Functional Compartmentation of the Golgi Apparatus of Plant Cells

Immunocytochemical Analysis of High-Pressure Frozen- and Freeze-Substituted Sycamore Maple Suspension Culture Cells

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ABSTRACT

The Golgi apparatus of plant cells is engaged in both the processing of glycoproteins and the synthesis of complex polysaccharides. To investigate the compartmentalization of these functions within individual Golgi stacks, we have analyzed the ultrastructure and the immunolabeling patterns of high-pressure frozen and freeze-substituted suspension-cultured sycamore maple (Acer pseudoplatanus L.) cells. As a result of the improved structural preservation, three morphological types of Golgi cisternae, designated cis, medial, and trans, as well as the trans Golgi network, could be identified. The number of cis cisternae per Golgi stack was found to be fairly constant at approximately 1, whereas the number of medial and trans cisternae per stack was variable and accounted for the varying number of cisternae (3–10) among the many Golgi stacks examined. By using a battery of seven antibodies whose specific sugar epitopes are secreted polysaccharides and glycoproteins are known, we have been able to determine in which types of cisternae specific sugars are added to N-linked glycans, and to xyloglucan and polygalacturonic acid/rhamnogalacturonan-I, two complex polysaccharides. The findings are as follows. The β-1,4-linked α-glucosyl backbone of xyloglucan is synthesized in trans cisternae, and the terminal fucosyl residues on the trisaccharide side chains of xyloglucan are partly added in the trans cisternae, and partly in the trans Golgi network. In contrast, the polygalacturonic/rhamnogalacturonan-I backbone is assembled in cis and medial cisternae, methylesterification of the carboxyl groups of the galacturonic acid residues in the polygalacturonic acid domains occurs mostly in medial cisternae, and arabinose-containing side chains of the polygalacturonic acid domains are added to the nascent polygalacturonic acid/rhamnogalacturonan-I molecules in the trans cisternae. Double labeling experiments demonstrate that xyloglucan and polygalacturonic acid/rhamnogalacturonan-I can be synthesized concomitantly within the same Golgi stack. Finally, we show that the xylosyl residue-linked β-1,2 to the β-linked mannose of the core of N-linked glycans is added in medial cisternae. Taken together, our results indicate that in sycamore maple suspension-cultured cells, different types of Golgi cisternae contain different sets of glycosyltransferases, that the functional organization of the biosynthetic pathways of complex polysaccharides is consistent with these molecules being processed in a cis to trans direction like the N-linked glycans, and that the complex polysaccharide xyloglucan is assembled exclusively in trans Golgi cisternae and the trans Golgi network.

The Golgi apparatus of plant cells serves two major synthetic functions: it assembles and processes the oligosaccharide side chains of glycoproteins and proteoglycans, and it synthesizes the complex polysaccharides of the cell wall matrix (3, 31). Whereas the first function is common to plants and animals, the second is a unique feature of plant cells. We are interested in determining how these two types of synthetic pathways are spatially and functionally organized within the confines of an individual plant Golgi stack.

The initial glycosylation of N-linked glycoproteins occurs in the ER and involves the transfer of a high mannose 14-sugar oligosaccharide from a dolichol carrier to defined asparagine residues of the nascent polypeptide. Following the removal of three terminal glucosyl residues, the glycoprotein is transported to the Golgi apparatus, where it is exposed to a battery of glycosidases and glycosyltransferases that process the oligosaccharide side chain(s) in a specific manner. Many of the enzymes involved in these reactions as well as their intermediary products have been identified, and the sequence in which they operate has been determined (21). Although this pathway was initially elucidated in animal cells, subsequent studies have shown that in plant cells, N-linked glycoproteins are processed in a nearly identical fashion, with most of the differences involving the use of different types of terminal sugars (9).

The two major classes of complex polysaccharides synthesized by the Golgi apparatus in dicotyledonous plants are the neutral hemicelluloses and the acidic pectic polysaccharides (3). XG2 is the most abundant hemicellulose of the cell wall of growing dicotyledonous plants. This large polysaccharide (degree of polymerization up to 2200) is composed of a β-

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Abbreviations: XG, xyloglucan; PBST, phosphate buffered saline plus Tween-20; PGA/RG-I, polygalacturonic acid/rhamnogalacturonan-I; TGN, trans Golgi network.
1.4-linked glucosyl backbone that is decorated at regular intervals with xylosyl and xylosyl-galactosyl-fucosyl side chains to form characteristic hepta- and nonasaccharide repeats (14). Its main function is to produce cross-links between cellulose fibrils and thereby contribute to the overall strength of the wall and permit cells to regulate the rate and degree of their expansion (14).

PGA/RG-I is the most abundant pectic polysaccharide. It consists of two covalently-linked domains, blocks of PGA and blocks of RG-I. Most of the PGA blocks are methylesterified prior to secretion, and are acted upon by a pectinmethylesterase after arrival in the cell wall (18). RG-I is composed of a backbone of alternating galactosyl and rhamnosyl residues, with about half of the rhamnosyl residues carrying oligosaccharide side chains (1). Pectic polysaccharides define the pore size and degree of hydration of plant cell walls and contribute to cell adhesion (2, 18). Specific fragments of XG and PGA/RG-I have also been shown to serve as hormone-like regulatory molecules (40).

Biochemical fractionation, histochemical, and immunocytochemical studies have demonstrated that the Golgi apparatus of animal cells is composed of four types of cisternae, cis, medial, and trans, and the TGN, based on their position within a stack and the different sets of enzymes they contain (7, 13). In conjunction with pulse chase experiments, these investigations led to the postulate that newly synthesized glycoproteins are processed sequentially in cis, medial, and trans cisternae, and that the final products are sorted and packaged in the TGN for transport to their final destination (33). For example, there is general agreement that mannosidase I is a cis cisterna enzyme, that N-acetyl-glucosaminytransferase II is resident in medial cisternae, and that galactosyltransferase is localized to trans cisternae of the animal Golgi apparatus. This knowledge of the functional organization of the Golgi apparatus has proven immensely valuable for designing experiments to explore mechanisms of trafficking through the Golgi apparatus (24, 39).

