Plant Physiol. (1993) 101: 1363–1373

Effect of Brefeldin A on the Structure of the Golgi Apparatus and on the Synthesis and Secretion of Proteins and Polysaccharides in Sycamore Maple (*Acer pseudoplatanus*) Suspension-Cultured Cells[1]

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Brefeldin A (BFA), a specific inhibitor of Golgi-mediated secretion in animal cells, has been used to study the organization of the secretory pathway and the function of the Golgi apparatus in plant cells. To this end, we have employed a combination of electron microscopical, immunocytochemical, and biochemical techniques to investigate the effects of this drug on the architecture of the Golgi apparatus as well as on the secretion of proteins and complex cell wall polysaccharides in sycamore maple (*Acer pseudoplatanus*) suspension-cultured cells. We have used 2.5 and 7.5 μg/mL of BFA, which is comparable to the 1 to 10 μg/mL used in experiments with animal cells. Electron micrographs of high-pressure frozen and freeze-substituted cells show that although BFA causes swelling of the endoplasmic reticulum cisternae, unlike in animal cells, it does not induce the disassembly of sycamore maple Golgi stacks. Instead, BFA induces the formation of large clusters of Golgi stacks, an increase in the number of *trans*-like Golgi cisternae, and the accumulation in the cytoplasm of very dense vesicles that appear to be derived from *trans* Golgi cisternae. These vesicles contain large amounts of xyloglucan (XG), the major hemicellulosic cell wall polysaccharide, as shown by immunocytochemical labeling with anti-XG antibodies. All of these structural changes disappear within 120 min after removal of the drug. In vivo labeling experiments using [^{3}H]leucine demonstrate that protein secretion into the culture medium, but not protein synthesis, is inhibited by approximately 80% in the presence of BFA. In contrast, the incorporation of [^{3}H]fucose into N-linked glycoproteins, which occurs in *trans*-Golgi cisternae, appears to be affected to a greater extent than the incorporation of [^{3}H]xylose, which has been localized to medial Golgi cisternae. BFA also affects secretion of complex polysaccharides as evidenced by the approximate 50% drop in incorporation of [^{3}H]xylose and [^{3}H]fucose into cell wall hemicelluloses. Taken together, these findings suggest that at concentrations of 2.5 to 7.5 μg/mL BFA causes the following major changes in the secretory pathway of sycamore maple cells: (a) it inhibits the transport of secretory proteins to the cell surface by about 80% and of hemicelluloses by about 50%; (b) it changes the patterns of glycosylation of N-linked glycoproteins and hemicelluloses; (c) it reduces traffic between *trans* Golgi cisternae and secretory vesicles; (d) it produces a major block in the transport of XG-containing, dense secretory vesicles to the cell surface; and (e) it induces the formation of large aggregates of Golgi stacks in the vicinity of the nucleus, possibly mediated by the fusion of Golgi matrix zones. Thus, although the Golgi apparatus of plant and animal cells share many functional and structural characteristics, the plant Golgi apparatus possesses properties that make its response to BFA unique.

BFA is a hydrophobic fungal antibiotic (Härri et al., 1963) that has been extensively used to investigate the function of the Golgi apparatus and the mechanisms regulating membrane trafficking in animal cells (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989, 1990). It was originally reported that BFA blocks the transport of secretory proteins from the ER to the Golgi complex (Misumi et al., 1986; Oda et al., 1987; Kato et al., 1989). However, the most striking effects of this drug are the rapid and dramatic disorganization of Golgi structure and the gradual redistribution of Golgi proteins to the ER (Fujiwara et al., 1988; Doms et al., 1989; Lippincott-Schwartz et al., 1989, 1990). The BFA-induced morphological changes of the Golgi apparatus are manifested by the complete disassembly of cisternal stacks and their replacement by swollen Golgi "vacuoles." These "vacuoles" give rise to long tubulovesicular structures through which Golgi enzymes move back to the ER (Lippincott-Schwartz et al., 1990). Membrane tubules induced by BFA have also been observed for other cellular organelles, including endosomes and lysosomes (Lippincott-Schwartz et al., 1991; Wood et al., 1991). Recent studies have identified specific biochemical changes of the Golgi apparatus in cells treated with BFA. Donaldson et al. (1991) have shown a rapid (approximately 30 s) dissociation of a 110-kD membrane protein from Golgi membranes, a protein that appears to correspond to the coat protein β-COP of nonclathrin-coated Golgi vesicles (Serafini et al., 1991). More recently, Robinson and Kreis (1992) have demonstrated that γ adaptin, a coat protein of clathrin-coated vesicles, also dissociates from the TGN following BFA treatment. This, in turn, may inhibit binding of regulatory coat

[1] This work was supported by National Institutes of Health grant GM 18639 and National Science Foundation grant DCB-8615763 to L.A.S. and a stipend by la Région de Haute Normandie, Rouen-France, to A.D.

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Abbreviations: BFA, brefeldin A; TGN, *trans* Golgi network; XG, xyloglucan.

proteins to their membrane targets (Klausner et al., 1992). Similarly, Orci et al. (1991) have reported that BFA prevents the assembly and the budding of nonclathrin-coated vesicles from Golgi cisternae and ER, which may explain the BFA-induced inhibition of protein secretion.

All of these BFA-induced alterations of the ER/Golgi system are reported to occur without affecting other cellular events such as endocytosis, lysosomal degradation, or protein synthesis (Misumi et al., 1986; Fujiwara et al., 1988). In addition, they are all fully reversible. After removal of the drug, normal protein transport and processing resume, and the Golgi complex reforms (Doms et al., 1989; Lippincott-Schwartz et al., 1989).

The Golgi apparatus of both animal and plant cells is the traffic center through which newly synthesized secretory and membrane proteins pass en route to their final destination inside or outside the cell. In animal cells, biochemical and immunocytochemical studies of the Golgi apparatus have demonstrated that its main function is to process glycoproteins synthesized in the ER and destined for lysosomes, the cell surface, or the extracellular matrix (Pfeffer and Rothman, 1987). Different sets of processing enzymes have been localized to subtypes of Golgi cisternae known as *cis*, medial, and *trans* cisternae, and the processing occurs in a *cis*-to-*trans* direction (Kornfeld and Kornfeld, 1985; Hirshberg and Snider, 1987). Sorting and packaging occurs in the TGN (Rothman and Orci, 1990). Clathrin-coated vesicles that are used to sequester and transport proteins to the lysosomal compartment bud from the TGN. In contrast, transport from the ER to the Golgi complex, between Golgi cisternae, between the *trans* Golgi cisternae and the TGN, and between the TGN and the plasma membrane are mediated by other types of vesicular carriers including nonclathrin-coated vesicles (Duden et al., 1991).

