

## Developmental Expression of PEPT1 and PEPT2 in Rat Small Intestine, Colon, and Kidney

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### ABSTRACT

Mammalian peptide transporters (PEPT1 and PEPT2) play a pivotal role in the absorption of small peptides from the intestine and kidney, respectively, and in the disposition and targeting of peptide or mimetic drugs. However, there are few reports on the molecular basis of their regulation, especially in the young. The aim of this study was to determine the developmental expression of intestinal and renal oligopeptide transporters in rats from embryonic to adult ages. Intestinal segments were collected (*i.e.* duodenum, jejunum, ileum, and colon) along with whole kidney, and their mRNA and protein levels were measured. Expression levels of PEPT1 were maximal 3–5 d after birth in the duodenum, jejunum, and ileum, and then declined rapidly. Expression was increased transiently at d 24, most notably in the ileum. Adult protein levels were approximately 70% of that observed on d 3–5. Significant PEPT1 expression was observed in colon during the first week of life, but levels were undetectable shortly thereafter through adulthood. PEPT1 and PEPT2 expression is less regulated in rat kidney and more pronounced in older

animals. Peptide transporters were also present as early as d 20 of fetal life for all tissues tested. These results are unique in providing the developmental expression of peptide transporter mRNA and protein in distinct regions of the small intestine, colon, and kidney in rat. Our findings suggest that intestinal expression of PEPT1 is induced postpartum, possibly by suckling, and again at the time of weaning, and that the colon may participate in peptide transport early in life. (*Pediatr Res* 49: 789–795, 2001)

#### Abbreviations:

**PEPT1**, intestinal oligopeptide transporter  
**PEPT2**, renal oligopeptide transporter  
**PHT1**, peptide/histidine transporter  
**GlyGly**, glycylglycine  
**GlyPro**, glycyl-L-proline  
**GlySar**, glycylsarcosine

Expression and molecular cloning studies have resulted in the identification of two distinct proton-coupled oligopeptide transporters in rabbit (1–3), rat (4–6), and human (7, 8) (*i.e.* PEPT1 as low-affinity carrier, PEPT2 as high-affinity carrier). The proteins range in size from 707 to 729 amino acids in various species, with high homology between species for a given transporter (approximately 80%) and less homology between transporters for a given species (approximately 50%). The gene products, as predicted by hydrophathy analysis, contain 12 membrane-spanning domains and a large extracellular loop between transmembrane domains 9 and 10. The encoded proteins have a number of potential *N*-glycosylation as well as protein kinase recognition sites, which suggests that the trans-

porters may be regulated by reversible phosphorylation. More recently, a third peptide transporter, PHT1, was cloned from rat brain (9). Encoding a protein of 572 amino acids, hydrophathy analysis predicts the presence of 12 transmembrane domains. There are also multiple *N*-glycosylation sites in the hydrophilic extracellular loop of PHT1, along with potential sites for protein kinase C-dependent phosphorylation. PHT1 is novel in that it transports histidine, as well as small peptides, with high affinity and in a proton gradient-dependent manner. Although expressed in the brain and eye, PHT1 is not found in the intestine or kidney and shows little homology to PEPT1 and PEPT2 (<20% amino acid identity). Its physiologic role remains to be determined.

Mammalian peptide transporters (PEPT1 and PEPT2) are steeped in nutritional, pharmacologic, and clinical importance. The localization of PEPT1 (in intestine and kidney) and PEPT2 (in kidney) is vital for their physiologic role in absorbing small peptides arising from digestion of dietary proteins in the small intestine, as well as in reabsorbing filtered peptides generated

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from luminal peptidases in the kidney (10–12). In addition, several pharmacologically active drugs are accepted by the intestinal and renal oligopeptide transporters (e.g. aminocephalosporins, aminopenicillins, angiotensin-converting enzyme inhibitors, nucleoside ester prodrugs) (13–16). As a result, this novel transport system is an important determinant of drug disposition, toxicity, and therapeutic efficacy. Some investigators have advocated the use of short-chain peptides as substitutes for free amino acids in enteral and parenteral solutions (17–19). This strategy may be particularly advantageous in newborns because amino acids were much more effectively absorbed in the intestine as peptide-bound nitrogen when compared with adults (17, 18). These studies and others (20, 21) suggest that the expression of PEPT1 may change markedly during development. However, the molecular basis for age-related alterations of peptide transport activity in the intestine is unclear inasmuch as previous studies did not measure protein levels of PEPT1 and sampling was limited to jejunal tissue alone (5). Furthermore, there is no information on the developmental aspects of PEPT1 and PEPT2 in the kidney. With this in mind, the objective of this study was to determine the developmental expression of intestinal and renal oligopeptide transporters in rats from embryonic to adult ages.

