Biosynthesis and maturation of lactase–phlorizin hydrolase in the human small intestinal epithelial cells

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The biosynthesis and maturation of the human intestinal lactase-phlorizin hydrolase (LPH; EC 3.2.1.23–3.2.1.62) has been studied in cultured intestinal biopsies and mucosal explants. Short time pulse labelling revealed one high mannose intermediate of $M_r$ 215000 which was converted upon endo-$\beta$-N-acetylglucosaminidase H (endo-H) digestion to a polypeptide of $M_r$ 200000. The brush border form of LPH was revealed after longer pulse periods and has $M_r$ 160000. It possesses mainly complex oligosaccharide chains and, owing to its partial endo-H sensitivity, at least one chain of the high mannose type. Leupeptin partially inhibited the appearance of the $M_r$-160000 polypeptide. Monensin treatment of biopsies resulted in the modification of the $M_r$-160000 species to the $M_r$-140000 molecule, which was endo-H sensitive. Pulse-chase analysis indicated a slow post-translational processing of the high mannose precursor ($M_r$ 215000) to yield the mature brush-border form ($M_r$ 160000) of LPH. Our results further indicate that LPH is synthesized as a single polypeptide precursor which is intracellularly cleaved to yield the mature brush border of LPH. The data presented suggest that this cleavage occurs during the translocation of the molecule across the Golgi complex.

INTRODUCTION

A large body of information has accumulated over the past few years about the biosynthesis of membrane bound proteins. The well-studied examples with the so-called enveloped viruses, such as Semliki forest and vesicular stomatitis viruses (for a review see Garoff et al., 1982) revealed the existence of highly organized pathways for proteins to enter the cell and follow a distinct sequential route through the cellular compartments during biosynthesis. Such studies have exploited the compartmentalization of the cell to an especially fine degree, assigning, thus, a unique role to each compartment. The epithelial cells of the small intestine provide an interesting model to study the biogenesis and traffic of membrane proteins, owing to their polarized structure and the expression of a large number of membrane glycoproteins on their microvillar domain. These glycoproteins are involved in the digestion of micrornutrients in food before absorption along with vitamins and fluids via a vast absorptive surface.

A number of studies has been published on the structure and biosynthesis of the microvillar membrane proteins (for a review see Kenny & Maroux, 1982). As many of these studies have been performed in animals and most of the human intestinal brush-border hydrolases have not been purified to homogeneity, the structure, function and biosynthesis of the human small intestinal membrane hydrolases remain an important goal. With the development of the hybridoma technology (Köhler & Milstein, 1975) it became possible to produce specific antibodies, which proved to be excellent tools in isolating highly purified forms of many proteins for structural and functional analysis as well as biosynthetic studies. Recently, the production of monoclonal antibodies against the hydrolases of the human small intestinal brush-border membrane has been reported (Hauni et al., 1985).

One major glycoprotein of the brush-border membrane of the human small intestine is lactase–phlorizin hydrolase (LPH) (EC 3.2.1.23–3.2.1.62). It belongs to a group of intestinal microvillar disaccharidases which includes also sucrase–isomaltase and maltase–glucosamylase. LPH consists of two strongly associated enzymic activities with partly independent catalytic sites: lactase ($\beta$-$\alpha$-galactosidase galactohydrolase, EC 3.2.1.23), which is responsible for the hydrolysis of lactose, the major carbohydrate component of milk, and phlorizin hydrolase (glycosyl-$N$-acylsphingosine glycohydrolyase, EC 3.2.1.62) (Colombo et al., 1972; Schlegel-Haueter et al., 1972; Birkenmeier & Alpers, 1974). In the rat, lactase activity is well developed in the fetus and reaches a maximum shortly after birth, but declines markedly thereafter to a low adult level (Doell & Kretchmer, 1962; Rubinio et al., 1964). In Caucasian man, however, lactase activity usually remains high throughout life (Antonowicz & Lebenthal, 1977; Welsh et al., 1978). Congenital lactose malabsorption or adult hypolactasia have been described, which stimulated research and debate on the molecular events leading to the decrease of lactase activity (Freiburghaus et al., 1976; Sai, 1978).

