Castanospermine inhibits glucosidase I and glycoprotein secretion in human hepatoma cells

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We studied the effect of the plant alkaloid castanospermine on the biosynthesis and secretion of human hepatoma glycoproteins. The HepG-2 cells, grown in the presence or absence of the alkaloid, were labelled with [2-³H]mannose and then the labelled glycopeptides were prepared by Pronase digestion. This material was analysed by gel filtration on Bio-Gel P-4 before and after treatment with endo- β -*N*-acetylglucosaminidase H. Castanospermine caused an accumulation of high-mannose oligosaccharides, by 70–75% over control. The major accumulated product, which could also be labelled with [³H]galactose and was only partially susceptible to α -mannosidase digestion, was identified by h.p.l.c. as a Glc₃Man₆GlcNAc. Thus the alkaloid inhibits glucosidase I in the human hepatoma cells. Analysis of total glycoproteins secreted by the cells into the medium revealed the presence of only complex oligosaccharides in both control and treated cultures, and the amount of the oligosaccharides labelled with radioactive mannose, galactose or *N*-acetylmannosamine, secreted by treated cells, was decreased by about 60%. The rate of secretion of total protein labelled with [³⁵S]methionine and precipitated from the medium with trichloroacetic acid was inhibited by up to 40% in the presence of castanospermine. Pulse-chase studies utilizing [³⁵S]methionine labelling were performed to study the effect of the alkaloid on secretion of individual plasma proteins. Immunoprecipitation at different chase times with monospecific antisera showed that castanospermine markedly decreased the secretion rates of α_1 -antitrypsin, caeruloplasmin and, to a lesser extent, that of antithrombin-III. Secretions of apolipoprotein E, a glycoprotein containing only *O*-linked oligosaccharide(s), and albumin, a non-glycosylated protein, were not affected by the drug. It is suggested that castanospermine inhibits secretion of at least some glycoproteins containing *N*-linked oligosaccharides, owing to the inhibition of oligosaccharide processing.

INTRODUCTION

A plant alkaloid, castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizidine), was shown to inhibit a number of commercial α - and β -glucosidases (Saul *et al.*, 1983) and also the processing of the oligosaccharide chains of the influenza-virus haemagglutinin (Pan *et al.*, 1983). Thus the alkaloid appears to impair the normal removal of glucose residues from the high-mannose-type precursor, which is the first step in the formation of glycoprotein complex-oligosaccharide chains (Hubbard & Ivatt, 1981). These oligosaccharides are synthesized by extensive post-translational modifications; subsequent removal of mannose residues (Tabas & Kornfeld, 1979) is followed by addition of other sugars, i.e. *N*-acetylglucosamine, galactose, fucose and sialic acid (Hubbard & Ivatt, 1981). The complex chains are commonly present in plasma glycoproteins synthesized and secreted by the liver.

Established human hepatoma cell lines HepG-2 and Hep3-B (Knowles *et al.*, 1980) have been shown to synthesize and secrete a large number of plasma glycoproteins. These cells have the capacity to metabolize carcinogens and also to express a set of cell-surface receptors, including those for asialoglycoprotein (Schwartz & Rup, 1983), transferrin and insulin (Ciechanover *et al.*, 1983a, b), as well as those for epidermal growth factor and

low-density lipoprotein (Knowles *et al.*, 1985). The cells are also hormone-responsive (Knowles *et al.*, 1985; Archer *et al.*, 1985). Thus the cells have retained a variety of properties characteristic of differentiated normal parenchymal liver cells, and they appear to be a relevant model of normal hepatocytes.

In the present study, we have examined the effect of castanospermine on the synthesis and secretion of glycoproteins in HepG-2 cells. We report here that the alkaloid inhibits glucosidase I and the rate of secretion of hepatoma glycoproteins.

