Further studies of glycosylation and intracellular transport of lactase–phlorizin hydrolase in rat small intestine

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Previous studies [Büller, Montgomery, Sasak & Grand (1987) *J. Biol. Chem.* **262**, 17206–17211] have demonstrated that lactase–phlorizin hydrolase is inserted into the microvillus membrane (MVM) as a large precursor of approx. 220 kDa, which then undergoes two proteolytic cleavage steps to become the 130 kDa mature MVM protein. In order to assess the role of glycosylation in intracellular transport, the processing of this enzyme has been studied in the presence of castanospermine, an inhibitor of *N*-linked oligosaccharide modification and subsequent treatment with two endoglycosidases, endo- β -*N*-acetylglucosaminidase (endo-H) and peptide:*N*-glycosidase-F (*N*-glycanase). We now show that the intracellular precursor (205 kDa) undergoes carbohydrate processing (220 kDa) and transport to the MVM where its further proteolytic cleavage is as described. Treatment of the intracellular 205 kDa precursor with either endo-H which cleaves only high-mannose *N*-linked oligosaccharides, or with *N*-glycanase, which cleaves both high-mannose and complex *N*-linked oligosaccharides, results in the conversion of the 205 kDa protein band to one of 195 kDa. These data suggest that the 205 kDa precursor contains only high-mannose *N*-linked carbohydrates, and that the unglycosylated nascent protein is 195 kDa. In the presence of castanospermine, an intracellular precursor of approx. 210 kDa is observed. When treated with endo-H or *N*-glycanase, this form also produces a protein of 195 kDa. The transport of the intracellular precursor to the MVM and further proteolytic processing is not blocked by the inhibitor. However, all MVM forms of lactase–phlorizin hydrolase show an increase of approx. 5 kDa. Treatment of these three MVM forms with endo-H indicates the increased presence of high mannose oligosaccharides in comparison with non-castanospermine-treated forms. The susceptibility to endo-H of the 130 kDa MVM band synthesized in the absence of castanospermine implies the presence of high-mannose *N*-linked oligosaccharides in the mature form of lactase–phlorizin hydrolase. Incubation of these MVM forms with *N*-glycanase further reduces their electrophoretic mobility, indicating the presence of complex *N*-linked oligosaccharides in the MVM forms, in contrast with the intracellular precursor. Altered glycosylation reduces but does not abolish intracellular transport of lactase–phlorizin hydrolase to the MVM.

INTRODUCTION

In recent years, advances have been made in the knowledge of the biosynthesis, processing, sorting and transport of several membrane-bound intestinal glycoproteins [1–4]. These, like most lysosomal and secretory glycoproteins, are synthesized on ribosomes attached to the endoplasmic reticulum (ER) and are co-translationally inserted into the lumen of the ER [5–7]. During this process, *N*-glycosylation begins with the transfer of one or more molecules of the lipid-linked oligosaccharide (Glc)₃(Man)₉(GlcNAc)₂ to asparagine residue(s) of the nascent protein [8,9]. These high-mannose oligosaccharides are then modified by a series of glycosidases and glycosyltransferases located in the ER and Golgi complex [6,8,9]. Further *O*-linked glycosylation may take place in the Golgi complex. The role these sugars may play in the sorting of membrane-bound glycoproteins is unclear [2,3]. Mannose 6-

phosphate in the *N*-linked oligosaccharide of lysosomal enzymes serves as a recognition marker for delivery of the enzymes to lysosomes [5,6,10]. If addition of *N*-linked sugars is blocked or the initial trimming of high-mannose oligosaccharide chains is inhibited, some glycoproteins fail to be secreted or expressed on the cell surface [9–12].