Conflicting results were obtained on the structure and identification of the precursor molecules of LPH from the pig small intestine. Whereas earlier work by Danielsen et al. (1981) provided evidence that the lactase precursor is a single polypeptide subunit of $M_r$ 150000, Sjöström

Abbreviations used: LPH, lactase–phlorizin hydrolase (EC 3.2.1.23–3.2.1.62); SI, sucrase–isomalstatase (EC 3.2.1.48–3.2.1.10); APN, aminopeptidase N (EC 3.4.11.2); SDS-PAGE, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; endo-H, endo-$\beta$-$\alpha$-N-acetylglucosaminidase H; endo-F, endo-$\beta$-$\alpha$-N-acetylglucosaminidase F; PBS, phosphate-buffered saline; NP-40, Nonidet P-40; DOC, sodium deoxycholate; PMSF, phenylmethanesulphonyl fluoride; Mab, monoclonal antibody.

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et al. (1983) reported that a major polypeptide of \( M_2 \) 20000 was revealed by pulse labelling of pig intestinal explants. More recently, however, the same group claimed that the earliest detectable form of the pig LPH was an intracellular, membrane-bound polypeptide of \( M_2 \), 225000, which is intracellularly cleaved after complex glycosylation to yield the mature brush-border membrane form of LPH of \( M_2 \), 160000 (Danielsen et al., 1984). Recent results obtained by Hauri et al. (1985) using a human colorectal carcinoma cell line showed post-translational processing similar to that observed in the pig. However, these events proceeded at such a slow rate that they may not represent those in normal small intestinal epithelial cells.

The biosynthesis of the human enzyme as well as the regulation of its expression are poorly understood. We have studied the biosynthesis and maturation of LPH in organ culture of normal human small intestinal explants using a monoclonal antibody (Mab) HBB 1/909/34/74 directed against the human LPH molecule. We present data on the identity of the precursor and the sequence of events that occur upon its maturation.

**EXPERIMENTAL PROCEDURES**

**Reagents**

- \(^{1}[^{35}S]\) Methionine (> 800 Ci/mmol) and Amplify were obtained from Amersham, England; tunicamycin, phenylmethanesulphonyl fluoride (PMSF), pepstatin, aprotonin, leupeptin, benzamidine, cycloheximide and molecular weight standards for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS-PAGE) were from Sigma Chemical Co.; SDS-PAGE purity reagents were from Bio-Rad. Organ culture equipment was from Falcon, organ culture media, antibiotics (streptomycin, penicillin) and fetal calf serum from Amimed-Basel, endo-\(\beta-N\)-acetylglucosaminidases H and F from New England Nuclear, cyanogen bromide-activated Sepharose CL-4B and protein A-Sepharose from Pharmacia Fine Chemicals. Monensin (90–95\% pure) was a gift from Dr. J. Roth, Biocenter, Basel; X-Omat SO-282 films were from Kodak. All other reagents were of analytical grade.

**Biological materials**

Human intestinal biopsies (5–10 mg) were obtained for routine diagnosis by suction with a pediatric Watson capsule. They appeared normal when examined by light microscopy and expressed normal levels of brush-border disaccharidase activities. Another source for the organ culture material was the small intestine of kidney donors taken directly after the respiratory support system has been switched off. Intestinal explants were prepared by dissection of the mucosa from the proximal jejunum. These were approximately 2 mm x 5 mm in size and had normal morphology and hydrolase activities.

**Immunochromal reagents**

Monoclonal antibodies against the human brush-border membrane hydrolases were produced according to established hybridoma techniques and are published elsewhere (Hauri et al., 1985). The anti-lactase–phlorizin hydrolase HBB 1/909/34/74 was obtained in the form of ascites from pristane-primed Balb/c mice. The immunoglobulins were partially purified from the ascites fluid by

two successive precipitations with 45\% ammonium sulphate. The precipitates were dissolved in coupling buffer and covalently coupled to CNBr-activated Sepharose according to the manufacturer's instructions. An average of 3–4 mg of immunoglobulins were coupled per millilitre of Sepharose.

