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The levels of lactase and of sucrase-isomaltase along the rabbit small intestine are regulated both at the mRNA level and post-translationally

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We determined along the small intestine of young and adult rabbits the activities of lactase (LPH) and sucrase (SI), the levels of their cognate mRNAs, and examined the in vitro biosynthesis of LPH and pro-SI. Lactase activity is low in the proximal 1/3 of the intestine, whereas the mRNA levels are high. However, the rates of *biosynthesis* of the LPH forms correlated well with the steady-state levels of LPH mRNA in all segments, indicating that factor(s) acting post-translationally produce a decline in brush border LPH in the proximal small intestine. These factor(s) are *not* involved in the processing of pro-LPH to mature LPH, since the relative amounts of the various forms of LPH are almost the same along the small intestine. Unexpectedly, we find that also for SI the ratio of activity to mRNA is low in proximal intestine. The biosynthesis of pro-SI correlates with the steady-state levels of its mRNA. Hence, the steady-state levels of LPH and SI along the small intestine are regulated both by mRNA levels *and* by posttranslational factor(s).

Lactase-phlorizin hydrolase; Sucrase-isomaltase; Regulation; Small intestine

1. INTRODUCTION

Small-intestinal lactase (also called lactase-phlorizin hydrolase, lactase-glycosylceramidase EC 3.2.1.23/62, or LPH) develops prior to birth and declines in most mammals at the time of weaning or shortly thereafter [1–3]. An exception is man: in about one half of mankind lactase does indeed decline in early youth (the so-called 'adult-type hypolactasia'), but in the other half, including northern Europeans, it remains at high levels in adulthood also.

The mechanism whereby lactase declines in some human populations and in other mammals is far from clear. Indeed, it is not even known whether the same mechanism(s) act in man and in other mammalian species. LPH-mRNA has been reported to remain at high levels after the decline of lactase enzymatic activity in rabbits [3], rats [4–6], and man [3]. Others have instead observed a decline in LPH-mRNA, in adult sheep [6] and also in rats [7] and man [8,9]. The rate of biosynthesis of LPH in adult rats in vivo has been reported to be reduced as compared to that in baby rats [10] or not to be reduced at all [11,12].

A potential source of these discrepancies may have been the segment(s) of the small intestine used in these studies. In fact, in adult rats, as compared to baby rats, the ratio of lactase enzymatic activity to LPH-mRNA varies widely along the small intestine [5]. Similarly, in humans with adult-type hypolactasia low levels of LPH-mRNA have been reported in biopsies stemming from duodenum (e.g. from peroral endoscopic biopsies) but high levels of LPH-mRNA are in general found in more distal segments of small intestine [3]. Another source of this discrepancy is undoubtedly the very large individual variability in the time of onset of LPH decline. For example, we have observed in the rabbit that lactase decline may in some individuals be nearly complete at 30 days [3], whereas in others lactase activity may still be high in adulthood.

We have now investigated the levels of lactase, sucrase-isomaltase (as a reference), their mRNAs, and their in vitro biosynthesis along the whole small intestine in rabbits (adult rat small intestine is notoriously difficult, if not impossible, to cultivate in vitro). In each animal the various intestinal segments would serve as the control for the others, thereby reducing or eliminating the variability related to genetic, hormonal, dietary, circadian, and perhaps other factors.

This paper addresses the following questions. (i) Does the ratio of lactase activity to LPH-mRNA change along the small intestine? (ii) Do the LPH-mRNA levels correlate with the rate of biosynthesis of LPH in organ culture? (iii) How do these observations compare in the rabbits of the two ages considered, i.e. shortly after weaning (36 days) and adult (3.5 years)? As a reference, the same questions were put for the sucrase-isomaltase complex (SI), an enzyme which in most mammals, including the rabbit, is expressed at the time of weaning [3].

