures for two-way factorial in completely randomized designs. Other data were evaluated using one-way ANOVA by using the general linear models procedure of SAS® software (SAS Institute, version 8.0) and orthogonal contrasts were used for post-hoc comparisons.

RESULTS

Sequence and structures of the turkey intestinal PepT1 cDNA.

The turkey intestinal tPepT1 cDNA was 2,921 bp long with an open reading frame of 2142 bp. A 72-bp 5' UTR and a 707-bp 3' UTR flanked the open reading frame. The initiation codon was consistent with the Kozak consensus sequence, GCCGCC(A/G)CATGG (Kozak 1987). At the 3' end, the cDNA had a polyadenylation signal (AATAAA) 19 nt preceding the polyA tail. The mRNA isolated from turkey small intestine hybridized to the tPepT1 cDNA probe at 2.9 kb. The encoded protein was predicted to have 714 amino acids with a molecular mass of

79.4 kDa and a pI of 5.9. Alignment results showed that the amino acid sequence of tPepT1 was 94.3% identical to cPepT1 and 63.2%, 64.9%, 64.6%, 63.4%, and 62.8% identical to PepT1 from mouse, rat, sheep, rabbit, and human, respectively (Figure 1).

Hydrophobicity analysis indicated that tPepT1 has 12 putative transmembrane domains with a large extracellular loop (203 amino acids) between transmembrane domains 9 and 10 (Figure 2). The alignment of turkey PepT1 with chicken PepT1 shows 94.8% identity in the transmembrane domain regions, whereas only 88.2% identity is present in the large extracellular loop. Like the other member of the PepT1 family, both the amino and the carboxyl termini of tPepT1 are on the cytoplasmic side of the membrane as predicted by the model. The large extracellular loop contained seven potential N-linked glycosylation sites at positions Asn 414 (NVT), Asn 423 (NVT), Asn 453 (NRS), Asn 485(NFT), Asn 508 (NIT), Asn 526 (NYT), and Asn 546 (NCT). An additional putative N-linked glycosylation site at position Asn 56 (NLS) in the extracellular loop between transmembrane domains 1 and 2 was observed. The predicted protein also contained one potential site for protein kinase C (PKC)-dependent phosphorylation in the putative intracellular loop between transmembrane domains 6 and 7 (Ser 272, S-E-K) and one potential site for protein kinase A (PKA)-dependent phosphorylation in the intracellular loop between transmembrane domains 8 and 9 (Thr 365, R-K-I-T).

Functional Characteristics of tPepT1.

Two-electrode voltage-clamp analysis demonstrated that control oocytes (antisense cRNA or H_2O injected) did not display any current when perfused with the substrates in all experiments. To determine the optimal amount of cRNA and optimal time for tPepT1 expression, oocytes were injected with 4, 8, 16, 32, or 64 ng sense tPepT1

cRNA or DEPC treated water (50 nl) and incubated for 7 d after injection. Peptide transport activity, indicated by inward currents when the oocytes were perfused with 1 mmol/L Gly-Sar in standard buffer at pH 6.0, was observed 1 d after injection (Figure 3). Inward current responded quadratically with the amount of cRNA injected into the oocytes ($P < 0.001$). The maximum inward currents were observed in the oocytes injected with 64 ng cRNA from d 3 to 6 (no differences in current responses from d 3 to 6, $P > 0.05$). Therefore, all functional expression experiments were carried out with oocytes injected with 64 ng cRNA and on d 3 to 6 after injection.

To investigate the pH- and ion-dependency of tPepT1, transport was measured in sense cRNA-injected oocytes perfused with 1 mmol/L Gly-Sar in the standard buffer, Na^+-free and K^+-free buffer (by substituting NaCl or KCl in the standard buffer with choline cloride) at pH 5.0, 5.5, 6.0, 6.5, and 7.0. There was a quadratic ($P < 0.001$) effect of pH on inward current with greatest uptake occurring at pH 6.5 (Figure 4). Inward currents in the cRNA-injected oocytes in 1 mmol/L Gly-Sar in Na^+- and K^+-free buffer did not differ from inward currents induced in the standard buffer at all pH levels ($P > 0.05$).

Transport kinetics of tPepT1 were investigated by perfusing the cRNA-injected oocytes with 0.05, 0.2, 0.5, 1, 5, and 10 mmol/L Gly-Sar in the standard buffer at pH 6.5. Gly-Sar induced currents were saturable and fit Michaelis-Menten kinetics ($r^2 = 0.997$), resulting in a substrate affinity (K_t) of 0.69 ± 0.14 mmol/L and a maximal transport rate (V_{max}) of 188.6 ± 8.8 nA (Figure 5A). The dipeptide Met-Met induced currents were also saturable over a Met-Met concentration range of 0.05-10 mmol/L. The K_t and V_{max} for Met-Met uptake were determined to be 0.23 ± 0.04 mmol/L and 194.4 ± 7.17 nA. Different from the dipeptide, tPepT1 transport of the tetrapeptide,

Met-Gly-Met-Met, showed increased substrate uptake at low substrate concentrations from 0.05 to 0.5 mmol/L followed by inhibition at higher concentrations from 1-10 mmol/L. The maximum current was 33.8 nA which was observed at a concentration of 0.5 mmol/L (Figure 5B).

Change in PepT1 mRNA Abundance in Turkey and Broiler Embryonic Intestinal Tissue

PepT1 mRNA was barely detected in turkey and broiler embryonic small intestine at e23 or e16 for turkey or broiler, respectively. Northern blot analysis indicated that there was a quadratic increase ($P < 0.001$) in PepT1 mRNA abundance with age in turkey and broiler embryonic small intestinal tissue from e23 or e16 to d0 for turkey or broiler, respectively (Figure 8). In broilers, cPepT1 mRNA abundance at d0 was approximately 14-fold that at e16. However, in turkeys, the increase in PepT1 mRNA abundance was only 3.2-fold from e23 to d0. No differences were observed ($P > 0.001$) in PepT1 mRNA abundance between male and female birds.

