Lactase–phlorizin hydrolase and aminopeptidase N are differentially regulated in the small intestine of the pig

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The longitudinal expression of two brush-border enzymes, lactase–phlorizin hydrolase (EC 3.2.1.23/62) and aminopeptidase N (EC 3.4.11.2), was studied in the small intestine of the post-weaned pig. Whereas the level of mRNA, encoding aminopeptidase N (relative to that of β -actin), only varied moderately from the duodenum to the terminal ileum, the amount of lactase–phlorizin hydrolase mRNA exhibited a sharp maximum in the proximal jejunum. For both enzymes, the level of protein synthesis, studied in cultured mucosal explants, correlated well with the level of mRNA, and no major variation in post-translational processing or intracellular transport was observed along the intestine. The mRNA/specific-activity ratio for both enzymes was markedly (3–5-fold) higher in the duodenum and proximal jejunum, compared with the ileum. This indicates an

increased proximal turnover rate, most likely caused by the presence in the gut lumen of pancreatic proteases. In neonatal animals, the level of mRNA for lactase–phlorizin hydrolase in both proximal and distal regions of the intestine was of the same magnitude as in the proximal jejunum of the post-weaned pigs. Our results point to two mechanisms that affect the expression of lactase–phlorizin hydrolase in the pig during development: (1) a primary regulation at the level of mRNA (predominantly in the ileum); (2) an increased rate of turnover of the enzyme, mainly in the duodenum and proximal jejunum, and most likely due to an increased secretion into the gut lumen of pancreatic proteases (a mechanism also affecting aminopeptidase N and probably other brush-border enzymes as well).

INTRODUCTION

In order to digest and take up dietary carbohydrate and protein, the small-intestinal enterocyte relies on a continued synthesis of brush-border hydrolases, including lactase–phlorizin hydrolase (EC 3.2.1.23/62) and aminopeptidase N (EC 3.4.11.2). It has been long known that the specific activity of brush-border enzymes, in particular lactase–phlorizin hydrolase, varies along the small intestine as well as with age (reviews: Semenza and Auricchio, 1989; Büller and Grand, 1990), but the level at which this is controlled is still a matter of controversy. Thus studies on the post-weaning decline of lactase activity have reported both persistent levels of lactase mRNA in adults of rabbit and man (Sebastio et al., 1989) as well as decreasing mRNA levels in rat (Büller et al., 1990) and man (Escher et al., 1992; Lloyd et al., 1992). Concerning the expression along the small intestine of the rat, varying mRNA levels have been observed in the post-weaned, but not the suckling, state (Freund et al., 1990, 1991). Studies at the protein level of expression have reported an unchanged rate of synthesis but an altered post-translational proteolytic processing in the rat (Castillo et al., 1990; Quan et al., 1990). Differences in processing have also been observed in the rabbit (Rossi et al., 1992), whereas biosynthesis studies on adult hypolactemic and lactase-persistent humans in most cases find a correlation between enzyme activity and biosynthetic rate (Sterchi et al., 1990; Witte et al., 1990; Lloyd et al., 1992).

In the pig, expression of lactase–phlorizin hydrolase along the small intestine and during development has been studied mainly at the level of enzyme activity (Shulman et al., 1988; Sangild et al., 1991). Freund et al. (1989) observed no correlation between the enzyme activity and mRNA level when comparing adult and suckling pigs. Recently, however, the gene promoter of the

enzyme was cloned and a trans-acting factor, NF-LPH1, was shown to co-vary with the enzyme activity (Troelsen et al., 1992).

In the present work, we studied the expression at the mRNA, protein synthesis and enzyme activity levels of lactase–phlorizin hydrolase and aminopeptidase N and provide evidence that the two brush-border enzymes are differentially regulated in the small intestine of the post-weaned pig.