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Both enzymes considered in the present study are synthesized as high molecular weight precursors, i.e. pre-prc-LPH with 1,926 amino acids [13] and pro-SI with 1,827 [14] (for the rabbit). They are proteolytically processed in different ways; however, pre-pro LPH is (most likely) first cleaved by signal peptidase in the endoplasmic reticulum; pro-LPH is then split in one or more steps to the 'final' LPH of the brush border membrane [15,16], and to one or more 'pro-sequences' of unknown cellular location(s) and biological role(s). Pro-SI instead reaches the brush border membrane intact, and is split by pancreatic protease(s) into the two homologous, but different, subunits of 'final' SI (reviewed in [17]). Both pro-LPH and pro-SI undergo the usual glycosylation steps during transit through the endoplasmic reticulum and Golgi apparatus.

2. MATERIALS AND METHODS

2.1. *Animals and preparation of whole small intestine*

New Zealand White rabbits (36 days and 3.5 years old) were fasted for 41 hours with free access to drinking water. After sacrifice (at 09.00 h) the small intestine, delimited by the pylorus and by the ileocecal junction, was excised and divided into 8 parts of equal length. A section about 1 cm long from the middle of each segment was used for organ culture. The remaining 16 pieces (named 1A and 1B, etc.) were extensively rinsed with 0.9% NaCl, frozen in liquid N_2, and then reduced to small pieces with a mortar and pestle (cooled with liquid N_2). Tissues were stored at −80°C. A sample of liver was taken as a negative control.

2.2. *Enzyme and protein assays*

Tissues were homogenised (2 min, half-maximal speed), with a Polytron mixer (Brinkmann Instruments Inc., Westbury, NY) at about 70 mg per ml homogenate buffer (0.9% NaCl, 0.1% Triton X-100, 0.5 mM PMSF, 0.5 mM *ortho*-Phenanthroline). Aliquots for total RNA determination were immediately frozen at −80°C. Protein was estimated by the method of Bradford [18]; the homogenates were then diluted to 1 mg protein per ml and immediately used for enzymatic assays and for more accurate protein determination by the method of Lowry et al. [19] as modified by Peterson et al. [20].

Total lactase activity, acid-β-galactosidase activity, and brush border lactase activity were determined as described by Villa et al. [21]. Sucrase activity was measured at 37°C in 33 mM Na-maleate buffer, pH 6.0, using 33 mM sucrose as substrate. After boiling for 2 min, the D-glucose liberated was determined with D-glucose dehydrogenase (Merck).

2.3. *RNA analysis*

Total RNA in crude homogenates was determined by the orcinol/$FeCl_3$ method, adapted from [22–24]. In short, 5 mg of homogenized tissue was diluted to 100 μl with water. Proteins and nucleic acids were precipitated by addition of an equal volume of 12% trichloroacetic acid. After centrifugation the pellet was dissolved in 100 μl of 0.2 N NaOH and incubated at 75°C for 60 min. Protein was precipitated by adding 15 μl of concentrated HCl. The supernatant was mixed with an equal volume of the orcinol reagent (1 mg $FeCl_3 \cdot 6\ H_2O$, 1 mg orcinol per ml concentrated HCl), and then boiled for 45 min in a water bath. Hydrolyzed RNA was determined at 610 nm. Yeast tRNA was used as a standard.

Total cellular RNA was purified by a urea-lithium chloride method [25]. Standard RNAs were prepared by in vitro transcription of plasmids containing LPH [13] and SI [14,26] sequences. Transcripts containing nucleotides 1–3601 and 1–713 of LPH and SI, respectively, were purified by agarose gel electrophoresis, eluted, and quantitated by UV absorption.

RNA (3 μg; standard RNA was diluted with yeast RNA) was denatured with formaldehyde/formamide [27] and applied to Gene Screen membrane (New England Nuclear) using a 'slot blot' apparatus (BioRad). Hybridization was carried out with ^{32}P-labelled [28] fragments of LPH and SI cDNA (same sequences as present in standard RNAs), with final washing in 0.2 × SSPE [27], 0.2% SDS, at 65°C. Appropriate autoradiographic exposures were quantitated by densitometry with a DeSaga CD50 chromatogram scanner.

