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Subcellular Localization of Glycosidases and Glycosyltransferases Involved in the Processing of N-Linked Oligosaccharides[1]

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ABSTRACT

Using isopycnic sucrose gradients, we have ascertained the subcellular location of several enzymes involved in the processing of the N-linked oligosaccharides of glycoproteins in developing cotyledons of the common bean, *Phaseolus vulgaris*. All are localized in the endoplasmic reticulum (ER) or Golgi complex as determined by co-sedimentation with the ER marker, NADH-cytochrome *c* reductase, or the Golgi marker, glucan synthase I. Glucosidase activity, which removes glucose residues from $Glc_3Man_9(GlcNAc)_2$, was found exclusively in the ER. All other processing enzymes, which act subsequent to the glucose trimming steps, are associated with the Golgi. These include mannosidase I (removes 1–2 mannose residues from $Man_{6-9}[GlcNAc]_2$), mannosidase II (removes mannose residues from $GlcNAcMan_5[GlcNAc]_2$), and fucosyltransferase (transfers a fucose residue to the Asn-linked GlcNAc of appropriate glycans). We have previously reported the localization of two other glycan modifying enzymes (GlcNAc-transferase and xylosyltransferase activities) in the Golgi complex. Attempts at subfractionation of the Golgi fraction on shallow sucrose gradients yielded similar patterns of distribution for all the Golgi processing enzymes. Subfractionation on Percoll gradients resulted in two peaks of the Golgi marker enzyme inosine diphosphatase, whereas the glycan processing enzymes were all enriched in the peak of lower density. These results do not lend support to the hypothesis that N-linked oligosaccharide processing enzymes are associated with Golgi cisternae of different densities.

Recent evidence from several laboratories indicates that plant glycoproteins contain both high mannose and complex glycans attached to the polypeptide via a GlcNAc → asparagine bond. The high mannose glycans have the general structure $Man_{5-9}(GlcNAc)_2$; the complex glycans usually have fewer mannose residues, additional GlcNAc, and other sugars, such as fucose, xylose, and/or galactose (4). The biosynthesis of the parent glycan occurs in the ER and culminates in the co-translational transfer of $Glc_3Man_9(GlcNAc)_2$ from dolichylpyrophosphate to specific asparagine residues on the nascent polypeptide chain. Modications of these N-linked oligosaccharides occur along the transport pathway (reviewed in [16]) as the glycoproteins move from the ER via the Golgi to their ultimate destinations, such as the extracellular matrix, the vacuoles, or the plasma membrane. These modifications require the participation of two classes of enzymes: glycosidases and glycosyltransferases. The glycosidases include glucosidases I and II, whose combined actions trim $Glc_3Man_9(GlcNAc)_2$ to $Man_9(GlcNAc)_2$; α-mannosidase I, which removes up to four α1-2 linked mannose residues from $Man_9(GlcNAc)_2$ to yield $Man_{5-8}(GlcNAc)_2$; and α-mannosidase II, which removes an α1-3 and α1-6 mannose from GlcNAc $Man_5(GlcNAc)_2$ to produce $GlcNAcMan_3(GlcNAc)_2$. The various glycosyltransferases transfer sugars (GlcNAc, fucose, xylose, and galactose) from their nucleoside diphosphate donors to specific N-linked glycan acceptors in a preferred sequence. GlcNAc-transferase I precedes while GlcNAc transferase II follows the action of α-mannosidase II to generate GlcNAc-$Man_5(GlcNAc)_2$ and $(GlcNAc)_2Man_3(GlcNAc)_2$, respectively. The latter product is an excellent acceptor for both fucosyl and xylosyltransferase (15), and presumably also for galactosyltransferase. Figure 1 shows the structure of the complex oligosaccharide that would result from the glycan-processing steps described above.