## METHODS

**Animals.** Pregnant Sprague Dawley rats were delivered at d 16 and 21 of gestation from Charles River Laboratories (Portage, MI, U.S.A.) and housed at the University of Michigan Animal Care Facility. Litters were born at 22 d gestation and were kept with their mothers until they were 21 d old. Animals were routinely weaned at 21 d to a standard laboratory diet (Rodent Diet 5001, PMI Nutrition International, St. Louis, MO, U.S.A.). Rats of either sex were killed at d 17 and 20 of gestation, and at 1, 3, 5, 7, 14, 21, 24, 28, 60, and 75 d (adult) after birth. Intestinal segments were then collected (*i.e.* duodenum, jejunum, ileum, and colon) along with whole kidney (owing to its smaller size). Embryonic colon samples were not collected (because of the uncertainty of obtaining intact tissue at this developmental stage). For each developmental age, samples were pooled using one to three pups per litter for a total of three to 18 animals per preparation. Data points represent the mean of two to three independently generated preparations. Animals were anesthetized under hypothermic conditions with CO<sub>2</sub> before decapitation. All animal procedures and study protocols were approved by the University of Michigan Committee on Use and Care of Animals.

**Northern blot analysis.** Total RNA was prepared from different regions of the small intestine, and from the colon and kidney of developing rats. These regions were homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH, U.S.A.), and RNA was obtained using a modification of the method of Chomczynski and Sacchi (22), as described by the manufacturer (23). Because of low-intensity signals in kidney, poly(A)<sup>+</sup> RNA was prepared from this tissue using guanidium isothiocyanate followed by cesium chloride gradient centrifugation and oligo(dT)-cellulose chromatography. Northern analyses were then performed for rat PEPT1 and PEPT2 as de-

scribed previously (12). Briefly, 15  $\mu$ g of total RNA from small intestine or colon or 4  $\mu$ g of poly(A)<sup>+</sup> RNA from kidney were electrophoresed overnight on 1% agarose gels containing 6% formaldehyde and transferred with 10 $\times$  SSC (1 $\times$  SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0) to nylon membranes by capillary action. cDNA probes of rat PEPT1, PEPT2, and  $\beta$ -actin were labeled with [<sup>32</sup>P]dCTP using a random labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany). Hybridization of the nylon membrane to <sup>32</sup>P-labeled cDNA was performed overnight in a solution of 50% formamide, 6 $\times$  SSC, 2.5 $\times$  Denhart's solution, 0.5% SDS, and 150  $\mu$ g/mL salmon sperm DNA at 45°C, and then washed several times at 65°C in 0.1 $\times$  SSC plus 0.1% SDS. Autoradiography was performed by using a phosphorimager, and signal intensities were quantified by computerized densitometric analysis (Imagequant v1.2 software, Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.). Data were normalized to  $\beta$ -actin to control for variability in RNA loading.

**Membrane preparation.** Brush border membranes were prepared from intestinal segments and from the colon and kidney of developing rats, as described previously (24). In brief, tissue samples were homogenized in buffer 1 (10 mM mannitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM Tris, pH 7.0) for 5 min in a Waring blender (VWR, Chicago, IL). Calcium chloride was added to the homogenate (final concentration, 10 mM), which was stirred and allowed to stand for 15 min (step 1). The suspension was then centrifuged at 500  $\times$  g for 12 min, and the resulting supernatant was centrifuged at 16,000  $\times$  g for 15 min (step 2). The pellet from this high-speed spin was suspended in buffer 1 using a glass-Teflon homogenizer. Steps 1 and 2 were repeated on this homogenate, and the resultant pellet was then suspended in buffer 2 (100 mM mannitol, 20 mM HEPES, pH 7.4). The suspension was centrifuged at 2,000  $\times$  g for 15 min, and the resultant supernatant was centrifuged at 47,000  $\times$  g for 15 min. The remaining pellet, which contained enriched brush border membranes, was then suspended in a small volume of PBS by repeated passage through an 18-gauge needle. All procedures were performed on ice or at 4°C. Protein concentrations were determined by the Coomassie blue binding method (25) with BSA as the protein standard.