**Bioisynthetic labelling**

**Continuous pulse.** Biopsies were washed with RPMI 1640 medium supplemented with streptomycin (100 \(\mu\)g/ml), penicillin (100 units/ml) and 10\% fetal calf serum, and placed on stainless-steel grids in organ culture dishes essentially as described elsewhere (Browning & Trier, 1969). They were then incubated in methionine-free RPMI 1640 medium for 2 h at 37°C in a \(\text{CO}_2+\text{O}_2\) (5:95, v/v) incubator before pulsing by the addition of 200 \(\mu\)Ci of \([^{35}\text{S}]\) methionine. Continuous labelling was performed for 15 min, 4 h and 18 h. When used, monensin (1 \(\mu\)mol/ml), tunicamycin (4 \(\mu\)g/ml) and leupeptin (5 \(\mu\)g/ml) were present during the preincubations of methionine-deficient medium and during pulse labelling. After appropriate incubation times, the biopsies were washed three times in RPMI 1640 medium and further processed as indicated in the 'Immunoprecipitations' section below.

**Pulse-chase.** Mucosa was prepared for labelling as described above and pulsed for 12 min with 250 \(\mu\)Ci of \([^{35}\text{S}]\) methionine. Samples were then extensively washed with methionine-containing medium and incubated for 2 min with RPMI medium containing cycloheximide (0.1 mg/ml). Samples were chased for various times with RPMI medium containing 2.5 mm-methionine. The explants were finally washed and prepared for immunoprecipitation.

**Immunoprecipitations**

The labelled biopsies or mucosal explants were homogenized with a Teflon–glass homogenizer in 1 ml of 25 mm-Tris–HCl, pH 8.1, supplemented with 50 mm-NaCl, 1 mm-PMSF, pepstatin (1 \(\mu\)g/ml), leupeptin (5 \(\mu\)g/ml), benzamidine (17.4 \(\mu\)g/ml) and aprotonin (1 \(\mu\)g/ml). The nuclei and debris were removed by centrifugation at 1000 \(g\) for 30 min. The supernatant was recovered and solubilized with Nonidet P-40 (NP-40) and sodium deoxycholate (DOC), 0.5\% final concentrations, by stirring on ice for 30 min. The solubilized material was either filtered through a 0.22 \(\mu\)m filter or spun at 100000 \(g\) for 1 h at 4°C.

In some experiments, microvillar and intracellular membranes were prepared as described by Schmitz et al. (1973).

The solubilized membranes were precleared twice by treatment with 35 \(\mu\)l of protein A-Sepharose beads, and transferred to tubes containing 40 \(\mu\)l of anti-LPH-Sepharose. After an incubation period of 1 h at 4°C, the beads were washed four times with phosphate-buffered saline containing 0.5\% NP-40, 0.05\% DOC and 0.01\% SDS and the protease-inhibitor cocktail mentioned above, and then twice with 125 mm-Tris–HCl, 500 mm-NaCl, 1 mm-EDTA, 0.5\% NP-40, pH 8.1.

**Endo-\(\beta-N\)-acetylglucosaminidase H treatment**

Digestion of immunoprecipitates with endo-\(\beta-N\)-acetylglucosaminidase H (endo-H) was performed essentially by the method described by Owen et al. (1981). In
brief, immunoprecipitated proteins were eluted from Sepharose beads by boiling in 0.1 M-Tris—HCl, pH 7.5, 1% SDS and 1% 2-mercaptoethanol for 4 min. This solution was diluted with 9 vol. of 0.15 M-sodium citrate buffer, pH 5.5, containing 4 mM-PMSF and 4 mM-i.u. of endo-H and incubated for 16 h at 37 °C. Proteins were recovered by precipitation with an equal volume of 30% (w/v) trichloroacetic acid and the pellet was washed twice with acetone and kept at −20 °C.

**Endo-β-N-acetylglucosaminidase F treatment**

The digestion of proteins by endo-β-N-acetylglucosaminidase F (endo-F) was carried out as follows. The immunoprecipitates were eluted from the Sepharose beads as described above for endo-H digestion and then diluted with 9 vol. of 0.1 M-sodium phosphate buffer, pH 6.1, containing 50 mM-EDTA, 1% NP-40, 1% 2-mercaptoethanol and 4 mM-PMSF, and incubated with 1.25 units of endo-F for 18 h at 37 °C. Proteins were further processed as described for the endo-H treatments.

**Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis**

Immunoprecipitates and proteins subjected to enzymic digestions with endo-H and endo-F were solubilized with 50 µl of 2-fold concentrated electrophoresis sample buffer, 10 µl of 20% SDS, 10 µl of 0.1 M-dithiothreitol, boiled for 4 min and submitted to electrophoresis on 5% acrylamide gels according to Laemmli (1970). The molecular weight standards were: myosin (202,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000) and ovalbumin (45,000). 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 films were developed in a 3M XP507 X-ray processor and scanned in a CAMAG TLC scanner-2.

