Processing of human pro-lactase–phlorizin hydrolase at reduced temperatures: cleavage is preceded by complex glycosylation

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Intracellular processing of human intestinal lactase–phlorizin hydrolase (LPH) includes an essential proteolytic cleavage step that generates the mature brush border enzyme from the single-chain polypeptide precursor (pro-LPH). Previous work in organ culture of small intestinal biopsy samples [Naim, Sterchi & Lentze (1987) *Biochem. J.* **241**, 427–434] has demonstrated that this cleavage occurs intracellularly. Since no intermediate forms of pro-LPH (trimmed or complex glycosylated) could be discerned in pulse–chase analyses it was suggested that the cleavage process occurs at a fast rate. To identify intermediate forms of pro-LPH prior to cleavage, I studied the biosynthesis of LPH by employing a pulse–chase protocol in mucosa explants (or biopsies) at reduced temperatures (22 °C). Here, I could identify by immunoprecipitation with monoclonal anti-LPH antibodies four LPH forms that exhibited a product–precursor relationship: mannose-rich precursor (pro-LPH_n), trimmed pro-LPH (LPH_t), complex glycosylated pro-LPH (pro-LPH_c) and cleaved, mature LPH (LPH_m). The results clearly indicate that the generation of mature LPH is preceded by complex glycosylation of the precursor form. The fact that this was not previously observed in the same experimental system under normal biosynthetic labelling conditions (37 °C) demonstrates that the cleavage process of pro-LPH occurs at a fast rate in the human small intestine.

INTRODUCTION

Lactase–phlorizin hydrolase (LPH, EC 3.2.1.22/62) is an integral glycoprotein of the small intestinal brush border membrane. It is abundant in almost all mammals and is responsible for the conversion of lactose, the main carbohydrate in mammalian milk, to its two monosaccharide components. LPH carries also a hydrolytic activity for β -glycosylceramides (Colombo *et al.*, 1973; Schlegel-Hauter *et al.*, 1972; Skovbjerg *et al.*, 1981), which is presumably located on the same polypeptide as the lactase activity (Mantei *et al.*, 1988).

LPH is synthesized as a large single-chain precursor (pro-LPH) that is cleaved to the final brush border form either intracellularly, in human and pig intestinal cells (Danielsen *et al.*, 1984; Hauri *et al.*, 1985; Naim *et al.*, 1987), or at the cell surface, in the rat small intestine (Büller *et al.*, 1987). The site of the intracellular cleavage of pro-LPH has not been localized with certainty yet, although the Golgi apparatus seems to be the potential organelle where this process occurs (Danielsen *et al.*, 1984; Naim *et al.*, 1987). The implication of this event in the function and transport of LPH has been recently investigated by expressing a full length cDNA encoding LPH in COS-1 cells (Naim *et al.*, 1991) and it was demonstrated that uncleaved pro-LPH is as transport-competent and functionally active as the brush border-cleaved LPH form.

Recent investigations in biopsy specimens of individuals with adult hypolactasia (Sterchi *et al.*, 1990; Witte *et al.*, 1990) have demonstrated that, in some cases, pro-LPH is improperly glycosylated and is not proteolytically processed to mature LPH. Moreover, the intracellular transport of pro-LPH is substantially delayed concomitant with the appearance of complex glycosylated pro-LPH molecules, which were not previously observed in the same organ culture system (Naim *et al.*, 1987). In this respect, it is noteworthy to mention that Hauri *et al.* (1985) have demonstrated that pro-LPH becomes complex-glycosylated in Caco-2 cells prior to its cleavage to mature LPH. However, the transport and processing of pro-LPH in these cells proceed at

extremely slow rates in comparison to those in normal intestinal cells. It is therefore reasonable to assume that the failure to identify intermediate forms of pro-LPH (trimmed or complex glycosylated) prior to intracellular cleavage in biosynthetically-labelled biopsy samples (Naim *et al.*, 1987) is due to a rapid cleavage of pro-LPH, which may occur in normal cells at a much faster rate than in Caco-2 cells. To investigate this issue I have studied the biosynthesis of LPH using a pulse–chase protocol of mucosa explants (biopsies) at reduced temperatures in an attempt to slow down the transport kinetics of pro-LPH and to identify processed intermediate forms of pro-LPH. I show that under these experimental conditions the proteolytic processing of pro-LPH to LPH is preceded by complex glycosylation of the precursor form.