2.4. *Organ culture and immunoisolation*

Explants were cultured as described by Browning and Trier [29] with modifications according to Naim et al. [30] and Lottaz et al. [31]. In brief, after 2 h in methionine-free medium explants were labelled continuously for 6 h with 150 μCi [^{35}S]methionine (Amersham) in 1 ml medium. After washing, tissues were solubilized by homogenization with 1% NP-40, 1% deoxycholate in the presence of protease inhibitors, cleared by centrifugation at 100,000 × g, 'precleared' by incubation with protein A-Sepharose (Pharmacia), and SI and LPH were immunoisolated using guinea pig anti-LPH and anti-SI sera bound to protein A-Sepharose, all as described in [31]. The products were analyzed by SDS-PAGE in 5% acrylamide gels [32].

2.5. *Calculations*

Amounts of standard RNA were converted to amounts of mRNA by multiplying by (length of mRNA ÷ length of standard RNA). Milliunits of enzyme activity per μg of the corresponding mRNA were calculated as $E/(R \times M)$, where E = milliunits of enzyme activity per ml homogenate, R = mg total RNA per ml homogenate, and M = μg cognate mRNA per mg total RNA.

To quantitate in vitro biosynthesis of LPH and SI, digital images of the gels produced with a PhosphorImager scanner were quantitated using Imagequant 3.2 software (both Molecular Dynamics, Sunnyvale, CA). The 'detector counts' in each band were divided by the number of methionine residues in the corresponding protein (25 for pro-LPH, about 23 for the 185–190 kDa forms, and 15 for mature LPH) to correct for size differences among the polypeptides, and the result divided by the acid-insoluble cpm in the total protein with which the immunoisolation was performed, to correct for differences in labelling efficiencies. These values are referred to as specific incorporation into LPH.

3. RESULTS AND DISCUSSION

Lactase and sucrase enzymatic activities were measured in 16 segments along the small intestine (Fig. 1A and D). Both activities peak in the mid-third of the small intestine, and are lower at the ends. This pattern is comparable to that in the rat [5], but the rabbit seems to show a steeper drop in activity in the proximal jejunum. Sucrase is higher in the adult animal and peaks in jejunum.

LPH and SI mRNAs were measured with a slot blot procedure (Fig. 2 and not shown), and by Northern blotting (not shown). Both LPH and SI mRNAs are at high levels in the proximal and mid small intestine (Fig. 1B, E), with the weight of the distribution being shifted proximally compared to the distribution of enzymatic activity. To more clearly relate mRNA levels to enzymatic activity, we proceeded as follows: total RNA was chemically quantified in the same homogenates used to measure enzyme activity, allowing expression of the specific activity as mU lactase or sucrase per mg total