The subcellular locations of several of these glycan-processing enzymes in plants have been ascertained indirectly through characterization of the oligosaccharides obtained from, and measurement of sugar incorporation into, glycoproteins isolated from specific subcellular fractions. Results from such experiments show that glycoproteins in the ER all have high mannose sidechains (24, 30), indicating that the glucose residues are removed early in the sequence. Glycoproteins in the Golgi have complex

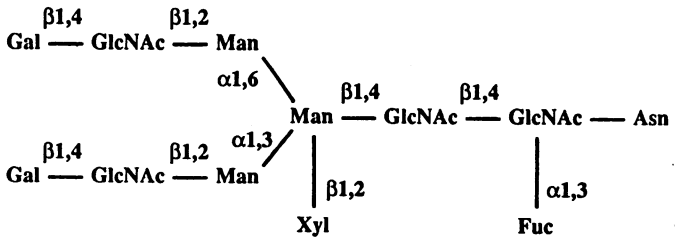

FIG. 1. Structure of a hypothetical complex oligosaccharide showing two important structural features of plant N-linked glycans: β(1→2) linked xylose and α(1→3) linked fucose.

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sidechains with fucose (3) and terminal GlcNAc residues (5, 31), indicating that these sugars are added in the Golgi complex. In the case of the storage glycoproteins in bean, terminal GlcNAc residues are removed in the protein storage vacuoles (31). Using exogenously supplied glycopeptide acceptors of known structure, Johnson and Chrispeels (15) described the sequence in which the various modifying enzymes act to convert a high mannose glycan into a complex glycan. Here we use the same approach to determine the subcellular location of the various enzymes and describe our attempt to subfractionate the Golgi cisternae on the basis of density in sucrose and Percoll gradients.

MATERIALS AND METHODS

Plant Material. Plants of *Phaseolus vulgaris* L. cv Greensleeves were grown in a greenhouse. Experiments were done with developing cotyledons weighing 125 to 175 mg, when accumulation of phaseolin and phytohemagglutinin is quite rapid. Organic chemicals were purchased from Sigma Chemical, and inorganic chemicals were obtained from Mallinckrodt (Paris, KY) unless otherwise indicated. D-[2-^{3}H]mannose (11 Ci/mmol), D-[1-^{3}H] galactose (12 Ci/mmol), GDP-L-[U-^{14}C]fucose, and UDP-D-[U-^{14}C]glucose (281 mCi/mmol) were obtained from Amersham Co; UDP-D-[1-^{3}H]xylose (9.9 Ci/mmol) and UDP-D-[4,5-^{3}H] galactose (36.3 Ci/mmol) were purchased from New England Nuclear.

Fractionation of Organelles on Sucrose Gradients. Cotyledons were ground with a mortar and pestle in grinding buffer (12% sucrose [w/w], 100 mM Tris/HCl, pH 7.8 and either 2 mM $MgCl_2$ or 1 mM EDTA). Cell walls and debris were removed by centrifugation at 1,000*g* for 5 min. The organelles were separated from the soluble fraction by passage through Sepharose 4B (Pharmacia, Uppsala, Sweden) columns as described (3), then fractionated by equilibrium density centrifugation on discontinuous or linear sucrose gradients containing $MgCl_2$ or EDTA grinding buffer. The discontinuous gradients consisted of layers of 54% sucrose (2 ml), 34% sucrose (4 ml), 16% sucrose (5 ml), and organelles (1.5 ml). The linear gradients contained 11 ml of either 16 to 54% or 22 to 44% sucrose with 1.5 ml of the organelle fraction. Equilibrium density flotation was carried out by increasing the sucrose concentration of the organelle fraction to 45% and overlaying it with a linear 22 to 40% sucrose gradient. The gradients were centrifuged up to 20 h at 35,000 rpm (150,000*g*) in a Beckman SW41 rotor. After centrifugation of the discontinuous gradients, the organelle bands overlaying the 34% and 54% sucrose layers were recovered with a Pasteur pipette. The linear gradient fractions (0.6 ml) were collected using a Buchler model IIC density gradient fractionator. The absorbance at 280 nm was recorded with an ISCO model UA-5 absorbance monitor. Separation of organelles by rate-zonal centrifugation was performed as described earlier (3). Briefly, 0.8 ml of organelles were layered onto shallow linear sucrose gradients (10 ml of 12–20% sucrose on a 1 ml cushion of 35% sucrose) and centrifuged in the SW41 rotor for 15 to 60 min at 20,000 rpm (48,000*g*).