It therefore seems appropriate to investigate whether castanospermine, by its ability to inhibit the processing of oligosaccharide chains, has any effects on the transport and processing of lactase–phlorizin hydrolase (EC 3.2.1.23–3.2.1.62), and to use this agent in combination with deglycosylating enzymes to elucidate glycosylation steps that are involved in the biosynthesis of lactase–phlorizin hydrolase. In the present study, we show the presence of high-mannose and complex-type oligosaccharides in lactase–phlorizin hydrolase; no previous study has identified a complex glycosylated precursor of this enzyme [2].

Abbreviations used: MVM, microvillus membrane; ER, endoplasmic reticulum; endo-H, endo- β -*N*-acetylglucosaminidase-H; *N*-glycanase, peptide:*N*-glycosidase-F; PAGE, polyacrylamide-gel electrophoresis; TCA, trichloroacetic acid; PMSF, phenylmethanesulphonyl fluoride.

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EXPERIMENTAL

Animals

Sprague-Dawley CD rats were purchased from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. All animals were housed in a constant temperature and humidity environment on a 12 h light-dark cycle. Animals were allowed to suckle *ad libitum*, and the dams were fed standard laboratory chow and water *ad libitum*. The times for all animal procedures were kept constant throughout the study.

Materials

Cell-labelling grade [³⁵S]methionine (> 670.8 Ci/mmol), and En³Hance were from Du Pont-New England Nuclear. Methionine-free Dulbecco's modified Eagle's medium was from Irvine Scientific, Santa Ana, CA, U.S.A. Castanospermine was a gift from Dr. Alan Elbein, University of Texas Health Science Center, San Antonio, TX, U.S.A., and was as previously used in our laboratory [12]. Castanospermine, a plant alkaloid, is a potent inhibitor of glycoprotein processing [9] by virtue of blocking the removal of glucose residues from newly synthesized oligosaccharide chains. Endoglycosidase H (endo- β -N-acetylglucosaminidase-H) and N-glycanase (peptide:N-glycosidase F) were purchased from Genzyme, Boston, MA, U.S.A.

Antibodies

The monoclonal antibody [13] used in these experiments is the same as described previously [14]. The hybridoma cells (YBB 2/61) were generously given by Dr. Andrea Quaroni, Cornell University, Ithaca, NY, U.S.A. The antibody was obtained in the form of ascites from pristane-primed Balb/c mice. The immunoglobulins (IgG₁) were purified from the ascites fluid by affinity chromatography on a Protein A-CL Sepharose 4B (Pharmacia) column [15], and covalently coupled to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions.

Organ culture

The organ culture of jejunal explants from 5-day-old rats was performed according to the previously described method [14]. All explants were preincubated for 30 min in methionine-free medium containing penicillin (100 units/ml) and streptomycin (100 μ g/ml). Labelling was performed during 45 min in [³⁵S]methionine (500 μ Ci/ml)-containing medium. After thorough washing with phosphate-buffered saline, explants were incubated in complete Dulbecco's modified Eagle's medium (containing 460 times the concentration of radioactive methionine) for 180 min. No proteinase inhibitors were added to the media.

When used, castanospermine (50 μ g/ml) was present during the preincubation in methionine-deficient medium and during pulse-chase labelling. Every hour, castanospermine was freshly added to the chase medium, because our preliminary results had shown a decreasing effect of the drug after approx. 1 h.

Fractionation of labelled explants

Labelled explants were separated into intracellular membrane (ER/Golgi) and MVM fractions using the Ca²⁺ precipitation technique as previously described [14]. Lactase activities were measured in the presence

of *p*-chloromercuribenzoate to inhibit lysosomal β -galactosidase activity [16]. The lactase assay was performed as described previously [14]. All buffers used during fractionation contained proteinase inhibitors at the following concentrations: leupeptin (50 μ g/ml), phenylmethanesulphonyl fluoride (PMSF; 1 mM) and soybean trypsin inhibitor (50 μ g/ml). Protein synthesis was determined as trichloroacetic acid (TCA)-precipitable radioactivity in 25 μ l samples of the different fractions, and expressed per mg of protein [14].