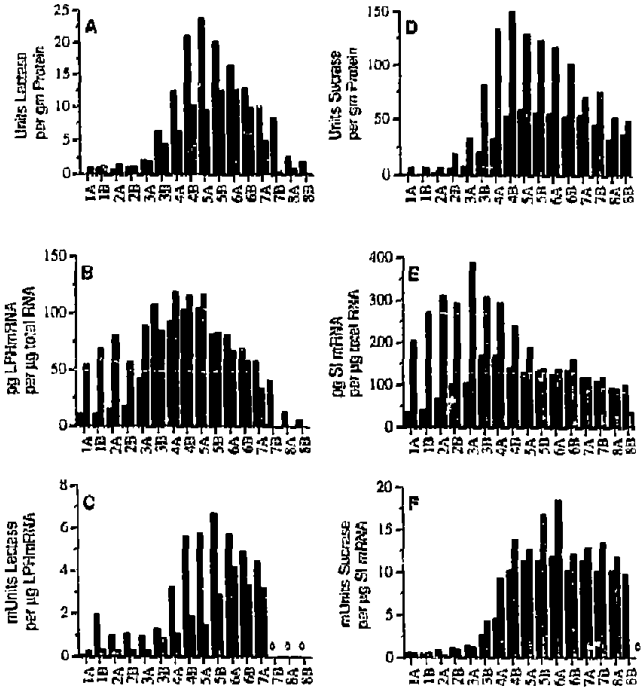

Fig. 1. LPH (A, B, C) and SI (D, E, F) enzymatic activity (lactase, A and sucrase, D), mRNA concentration (B and E) and milliunits of enzyme activity per μg of the corresponding mRNA (C and F), determined for 16 segments along the small intestine. Black bars, 36-day-old rabbit; shaded bars, 3.5-year-old rabbit. ◇, these ratios (not reported) were inaccurate, due to the small magnitudes involved.

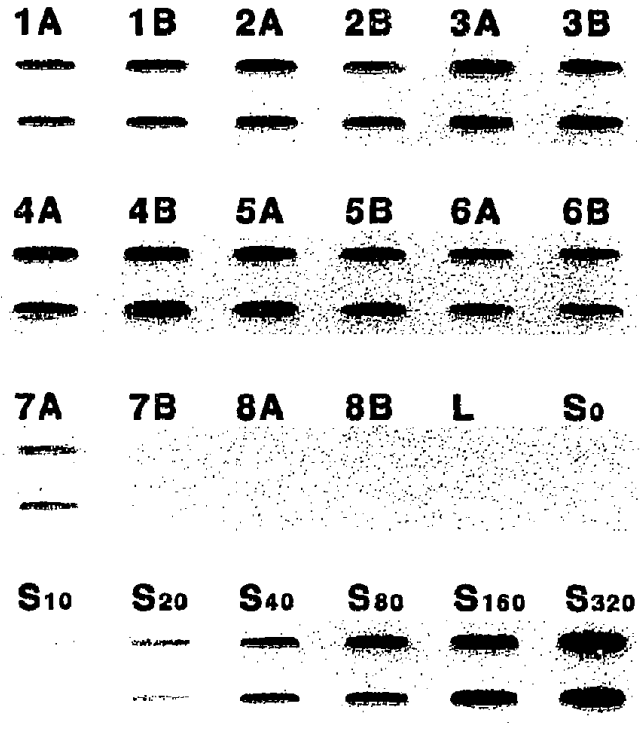

Fig. 2. Slot blot analysis of LPH-mRNA in 16 segments of small intestine from a 3.5 year old rabbit. Segment numbers as in Fig. 1. L = liver RNA. S-0 to S-320 are amounts of standard RNA corresponding to 0 to 320 pg full-length LPH-mRNA per μg total RNA.

RNA. Dividing this result by the amount of mRNA per mg total RNA, determined in the slot blot assay with in vitro-transcribed mRNA as standard (Fig. 2), we arrive at mU lactase or sucrase activity per μg LPH- or SI-mRNA, respectively (Fig. 1C and F). Possibly confounding variables originating in part from cells other than enterocytes, such as the amount of total protein (which enters into the calculation of specific activity as units per g protein) or amount of total RNA (in the calculation of pg mRNA per μg total RNA), are thus eliminated. The enzyme activity/mRNA ratios so obtained are most instructive. Disregarding for the moment the ratios in the distal ileum, which were inaccurate due to the low absolute figures involved, the main conclusions are: (i) the ratio of lactase to LPH-mRNA *differs* along the small intestine, being much lower in the proximal third of the intestine. This effect is even more pronounced in the 3.5-year compared to the 36-day-old rabbit. (ii) Unexpectedly, we find that also the ratio of sucrase to SI-mRNA is very low in the proximal half of the intestine (Fig. 1F). At both ages the ratio changes abruptly approximately at the transition between the first and the second third of the intestine.

The preceding observations strongly indicate (but do not necessarily prove) that the variations in lactase and sucrase along the intestine are due to a post-transcriptional event. (Note that the time of onset (see e.g. [3]) and the pattern of decline of lactase along the intestine are likely to vary widely among species and among individuals, which may well be one reason for the contradictions in the literature quoted in the introduction.)