Subfractionation of Golgi Membranes on Percoll Gradients. The subfractionation of Golgi membranes using Percoll gradients was carried out as described (7). Briefly, 0.5 ml of a Golgi-enriched fraction obtained from the 16/34% interface of the $MgCl_2$-containing discontinuous sucrose gradient was diluted 3-fold with grinding buffer and centrifuged in a Beckman 50Ti rotor for 90 min at 40,000 rpm. The membrane pellet was resuspended in 1 ml 0.25 M sucrose using a Dounce homogenizer. The homogenate was layered on top of 9 ml of 8.5% Percoll (v/v) made from a stock solution of 0.25 M sucrose in 100% Percoll (Pharmacia, Uppsala, Sweden) and 0.25 M sucrose in water. Gradients were formed by centrifugation in a Beckman 50Ti rotor for 20 min at 25,000 rpm (42,000*g*). Fractions (0.6 ml) were collected and the Percoll density in each fraction was monitored by measuring the refractive index which was converted into density values as described in the Pharmacia Percoll booklet.

Enzyme Assays. Gradient fractions were assayed for NADH-Cyt *c* reductase (8), Cyt *c* oxidase (17), glucan synthase I (19), IDPase[4] (14), aryl-mannosidase (29), glucosidase (25, 26), α-mannosidase I (27), GlcNAc transferase (5), fucosyltransferase (15), xylosyltransferase (15), and galactosyltransferase with ovalbumin as the sugar acceptor (22). Galactosyltransferase was also assayed with $(GlcNAc)_2Man_3(GlcNAc)_2Asn$ as the acceptor according to the procedure described for fucosyltransferase (15).

The incubation mixture for the α-mannosidase II assay contained 120 mM Mes/NaOH (pH 6.0), 0.1% Triton X-100, GlcNAc[^{3}H]$Man_5GlcNAc$ (4000 cpm), and various amounts of gradient fraction (10–50 μl) in a final volume of 200 μl. The assay mixtures were incubated at 37°C for 1 h. Free [^{3}H]Man was determined as described for the α-mannosidase I assay. [^{3}H] $Glc_3Man_9GlcNAc$ was prepared as described (25) and [^{3}H] $Man_9GlcNAc$ was obtained from phaseolin isolated from bean cotyledons labeled for 24 h with [^{3}H]mannose (24). GlcNAc[^{3}H] $Man_5GlcNAc$ was prepared as follows. [^{3}H]$Man_9GlcNAc$ was incubated with purified α-mannosidase I and the product [^{3}H] $Man_5GlcNAc$ was isolated. The product was incubated with GlcNAc transferase I partially purified from mung bean seedlings. The second incubation was carried out in the presence of swainsonine to inhibit the action of α-mannosidase II. The product, GlcNAc[^{3}H]$Man_5GlcNAc$, was reisolated. The details of this procedure will be described in a publication dealing with the purification and properties of α-mannosidase II (T Szumilo, GP Kaushal, AD Elbein, unpublished data). $GlcNAc_2$ $Man_3GlcNAc_2$ was prepared from human transferrin (23). Reaction products of α-mannosidase I and α-mannosidase II were analyzed according to Szumilo *et al.* (26).

RESULTS

Localization of ER and Golgi Marker Enzymes in Linear Sucrose Gradients. When cotyledons are homogenized in buffered sucrose containing 1 mM EDTA, ribosomes detach from the ER, yielding smooth membranes having a density of 1.125 g/ml on sucrose gradients (3). In the presence of $MgCl_2$, ribosomes remain associated with the rough ER, which has a density of 1.18 g/ml (3). The density of Golgi membranes (approximately 1.13 g/ml) remains constant under these conditions. Figure 2 shows that the distribution of bean cotyledon glucan synthase I, a Golgi marker enzyme, is unaffected by the presence or absence of $MgCl_2$ in the gradient, whereas the activity of NADH-Cyt *c* reductase, a marker for the endoplasmic reticulum (8), undergoes its characteristic shift to higher sucrose densities in the presence of $MgCl_2$. IDPase, a second Golgi marker enzyme (6), has the same activity profile as glucan synthase I on both gradient types, but a slightly broader peak (data not shown, but identical to the one shown in [2]). Aryl-mannosidase, which cleaves any terminal α-linked mannose residue, behaves like NADH-Cyt *c* reductase (Fig. 2, lower 2 panels). This confirms earlier findings (29) that aryl-α-mannosidase is localized in the ER of bean cotyledons.