EXPERIMENTAL

Materials

Organ tissue culture dishes (60 mm $\times$ 15 mm with centre well) were obtained from Falcon, Becton, Dickinson & Co. RPMI 1640, methionine-deficient RPMI 1640, streptomycin, penicillin, and fetal calf serum were purchased from Gibco. [³⁵S]Methionine (> 1000 Ci/mmol) and Amplify were purchased from Amersham. Acrylamide, *NN'*-methylenebisacrylamide, SDS, Tris, TEMED, ammonium persulphate, 2-mercaptoethanol, dithiothreitol and Triton X-100 were obtained from Bio-Rad laboratories. Pepstatin, leupeptin, benzamidin, aprotinin, and molecular mass standards for SDS-PAGE were purchased from Sigma. Endo- β -*N*-acetylglucosaminidase H (endo H) and phenylmethanesulphonyl fluoride (PMSF) were obtained from Boehringer-Mannheim. Protein A–Sephadex was from Pharmacia. All other reagents were of superior analytical grade.

Biological materials

The results presented in this paper are representative of three experiments. The tissue used in the organ culture studies in two of these experiments was the small intestine of kidney donors

taken directly after the respiration support system had been switched off. Each experiment was performed with tissue samples taken from a different person (kidney donor). Explants (biopsies) were taken from the upper jejunum and each sample was about 10–20 mg wet weight. In each experiment 20 explants (biopsies) were labelled in 20 different organ culture dishes. In a third experiment we used biopsy samples taken from one single volunteer. Here the biopsy samples were cut into 10 similar sized pieces that were biosynthetically-labelled in 10 dishes. Based on the results obtained with the tissue explants obtained from kidney donors and due to tissue limitation in the third experiment we omitted some chase points that had proven not to be of importance in the previous two experiments. The results in all three experiments were similar. The biopsies from both sources, i.e. kidney donors and volunteers, appeared normal when examined by light microscopy and expressed normal levels of brush-border disaccharidase activities [sucrase: 28–80 units/g and lactase: 16–49 units/g, determined according to Asp *et al.* (1975)].

Immunochemical reagents

The mouse anti-(human lactase-phlorizin hydrolase) monoclonal antibody [anti-LPH; product of hybridoma HBB 1/909/34/74 (Hauri *et al.*, 1985)] was a generous gift from Dr. Hans-Peter Hauri, Biocenter, Basel, Switzerland, and was used in the form of ascites prepared from hybridoma-bearing pristane-primed Balb/c mice. For immunoprecipitations, 2–3 μ l of the ascites fluid was added to 1 ml of 0.1 M-sodium phosphate buffer, pH 8.1, and 60 μ l of 50 % protein A–Sepharose suspension in phosphate-buffered saline. After 1 h at 4 °C, the beads were washed with phosphate buffer and used to isolate LPH.

Biosynthetic labelling of intestinal mucosa in organ culture

Biopsies or explants were washed three times in methionine-free RPMI 1640 medium supplemented with streptomycin (100 μ g/ml), penicillin (100 units/ml) and 10 % dialysed fetal calf serum (FCS) and placed on stainless-steel grids in organ culture dishes (Browning & Trier, 1969). After preincubation in the same medium at 1 ml/explant for 2 h at 22 °C in a CO₂/O₂ (1:19) incubator, the tissue was labelled with 150 μ Ci of [³⁵S]methionine for 1 h. After the labelling periods, the specimens were washed three times in RPMI 1640 medium containing an excess of unlabelled methionine and chased for different periods of time up to 24 h at 22 °C. After each time point the explants were washed in cold phosphate-buffered saline and homogenized with a Teflon/glass homogenizer in 1 ml of 25 mM-Tris/HCl (pH 8.1)/50 mM-NaCl, and a cocktail of protease inhibitors containing 1 mM-PMSF, 1 μ g of pepstatin, 5 μ g of leupeptin, 17.4 μ g of benzamide and 1 μ g of aprotinin. The homogenates were further processed for immunoprecipitation with monoclonal anti-LPH antibodies according to Naim *et al.* (1987, 1988). In another experiment biopsy samples were continuously labelled with 150 μ Ci of [³⁵S]methionine for 5 h and 8 h at 22 °C. Under these conditions the intermediate forms of pro-LPH could be adequately labelled for further analysis with endo H.