MATERIALS AND METHODS

Culturing and labelling of cells

Human hepatoma cells (HepG-2) were cultured in minimal essential medium (Gibco, Grand Island, NY, U.S.A.) as previously described (Knowles *et al.*, 1980). The cells were incubated with various concentrations of castanospermine (0–500 μ g/ml) for 1 h at 37 °C, and then for an additional time (30 min to 8 h) in the low-glucose medium (containing only one-twentieth of the normal glucose concentrations) in the presence or absence of castanospermine. The alkaloid was extracted from seeds of *Castanospermum australe* and was purified by repeated chromatography on Dowex-50 (NH₄⁺ form)

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(Pan *et al.*, 1983). The cells were labelled with 5 μ Ci of D-[2-³H]mannose (24.3 Ci/mmol)/ml, 5 μ Ci of D-[6-³H]galactose (29.2 Ci/mmol)/ml or 10 μ Ci of N-acetyl-D-[6-³H]mannosamine/ml (22.7 Ci/mmol) (all from New England Nuclear, Boston, MA, U.S.A.), along with 100 kallikrein-inhibitor units of Trasylol/ml (Sigma Chemical Co., St. Louis, MO, U.S.A.). After the labelling period, the medium was removed and centrifuged (1000 g for 10 min) to pellet any detached cells. Radioactively labelled secreted proteins were precipitated with cold 10% (w/v) trichloroacetic acid in the presence of 0.5 mg of bovine serum albumin/ml (fraction V; Sigma) for 24 h at 4 °C. The pellet was washed four times with cold 10% trichloroacetic acid and once with diethyl ether. The cells attached to plastic dishes were extensively washed with phosphate-buffered saline, pH 7.6 (0.4 g of KCl/l, 0.06 g of KH₂PO₄/l, 8 g of NaCl/l, 0.09 g of Na₂HPO₄·7H₂O/l) and extracted with chloroform/methanol (2:1, v/v), water and chloroform/methanol/water (10:10:3, by vol.). The protein fractions were digested with Pronase to obtain glycopeptides (Sasak *et al.*, 1982), and the material was analysed on Bio-Gel P-4 columns before and after endoglucosaminidase H treatment.

This enzyme cleaves high-mannose oligosaccharides between the two N-acetylglucosamine residues of the inner core (Tai *et al.*, 1977; Chandrasekaran *et al.*, 1983). Fractions from the Bio-Gel columns were collected and radioactivity was determined in a liquid-scintillation counter. The efficiency of counting was 48–52%. The presence of low-glucose medium did not lead to the formation of shorter oligosaccharides in HepG-2 cells as compared with the medium containing normal glucose concentration (1 g/l). Truncated oligosaccharides were shown in rat hepatoma cells during glucose starvation in glucose-free medium (Baumann & Jahreis, 1983).

For the pulse-chase experiments, the cells grown in 60 mm-diameter dishes were preincubated with or without 500 μ g of castanospermine/ml for 1 h in 4 ml of methionine-free minimal essential medium. Then 50 μ Ci of L-[³⁵S]methionine (1120 Ci/mmol; New England Nuclear) was added to each dish for 10 min. After this time, the medium was removed and the cells were quickly washed twice with minimal essential medium containing 10 mg of methionine/l. The pulse was chased with 4 ml of the latter medium for the times indicated. The medium was collected and centrifuged (1000 g for 10 min) to pellet any detached cells. The resulting supernatant was distributed into four 1 ml portions for immunoprecipitation. Precipitation with trichloroacetic acid of L-[³⁵S]methionine-labelled protein fraction was carried out as described above for precipitation of labelled glycoproteins.

Immunoprecipitation

To each 1 ml of supernatant obtained from the cell medium, 3 ml of buffer containing 20 mM-Tris/HCl, pH 7.6, 0.14 M-NaCl, 5 mM-EDTA, 1% Triton X-100, 1 mM-phenylmethanesulphonyl fluoride and 100 kallikrein-inhibitor units of Trasylol/ml was added (Gross *et al.*, 1983b). The antibodies prepared against human proteins in goats were from Sigma, except that antithrombin III, prepared in rabbits, was from Calbiochem-Behring (San Diego, CA, U.S.A.), and anti-apolipoprotein E was obtained from Dr. S. Karathanasis of Children's Hospital, Boston, MA, U.S.A. After addition of 10 μ l of a specific antiserum and

incubation at 4 °C overnight, the antigen-antibody complex was bound to 5 mg of Pansorbin cells (Calbiochem-Behring) by incubation for 1 h at 4 °C. The pellet was washed four times with the same buffer and radioactivity was determined in a liquid-scintillation counter, by using Ready-Solv scintillation cocktail (Beckman Instruments, Fullerton, CA, U.S.A.). The efficiency of counting was 67–70%.