Although much less is known about the functional organization of plant Golgi stacks, the major steps of the processing pathway for N-linked glycoproteins are similar to those reported for animal systems (Faye et al., 1989). The main differences involve the use of different terminal sugars. The processing of N-glycans also occurs in a *cis*-to-*trans* direction, and sorting and transport of vacuolar proteins is mediated by TGN-derived clathrin-coated vesicles (Harley and Beevers, 1989; Chrispeels, 1991). As in animal cells, the secretion of proteins to the cell surface in plants occurs via a default pathway (Denecke et al., 1990; Hunt and Chrispeels, 1991), but evidence for regulated secretion is still missing (Griffing, 1991).

What makes the plant Golgi apparatus unique, however, is its ability to synthesize complex matrix polysaccharides of the cell wall in addition to N- and O-linked glycoproteins. Unlike cellulose, which is synthesized at the plasma membrane (Delmer, 1987), and glycoproteins, whose protein backbones are produced in the ER, the cell wall matrix polysaccharides, hemicelluloses and pectins, are assembled exclusively in Golgi cisternae and the TGN. Recent immunocytochemical studies have provided the first evidence on how the synthesis of these polysaccharides is organized within the Golgi stacks (Moore et al., 1991; Zhang and Staehelin, 1992). Thus, the synthesis of pectic polysaccharides in sycamore cultured cells involves assembly of the backbone in *cis* and medial cisternae, methylesterification of polygalacturonic acid domains in medial cisternae, and the addition of side chains in the *trans* cisternae. In contrast, the synthesis of the backbone and the side chains of the hemicellulose XG is confined to the *trans* Golgi cisternae and the TGN.

As in animal cells, pharmacological drugs such as monensin have also been used to investigate the trafficking through and around the Golgi apparatus of plant cells (Chrispeels, 1983; Morré et al., 1983; Mollenhauer et al., 1988; Driouich et al., 1989). However, the monensin data have created as much confusion as enlightenment (see discussion in Moore et al., 1991), possibly due to the fact that the glycoprotein and complex polysaccharide assembly pathways are differentially affected by this drug (Zhang et al., 1993). In contrast to the extensive literature on the effects of BFA on the Golgi apparatus of animal cells, very few investigations on the effects of BFA on the organization of the Golgi apparatus and the secretory pathway of plant cells have been made to date (Satiat-Jeunemaitre and Hawes, 1992, 1993).

In the present study, we have examined the effects of BFA on the structure of the Golgi apparatus as well as the secretion of proteins and hemicellulosic polysaccharides in sycamore maple (*Acer pseudoplatanus*) suspension-cultured cells, using a combination of electron microscopical, immunocytochemical, and biochemical techniques. Our studies demonstrate that under conditions that lead to an 80% reduction in protein secretion and a 50% reduction in the secretion of hemicelluloses (7.5 μg/mL of BFA), the Golgi stacks do not disintegrate. Instead, the main changes include swelling of ER cisternae, the accumulation of dense, XG-containing vesicles, and the clustering of Golgi stacks.

MATERIALS AND METHODS

Cell-Suspension Cultures

Sycamore maple (*Acer pseudoplatanus*) suspension cells were grown as described by Zhang and Staehelin (1992).

Chemicals

Organic chemicals were obtained from Sigma Chemical Co. unless otherwise indicated. L-[3,4,5-^{3}H]Leu (5735 GBq/mmol), UDP-D-[1-^{3}H]xylose (329 GBq/mmol), L[6-^{3}H]Fuc (2690 GBq/mmol), and D-[2-^{3}H]Man (851 GBq/mmol) were purchased from New England Nuclear.

BFA Treatment and Radiolabeling Experiments

Cells were harvested, filtered, and washed with medium containing no Suc and then resuspended in this medium (3 g of cells/10 mL of medium) as previously described (Driouich et al., 1989). Radioactive labeling was done for 60 min with [^{3}H]Leu, [^{3}H]Fuc, UDP-[^{3}H]xylose, or [^{3}H]Man in the presence or absence of 7.5 μg/mL BFA. The drug was present at the same concentration during the labeling.

Precipitation of Proteins Secreted into the Culture Medium

After labeling and treatment, the culture medium was collected by filtration. Proteins secreted into the culture me-

dium were precipitated with TCA as previously described (Driouich et al., 1992). This protein fraction was named "culture medium-secreted proteins." The protein precipitate was resuspended in the following buffer: 20 mM Tris-HCl, pH 8.6, 1% (w/v) SDS, and 0.3% (w/v) β-mercaptoethanol, for better solubilization than in distilled water. An aliquot of the protein fraction was mixed with scintillation liquid in plastic vials and the radioactivity measured in a Beckman LS 6800 Scintillation Counter.

Precipitation of Cellular Proteins and Extraction of Cell Wall Hemicelluloses

After removal of the culture medium (see above), the cells were washed once with fresh culture medium, and then resuspended and stirred in phenol:acetic acid:H_2O (2:1:1, w/v/v) for 16 h at 25°C to remove "cytoplasmic material" as described by Fry (1988). The insoluble residues (cell walls: polysaccharides plus covalently bound proteins) were washed extensively with H_2O and then used to extract hemicelluloses with 6 M NaOH containing 1% (w/v) $NaBH_4$ (Fry, 1988). The cellular proteins were precipitated from the cytoplasmic material by acetone and ammonium formate (Fry, 1988). The bulk of this fraction consists of intracellular proteins, but some soluble cell wall proteins are also present, since the phenol:acetic acid:H_2O solution is capable of extracting some of the noncovalently/ionically bound cell wall proteins but not the covalently bound cell wall proteins or the polysaccharides (Fry, 1988). This protein fraction was named "cellular proteins."