**Immunoblot analysis.** Brush border membranes were solubilized in sample loading buffer (1% SDS, 50 mM Tris-HCl, pH 7.0, 20% glycerol, 5% mercaptoethanol, 0.01 mg/mL bromphenol blue) and heated at 100°C for 3 min. Samples were subjected to 7.5% SDS-PAGE, and resolved proteins were transferred to nitrocellulose membranes. Immunoblotting was then performed with specific affinity-purified rat PEPT1 and PEPT2 antisera developed in our laboratory (12). Membranes were blocked with 6% nonfat dry milk in TBS-T (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 2 h and then probed with polyclonal antibody (1:1000 dilution in blocking buffer) for 1.5 h, both steps at room temperature. The membranes were washed three times in TBS-T, blocked again, and then incubated with the second antibody, peroxidase-conjugated goat anti-rabbit IgG, 1:5000 dilution (Vector Laboratories, Burlingame, CA, U.S.A.). The membranes were then washed several times in TBS-T, and the bound antibody was detected with an enhanced chemiluminescence immunoblot-

ting system (ECL Plus, Amersham Life Science, Arlington Heights, IL, U.S.A.). Intensity of the signal was quantified using National Institutes of Health Image v1.61/PPC software (Bethesda, MD, U.S.A.). Preliminary studies showed linearity of the immunoblot assay from 1 to 200  $\mu\text{g}$  of intestine and kidney membrane protein.

## RESULTS

### *Expression of PEPT1 in the rat small intestine and colon.*

Quantitative Northern analyses of PEPT1 RNA were performed in intestinal tissue segments from embryonic d 17 through postpartum d 75 (adult) rats. As shown in Figure 1, PEPT1 transcripts were evident in the small intestine as early as d 20 of fetal life. PEPT1 mRNA levels increased rapidly at birth in the duodenum, jejunum, and ileum, and were maximal by d 3–5. PEPT1 levels then fell rapidly to 11–13% of maximal expression by d 14 and then rose again to 23–58% of maximal expression by d 24, about the time of weaning. This increase was most notable in the ileum. Adult mRNA levels of PEPT1 were approximately 25% of the maximal expression observed at d 3–5. Significant expression of PEPT1 mRNA was observed in the colon at d 1–5, followed by a rapid decrease to very low levels at d 7 and to undetectable levels at d 14. A similar pattern of expression was observed for PEPT1 protein. In all small intestinal segments (Fig. 2), embryonic d 20 levels were detectable but low and expression was maximal 3–5 d after birth. PEPT1 protein then decreased sharply, but at d 24 levels were 59–88% of maximal expression. At d 75 (adult), levels were approximately 70% of maximal expression. PEPT1 protein was observed in the colon at d 1–5, but expression was undetectable thereafter.

***Expression of PEPT1 and PEPT2 in the rat kidney.*** The expression pattern of PEPT1 and PEPT2 were similar in kidney but in sharp contrast to that observed for PEPT1 in the small intestine and colon. As observed in Figure 3, the mRNA of both peptide transporters was readily detected in fetal kidneys as early as embryonic d 17. mRNA levels then climbed steadily to a postpartum value of 70% maximal expression at d 14. An abrupt increase was then observed, reaching a maximal expression at d 24. Peptide transporter mRNA then declined to reach values as an adult of 63–72% maximal expression. PEPT1 and PEPT2 proteins showed a monotonic increase in their expression from fetal d 20 to d 75 (adult). By d 7, protein levels were approximately 86% of maximal expression, and by d 14, protein levels were 92–98% of maximal expression (Fig. 4).