H.p.l.c.

A column (5 μ m) (0.46 cm $\times$ 25 cm; Alltech Associates, Deerfield, IL, U.S.A.) of Econosphere-NH₂ was utilized along with a Beckman model 344 liquid chromatograph, consisting of two model 112 pumps, cathode-ray-tube controller and a model 210 universal sample injector. The column, preceded by a cartridge guard column (0.46 cm $\times$ 3 cm), with the same packing as for the analytical column, was equilibrated with the initial solvent acetonitrile/water (29:21, v/v). Isocratic conditions were maintained for 15 min, followed by a linear gradient to acetonitrile/water (12:13, v/v) for 30 min. The flow rate was 1.5 ml/min. Fractions (1 ml) were collected, and radioactivity was determined in a liquid-scintillation counter. The injection valve was equipped with a 20 μ l loop. Samples of oligosaccharides collected from Bio-Gel P-4 columns were freeze-dried and dissolved in the initial solvent of acetonitrile/water. Between 10 and 15 μ l was usually injected into the column, and the recovery of radioactivity was 85–90%. The efficiency of counting was about 45%.

Glycosidase treatment

Treatment with endo- β -N-acetylglucosaminidase H, jack-bean α -mannosidase and *Vibrio cholerae* sialidase was performed as described previously (Sasak *et al.*, 1982, 1983).

Preparation of oligosaccharide standards

The oligosaccharide standard [³H]Man₆GlcNAc was obtained by labelling of rat intestinal-epithelial cells (IEC-6) with [2-³H]mannose (Sasak *et al.*, 1982). The lipid-linked [³H]oligosaccharides were extracted with chloroform/methanol/water and the oligosaccharides were released by mild acid treatment (0.01 M-HCl, 30 min, 90 °C). After digestion with endo- β -N-acetylglucosaminidase H, the oligosaccharides were isolated by h.p.l.c. and compared with standard oligosaccharides isolated from human mannosidosis urine (Warren *et al.*, 1983). Standard [¹⁴C]Glc₃-Man₆GlcNAc isolated from calf pancreas microsomal fractions (Kilker *et al.*, 1981) was obtained from Dr. Annette Herscovics and Dr. Brigitte Saunier (Massachusetts General Hospital, Boston, MA, U.S.A.).

Gel-filtration analysis

The Bio-Gel P-4 (200–400 mesh) column (1.5 cm $\times$ 180 cm) was eluted with 0.1 M-NH₄HCO₃, pH 8.0. Flow rate was 5 ml/h. Recovery of radioactivity from the column was 95–103%.

Concanavalin A-Sepharose chromatography

A procedure described by Finne *et al.* (1980) was used. The buffer, 5 mM-sodium acetate, pH 5.2, containing 0.1 M-NaCl and CaCl₂, MnCl₂ and MgCl₂ (1 mM each), was used to wash the column, and to elute the glycopeptides with 20 mM- and 200 mM-methyl α -D-

Fig. 1. Effect of castanospermine on the synthesis of oligosaccharide chains of hepatoma glycoproteins

Bio-Gel P-4 column chromatography of [$2\text{-}^3\text{H}$]mannose-labelled glycopeptides (a) before and (b) after endoglucosaminidase H treatment. The cells grown in the absence (—) or presence of $500\text{ }\mu\text{g}$ of castanospermine/ml (---) were labelled for 30 min and then extracted with chloroform/methanol, water and chloroform/methanol/water to remove mannose-labelled lipid-linked mono- and oligo-saccharides as well as water-soluble compounds. Delipidated residue was treated with Pronase to obtain glycopeptides. Fractions (1.2 ml) were collected. The arrows indicate the void volume and the elution position of oligosaccharide standard $\text{Glc}_3\text{Man}_6\text{GlcNAc}$ ($\text{G}_3\text{M}_6\text{GN}$).

mannopyranoside. Concanavalin A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) contained 10–16 mg of concanavalin A/ml of gel. Recovery of radioactivity from the columns ($0.5\text{ cm} \times 2.5\text{ cm}$) was 75–88%.