The fact that all plant Golgi cisternae possess very similar densities, together with the lack of proven enzymic markers, has hampered efforts to study subcompartmentation of Golgi stacks derived from plant cells (4, 42), even though they exhibit a pronounced morphological polarity in electron microscopic images (36). Furthermore, because the structure of many types of complex polysaccharides is still poorly defined, it is difficult to even contemplate in a more than superficial manner how the glycoprotein and complex polysaccharide synthesis pathways might be integrated in plants. Complicating matters further, a study of high-pressure frozen/freeze-substituted root tips of Arabidopsis and Nicotiana has demonstrated that the architecture of plant Golgi stacks varies in a cell type-specific manner (41). This observation suggests that the functional organization of plant Golgi stacks might be tailored to meet the specific needs of different types of cells, an hypothesis that has received support from two recent immunocytochemical investigations. Thus, polyclonal antibodies raised against the pectic polysaccharide, PGA/RG-I, have been reported to bind specially to cis and medial Golgi cisternae of root tip cortical cells (31) and to trans cisternae and the TGN of peripheral root cap cells of clover (25).

To circumvent potential problems arising from tissue-specific differences in plant Golgi stacks, we have focused our current investigation on the functional organization of the Golgi apparatus of suspension-cultured sycamore maple cells. Using a battery of well-characterized antibodies, we have immunolabeled the high-pressure frozen/freeze-substituted cells to determine in which types of morphologically defined Golgi cisternae specific sugars are added to glycoproteins and complex polysaccharides. Our data imply that the assembly of pectic polysaccharides involves cis, medial, and trans types of Golgi cisternae, whereas the synthesis of the hemicellulose, XG, is confined to trans Golgi cisternae and the TGN. Moreover, the addition of xylose to N-linked glycoproteins in the medial cisternae suggests that the enzymes of the N-linked glycosylation pathway are similarly organized in the plant and animal Golgi apparatus.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

Suspension-cultured sycamore maple (Acer pseudoplatanus L.) cells were kindly provided by Dr. Michael G. Hahn (Complex Carbohydrate Research Center, University of Georgia, Athens, GA) and cultured in modified M6 medium (44) on a shaker (125 rotations/min) at 25°C in the dark. The cells were subcultured every 7 to 10 d.

High-Pressure Freezing

Five- to seven-day-old cultures were harvested by centrifugation (1000g) and mixed in a ratio of 1:10 (v/v) with 25% (w/v) aqueous dextran (Mr 38,800) or 200 mM sucrose in fresh culture medium to optimize the percentage of well-frozen cells. The concentrated cell suspension was then poured onto a 30 μm nylon mesh in a Petri dish, and immediately prior to their transfer to the specimen cups for freezing, the cells were further concentrated by lifting a corner of the nylon mesh out of the fluid. Specimens were frozen in a Balzers HPM 010 high-pressure freezing apparatus as described by Craig and Staehelin (6), and were stored in liquid nitrogen prior to freeze-substitution.

Freeze-Substitution, Infiltration, and Embedding

The frozen specimen cups were transferred to precooled 1.8-mL cryogenic vials filled with 2.0% (w/v) osmium tetroxide and 8.0% 2,2-dimethoxypropane in acetone as a substitution medium. Substitution was carried out at −78.6°C in a dry ice/acetone bath for 2.5 to 3.0 d. After slow warming (2 h at −20°C, 2 h at 4°C, and 2 h at room temperature), the substitution medium was discarded and the samples rinsed in acetone. To ensure proper infiltration of the cell walls with the resin, the following infiltration schedule was used: 10% Epon 812:acetone for 14 to 16 h, 25%, 50%, 75% Epon 812:acetone each for 24 h, and 100% Epon 812 resin for 48 h with a change of resin after 24 h. The Epon-embedded samples were polymerized under vacuum at 55°C for 22 h. Specimens to be embedded in LR White were first subjected to an ethanol wash after the acetone rinse and then infiltrated with mixtures of the same percentages of LR White:ethanol and times as for the Epon-embedded samples. Polymerization was carried out at 52°C for 24 h.
Thin sections (about 0.1 μm) were cut on a Reichert Ultracut E (American Optical, Buffalo, NY) and picked up on formvar-carbon-coated copper or nickel grids (300 mesh, Polysciences, Warrington, PA). The Epon-embedded sections were mounted on copper grids and counterstained with 2% (w/v) uranyl acetate in water for 5 min and lead citrate for 50 s, and examined at 80 kV in a Philips electron microscope. Nickel grids were used exclusively for the antibody labeling experiments.

Antibody Probes

The rabbit polyclonal anti-XG and anti-PGA/RG-I antibodies were raised against purified sycamore maple XG and RG-I, respectively (29). Their characterization has been described in great detail by Lynch and Staehelin (25). The polyclonal anti-βXyl antisera was kindly provided by Dr. Maarten Chrispeels (University of California San Diego, La Jolla, CA) and has been characterized as described in Laurière et al. (22). The rat monoclonal JIM7 antibody that recognizes methylesterified PGA (19) was a generous gift of Dr. Paul Knox (John Institute, Norwich, England). Dr. Michael Hahn kindly supplied us with aliquots of the CCRC-M1, CCRC-M2, and CCRC-M7 mouse monoclonal antibodies. The CCRC-M1 antibody (35) recognizes the terminal fucosyl residue of the trisaccharide side chain of XG in the context of an extended XG chain, but not in the context of an isolated nonasaccharide fragment. CCRC-M2 is an RG-I-specific antibody that does not bind PGA. CCRC-M7 recognizes an arabinosyl-containing epitope on RG-I, which suggests that it recognizes a side chain of RG-I, but the precise structure of the epitope has not yet been determined (M. Hahn, personal communication).