EXPERIMENTAL

Materials

Radioactive chemicals ($[\alpha\text{-}^{32}\text{P}]\text{dATP}$, 3000 Ci/mmol; $[\text{S}^{35}]\text{-methionine}$, 1000 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K.). Oligo(dT)₂₅ beads were from Dynal (Oslo, Norway).

Pig small intestines from post-weaned animals were kindly given by the Department of Experimental Pathology, Rigshospitalet, Copenhagen. Small intestines from newborn pigs were kindly provided by Dr. Per Sangild, Department of Animal Physiology, the Royal Veterinary University, Copenhagen.

Isolation of mRNA

Small segments of intestine were excised from the duodenum, proximal jejunum (taken approx. 1 m from the duodenum), proximal ileum (taken approx. 4 m from the duodenum) and terminal ileum (taken < 0.5 m from the caecum) of (post-weaned) 4-month-old pigs (weight 30–35 kg) and immediately frozen in liquid N₂. The small intestine of newborn animals was divided into a proximal and a distal part, and segments from the middle of both proximal and distal parts were taken and frozen in a similar way. Total RNA was extracted from 1 g samples of

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frozen tissue by the guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). Poly(A)⁺ RNA was isolated from 100 µg samples of total RNA by adsorption to magnetic oligo(dT)₂₅ Dynabeads, by the protocol supplied by the manufacturer.

Northern-blot analysis

Samples of mRNA were denatured by incubation at 65 °C for 5 min in the presence of 9 % formaldehyde and 66 % formamide, electrophoresed in 1 %-agarose gels in the presence of 2 % formaldehyde and transferred to nitrocellulose filters by vacuum blotting (LKB 2016 VacuGene). After prehybridization, hybridization with ³²P-labelled cDNA probes was carried out at 37 or 42 °C overnight, essentially as described by Meinkoth and Wahl (1984). For lactase-phlorizin hydrolase, the probe was a pig cDNA fragment of 0.45 kb (Troelsen et al., 1992). For aminopeptidase N, a 0.55 kb fragment from the 5'-end of the pig gene was used (Olsen et al., 1989), and for β-actin, a 0.70 kb fragment from the untranslated 3'-end of the cDNA (from human fibroblasts) was used (Ponte et al., 1983). After hybridization, the filters were washed and then wrapped in plastic film and autoradiographed. Exposed X-ray films were developed and scanned in a densitometer (LKB Ultrosan XL). For repeated hybridizations, the filters were stripped by heating to 95 °C for 90 min in the presence of 0.1 % SDS.

Enzyme activity

Samples (1 g) of frozen intestinal tissue were thawed and homogenized in 10 ml of 10 mM Na₂HPO₄/150 mM NaCl, pH 7.5, in a Potter-Elvehjem homogenizer and centrifuged at 500 g for 5 min. The supernatants were used for determination of lactase activity (Dahlqvist, 1968) and aminopeptidase N (alanine p-nitroanilide hydrolysing) activity (Sjöström et al., 1978). Protein was determined as described by Lowry et al. (1951).

Organ culture and fractionation of mucosal explants

Small-intestinal mucosal explants of about 100 mg wet wt. were excised from freshly obtained duodenum, proximal jejunum, proximal ileum and terminal ileum and cultured in Trowell's T-8 medium essentially as previously described (Danielsen et al., 1982). Explants obtained from different intestinal segments of the same animal were cultured and labelled with [³⁵S]methionine in parallel. After culture, the explants were fractionated by the bivalent-cation precipitation method (Booth and Kenny, 1974) into a Mg²⁺-precipitated fraction (intracellular and basolateral membranes) and a microvillar fraction and solubilized with Triton X-100 (1 %) as described previously (Danielsen, 1982). In some experiments, the explants were homogenized and solubilized in 500 µl of 25 mM Tris/HCl/75 mM NaCl/5 % (w/v) Triton X-100, pH 7.5, followed by centrifugation at 20000 g for 10 min, to obtain a supernatant of total detergent-extracted mucosal proteins.