Preparation of the Microsomal Fraction

A crude microsomal fraction was prepared as follows: after radiolabeling and treatment, the cells were gently homogenized by grinding in 50 mM Hepes-KOH buffer, pH 7.5, 1 mM EDTA and 15% Suc, filtered through two layers of Miracloth, and centrifuged at 1000*g* for 10 min. Membranes in the supernatant were pelleted by ultracentrifugation at 100,000*g* using a Beckman Ti 60 rotor type. The pelleted membranes (microsomes) were resuspended in 2 mL of the homogenization buffer and the microsomal proteins precipitated with 10% (w/v) TCA (final concentration). The radioactivity incorporated in these fractions was measured as described above for secreted proteins of the culture medium.

EM

After treatment with BFA (2.5 μg/mL or 7.5 μg/mL) for 15 to 60 min, sycamore maple cells were fixed by high-pressure freezing then freeze substituted, infiltrated, and embedded for EM as described by Zhang and Staehelin (1992). Immunolabeling with anti-XG antibodies was also performed as described previously (Zhang and Staehelin, 1992).

RESULTS

BFA Affects the Structure of ER, *trans* Golgi Cisternae, and the TGN

To examine the effects of BFA on the general architecture of plant cells, we have analyzed the ultrastructural changes in high-pressure-frozen/freeze-substituted sycamore maple suspension-culture cells exposed to 2.5 and 7.5 μg/mL of BFA for 15 to 60 min. These concentrations were chosen based on the effects of BFA on animal cells, where concentrations of 1 to 10 μg/mL have been commonly employed (Fujiwara et al., 1988; Lippencott-Schwartz et al., 1989, 1990, 1991). In general, all of the BFA effects reported in this paper can be observed at a concentration of 2.5 μg/mL, but the onset of the cellular changes is somewhat slower than when 7.5 μg/mL is used. In addition, the response of the 2.5-μg/mL samples tends to be somewhat less uniform during the shorter exposure times than when 7.5 μg/mL of BFA is used. Based on these findings, 7.5 μg/mL of BFA was used for all the biochemical studies.

Figure 1 illustrates the morphology of a Golgi stack and ER of an untreated sycamore maple cell. The dispersed organization of Golgi stacks typical of control cells is highlighted by the absence of neighboring Golgi stacks in this image. Note the discrete morphological differences in staining patterns of the *cis*, medial, and *trans* Golgi cisternae (Zhang and Staehelin, 1992) and the TGN, the general morphology of the ER, and the lack of dense vesicles in the cytoplasm. The first organelle to show BFA-induced changes in its morphology is the ER, whose cisternae become swollen and display more tubular profiles within 15 min of exposure to 2.5 μg/mL of BFA (Fig. 2A). In contrast, the Golgi stacks retain their normal morphology and cisternal staining patterns, even in the presence of 7.5 μg/mL of BFA (Fig. 2B). The second major change in the 15-min samples pertains to the appearance of round and dense vesicles that accumulate in the normal location of the TGN. More of these dense vesicles are seen in the 7.5-μg/mL samples than in the 2.5-μg/mL 15-min samples (compare Fig. 2, A and B).

After 1 h in BFA, all Golgi stacks still exhibit a normal type of morphology with clearly distinguishable *cis*, medial, and *trans* types of cisternae (Fig. 2C). However, as most clearly documented in Figures 2C and 3B, the number of *trans*-type cisternae with collapsed central lumina and very densely staining products increases from an average of about 2 to over 4. A second major change pertains to the formation of large clusters of Golgi stacks as evidenced in Figure 3A. Such clusters are most prominent in the cytoplasm around the nucleus of vacuolated cells and are never seen in untreated cells (Fig. 1), or in cells treated for only 15 min (Fig. 2A). The third alteration involves the accumulation of a large number of round dense vesicles in the cytoplasm, most of which appear to remain associated with the Golgi stack clusters (Figs. 2C and 3A). Careful examination of these dense vesicles in the 1-h samples demonstrates a considerable amount of variability in the actual staining of the individual vesicles. Over time, the percentage of more lightly staining vesicles seems to increase. Using immunolabeling techniques in conjunction with anti-XG (a neutral polysaccharide that binds to cellulose microfibrils) antibodies, we show in Figure 3C that the dense vesicles are highly enriched in this polysaccharide that is known to be assembled in *trans* Golgi cisternae and the TGN (Moore et al., 1991; Zhang and Staehelin, 1992).

Upon removal of the BFA and incubation of the cells in fresh culture medium, all cellular organelles regain their normal appearance. Thus, the ER cisternae unswell, the num-

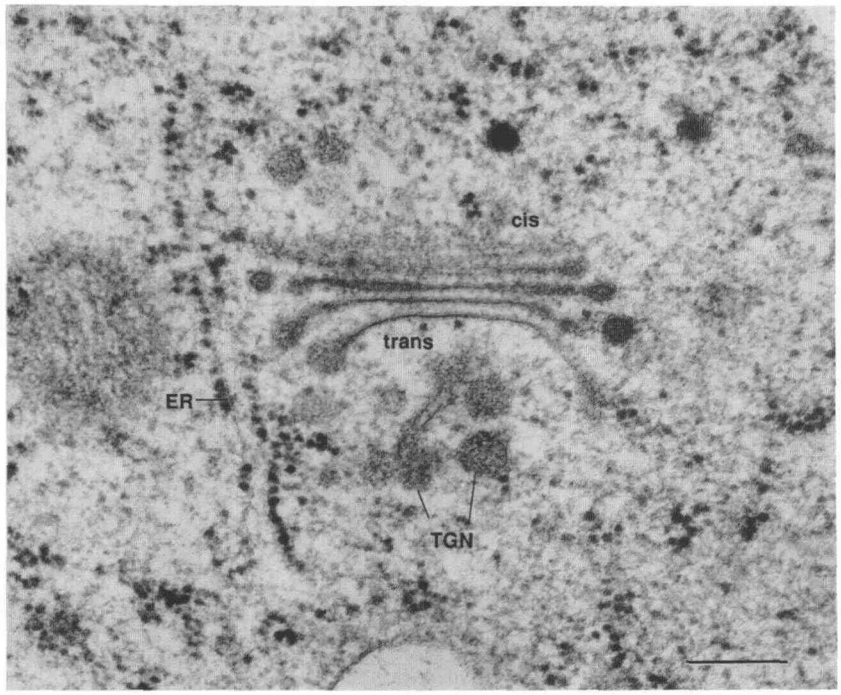

Figure 1. Morphology of Golgi stacks and associated TGN and the ER observed in high-pressure-frozen and freeze-substituted control sycamore maple suspension-culture cells. *cis*, medial, and *trans* types of Golgi cisternae can be distinguished. Bar = 0.2 μm.