DISCUSSION

Chickens and turkeys are two major domestic fowls. Comparison of the amino acid sequence between our tPepT1 and cPepT1 reveals a high degree of homology (94.3% similarity). Turkey PepT1 has 714 predicted amino acid residues, same as the chicken counterpart. Amino acids in the membrane-spanning regions are especially well conserved between the two species (94.8% in similarity), whereas the large extracellular loop between transmembrane domains 9 and 10 shows an 88.2% sequence match. A reduced similarity in the large extracellular loop between other species has also been reported (Miyamoto et al., 1996; Chen et al., 2002). It remains

to be clarified if the difference of the amino acid sequence in the large extracellular loop has any effect on the function of peptide transporters of different species. It has been suggested that the putative substrate binding site in mammalian PepT1 resides in the region which is comprised of transmembrane domains 7, 8, and 9 and the loops in between (Fei et al., 1998). Histidyl residues His-57 and His-121 have been shown to be essential to substrate recognition and transport activity of PepT1 (Terada et al., 1996; Fei et al., 1997). Although tPepT1 and cPepT1 show only ~ 60% identity to mammalian PepT1, these regions are well conserved among the species. The PKC and PKA phosphorylation sites, which have been demonstrated to have an important role in regulation of the maximum transport rate of PepT1 (Brandsch et al., 1994), are also well conserved within cPepT1 and tPepT1 proteins. An important difference between these two proteins is that the tPepT1 has an isoelectric point (pI) of 5.9, whereas the pI of cPepT1 is 7.5. The difference in pI values is attributed to the different composition of basic and acidic amino acids of the two proteins. There are 56 strongly basic amino acids (Lys and Arg) and 63 strongly acidic amino acids (Asp and Glu) in tPepT1. Whereas in cPepT1, there are 59 strongly basic amino acids and 58 strongly acidic amino acids. No information is available at the moment about how the function of the peptide transporter is related to pI value. The pI of tPepT1 is the lowest among the PepT1 cloned so far. The comparison of sequence and protein characteristics of tPepT1 with other PepT1 from other species is shown in Table 1.

The *Xenopus* oocyte is a well-defined experimental model used for in vitro expression. Oocytes have the ability to accept foreign mRNA and later translate it into functional proteins. Characteristics of the proteins can then be studied through functional examination of the synthesized proteins in *Xenopus* oocytes (Soreq and

Seidman, 1992). The functional characteristics of PepT2 determined in *Xenopus* oocytes were found to be very similar to, if not identical with, the peptide transport activity found in the apical membrane vesicles of rabbit kidney tubular cells (Boll et al., 1996). Previous studies indicated that transport kinetics determined in the oocyte expression system were comparable to that determined in a mammalian cell line, Chinese Hamster Ovary (CHO) cells (Chen et al., 2002). Expression of tPepT1 in *Xenopus* oocytes revealed that Gly-Sar transport was pH-dependent with an optimal pH of 6.5. Compared to tPepT1, cPepT1 has about the same optimal pH (6.0 to 6.5). In turkey small intestine, the pH ranges from 5.8 to 6.9 which is similar to the intestinal pH of the chicken, which is between 5.7 to 6.4 (Sturkie, 1986). Whether the optimal pH for PepT1 from different species is dependent upon pH environment of the small intestine is unknown. Substitution of Na^+ and K^+ did not have any effect on peptide transport activity of tPepT1 at any pH tested, which demonstrated that tPepT1 operates independently from these ions, but is coupled to H^+. These results are consistent with that of previous studies, which indicated that peptide transport activity of PepT1 was driven by an inwardly directed H^+ gradient and was independent of Na^+, K^+, Cl^- or Ca^+ (Pan et al., 2001; Chen et al., 2002).

Early efforts to characterize PepT1 were focused on uptake of peptidomimetic drugs. Recent studies in our lab evaluated the transport characteristics of ovine and chicken PepT1 using peptides containing essential amino acids (mainly Met and Lys; Pan et al., 2001; Chen et al., 2002). The studies on cPepT1 indicated that a wide range of di- or tripeptides, regardless of their molecular weight, electrical charge and hydrophobicity, could be transported, demonstrating the importance of peptide transport in chickens. In the present study, transport of the hydrolysis-resistant

dipeptide, Gly-Sar, in oocytes expressing tPepT1 obeyed Michaelis-Menten-type kinetics with a K_t of 0.69 ± 0.14 mmol/L and I_{max} of 189 ± 8.8 nA, which is comparable to that of cPepT1 (K_t 0.47 ± 0.03 nA; I_{max} 206 ±3.1 mmol/L). The kinetic study also showed that tPepT1 transport of the dipeptide Met-Met has a lower affinity (K_t = 0.23 ± 0.04 vs. 0.08 ± 0.01 mmol/L) and a greater transport velocity (I_{max} = 194 ± 7.2 vs. 113 ± 3.2 nA) when compared to cPepT1. According to NRC (1994), the methionine requirement of turkey is 0.25-0.55%, which is generally greater than that of chicken (0.19 to 0.3%). It is not clear if PepT1 transport kinetics is related to the amino acid requirements of animals.

Dipeptides as well as tripeptides are excellent substrates for the intestinal peptide transport system (Pan et al., 2001; Chen et al., 2002). Transport of tetrapeptides occurs to only a small extent, if at all (Ferraris, 1994). In the present study, transport of the tetrapeptide Met-Gly-Met-Met was observed in oocytes expressing tPepT1. However, the transport activity of Met-Gly-Met-Met did not fit with Michaelis-Menten kinetics. It showed increased transport at low substrate concentrations from 0.01 to 0.5 mmol/L, with a maximum transport velocity of 33.8 nA at 0.5 mmol/L, then followed by substrate inhibition at higher concentrations from 1 to 10 mmol/L. The substrate inhibition phenomenon has also been reported for the transfer of nitrate across the membrane of *Xenopus* oocytes expressing a high affinity fungal nitrate carrier (Zhou et al., 2000). Whether the substrate inhibition reaction is related to a conformational change of tPepT1 at high extracellular tetrapeptide concentrations, which affect the transport affinity and velocity, or because of a second binding site on tPepT1 remains to be studied. Methionine is an essential amino acid but a potentially toxic substance to animals at high dietary substrate levels. Toxic nutrients tend to

repress their transporters at a high substrate level (Ferraris, 1994). An L-methionine enriched (0.4%) diet reduced L-methionine uptake in chicken jejunum (Soriano-Garcia et al., 1999) through reducing the V_{max} and/or K_m of amino acid transport systems. It might be postulated that the substrate inhibition of Met-Gly-Met-Met transport by tPepT1 might play a significant physiologic role to protect the organism against the risk of methionine toxicosis.