Immunoprecipitation and SDS/PAGE analysis of brush-border enzymes

Lactase-phlorizin hydrolase and aminopeptidase N were immunopurified from detergent-solubilized labelled mucosal explant membrane fractions by line immunoelectrophoresis against their respective rabbit antisera as previously described

(Danielsen and Cowell, 1983). In order to obtain directly comparable data, the two brush-border enzymes from the same fractions were immunoprecipitated in one single electrophoretic procedure. The excised immunoprecipitates were subjected to SDS/PAGE in 10 %-acrylamide gels under reducing conditions (Laemmli, 1970), and the labelled bands were revealed by fluorography (Bonner and Laskey, 1974).

RESULTS

mRNA levels and specific activity of lactase-phlorizin hydrolase and aminopeptidase N along the small intestine

As shown in Figure 1, the 3.5 kb mRNA encoding aminopeptidase N (Olsen et al., 1988) was present from the duodenum to the terminal ileum. In contrast, the 6.2 kb mRNA

Figure 1 Northern-blot analysis of aminopeptidase N, lactase-phlorizin hydrolase and β-actin

mRNA from the duodenum (1), proximal jejunum (2), proximal ileum (3) and terminal ileum (4) was extracted, isolated and analysed by agarose-gel electrophoresis, followed by hybridization to ³²P-labelled cDNA probes to aminopeptidase N (a), lactase-phlorizin hydrolase (b, c; two different animals) and β-actin (d). The molecular size (kb) is indicated.

Table 1 Specific activity of lactase-phlorizin hydrolase and aminopeptidase N

Lactase and aminopeptidase N activities and protein concentration were determined in mucosal homogenates of post-weaned and neonatal animals as described in the Experimental section. The values listed are means $\pm$ S.E.M. of results obtained from three post-weaned and three neonatal animals.

Animals	Organ	Lactase (nmol hydrolysed/min per mg)	Aminopeptidase N (nmol hydrolysed/min per mg)
Post-weaned	Duodenum	2 $\pm$ 0	44 $\pm$ 10
	Proximal jejunum	32 $\pm$ 7	138 $\pm$ 28
	Proximal ileum	34 $\pm$ 10	333 $\pm$ 59
	Distal ileum	4 $\pm$ 0	243 $\pm$ 10
Neonatal	Proximal intestine	148 $\pm$ 15	186 $\pm$ 32
	Distal intestine	70 $\pm$ 20	186 $\pm$ 47

of lactase-phlorizin hydrolase (Mantei et al., 1988) was mainly present in the proximal jejunum and proximal ileum, much less abundant in the duodenum and hardly detectable in the terminal ileum. In blots of mRNA of some animals, a second hybridization to a mRNA band of about 3 kb by the lactase-phlorizin hydrolase cDNA probe could be detected (Figure 1). When present, this mRNA species was most abundantly present in the proximal ileum and terminal ileum.

Table 1 shows the specific activities of lactase-phlorizin hydrolase and aminopeptidase N at the positions in the small intestine from which mRNA was extracted and isolated. This distribution agrees well with a previous report on the longitudinal and developmental profile of specific activity in the pig (Shulman et al., 1988).

In the present work β -actin was chosen as a marker for standardization of the amounts of mRNA applied to the gel tracks, and the cDNA probe hybridized to a single 2.0 kb mRNA which was present the whole length of the small intestine (Figure 1). Actin is the most abundant protein in the mucosal epithelium, and in SDS/PAGE of labelled mucosal explants about 15% of the [35 S]methionine incorporated into the protein of a homogenate appeared in the 42 kDa band of actin, regardless of the location along the small intestine (results not shown).

Figure 2 shows the mRNA levels for aminopeptidase N and lactase-phlorizin hydrolase, relative to that of β -actin, along the small intestine. For aminopeptidase N, a fairly constant level of expression of mRNA was seen from the proximal jejunum to the terminal ileum. The expression in the duodenum was at about 40% of this level. This correlated roughly with a profile of the relative specific enzyme activity in the proximal ileum and terminal ileum, whereas the duodenum and proximal jejunum had a much higher ratio of mRNA/specific enzyme activity.