Antibody Labeling Procedures

The LR White sections were first blocked for 30 min in a 5% (w/v) (for polyclonal antibodies) or a 3% (w/v) (for monoclonal antibodies) solution of nonfat dried milk in PBST (10 mM Na-phosphate, 500 mM NaCl, pH 7.2, 0.1% Tween-20; however, the salt concentration in PBST for monoclonal antibodies was 50 mM NaCl). After wicking away the blocking solution, the grids were incubated on the primary antiserum diluted either 1:10 (anti-XG, anti-PGA/RG-I, anti-βXyl, CCRC-M1, CCRC-M2, and CCRC-M7) or 1:1 (JIM7) in PBST. Incubation was for 2 h at room temperature for the polyclonal antibodies, and overnight at 4°C for the monoclonal antibodies. The grids were washed in a continuous stream of PBST containing 0.5% Tween-20 for 30 s and then transferred onto a secondary dilution in PBST containing either colloidal gold (10, 15, or 20 nm) coupled to protein A (Janssen Pharmaceuticals, B-2430 Olen, Belgium) for the anti-XG, anti-PGA/RG-I, and anti-βXyl antibodies, and to goat anti-rat antibodies (Biocell Research Laboratories, Cardiff, UK) for the JIM7 antibodies, or to goat anti-mouse immunoglobulin G + immunoglobulin M (Biocell Research Laboratories) for the CCRC-M1, M2, and M7 antibodies. After 45 min on the secondary solution, the grids were first rinsed for 30 s with PBST (0.5% Tween-20) and then with dH2O for 30 s. After immunolabeling, the sections were stained with 2% aqueous uranyl acetate for 4 min, and Reynolds’s lead citrate for 15 to 20 s. For the deesterification experiments, the sections were first incubated with 0.1 mM Na2CO3 overnight at 4°C prior to the blocking step. Unless otherwise noted, all incubations were at room temperature.

The double labeling protocol for JIM7 and anti-XG antibodies is a modification of the procedure of Moore (28). Sections were incubated with 3% milk in PBST solution, JIM7 antibody, and washed as above. Following this primary antisera, the grids were incubated in the smaller size gold probe (10 nm) for 45 min, washed with PBS (as above) containing 0.5% Tween-20, and blocked with an excess of protein A (0.15 mg/mL) for 20 min followed by 20 min incubation in a 5% milk solution. The grids were then incubated with the second primary antisera for 2 h, washed, and treated with the large gold probe (20 nm) for 45 min. The grids were washed and counterstained as above.

Quantitative Analysis

The statistical analyses were performed on micrographs of cross-sectioned Golgi stacks randomly selected from whole sections, and which possessed the clarity with which the cisternae could be discerned. The morphological criteria used for identifying cis, medial (early and late), and trans types of Golgi cisternae, as well as elements of the TGN, are defined in “Results.” Generally, cis cisternae possess the most irregular contour and are the most difficult to visualize. Some small-sized Golgi stacks lack identifiable cis cisternae due to the absence of differences in staining pattern between the cis face region and adjacent regions in the cytoplasm. Medial and trans cisternae were easily distinguished. Identification of the TGN was based on the intercisternal spacing between the two structures and the presence of rounded, branched vesicles.

To count the number of gold particles over the Golgi stack and the associated TGN, about 50 micrographs of Golgi stacks with clearly defined cisternae and heavy labeling (except in the case of the anti-PGA/RG-I antibody-labeled samples before Na2CO3 treatment) were chosen for each analysis. Gold particles over intercisternal spaces were counted as “half” particles for each type of compartment.

RESULTS

Variation in Golgi Stack Size and Morphology

The morphology of the Golgi stacks of suspension-cultured sycamore maple cells is remarkably variable (Figs. 1–5). This variability expresses itself most notably in the number of cisternae, but also in their diameter, the ratios of cisternal types, and the presence/absence and size of the TGN. Whereas the greatest differences are seen between Golgi stacks of different cells, it is not uncommon to observe Golgi stacks with different numbers of cisternae and with different diameters within a single cell.

Figures 1 and 2 depict typical images of Golgi stacks of freeze-substituted and high-pressure frozen suspension-cultured sycamore maple cells embedded in Epon 812. Their
cisternae display a cis to trans variation in morphology, and with five cisternae they fall into the category of "average" Golgi stacks in terms of size. Characteristic of the Golgi stacks of the suspension-culture cells used in this study, the one shown in Figure 1 displays a distinct TGN close to the transmost cisterna, whereas the stack illustrated in Figure 2 exhibits no TGN. Indeed, within a random sample of 236 Golgi stacks examined, only 137 (52%) displayed any association with membrane structures resembling a TGN on the trans side of the cisternal stack. To what extent the TGN can become detached from a Golgi stack and float around as a separate unit in the cytoplasm, as suggested by Hilmer et al. (16), has not been determined for these cells. As documented in Figures 3 and 4 and quantitated in Figure 5, the number of cisternae per Golgi stack and the ratios of cisternal types (see below) can vary dramatically in sycamore maple suspension culture cells. Thus, about 75% of the stacks contain 5 or 6 cisternae, less than 10% 3 or 4 cisternae, and close to 20% as many as 7 to 10 cisternae.

**Definition of cis, Medial, and trans Golgi Cisternae**

The assignment of cis, medial, and trans labels to subtypes of Golgi cisternae in the micrographs of high-pressure frozen, freeze-substituted, and Epon-embedded sycamore maple cells is based largely on the morphological criteria defined by Staehelin et al. (41) for the Golgi stacks of similarly processed root tips of Arabidopsis and Nicotiana, and not on marker enzymes as has been done for animal cells (21). These criteria are as follows.