RESULTS

Effect of castanospermine on the oligosaccharide chain syntheses

Castanospermine ($500\text{ }\mu\text{g}/\text{ml}$) affected the composition of [$2\text{-}^3\text{H}$]mannose-labelled glycopeptides, as judged from the gel-filtration analysis (Fig. 1). The cells were incubated with the label for only 30 min. Three major glycopeptide peaks were obtained from both control and castanospermine-treated cells. The glycopeptides (Fig. 1a) were susceptible to endoglucosaminidase H treatment (Fig. 1b), indicating that the great proportion of newly synthesized glycoproteins contained high-mannose oligosaccharide chains. Since endoglucosaminidase H digestion of glycopeptides from both control or treated cells generated mainly one broad peak of a lower M_r , it was presumed that the glycopeptides differed

greatly in the length of their peptide moieties. In the presence of castanospermine, an accumulation of a longer oligosaccharide fraction was evident (Fig. 1b). This fraction was eluted from the column in the area corresponding to a standard $\text{Glc}_3\text{Man}_6\text{GlcNAc}$; however, the possibility of a mixture of oligosaccharides could not be ruled out. Only a small proportion of the labelled glycopeptides (less than 5% of the total), which were eluted at or close to the void volume of the column, were resistant to the endoglycosidase treatment (Fig. 1b).

The elution profile of glycopeptides labelled with [$2\text{-}^3\text{H}$]mannose for 8 h is shown in Fig. 2(a). Endoglucosaminidase H treatment cleaved high-mannose-type glycopeptides, yielding a series of oligosaccharides (Fig. 2b, fraction III). This fraction, as well as fractions I and II, pooled separately as indicated in the Figure, were analysed on concanavalin A-Sepharose. About 50% of fraction I was not retained by the column. The remaining 50% of the radioactivity bound to the column and was eluted with 20 mM-methyl mannoside. Fractions II and III were retained by the lectin, and were eluted with 20 mM- and 200 mM-methyl mannoside respectively. Under these conditions, glycopeptides not retained by the column are characterized as possessing triantennary and higher branched complex oligosaccharides, whereas glycopeptides bound by the column and eluted with 20 mM-methyl mannoside are characterized as containing biantennary complex chains, and those eluted with 200 mM-methyl mannoside as high-mannose oligosaccharides (Krusius, 1976; Krusius & Finne, 1977; Finne *et al.*, 1980). In addition, fraction I was completely resistant to α -mannosidase, whereas fraction III was substantially degraded by the enzyme. This confirms that fraction III is composed of high-mannose oligosaccharides, and indicates the presence of complex chains in fraction I. The nature of fraction II has not been investigated.

After incubation (8 h) of the control cells with [$2\text{-}^3\text{H}$]mannose, the amount of radioactivity in the endoglucosaminidase-H-sensitive oligosaccharides was approximately the same as that in the resistant structures (Fig. 2b). Incubation of the cells with castanospermine (250 or $500\text{ }\mu\text{g}/\text{ml}$) increased the amount of radioactivity in the sensitive oligosaccharides by 50% and 72% respectively; however, the amount of radioactivity in the resistant oligosaccharides (fraction I) was decreased by only 6–8% (Fig. 2b). Thus the increase in radioactive high-mannose oligosaccharides induced by castanospermine was not at the expense of the complex chains. These concentrations of the alkaloid were not toxic to the cells, nor did they affect protein and DNA synthesis as measured by incorporations of [^{14}C]leucine (Pan *et al.*, 1983) and [^3H]thymidine (Stubblefield *et al.*, 1967) respectively. Concentrations of castanospermine up to $100\text{ }\mu\text{g}/\text{ml}$ did not significantly change the elution profile of the mannose-labelled glycopeptides. Owing to the limited amounts of the drug, concentrations higher than $500\text{ }\mu\text{g}/\text{ml}$ were not tested. It was shown, however, that even very high concentrations of the drug did not completely inhibit the formation of complex chains (Pan *et al.*, 1983).

