

Spatial Organization of the Assembly Pathways of Glycoproteins and Complex Polysaccharides in the Golgi Apparatus of Plants

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Abstract. The Golgi apparatus of plant cells is the site of assembly of glycoproteins, proteoglycans, and complex polysaccharides, but little is known about how the different assembly pathways are organized within the Golgi stacks. To study these questions we have employed immunocytochemical techniques and antibodies raised against the hydroxyproline-rich cell wall glycoprotein, extensin, and two types of complex polysaccharides, an acidic pectic polysaccharide known as rhamnogalacturonan I (RG-I), and the neutral hemicellulose, xyloglucan (XG). Our micrographs demonstrate that individual Golgi stacks can process simultaneously glycoproteins and complex polysaccharides. O-linked arabinosylation of the hydroxyproline residues of extensin occurs in *cis*-cisternae, and glycosylated molecules pass through all cisternae before they are packaged into secretory vesicles in the

monensin-sensitive, *trans*-Golgi network. In contrast, in root tip cortical parenchyma cells, the anti-RG-I and the anti-XG antibodies are shown to bind to complementary subsets of Golgi cisternae, and several lines of indirect evidence suggest that these complex polysaccharides may also exit from different cisternae. Thus, RG-I type polysaccharides appear to be synthesized in *cis*- and medial cisternae, and have the potential to leave from a monensin-insensitive, medial cisternal compartment. The labeling pattern for XG suggests that it is assembled in *trans*-Golgi cisternae and departs from the monensin-sensitive *trans*-Golgi network. This physical separation of the synthesis/secretion pathways of major categories of complex polysaccharides may prevent the synthesis of mixed polysaccharides, and provides a means for producing secretory vesicles that can be targeted to different cell wall domains.

THE Golgi apparatus of both plant and animal cells is a highly polar organelle consisting of stacks of flattened cisternae (Robinson and Kristen, 1982; Farquhar, 1985). In animal cells, the Golgi apparatus forms a cap-like structure around one side of the nucleus and is comprised of many stacks interconnected by tubular elements (Rambourg and Clermont, 1990). In contrast, plant cells contain up to several hundred individual Golgi stacks, often referred to as dictyosomes, dispersed throughout the cytoplasm (Molenhauer and Morré, 1980). At the level of the stack, however, plant and animal Golgi apparatus exhibit many similarities, including the structural and cytochemical distinction of a *cis*-, or forming, face and a *trans*-, or maturing, face (Mollenhauer and Morré 1980; Farquhar and Palade, 1981; Robinson and Kristen, 1982).

The major function of the Golgi apparatus in animal cells is to receive proteins exported from the RER, to process these proteins biochemically, and then sort and package them before they are transported to their final destinations (Farquhar, 1985). Proteins enter the stack at its *cis*-face and appear to depart from the opposite *trans*-face (Tartakoff, 1980; Dunphy, 1985), and transport between the cisternal compartments is mediated by transport vesicles (Rothman and Orci, 1990). Each Golgi stack is comprised of three types of cisternae, termed *cis*, medial, and *trans*, based on

their position within the stack and their complement of enzymes, and processing of N-linked oligosaccharides of glycoproteins has been shown to proceed in a stepwise manner in a *cis*-to-*trans* direction (Hirschberg and Snider, 1987; Pfeffer and Rothman, 1987). The completed products destined for the plasma membrane, secretory granules, and lysosomes then leave the actual Golgi stack and enter yet another cisternal compartment, the *trans*-Golgi network, where they are sorted and packaged into separate types of transport vesicles for delivery to their appropriate cellular destination (Griffiths and Simons, 1986; Huttner and Tooze, 1989).

In plants, the synthesis and the processing of N-linked glycoproteins has been studied most intensively in seed tissues that produce copious amounts of storage proteins and lectins. The take-home message from these investigations is that the major processing steps and processing sites for N-linked glycoproteins are very similar in plant and animal cells (Chrispeels, 1985; Kaushal et al., 1988; Faye et al., 1989; Jones and Robinson, 1989). Thus, these proteins are cotranslationally glycosylated in the ER, transferred to the Golgi stacks in vesicles (Morre et al., 1989), and processed stepwise in a *cis*-to-*trans* direction, and, in the case of seed lectins and storage proteins, sorted into clathrin-coated vesicles (Harley and Beevers, 1989; Robinson et al., 1989) for

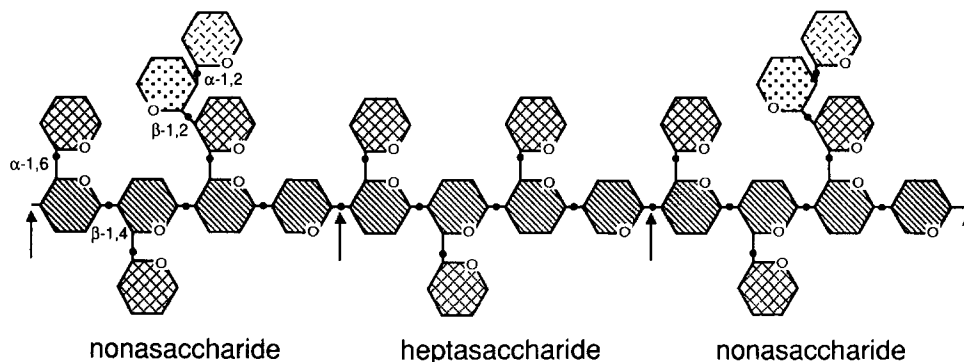


Figure 1. Schematic model of the proposed partial structure for XG. The heptasaccharide and nonasaccharide repeats are released upon endoglucanase treatment. (●) D-glucose; (⊗) D-xylose; (⊙) D-galactose; (⊕) L-fucose; and (—●) O-glycosidic bond.

transport to the storage vacuoles. Constitutive secretion of proteins occurs via a default pathway as in animal cells (Burgess and Kelly, 1987; Denecke et al., 1990).

The major synthetic products of the Golgi apparatus of most plant cells, however, are not glycoproteins but complex polysaccharides that are components of the cell wall (Robinson and Kristen, 1982; Moore and Staehelin, 1988). In dicots, the two main types of plant cell wall matrix polysaccharides are the neutral hemicelluloses and the acidic pectic polysaccharides. The most abundant hemicellulose is xyloglucan (XG;¹ Fig. 1), and the most abundant pectic polysaccharide is polygalacturonic acid/rhamnogalacturonan-I (PGA/RG-I; Fig. 2; Bacic et al., 1988; Fry, 1988). The major constituents of XG of young cell walls (mol wt 250–350 kD; D.P. 800–1,200; Hayashi and MacLachlan, 1984) are the hepta and nonasaccharide repeats shown in Fig. 1. In contrast, the RG-I portion of PGA/RG-I (D.P. 2,000; Fig. 2) contains up to 300 alternating galactosyl and rhamnosyl residues, and half of the rhamnosyl residues are branched at C4 with side chains of up to 15 glycosyl residues (McNeil et al., 1984). Only a small fraction of these side chains has been characterized to date (Bacic et al., 1988).

Both XG and PGA/RG-I serve structural roles. In addition, chemically defined fragments of these polysaccharides, termed oligosaccharins, have been shown to possess regulatory activities (Ryan, 1987). Since the biological activities of oligosaccharins are dependent on a precise molecular configuration, one must assume that error-free synthesis of at least some polysaccharides is essential. Conceptually, error-free production of complex polysaccharides may be achieved in two ways: one, by synthesis of polysaccharides by extremely specific enzymes capable of distinguishing between large numbers of very similar precursor molecules, or two, by segregating enzymes involved in the synthesis of different polysaccharides into separate compartments of the Golgi apparatus. This second approach still requires specific enzymes, but the degree to which they would have to distinguish between different types of precursors to prevent the synthesis of hybrid polysaccharides would be reduced.

A few of the enzymes involved in the synthesis of cell wall matrix polysaccharides have been identified, partially characterized, and determined to be localized to the Golgi apparatus (Bolwell and Northcote, 1984; Camirand and MacLachlan, 1986; Camirand et al., 1987). However, virtually

nothing is known about the organization of these enzymes in Golgi stacks, how polysaccharide synthesis is initiated, and how complex polysaccharides are assembled.

To shed light on some of these questions we have employed polyclonal antibodies raised against XG and PGA/RG-I (Moore et al., 1986; Moore and Staehelin, 1988) as well as the hydroxyproline-rich, structural cell wall glycoprotein, extensin-I (Stafstrom and Staehelin, 1988) in conjunction with immunocytochemical techniques to localize these molecules within plant cells actively involved in cell wall synthesis. In particular, our main goals were to (a) determine if a given higher plant Golgi stack can simultaneously synthesize glycoproteins and complex polysaccharides; and (b) elucidate the sites of assembly, sorting and packaging of XG and PGA/RG-I. Our findings indicate that a given Golgi stack can produce simultaneously glycoproteins and polysaccharides, and that the synthesis of different complex polysaccharides occurs in different Golgi cisternae. We also demonstrate that in expanding cortical parenchyma root tip cells most XG and RG-I molecules are packaged into separate vesicles and probably exit Golgi stacks at different sites. Thus, the traffic through and around Golgi stacks associated with the synthesis and packaging of complex polysaccharides in these parenchyma cells appears to differ from the traffic associated with the processing and packaging of glycoproteins.

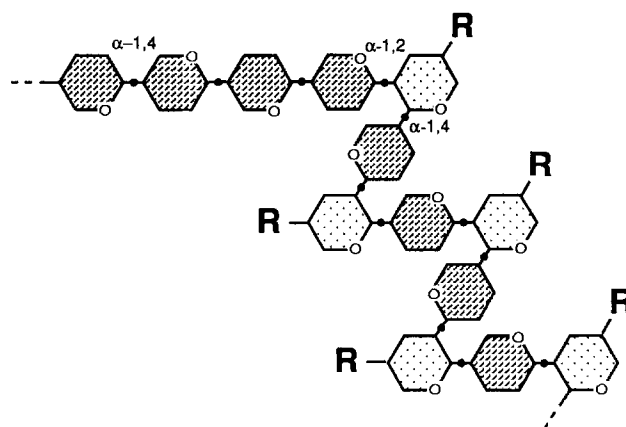


Figure 2. Schematic model of the proposed structure for a segment of a mixed PGA/RG-I polymer. The side chains (R) are composed primarily of arabinose, galactose, and small amounts of fucose and contain up to 15 sugar residues. (⊗) D-galacturonic acid; (⊕) L-rhamnose; (R) potential sidechains, up to 15 glycosyl residues; and (—●) O-glycosidic bond.