## DISCUSSION

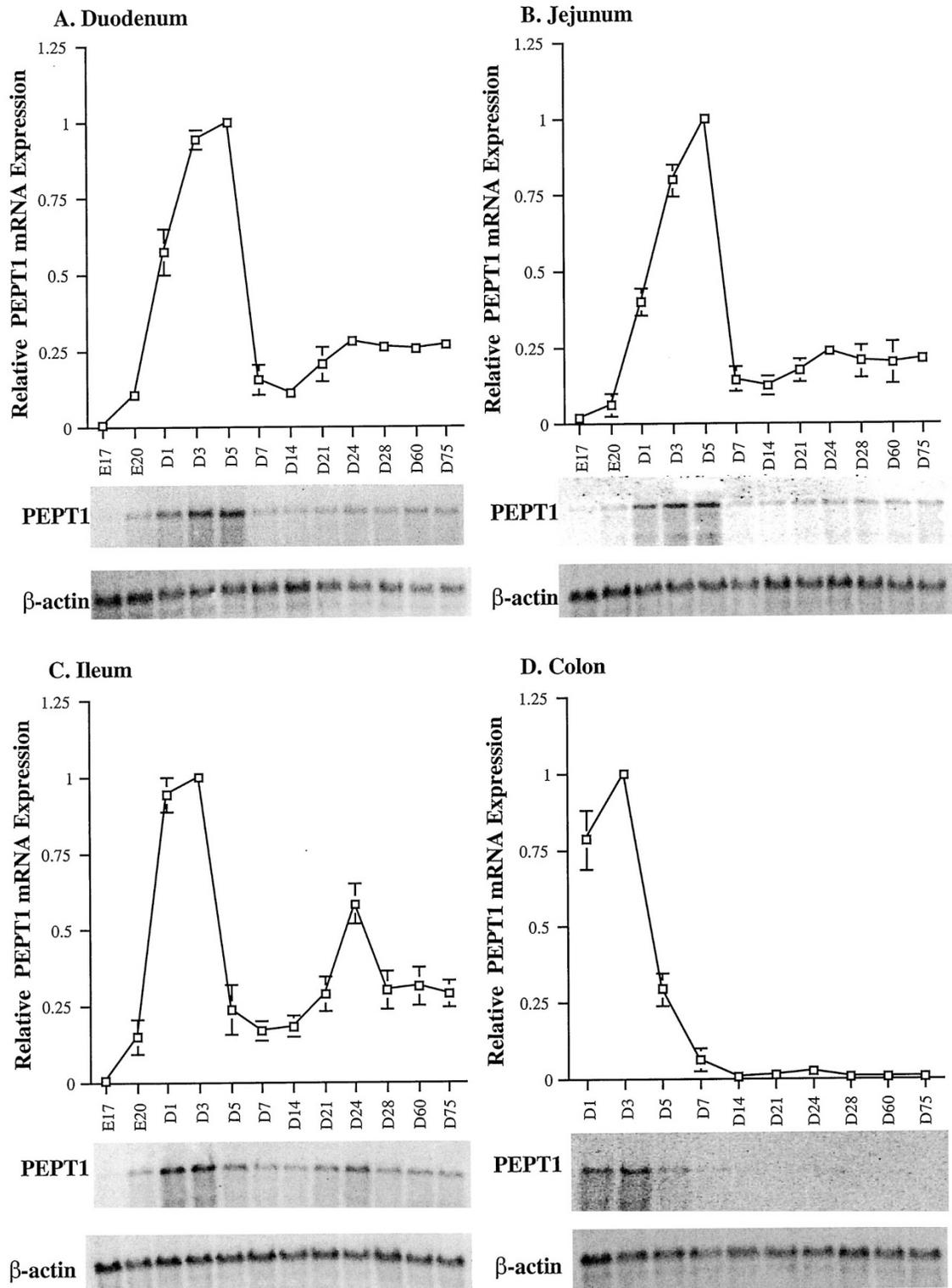
Few studies have investigated the ability of the small intestine to transport peptides in developing animals (17, 18). In one study (17), age-dependent changes in the absorption of dipeptides and their constituent amino acids were evaluated in isolated everted intestinal segments (*i.e.* jejunum and ileum) of guinea pig. It was observed that GlyGly and glycyl-L-leucine influxes were significantly greater in sucklings (3–4 d, 80–120 g) than in weanlings (10–14 d, 160–220 g), which, in turn, were greater than in adults (350–500 g). Kinetically, the developmental change in jejunal GlyGly influx was related to