Localization of the Oligosaccharide Processing Enzymes. Figure 3 shows the distribution patterns of glucosidase, α-mannosidases I and II, and fucosyltransferase activities on the EDTA and Mg^{2+} sucrose gradients. Glucosidase I, the first enzyme in the glycan modification pathway, is associated with membranes which show a density shift in the sucrose gradients similar to the ER marker enzyme NADH-Cyt *c* reductase. The assay used here is optimal for glucosidase I since it uses the $Gl_3Man_9GlcNAc$ substrate, but after removal of the terminal glucose, glucosidase II could remove the next two glucoses (26). Most likely, all

[4] Abbreviation: IDPase, inosine diphosphatase.

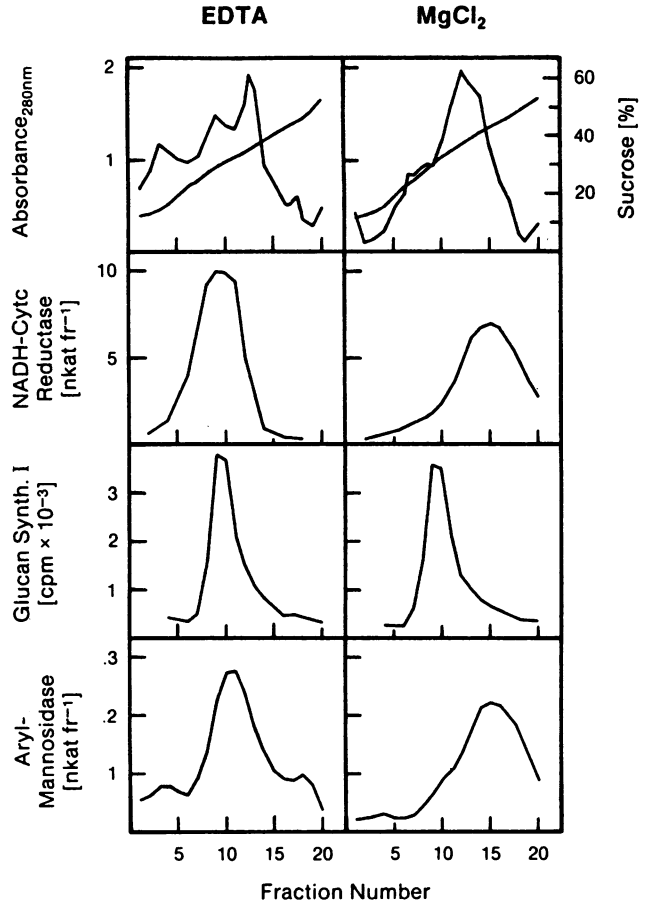

FIG. 2. Fractionation of organelles on isopycnic sucrose gradients. Cotyledons (1 g per sample) were homogenized in medium containing 1 mM EDTA or 2 mM $MgCl_2$, and all subsequent manipulations (Sepharose 4B chromatography, isopycnic sucrose gradient centrifugation) were done in the same media. Sucrose gradients (16–54% w/w sucrose) were centrifuged at 150,000*g* for 2.5 h. Fractions were assayed for absorbance at 280 nm, sucrose concentration, NADH-Cyt *c* reductase, glucan synthase I, and aryl-mannosidase.