* cis cisternae occupy the end of the stack opposite the most densely staining trans cisternae. Both their membranes and their contents pick up little stain, which often makes them difficult to discern (compare Figs. 1-4), and difficult to distinguish from ER membranes, which stain equally poorly in freeze-substituted cells processed according to standard protocols. cis cisternae also possess the widest lumen, but the fenestrations even in their central region often make them appear like rows of distorted vesicles. As illustrated in Figure 5, nearly all Golgi stacks contain only one cis-type cisterna.

As their name suggests, the medial cisterna occupy the central region of the stack. They consist of a well-defined central domain where the spacing between the two membranes of each cisterna, as well as the spacing between adjacent cisternae, is remarkably uniform. The staining of the lumenal contents tends to be mottled and in most instances the medial cisterna adjacent to the first trans cisterna is much

Figures 1 and 2. Thin section electron micrographs of Golgi stacks with (Fig. 1) and without (Fig. 2) a typical TGN in suspension-cultured sycamore maple cells preserved by high-pressure freezing/freeze-substitution techniques (embedded in Epon 812). Both of the stacks consist of five cisternae and display a distinct structural polarity that can be used to distinguish one cis, two medial (one early [E] medial and one late [L] medial), and two trans cisternae. The intercisternal spacing and stainability of cisternal lumina increase sequentially from the cis to the trans face, whereas the intracisternal spacing decreases in the same direction. Note that the late medial cisterna possesses nearly the same intracisternal spacing as the early medial cisterna, but its lumen is filled with more densely staining material. Bar, 0.2 μm.
of the swollen margins carry nonclathrin-type fuzzy coats, but in these sycamore cells, the coats are never as clearly resolved as those seen in the root tips of Nicotiana (41), even though the same staining procedures were employed in both studies. Unfortunately, we have not yet been able to incorporate the distinction of 'early' and 'late' medial cisternae to the immunocytochemical studies (see below) due to the loss of the characteristic staining patterns in LR White-embedded samples.

Typical of trans cisternae is a collapsed lumen, where the cisternal membranes are tightly appressed and the lumen is filled with uniformly dense products, giving rise to 4 to 6 nm dark lines in cross-sectional views. The spacing between the cisternae is quite uniform and slightly greater than between the medial cisternae (Figs. 2–4). Blebbing vesicles with densely staining contents are often associated with the cisternal margins.

In some instances, such as in Figures 1 and 3, it is fairly easy to distinguish the TGN from the trans cisternae based on the spacing between the two structures, the more rounded and often branched configuration of the vesicles budding off the TGN, and the clathrin coats on some of them. Where no distinct TGN is seen, the trans-most trans cisterna often assumes an intermediate morphology by displaying some rounded and/or branching vesicular configurations (Figs. 2 and 4), suggesting that in these Golgi stacks the trans-most cisterna(e) may assume some TGN function. However, more detailed studies are needed to clarify this point.

The histograms shown in Figure 5 illustrate not only the darker than those closer to the cis cisterna (Figs. 1–4). Because of the consistency with which these differences are displayed, we propose that the more cis located, and more lightly stained medial cisternae be called “early medial” cisternae, and the more trans located, and more darkly stained medial cisternae, “late medial” cisternae. Occasionally, the late medial cisternae exhibit a central domain with a staining pattern of an early medial cisterna (Figs. 1–3). The contents of the bulbous margins of the medial cisternae tend to appear darker than the contents of the more appressed central domains. Some

**Figures 3 and 4.** Two sizes of Golgi stacks observed in sycamore culture cells. The smaller stack (Fig. 3) consists of four cisternae, one cis, one early medial, one late medial, and one trans cisterna; and the large-sized one (Fig. 4) consists of eight cisternae, one cis, two early medial, one late medial, and four trans cisternae. The cis cisternae possess a relatively wide, unstained lumen and irregular contours. Early medial cisternae have lumina that are slightly narrower than cis cisternae and partially filled with stainable products. Late medial cisternae exhibit a more consistently dense staining pattern than early medial cisternae but the same intracisternal spacing. Trans cisternae can be recognized by their tightly appressed membranes that form a single densely stained line. CW, Cell wall. Bar, 0.2 μm.

**Figure 5.** Histograms illustrating the variability in Golgi structure of suspension-cultured sycamore maple cells in terms of total numbers of cisternae per stack and numbers of specific types of cisternae. Stacks of all sizes usually have only one cis cisterna. Larger stacks have increased numbers of medial (especially early) and trans cisternae. For medical cisternae, dark shading = early; light shading = late.
percentages of Golgi stacks in sycamore maple suspension-culture cells containing specific numbers of cisternae, but also a breakdown of the average number of cis, medial, and trans cisternae in Golgi stacks of different size. Most Golgi stacks, irrespective of size, possess only one and never more than two cis-type cisterna. Thus, the increased number of cisternae of larger stacks is due to a proliferation of both medial and trans types of cisternae in roughly equal proportions. The average number of less than one cis cisterna for small Golgi stacks is due to the fact that very small cis cisternae are difficult to discern in our electron micrographs.