Immunoprecipitation

ER/Golgi and MVM fractions were solubilized in 1 ml of a buffer containing 10 mM-Hepes/HCl, pH 7.5, 20 mM-NaCl, 0.5 % Triton X-100, 0.02 % sodium azide and the proteinase inhibitors as mentioned above. Following centrifugation at 30000 *g* for 10 min, the supernatant was incubated overnight at 4 °C with 150 μ l of monoclonal antibody bound to Sepharose. Washing and analysis of the immune complex by SDS/polyacrylamide-gel electrophoresis (PAGE) were performed as described [14].

Endo-H treatment

Digestion of the immunoprecipitates with endo-H was performed essentially as described by the manufacturer. After extensive washing as described [14], the immune complex bound to the Sepharose beads was boiled for 3 min in a buffer containing 50 mM-sodium phosphate, pH 6, and 0.1 % SDS. This mixture was incubated overnight at 37 °C with 50 munits of endo-H/ml and 2 mM-PMSF. The sample was diluted in 100 μ l of Laemmli sample buffer [17], boiled for 5 min and centrifuged. An aliquot (10 μ l) of the supernatant was added to Ready-Solv scintillation mixture (Beckman) and radioactivity was determined in a liquid-scintillation counter. The remainder was analysed by SDS/PAGE according to the method of Laemmli [17].

N-Glycanase treatment

N-Glycanase hydrolyses asparagine-linked oligosaccharides (high-mannose, complex and hybrid chains) of glycoproteins [18]. The digestion of immunoprecipitates was carried out as follows. The protein bound to the Sepharose beads was boiled for 3 min in a buffer containing 0.5 % SDS and 0.1 M- β -mercaptoethanol. The sample was diluted with 0.55 M-sodium phosphate buffer, pH 8.6, containing albumin (6 mg/ml) and Nonidet P-40 in a final concentration of 1.25 % (as outlined in the manufacturer's instructions). Incubation with N-glycanase (0.25 units) was done overnight at 37 °C. After incubation, the sample was analysed as described above for the endo-H digestion.

RESULTS

Effects of castanospermine on protein synthesis

Castanospermine (50 μ g/ml) caused no decrease in overall protein synthesis as judged from the incorporation of radioactivity into TCA-precipitable protein in total homogenates (Table 1); similar results were obtained with 100 μ g of the drug/ml. However, concentrations of 250 and 500 μ g/ml significantly inhibited protein synthesis (results not shown). Thus, 50 μ g of castanospermine/ml was used in all subsequent experiments.

Table 1. Effects of castanospermine on protein synthesis in jejunal explants of 5-day-old rats

Explants were incubated, labelled and analysed as described in the Experimental section in the presence or absence of castanospermine (50 μ g/ml).

Fraction	$10^{-5} \times$ Protein synthesis		
	Control (c.p.m./mg)	Castano- spermine (c.p.m./mg)	Inhibition (%)
Total homogenate	244 $\pm$ 14	246 $\pm$ 19	0
ER/Golgi	18 $\pm$ 3	14 $\pm$ 2	22
MVM	3 $\pm$ 0.3	1.7 $\pm$ 0.2	44

Castanospermine had a moderate effect on the TCA-precipitable counts in the ER/Golgi fraction with a reduction of 22% as compared with the control. The effect of castanospermine on the TCA-precipitable radioactivity in the MVM fraction was more profound, with a reduction of 44%. These results correlated very well with those obtained with immunoprecipitated lactase-phlorizin hydrolase from castanospermine-treated and untreated explants. Radioactivity in lactase-phlorizin hydrolase immunoprecipitates (adjusted for lactase units of activity) from castanospermine-treated ER/Golgi and MVM fractions measured 85–94% and 46–51% respectively of controls. These values indicate that approx. 50% of newly synthesized lactase-phlorizin hydrolase reached the MVM in castanospermine-treated explants. While the data depend on the assumption that castanospermine does not influence lactase activity in subcellular fractions, castanospermine does not affect total mucosal lactase specific activity (results not shown).