In the very same small-intestinal segments used for the experiments of Figs. 1 and 2, we investigated the in vitro biosynthesis of both LPH and SI. Samples numbered 1 to 8 in Fig. 3 and 4 were located in the original tissue between the positions 1A and B, 2A and B, etc., indicated in Fig. 1. We measured the incorporation of [^{35}S]methionine into acid insoluble material ('total protein'), into immunoisolated LPH, and into immunoisolated SI, after 6 h of continuous labelling in organ culture. Fig. 3 shows an example of SDS-PAGE analysis of immunoisolated LPH.

As mentioned in the introduction, LPH is synthesized as pre-pro-LPH. Pro-LPH is 'high mannose' glycosylated to an apparent molecular weight of ca. 200 kDa, and then 'complex glycosylated' to an apparent molecular weight of ca. 220 kDa. Mature rabbit LPH has a molecular weight of 150 kDa. The 185–190 kDa forms observed in Fig. 3 (probably the same as the 180 kDa form described in [33]) may represent intermediates in the conversion of pro-LPH to LPH, or may be dead end products which are eventually degraded. In the quantitation we considered the 200 and 220 kDa products together ('pro-LPH') and the 185–190 kDa products

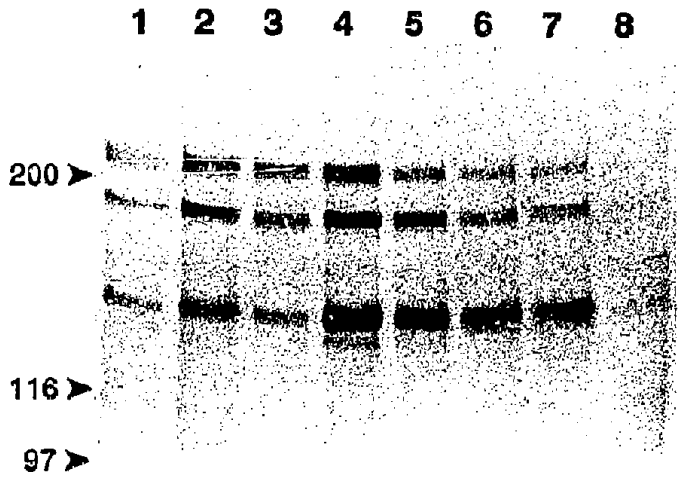

Fig. 3. SDS-PAGE analysis of LPH synthesized by explants of small intestine of a 3.5-year-old rabbit. Lanes 1 to 8 correspond to segments 1 to 8 in Fig. 1. Molecular weights of marker proteins indicated at the left.