1. Abbreviations used in this paper: PGA/RG-I, polygalacturonic acid/rhamnogalacturonan-I; XG, xyloglucan.

Materials and Methods

Plant Material

Red clover (*Trifolium pratense*) and onion (*Allium cepa*) seeds were obtained from a local food market and germinated on moist filter paper for 48 h. Carrot suspension culture cells were obtained from Dr. Steven Wallner (Colorado State University, Fort Collins) and maintained as a callus culture on Murashige and Skoog (M & S) medium supplemented with 1 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D). Suspension cultures were grown in liquid M & S medium plus 2,4-D on a rotary shaker (150 rpm) at 25°C and were subcultured every 7 d.

EM

Root tips were fixed in 2.5% glutaraldehyde in 10 mM Na-phosphate buffer (pH 7.2) for 2 h at room temperature. After rinsing with the same buffer, the root tips were postfixed in 1% OsO₄ in ddH₂O for 1 h, washed three times with ddH₂O, and dehydrated in an ethanol series (30, 50, 70, 90, 100%; 10 min each step, room temperature). The root tips were infiltrated in 2:1 (vol/vol) ethanol/LR White resin (Polysciences Inc., Warrington, PA) for 1 h, 1:2 (vol/vol) ethanol/LR White for 2 h and 100% LR White overnight at 4°C (all other steps were carried out at room temperature). The infiltrated samples were embedded in gelatin capsules and allowed to polymerize overnight at 50°C. The carrot suspension cells were initially fixed for 5 min in 0.25% glutaraldehyde, 100 mM Na phosphate (pH 7.0), 0.2 M sorbitol and then for 4 h in 2.5% glutaraldehyde in the same buffer at room temperature. After rinsing in buffer plus sorbitol, the cells were dehydrated in a graded acetone series, treated with 0.5% hafnium chloride in 100% acetone for 20 min, washed with acetone, and transferred to 100% ethanol. The cells were infiltrated through increasing concentrations of the resin Lowicryl K4M (Polysciences Inc.): 2:1 and 1:2 ethanol/Lowicryl K4M and two times 100% Lowicryl K4M, 1 h each step. UV polymerization was for 16 h at -20°C, and 24 h at room temperature. We chose to embed in Lowicryl K4M rather than LR White, partly because suspension-cultured cells are often extracted after embedding in LR White, and partly because Lowicryl K4M increases the density of labeling.

Antibody Labeling

The anti-XG and anti-PGA/RG-I antibodies have been characterized as described in Moore et al. (1986), Moore and Staehelin (1988), Moore (1989), and Lynch, M.A., and L. A. Staehelin (manuscript submitted for publication), and the antiextensin-I antibodies as in Stafstrom and Staehelin (1988). The sections were treated with 0.1 N HCl for 10 min to remove glutaraldehyde (Craig and Goodchild, 1984), and then incubated in a blocking solution of 5% low fat dried milk (Carnation Co., Los Angeles, CA) in PBST (10 mM Na-phosphate, 500 mM NaCl, 0.1% Tween-20; blocking solution was made fresh daily) for 20 min. After the grids were blotted dry, they were incubated on primary antiserum diluted in PBST (anti-XG 1:10; anti-PGA/RG-I 1:4) for 30 min. The grids were then washed in a continuous stream of PBS containing 0.5% Tween-20 for 30 s and transferred to a solution of protein A-colloidal gold (prepared according to Slot and Geuze, 1985) diluted in PBST for 20 min. Excess protein A-gold was removed by rinsing with PBS containing 0.5% Tween-20 followed by a ddH₂O wash. After immunolabeling, the sections were stained with 2% uranyl acetate for 5 min and Reynolds lead stain (Reynolds, 1963) for 10 s. All steps were carried out at room temperature.

The double-labeling protocol for XG and PGA/RG-I is a modification of the procedure of Titus and Becker (1985; Moore, 1989). Sections were treated with 0.1 N HCl, 5% milk, the first primary antibody, and washed as above. Following this first primary antiserum, the grids were incubated in the smaller size gold probe (7 or 7.5 nm) for 30 min, washed with PBST, and blocked with an excess of protein A (0.2 mg/ml). The grids were then incubated with the second primary antiserum for 1 h, washed, and treated with the larger gold probe (12 or 17 nm) for 30 min. The grids were washed and stained as above. For the double-labeling experiments with antiextensin-I and anti-XG antibodies on carrot suspension culture cells, the same procedures were used except that the antiextensin antibodies were incubated overnight at 4°C in a humid chamber.

Monensin Treatment of Carrot Cells

Root tips were not used for the monensin studies because the drug does not readily diffuse into tissues (Morré et al., 1983). Therefore, carrot suspen-

sion-cultured cells were employed for these experiments. These cells have previously been used in monensin studies, and their response to the drug is well documented (Morré et al., 1983; Boss et al., 1984). Carrot cell clumps between 205 and 62 µm in diameter were collected from logarithmically growing cultures by sieving through two sizes of nylon mesh. The cells were washed in culture media without 2,4-D and concentrated by centrifugation. The cells were divided into flasks containing 15 ml of medium (minus 2,4-D) and either 30 µl 5 mM monensin in methanol (final concentration 10 µM) or 30 µl methanol alone (control). Flasks were returned to the rotary shaker and cells harvested and fixed for EM at 15-, 30-, and 60-min timepoints; the methanol control was processed at 30 min.

Statistical Analysis

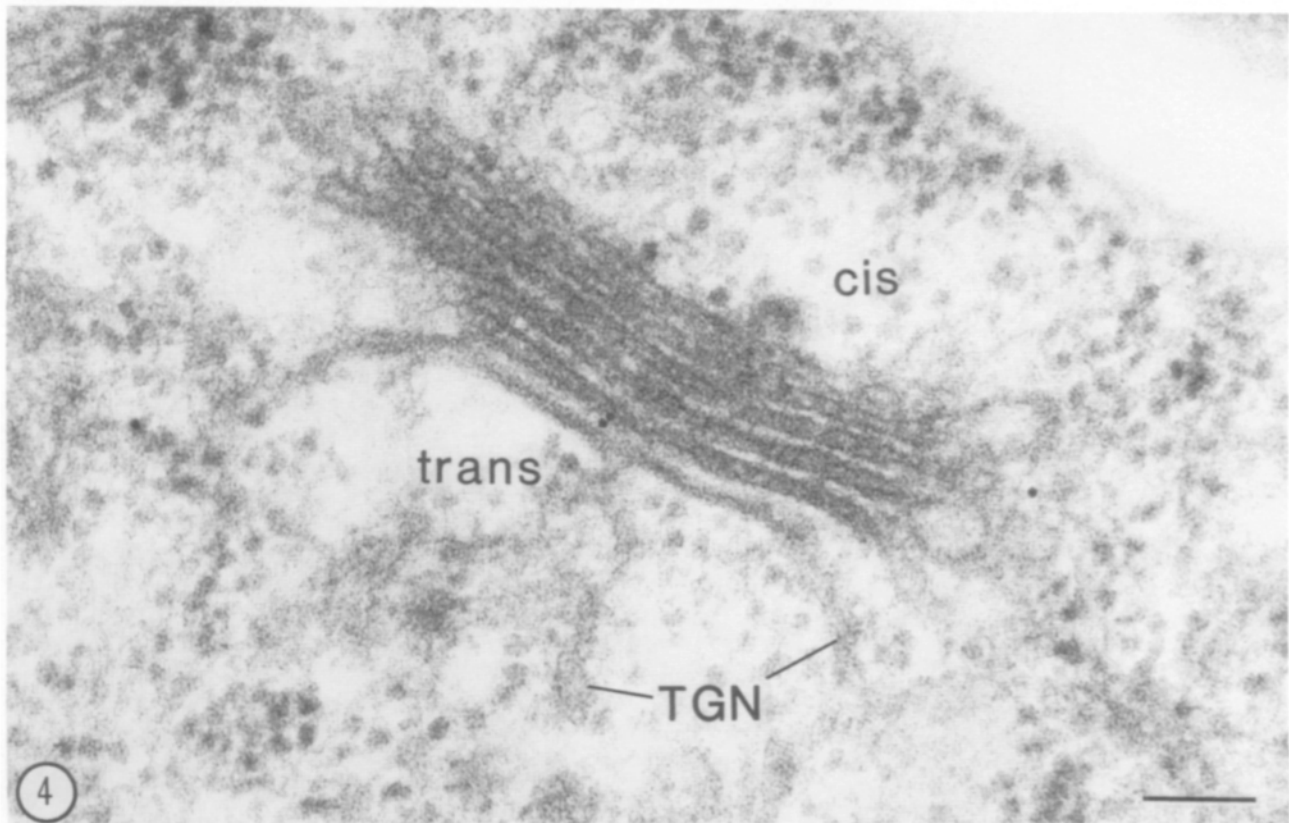
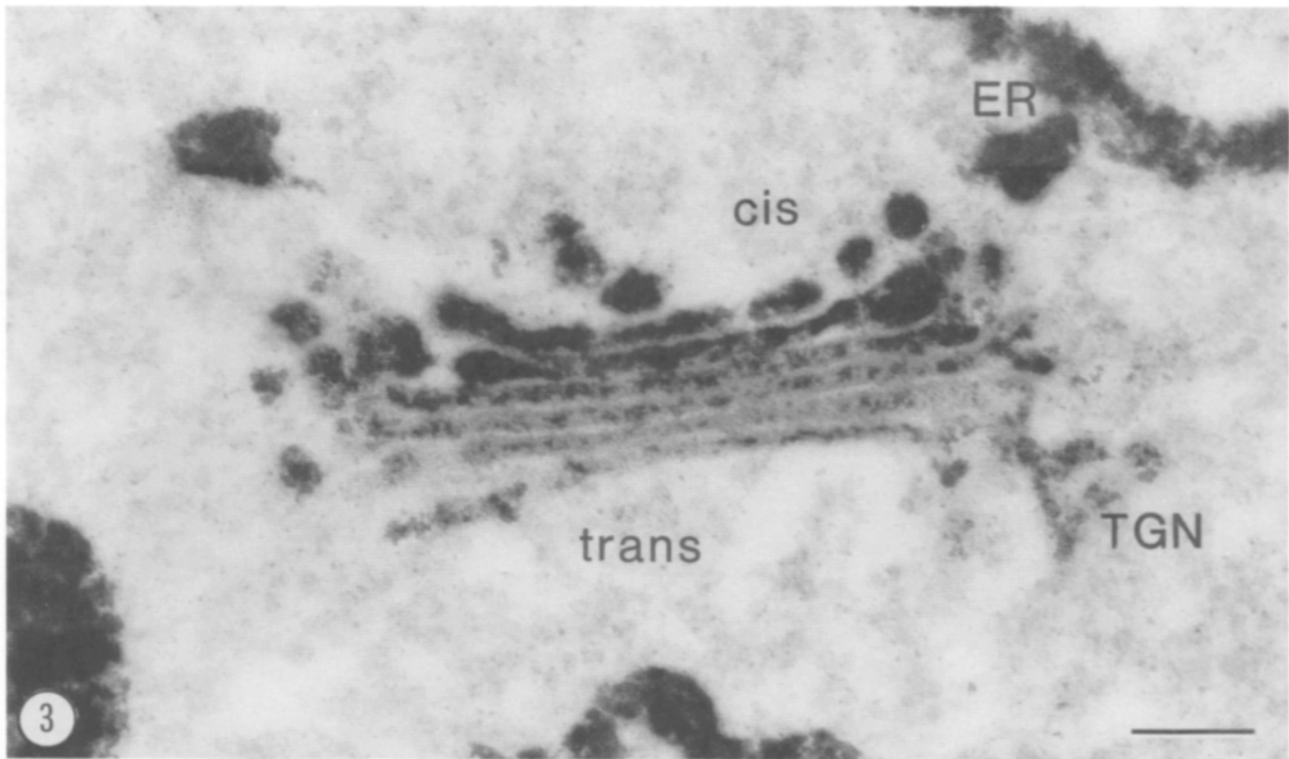
Standard goodness of fit tests (G-test; Sokal and Rohlf, 1981) were used to determine significant differences in the distribution of antibody label. The *cis-trans*-orientation of Golgi stacks was determined by morphological observations of cisternal width and spacing. The stacks were then subdivided into *cis*-, medial, and *trans*-compartments, each consisting of two cisternae. The number of gold particles over each type of cisterna was counted in order to localize the label to *cis*-, medial, or *trans*-cisternae. For the double-labeling experiments of secretory vesicles, only vesicles with two or more particles were counted. The measurements were performed blind by an independent observer.