Lactase-phlorizin hydrolase in the ER/Golgi and MVM

Fig. 1 shows the SDS/PAGE analysis of immunoprecipitated lactase-phlorizin hydrolase from the intracellular and MVM fractions of labelled jejunal explants. The labelled ER/Golgi form (lane 1) is approx. 205 kDa, while the labelled MVM bands (lane 2) are 220, 180, and 130 kDa. As shown previously [14], the fully glycosylated 220 kDa intracellular precursor arrives at the MVM and, after insertion, this form is proteolytically cleaved virtually completely (> 95%) in two separate steps: first from the 220 to the 180 kDa form and then to the final 130 kDa form. The inserted 220 kDa form completely disappears during this process [14]. The difference between the precursor form in the ER/Golgi and that inserted into the MVM was not distinguished in our previous study [14].

Intracellular (ER/Golgi) lactase-phlorizin hydrolase treated with castanospermine

Fig. 2 shows the immunoprecipitated lactase-phlorizin hydrolase from the ER/Golgi fraction. In lane 1, in the absence of castanospermine, the same 205 kDa precursor is seen as in Fig. 1. However, if, as seen in lane 2, castanospermine was added to the incubation mixtures, this band increased to approx. 210 kDa. When these two

Fig. 1. SDS/PAGE and fluorography of immunoprecipitated lactase-phlorizin hydrolase from jejunum of 5-day-old rats

Jejunal explants were pulse-labelled with [35 S]methionine for 45 min and the label was chased for either 0 min (ER/Golgi) or 180 min (MVM). Explants were then processed and analysed on 5% polyacrylamide gels as described in the Experimental section. Lane 1 shows the ER/Golgi fraction and lane 2, MVM. The bars at the left indicate the positions of the molecular mass standards; the sizes of the labelled jejunal MVM bands are given at the right (arrows). Despite the presence of proteinase inhibitors in all incubation mixtures, we infrequently observed minor labelled bands, probably due to proteolysis.

precursors were treated with endo-H, as shown in lanes 3 and 4, the effect of castanospermine was eliminated, and both forms were equally reduced to a size of approx. 195 kDa. Treatment with *N*-glycanase (lanes 5 and 6) showed no further effect on the mobility of the labelled bands compared with the endo-H treated bands, indicating the presence of only high-mannose oligosaccharides in the intracellular precursor.

MVM lactase-phlorizin hydrolase treated with castanospermine

Analysis of the immunoprecipitated lactase-phlorizin hydrolase from MVM is shown in Fig. 3. The [35 S]-methionine labelling and deglycosylation treatments were as for Fig. 2, except that the MVM fractions were prepared after a chase period of 180 min in order to obtain maximal labelling of the three MVM bands [14]. In lane 1, i.e. the control lane with no castanospermine added to the medium, the three labelled bands are the same as seen in Fig. 1: 220, 180 and 130 kDa. The effect

Fig. 2. SDS/PAGE and fluorography of immunoprecipitated ER/Golgi lactase-phlorizin hydrolase

Explants were labelled as shown in Fig. 1 and processed and analysed on 5% polyacrylamide gels as described in the Experimental section. The presence of castanospermine (50 µg/ml) and treatment with deglycosylating enzymes (see the Experimental section) are indicated above the gel. The bars at the left indicate the positions of molecular mass standards.