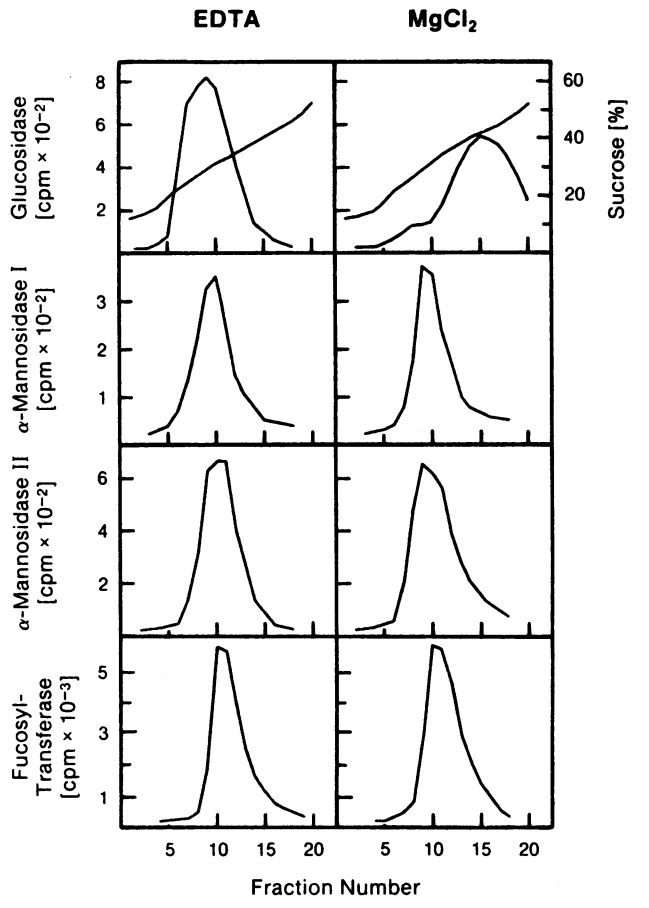

FIG. 3. Fractionation of organelles on isopycnic sucrose gradients. Details as in Figure 1. Fractions were assayed for sucrose concentration, glucosidase, α-mannosidase I, α-mannosidase II, and fucosyl-transferase.

glucosidase activity is associated with the ER.

When assaying the glycan-processing α-mannosidases, one must take care to use assay conditions that specifically differentiate among α-mannosidase I, α-mannosidase II, and aryl-mannosidase. α-Mannosidase I, which splits α1-2-linked mannose residues from high mannose oligosaccharides, requires Ca^{2+}, is inhibited by Zn^{2+} but not by swainsonine; it has a pH optimum between 5.5 and 6.0, and shows virtually no activity toward *p*-nitrophenyl-α-mannoside (13, 27). In contrast, aryl-mannosidase requires Zn^{2+}, has a pH optimum near 4.5, and is sensitive to swainsonine (27). Under our assay conditions for α-mannosidase I, the release of mannose from the substrate, $Man_9(GlcNAc)_2Asn$, was completely insensitive to swainsonine at a concentration (0.2 μg/ml) that inhibited aryl-mannosidase activity by 90% (data not shown). In addition, prolonged incubations resulted in the accumulation of $Man_5(GlcNAc)_2Asn$ as the sole glycopeptide product (data not shown). The results (Fig. 3) show that α-mannosidase I does not undergo a density shift in the sucrose gradients and is therefore associated entirely with the Golgi.

α-Mannosidase II of plants has no cation requirement and a pH optimum of 6.0 (data not shown). The analysis of reaction products under our assay conditions for α-mannosidase II with $GlcNAc[^3H]Man_5GlcNAc$ as a substrate identified the products as $GlcNAc[^3H]Man_3GlcNAc$ and free [^{3}H]mannose (data not shown). α-Mannosidase II, like α-mannosidase I, co-distributes with the Golgi membrane fraction on the EDTA- and Mg^{2+}-containing sucrose gradients.

Fucosyltransferase (Fig. 3, lower panels) as well as GlcNAc- and xylosyltransferases (data not shown) all co-distribute with glucan synthase I (Fig. 2) in both the EDTA and Mg^{2+} sucrose gradients, confirming earlier studies (15) that these glycosyltransferases from bean are Golgi-localized. We were unable to detect galactosyltransferase activity in any of the membrane fractions using either ovalbumin or $(GlcNAc)_2Man_3(GlcNAc)_2Asn$ as acceptors.