Results

Selection of Cells for the Immunocytochemical Labeling of Golgi Stacks

Vascular plants appear to operate only constitutive secretory pathways; no regulated secretion has been found to date in any vascular plant system (Jones and Robinson, 1989). Most plant secretions involve cell wall components, polysaccharides and glycoproteins, and these macromolecules are only made and secreted during specific stages of cell expansion and/or cell differentiation (Bolwell and Northcote, 1981). Thus, in any given sample only a limited number of cells are at any one time actively involved in the secretion of a specific type of molecule, and even fewer cells are producing simultaneously two or more cell wall components at rates that are optimal for coimmunolocalization in Golgi stacks. These factors severely restrict both the numbers and types of cells available for analysis, as well as the combinations of molecules that can be studied in double-labeling experiments. To minimize possible problems associated with this type of variability, all of the micrographs of Golgi stacks used for the quantitative analysis of anti-XG and anti-PGA/RG-I labeling patterns were from expanding cortical parenchyma cells of similar developmental stages based on the morphology of the vacuoles. Cells chosen for these studies displayed several round vacuoles which occupied 30–50% of the cell volume. All micrographs of root tip Golgi stacks included in this paper are from red clover samples. The results from the onion root tip studies are presented only in a quantitative summarized form in the histogram (see Fig 9 b).

In growing tissues, extensin secretion commences just before the cessation of cell elongation and has therefore been implicated in growth control (Sadava et al., 1973; Lampport, 1980). Enhanced rates of extensin synthesis have also been reported in response to stress-related stimuli such as ethylene exposure, mechanical wounding, cold temperatures, and heat stress (Swords and Staehelin, 1989). Tentative observations (data not shown) suggest that whenever the synthesis of stress-related proteins such as extensin or β-1,3 glucanase is enhanced, immunolabeling of Golgi stacks with our anti-

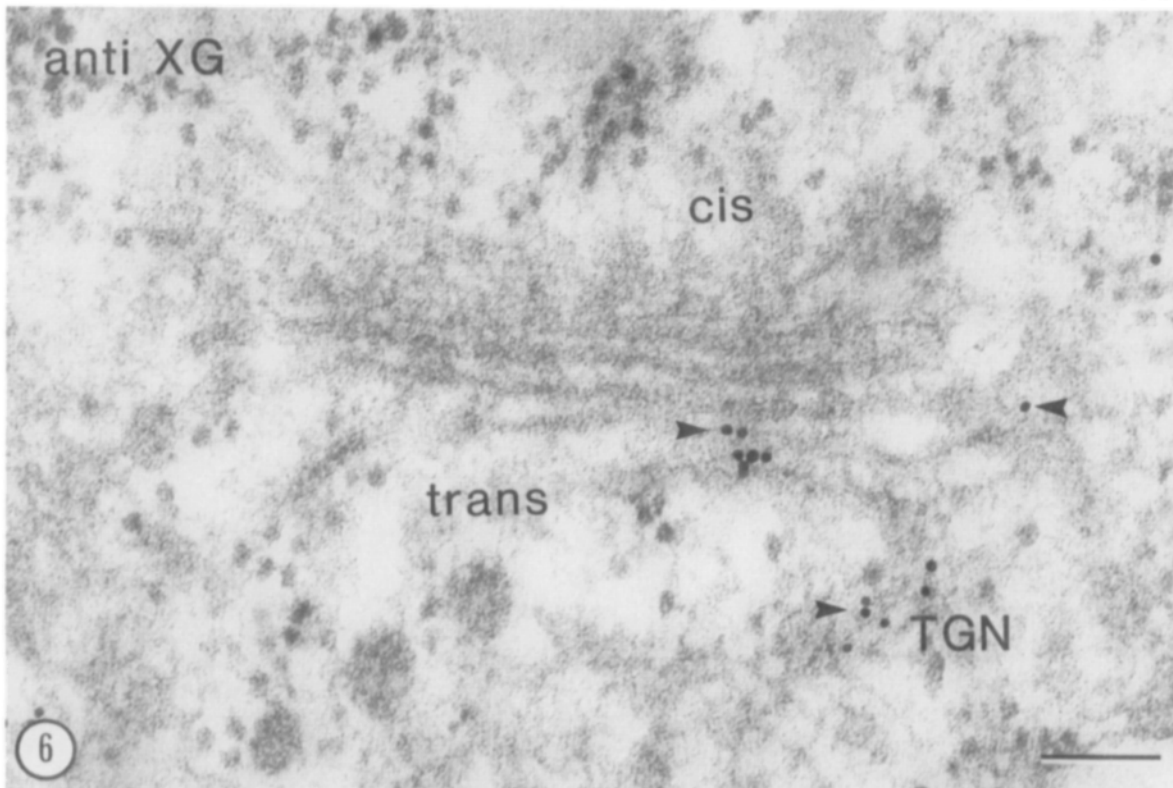
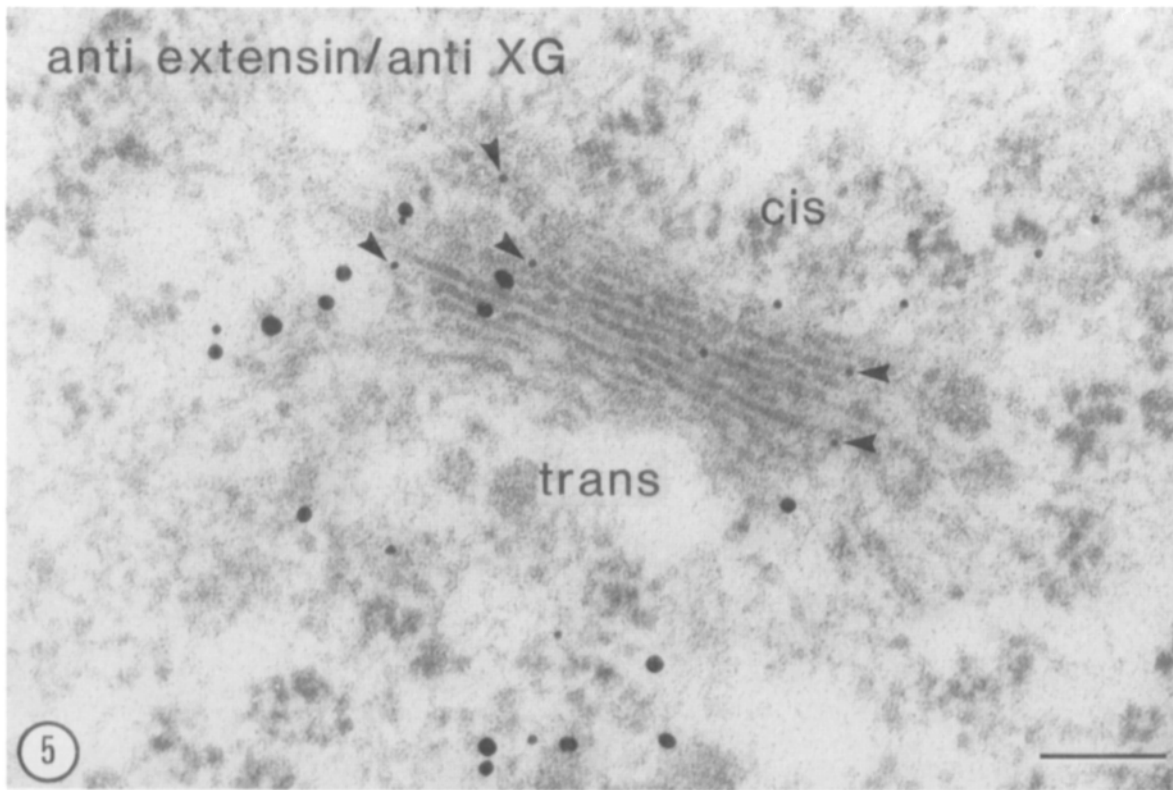


Figures 3 and 4. Thin-section electron micrographs of Golgi stacks in clover root tip cortical cells stained with zinc-iodine-osmium (Fig. 3) or by conventional means (Fig. 4). There is both a change in the staining pattern and a progressive decrease in luminal width of the cisternae in the *cis*-to-*trans*-direction. *Trans*-Golgi network (*TGN*). Bars, 0.1 μ m.