a decrease in maximal transport capacity (*i.e.*  $V_{\text{max}}$ ; 51 and 20  $\text{nmol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$  in sucklings and adults, respectively) as opposed to a change in substrate affinity (*i.e.*  $K_m$ , 2.4 and 2.1 mM in sucklings and adults, respectively). In the other study (18), uptake of GlyPro and glycine was evaluated *in vitro* in the jejunum and ileum of rabbits from the 25th day of gestational age into adulthood. Results show a gradual increase of GlyPro influx in both jejunum and ileum from the earliest fetal period studied, with a striking perinatal peak. After the first 6 d of postnatal life, the uptake of GlyPro declined continually, reaching minimum values as an adult (>3 mo). In contrast, glycine uptake did not show a well-defined developmental pattern in jejunal and ileal tissues. Further, the uptake of GlyGly was substantially higher than that of glycine in fetal (25–30 d gestational age), newborn (1–6 d old), and suckling or weaned (10–50 d old) rabbits. These two studies point to developmental changes in the activity of the intestinal dipeptide carrier, and the preferential uptake of small peptides over their free amino acids in newborns and infants.

In a subsequent study (5), the cloning, tissue distribution, and expression of rat PEPT1 in jejunum was examined during development. Northern blot analyses revealed that intestinal PEPT1 mRNA levels were highest in 4-d-old rats, and then decreased reaching the adult level (d 70) by d 28 after birth. Although providing the first molecular evidence of developmental changes in the rat intestinal oligopeptide transporter, this study was limited in that only jejunal tissue was sampled and protein levels were not determined.

In the present study, we characterized the developmental expression of PEPT1 mRNA and protein in the duodenum, jejunum, ileum, and colon of the rat. We also characterized PEPT1 and PEPT2 expression (mRNA and protein) in the developing rat kidney. With respect to the small intestine, our findings support a molecular basis for age-related alterations in dipeptide transport. In this regard, our mRNA and protein results in rat are similar to the activity data reported in guinea pig (17) and rabbit (18). In general, maximal effects are observed in the small intestine of newborns (within 1 wk of birth) and rapidly fall during the next week. Expression levels then begin to increase at about d 21, especially in the ileum. Interestingly, PEPT1 was expressed and developmentally regulated in the colon during the first few days of life. This is a novel finding because PEPT1 is not expressed in the colon of adult rats [(26), this study]. It should be appreciated, however, that amino acid transporters are also expressed in the colon of newborn pigs and, so it appears, seem to parallel that of PEPT1 (27–29).

To our knowledge, no studies have examined whether or not oligopeptide transporters are developmentally regulated in the kidney. This information is important given the role of the kidney in peptide retention and, in concert with the intestine, the regulation of di- and tripeptide levels in plasma. In the present study, mRNA and protein levels of PEPT1 and PEPT2 showed a similar pattern in their age-dependent expression. In general, expression levels increased steadily over time to reach an asymptote by 2 wk after birth. However, the abrupt change in mRNA at d 24 was not reflected by a similar change in protein, suggesting posttranscriptional control of this process.