The activity of nutrient transport is known to be regulated by development. Intestinal glucose and amino acid transporters are suggested to be present prenatally in humans, guinea pigs, sheep, rabbits, and rats (Pácha, 2000). In rabbits, active uptake of glucose and galactose was increased 3-fold during the final 7 d of gestation with the highest transport rate occurring straight after birth, but then decreasing gradually thereafter (Ferraris, 2001). In rats, expression levels of PepT1 increased dramatically at birth and were maximal 3 to 5 d after birth in the duodenum, jejunum, and ileum, and then declined rapidly (Shen et al., 2001). Although considerable progress has been made in understanding the mechanisms for developmental regulation of nutrient transport in mammals, little has been done in poultry. A recent study with broilers in our lab showed that cPepT1 mRNA abundance increased dramatically by the time of hatch when compared with 3 d before hatch (E18; Chen et al, 2001). In the present study, the PepT1 mRNA abundance in turkey and broiler embryonic small intestinal tissue was detected day by day from 5 d before hatch to d 0 to investigate the regulation pattern of the PepT1 gene in turkey and broiler embryos. The results of our study indicated that there was a quadratic increase of PepT1 mRNA abundance with age. In broilers, cPepT1 mRNA abundance increased approximately 14-fold from e16 to d0. However, the increase in tPepT1 mRNA abundance in the

turkey was only 3.2-fold from e23 to d0. A recent study of expression and cellular distribution of PepT1 during development in rat small intestinal epithelium indicated that distribution of PepT1 is exclusively in the apical brush border of enterocytes from both prenatal and mature animals. However, immediately after birth immunolocalization of PepT1 extends to the subapical cytoplasm and to the basolateral membrane of enterocytes (Hussain, 2002). At birth or hatch, the digestive tract of an animal faces a serious challenge because of the dramatic change of the luminal environment. In another study conducted with broiler chicks, the specific activities of digestive enzymes, like maltase, sucrase and aminopeptidase N, were reported to be maximal at hatch (Iji et al., 2001). The developmental regulation of PepT1 gene expression and digestive enzymes at birth or hatch may have a significant physiologic role for adaptation of an animal to the new environment. More work needs to be done to clarify the molecular and cellular mechanisms underlying developmental regulation.

In summary, we have cloned a turkey intestinal peptide transporter, tPepT1, which encodes for a 714-amino acid protein with 12 transmembrane domains and a pI of 5.9. tPepT1 has high homology with cPepT1. Functional characterization of tPepT1 in *Xenopus* oocytes demonstrated that the transport process was pH-dependent but Na^+-and K^+-independent. The transport of the dipeptides, Gly-Sar and Met-Met, into oocytes was saturable and the affinity was high. Transport of the tetrapeptide Met-Gly-Met-Met by tPepT1 was observed. The transport activity did not fit Michaelis-Menten kinetics but showed a substrate inhibition pattern. The PepT1 gene was expressed in turkey and broiler embryos 4 to 5 d before hatch. There was developmental regulation of PepT1 transcription in turkey and broiler embryos with a

quadratic increase in PepT1 mRNA abundance from 5 d before hatch to d0. These studies provide more information on both molecular and nutritional aspects of intestinal peptide transport. It also sheds light on the importance of intestinal peptide absorption for neonatal animals and implies the possibility for modulating PepT1 activity with peptides in neonates for better growth and performance.