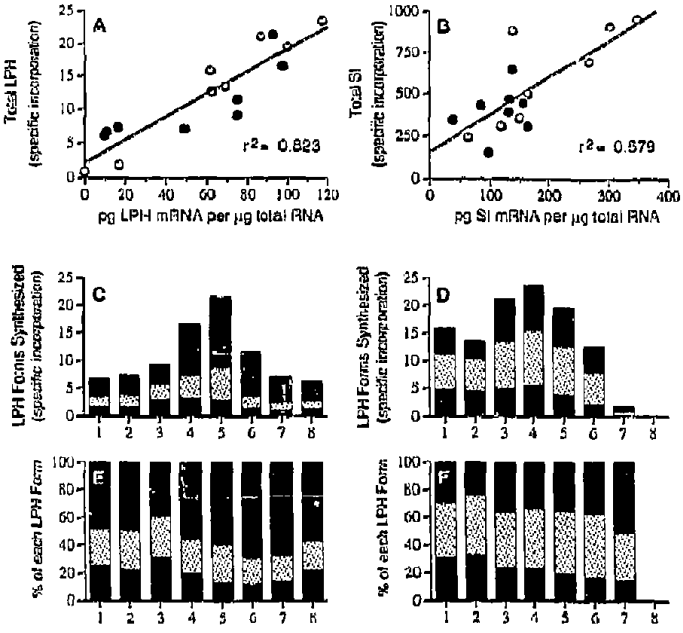

Fig. 4. Analysis of in vitro biosynthesis of LPH and SI. 'Specific incorporation' (see Materials and Methods) into all forms of LPH (A) and into pro-SI (B) was plotted as a function of the cognate mRNA. Filled circles, 36-day; open circles, 3.5-year-old rabbit. 'Specific incorporation' (C and D) into various forms of LPH (black bars, pro-LPH; dotted bars, 185–190 kDa forms; shaded bars, mature LPH), as well as the percent of each form (E and F) was calculated for segments 1 to 8 of small intestine of 36-day (C and E) and 3.5-year-old (D and F) rabbits.

together. The radioactivity incorporated was corrected as described in section 2.4.

Fig. 4A relates the in vitro biosynthesis of LPH with the levels of LPH-mRNA (calculated by averaging the values of the two neighboring segments; Fig. 1B). There is a very satisfactory correlation, r^2 being 0.82 for the pooled data, and 0.69 and 0.95 for the 36-day and 3.5-year animals, respectively. Clearly, throughout the small intestine the *biosynthesis* of pre-pro-LPH and its derivatives is regulated by the level of LPH-mRNA. Contrary to this, as already pointed out, the steady-state levels of lactase activity along the small intestine do *not* parallel the levels of LPH-mRNA (Fig. 1A, B and C). One or more post-translational events must therefore be responsible for the decline of lactase activity in the proximal third (36-day animal) or two-thirds (3.5- year animal) of the small intestine.

What is (or are) these posttranslational event(s). A non-exhaustive list of possibilities includes: (i) LPH might be degraded by luminal, pancreatic proteases, a possibility already indicated in connection with the post-weaning decline of lactase (see, for example, [34,35]). Indeed, pancreatic proteases could be detected in the luminal contents of these proximal segments of small intestine (not shown). (ii) LPH might be degraded by one or more enterocyte proteases, either of the intracellular pathway or of the brush border membrane. (iii) Two or three closely related chromosomal LPH genes occur in the rabbit (Brunschwiler, Villa, G.S. and N.M., in preparation). Possibly the gene(s) expressed in proximal small intestine are not identical with those expressed more distally. Subtle differences in messenger sequence, not detectable in filter hybridization assays, may lead to LPH enzymes of different stability and/or different susceptibility to proteolysis and/or with different targeting. (iv) It may also be that in the proximal small intestine the migration of enterocytes along the villus proceeds faster, so that the mRNAs are not given enough time to synthesize as much LPH as in the middle of the intestine.

We conclude, therefore, that both the mRNA levels and a post-translational mechanism determine the steady-state levels of lactase along the small intestine of young and adult rabbits. We have already suggested two regulatory mechanisms to operate in the biosynthesis of LPH in fetal small intestine [21].

A nuclear factor has been recently reported to interact with the promoter of the pig LPH gene; this factor occurs in higher amounts in newborn than in adult pigs [36].

As to the processing of pro-LPH to LPH, we find no significant difference in the relative amounts of the major forms of LPH synthesized along the small intestine of young or adult rabbits (Fig. 4E, F). This shows that the post-translational mechanism(s) responsible for converting pro-LPH to LPH + pro sequence is or are not identical with those leading to the decline of LPH in proximal small intestine (Fig. 1A).

A final comment on the in vitro biosynthesis of pro-sucrase-isomaltase: it also correlated with the steady-state levels of its cognate mRNA along the small intestine (Fig. 4B), although less well than lactase with its mRNA. This is germane to what we have reported on

the site of control of sucrase-isomaltase biosynthesis in vivo in fetal life (in man [37]) or during weaning (in rabbits [38]). As pointed out in a previous section, we unexpectedly find in addition a post-translational mechanism that reduces sucrase activity in the proximal third of the small intestine (Fig. 1D, E and F). Whether this mechanism(s) is(are) identical with those leading to the decline of lactase remains to be seen.

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