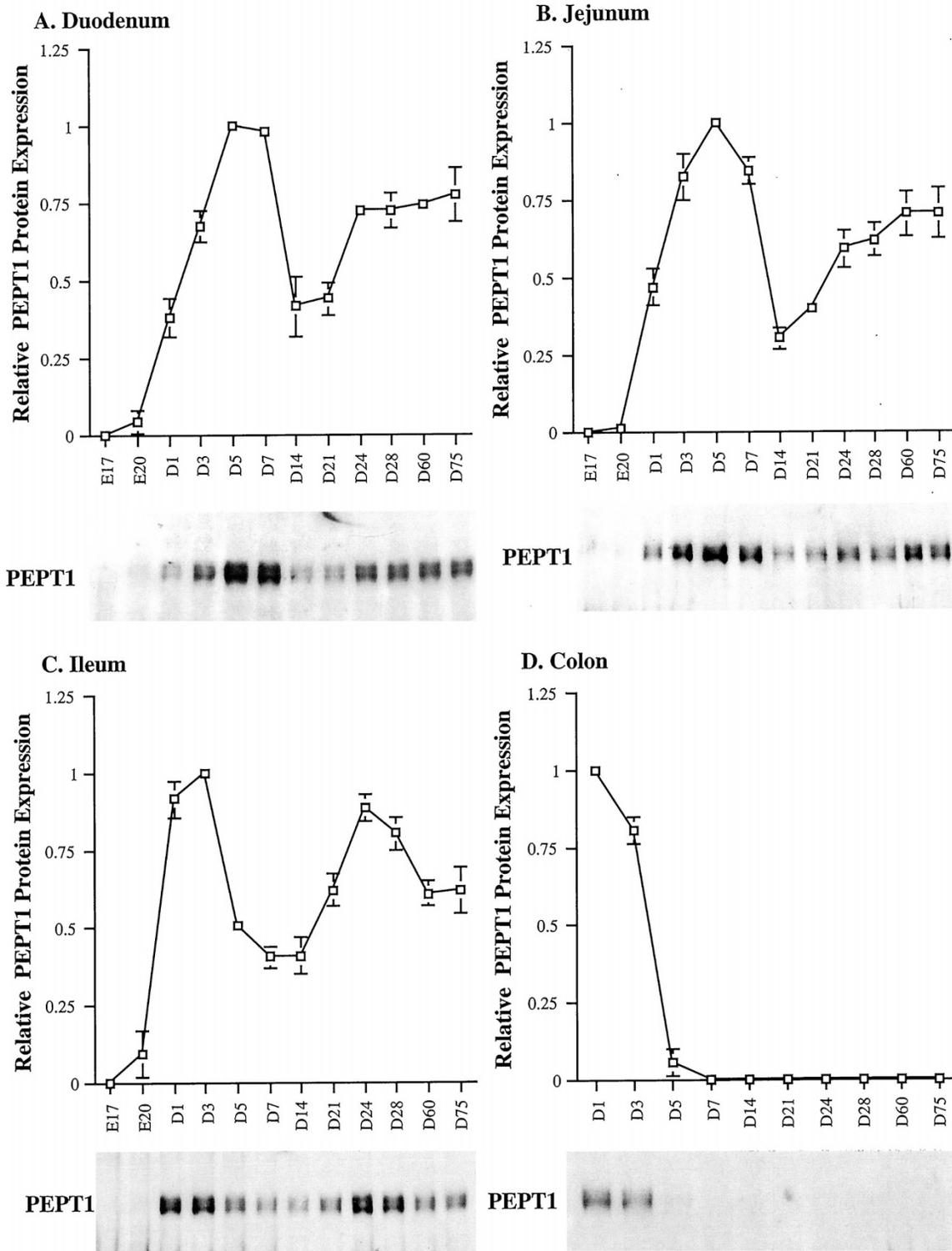


**Figure 1.** High-stringency Northern blot analysis of PEPT1 mRNA in the small intestine (duodenum, jejunum, and ileum) and colon of developing rats. Total cellular RNA was extracted from pooled tissue samples at 17 and 20 d of gestation (E17, E20) and at 1–75 d after birth (D1–D75). Each lane contains 15  $\mu$ g of total RNA. PEPT1 mRNA levels were standardized to  $\beta$ -actin mRNA. Autoradiographs were subjected to scanning densitometry, and mRNA abundance was expressed relative to that of maximal expression (*i.e.* d 5 for duodenum and jejunum; d 3 for ileum and colon). Data are reported as mean  $\pm$  SEM of two to three preparations, with three to 18 animals per preparation.

Overall, it appears that nutrition may play a less prominent role in the renal expression of PEPT1 and PEPT2.