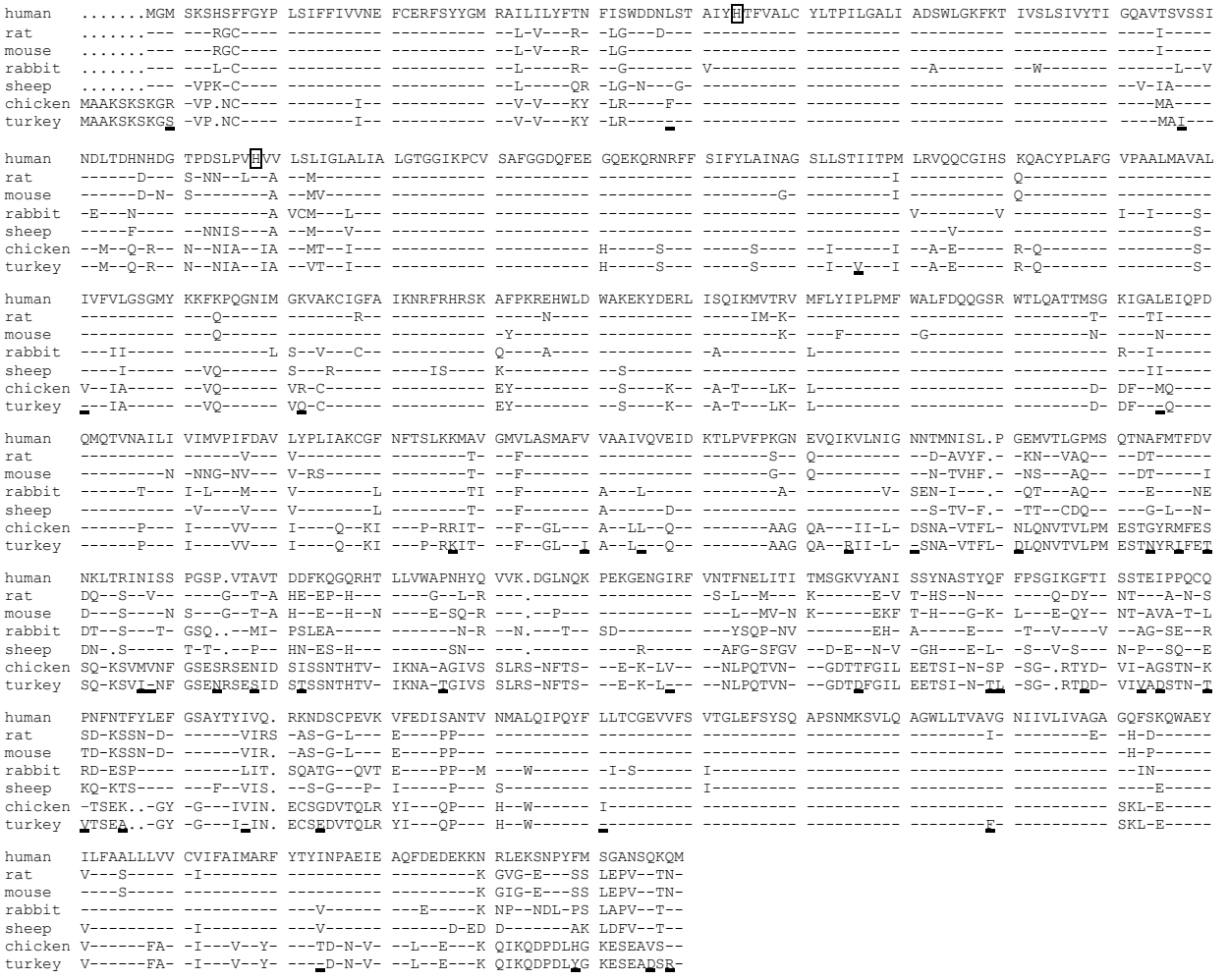

FIGURE 1. Predicted amino acid sequence of turkey PepT1 and alignment with human, rat, mouse, rabbit, sheep and chicken PepT1. Amino acids identical to the human sequence are indicated by dashes (-). The amino acids in turkey PepT1 different from chicken PepT1 are underlined. Histidyl residues His-57 and His-121 are boxed.

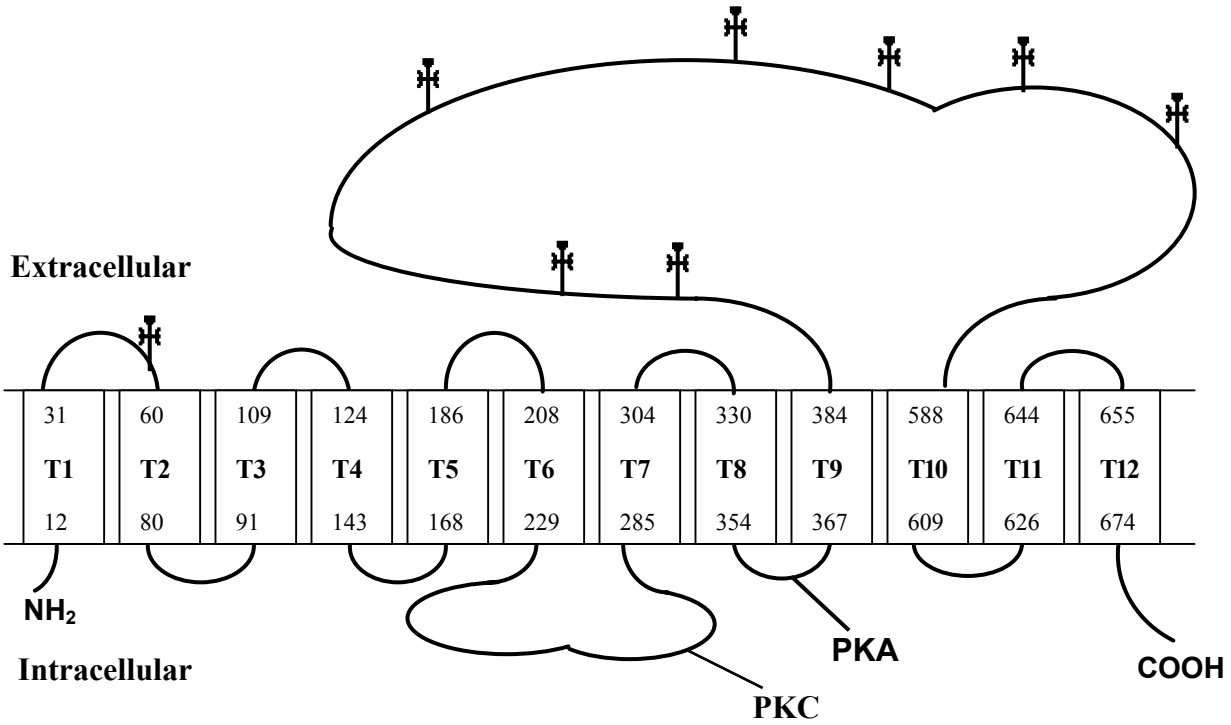

FIGURE 2. Putative membrane-spanning model of turkey peptide transporter (tPepT1). The predicted tPepT1 has 12 putative transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10. Potential N-linked glycosylation sites are indicated by the symbol (†). Potential protein kinase C (PKC) phosphorylation sites and protein kinase A (PKA) phosphorylation sites are also indicated.