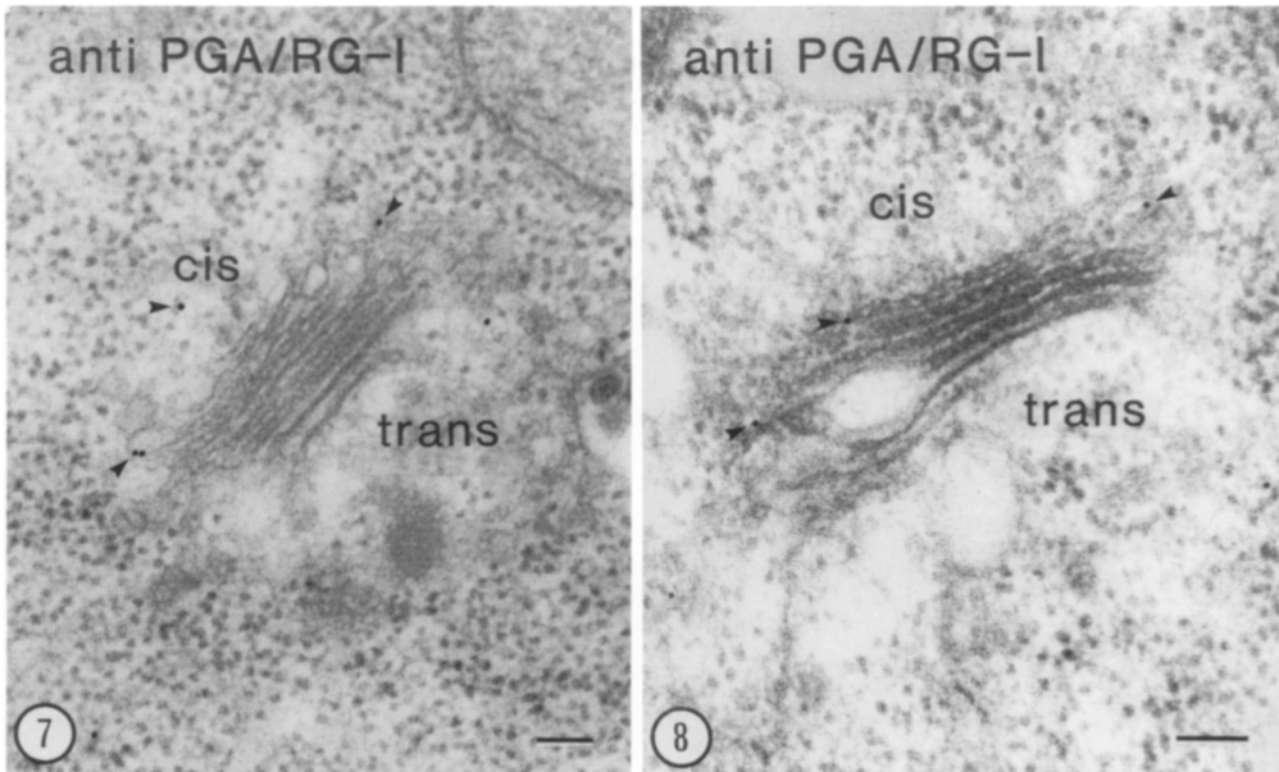
XG or anti-PGA-RG-I antibodies is significantly decreased. To circumvent these problems we had to switch to a carrot suspension culture system for our antiextensin-1/anti-XG-I double-labeling experiments.

Polarity Markers of Plant Golgi Stacks

Typical Golgi stacks of plant cells possess between four and eight flattened cisternae, and usually display a distinct struc-



Figures 5 and 6. (Fig. 5) Golgi stack of a carrot suspension culture cell double labeled with anti-extensin-1 (7 nm gold) and anti-XG (17 nm gold) antibodies. Note that the same Golgi stack labels with both antibodies with the extensin-1 labeling extending across the entire stack and the XG labeling being confined to the *trans*-side of the stack and the *trans*-Golgi network. (Fig. 6) Golgi stack of a clover root tip cortical cell labeled with anti-XG antibodies. All of the label is seen over *trans*-Golgi cisternae and the *trans*-Golgi network (*TGN*). Bars, 0.1 μ m.



Figures 7 and 8. Anti-PGA/RG-I-labeled Golgi stacks of clover root tip cortical cells. The antibodies do not label these Golgi stacks very strongly, but the consistent, preferential labeling of *cis* and medial cisternae (see histogram Fig. 9) can be discerned. Bars, 0.1 μm .

tural polarity (Figs. 3 and 4). This polarity is expressed in a variety of morphological parameters that can be examined in the electron microscope, including width of cisternae and stainability of cisternal contents, intercisternal spacing, thickness and stainability of cisternal membranes, location of intercisternal elements, density of freeze-fracture particles, and size and contents of attached secretory vesicles (Robinson and Kristen, 1982; Staehelin et al., 1990). However, in contrast to animal cells, a close spatial relationship between ER and *cis*-Golgi cisternae is only observed in a limited number of plant cell types. Two reliable polarity markers for plant Golgi stacks are histochemical stains such as the zinc-iodine-osmium stain illustrated in Fig. 3, and the width of cisternae in conventionally fixed and stained samples (Fig. 4). Zinc-iodine-osmium staining is most pronounced in ER and *cis*-Golgi cisternae, decreases progressively towards the *trans*-end of a stack, and often increases slightly again in the *trans*-Golgi network (Fig. 3). In conventionally fixed specimens the cisternal width decreases in a *cis*-to-*trans*-direction (Fig. 4).

Colocalization of Extensin and XG in Golgi Stacks

Glycoproteins destined for export, or for transfer to lysosomes or to vacuoles, are synthesized in the ER, and then sequentially transferred to *cis*-, medial and *trans*-cisternae for processing before being sorted in the *trans*-Golgi network (Hirschberg and Snider, 1987; Faye et al., 1989). This model predicts that antibodies raised against appropriate domains of a secretory glycoprotein should be able to immunolabel all cisternae of a Golgi stack actively involved in the process-

ing of this glycoprotein. Consistent with this model, our antiextensin-1 antibodies are seen to label virtually all cisternae of the carrot suspension culture cell Golgi stack shown in Fig. 5 (small gold particles), thereby confirming the usefulness of immunocytochemical techniques for localizing specific secretory products in plant Golgi stacks.

To determine whether a given Golgi stack is capable of processing glycoproteins and complex polysaccharides concomitantly, the same thin section was subjected to a second round of labeling with anti-XG antibodies (large gold particles). As seen in Fig. 5, several cisternae as well as possible elements of the *trans*-Golgi network are marked by both types of gold particles, suggesting that this Golgi stack was processing both types of molecules at the time of fixation.

Polarity of Golgi Stack Labeling with Anti-XG and Anti-PGA/RG-I Antibodies

As already demonstrated by Moore and Staehelin (1988) and confirmed in Figs. 5-7, our polyclonal anti-XG and anti-PGA/RG-I antibodies can be used to immunolocalize these complex polysaccharides to Golgi stacks. In such samples (Figs. 5-7) most of the labeling occurs over the swollen margins and not the interior regions of the individual cisternae, suggesting that the concentration of these molecules is highest in the inflated marginal domains of the cisternae. In addition, labeling with the anti-PGA/RG-I antibodies is always less dense than for the anti-XG antibodies. However, of greatest import appears to be the differential labeling across the stacks. Thus, as illustrated in Figs. 5, 6, and 10, virtually all of the anti-XG gold label appears to be concentrated over

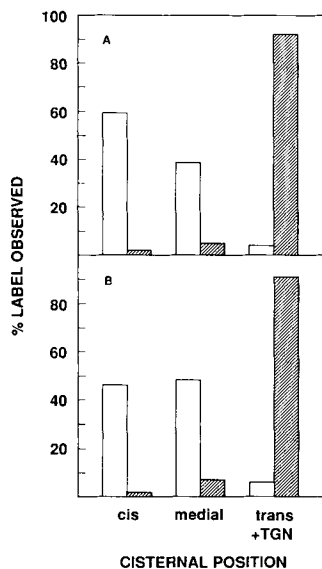


Figure 9. Histograms illustrating the immunolabeling patterns of Golgi stacks of expanding clover (A) and onion (B) root tip cortical parenchyma cells with the anti-XG and the anti-PGA/RG-I antibodies. The two antibodies appear to label complementary sets of cisternae, the anti-PGA/RG-I antibodies mostly *cis*- and medial cisternae, and the anti-XG antibodies preferentially *trans*-cisternae and the *trans*-Golgi network. (□) anti-PGA/RG-I; (▨) anti-XG.

trans-cisternae and the *trans*-Golgi network. In contrast, the anti-PGA/RG-I antibodies appear to label *cis*- and medial cisternae preferentially (Figs. 7 and 8). To confirm these qualitative observations, we have quantified the distribution of the anti-PGA/RG-I and anti-XG labels by counting the number of gold particles present over *cis*-, medial or *trans*-cisternae (including swollen margins) and *trans*-Golgi network in selected root tip cortical cells ($N = 50$ for each antibody). As seen in the histograms in Fig. 9, A and B (red clover and onion root tip samples), >90% of the anti-PGA/RG-I label was present over *cis*- and medial cisternae, and >90% of the anti-XG label over the *trans*-cisternae (including closely applied elements of the *trans*-Golgi network). Based on a statistical analysis of our data, we can reject the null hypothesis that label was uniformly distributed over the Golgi stack (G-test of significance [red clover samples]): for PGA/RG-I, $G = 76.255$, d.f. = 2, $P < 0.001$; for XG, $G = 409.245$, d.f. = 2, $P < 0.001$).