Identification of oligosaccharide accumulated in the presence of castanospermine

The longest endoglucosaminidase-H-susceptible oligosaccharide that accumulated in the presence of the

Fig. 2. Effect of castanospermine on the oligosaccharide composition of hepatic glycoproteins

Bio-Gel P-4 column chromatography of [2-³H]mannose-labelled glycopeptides (a) before and (b) after endoglucosaminidase H treatment. The cells were labelled for 8 h. The shaded area (fractions 152–157) was pooled for further analysis by h.p.l.c. The horizontal bars indicate material which was pooled into fractions I, II and III for analysis on concanavalin A–Sephadex.

alkaloid migrated on the Bio-Gel column in the position of Glc₃Man₉GlcNAc. This oligosaccharide could also be labelled after an incubation with [³H]galactose and was only partially degraded by α -mannosidase. These findings confirm that the oligosaccharide contains glucose residues. This oligosaccharide (fractions 152–157; Fig. 2b), which was not present in control cells, was further analysed by h.p.l.c. on an aminopropylsilyl column, by using a shallow gradient of acetonitrile/water for elution in order to enhance the resolution of longer oligosaccharides. Inasmuch as these conditions generated relatively broad peaks, the oligosaccharide was eluted as a single peak in the position of Glc₃Man₉GlcNAc, separated by 21 fractions from a standard Man₉GlcNAc (Fig. 3). It should be mentioned that oligosaccharide Glc₁Man₈GlcNAc isolated from lymphoma cells (G. Palamarczyk & A. D. Elbein, unpublished work) was eluted in the same position as Man₉GlcNAc. Although Glc₂Man₈GlcNAc and Glc₁Man₈GlcNAc were not tested, it is unlikely that they could be eluted in the same area as the accumulated hepatoma oligosaccharide.

Effect of castanospermine on glycoprotein secretion

The HepG-2 cells grown in the absence or presence of castanospermine were labelled with [2-³H]mannose and secreted glycoproteins were precipitated with trichloroacetic acid. Glycopeptides prepared from the precipitate were separated on a Bio-Gel P-4 column. All the glycopeptides were eluted near the void volume of the column and were resistant to endoglucosaminidase H and α -mannosidase, suggesting the presence of complex

oligosaccharides. The amount of the labelled glycoproteins secreted by the cells grown with 250 and 500 μ g of castanospermine/ml was decreased by 35% and 60% respectively. The time course of synthesis and secretion of the mannose-labelled glycoproteins is shown in Fig. 4. At 4 h and 8 h of incubation in the presence of the drug (500 μ g/ml), the amount of radioactivity in intracellular glycoproteins was enhanced by 28% and 66% respectively, whereas the amount of the labelled secreted glycoproteins was decreased by 51% and 63% respectively. Similar results were obtained after incubation of the cells with [³H]galactose and *N*-acetyl[³H]mannosamine. These sugar precursors label complex oligosaccharides. About 50% of the radioactivity derived from glycopeptides labelled with *N*-acetyl[³H]mannosamine could be released by sialidase treatment. Thus *N*-acetyl[³H]mannosamine was converted by the cells into [³H]sialic acid and incorporated into glycoproteins, presumably from CMP-sialic acid. Castanospermine (500 μ g/ml) did not markedly affect the amounts of intracellular glycoproteins labelled with either [³H]galactose or *N*-acetyl[³H]mannosamine; however, the amounts of the labelled secreted glycoproteins were decreased by about 55–60%.

In order to exclude the possibility that the observed differences between control and treated cells in the amount of radiolabelled carbohydrate portions of secreted proteins were due primarily to a decrease in number of the oligosaccharide chains (Gross *et al.*, 1983b), the media obtained from the [³⁵S]methionine-pulse-chased cells at various time intervals were subjected to precipitation with trichloroacetic acid. As

Fig. 3. Identification of major oligosaccharide accumulated in the presence of castanospermine

H.p.l.c. was carried out on Econsphere amino-bonded silica column (0.46 cm $\times$ 25 cm) in a gradient of acetonitrile/water. Flow rate was 1.5 ml/min. Fractions (1 ml) were collected and counted for radioactivity. The oligosaccharide was isolated on a Bio-Gel P-4 column after endoglucosaminidase H treatment of [^{3} H]mannose-labelled glycopeptides (see Fig. 2). The arrows indicate the elution positions of oligosaccharide standards: M_9GN , $Man_9GlcNAc$; G_3M_9GN , $Glc_3Man_9GlcNAc$.