**RESULTS**

**Identification of the molecular species recognized by the monoclonal antibody**

Microvillar and intracellular membranes were prepared from human intestinal biopsies metabolically labelled with [35S]methionine for 4 h. Both fractions were solubilized in NP-40 and DOC and subjected to immunoprecipitation with the Mab HBB 1/909/34/74. As shown in Fig. 1, one main band was obtained (Mr 160,000) when microvillar membranes were precipitated. In addition, a faint band of Mr 215,000 can also be identified (Fig. 1, lane b). In the intracellular membrane fraction, however, both bands are revealed and their relative labelling intensity was similar (Fig. 1, lane a). This result indicates that (i) the antibody HBB 1/909/34/74 recognizes epitopes present on both polypeptides and (ii) the brush-border membrane form of the LPH molecule is predominantly represented by the Mr 160,000 species.

**Fig. 1. Identification of lactase–phlorizin hydrolase (LPH) by the monoclonal HBB 1/909/34/74 in the intracellular and brush-border membranes**

Biopsies were pulse-labelled for 4 h with [35S]methionine. The biopsies were subsequently homogenized and intracellular and brush-border membrane fractions were prepared by the method of Schmitz et al. (1973). The LPH molecule was immunoprecipitated with the monoclonal antibody HBB 1/909/34/74. The immunoprecipitates were resolved by electrophoresis (Laemmli, 1970) on 5% gels, followed by fluorography. Exposure time: 3 days. (a) LPH purified from intracellular membranes; (b) LPH purified from brush-border membranes.

**Fig. 2. Endo-β-N-acetylglucosaminidase H and F treatment of lactase–phlorizin hydrolase (LPH)**

Biopsies were pulse-labelled for 15 min and 4 h with [35S]methionine in methionine-free RPMI 1640 medium. After homogenization and solubilization, LPH was purified by monoclonal antibody HBB 1/909/34/74. The immunoprecipitates were treated with endo-H (4 m-i.u.), or, endo-F (1.24 units) or not treated. The samples were further analysed by SDS-PAGE electrophoresis on 5% gels and processed for fluorography. Exposure time: 3 days. (a–c) Biopsies pulse-labelled for 15 min; (d–f) biopsies pulse-labelled for 4 h; (b,e) endo-H-treated immunoprecipitates; (c,f) endo-F-treated immunoprecipitates (a,d) no treatment.
Identification and size of the precursor polypeptides of the LPH molecule

In order to determine precisely the size of the precursor forms of LPH, biopsies were pulsed with \[^{35}S\]methionine for 15 min and 4 h. The homogenized biopsies were solubilized with NP-40 and DOC and immunoprecipitated with anti-LPH. The precipitates were either subjected to electrophoresis after treatment with endo-H, which cleaves carbohydrates of the high mannose type (Tarentino & Maley, 1974), or with endo-F, which hydrolyses both the high mannose glycan units and complex carbohydrates (Elder & Alexander, 1982), or without enzymic treatments. Fig. 2 shows results typical for such an experiment (see also Table 1). After a pulse period of 15 min one single polypeptide of \(M_r = 215000\) was revealed (Fig. 2, lane a). This polypeptide was endo-H sensitive, since it was converted to a band of \(M_r = 200000\) upon digestion with endo-H (Fig. 2, lane b). A similar molecular species was revealed upon endo-F treatment (Fig. 2, lane c). An additional, rather diffuse polypeptide appears of \(M_r = 160000\) when biopsies were pulsed for 4 consecutive hours with \[^{35}S\]methionine (Fig. 2, lane d). This molecule was transformed to a band of \(M_r = 155000\) upon endo-H treatment (Fig. 2, lane e). However, digestion of LPH precipitated from 4 h labelled biopsies with endo-F, generated a polypeptide of \(M_r = 200000\) corresponding to the \(M_r = 215000\) species and a \(M_r = 125000\) band corresponding to the \(M_r = 160000\) species (Fig. 2, lane f). Owing to similar sensitivities to both endo-H and endo-F treatments, this result implies that the \(M_r = 215000\) carries \(N\)-linked carbohydrates exclusively of the high mannose type and represents therefore the high mannose precursor form of LPH. The \(M_r = 160000\), on the other hand, carries mainly complex carbohydrates in addition to at least one chain of the high mannose type. This result, together with the predominant expression of the \(M_r = 160000\) in the microvillar membrane, indicates that the \(M_r = 160000\) corresponds to a mature LPH form. No significant changes in the glycosylation pattern were obtained by longer pulse periods (not shown). Similar electrophoretic patterns were revealed when the immunoprecipitates were run on SDS gels under non-reducing conditions (not shown). The size of the non-glycosylated LPH-precursor polypeptide was determined by labelling biopsies with \[^{35}S\]methionine in the presence of tunicamycin, which inhibits \(N\)-linked glycosylation of proteins (for review see Yamada and Olden, 1978). LPH purified from these biopsies was revealed as a polypeptide of \(M_r = 200000\) after 15 min of pulse (Fig. 3, lane a), which is equivalent to the endo-H and endo-F treated forms of the \(M_r = 215000\) band (Fig. 2, lanes b, c) (see also Table 1). The same molecule was obtained when LPH was immunoprecipitated from 60 min pulsed biopsy (Fig. 3, lanes b or c). We were unable to detect the LPH molecule upon prolonged pulse periods (2–4 h) in the presence of tunicamycin, presumably due to the reported inhibitory effect on protein synthesis (Mahoney & Duksin, 1979) or to enhanced degradation or proteolysis of the non-glycosylated protein. Such a processing has been reported for fibronectin in chick embryo fibroblasts cultures grown in the presence of tunicamycin (Olden et al., 1978).