This nonuniform labeling of our anti-XG and anti-PGA/RG-I antibodies across plant Golgi stacks can be interpreted in several ways. For example, the anti-XG antibodies may not recognize precursor forms of XG, and therefore not label *cis*- and medial cisternae. Similarly, the anti-PGA/RG-I antibodies may not bind tightly to mature (esterified?) forms of these pectic polysaccharides and therefore label *trans*-cisternae only rarely. A second explanation would be that XG passes very quickly through *cis*- and medial cisternae and accumulates in *trans*-cisternae before secretion, giving rise to the heavy labeling of the latter cisternae with the anti-XG antibodies. For pectic polysaccharides (PGA/RG-I) the postulated rates of transfer would be the reverse, slow through *cis*- and medial cisternae, and fast through the *trans*-cisternae. A third possibility is illustrated in the model shown in Figure 14. This model predicts that the pectic polysaccharides are assembled in *cis*- and medial cisternae, and have the ability to leave in vesicles derived from medial cisternae, whereas the hemicellulose molecules such as XG are synthesized in *trans*-cisternae and are packaged into secretory vesicles in the *trans*-Golgi network. We have tested two features of this model, the packaging of PGA/RG-I and of XG into separate

secretory vesicles, and the effects of blocking transport from the *trans* cisternae, which should lead to the preferential accumulation of XG in the cytoplasm. The results of experiments designed to test these predictions are reported below.

Double Labeling of Secretory Vesicles with Anti-XG and Anti-PGA/RG-I Antibodies

To determine if XG and PGA/RG-I are packaged into separate secretory vesicles as suggested in the model shown in Fig. 14, sections of clover root tips were double immunolabeled with both anti-PGA/RG-I and anti-XG antibodies. Micrographs were taken of every Golgi stack encountered on every section examined. The number of vesicles with either large (12 nm) or small (7.5 nm) gold particles only, or both size classes of gold particles were counted, and only vesicles with two or more particles were scored. In instances where the vesicle membrane was not clearly seen, a vesicle was considered to be an area in a zone of ribosome exclusion in which two or more gold particles were present in close proximity. As seen in Table I, ~90% of the scored vesicles bound only one size class of gold particles, consistent with the hypothesis that in cortical parenchyma root tip cells most PGA/RG-I and XG molecules are packaged into separate secretory vesicles. The statistical analysis shows these results to be significantly different from an expected uniform distribution of labeling ($G = 105.86$, d.f. = 2, $P < 0.001$).

As a control experiment we tested whether the nonuniform distribution of label could be due to an inability of the small and large gold particles to bind in proximity to one another in the same vesicle. This was done by treating sections with the anti-XG antibody at both primary antibody incubation steps in the double immunolabeling protocol. Distribution of label within vesicles was determined as above. Of the 73 vesicles scored in this experiment, 54 (73%) were labeled with both sized gold particles. Therefore, our finding that most vesicles in the vicinity of anti-XG anti-PGA/RG-I double-labeled Golgi stacks bound only one size gold particle is not due to an inability to both size particles to bind to a single vesicle. Packaging of pectic polysaccharides into a subset of Golgi-derived secretory vesicles has also been observed in pollen of common poppy and honeysuckle using the JIM-5 mAbs against PGA (Pennell, R. I., G. N. Scofield, and K. Roberts, personal communication).

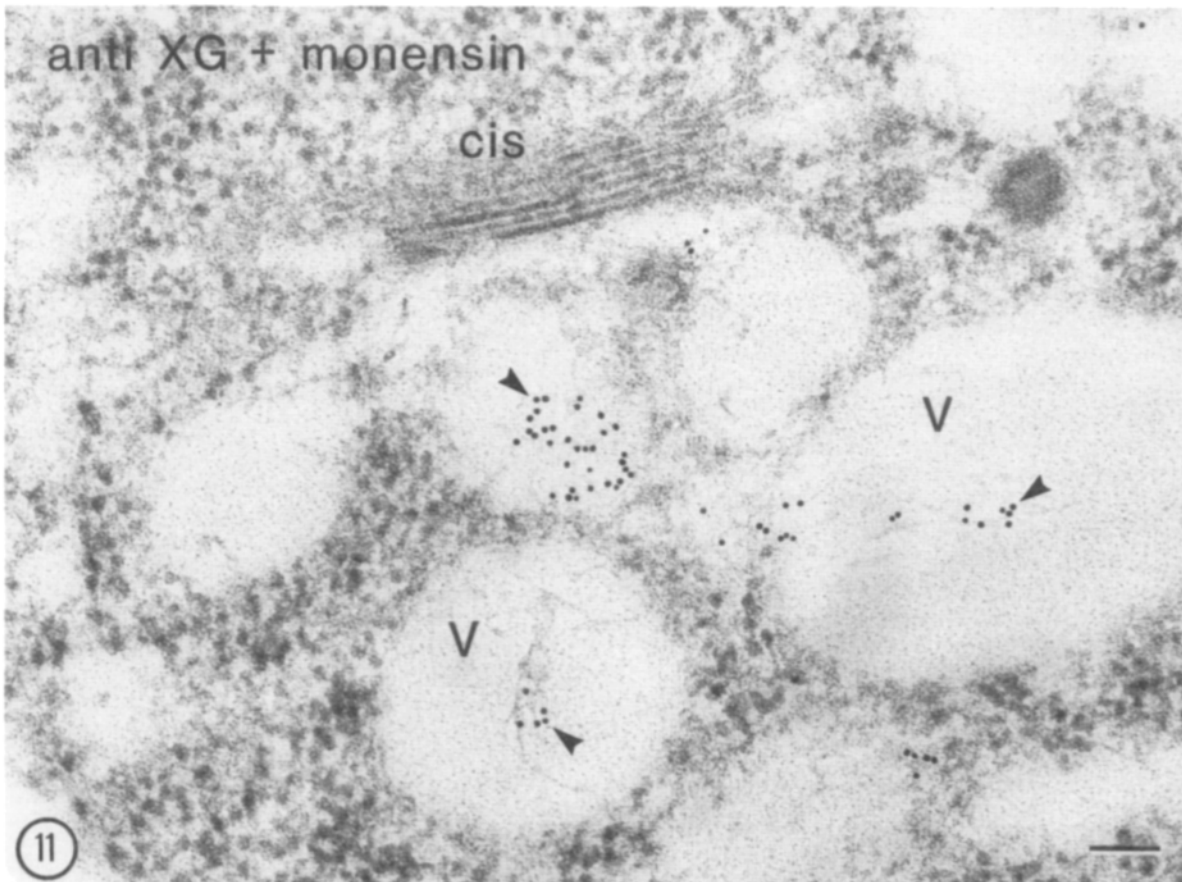
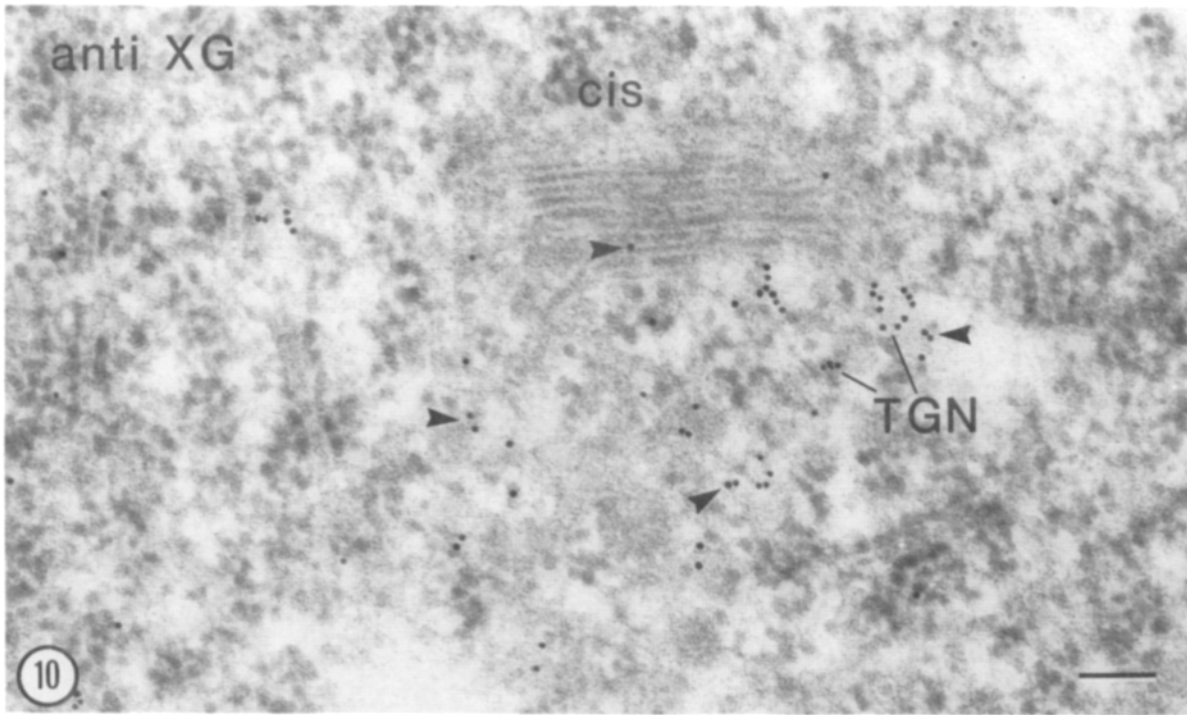
Immunolabeling of Golgi Stacks in Monensin-treated Cells

In animal cells, the carboxylic acid ionophore monensin has been shown to inhibit the transfer of secretory molecules from the Golgi apparatus to the cell surface, apparently by