Fig. 4. Effect of castanospermine on the synthesis and secretion of hepatoma glycoproteins

Distribution of radioactivity into cellular glycoproteins of HepG-2 cells (left ordinate) grown in the presence (○) and absence (●) of 500 μ g of castanospermine/ml, and into secreted glycoproteins (right ordinate) from castanospermine-treated (△) and control (▲) cells. The cells were incubated with [^{3} H]mannose for periods of time indicated. At the end of incubation, the medium was separated from the cells and subjected to trichloroacetic acid precipitation. The cells were washed with phosphate-buffered saline and extracted with chloroform/methanol and chloroform/methanol/water. Denatured protein fractions were digested with Pronase and applied to the Bio-Gel column. The radioactivity contained in the glycopeptide fractions was taken for calculation.

shown in Fig. 5(a), the drug decreased the rate of secretion of total labelled proteins by 25–40%.

To follow the effect of castanospermine on secretion of individual glycoproteins, we have employed pulse-chase studies, followed by immunoprecipitation with specific antisera. The rate of secretion of albumin, a non-

glycosylated protein, was not changed (Fig. 5b); however, the secretion of newly synthesized α_1 -antitrypsin (Fig. 5c) and caeruloplasmin (Fig. 5d) was decreased after 2 h of chase by about 60% and 40% respectively. Each of these plasma proteins has three *N*-linked complex-oligosaccharide chains (Mega *et al.*, 1980; Endo *et al.*, 1982). The rate of secretion of antithrombin III, a major blood anticoagulant containing four *N*-linked sugar chains (Björk *et al.*, 1981), was also affected by the drug, but to a lesser degree (Fig. 5e). The time required to chase completely radioactive antithrombin III from the cells to the medium was much longer (120 min or more) than that to chase α -antitrypsin (30 min) and caeruloplasmin (about 90 min). No change in the secretion pattern of apolipoprotein E (Fig. 5f) was observed. Although the oligosaccharide structure of apolipoprotein E is not known, the amino acid sequence of the whole molecule (Rall *et al.*, 1982) indicates the presence of only one asparagine residue and lack of the amino acid sequence Asn-Xaa-Ser/Thr, which is required for *N*-glycosylation. This finding, as well as the known carbohydrate content of apolipoprotein E (Jain & Quarfordt, 1979), suggest that the protein contains only *O*-linked carbohydrate chain(s).

These results confirm that the drug affects secretion of plasma glycoproteins, presumably as a result of its inhibitory effect on the oligosaccharide processing.

DISCUSSION

We have shown that in human hepatoma cells (HepG-2) castanospermine inhibits glucosidase I, the enzyme that removes the outermost glucose from the *N*-linked oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$. Therefore, this drug inhibits the normal processing of high-mannose chains to the complex-type oligosaccharides. Glycoproteins which retained their high-mannose chains were not secreted by the cells and accumulated intracellularly.

It is interesting that the intracellular accumulation of high-mannose oligosaccharides was not associated with a significant decrease in complex oligosaccharides. It is possible that an alternative pathway of complex-oligosaccharide synthesis exists. One likely explanation would involve a transfer of unglucosylated oligosaccharides from the lipid to protein, which is then normally processed. This is not in agreement with the generally accepted view, in which glucose residues are required for the transfer (Hubbard & Ivatt, 1981). Another possibility would exist if castanospermine inhibited only glucosidase I and did not have an inhibitory effect on glucosidase II, which removes two inner-core glucose residues. In that case, the incompletely glucosylated oligosaccharides with only two glucose residues could be transferred to protein and then processed. This mechanism would not have to include the inhibition of glucose transfer to the lipid-linked oligosaccharide by castanospermine. It is feasible that under appropriate conditions, such as an accumulation of dolichol-linked $Glc_3Man_9GlcNAc_2$, the transfer of incomplete oligosaccharides from lipid to protein takes place. It has been reported that lipid-linked trisaccharide can be a direct donor of its carbohydrate unit in glycosylation of hen oviduct glycoproteins (Chen & Lennarz, 1976).