SDS/PAGE

One-dimensional gel electrophoresis was conducted in 5 % or 6 % polyacrylamide slab gels containing 0.1 % SDS according to Laemmli (1970). Immunoprecipitates and proteins subjected to endo H treatment were dissolved in 80 mM-Tris/HCl (pH 6.8), 0.1 M-dithiothreitol (DTT), 4 % SDS, 10 % glycerol and 0.01 % Bromophenol Blue (sample buffer) at 100 °C for 5 min before layering onto the gel. The following M_r standards were used: myosin, 202000; β -galactosidase, 116000; phosphorylase b, 975000; bovine serum albumin, 68000; ovalbumin, 43000. The gels were stained with Coomassie Blue, destained, and then

treated with Amplify, dried and exposed on Kodak SO-282 films at –80 °C. The fluorograms were finally scanned in a Molecular Dynamics scanner.

Other procedures

Immunoprecipitations and endo H treatment were performed according to Naim *et al.* (1987, 1988).

RESULTS

Biosynthesis of human intestinal LPH at room temperature and identification of complex glycosylated uncleaved precursor LPH forms

Tissue explants were labelled with [³⁵S]methionine for 1 h and chased for several periods of time with unlabelled methionine essentially as described by Naim *et al.* (1987, 1988), except that the labelling temperature was reduced to 22 °C. The explants were homogenized and the detergent extracts were immunoprecipitated with anti-LPH antibodies and analysed by SDS/PAGE and fluorography (Naim *et al.*, 1987). A total of 20 chase points were performed within 24 h. Fig. 1 depicts the results obtained with the most important time points. The first LPH form that could be identified in these experiments at 1 h of pulse

Fig. 1. Pulse-chase analysis of LPH at 22 °C

Small intestinal mucosa explants were pulse-labelled with [³⁵S]methionine for 1 h and chased for the indicated times at 22 °C. LPH was immunoprecipitated with a monoclonal antibody and analysed by SDS/PAGE and fluorography. Shown are results of one single experiment, which are representative of three experiments.

Fig. 2. Endoglycosidase H treatment of LPH

Biopsy samples were labelled with [³⁵S]methionine continuously for 5 h (lanes 1 and 2) and 8 h (lanes 3 and 4) at 22 °C. Under these conditions the M_r -205000 (lane 1) and the M_r -230000 (lane 3) forms become adequately labelled for further analysis with endo H (lanes 2 and 4).

Fig. 3. Densitometric scanning of the fluorograms shown in Fig. 1

(a) pro-LPH_n, (b) LPH_m, (c) pro-LPH_t, (d) pro-LPH_c.

is the mannose-rich pro-LPH polypeptide of M_r 215 000 (Fig. 1, lane 1) (Naim *et al.*, 1987). This component remained the most predominantly labelled band throughout the chase. The second LPH form could be discerned at 4 h of chase as a faint, but definite, polypeptide of M_r 205 000 (Fig. 1, lane 4). The proportion of this form in the total LPH protein increased with increasing chase times and reached a maximum at 8 h of chase (Fig. 1, lane 8 and Fig. 3, panel c). At 5 h of chase a polypeptide of M_r 230 000 appeared (Fig. 1, lane 5). The labelling intensity of the M_r -230 000 form and its proportion in the total LPH protein increased substantially to reach a maximum at 12 h of chase (Fig. 3, panel d) concomitant with decrease in the proportion of the M_r -205 000 species (Fig. 1, lane 10 and Fig. 3, panel c). The final LPH form, the M_r -160 000 form, which corresponds to the cleaved mature LPH protein (Naim *et al.*, 1987), appeared after 8 h of chase (Fig. 1, lane 8). The proportion of this polypeptide in the total LPH protein increased with prolonged chase points (Fig. 1, lanes 9–13 and Fig. 3, panel b). After 20 h of labelling the M_r -160 000 form became one of two most predominantly labelled bands with concomitant disappearance of the M_r -205 000 and the M_r -230 000 forms (Fig. 1, lane 13). Treatment of these forms with endo H, which cleaves glycan units of the high-mannose type (Tarentino & Maley, 1974) revealed that the M_r -215 000 and the M_r -205 000 forms are endo H sensitive as assessed by their conversion to the M_r -200 000 species (Fig. 2, lanes 1 and 2). The M_r -230 000 form on the other hand is endo H-resistant since no substantial shift in its apparent molecular mass was discerned upon endo H treatment (Fig. 2, lanes 3 and 4) and the M_r -160 000 form has been shown previously to bear complex type of glycans (Naim *et al.*, 1987). Altogether, the results indicate that mannose-rich pro-LPH (M_r 215 000) has been converted to the complex glycosylated pro-LPH of M_r 230 000 before the appearance of the M_r -160 000 form. For simplicity the various forms of LPH will be referred to as follows: pro-LPH_n represents the mannose-rich precursor (M_r 215 000); pro-LPH_c represents the complex glycosylated form (M_r 230 000); pro-LPH_t represents the trimmed form (M_r 205 000) and finally LPH_m is the mature brush border form of M_r 160 000.