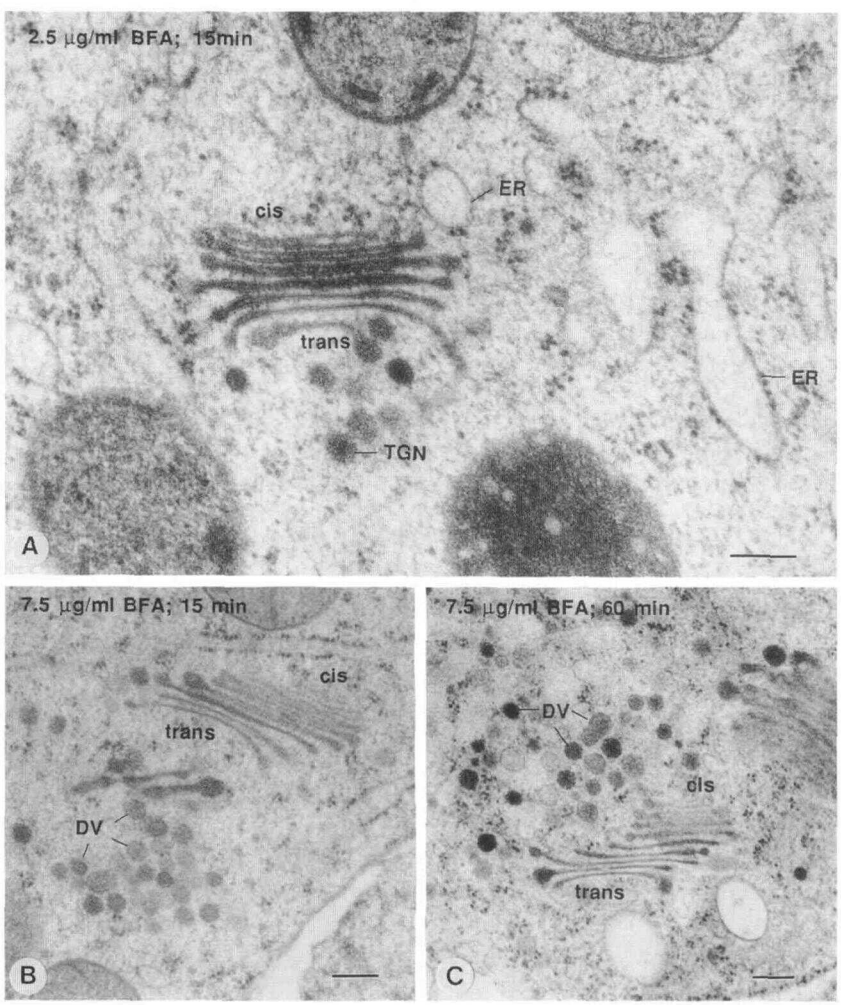

Figure 2. Morphology of Golgi stacks and ER of sycamore maple cells observed after 15 min of treatment with 2.5 μg/mL of BFA (A), 15 min of 7.5 μg/mL of BFA treatment (B), or 60 min of 7.5 μg/mL of BFA treatment (C). Note that despite the large swelling of the ER and the accumulation of small dense vesicles (DV) in the vicinity of the *trans* face of the Golgi stacks, the Golgi apparatus does not disassemble. Bar = 0.2 μm.