For lactase-phlorizin hydrolase, the corresponding profile showed a distinct maximum level of mRNA in the proximal jejunum, intermediate expression in the proximal ileum and very low levels in both duodenum and terminal ileum (Figure 2b). The specific activity of the enzyme was at a similar maximal level in the proximal jejunum and the proximal ileum, but very low levels were found in the duodenum and the terminal ileum. As with aminopeptidase N, the relative levels of the mRNA and specific activity correlated well in the proximal ileum and terminal ileum, whereas the proximal jejunum and the duodenum had a much higher mRNA/specific-activity ratio. It is thus evident that the

Figure 2 Relative mRNA levels and specific activities of aminopeptidase N and lactase-phlorizin hydrolase along the small intestine

Northern blots of mRNA for aminopeptidase N, lactase-phlorizin hydrolase and β -actin from the duodenum (1), proximal jejunum (2), proximal ileum (3) and terminal ileum (4) obtained from four animals were densitometrically scanned, and the aminopeptidase N-mRNA/ β -actin-mRNA (a) and lactase-phlorizin hydrolase-mRNA/ β -actin-mRNA (b) ratios determined. (The ratios obtained at the proximal ileum were arbitrarily set at 100%. The profiles of the specific activities, listed in Table 1, are shown for comparison with the value of the proximal ileum = 100%.) Bars indicate S.E.M.

two brush-border enzymes differ dramatically in variability of expression of mRNA along the small intestine; whereas the mRNA of aminopeptidase N only varies 2–3-fold from the lowest (duodenum) to the highest (proximal jejunum) level, that

Figure 3 Biosynthesis of aminopeptidase N and lactase-phlorizin hydrolase in mucosal explants

Mucosal explants of similar size (about 100 mg wet wt.) were excised from duodenum (1), proximal jejunum (2), proximal ileum (3) and terminal ileum (4) and labelled for 30 min with [35 S]methionine (100 μ Ci/ml). After culture, aminopeptidase N (a) and lactase-phlorizin hydrolase (b) were purified from detergent extracts of whole mucosa by line immunoelectrophoresis, and the excised precipitates were analysed by SDS/PAGE and fluorography. (Samples of the detergent extracts were electrophoresed in parallel, and the intensity of the 42 kDa band of actin determined by densitometry.)

of lactase-phlorizin hydrolase varies by about two orders of magnitude.

Biosynthesis of aminopeptidase N and lactase-phlorizin hydrolase along the small intestine

Figure 3(a) shows [35 S]methionine-labelled aminopeptidase N, immunopurified from mucosal explants of duodenum, proximal jejunum, proximal ileum and terminal ileum, that were pulsed for 30 min. The explants were of comparable size and were labelled in parallel, and it is evident that the transient high-mannose-glycosylated 140 kDa-form of the enzyme (Danielsen, 1982) was synthesized roughly at an equal level all along the small intestine. In contrast, lactase-phlorizin hydrolase immunoprecipitated in parallel from the same explants showed a much more variable level of synthesis (Figure 3b). The 225 kDa high-mannose-glycosylated precursor (Danielsen et al., 1984) (together with proteolytic fragments of 205 and 150 kDa) was most abundantly synthesized in the proximal jejunum, with much less incorporation of label in the duodenum and proximal ileum. In the terminal ileum, the precursor form was hardly detectable. For both aminopeptidase N and lactase-phlorizin hydrolase, there seems to be a fairly close correlation between the levels of mRNA and protein synthesis along the small intestine.