Fig. 3. SDS/PAGE and fluorography of immunoprecipitated MVM lactase-phlorizin hydrolase

Explants were labelled as shown in Fig. 1 and processed and analysed on 5% polyacrylamide gels as described in the Experimental section. The presence of castanospermine (50 µg/ml) and treatment with deglycosylating enzymes (see the Experimental section) are indicated above the gel. The arrows at the left indicate the positions of the three forms of MVM lactase-phlorizin hydrolase without castanospermine or enzymes. The positions of molecular mass standards are shown at the right.

of castanospermine, as depicted in lane 2, was a slight increase in the molecular mass of each of the three bands. The effect of castanospermine on lactase-phlorizin hydrolase was more clearly visible if the immunoprecipitates were treated with endo-H as shown in lanes 3 and 4. There was a distinct difference in the mobility of all three bands between the control (lane 3) and the castanospermine-treated (lane 4) explants. Further decreases in molecular mass were found when the MVM precipitates were treated with *N*-glycanase, as shown in lanes 5 and 6.

DISCUSSION

After biosynthesis of the intracellular precursor, many secretory and membrane glycoproteins undergo proteolytic processing in different compartments of the cell [5-8]. The acquisition and modification of oligosaccharides, however, is very similar for different proteins and encompasses two separate steps, the co-translational attachment of *N*-linked oligosaccharides of the high-mannose type in the endoplasmic reticulum, and trimming and complex glycosylation, as well as *O*-linked glycosylation, in the Golgi apparatus [6,8]. Several authors [9,11,12] have shown that glycosylation in the ER functions in the sorting and transport of glycoproteins to their final destination, while others [19,20] have suggested that carbohydrates only play an indirect role in intracellular transport and that the primary role is in the folding of the polypeptide.

The major intestinal microvillus glycoprotein disaccharidases, sucrase-isomaltase, maltase-glucoamylase and lactase-phlorizin hydrolase have been studied in varying detail. Sucrase-isomaltase has been examined extensively (for review see [1]). The complete amino-acid sequence, including potential glycosylation sites, of the rabbit enzyme [21], and the partial sequence of human sucrase-isomaltase have been reported [22]. Maltase-glucoamylase biosynthesis and processing are less well understood [1,23]. Although some studies [1-4, 14] have dealt with the biosynthesis of lactase-phlorizin hydrolase, and the sequences of the rabbit and human enzyme have been published recently [24], very little is known about the glycosylation and processing of this important enzyme.

In earlier studies [14] we showed that the initial intracellular form of lactase-phlorizin hydrolase could readily be labelled with [2-³H]mannose, and we suggested that this form represents the high-mannose-type glycosylated precursor. The present study confirms this by the susceptibility of the 205 kDa intracellular precursor to both endo-H and *N*-glycanase; no shift in mobility is seen when the two treatments are compared (Fig. 2), providing evidence for the presence of only high-mannose oligosaccharide chains. The increase in molecular mass of the intracellular precursor of approx. 5 kDa after incubation with castanospermine may arise from the extended length of the unprocessed chains. This somewhat higher precursor band is identically reduced to a 195 kDa band after removal of the *N*-linked sugars. This is in accordance with the studies of Naim *et al.* [2], which demonstrated that the deglycosylated precursor of lactase-phlorizin hydrolase in human explants is approx. 200 kDa. These data, taken together, strongly indicate that the unglycosylated, nascent protein has a molecular mass of 195 kDa. The precursor of lactase-phlorizin

hydrolase that is inserted in the MVM is clearly different from the initial intracellular form in several ways. This band (220 kDa), although still somewhat endo-H-sensitive, is also susceptible to *N*-glycanase, demonstrating the presence of complex oligosaccharide chains. The difference of approx. 10 kDa that remains between the intracellular precursor and the MVM precursor after treatment with *N*-glycanase (Figs. 2 and 3) is most likely due to *O*-linked glycosylation, although other post-translational modifications cannot yet be excluded.

In a human study [2], the lactase-phlorizin hydrolase precursor found in the MVM showed the same susceptibility to endo-H and endo-F as the intracellular form; however, no explanation of the insertion of this non-complex glycosylated form into the MVM was given. The present study is the first to identify the complex glycosylated form of lactase-phlorizin hydrolase in the MVM.