Subfractionation of Organelles on Sucrose Gradients. We tried to enhance any density differences between membrane fractions containing α-mannosidase I and fucosyltransferase by centrifuging the total organelle material in shallow sucrose gradients (22–44%). Samples were loaded either below the gradients (flotation) or above the gradients (centrifugation). In all cases, no significant differences in α-mannosidase I and fucosyltransferase distributions could be obtained (data not shown). Rate zonal centrifugation of organelles on shallow sucrose gradients (12–20% sucrose) showed that α-mannosidase I and fucosyltransferase containing membranes migrated with the same speed (data not shown).

Preparation of a Golgi-Enriched Fraction and Its Subfractionation on Percoll Gradients. When the organelle fraction from bean cotyledons is subjected to discontinuous Mg^{2+}-sucrose gradient centrifugation, the membrane band appearing at the 16/34% sucrose interface is enriched for Golgi enzymes. Table I shows that this fraction contains 83% of the total fucosyltransferase, 59% of the total IDPase, and only small amounts of

Table I. *Protein Content and Distribution of Enzyme Activities in the Membrane Fractions of Bean Cotyledons*

Cotyledon extracts were fractionated on discontinuous gradients made in media containing 2 mM $MgCl_2$. Membranes were collected from the 16%/34% interface, the 34%/54% interface, and the pellet. The fractions were assayed for protein, NADH-Cyt *c* reductase, fucosyl-transferase, Cyt *c* oxidase, and IDPase. Protein content and enzyme activities are expressed in total amount or total activity per fraction (fr).

Enzyme Activity	Membrane Fraction		
	16/34	34/54	Pellet
NADH-Cyt *c* reductase (nkat/fr)	2.5	21.7	0.1
Fucosyl-transferase (cpm/fr)	184×10^3	39×10^3	8×10^3
Cyt *c* oxidase (nkat/fr)	6.9	290.0	4.8
IDPase (nkat/fr)	27.1	14.3	4.8
Protein (μg/fr)	705	1758	396

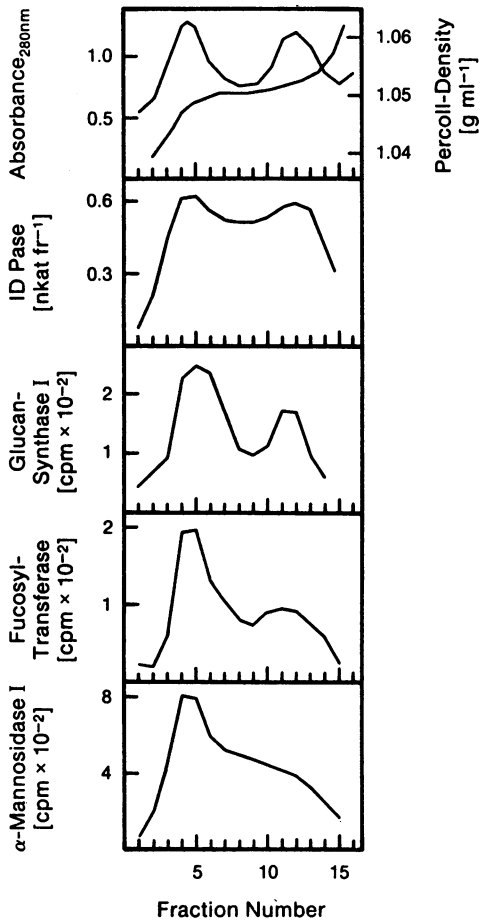

FIG. 4. Fractionation of Golgi vesicles on a Percoll gradient. A Golgi enriched fraction was prepared from cotyledons (3 g per sample) on a step gradient (16/34% w/w sucrose, containing 1 mM $MgCl_2$). The Golgi vesicles were resuspended in 0.25 M sucrose and fractionated on a linear Percoll gradient. Fractions were assayed for Percoll density, IDPase, glucan-synthase I, fucosyl-transferase, and α-mannosidase I.