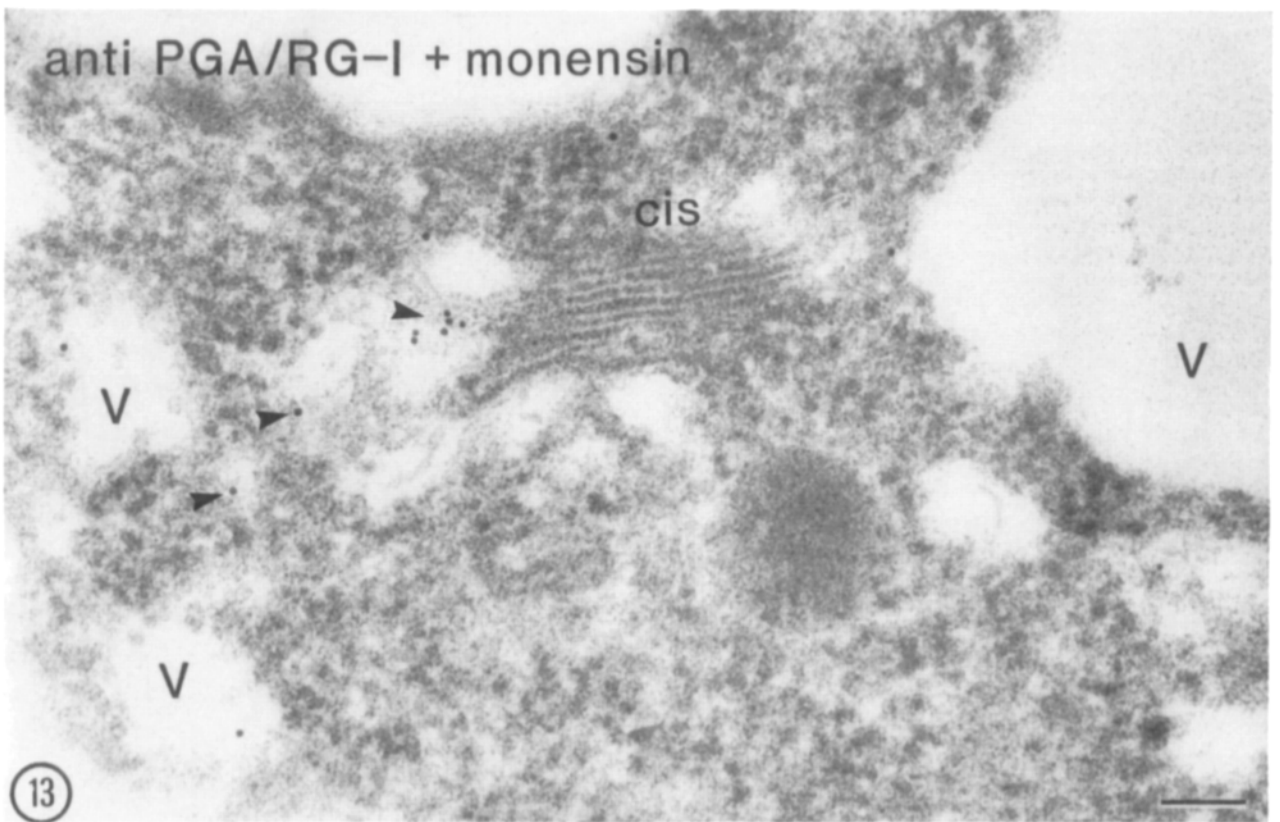
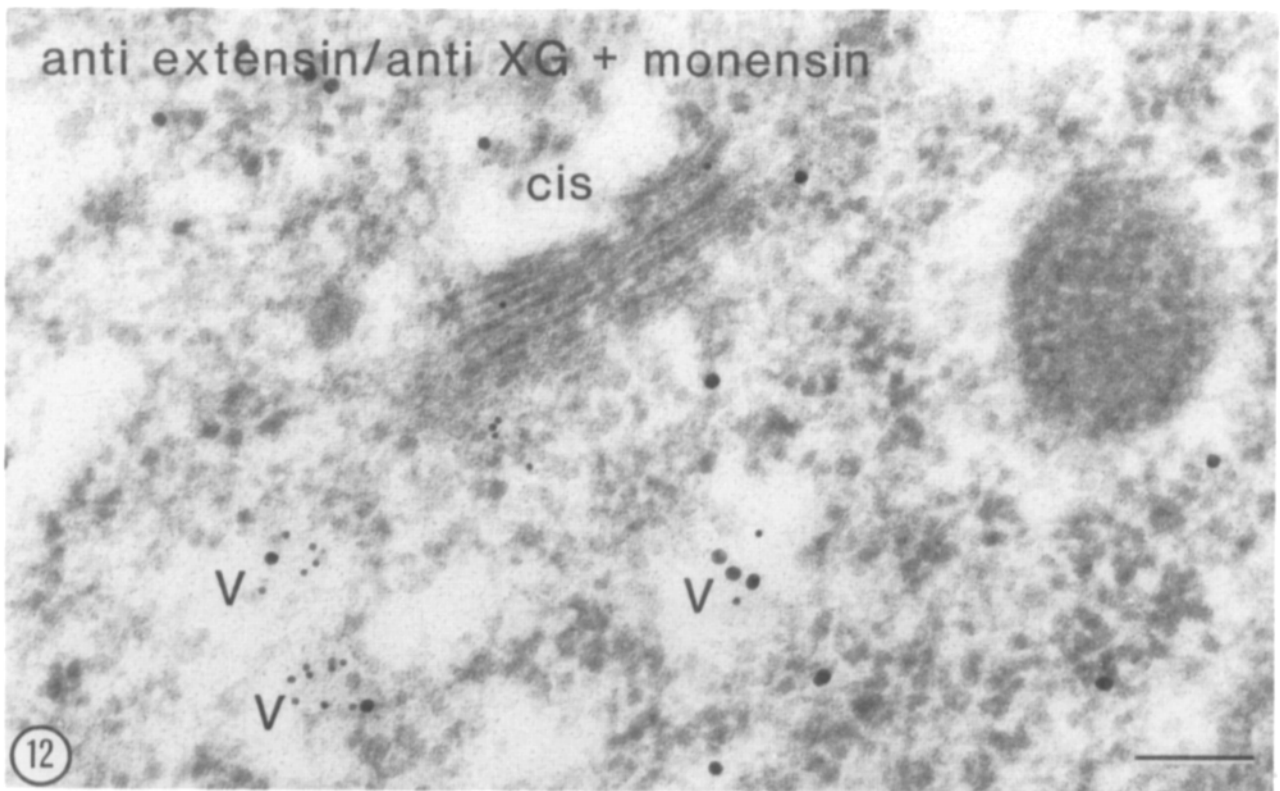
Table I. Double Labeling of Golgi Complexes with Anti-PGA/RG-I and Anti-XG Antiserum

Size of label	No. vesicles labeled*	Percent labeled
		%
12 nm (XG)	150	48
7.5 nm (PGA/RG-I)	137	43
Both	28	9

* With two or more gold particles.



Figures 10 and 11. (Fig. 10) Golgi stack and associated *trans*-Golgi network (*TGN*) of a carrot suspension culture cell labeled with anti-XG antibodies. All but one of the gold particles are seen over *trans*-cisternae and the *trans*-Golgi network. (Fig. 11) Anti-XG antibody-labeled Golgi stack of a carrot suspension culture cell exposed to monensin for 30 min. Note the heavy labeling of filamentous inclusions of the large swollen vesicles (*V*) associated with the *trans*-side of the Golgi stack that are not seen in control cells (Fig. 10). Bars, 0.1 μm .



Figures 12 and 13. (Fig. 12) Golgi stack and associated swollen vesicles of a monensin-treated (30 min) carrot suspension culture cell double labeled with antiextensin-1 (7 nm gold) and anti-XG (17 nm gold) antibodies. Note the presence of both sizes of gold particles in swollen vesicles (V) in the vicinity of the Golgi stack. (Fig. 13) Anti-PGA/RG-I antibody-labeled Golgi stack of a carrot suspension culture cell treated with monensin for 30 min. In contrast to the labeling with the anti-XG antibodies (Fig. 11), most of binding of these antibodies appears over small vesicles that appear to arise from the medial cisternae. Very few gold particles are seen over the large, swollen vesicles (V) that label heavily with the anti-XG antibodies. Bars, 0.1 μm .

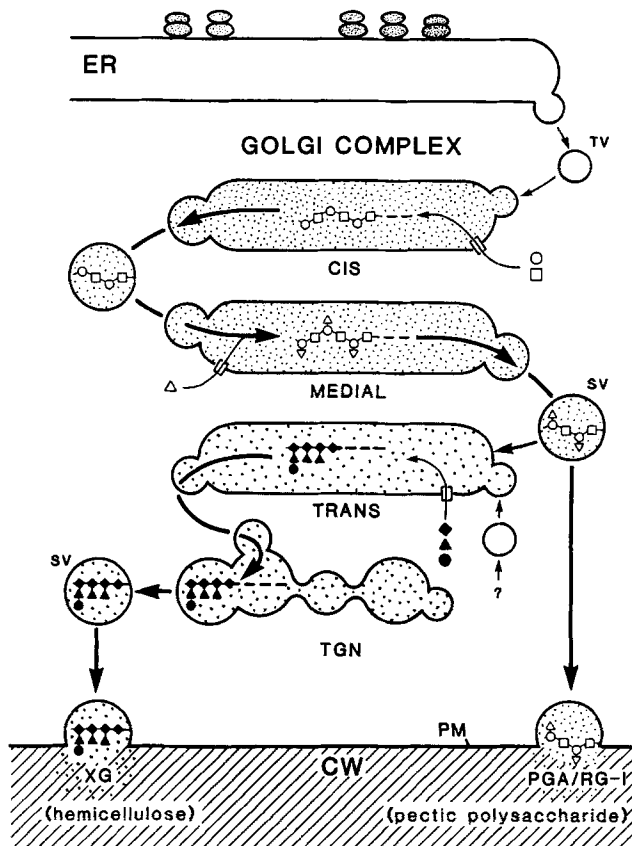


Figure 14. Diagrammatic model of the sites of synthesis and the traffic patterns of complex polysaccharides in the Golgi stacks of plant cells. The enzymes associated with the synthesis of the pectic polysaccharide RG-I are postulated to be confined to *cis*- and medial cisternae, and those involved in the synthesis of XG are postulated to be located in *trans*-cisternae and the *trans*-Golgi network. The model also suggests that RG-I type pectic polysaccharides have the potential to exit Golgi stacks directly from medial cisternae, whereas the XG molecules are shown to leave the Golgi complex from the *trans*-Golgi network, probably together with glycoproteins.

blocking Golgi functions such as sorting of products at or near the *trans*-face (Tartakoff, 1983; Morré et al., 1982). In carrot suspension culture cells, monensin produces an exchange of H^+ for Na^+ and K^+ ions, and thereby causes osmotic swelling of *trans*-Golgi compartments, their separation from Golgi stacks, and their accumulation in the cytoplasm (Boss et al., 1984). We have employed monensin to test the prediction of the model in Fig. 14 that XG but not PGA/RG-I exits Golgi stacks exclusively from *trans*-cisternae.