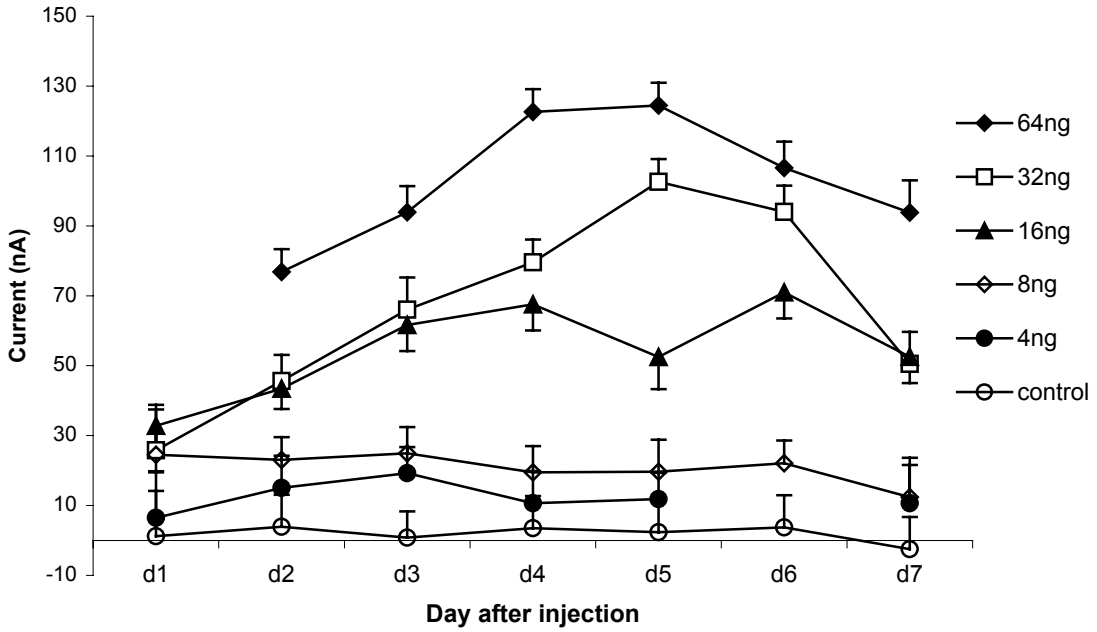

FIGURE 3. Dependence of uptake of 1 mM Gly-Sar at pH 6.0 on amount of tPepT1 cRNA injected and time of injection. Values are means ± SE, n = 2 to 4 for each data point.

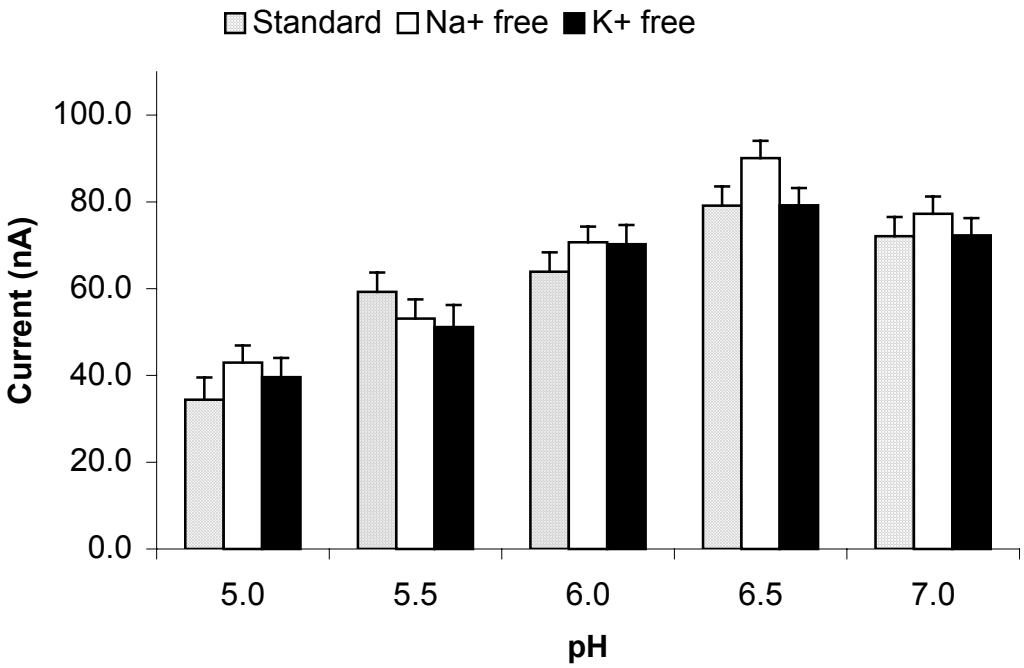

FIGURE 4. pH and ion-dependency of turkey peptide transporter (tPepT1) in cRNA-injected *Xenopus* oocytes. Oocytes were clamped at - 60 mV and perfused with 1 mmol/L glycyl-sarcosine (Gly-Sar) in standard measurement buffer at pH 5.0, 5.5, 6.0, 6.5, and 7.0. Ion-dependent study was performed in Na^+-free and K^+-free buffers to determine whether the transport process was dependent on Na^+ and K^+. Na^+-free and K^+-free buffers were prepared by replacing NaCl or KCl in the standard buffers with choline chloride. Values are means ± SE, n = 3 to 6. There was a quadratic ($P < 0.001$) effect of pH with greatest uptake occurring at pH 6.5. Inward currents did not differ among ions ($P > 0.05$).