**Nature of the Epitopes Recognized by the Anti-Glycan Antibodies**

The greatly improved structural preservation of the Golgi stacks in freeze-substituted samples (Figs. 1-4), and the resulting ability to identify subtypes of cisternae based on their position, dimensions, and staining patterns offers researchers unique opportunities to determine the functional organization of Golgi stacks by immunocytochemical means. To this end, we have employed a battery of both monoclonal and polyclonal antibodies raised against defined complex polysaccharides and glycoproteins. The specific epitopes recognized by the antibodies are illustrated in Figures 6 to 8. The polyclonal anti-XG antibodies have been shown to recognize nearly exclusively the \( \beta-1,4 \)-linked glucose backbone of XG (25), most likely in its twisted solution form (23); they also bind to solubilized cellulose molecules but not to cellulose fibrils. The CCRC-M1 monoclonal antibody appears to recognize specifically the terminal fucosyl residue of the trimeric side chain of XG in the context of an extended XG chain (35). The polyclonal anti-PGA/RG-I antibodies appear to recognize the transition region between deesterified PGA and the first \( \alpha-1,2 \)-linked rhamnosyl residue of RG-I (25, 29). The JIM7 monoclonal antibodies recognize methylesterified PGA domains (19). The monoclonal antibody CCRC-M7 recognizes an arabinosyl-containing epitope on RG-I, consistent with this epitope being part of a side chain, but the exact nature of the epitope remains to be determined (M. Hahn, personal communication). Similarly incomplete is the information on CCRC-M2, which binds to RG-I. The major

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**Figure 6.** Schematic diagram of a XG molecule depicting the binding epitopes of the anti-XG and CCRC-M1 antibodies.

**Figure 7.** Highly schematic diagram of a PGA/RG-I molecule illustrating the binding epitopes of the anti-PGA/RG-I, JIM7, and CCRC-M2 and -M7 antibodies. Note that in the case of real molecules, the PGA domain would be either fully deesterified and bind only anti-PGA/RG-I antibodies, or be fully methylesterified and bind only JIM7 antibodies.

**Figure 8.** Schematic diagram of a typical plant N-linked glycan demonstrating the binding epitope of the anti-\( \beta \)Xyl antibodies.
 (>95%) epitope recognized by the anti-βXyl antibodies is the xylose residue linked β-1,2 to the β-linked mannose of the glycan core of N-linked oligosaccharides (22); the remaining <5% of the polyclonal antiserum binds to another but undetermined epitope of complex glycans (M. Chrispeels, personal communication).

Because all of the antibody probes employed in this study were directed against sugar residues, we have been able to use the same freeze-substitution protocol, including the 2% OsO₄ as for the morphological studies, without destroying the antigenicity of the glycoprotein and complex polysaccharide epitopes. This has ensured an overall excellent structural preservation of the Golgi stacks despite the use of LR White as an embedding medium for the immunolabeling experiments. Thus, as shown in Figures 9, 10, 12, 13, 15, 17, 19, and 20, even though the structural preservation of the LR White-embedded specimens does not quite match the quality of the Epon-embedded samples (compare Figs. 1–4 with Figs. 9 and 10), it is still possible to structurally identify the subtypes of Golgi cisternae in the LR White sections after immunolabeling.

**Immunolocalization of XG in Golgi Stacks**

As already demonstrated by Moore and Staehelin (30) and mentioned above, the polyclonal anti-XG antibodies and the monoclonal antibody CCRC-M1 (Fig. 6) can be used to immunolocalize the sites for synthesizing and assembling the neutral hemicellulose, XG. Figure 9 illustrates the immuno-labeling pattern of the anti-XG antibodies over a Golgi stack of a suspension-cultured sycamore maple cell. Note that all of the gold particles appear over trans cisternae and the TGN. A similar distribution of label over trans cisternae and the TGN is also observed with the CCRC-M1 (anti-terminal fucose of XG) antibodies (Fig. 10). The results of a quantitative analysis of these binding patterns can be seen in the histogram Figure 11, which confirms that >90% of the labeling of both types of anti-XG antibodies is confined to the trans cisternae and the TGN. Interestingly, the anti-XG backbone antibodies label the trans cisternae more heavily than the elements of the TGN (approximately 60% versus approximately 40%), whereas CCRC-M1 antibodies exhibit the opposite distribution, i.e. a proportionally greater amount of labeling over the TGN.

**Distribution of Deesterified, Methylesterified, and Mature Pectic Polysaccharides in Golgi Stacks**

Pectic polysaccharides are a very complex constituent of plant cell walls. A number of antibodies that recognize different domains of pectic polysaccharides (Fig. 7) are available for immunolabeling experiments. The binding patterns of the polyclonal anti-PGA/RG-I antibodies before and after Na₂CO₃ treatment are shown in Figures 12 and 13. The binding of anti-PGA/RG-I antibodies to the Golgi cisternae and the TGN is altered by treatment with Na₂CO₃, which deesterifies methylesterified PGA. Thus, in untreated samples, most of the meager (only 30% of the stacks carried two

**Figures 9 and 10.** Anti-XG (Fig. 9) and CCRC-
M1 (Fig. 10) labeled Golgi stacks of suspension-
cultured sycamore maple cells embedded in LR White. The majority of anti-XG and CCRC-
M1 antibody label is seen over trans cisternae and the TGN (arrows). As confined in Figure
11, the CCRC-M1 antibodies bind more heavily to the TGN than the trans cisternae. Bar, 0.2 µm.
or more gold particles) labeling is seen over cis and medial cisternae of the Golgi stacks, whereas after the Na$_2$CO$_3$ treatment, the much heavier labeling (about 100% of Golgi stacks labeled) shifts more toward the medial and trans cisternae as well as the TGN (Fig. 13). This suggests that the deesterification treatment uncovers cryptic anti-PGA/RG-I epitopes in the later compartments (Fig. 13). These qualitative results are confirmed in the quantitative analysis shown in the histogram Figure 14.