Fig. 10 illustrates the general morphology of a Golgi stack of a carrot suspension culture cell immunolabeled with anti-XG antibodies. As already shown for root tip Golgi stacks (Fig. 6), nearly all of the gold particles are localized over *trans*-cisternae and elements of the *trans*-Golgi network. Exposure of such cells to monensin leads to the accumulation of swollen vesicles in the vicinity of the *trans*-face of Golgi stacks (Fig. 11). Upon labeling with our anti-XG antibodies, large clusters of gold particles are seen over filamentous aggregates within these swollen vesicles (Fig. 11). Similarly, both extensin and XG appear to accumulate together in the swollen vesicular structures on the *trans*-side of Golgi stacks

in monensin-treated cells (Fig. 12). In contrast to these expected results, our anti-PGA/RG-I antibodies detect only a small amount of accumulation of this pectic polysaccharide in the swollen vesicles even after a 1-h exposure to monensin. Rather, much of the anti-PGA/RG-I label is still observed over small vesicles that are labeled with one or two gold particles as in control cells, and that appear to arise from the medial cisternae (Fig. 13). Thus, monensin appears to essentially block the secretory pathway for the glycoprotein, extensin, and for XG, but affect the secretory pathway for PGA/RG-I only to a limited extent.

Discussion

Current Limitations of Our Understanding of the Functional Organization of Plant Golgi Stacks

In animal cells, the Golgi apparatus processes N-linked and O-linked glycoproteins and assembles complex carbohydrate chains onto the protein core of proteoglycans. The only pure polysaccharide secreted by animals, hyaluronate, appears to be synthesized at the plasma membrane (Prehm, 1984). In contrast, the Golgi apparatus in plant cells not only is the site of processing of glycoproteins and proteoglycans, but also is the site of synthesis of complex matrix polysaccharides of the cell wall. It therefore possesses an additional set of synthetic capabilities that the animal cell Golgi apparatus appears to lack.

During the past ten years, remarkable progress has been made in the understanding of the biosynthetic pathways of secretory and lysosomal proteins in the Golgi apparatus of animal cells (reviewed by Kornfeld and Kornfeld, 1985; Burgess and Kelly, 1987; Huttner and Tooze, 1989; Rothman and Orci, 1990). These studies have not only identified the biochemical steps associated with the synthesis of numerous glycoproteins, and particularly N-linked glycoproteins, but have also shown where specific reactions occur, and have produced information on the transport and sorting machinery associated with this multifaceted organelle. Although parallel studies of the synthesis and assembly of N-linked glycoproteins of plant cells (mostly storage proteins and lectins) have progressed much more slowly, it appears as if most of the major processing steps have been evolutionarily conserved in plant and animal systems (reviewed by Chrispeels, 1985; Kaushal et al., 1988; Faye et al., 1989; Jones and Robinson, 1989). Thus, following their synthesis and cotranslational glycosylation in the ER, these proteins exit the ER in transition vesicles, enter the *cis*-face cisternae, move through the Golgi stack, and exit from the *trans*-Golgi network, where vacuolar proteins are sorted from proteins destined for the plasma membrane and the cell wall. However, the lack of a plant experimental system equivalent to the VSV G-glycoprotein system of animal cells, together with an inability to date of plant researchers to subfractionate isolated Golgi stacks into *cis*-, medial, and *trans*-cisternal fractions (Sturm et al., 1988), have severely hampered efforts to elucidate the spatial organization of enzymes and the traffic patterns associated with the processing of N-linked oligosaccharides.

This lack of detailed knowledge of the functional organization of plant Golgi stacks is even more evident in studies of the glycosylation of O-linked glycoproteins, which constitute

the bulk of the proteins secreted into plant cell walls (Jones and Robinson, 1989), and is most evident in investigations of the assembly of complex polysaccharides (Camirand et al., 1987; Bolwell, 1988; Carpita and Gibeau, 1988). Furthermore, since a given plant cell has the potential for synthesizing all these different types of molecules, although some may be produced sequentially (Bolwell and Northcote, 1981; Staehelin et al., 1990), it is difficult to narrow down the number of hypotheses of how the plant Golgi apparatus might work without knowing where specific types of molecules are made. With these questions in mind, we have used immunocytochemical methods to map the cisternal distribution of an O-linked glycoprotein and of representatives of two major classes of complex polysaccharides to learn more about the functional organization of plant Golgi stacks.

O-linked Arabinosylation of Extensin Commences in Cis-Cisternae

The polyclonal antiextensin-1 antibodies used in this study were raised against the wounding-stimulated, structural cell wall glycoprotein, extensin-1, from carrot discs (Stafstrom and Staehelin, 1988). Extensin belongs to the family of hydroxyproline-rich glycoproteins, and contains 45% hydroxyproline, nearly all in the sequence Ser Hyp₄. Carbohydrate makes up approximately two thirds of the glycoprotein's mass, 90% of which is arabinose linked to hydroxyproline as tetra-arabinosides (Swords and Staehelin, 1989). Our antiextensin-1 antibodies appear to bind nearly exclusively to the α -1,2-linked terminal arabinose residues of the homoarabinose side chains, and faintly recognize either the deglycosylated protein backbone or intermediate β -1,3-linked arabinose residues in oligosaccharide chains whose non-reducing terminal sugar ring has been opened by treatment with Na-periodate (Stafstrom and Staehelin, 1988; Swords, K. M. M., and L. A. Staehelin, manuscript submitted for publication). For this reason it was somewhat unexpected for us to see our antiextensin-1 antibodies bind not only to medial and *trans*-cisternae, but also to *cis*-cisternae. Based on this finding, we conclude that the *cis*-cisternae of plant Golgi stacks contain all the arabinosyl transferase enzymes needed not only to initiate but also complete the O-linked glycosylation of hydroxyproline residues of hydroxyproline-rich glycoproteins. In animal cells, the initial step of O-linked glycosylation involving the acquisition of N-acetyl-galactosamine has also been localized to *cis*-Golgi cisternae, but completion of the chains appears to occur in medial and *trans*-cisternae. Using cytochemical labeling techniques, Roth (1984) has shown this to be the case for mucin-type glycoproteins in intestinal goblet cells, and Tooze et al. (1988) have deduced a similar sequence of events for the glycosylation of the E1 glycoprotein of mouse hepatitis virus-A59 based on the differential temperature sensitivity of the different processing steps.

Since the antiextensin-1 antibodies can only detect arabinosylated products, but not the arabinosylation enzymes, our immunolabeled micrographs do not allow us to determine if the arabinose residues are added exclusively in *cis*-cisternae, or if some of the homoarabinosyl side chains are extended in medial and *trans*-cisternae. However, the observation of Kawasaki (1981) that two Golgi fractions derived from sycamore maple suspension cultured cells contain extensin-like molecules with different length arabinoside side chains sug-

gests that although arabinosylation is initiated in *cis*-cisternae, some of the oligosaccharide side chains may only be completed in medial or *trans*-cisternae.

Interpretation of the Antibody Labeling Patterns in the Context of Antibody Specificity

The polyclonal anti-PGA/RG-I antibodies were raised against purified RG-I-molecules derived from a fraction of deesterified sycamore maple pectic polysaccharides treated with endogalacturonase (Moore et al., 1986). Although the endogalacturonase treatment removes nearly all PGA tails from the RG-I molecules (Fig. 2), the bulk of our anti-PGA/RG-I antibodies can be preabsorbed with commercial preparations of deesterified PGA (Moore and Staehelin, 1988). Interestingly, the JIM-5 mAb that binds strongly to deesterified PGA does not crossreact with RG-I (Knox et al., 1990). Binding of the anti-PGA/RG-I antibodies to purified RG-I or deesterified PGA is not affected by galacturonic acid, galactose or rhamnose (Moore and Staehelin, 1988). Based on these results, we conclude that the bulk of our anti-PGA/RG-I antibodies recognize either the short deesterified PGA tails left on some of the RG-I molecules after the endogalacturonase treatment, and/or the PGA to RG-I transition region of these molecules (Fig. 2). More recent studies have shown that the anti-PGA/RG-I antibodies also recognize commercial preparations of pectins that contain between 27 and 71.5% esterified residues, the reaction being weaker with these esterified samples than with deesterified PGA (Lynch, M. A., and L. A. Staehelin, manuscript submitted for publication). No crossreactivity to XG, extensin-1, arabinogalactan, or the pectic polysaccharide RG-II has been detected in dot blots (Moore and Staehelin, 1988; Lynch, M. A., and L. A. Staehelin, manuscript submitted for publication).

One reasonable interpretation of the preferential binding of the anti-PGA/RG-I antibodies to *cis*- and medial cisternae (Fig. 9) is that the antibodies recognize "immature" but not "mature" forms of PGA/RG-I. The postulated final step in the processing of pectic polysaccharides involves esterification of >70% of the galacturonic acid residues, which makes the molecules soluble and readily transportable (Jarvis, 1984). As mentioned above, our anti-PGA/RG-I antibodies bind less efficiently to esterified pectins (Lynch, M. A., and L. A. Staehelin, manuscript submitted for publication), which could partly explain the virtual absence of labeling of the *trans*-face cisternae. To study this question further we have attempted to deesterify the pectic polysaccharides in our sections by both chemical and enzymatic procedures to render them more reactive with our antibodies. Unfortunately, these experiments have not led to clearcut results. Nevertheless, the fact that our anti-PGA/RG-I antibodies do bind to secretory vesicles, even where labeling of *trans*-Golgi compartments is negligible, tends to support the idea that at least some pectic polysaccharides may be able to bypass these latter compartments on their way to the cell surface.