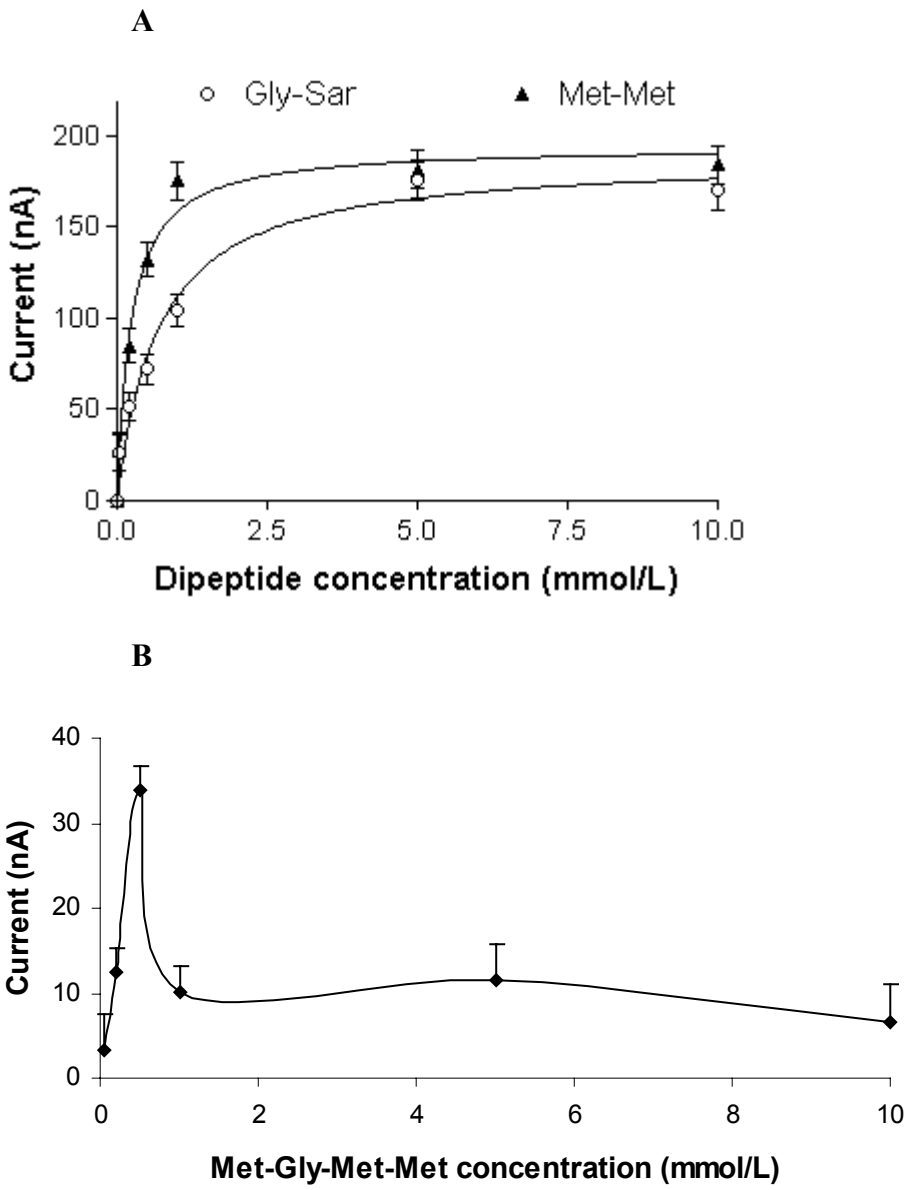

FIGURE 5. Kinetic study of dipeptide (Gly-Sar and Met-Met) and tetrapeptide (Met-Gly-Met-Met) transport by turkey peptide transporter (tPepT1). Oocytes expressing tPepT1 were clamped at - 60 mV and perfused with substrates with concentrations ranging from 10 μmol/L to 10 mmol/L in standard measurement buffer at pH 6.5. Values are means ± SE. (*A*) tPepT1 transport of the dipeptides Gly-Sar and Met-Met was saturable and fit Michaelis-Menten kinetics. n = 4 to 8. (*B*) tPepT1 transport of the tetrapeptide Met-Gly-Met-Met showed substrate inhibition. n = 2 to 4.

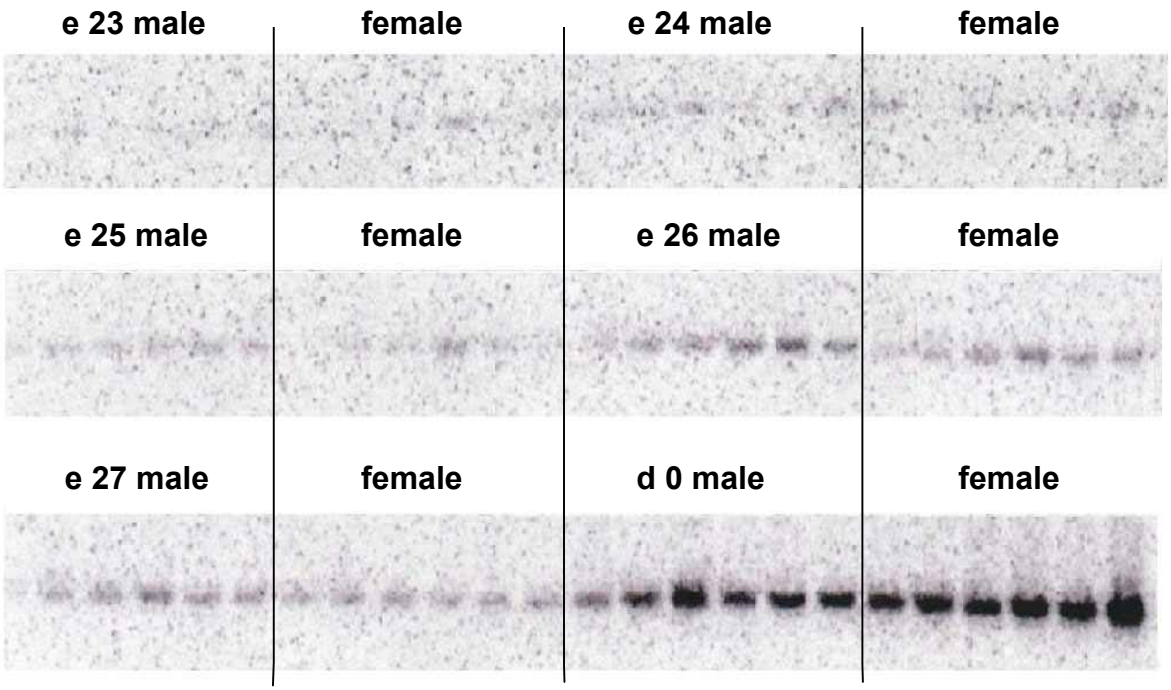

FIGURE 6. High stringency northern blot analysis of tPepT1 mRNA expression in turkey embryonic intestinal tissue from e 23 to d 0 for both males and females. n = 6.