Several recent studies have examined the regulatory influence of diet on the expression or activity of peptide transport-

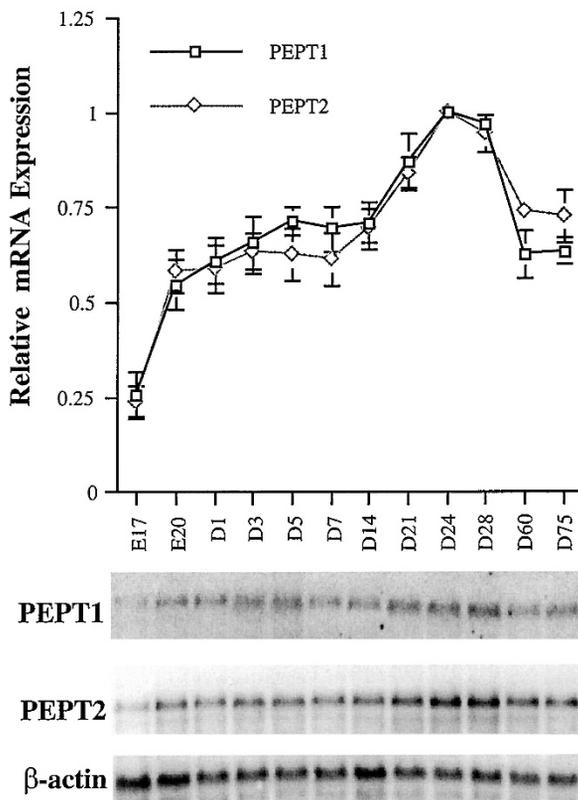
ers. Thus, it was observed that PEPT1 activity ( $V_{\max}$  increased,  $K_m$  unchanged) and protein and mRNA levels were up-regulated when Caco-2 cells were incubated in a peptide-rich medium (4 mM glycyl-L-glutamine replacing 4 mM glutamine)



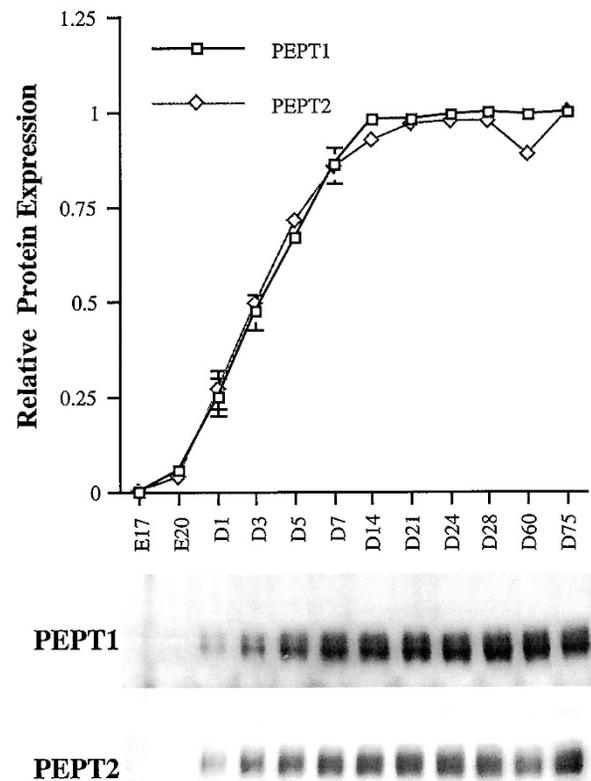
**Figure 2.** Immunoblot analysis of PEPT1 protein in the small intestine (duodenum, jejunum, and ileum) and colon of developing rats. Brush border membranes were purified from pooled tissue samples at 17 and 20 d of gestation (E17, E20) and at 1–75 d after birth (D1–D75). Each lane contains 100  $\mu\text{g}$  of membrane protein, which was incubated with polyclonal antibodies raised against a PEPT1 synthetic peptide (residues 699–710). Autoradiographs were subjected to scanning densitometry, and protein abundance was expressed relative to that of maximal expression (*i.e.* d 5 for duodenum and jejunum; d 3 for ileum; d 1 for colon). Data are reported as mean  $\pm$  SEM of two to three preparations, with three to 18 animals per preparation.