Figure 4(a) shows the distribution of newly synthesized aminopeptidase N, after 2 h of chase, between a Mg^{2+} -

Figure 4 Molecular processing and intracellular transport of newly synthesized brush-border enzymes

Mucosal explants, obtained and labelled for 30 min as described in the legend to Figure 3, were chased for 2 h in the presence of 1 mM (non-radioactive) methionine. After culture, the explants were fractionated into a Mg^{2+} -precipitated (Mg^{2+}) and a microvillar (Mic) fraction, and aminopeptidase N (a) and lactase-phlorizin hydrolase (b) were immunopurified from the detergent-solubilized fractions and subjected to SDS/PAGE and fluorography as described in the Experimental section.

precipitated membrane fraction and a microvillar fraction. Regardless of the longitudinal intestinal origin of the explant, the major portion of the enzyme was in the complex-glycosylated mature 166 kDa form (Danielsen, 1982) and was present in the microvillar fraction, with only small amounts of mature form and transient form remaining in the Mg^{2+} -precipitated fraction. For lactase-phlorizin hydrolase immunoprecipitated from the same fractions of pulse-chase-labelled explants, the major portion of the enzyme was likewise in the microvillar fraction in the form of the mature 160 kDa polypeptide (Danielsen et al., 1984) or the complex glycosylated precursor of 245 kDa (together with a cleaved precursor of 215 kDa) (Figure 4b). In the terminal ileum, however, the small amounts of newly synthesized enzyme remained largely as a precursor in the Mg^{2+} -precipitated fraction, suggestive of an impaired molecular processing and intracellular transport. With this exception, it can therefore be concluded that the time required for molecular processing and intracellular transport does not differ markedly along the small intestine. In particular, no 'alternative' degradative processing seems to occur for either of the two enzymes.

Expression of lactase-phlorizin hydrolase in the neonatal pig

Figure 5(a) shows a Northern blot of lactase-phlorizin hydrolase mRNA obtained from the proximal and distal regions of the small intestine of neonatal pigs. The neonatal-pig mRNA was of

Figure 5 mRNA levels of lactase-phlorizin hydrolase in neonatal pigs

(a) Northern blot of mRNA for lactase-phlorizin hydrolase and β -actin from the proximal jejunum of three post-weaned animals (lanes 1–3) and the proximal (lanes 4, 6, 8) and distal (lanes 5, 7, 9) parts of the small intestine from three neonatal pigs. The molecular size (kb) is indicated. (b) Lactase-phlorizin hydrolase-mRNA/ β -actin-mRNA ratio, determined by densitometric scanning for proximal jejunum from post-weaned animals (1) and from proximal (2) and distal (3) parts of neonatal small intestine (stippled bars) (the mean value obtained for proximal jejunum from post-weaned pigs = 100%). The corresponding specific activities (also listed in Table 1) are shown for comparison (hatched bars). Bars indicate S.E.M.

Figure 6 Biosynthesis of lactase-phlorizin hydrolase in neonatal-pig small intestine

Mucosal explants from neonatal-pig small intestine were labelled continuously for 0.5 h or 1.5 h in the presence (+) or absence (–) of 50 μ g/ml leupeptin. After culture, lactase-phlorizin hydrolase was immunopurified from detergent extracts of whole mucosa and analysed by SDS/PAGE. For comparison, lactase-phlorizin hydrolase from a labelled explant obtained from a post-weaned animal is shown in the gel track furthest to the right.

the same size as that of post-weaned animals and was present at comparable levels at the two locations analysed. Standardized to actin mRNA, the neonatal level of lactase-phlorizin hydrolase mRNA was of the same magnitude as that in the proximal jejunum of a post-weaned animal. In contrast, the corresponding levels of specific enzyme activity were well above the post-weaned level (Table 1, Figure 5b). Similarly, for aminopeptidase N the mRNA levels in the proximal and distal parts of the

neonatal intestine were comparable with each other and with that of the proximal jejunum of post-weaned animals (results not shown).