The immunolabeling patterns with anti-pectic polysaccharide antibodies JIM7, CCRC-M2, and -M7 are depicted in Figures 15 to 17. As seen in Figure 15, the JIM7 antibody, which recognizes methylesterified PGA residues (Fig. 7), bind most heavily to the medial cisternae and in decreasing amounts to the trans cisternae and the TGN. The validity of this pattern is confirmed by the quantitative analysis shown in the histogram Figure 18. Thus, approximately 45% of the JIM7 labeling occurs over medial cisternae, about 40% over trans cisternae, and about 15% over the TGN. Virtually no label was present over the cis cisternae. In contrast, the CCRC-M2 and the CCRC-M7 antibodies that both appear to recognize side chains of RG-I (Fig. 7) only detect their epitopes in trans cisternae and the TGN. The labeling patterns for both types of antibodies appear very similar on the electron micrographs (Figs. 16 and 17), and this is confirmed in the histogram Figure 18.

To determine whether hemicellulose and pectic polysaccharides are synthesized in the same Golgi stack, the same section was subjected to two rounds of labeling with JIM7 and anti-XG antibodies. Both types of label were observed over individual Golgi stacks (data not shown), indicating that a given Golgi stack can produce both hemicellulose and pectic polysaccharides simultaneously.

**Figures 11 and 13.** Golgi stacks of sycamore maple culture cells immunolabeled with anti-PGA/RG-I antibodies without (Fig. 12) and with (Fig. 13) Na$_2$CO$_3$ treatment that deesterifies the PGA domains of pectic polysaccharides. The gold particles appear over cis and medial cisternae (arrow) in the absence of Na$_2$CO$_3$. After treatment, they are found across the whole stack as well as over the TGN (arrows). Bar, 0.2 µm.
Characterization of Antibody Labeling with Anti-βXyl in Golgi Stacks

In plants, most complex N-linked oligosaccharides contain a terminal β-1,2-xylosyl residue (Fig. 8; see ref. 9). This xylosyl residue, which is not found in animal glycoproteins, appears to be a major antigenic site of N-linked glycoproteins of plants (M. Chrispeels, personal communication), and is the dominant epitope recognized by the anti-βXyl antibodies. Figure 19 demonstrates binding of these antibodies throughout the stack, but with a slightly greater amount of labeling over medial cisternae. This impression is validated by the quantitative analysis of the anti-βXyl antibody binding patterns shown in Figure 20, which demonstrate little labeling over cis cisterna, the highest percentage of labeling (approximately 45%) over medial cisternae, and decreasing percentages of gold label over the trans cisternae and the TGN.

DISCUSSION

Preservation of Ultrastructure and Antigenic Sites of Golgi Stacks in High-Pressure Frozen Cells

Like all ultrarapid freezing techniques, high-pressure freezing is superior to chemical fixation in its ability to preserve the ultrastructure of plant cells and tissues (6, 41). This improved structural preservation is due largely to the fact that ultrarapid freezing techniques can stabilize cells' orders of magnitude faster than chemical fixatives, and that during ultrarapid freezing all molecules become immobilized simultaneously, in contrast with the selective immobilization of molecules with chemical fixatives (11). These features are of particular importance for the preservation of highly dynamic organelles such as the Golgi apparatus, where membrane fission and fusion events occur on a time scale of milliseconds, and where co- and postfixational osmotic events can produce major structural rearrangements (34). The advantage of high-pressure freezing for preserving the macromolecular architecture of plant Golgi stacks in intact tissues was recently demonstrated by Staehelin et al. (41). Here we illustrate how the much improved structural preservation obtained using high-pressure freeze/freeze-substitution techniques can be exploited in conjunction with immunocytochemical techniques to obtain remarkably detailed information on the cisternal distribution of putative functional activities in Golgi stacks. We show that both polyclonal and monoclonal antibodies that recognize carbohydrate epitopes of either complex polysaccharides or glycoproteins all bind with high specificity to the Golgi stacks of cryofixed and freeze-substituted cells.

Variability in Size and Morphology of Golgi Stacks

We chose the suspension-cultured sycamore maple cells for this study because they are the source of complex polysaccharide antigens used for making several of the antibody probes employed in this study. In addition, the cell line appealed to us because of the uniform appearance of the cells, which have been in culture for over 30 years and have lost their ability to differentiate. These latter features seemed important considering the findings of Staehelin et al. (41) that in high-pressure frozen and freeze-substituted root tips

Figures 15–17. Immunolabeling of Golgi stacks and the TGN of sycamore maple cells with monoclonal JIM7 (Fig. 15), CCRC-M2 (Fig. 16), and CCRC-M7 (Fig. 17) antibodies. With JIM7 (Fig. 15), labeling is observed mostly over medial and trans Golgi cisternae and lightly over the TGN (arrows). With the CCRC-M2 and -M7 antibodies (Figs. 16 and 17), the heavier labeling is seen over the trans cisternae and the TGN (arrows). Bar, 0.2 μm.
of *Arabidopsis* and *Nicotiana*, the morphology of Golgi stacks differed substantially in a cell type-specific manner. The observed variability of size and morphology of the Golgi stacks of our cultured sycamore maple cells was somewhat unexpected, but not totally unpredictable. Hirose and Komamine (17) have demonstrated in conventionally fixed samples that the number and diameter of Golgi cisternae of cultured *Catharanthus roseus* cells changes systematically in a cell cycle-dependent manner. The morphological variability of the Golgi stacks in our unsynchronized sycamore maple culture cells falls within the parameters defined for the *Catharanthus* cells. To what extent the absence of a clear-cut TGN in a high proportion of the Golgi stacks of sycamore maple cells is typical of cultured cells is unclear at the present time because this aspect of the TGN has not been addressed in comparable studies of cultured cells (16, 17). However, even though the morphological variability of the sycamore maple Golgi stacks was greater than anticipated, our ability to distinguish subtypes of cisternae by morphological criteria other than their position within a stack has enabled us to use these cells to define functional differences between these compartments by means of immunocytochemical techniques.