The proportions of these forms in the total LPH protein were determined by densitometric scanning of the fluorograms and plotted versus chase times. Fig. 3 demonstrates that the proportion of pro-LPH_n decreased throughout the chase periods and remained constant (around 40%) after 14 h of chase (panel a). The proportions of pro-LPH_t (panel c) and pro-LPH_c (panel d) increased with increasing chase times and exhibited almost similar patterns of appearance, with pro-LPH_c trailing pro-LPH_t by about 2 h, indicating that pro-LPH_c is a processed form of pro-LPH_t. The proportions of pro-LPH_c and pro-LPH_t reached maximal values after 12 h of chase, before the appearance of the mature LPH form (panel b), but then declined and eventually disappeared with a concomitant increase in the proportion of LPH_m.

Collectively, these results demonstrate a precursor-product relationship between all the four LPH forms as assessed by the partial conversion of newly synthesized pro-LPH_n to pro-LPH_t and complete conversion of pro-LPH_t to pro-LPH_c and pro-LPH_c to LPH_m. The process of cleavage to mature LPH (LPH_m) is rapid and could be only detected by slowing down the transport kinetics of the molecule by performing pulse chase labelling at room temperature.

DISCUSSION

Recent analyses of precursor and processed forms of LPH in biopsy samples from individuals with adult hypolactasia have suggested that in some cases the decline in lactase activity is linked to slow intracellular transport of pro-LPH and delayed intracellular cleavage of pro-LPH to mature LPH takes place (Sterchi *et al.*, 1990; Witte *et al.*, 1990). It is important to note that in these cases the generation of mature LPH from pro-LPH is preceded by complex glycosylation of the latter, an event that has not been previously observed in intestinal biopsy samples from individuals with normal lactase activities (Naim *et al.*, 1987). Interesting is that in Caco-2 cells, in which the intracellular processing of pro-LPH occurs at a slow rate, complex glycosylated pro-LPH molecules are discerned that are ultimately

cleaved to mature brush border LPH (Hauri *et al.*, 1985). It seems therefore likely that the failure to detect complex glycosylated or intermediary forms in intestinal biopsy specimens prior to cleavage (Naim *et al.*, 1987) is conferred by the rapid rate at which mannose-rich pro-LPH is converted to complex glycosylated pro-LPH and proteolytically cleaved. In this study the transport kinetics of newly synthesized pro-LPH in individuals with normal LPH levels were slowed down by applying a pulse-chase protocol at room temperature. It has been shown that temperature variations elicit significant effects on the intracellular transport rates of glycoproteins, leading in many cases to their arrest after terminal glycosylation in the *trans*-Golgi (Matlin & Simons, 1983; Saraste & Kuismanen, 1984) or as hybrid-type glycosylated proteins between transitional elements of the endoplasmic reticulum and the *cis*-Golgi (Danielsen *et al.*, 1989). The delayed intracellular transport and processing of LPH enabled me to demonstrate unequivocally that some proportions of newly synthesized mannose-rich pro-LPH molecules undergo trimming followed by complex glycosylation and are ultimately cleaved to mature brush border LPH. Since trimmed pro-LPH (pro-LPH_t) and complex glycosylated pro-LPH (pro-LPH_c) could only be detected in tissue in which protein transport has been delayed, we conclude that post-translational modifications of pro-LPH_t after its exit from the endoplasmic reticulum, which include trimming, complex glycosylation and proteolytic cleavage, occur at a fast rate. This is consistent with recent data in which inconsiderable and variable amounts of complex glycosylated pro-LPH were observed, suggesting that this is due to a rapid processing of this form to mature LPH (Witte *et al.*, 1990). The facts that no cleaved products corresponding to mannose-rich or trimmed pro-LPH could be discerned in my pulse-chase experiments and that LPH_m appears after pro-LPH_c demonstrate that the proteolytic cleavage takes place in the *trans*-Golgi after complex glycosylation of pro-LPH has been terminated. Therefore, these results are in line with previous observations obtained in the pig small intestine (Danielsen *et al.*, 1984) and in the human colorectal cell line Caco-2 cells (Hauri *et al.*, 1985).

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