NADH-Cyt *c* reductase (10%) and Cyt *c* oxidase (2%). This fraction is thus relatively free of ER and mitochondria. When these Golgi-enriched membranes were fractionated on an 8.5% Percoll gradient, two protein peaks were evident (Fig. 4). IDPase activity was evenly distributed in these peaks, whereas the activities of glucan synthase I, fucosyltransferase, and α-mannosidase I were clearly enriched in the lower density fraction. Xylosyltransferase and GlcNAc-transferase activities had profiles similar to that for fucosyltransferase (data not shown).

DISCUSSION

The purpose of this study was to determine the subcellular location of enzymes involved in the processing of N-linked oligosaccharides of plant glycoproteins and to find out whether these enzymes are localized in cisternae with different densities, thereby obtaining evidence for compartmentation within the Golgi complex. The modification of N-linked oligosaccharides in both plant and animal cells starts with the removal of 3 Glc residues by glucosidases I and II, followed by the removal of α,1 → 2 linked Man residues by α-mannosidase I. In bean cotyledons, glucosidase is associated with the ER, as it is in a number of animal tissues (12), while α-mannosidase I is associated with the Golgi. In animal cells, this enzyme is found in both the ER and the Golgi, and the removal of Man starts in the ER (2). We have isolated phytohemagglutinin (30) and phaseolin (24) from the ER, and found invariably that their glycans have a $Man_9(GlcNAc)_2$ structure. This agrees with the observation that the removal of Man does not start till the proteins reach the Golgi where α-mannosidase I is located. The enzymes for subsequent modifications of the N-linked glycans are also associated with the Golgi complex with the final removal of terminal GlcNAc residues in the protein bodies (31). The absence of Glc from the high mannose glycans in the ER shows that the removal of Glc occurs very fast, possibly co-translationally.

It has been proposed (11) that the sequential nature of the modifying reactions, which N-linked oligosaccharides undergo in the Golgi complex, is accomplished by the compartmentation of these enzymes in different cisternae of the Golgi. Thus, in some animal cells, early acting enzymes (α-mannosidase I) have been found in the cis-Golgi (18), GlcNAc transferase I in the medial Golgi (9), and late acting enzymes (galactosyltransferase) in the trans-Golgi (20). In addition, these different cisternae have been shown to have different densities in equilibrium density gradients (7, 10). Interestingly, not all cells conform to this pattern. While an *in situ* cytochemical technique has localized sialyltransferase to the trans cisternae of rat intestinal goblet cells, this enzyme is detected throughout the Golgi stack in adjacent absorptive cells (21).

Our attempts to find subcompartmentation of the Golgi processing enzymes in bean cells by density gradient centrifugation methods were unsuccessful. Although two major protein peaks of Golgi membranes were resolved in the Percoll gradient, a result for which there is precedence in the literature (7), we did not observe a differential distribution of the processing enzymes between these two subfractions. There are several possible explanations for this. First, the Golgi complexes of bean cells may have functionally compartmentalized cisternae which simply do not vary in density sufficiently to separate on gradients. Second, bean Golgi may have denser trans cisternae, but the enzymes we have measured are not sufficiently late in the modification pathway to show a different distribution on the gradients. The recent demonstration (28) that plant cells contain quite complex N-linked oligosaccharides suggests that plant Golgi complexes must contain such late acting enzymes. Whether they are in cisternae of greater densities remains to be determined. In a recent article, Ali *et al.* (1) claim to have shown that processing enzymes of N-linked oligosaccharides are associated with Golgi cisternae of different densities. However, to assay glycosyltransferases, they relied on endogenous acceptors or exogenous GlcNAc (for galactosyltransferase). It is not at all clear how the glycosyltransferase activities measured relate to the oligosaccharide processing enzymes, or whether they represent activities involved in polysaccharide biosynthesis as well as glycan processing. The third possibility is that the model for the compart-

mentation of processing enzymes across the cisternae stack according to their position in the processing pathway is not universally applicable to Golgi complexes. Recent immunocytochemical data with sialyltransferase (21) support this conclusion. Thus, in the bean cotyledons, all enzymes may be present in all cisternae in the Golgi complex.

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