**Fig. 3. Effect of tunicamycin on pulse-labelling of biopsies**

Biopsies were pulse-labelled in the presence of tunicamycin (4 \(\mu g/ml\) for 15 min (lane a) and 60 min (lanes b, c)) with \[^{35}S\]methionine. Lactase–phlorizin hydrolase (LPH) was purified by immunoprecipitation and further analysed by electrophoresis and fluorography. Exposure time: 3 days (a, b) or 12 days (c).

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<th>Table 1. Lactase–phlorizin hydrolase (LPH) forms</th>
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<td>High mannose</td>
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<td>Complex glycosylated</td>
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<tr>
<td>Endo-H-treated</td>
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<td>155000</td>
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<tr>
<td>Endo-F-treated</td>
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<tr>
<td>Tunicamycin</td>
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<td>Not observed</td>
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<tr>
<td>Monensin</td>
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<td>Endo-H-treated monensin forms</td>
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<td>Ca(^{2+})-precipitated membranes</td>
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<tr>
<td>Brush-border membranes</td>
<td>Faint 215,000</td>
<td>160000</td>
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In order to investigate the processing and subsequent maturation of LPH, pulse-chase experiments with \[^{35}S\]methionine combined with endo-\(\beta\)-\(N\)-acetylglucosaminidase H treatments on mucosa explants were performed. The selectivity of endo-H in hydrolysing only Asn-linked carbohydrates of the high mannose type provides a useful tool in localizing glycoproteins to the rough endoplasmic reticulum before they are translocated across the Golgi, where they acquire complex types of carbohydrates. In this case they become endo-H insensitive (Strous & Lodish, 1980). LPH was isolated by immunoprecipitation and analysed on SDS-PAGE with or without endo-H treatment. The high mannose form of LPH was detectable after 12 min pulse (Fig. 4a, lane a). Endo-H digestion generated the previously described precursor polypeptide of \(M_r = 200000\) (Fig. 4a, lane b).
The much diminished endo-H sensitivity of the $M_r$-160000 species (a shift of $M_r$ 5000 in comparison with $M_r$ 35000 upon endo-F treatment) indicates that the $M_r$-160000 molecule has crossed the Golgi apparatus and has acquired carbohydrates of the complex type.