The biosynthesis and molecular processing of lactase-phlorizin hydrolase in explants of neonatal-pig mucosa is shown in Figure 6. A 225/205 kDa precursor electrophoretically indistinguishable from that of a post-weaned animal was seen after 30 min of labelling, and after 1.5 h the mature 160 kDa polypeptide was visible. As is the case in adult pig and human intestine (Danielsen et al., 1984; Naim et al., 1987), leupeptin added to the culture medium could prevent the proteolytic processing of the precursor, in which case the complex-glycosylated precursor of 245 kDa became visible on the gel. These data thus indicate that the post-translational processing and intracellular transport of lactase-phlorizin hydrolase at least are qualitatively similar in the neonatal and the post-weaned pig. Aminopeptidase N obtained from the same fractions showed a transient (140 kDa) and a mature form (166 kDa) electrophoretically indistinguishable from the corresponding 'post-weaned' molecular forms (results not shown).

DISCUSSION

The purpose of the present work was to study the levels of mRNA, protein synthesis and specific activities of the two brush-border enzymes lactase-phlorizin hydrolase and aminopeptidase N along the small intestine and to compare these levels in neonatal and post-weaned pigs. The results obtained point to differences as well as similarities in the expression of these two digestive enzymes. Only lactase-phlorizin hydrolase is differentially expressed to a great extent at the mRNA level in post-weaned pigs, and it was a surprising finding that the proximal jejunum has a level of mRNA comparable with that of the neonatal-pig intestine, despite its much lower specific activity. In the rat, the transcriptional down-regulation during weaning has been reported to proceed from the distal towards the proximal end of the intestine (Freund et al., 1990, 1991). Considering the age of the post-weaned pigs used for these experiments (about 4 months), they may therefore represent a stage of development where this down-regulation is not yet complete. Interestingly, the 3 kb mRNA species which also hybridized with the lactase-phlorizin hydrolase cDNA probe had an opposite longitudinal distribution. It is thus unlikely to be an artefactual fragment of the 6.3 kb mRNA, and it can be speculated that it is a product of an alternatively spliced transcript. However, it may equally well represent a different gene product.

The pulse-chase-labelling experiments with mucosal explants indicated a close correlation between the levels of mRNA and protein synthesis for both brush-border enzymes. Again, the fairly constant level of synthesis of aminopeptidase N along the small intestine contrasted sharply with the peak of synthesis in the proximal jejunum, observed for lactase-phlorizin hydrolase. With the possible exception of lactase-phlorizin hydrolase synthesis in the terminal ileum, no major variation in the molecular processing was detected along the small intestine for any of the brush-border enzymes, indicating that in the pig the newly synthesized brush-border enzymes are not subjected to any major type of degradatory down-regulation during their intracellular transport.

For both aminopeptidase N and lactase-phlorizin hydrolase, the mRNA/specific activity ratio was 3–5-fold higher in the duodenum and proximal jejunum than in the ileum. Given the correlation between the levels of mRNA and protein synthesis, this most likely indicates a substantially higher rate of proteolytic degradation in the proximal end of the small intestine. The

presence in the gut lumen of pancreatic proteases, known to cleave brush-border enzymes *in situ* in the pig (Sjöström et al., 1978, 1980), would be the most obvious cause of such an accelerated turnover.

In summary, the results obtained in the present work thus point to two different mechanisms which both affect the expression of lactase-phlorizin hydrolase after weaning: (1) a reduced level of mRNA, predominantly occurring in the ileum; (2) an increased turnover of enzyme in the brush-border membrane, mainly occurring in the duodenum and proximal jejunum. This increase is most likely caused by an increased secretion of pancreatic proteases (Sangild et al., 1991) and affects aminopeptidase N (and probably other brush-border enzymes) as well.

Essentially the same conclusions concerning the control of expression of lactase-phlorizin hydrolase have been reported for the rat (Büller et al., 1990; Freund et al., 1990, 1991) and rabbit (Keller et al., 1992) enzymes, indicating that this dual type of regulation may well be a general phenomenon among mammals.

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