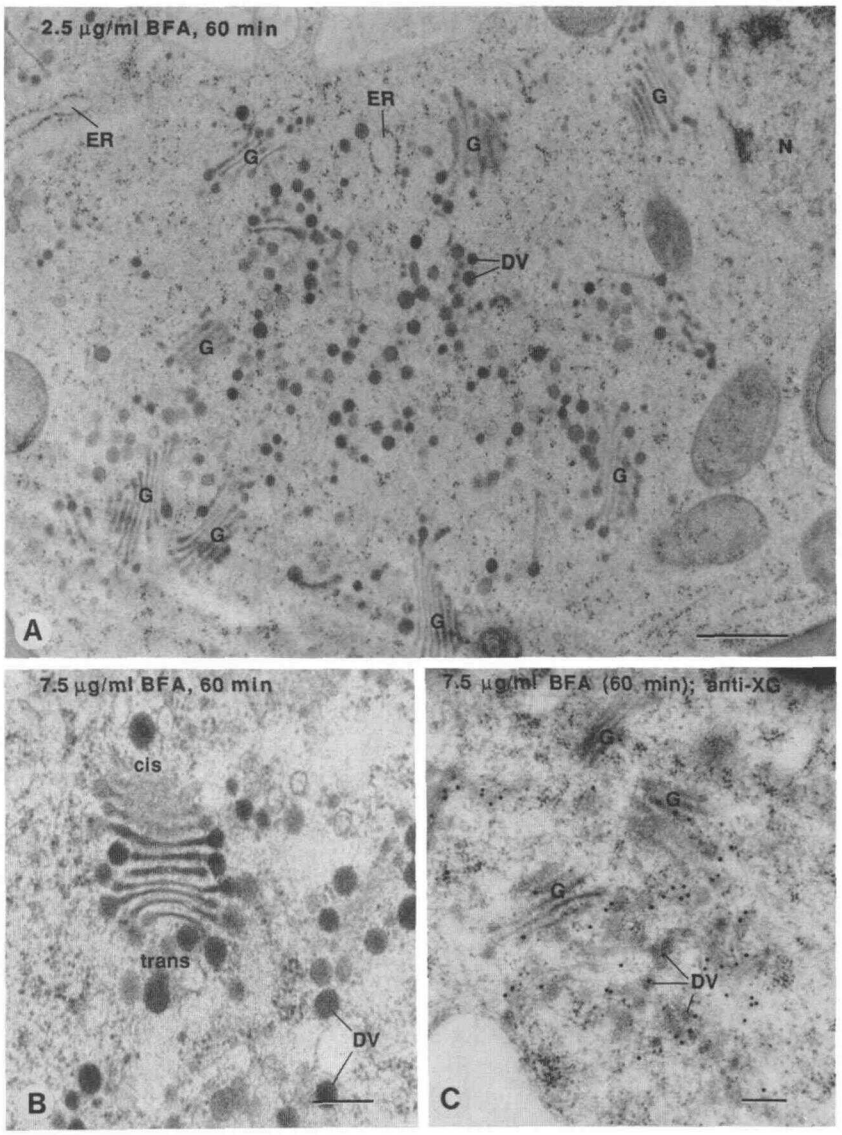

Figure 3. Morphology of Golgi stacks of sycamore maple cells treated with BFA. A, Sycamore maple cells treated with 2.5 µg/mL of BFA for 60 min, showing the formation of a cluster of Golgi that surround a large number of dense vesicles (DV) apparently derived from *trans* Golgi cisternae. B, Sycamore maple cells treated with 7.5 µg/mL of BFA for 60 min, illustrating a typical increase in the number of *trans* cisternae (four *trans* cisternae instead of an average of two in control cells). C, Sycamore maple cells treated with 7.5 µg/mL of BFA for 60 min and labeled with anti-XG antibodies. Note the labeling of the dense vesicles located around the Golgi stacks. G, Golgi stacks; N, nucleus. Bar = 0.5 µm (A) and 0.2 µm (B and C).

ber of *trans* Golgi cisternae decreases to control cell levels, and the Golgi stack clusters disappear in conjunction with the disappearance of the dense vesicles (Fig. 4).

BFA Inhibits Protein Secretion

Because the effects of BFA on the morphology of the ER/Golgi system of sycamore maple cells were more pronounced at the concentration of 7.5 µg/mL and at 60 min of treatment, we chose these conditions for biochemical radiolabeling studies.

To investigate the effect of BFA on protein synthesis and secretion, the sycamore maple cells were labeled with [^{3}H]-Leu for 60 min in the presence or absence of BFA (7.5 µg/mL), and the amount of radiolabeled, newly synthesized cellular proteins and secreted proteins of the culture medium determined as described in "Materials and Methods." As shown in Table I, treatment with BFA reduces the accumulation of newly synthesized proteins in the culture medium by 78%. On the other hand, BFA had only a small effect on the incorporation of [^{3}H]Leu into cellular proteins (Table I), the measured radioactivity of these proteins being reduced by only 16%. These results clearly suggest that BFA is an effective inhibitor of protein secretion in sycamore maple cells, and that this inhibition of secretion in the presence of BFA is not due to a general decrease of protein biosynthesis. The fact that the cellular protein fraction contains a minor amount of contaminating soluble cell wall proteins (Fry, 1988) does not materially affect this conclusion. These data are also supported by the finding that the incorporation of [^{35}S]Met into noncovalently/ionically bound cell wall proteins and culture medium proteins is greatly reduced in the presence of BFA (not shown).

Table I also shows that the incorporation of [^{3}H]Leu into proteins of the microsomal fraction in the presence of BFA is increased. This result suggests a possible accumulation of proteins in the endomembrane system, e.g. ER/Golgi system,

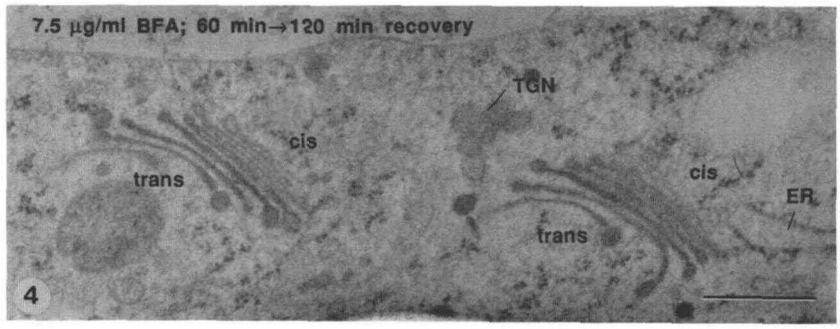

Figure 4. Golgi stacks of sycamore maple cells following a 120-min recovery from BFA treatment. After 60 min of BFA treatment (7.5 µg/mL), sycamore maple cells were washed with fresh culture medium and reincubated in the same medium without BFA. The Golgi stacks have regained a normal appearance and very few dense vesicles are seen. Bar = 0.5 µm.

which is consistent with the morphological changes of these organelles (Figs. 2A and 3A).

Effect of BFA on the Incorporation of [^{3}H]Man, [^{3}H]Fuc, and [^{3}H]Xylose into Glycoproteins

Because the structural studies showed that BFA induces a marked swelling of the ER (Fig. 2), we wanted to gain information about the effect of this drug on the N-glycosylation of proteins, which is one of the major ER functions. N-glycosylation occurs via a cotranslational transfer of high-Man oligosaccharides to nascent proteins in the ER lumen (Faye et al., 1989). Our assay consisted of measuring the incorporation of [^{3}H]Man into proteins in the presence or absence of BFA. The results presented in Table II show that BFA has a strong inhibitory effect on the incorporation of [^{3}H]Man into newly synthesized proteins. Thus, the incorporation of this label into cellular proteins and secreted proteins of the culture medium of BFA-treated cells over a 1-h period was inhibited by 76 and 45%, respectively (Table II). Whether this inhibition results from an inhibition of the assembly of high-Man glycans on the dolichol P-P carrier, the transfer of high-Man glycan onto the nascent polypeptide, or the trimming of the high-Man oligosaccharides by α-mannosidase(s) in the ER or Golgi, was not determined.

The observation that the structure of Golgi stacks in suspension-cultured sycamore maple cells is altered in response to BFA (Figs. 2C and 3B) suggests that functions associated with this organelle, principally the assembly and transport of glycoproteins and polysaccharides, might also be perturbed. To test this hypothesis, we examined the effect of BFA on the incorporation of radiolabeled sugar precursors ([^{3}H]xylose from UDP-[^{3}H]xylose and [^{3}H]Fuc) into the cellular and culture medium protein fractions in the presence or absence of 7.5 µg/mL of BFA for 60 min (Table III). As shown in Table III, when cells were labeled with [^{3}H]Fuc, the incorporation of radioactivity into cellular and secreted culture medium proteins was inhibited by 47% in BFA-treated cells compared with the controls lacking BFA. In contrast, when cells were incubated with UDP-[^{3}H]xylose, BFA reduced the incorporation of [^{3}H]xylose into cellular and secreted protein fractions by only 13 and 18%, respectively (Table III). Taken together, these results indicate that BFA interferes with the synthesis and/or processing of N-linked glycans in the ER/Golgi cisternae, and that it has a more pronounced inhibitory effect on the incorporation of Fuc than xylose into newly synthesized glycoproteins.