**Effect of monensin on the maturation of LPH**

Monensin is a carboxylic ionophore which interferes with post-translational processing and inhibits complex glycosylation of membrane and secretory proteins in the Golgi complex (Tartakoff, 1983). We have investigated the effect of this reagent on the appearance of the brush-border forms of LPH by labelling biopsies with [³⁵S]methionine for 15 min and 4 h in the presence of monensin. Fig. 5 shows that the mobility of the $M_r$-215000 polypeptide was not affected by monensin (lane a). After 4 h of pulse two polypeptides were identified, with estimated apparent $M_r$ values of 215000 and 140000 (Fig. 5, lane c). The latter corresponds to the $M_r$-160000 species in control biopsies without monensin and is endo-H sensitive, being transformed upon such treatment to a polypeptide of $M_r$ 125000 (Fig. 5, lane d), in contrast with the $M_r$ 160000, which is only partially endo-H sensitive (see above). The $M_r$ 215000 showed the same sensitivity to digestion with endo-H in both monensin-treated or non-treated biopsies (Fig. 5, lanes b,d) (see also Table 1). This result indicates that the $M_r$-140000 species carries glycan units of the high mannose type, which means that the high $M_r$ precursor must have been proteolytically processed before complex glycosylation in the Golgi apparatus has occurred.

**Fig. 4.** (a) Pulse-chase analysis of lactase–phlorizin hydrolase (LPH) and (b) densitometric scan of track k in Fig. 4(a)

Mucosal slices were pulse-labelled for 12 min with [³⁵S]methionine and subsequently chased for the indicated time points with 2.5 mM unlabelled methionine. LPH was purified by immunoprecipitation as described before and each sample divided into two parts, one of which was treated with endo-H (4 m-i.u.). The treated and non-treated samples were further analysed by electrophoresis and fluorography. Exposure time: 3 days.

This pattern was essentially the same after chase periods of up to 90 min (Fig. 4a, lanes c,e,g), with similar endo-H cleavage pattern (Fig. 4a, lanes d,f,h). This indicates that the LPH molecule is still located in the rough endoplasmic reticulum.

After a chase period of 3 h, the $M_r$-160000 species, i.e. microvillar membrane form of LPH, starts to appear (Fig. 4a, lane i). Upon longer chases, this molecule becomes the predominant species with a concomitant decrease in the labelling intensity of the $M_r$-215000 band (Fig. 4a, lane k). In fact, densitometric scanning of track k (Fig. 4b) revealed a 3-fold labelling intensity of the $M_r$-160000 compared with the $M_r$-215000. This implies that a large proportion of the high mannose precursor molecule has been transformed by cellular cleavage to the mature microvillar membrane species. However, no complete disappearance of the $M_r$ 215000 was achieved even after 18 h of chase (not shown).

**Fig. 5.** Effect of monensin on the maturation of lactase–phlorizin hydrolase (LPH)

Biopsies were pulse-labelled for 15 min (a,b) and 4 h (c,d) with [³⁵S]methionine in the presence of monensin (1 µM). LPH was purified by immunoprecipitation and each sample divided into two parts, one of which was treated with 4 m-i.u. of endo-H (b,d). Treated and non-treated immunoprecipitates were analysed by SDS-PAGE and fluorography. Exposure time: 3 days.
Effect of leupeptin on the post-translational processing of LPH

Leupeptin (acetyl-L-leucyl-L-leucyl-L-arginyl) of bacterial origin is an inhibitor of many serine and thiol proteases (Umezawa, 1976). We investigated its effect on the possible cleavage of the high mannose precursor by labelling biopsies for 15 min and 4 h in culture medium to which leupeptin was added. The LPH molecule immunoprecipitated from such biopsies revealed reduced amounts of the Mr-160000 microvillar membrane form (Fig. 6a, lane a), as compared with non-treated biopsies (Fig. 6a, lane c). Densitometric scans of LPH precipitated from the two types revealed similar labelling intensities of the Mr-215000 and Mr-160000 bands in the presence of leupeptin (50%; 49%) (Fig. 6b). In the absence of leupeptin, 2.3-fold more labelling of the Mr-160000 in comparison with the Mr-215000 was obtained (Fig. 6b). The susceptibility to endo-H of the Mr-215000 and the Mr-160000 isolated from leupeptin-treated biopsies was similar to that of their counterparts from biopsies cultivated in the absence of leupeptin (Fig. 6a, lanes b,d). Therefore, leupeptin did not interfere with other post-translational events such as glycosylation. Taken together, these results indicate that leupeptin partially inhibited the cleavage of the high mannose precursor under the experimental conditions followed, providing further evidence for an enzymic proteolysis of the LPH precursor.

DISCUSSION

The present study has focused on the identification of the subunit structure and precursor forms of the lactase–phlorizin hydrolase (LPH) molecule from the human small intestine and the determination of the sequence of events that occur upon its maturation. Such a study was made possible by production of a monoclonal antibody (Mab) HBB 1/909/34/74 (Hauri et al., 1985), which recognized both precursor and mature forms of the enzyme.