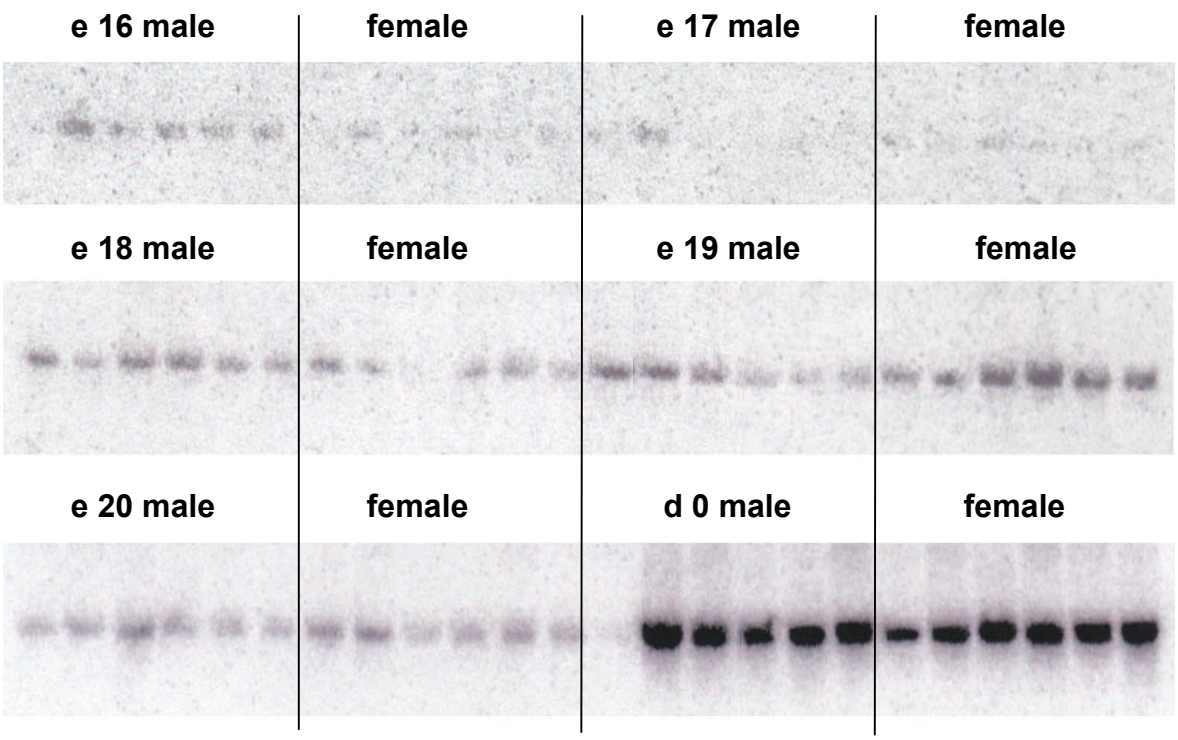

FIGURE 7. High stringency northern blot analysis of cPepT1 mRNA expression in broiler embryonic intestinal tissue from e 16 to d 0 for both males and females. n = 6.