Effect of BFA on the Incorporation of [^{3}H]Fuc and [^{3}H]Xylose into Cell Wall Polysaccharides

Table IV shows the effect of BFA on the incorporation of [^{3}H]Fuc and [^{3}H]xylose into alkali-extractable hemicelluloses from the cell wall. It is apparent from these data that the amount of [^{3}H]xylose labeling of the hemicellulose fraction is much greater than the amount of [^{3}H]Fuc labeling. This reflects the high amount of xylose found in XG and xylans, which constitute the hemicellulosic polysaccharides of the primary cell wall of sycamore maple cells (Darvill et al., 1980). When the cells were labeled with UDP-[^{3}H]xylose in the presence of BFA, the amount of [^{3}H]xylose incorporated into cell wall-extractable hemicelluloses decreased by 50% (Table IV). Under the same conditions, the incorporation of [^{3}H]Fuc into hemicelluloses extracted from the cell wall was

Table I. *Effect of BFA on protein secretion*

Three grams of cells were labeled with [^{3}H]Leu in the presence or absence of BFA (7.5 µg/mL) for 60 min. Cellular and microsomal proteins, as well as proteins secreted into the culture medium, were precipitated as described in "Materials and Methods," and the total radioactivity incorporated into each fraction was measured.

Sample Type	Incorporation of [^{3}H]Leu		
	Cellular proteins	Culture medium-secreted proteins	Microsomal proteins
		cpm	
Control	556,425	10,657	51,727
BFA	467,776 (−16%)	2,405 (−78%)	77,507 (+50%)

Table II. *Effect of BFA on protein glycosylation*

Three grams of cells were labeled with [^{3}H]Man in the presence or absence of BFA (7.5 μg/mL) for 60 min. Total radioactivity incorporated into cellular proteins and culture medium-secreted proteins was determined as described in "Materials and Methods."

Sample Type	Incorporation of [^{3}H]Man	
	Cellular proteins	Culture medium-secreted proteins
	cpm	
Control	270,750	26,168
BFA	64,914 (−76%)	14,535 (−45%)

Table IV. *Effect of BFA on the incorporation of [^{3}H]xylose and from UDP-[^{3}H]xylose and [^{3}H]Fuc into cell wall hemicelluloses*

Total radioactivity incorporated from UDP-[^{3}H]xylose and from [^{3}H]Fuc was measured in the hemicellulose fraction extracted from the cell wall (3 g of cells and 10 mL of medium) as described in "Materials and Methods." Time of labeling and the concentration of BFA are as in Table I.

Sample Type	Incorporation into Cell Wall Hemicelluloses	
	[^{3}H]Xylose	[^{3}H]Fuc
	cpm	
Control	17,223	8,011
BFA	8,546 (−50%)	4,740 (−40%)

reduced by 40% in BFA-treated cells (Table IV). These data suggest that the transport of polysaccharides (e.g. hemicellulosic XG) to the cell surface is affected by BFA. This is consistent with the accumulation of XG in BFA-induced dense vesicles shown in Figure 3C. We also found that the incorporation of [^{3}H]Glc into cellulose residues of the cell wall was reduced, suggesting an inhibition of cellulose synthesis, possibly resulting from an inhibition of the transfer of new cellulose synthase enzymes to the plasma membrane (data not shown).

DISCUSSION

Our analysis of the effect of BFA on plant cells has demonstrated that, as in animal cells, the ultimate effect of this fatty acid derivative is to block protein secretion and to affect the processing of N-linked glycans. However, in contrast with its effect in animal cells, BFA does not lead to the disassembly of Golgi stacks by resorption into the ER (Lippincott-Schwartz et al., 1989, 1990). We also show that BFA induces a proliferation of *trans*-like Golgi cisternae, the accumulation of dense, *trans* Golgi cisternae-derived vesicles, and the clustering of Golgi stacks. Finally, we report that BFA affects the incorporation of [^{3}H]xylose from UDP-[^{3}H]xylose and [^{3}H]Fuc into the hemicellulosic polysaccharides of the cell wall. Taken together, these findings indicate that although the plant Golgi apparatus shares many structural and functional features with its animal counterpart, it possesses properties that make its response to BFA unique.

Site(s) of BFA-Caused Blockage in the Secretory Pathway

One of the first morphological effects of BFA on sycamore maple suspension-culture cells is the swelling of the ER cisternae (Fig. 1). Dilation of the ER lumen in the presence of BFA is commonly observed in animal cells and has been shown to coincide with the accumulation of proteins in this organelle due to the blockade of protein transport to downstream compartments of the secretory system (Misumi et al., 1986). The swelling of the ER lumen in our samples could also be due to an inhibition of protein export from this organelle, since BFA inhibits the accumulation of secreted proteins in the culture medium and increases the amount of radiolabeled proteins retained in the microsomal fraction (Table I). However, preliminary subcellular fractionation experiments using Suc gradient centrifugations indicate that most of the accumulation of proteins occurs in the Golgi fraction and not in the ER fraction of BFA-treated cells (not shown). These results suggest that the main BFA-caused block in protein transport in sycamore maple suspension-cultured cells is associated with the Golgi complex. The cause of swelling of the ER cisternae is not known, but could be related to altered ion fluxes and resulting changes in the osmotic properties of the cisternae.

Based on the structural and immunocytochemical findings reported in this paper (Fig. 3, A–C), the most likely sites of transport inhibition are between *trans* Golgi cisternae and secretory vesicles, and between secretory vesicles and the

Table III. *Effect of BFA on the incorporation of [^{3}H]xylose from UDP-[^{3}H]xylose and [^{3}H]Fuc into glycoproteins*

Total radioactivity incorporated from UDP-[^{3}H]xylose and from [^{3}H]Fuc was determined in cellular and culture medium-secreted proteins obtained from a culture of 3 g of cells and 10 mL of medium. Time of labeling and the concentration of BFA are as in Table I.