Assembly of XG Is Confined to *trans* Cisternae and the TGN

XG is the major type of hemicellulose of dicot cell walls. It is composed of β-1,4-linked d-glucosyl residues of which 60 to 75% of residues are substituted at C6 with side chains of either a single xylosyl residue or with a xylose, galactosyl, and fucosyl trisaccharide (14). Treatment of XG from soybean, pea, and sycamore cell walls with endoglucanase yields primarily the heptasaccharide and nonasaccharide repeating subunits shown in Figure 6 in equal amounts, and these repeats appear to form alternating sequences (14). Another larger-scale repeat of XG has been recently reported by McCann and colleagues (26), who have shown that XG molecules isolated from onion cell walls appear to be assembled from about 30-nm building blocks.

The mechanism of XG biosynthesis is still poorly understood. The *in vitro* incorporation of glucose and xylose into the XG backbone occurs efficiently only if both UDP-glucose and UDP-xylose are present together in the reaction mixture, suggesting that the two sugars are added in a cooperative manner (15). Whether the glucan synthase I activity (43), which is often used as a marker enzyme for Golgi fractions, corresponds to the XG glucosyl transferase is still a matter of debate. Neither galactose nor fucose seem to be required for

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**Figure 18.** Histogram showing the labeling patterns of the Golgi cisternae and the TGN in suspension-cultured sycamore maple cells with monoclonal JIM7, CCRC-M2, and CCRC-M7 antibodies. JIM7 antibodies heavily label medial and trans cisternae and slightly label the TGN, whereas CCRC-M2 and CCRC-M7 antibodies do not label the medial cisternal compartment but heavily label the trans cisternae and the TGN.

**Figure 19.** Immunolabeling of Golgi stacks of sycamore maple culture cells with anti-βXyl antibodies. Gold particles are found mostly over medial cisternae and lightly over *trans* cisternae and the TGN (arrows). *cis* cisternae are rarely labeled. Bar, 0.2 μm.

**Figure 20.** Histogram showing the labeling pattern over the Golgi cisternae and the TGN of sycamore maple culture cells with anti-βXyl antibodies. The antibodies bind most heavily to medial cisternae and less to *trans* cisternae and the TGN.
the elongation of the XG backbone (12), and although galactosylation precedes fucosylation, there is no cooperative linkage between the two enzymic reactions (5, 8).

Much current interest is focused on how these functional activities are organized in the Golgi stacks. The most recent model of Brummell et al. (4) suggests that the glucose backbone is initiated in the cis cisternae, continued in medial cisternae, and completed in the trans cisternae, where fucosylation is also initiated. Completion of the fucosylation reactions is postulated to occur in the secretory vesicles during transport to the cell wall.

In this study, we confirm some elements of the Brummell et al. (4) model, and we contradict others. The most notable difference pertains to the observation that our anti-XG backbone antibodies only label trans cisternae and the TGN, but not cis and medial cisternae (Fig. 11). This result suggests that the assembly of the β-1,4-linked glucosyl backbone of XG occurs exclusively in the trans cisternae, and that no precursor forms of XG are made in cis and medial cisternae (Fig. 21). Figure 11 also demonstrates that the terminal fucosyl residues on the trisaccharide side chains of XG are partly added in the trans cisternae and partly in the TGN (Fig. 21). These data are consistent with the biochemical observation of Brummell et al. (4) that the XG fucosyl transferase activity of pea microsomal membranes is partitioned between the dictysosome (Golgi stack) fraction and the secretory vesicle fraction. Obviously, the ‘dictysosome fraction’ would include the trans Golgi cisternae, where the first fucosylation reactions occur, and the ‘secretory vesicle fraction’ most likely encompasses vesicles derived from the TGN, where the final fucosyl transferase reactions appear to take place. Because our immunocytochemical data are based on the localization of Golgi products carrying specific epitopes and not on the localization of specific enzymes, our results do not provide conclusive evidence for the confinement of the XG synthesis pathway to trans cisternae and the TGN.

Spatial Organization of the Assembly Pathways of Pectic Polysaccharides

Pectic polysaccharides are a major component of the matrix of the primary cell wall of dicotyledonous plants. They have been postulated to play an important role in cell wall hydration, filtering, adhesion between cells, and wall plasticity during growth (2, 18). In addition, pectic fragments can serve as elicitors for plant defense responses (40). Paralleling this diversity of function is a variability in structure that makes pectin the most complex class of cell wall polysaccharides (27). This complexity is further enhanced by differences between cell types within a plant tissue and changes associated with cell wall development (32).

Pectic polysaccharides are composed of distinct structural domains that are covalently linked together in characteristic patterns. The backbone consists of two types of domains, blocks of 15 to 70 α-1,4-linked β-galacturonosyl residues (the PGA or homogalacturonan domains) that are interspersed periodically with an α-1,2-linked l-rhamnosyl residue (20), and domains containing up to 300 repeats of alternating α-1,4 galacturonosyl-α-1,2 rhamnosyl residues (the RG-I and RG-II domains). About half of the latter rhamnosyl residues are substituted mostly at C4, with side chains containing up to 15 glycosyl residues (27). Few of the 30 plus arabinosyl-
and galactosyl-rich side chains have been characterized to date (1). The mol wt of isolated pectic polysaccharides ranges from 30,000 to 300,000.

The physical properties of the pectic polysaccharides are dominated by the charged PGA blocks that can bind calcium and form Ca⁺⁺-PGA junctions, which induce gelling and adds to the overall strength of the cell wall (18). Methylesterification and acetylation of the carboxyl groups at C6 prevents the formation of these calcium bridges, and it is generally believed that PGA is synthesized and secreted into the wall in a highly methylesterified form, thereby aiding its diffusion throughout the tightly knit mesh of cell wall molecules. Upon arrival in the cell wall, individual blocks of PGA are deesterified by a pectinmethylesterase, which apparently cannot cross the α-1,2 rhamnose bridges between the PGA blocks (18).