The purification of LPH resulted in the identification of two polypeptides of apparent Mr values 215000 and 160000 (Fig. 1). These two species are not linked by disulphide bridges, since no effect of reducing agents on the electrophoretic pattern has been observed (not shown). Both molecules are glycosylated as determined by endo-H and endo-F treatments (Fig. 2). The Mr-215000 species carries exclusively glycan units of the high mannose type and corresponds to the high mannose precursor of LPH whereas the Mr-160000 species is the complex glycosylated, brush-border membrane form of the molecule. The significant difference in the size of the carbohydrate moiety between the Mr-160000 (Mr, 35000) and the Mr-215000 (Mr, 15000) species may be due to an overestimation in the molecular weight of the Mr-160000 as a result of interference of sialic acid residues with SDS binding (Andersson & Gahmberg, 1978). In addition glycan chains of the complex type are somewhat heavier than those of the high mannose type.

The Mr-160000 is derived from the Mr-215000 species by intracellular proteolytic cleavage, since (1) at early pulse periods the only LPH component which could be detected is of Mr-215000 (Fig. 2); (2) treatment of biopsies with tunicamycin resulted in the identification of a single polypeptide, which has Mr similar to that of the deglycosylated endo-H form of the Mr-215000 species (Fig. 3); (3) pulse chase analysis indicated a time-dependent appearance of the Mr-160000 with a concomitant decrease in the labelling intensity of the Mr-215000 (Fig. 4); (4) leupeptin inhibited to a great extent the

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Fig. 6. (a) Effect of leupeptin on the maturation of lactase–phlorizin hydrolase (LPH) and (b) densitometric scans of tracks a (+ leupeptin) and c (−leupeptin) of Fig. 6(a)

Biopsies were pulse-labelled for 4 h with [35S]methionine in the presence (a,b) or absence (c,d) of leupeptin (5 μg/ml). LPH was isolated by immunoprecipitation and subjected to SDS-PAGE electrophoresis after endo-H treatment (b,d) or without treatment (a,c). Exposure time: 3 days.
appearance of the $M_r$-160000 (Fig. 6); (5) monensin, whose site of action is the Golgi membranes, modified the $M_r$-160000 to $M_r$-140000 species (Fig. 5). Furthermore, the biosynthetic studies were performed in organ culture, thus excluding the effect of external factors such as pancreatic secretion. Such a cleavage is in contrast with the extracellular splitting reported for another major disaccharidase of the brush-border membrane, the sucrose–isomaltase complex (SI) (EC 3.2.1.48–3.2.1.10) (Hauri et al., 1979; Sjöström et al., 1980). In this case, the polypeptide precursor, pro-SI, after acquiring complex carbohydrates is inserted into the membrane and cleaved by pancreatic secretions to yield the two subunits, sucrase and isomaltase. Our observation is that sucrase–isomaltase is not proteolytically cleaved to its two subunits in the organ culture system, indicating that no pancreatic proteases adhered to the mucosal surface of the cultured biopsies (Naim et al., 1986). In fact, SI purified from long pulse-labelled biopsies is revealed by SDS-PAGE analysis as a molecular species of $M_r$ 245000. This polypeptide corresponds to the pro-SI as recently shown by Hauri et al. (1985) in an epithelial colon cancer cell line. It is therefore unlikely that the expression of the $M_r$-160000 polypeptide in the brush-border membrane is the result of artificial proteolysis during our experimental procedures.

It is not clear why not all the high mannosic precursor pool, in the pulse-chase experiment, has been converted into mature LPH forms. Similar results were obtained on the maturation of human SI (unpublished work). In contrast, in the same experiment, another hydrolase, aminopeptidase N (APN) (EC 3.4.11.2) lost all its high mannosic precursor molecules during the acquisition of complex carbohydrates. Explanations for these differences are so far not available.

The proteolytic cleavage of the LPH precursor molecule apparently occurs at a fast rate. This may explain why neither a complex glycosylated precursor form nor a cleaved endo-H-sensitive form has been detected during the pulse-chase experiments. In fact, the precursor molecule was always found in an endo-H-sensitive form and the $M_r$-160000 species, which appeared after 3 h of chase, was only partially endo-H-sensitive. Such a cleavage could also be generated in vitro by homogenization of short pulse biopsies in buffer not containing leupeptin. In such a case, two polypeptides can be detected, of $M_r$ 215000 and $M_r$ 140000, which are both endo-H sensitive (unpublished work).