Sample Type	Cellular Proteins		Culture Medium-Secreted Proteins	
	[^{3}H]Xylose	[^{3}H]Fuc	[^{3}H]Xylose	[^{3}H]Fuc
	cpm		*cpm*	
Control	72,292	73,104	1,625	2,417
BFA	62,865 (−13%)	38,845 (−47%)	1,308 (−18%)	1,270 (−47%)

plasma membrane. Of these two sites, the secretory vesicles-plasma membrane block appears to be the more significant as judged by the accumulation of large numbers of dense, XG-containing vesicles in the vicinity of the Golgi stacks. The presence of a second, lesser block is suggested by the increase in the number of *trans*-like Golgi cisternae in Golgi stacks of BFA-treated cells. Such an increase in *trans*-like Golgi cisternae would be expected if the influx of new membrane molecules from the ER exceeded the number of molecules used for packaging of products exiting from the *trans* cisternae. Both of these features are highlighted in the summarizing diagram in Figure 5.

Effects of BFA on the Processing of N-Linked Glycans

As documented for animal cells (Perkel et al., 1988; Ulmer and Palade, 1991b), BFA also alters the glycosylation patterns of N-linked glycoproteins in plant cells. This is evidenced in our study by the changes in the incorporation of [^{3}H]Man, [^{3}H]xylose, and [^{3}H]Fuc into cellular and secreted glycoproteins of the culture medium (Tables II and III). To what extent these changes are due to direct effects of BFA on specific glycosyltransferases or are secondary effects of the general slowdown in trafficking between the different compartments has not been determined in this study. The longer a given glycoprotein is retained in a specific compartment of the secretory pathway, the greater the probability that resident glycosidases will act on the waiting protein and thereby change its glycosylation pattern. This may be the main cause for the significant reduction in the incorporation of [^{3}H]Man into N-linked glycans.

In terms of understanding the effects of BFA on the functional activities of different compartments within the Golgi apparatus, however, the differences in the incorporation of [^{3}H]xylose (−13%) and [^{3}H]Fuc (−47%) into cellular glycoprotein fractions are potentially more informative than the [^{3}H]Man incorporation data (Table III). As shown recently, xylose is added to N-glycans in medial Golgi cisternae (Lainé et al., 1991; Zhang and Staehelin, 1992), whereas the addition of Fuc takes place in *trans* Golgi cisternae (Faye et al., 1992). Thus, it appears that BFA affects the *trans* Golgi cisternae-associated fucosylation reactions to a much greater extent than the medial cisternae-associated xylosylation reactions. This result is consistent with the electron microscopic data (Figs. 2 and 3) that show a much greater BFA-induced alteration of the *trans* Golgi compartments than the medial cisternal compartments, and budding of dense secretory vesicles directly from the *trans* Golgi cisternae. Further studies are needed to determine if the reduced fucosyltransferase activity is caused by a loss of enzymes from the *trans* cisternae to the dense vesicles, to a change in cisternal pH, or to other alterations in the *trans* Golgi membranes such as a reduction in uptake of sugar nucleotides into the cisternae.

Preservation of Golgi Stacks in BFA-Treated Sycamore Maple Cells

Of all of the unexpected results of this study, none is more striking than the retention of normal-looking Golgi stacks in BFA-treated sycamore maple cells (Fig. 2). Does this mean that retrograde transport of membranes from Golgi to ER does not occur in plant cells or that plant Golgi stacks possess properties that prevent them from being resorbed into the ER membrane system? We postulate that plant Golgi stacks are less likely to be resorbed into the ER both because their association with the ER is of a more transient nature and because they have an inherently more stable structure than their animal counterpart in vivo.

The nature of the relationship between ER and Golgi stacks in plant cells has been debated for many years. In some tissues, such as the storage parenchyma cells of pea cotyledons (Craig and Goodchild, 1984), the Golgi stacks are always located close to ER cisternae, whereas in root tip cells, Robinson (1980) was unable to detect any close spatial relationship between several Golgi stacks and ER cisternae analyzed by serial sectioning. The simplest explanation for these findings is that because the major function of the Golgi apparatus of most types of plant cells is to produce cell wall polysaccharides and not glycoproteins (Bolwell, 1988), there is less need for maintaining a stable association between the

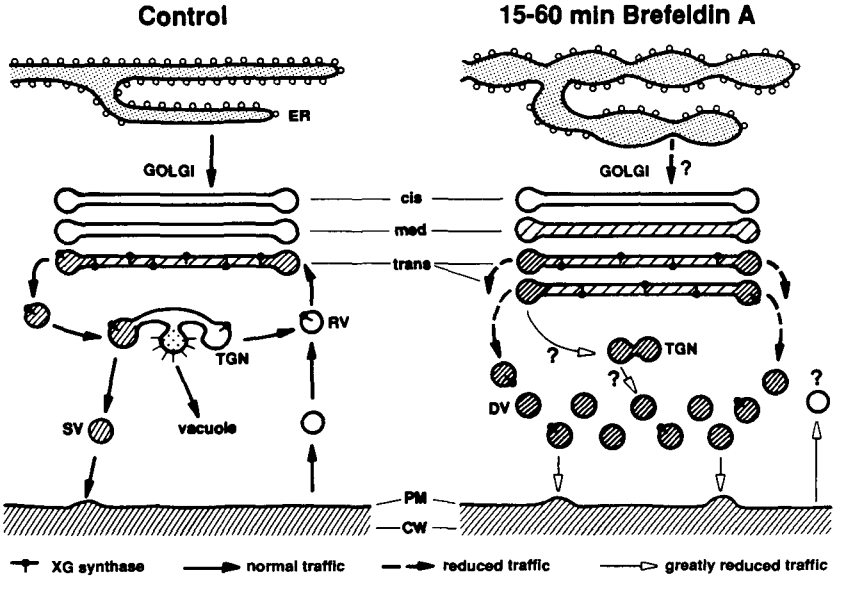

Figure 5. Model of the effects of BFA on the ER and Golgi stacks of sycamore maple suspension-culture cells. BFA causes (a) swelling of the ER, (b) an increase in *trans* cisternae-like staining of medial cisternae, (c) an increase in the number of *trans* cisternae that accumulate XG in dense, marginal blebs, (d) a diminution, if not loss, of the TGN, and (e) the accumulation of large numbers of dense XG-containing vesicles in the vicinity of the Golgi stacks. It is postulated that the increase in the number of *trans* cisternae and the accumulation of dense, XG-containing vesicles reflect both the continuing synthesis of XG by *trans* cisternae and the packaging of XG into vesicles whose ability to fuse with the TGN and the plasma membrane is impaired. SV, Secretory vesicle; RV, recycling vesicles; DV, dense vesicle.