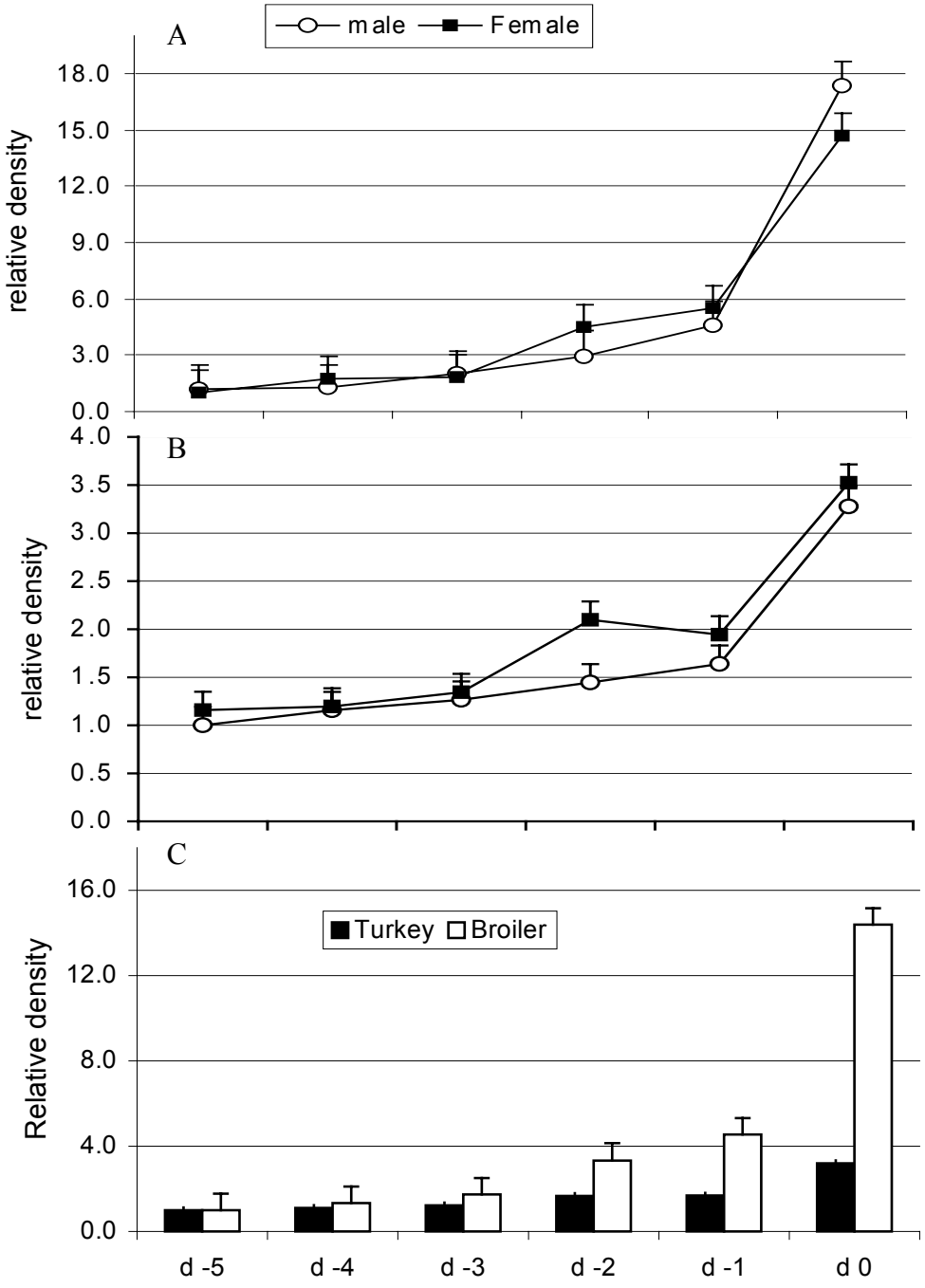

FIGURE 8. Densitometric analysis of PepT1 mRNA abundance in turkey and broiler embryonic small intestine from 5 d before hatch (d -5) to day of hatch (d0). (*A*) and (*B*) PepT1 mRNA abundance in broiler (A) and turkey (B) embryonic small intestine. cPepT1mRNA abundance was normalized to 18s rRNA. mRNA abundance was presented relative to the minimum in the group. n = 5 to 6, mean ± SE. (*C*) PepT1 mRNA abundance is presented as pooled data from male and female birds in turkeys and broilers. n = 11 to 12, mean ± SE. There was a quadratic increase ($P < 0.001$) in intestinal PepT1 mRNA abundance with age in turkeys and broilers from d -5 to d0.

TABLE 1

Comparison of PepT1 cDNA and protein structures between turkey and other species

Species	Amino acid similarity (%)	cDNA (kb)	Amino acid composition	pI[1]	Topology	Citation
Turkey PepT1	100.0	2.9	714	5.9	12 TM[2], 1 PKA[3], 1 PKC[4]	This thesis
Chicken PepT1	94.3	2.9	714	7.5	12 TM, 1 PKA, 1 PKC	Chen et al., 2001
Ovine PepT1	64.6	2.8	707	6.6	12 TM, 3 PKA, 4 PKC	Pan et al., 2000
Rabbit PepT1	63.4	2.7	707	7.5	12 TM, 1 PKA, 1 PKC	Fei et al., 1994
Rat PepT1	64.9	3.0	710	7.4	12 TM, 1 PKA, 1 PKC	Saito et al., 1995
Mouse PepT1	63.2	3.1	709	8.1	12 TM, 1 PKA, 1 PKC	Fei et al., 2000
Human PepT1	62.8	3.1	708	8.6	12 TM, 0 PKA, 2 PKC	Liang et al., 1995

[1]pI = isoelectric point.
[2]TM = transmembrane domain
[3]PKA = protein kinase A phosphorylation site
[4]PKC = protein kinase C phosphorylation site

Chapter IV

EPILOGUE

The molecular cloning and characterization of tPepT1 provide more information on both molecular and nutritional aspects of intestinal peptide transporters. It was shown that a wide range of di- or tripeptides, regardless of their molecular weight, electrical charge and hydrophobicity could be transported by ovine and chicken PepT1 (Pan et al., 2001; Chen et al., 2002). The kinetics of the uptake of dipeptides Gly-Sar and Met-Met across *Xenopus* oocyte membrane differed between tPepT1 and cPepT1. Kinetic studies on more di- or tripeptides are needed for better understanding the substrate specificity of tPepT1. Small peptides are major digestion products of protein in the small intestine and are of significant nutritional value to animals (Ganapathy et al, 1994). Dietary regulation of PepT1 activity has been reported (Ferraris et al., 1998; Thamotharan et al., 1998). Although addition of peptides in the diet is common now, with the improvement of protein hydrolysis and peptide production techniques, small peptides will become another important source of amino acids for animals. From this point, clarification of substrate specificity of PepT1 from different species will help in the regulation of peptide transport and amino acid assimilation in animals.

The present study also indicated that PepT1 was transcriptionally regulated in turkey and broiler embryonic small intestine with mRNA levels very high. PepT1 gene expression was found to have an unique cellular distribution in the enterocytes in

rats at birth. PepT1 presented not only on brush border membrane, but also in cytoplasm and on the basolateral membrane of enterocytes (Hussain, 2002). Elucidating the mechanisms and intracellular signaling pathways regulating this process is of interest. In addition, studies of the distribution of the peptide transporter protein in turkey and broiler embryos at tissue and cellular levels are necessary. Immunolocalization and in situ hybridization of embryonic intestinal tissue with anti-transporter antibodies can be used to investigate if PepT1 expression is also post-transcriptionally regulated. In rats, significant PepT1 mRNA and protein expression was observed in the colon during the first week of life. The expression of PepT1 in broiler and turkey embryonic and postnatal cecum needs to be examined to clarify if the cecum aids in peptide absorption at early ages in avian species.

The present study, for the first time, revealed that the tetrapeptide Met-Gly-Met-Met was transported across the apical membrane of *Xenopus* oocytes expressing tPepT1 not with Michaelis-Menten kinetics, but in a substrate inhibition manner. In *Xenopus* oocytes expressing a high affinity fungal nitrate transporter, substrate inhibition was observed at higher anion substrate concentrations (Zhou et al., 2000). The substrate inhibition of the currents was explained by a transport reaction cycle that included two routes for the transfer of nitrate across the membrane. Further studies need to be done to elucidate the mechanism of tetrapeptide transport by PepT1.

In summary, we have cloned and characterized a turkey intestinal peptide transporter, tPepT1. There was developmental regulation of PepT1 transcription in

turkey and broiler embryos with a quadratic increase in PepT1 mRNA abundance from 5 d before hatch to d0. These studies provide more information on both molecular and nutritional aspects of intestinal peptide transporter and sheds light on the importance of intestinal peptide absorption for neonatal birds.

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