Due to the above-mentioned complexity of pectic polysaccharides and their tissue-specific expression, very little is known about their biosynthesis (32). Moore et al. (31) have recently reported in an immunocytochemical study that PGA/RG-I epitopes are localized to the cis and medial cisternae of Golgi stacks as well as to a subset of secretory vesicles in cortical parenchyma root tip cells of clover, but due to a lack of knowledge of the exact nature of the epitopes, the specific type of product being localized in these compartments could not be determined. With the further characterization of these antibodies by Lynch and Staehelin (25), we know now that they bind mostly to the transition region of the deesterified PGA/RG-I domains, and probably to the transition region of the PGA blocks and the interspersed α-1,2-linked rhamnosyl residues. These latter authors also showed that in mucilage-secreting root cap cells of clover, the PGA/RG-I epitopes are shifted to the trans cisternae and the TGN, consistent with the idea that plant Golgi stacks can be functionally reorganized in a cell type-specific manner (41).

In suspension-cultured sycamore maple cells, the anti- 
PGA/RG-I antibodies label nearly exclusively cis and medial cisternae (Fig. 12) as in the root-tip cortical cells of clover (31), but essentially no secretory vesicles. However, the density of labeling was always very low and only about 30% of the Golgi stacks carried two or more gold particles. This contrasts with the labeling pattern of the JIM7 antibodies (Fig. 15) and with the anti-PCA/RG-I labeling of the Na₂CO₃-desterified samples (Fig. 13), where close to 100% of the stacks bound antibodies and the general density of gold particles was higher. These results suggest that the PGA/RG-I backbone is assembled in cis and medial cisternae and that the methylesterification of the carboxyl groups of the galacturonic acid residues occurs nearly simultaneously and very efficiently in the same Golgi compartments (Figs. 14 and 18). The deesterification experiment demonstrates, furthermore, that the lack of binding of the PGA/RG-I antibodies to trans cisternae and the TGN in the control cells is due to the esterification, and that PGA/RG-I epitope-containing molecules traverse the whole Golgi stack.

The precise epitopes recognized by the CCRC-M2 and -M7 antibodies have not yet been determined, but the characterization to date suggests that they bind to side chains of the RG-I domain (Fig. 7; M. Hahn, unpublished results). Our immunolabeling results with these antibodies (Fig. 18) provide strong support for the hypothesis that these chains are added to the nascent PGA/RG-I molecules in the trans Golgi cisternae.

Taken together, our immunolabeling experiments of sycamore maple suspension-cultured cells demonstrate (Fig. 22) (a) that the backbone of PGA/RG-I is assembled in a deesterified form in cis and medial Golgi cisternae, (b) that methylesterification of carboxyl groups occurs nearly simultaneously in the same compartments, (c) that the side chains of RG-I are added in the trans cisternae, and (d) that the finished products pass through the TGN on their way to the cell wall.

Sites of Processing of N-Linked Oligosaccharides

Of all the synthetic activities of the Golgi apparatus of animal cells, the processing pathway for N-linked glycopro-

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**Figure 22.** Model of the biosynthesis pathway of PGA/RG-I in the Golgi apparatus of sycamore maple suspension-cultured cells. The model postulates that the backbone is initiated in cis cisternae and extended in medial cisternae, that methylesterification of the galacturonic acid residues of the PGA domains occurs in medial cisternae, and that addition of the side chains to the RG-I domains occurs in trans cisternae.
teins is probably the best understood (reviewed in refs. 21 and 33). These studies have led to the division of the Golgi stacks into three functionally distinct compartments, the cis, medial, and trans types of cisternae, based on the specific oligosaccharide processing enzymes they contain. Thus, marker enzymes for cis cisternae include Golgi α-mannosidase I and N-acetylglucosaminyl-phosphotransferase, for medial cisternae, N-acetylglucosaminyl-transferase I and II, and for trans cisternae, galactosyltransferase and sialyltransferase (21). The localization of many of these enzymes has been determined both by biochemical and immunocytochemical techniques, and in most instances the two approaches have led to fairly consistent findings (37). However, as shown by Roth et al. (38), there are some tissues such as the absorptive cells of the rat colonic epithelium where the distinct subcompartmentation of certain glycosyltransferases (e.g. the α-2,6 sialyltransferase) does not conform to the generally accepted model.

In general terms, the processing of N-linked oligosaccharides in plants follows the pathways established for animal cells with several important modifications (9). For example, they do not employ the mannose-6-phosphate system for targeting proteins to the vacuole, xylose is often linked to the β-linked core mannose, and sialic acid is not used as a terminal sugar. Because all attempts to subfractionate plant Golgi stacks into cis, medial, and/or trans cisternal fractions have failed to date (42), it has not been possible to define in biochemical terms the enzymic composition of these subcompartments. By using antibodies that recognize specifically the xylose residue attached to the β-linked mannose of the N-linked oligosaccharide core (Fig. 8; see ref. 22) in conjunction with immunocytochemical techniques, we have been able to circumvent the fractionation problems and localize the site of addition of this xylose to the medial cisternae (Fig. 20). Because the xylosyl transferase that carries out this reaction operates just after the N-acetylgalactosamine transferase II (9), which in animal is one of the later-acting medial cisterna enzymes (21), our localization would be consistent with this latter enzyme also being present in medial cisternae of plant Golgi stacks. This would confine the possible sites of addition of the terminal fucosyl and galactosyl residues to more trans-located cisternae of Golgi stacks. Thus, it seems plausible to suggest that the terminal fucosyl and galactosyl residues of N-linked oligosaccharides might also be added in trans Golgi cisternae of plants. Further studies with appropriate antibodies both against specific glycosyl residues and specific processing enzymes of N-linked oligosaccharides will, hopefully, enable us to verify and expand on these hypotheses.

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LITERATURE CITED

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