Furthermore, the results suggest that the proteolytic cleavage takes place in the Golgi membranes. In monensin-treated biopsies only the carbohydrate portion of the $M_r$-160000 species has been modified. Since no such modified molecules could be detected in the brush-border membrane (not shown) it can be assumed that monensin has affected the further transport of LPH, and consequently the proteolytic cleavage has occurred at the site where monensin exerts its function, which is the Golgi apparatus. Such a mechanism has been reported for other proteins as well as pro-polypeptide hormones, which are known to be processed either in the Golgi apparatus or in secretory granules (Dockerty & Steiner, 1982; Hedo et al., 1983). It is unlikely, though, that it cannot be excluded, that a proteolytic cleavage has occurred in the endoplasmic reticulum as that reported for $\gamma$-glutamyltranspeptidase (EC 2.3.2.2) in hepatoma tissue culture cells (Barouki et al., 1984), and for many other proteins (Hanley et al., 1983; Chow et al., 1983). However, if that were the case then endo-H-sensitive cleaved forms should have been detected before the mature $M_r$-160000 species has appeared and they were not. Recently, the biosynthesis of the LPH molecule has been investigated in CaCo-2 cells (Hauri et al., 1985), a cell line derived from a colon adenocarcinoma, and in the pig small intestine (Danielsen et al., 1984). Both studies suggested an intracellular cleavage of the precursor after complex glycosylation, leading to the formation of the brush-border form of LPH. We were unable to detect complex glycosylated precursor before the appearance of the $M_r$-160000 in our system. LPH processing in CaCo-2 cells may be different from that in normal small intestinal epithelial cells. The reported terminal glycosylation of the precursor and its consequent cleavage to the brush-border form proceeded at such a slow rate (8–16 h) that it is difficult to correlate these findings with those for normal cells. It is also possible that the distribution of the protease responsible for the cleavage process in CaCo-2 cells differs from that in normal epithelial cells.

Although Danielsen et al. (1984) suggested a complex glycosylation of the precursor before cleavage, they were not able to monitor the appearance of the complex glycosylated form of LPH precursor in their pulse-chase experiments. This supports our hypothesis that the cleavage process occurs at such a fast rate that no intermediate glycosylated precursor forms or endo-H-sensitive cleaved products could be observed. It is also possible that molecules with similar specificities in different species have several differences in their structural features and in their post-translational processing. Evidently many secretory and membrane glycoproteins follow different routes in their proteolytic processing, which could be localized in different compartments of the cell such as the endoplasmic reticulum, the Golgi apparatus or at the cell surface after maturation of the precursor molecule. The glycosylation, however, is apparently similar in all these cases and includes two main steps, the co-translational acquisition of glycan units of the high mannosic type at the endoplasmic reticulum and trimming and complex glycosylation in the Golgi apparatus. Though these processes are the same, the rates differ considerably from one protein to the other. Indeed, several authors have shown that proteins are transferred from the endoplasmic reticulum to the Golgi at characteristic rates (Fitting & Kabat, 1982; Ledfort & Davis, 1983; Lodish et al., 1983; Hauri et al., 1985), indicating thus that a certain degree of specificity in the post-translational processing of proteins must exist.

In conclusion, the LPH molecule is synthesized as a single precursor polypeptide which undergoes proteolytic cleavage and complex glycosylation in the Golgi apparatus followed by insertion of the $M_r$-160000 into the microvillar membrane. Our results do not resolve the question whether the high-mannose precursor molecule is cleaved into two identical subunits (based on the size of the deglycosylated precursor, $M_r$ 140000, and the mature, $M_r$-125000) or whether one single polypeptide is generated per precursor molecule in addition to a species of lower $M_r$. Although, in all the experiments performed, no such species could be detected by using Mab HBB 1/909/34/74, its generation must be excluded. One possibility to detect such a
species would be to raise polyclonal antibodies against the precursor molecule of LPH. In addition, detailed peptide mapping analyses and amino acid sequencing of purified components will be required to determine the precise structural relationship between the high mannose precursor molecule and its mature product. Furthermore, the precise localization of the cleavage site and the determination of the size of the resulting molecules are still questions which need to be resolved.

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