two membrane systems. The very nature of the Golgi resorption process as defined in animal cells requires a relatively stable physical relationship between Golgi stacks and nearby ER cisternae. In the absence of such a relationship, resorption could occur only on an intermittent basis, which would obviously be a less efficient process. General support for this idea has come also from a preliminary study of the effects of BFA on maize suspension-cultured cells, which show much more Golgi fragmentation and vesiculation than is evident in the sycamore maple cells. Nevertheless, we have found no evidence for the resorption of Golgi membranes into the ER in these cells (A. Driouich, G.F. Zhang, and L.A. Staehelin, unpublished results).

The greater structural stability of plant Golgi stacks is highlighted by the following facts. A typical interphase plant cell contains about 400 individual Golgi stacks distributed throughout the cytoplasm (Garcia-Herdugo et al., 1988). These stacks multiply by fission in a *cis*-to-*trans* direction, yielding as many as 800 to 900 Golgi stacks just prior to cytokinesis (Garcia-Herdugo et al., 1988; Hirose and Komamine, 1989). The large number of Golgi stacks ensure that both daughter cells receive a full complement of Golgi membranes during cytokinesis. Retention of functional Golgi stacks during mitosis and cytokinesis is of critical importance for plant cells because these stacks are needed to produce the secretory vesicles that give rise to the cell plate and, subsequently, the plasma membrane and the cell wall domains that separate the daughter cells. Plant cells, therefore, always have to maintain a full complement of Golgi stacks throughout the cell cycle. Consistent with this idea is the fact that neither colchicine nor cytochalasins, two cytoskeletal-disturbing agents, seem to have an effect on the basic structural organization of plant Golgi stacks (Shanon and Steer, 1984). In contrast, the Golgi apparatus of animal cells is programmed to vesiculate and to cease its secretory activities at the onset of mitosis and to reassemble and resume its functions upon completion of cytokinesis. These changes ensure that both daughter cells receive a full complement of Golgi membranes, and that secretory product can be targeted to the correct plasma membrane domains (Warren, 1985). In addition, both the reassembly of the Golgi apparatus at the cell center after mitosis and its maintenance as a compact organelle have been shown to require intact, functional microtubules (Kreis, 1990). Taken together, these observations support the hypothesis that, although some "BFA-resistant" animal cells, e.g. PtK_1 cells, have been reported (Ktistakis et al., 1991), the Golgi apparatus of animal cells is designed to be an inherently more labile structure than its plant counterpart and that this lability may make it more sensitive to perturbations by drugs such as BFA.

During the final phase of writing of this paper, Satiat-Jeunemaitre and Hawes (1992, 1993) reported that BFA causes a nearly complete vesiculation of Golgi stacks in root cells of maize and onion but apparently no fusion of these vesicles with the ER. However, the BFA concentrations used in those studies (50–200 μg/mL) were 7 to 27 times higher than the highest concentration employed in our experiments, and at least 5 to 20 times higher than the highest concentrations used in BFA experiments with animal cells (Klausner et al., 1992). Because BFA readily partitions into bilayer membranes, it is impossible to determine if the high BFA concentration-induced vesiculation of Golgi stacks observed by Satiat-Jeunemaitre and Hawes (1992) is caused by a primary or a secondary effect of BFA on these membranes. Until this point is clarified, it is difficult to relate their findings to ours in a more than superficial manner.

Accumulation of Dense Vesicles and Clumping of Golgi Stacks: A Hypothesis

As illustrated in Figure 3, the accumulation of dense vesicles and the clumping of Golgi stacks represent two interesting responses of plant cells to BFA. Why do the XG-containing dense vesicles accumulate in the cytoplasm adjacent to the Golgi stacks and largely within the ribosome-free Golgi matrix zone (Figs. 2C and 3A)? The formation of unusually densely stained, round vesicles of fairly uniform size from *trans* and possibly modified medial cisternae (Fig. 3B) suggests that in plant cells BFA has an effect on the budding/packaging mechanism of Golgi cisternae, as has been reported for animal cells (Donaldson et al., 1990; Klausner et al., 1992). However, the dense vesicles that are produced under these conditions seem to lack the ability to fuse efficiently with the TGN and the plasma membrane. This is evidenced by their accumulation in the cytoplasm and by the reduced fucosylation of secreted glycoproteins of the culture medium and hemicellulosic cell wall polysaccharides (Tables III and IV), a function most likely confined to *trans* Golgi cisternae and the TGN (Lainé et al., 1991; Faye et al., 1992; Zhang and Staehelin, 1992). The accumulation of these dense vesicles in the cytoplasm and their lack of ability to fuse with the plasma membrane may explain the impairment of XG delivery to the cell surface.

Another feature of these improperly budded vesicles is their apparent continuing affinity for the ribosome-free Golgi matrix (Mollenhauer and Morré, 1980; Staehelin et al., 1990), which causes them to accumulate around the Golgi stacks. As more of these vesicles accumulate, the matrices of individual Golgi stacks become extended beyond their normal capacity and begin to fuse with each other to form large clusters of Golgi stacks and dense vesicles characteristic of sycamore maple cells exposed to BFA more than 30 min. This model postulates a role for the Golgi matrix, namely that it serves to hold "immature" Golgi vesicles in the vicinity of the Golgi stacks. It also predicts that immature Golgi vesicles may carry proteins on their cytoplasmic surface that could mediate binding to the Golgi matrix. In this context, it is of interest to note that in the electron microscopical studies of BFA-induced structural changes in erythroleukemia cells (Ulmer and Palade, 1991), a filamentous, ribosome-excluding matrix is also evident between the remnants of the vesiculated Golgi stacks.

ACKNOWLEDGMENTS

We are grateful to Drs. A.M. Delaunay, S. Freyermuth, T. Giddings, S. Levy, and L. Faye for helpful suggestions and comments on the manuscript.

Received October 15, 1992; accepted January 4, 1993.

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