It appears that sugar portions of glycoproteins may play a variety of biological roles, which are not well

Fig. 5. Effect of castanospermine on secretion of hepatoma proteins

The HepG-2 cells were preincubated with or without 500 μ g of castanospermine/ml for 1 h in methionine-free minimal essential medium, and then [^{35} S]methionine was added for 10 min. After quick washing of the cells with minimal essential medium, the label was chased with methionine for the times indicated and the medium was separated from the cells. Total protein secretion was determined by trichloroacetic acid precipitation. Individual proteins were immunoprecipitated with specific antisera. The antigen/antibody complex was bound to fixed *Staphylococcus* cells. The pellet was extensively washed and dissolved in 1 M-NaOH, neutralized with HCl and counted for radioactivity. Variations between the duplicate samples were 1–14%.

understood. Studies with tunicamycin, an antibiotic that blocks glycosylation, have suggested that certain oligosaccharide chains are required for efficient glycoprotein secretion (Hickman & Kornfeld, 1978; Firestone, 1983; Bartalena & Robbins, 1984). However, the effect of tunicamycin does not seem to be a general phenomenon, since other studies showed little or no effect of the antibiotic on glycoprotein secretion (Bell-Quint *et al.*, 1981; Mizraki *et al.*, 1978; Keller & Swank, 1978). In yet other published investigations, the effect of tunicamycin could not be clearly defined, owing to the inhibitory effect of the drug on protein synthesis (Struck *et al.*, 1978; Edwards *et al.*, 1979).

Utilization of oligosaccharide-processing inhibitors has provided additional information on the role of sugar chains. It was shown that inhibition of processing of the asparagine-linked oligosaccharides of human chorionadotropin resulted in an intracellular accumulation of precursors and an inhibition of secretion of the mature form of the hormone (Peters *et al.*, 1983). 1-Deoxynojirimycin, which blocks removal of glucose residues (Saunier *et al.*, 1982), was shown in HepG-2 cells to delay secretion of α_1 -antitrypsin, α_1 -antichymotrypsin and complement component C3, whereas secretion of albumin and transferrin was not changed. It was also demonstrated that the drug caused accumulation of

α_1 -antitrypsin and α_1 -antichymotrypsin in the rough endoplasmic reticulum (Lodish & Kong, 1984). 1-Deoxy-*nojirimycin* also inhibited secretion of α_1 -antitrypsin in rat hepatocytes (Gross *et al.*, 1983b) and glycoprotein processing in rat intestinal-epithelial cells (IEC-6) (A. Herscovics & W. Sasak, unpublished work). The same drug delayed proteolytic processing of precursors of β -hexosaminidase and cathepsin D to their mature forms. The precursors also accumulated in the endoplasmic reticulum (Lemansky *et al.*, 1984). Our results indicate that normal oligosaccharide processing, which involves removal of the outermost glucose residue, is required for effective secretion of α_1 -antitrypsin, caeruloplasmin and, to a lesser extent, that of antithrombin III. All of these proteins contain multiple *N*-linked oligosaccharides. It seems, however, that secretion of antithrombin III requires a longer time than that of α_1 -antitrypsin and caeruloplasmin. It has been shown (Lodish *et al.*, 1983; Ledford & Davis, 1983; Fitting & Kabat, 1982) that different proteins migrate and mature at different rates through the intracellular secretory system. Since castanospermine only inhibits and does not completely block removal of the glucose residue, it is likely that slower-migrating macromolecules which reside longer in the rough endoplasmic reticulum can be more efficiently processed and secreted. It is also possible that, in the presence of the inhibitor, the processing on one branch only occurs, which may be a satisfactory requirement for secretion. Studies with swainsonine, an inhibitor of the processing enzyme mannosidase II (Tulsiani *et al.*, 1982), have shown the presence of abnormal oligosaccharides (hybrid-type) on vesicular-stomatitis-virus G protein (Kang & Elbein, 1983) and on secreted α_1 -antitrypsin (Gross *et al.*, 1983a) and fibronectin (Arumugham & Tanzer, 1983). These changes in the oligosaccharide structure had no effect on the glycoprotein secretions.

We have also studied and found no effect of castanospermine on the secretion pattern of plasma proteins which do not possess *N*-linked oligosaccharide chains (albumin and apolipoprotein E).

Taken together, these results support the hypothesis that normal oligosaccharide processing may play a role in selective intracellular transport and proteolytic processing of glycoproteins.

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