The anti-XG antibodies were raised against an XG fraction derived from cell walls of sycamore maple suspension cultures (Moore et al., 1986). They do not crossreact with cellulose, PGA/RG-I, deesterified PGA, RG-II, arabinogalactan, or extensin-1, nor are they inhibited by fucose or xylose (Moore and Staehelin, 1988; Lynch, M. A., and L. A. Staehelin, manuscript submitted for publication). However, preabsorption with tamarind XG completely abolishes binding

to sycamore maple XG (Lynch, M. A., and L. A. Staehelin, manuscript submitted for publication). This finding is important in that tamarind XG differs from sycamore maple XG by the absence of terminal fucose residues on the side chains of the nonasaccharide-type segments shown in Fig. 1 (Farkas and Maclachlan, 1988). Based on these findings, we postulate that the anti-XG antibodies primarily bind to the glucose-xylose containing core domains of XG that constitute the bulk of these polymers.

To explain the minimal labeling of *cis*- and medial, and the heavy labeling of *trans*-Golgi compartments with our anti-XG antibodies (Fig. 9) one can postulate that generation of immunoreactive forms of XG may not occur until the XG precursor reaches the *trans*-cisternae, where further steps of synthesis are completed. Hayashi and Matsuda (1981a,b) have demonstrated, however, that xylosyl transfer does not occur onto preformed 1,4- β -glucan during XG synthesis; rather concurrent assembly of both glucose and xylose are obligatory. Thus, since the anti-XG antibodies seem to bind preferentially to the glucose-xylose core of XG, the anti-XG labeling pattern appears consistent with the idea that synthesis of XG occurs exclusively in *trans*-Golgi cisternae and possibly the *trans*-Golgi network.

Effects of Monensin on the Golgi Apparatus and on Secretion by Plant Cells

Monensin exchanges Na^+ , K^+ , and protons, and has been used extensively to study the function of the Golgi apparatus in a variety of animal and plant systems (Pressman and Fahim, 1982; Tartakoff, 1983; Boss et al., 1984; Sticher and Jones, 1988). In animal cells, monensin inhibits the secretion of proteins by impeding their transfer from the Golgi apparatus to secretory vesicles (Tartakoff, 1983). Most likely, monensin interferes with sorting of proteins in the *trans*-Golgi network by affecting the acidification of this compartment (Orci et al., 1984; Anderson and Parthak, 1985).

The literature on the effects of monensin on secretion by plant cells is very confusing. As shown by Boss et al. (1984), monensin-induced swelling of cisternae at the *trans*-face of Golgi stacks is dependent on the active pumping of protons into those cisternae, and the exchange of luminal protons with more osmotically active cations such as Na^+ and K^+ from the cytoplasm. The swollen cisternae detach from the Golgi stacks and accumulate in the cytoplasm (Fig. 11). Biochemical studies have demonstrated for barley aleurone cells that monensin blocks the secretion but not the synthesis of α -amylase and other enzymes (Heupke and Robinson, 1985; Melroy and Jones, 1986). In contrast, Sticher and Jones (1988) were unable to detect any effect of monensin on the secretion of polysaccharide slime by root tips of corn, even under conditions that inhibited secretion of α -amylase by corn scutellum cells. Driouch et al. (1989) have reported that monensin does not inhibit the secretion of acid hydrolases by sycamore maple suspension culture cells, even though it prevents the incorporation of fucose and N-acetylglucosamine into complex glycans. Yet another response to monensin has been described for developing cotyledons producing seed storage proteins. In such tissues monensin causes newly formed storage proteins to be secreted instead of being transferred to storage vacuoles (Craig and Goodchild, 1984; Bowles et al., 1986).

In this study, we demonstrate that in carrot suspension cul-

ture cells monensin leads to the accumulation of extensin-1 and XG in swollen vesicles on the *trans*-side of Golgi stacks (Fig. 12). This finding is consistent with the theory that monensin blocks secretion by interfering with sorting and/or packaging reactions required for the formation of secretory vesicles by the *trans*-Golgi network (Orci et al., 1984). However, at the same time that monensin appears to be blocking the export of XG and extensin-1, at least some secretion of PGA/RG-I seems to continue unaffected as evidenced by the presence of small, PGA/RG-I-containing vesicles in the cytoplasm and the minimal binding of the anti-PGA/RG-I antibodies to the large swollen XG-rich vesicles. Furthermore, as suggested by Fig. 13, the small PGA/RG-I vesicles may originate from medial cisternae, thereby providing a route for circumventing the monensin block of *trans*-face activities.

This postulate of plant Golgi stacks having two exit sites for secretory products can explain all of the seemingly contradictory effects of monensin on secretion by different types of plant cells as listed above. Thus, the observation of Sticher and Jones (1988) that monensin prevents the secretion of α -amylase, but not polysaccharide slime, suggests that polysaccharide slime can exit Golgi stacks from cisternae located ahead of the site of the monensin block, whereas the secretion of α -amylase depends on sorting and packaging functions associated with the monensin-sensitive *trans*-Golgi network. Similarly, the discovery that monensin inhibits the incorporation of the terminal sugars fucose and N-acetylglucosamine into complex glycans without preventing the secretion of acid hydrolases (Driouch et al., 1989) is consistent with monensin interfering with the access to and the functioning of *trans*-Golgi cisternae, but leaving the exit site associated with the medial cisternae unperturbed. Thus, secretion of the incompletely glycosylated hydrolases that continue to pile up across the Golgi stack would still be possible through secretory vesicles of the RG-I pathway that appear to originate from the medial cisternae. A similar "escape" of backed up seed storage proteins from medial cisternae to the cell surface would explain the monensin-caused redirection of these proteins from storage vacuoles to the cell wall (Craig and Goodchild, 1984; Bowles et al., 1986).

Functional Organization of Complex Polysaccharide Synthesis in Plant Golgi Stacks: a Novel Hypothesis

In our opinion, Fig. 14 presents in diagrammatic form the simplest explanation for the anti-XG and anti-PGA/RG-I labeling data reported in this paper. The main findings to be explained are (a) the localization of PGA/RG-I and XG to complementary cisternae of Golgi stacks; (b) the apparent segregation of PGA/RG-I and XG into separate types of secretory vesicles in cortical parenchyma root tip cells; and (c) the fact that XG accumulates exclusively in compartments that respond to monensin treatment by swelling, whereas PGA/RG-I appears to be found preferentially in compartments that do not swell.

The two main postulates of Fig. 14 are (a) that the RG-I type pectic polysaccharides are assembled in *cis*- and medial cisternae and that they can be packaged into secretory vesicles at the level of medial cisternae; and (b) that the neutral hemicelluloses (represented by XG) are synthesized exclusively in *trans*-cisternae and possibly the *trans*-Golgi network, and are packaged into secretory vesicles in this latter

compartment. The suggested separation of the assembly and secretion pathways for these two categories of complex polysaccharides of plant cell walls readily explains both the high degree of segregation of the two types of polysaccharides into different secretory vesicles, and the differential effects of monensin on the secretion of PGA/RG-I and XG.

At present, the evidence in support of the postulated XG pathway is stronger than for the postulated PGA/RG-I pathway. As discussed above, the anti-XG antibodies appear to bind to the glucose-xylose core of XG, and the assembly of glucose and xylose residues into the XG core polysaccharides occurs concurrently (Hayashi and Matsuda, 1981a,b). Thus it seems unlikely that the nonlabeling of *cis*- and medial cisternae with the anti-XG antibodies is due to their inability to bind to precursor forms of XG molecules. Furthermore, the accumulation of both XG and extensin-I in the same swollen *trans*-Golgi derived vesicles in monensin-treated cells is consistent with XG being sorted and packaged into secretory vesicles in the *trans*-Golgi network.

At this point, we cannot completely rule out the possibility that our anti-PGA/RG-I antibodies do not label the *trans*-compartments of Golgi stacks in cortical parenchyma cells of root tips because they are unable to bind efficiently to the mature, esterified forms of PGA/RG-I. This argument, however, is weakened by the findings that our anti-PGA/RG-I antibodies recognize highly esterified pectins on dot blots, and that they regularly label what appear to be secretory vesicles adjacent to Golgi stacks with labeled *cis*- and medial, but unlabeled *trans*-compartments. Furthermore, monensin seems to have little effect on the formation of small, PGA/RG-I-containing secretory vesicles that appear to arise in the vicinity of medial cisternae of Golgi stacks (Fig. 13), and only limited amounts of accumulation of PGA/RG-I can be detected in the swollen vesicles that form at the *trans*-face of monensin-treated Golgi stacks, even after a 1-h treatment (data not shown). Finally, the packaging of XG and PGA/RG-I into separate secretory vesicles is also most readily accounted for by the postulated physical separation of the two pathways.

The proposed model for the biosynthesis of complex polysaccharides in plant Golgi stacks differs from the widely accepted model for the N-glycosylation of animal and plant proteins as detailed by Kornfeld and Kornfeld (1985). During synthesis all glycoproteins pass through all compartments of the Golgi complex, and are successively modified by sets of enzymes that reside in each compartment. The separation of these oligosaccharide modifying enzymes into *cis*-, medial and *trans*-cisternae is a mechanism for controlling the sequential actions of the enzymes in time and space (Hirshberg and Snider, 1987). Control is also exerted by differences in substrate specificity of processing enzymes between tissues and species (Williams and Lennarz, 1984), and on the conformation of the peptide backbone (Green, 1982; Hunt et al., 1983).

In the absence of a peptide backbone, the precise synthesis of complex polysaccharides may be even more dependent on compartmentalization of the specific glycosyltransferases than is the case for the assembly of the oligosaccharide side chains of glycoproteins. Thus, the segregation of groups of enzymes within the Golgi complex, and the removal of finished polymers before they reach other groups of enzymes, appears to be the mechanism employed by plant cells

to produce some forms of highly specific complex polysaccharides, and to ensure that no hybrid polysaccharides are formed. Physical segregation of the biosynthetic pathways for pectic polysaccharides and hemicelluloses also reduces the need for specific polysaccharide sorting devices in the *trans*-Golgi network, and provides a simple mechanism for packaging the different polysaccharides into separate types of secretory vesicles that can be targeted to different cell wall domains. In the same manner, the redirection of seed storage proteins from vacuoles to cell corners in monensin-treated cotyledon cells (Craig and Goodchild, 1984) indicates that targeting of different types of complex polysaccharides to different cell wall domains could be important for normal growth and development of plants.

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