(30). Increased levels of human PEPT1 mRNA were a reflection of both increased transcription and mRNA stability *via* a signaling pathway acting directly on the enterocyte. In another study (31), it was found that increasing amounts of dietary

protein (*i.e.* casein) enhanced the transport of GlySar in rat ileal brush border membrane vesicles. Feeding of a dipeptide (*i.e.* glycyl-phenylalanine) or an amino acid (*i.e.* phenylalanine but not glycine) also stimulated GlySar transport activity ( $V_{\text{max}}$



**Figure 3.** High-stringency Northern blot analysis of PEPT1 and PEPT2 mRNA in the whole kidney of developing rats. Poly(A)<sup>+</sup> RNA was extracted from pooled tissue samples at 17 and 20 d of gestation (E17, E20) and at 1–75 d after birth (D1–D75). Each lane contains 4  $\mu$ g of poly(A)<sup>+</sup> RNA. PEPT1 and PEPT2 mRNA levels were standardized to  $\beta$ -actin mRNA. Autoradiographs were subjected to scanning densitometry, and mRNA abundance was expressed relative to that of maximal expression (*i.e.* d 24). Data are reported as mean  $\pm$  SEM of three preparations, with three to 18 animals per preparation.



**Figure 4.** Immunoblot analysis of PEPT1 and PEPT2 protein in the whole kidney of developing rats. Brush border membranes were purified from pooled tissue samples at 17 and 20 d of gestation (E17, E20) and at 1–75 d after birth (D1–D75). Each lane contains 100  $\mu$ g of membrane protein, which was incubated with polyclonal antibodies raised against a PEPT1 or PEPT2 synthetic peptide (residues 699–710 or 718–729, respectively). Autoradiographs were subjected to scanning densitometry, and protein abundance was expressed relative to that of maximal expression (*i.e.* d 75). Data are reported as mean  $\pm$  SEM of three preparations, with three to 18 animals per preparation.

increased but not  $K_m$ ) compared with rats fed a protein-free diet, along with concomitant changes in PEPT1 mRNA and protein levels. This finding suggested that up-regulation of dipeptide transport activity by dietary protein was caused by transcriptional activation of the PEPT1 gene by selective amino acids and dipeptides in the diet. In a third study (32), it was observed that short-term starvation (4-d fast but free access to water) markedly increased the amount of PEPT1 protein present in rat jejunum, whereas dietary administration of amino acids reduced PEPT1 protein. These results were subsequently confirmed by a preliminary report (33) in which the functional and molecular expression of PEPT1 was up-regulated after a brief fast (*i.e.* 1-d fast but free access to water) in rat jejunal brush border membrane vesicles. As a whole, these studies (30–33) confirm and extend the existing evidence that dietary regulation has a profound effect on peptide absorption and expression in the intestine.

After birth, the developing small intestine and colon face major changes in the functional demands that are placed on them. In particular, the placenta is replaced by the intestines as the site of nutrient procurement, and, at the time of weaning, animals make major changes in their diet. In the case of rats, a high-protein milk diet is often replaced with an adult diet

typically containing more carbohydrate than protein. Our findings, showing up-regulation of PEPT1 in the first week of life, are consistent with the influence of a high-protein diet on peptide expression. In addition, the increased expression of PEPT1 at weaning (especially in ileum) may be related to an adaptive response to the reduced protein diet or nourishment at this time period. In both cases, it appears that developmental changes occur such that the chances of survival are optimized. Although the kidney is not as regulated as intestine and colon with respect to diet, it is unclear what effect ontogenesis may have on peptidase activity and the associated retention of constituent amino acids in this organ. It is also unclear how hormonal or neural pathways affect oligopeptide regulation in the developing intestine and kidney. These factors were beyond the scope of the present study, but may be important as suggested previously (11, 20, 34).

In summary, our studies have provided, for the first time, the developmental expression of peptide transporter mRNA and protein in distinct regions of the small intestine, colon, and kidney in rat. Our findings suggest that intestinal expression of PEPT1 is induced postpartum, possibly by suckling, and again at the time of weaning, and that the colon may participate in

peptide transport early in life. PEPT1 and PEPT2 expression is less regulated in rat kidney and more pronounced in older animals. Overall, these results may have important implications for protein nutrition, as well as for drug disposition and targeting of peptides or mimetics in the young.

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