We have shown here that, after incubation with castanospermine, lactase-phlorizin hydrolase is still transported to the MVM and proteolytically converted to the mature form (135 kDa). Similarly, Danielsen & Cowell [3] found that castanospermine did not block the transport of sucrase-isomaltase and maltase-glucoamylase to the MVM, and concluded that processing of *N*-linked carbohydrate is not required for surface expression of microvillus enzymes. In contrast to the two disaccharidases they described, we found lactase-phlorizin hydrolase to have a higher molecular mass at all processing stages after exposure to castanospermine. Thus, for the three major disaccharidases, lactase-phlorizin hydrolase, sucrase-isomaltase and maltase-glucoamylase, intracellular processing and transport are not blocked by the inhibitor of the initial trimming enzyme, glucosidase I. However, the reduction in TCA-precipitated radioactivity and the intensity of the labelled bands after incubation with castanospermine indicate, as suggested by Danielsen & Cowell [3], that removal of the glucose residues is important for achieving normal function, and that the cell rapidly recognizes and degrades unprocessed molecules.

In contrast, some secretory proteins are particularly sensitive to alterations in glycosylation. Lodish & Kong [11] showed accumulation of α_1 -antitrypsin and α_1 -antichymotrypsin in the ER and markedly reduced secretion of the proteins after incubation with another glucosidase I inhibitor, 1-deoxynojirimycin. They concluded that the removal of the initial glucose residues provides the signal for transport from the ER to the Golgi complex. It is noteworthy that in experiments we performed in the presence of 1-deoxynojirimycin, no effect on lactase-phlorizin hydrolase was observed. It is apparent that the effect of inhibition of glucosidase I, by castanospermine or 1-deoxynojirimycin, on secretory proteins [9,11,12] is either no reduction in synthesis and secretion, or an accumulation in the ER, resulting in decreased secretion rates. In contrast, the results of this and other reports [3] indicate that membrane-bound proteins are more susceptible to intracellular degradation if the outer glucoses are not removed [9].

In the present study, the decrease in molecular mass of the mature 130 kDa band after endo-H treatment implies the presence of high-mannose *N*-linked carbohydrates, which means that not all the high-mannose units that are initially attached to the nascent protein are processed to the complex type (Fig. 3, lane 1 compared with lane 3).

The same phenomenon has been seen after endo-H digestion of mature sucrase-isomaltase and glucoamylase in pig intestine [3] and for lactase-phlorizin hydrolase in human explants [2]. That the mature 130 kDa protein also has complex-type oligosaccharides is shown by an additional decrease in molecular mass after *N*-glycanase incubation, which removes all *N*-linked oligosaccharides (Fig. 3, lane 3 compared with lane 5). The removal of high-mannose oligosaccharides from castanospermine-treated lactase-phlorizin hydrolase yields a shift in electrophoretic mobility (Fig. 3, lane 2 compared with lane 4). There is also a clear mass difference between the lactase-phlorizin hydrolase bands in control and castanospermine-treated explants after endo-H exposure (Fig. 3, lane 3 compared with lane 4), indicating that the forms which arrive in the MVM are affected by castanospermine. It is noteworthy that there still remains a slight mass difference between the forms exposed to both castanospermine and endo-H compared with those exposed to castanospermine and *N*-glycanase (Fig. 3, lane 4 compared with lane 6). This difference could be due to the presence of *N*-acetylglucosamine residues after endo-H treatment, resulting in altered electrophoretic mobility.

In our previous study, we found evidence to suggest that lactase-phlorizin hydrolase undergoes its proteolytic conversion to the mature form after insertion into the MVM; this is in contrast with other studies [2-4], which indicate that this proteolytic cleavage takes place intracellularly. Our present results again strongly suggest that lactase-phlorizin hydrolase is synthesized as a large precursor (nascent protein, 195 kDa) which undergoes glycosylation (205 kDa), trimming and translocation through ER and Golgi complex, and is subsequently transported and inserted (220 kDa) into the MVM.

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