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Effects of brefeldin A on the structure and function of the Golgi apparatus in the marine red alga *Erythrocladia subintegra* Rosenvinge

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The fungal macrocyclic lactone brefeldin A (BFA) was used to investigate the functional organization and trafficking through the Golgi apparatus of the *Erythrocladia subintegra* thallus cells. Treatment with increasing concentration and incubation time leads in a distortion of Golgi cisternae which gradually curve until they become round and the Trans-Golgi-Net (TGN) became replaced by the BFA compartment. Examination of the plasma membrane of BFA-treated cells after freeze-fracture revealed a decreased number of linear terminal complexes (TCs). After a 24-hour-incubation in BFA, significant structural and functional changes in the Golgi apparatus are evident. Dictyosomes are no longer visible in their typical form and having almost entirely been replaced by vesicles encountered in the BFA area. TCs and "tetrads" appeared in the plasma membrane. Thus, it is obvious that on the one hand no zymogenic particles (globules) responsible for cellulose synthesis (zymogen precursor of the TC) exist, and on the other hand no synthesis of amorphous polysaccharidic matrix takes place due to the absence of "tetrads". Both the zymogenic particles and the tetrads, which initially were formed in the Golgi complex, are transported to the plasma membrane via Golgi vesicles.

Key words: *Erythrocladia subintegra*, red alga, brefeldin A, Golgi apparatus.

INTRODUCTION

Two drugs, brefeldin A and monensin have been successfully applied in studies on the relationship between structure and function of the Golgi apparatus in animal and plant cells (Klausner *et al*., 1992; Salomon & Meindl, 1996; Staehelin & Driouich, 1997; Noguchi *et al*., 1998; Kartusch *et al*., 2000). Brefeldin A (BFA), a fungal macrocyclic lactone, results in the vesiculation of the Golgi apparatus and in the inhibition of protein and complex polysaccharide secretion (Driouich *et al*., 1993; Satiat-Jeunemaitre *et al*., 1996; Staehelin & Driouich, 1997; Ritzenthaler *et al*., 2002; reviewed in Nebenführ *et al*., 2002). BFA (10 μg ml^{-1}) also inhibits both cellulose synthesis (approximately by 77%) and cell wall matrix polysaccharides (approximately by 43%) synthesis in pea root seedlings (Lanubile *et al*., 1997).

In the carpospores of *Gigartina teedii* and the tetraspores of *Chondria tenuissima* acid phosphatases were found in the Golgi vesicles (Tsekos & Schnepf, 1991) where they were presumably reglycosylated (Dunphy & Rothman, 1985; Farquhar, 1985; Griffiths & Simons, 1986). Cell wall material in the carpospores of *G. teedii* and *C. tenuissima* is likewise formed in the dictyosomes and becomes extruded by Golgi-derived vesicles. The initial steps resemble scale formation in flagellates (Brown *et al*., 1970). Unexpectedly, however, acid phosphatase activity is irregularly distributed within the Golgi stack in the carpospores of *G. teedii* (Tsekos, 1981; Tsekos & Schnepf, 1991). The cause and the consequences of this remain enigmatic (cf. Novikoff *et al*., 1962; Farquhar & Palade, 1981; Farquhar, 1985).

Dictyosomal structure, particularly during the formation of "cored vesicles in developing carpospores

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of *Gigartina*, as well as distribution of the contents of the Golgi compartments and their enzymic activity, indicate that a dictyosomal model with stationary cisternae and migrating peripheral vesicles (Rothman, 1981, 1994; Dunphy & Rothman, 1985) is improbable for the dictyosome of that species. Particularly in older carpospores the dictyosomes seem to have *cis* to *trans* migrating cisternae (Morré & Mollenhauer, 1974; Tsekos, 1981, 1996; Tsekos & Schnepf, 1991); in the medial and *trans* regions the small, presumably non-clathrin coated vesicles at the edge of the stack and between the cisternae (Tsekos, 1996) presumably transfer certain enzymes from the *trans* to the medial region (Rambourg *et al*., 1987). Dictyosomal cisternae of the developing carpospores are directly and completely transformed into export vesicles involved in cell wall formation, whereas the cored vesicles secrete glycoproteins which are believed to function in spore adhesion and may have additional antibacterial functions (Tsekos, 1982). These results on red algal cells are believed to contribute to the renaissance of the cisternal progression model now called the *cisternal maturation model of intra-Golgi transport* (Nebenführ, 2003).

Freeze-fracture of the discoid thallus cells of *E. subintegra* allows the detailed analysis of the plasma membranes, as well as other cell membranes and provides further information on the origin of the terminal complexes (TCs) compared to all other protein particles. Protein-globules (zymogenic precursors of cellulose synthase) were observed in Golgi cisternae and vesicles and in depressions of the plasma membrane, which probably arose from the fusion of the transport vesicles with the plasma membrane (Tsekos *et al*., 1996; Tsekos & Dimopoulou, 2006). The TCs in *E. subintegra* seem to assemble from the zymogenic precursors, named globules (Tsekos *et al*., 1996). In addition to the globules and the regular particles, "membrane tetrads" were found in the Protoplasmic Fracture (PF) face of the Golgi cisternal membranes (Tsekos & Reiss, 1992; Tsekos *et al*., 1996; Tsekos & Dimopoulou, 2006), which are probably involved in matrix polysaccharide synthesis.

In the present study, the action mechanism of BFA in thallus cells of *E. subintegra* is analyzed and the ultrastructural changes of the Golgi apparatus in ultrathin sections and in replicas after freeze-fracture are described.

MATERIALS AND METHODS

Culture

A strain of *Erythrocladia subintegra* Rosenvinge was provided by Prof. G. Tripodi (Gargiulo *et al*., 1987). The algae were grown on mica sheets or the bottom of petri dishes containing the von Stosch culture medium, modified by Guiry & Cunningham (Guiry & Cunningham, 1984) with enriched seawater (ES) (Starr & Zeikus, 1987) at 20-23°C in cabinets under 16:8 hrs long day (LD) light regimes provided by cool white fluorescent lamps with a photon flux density at the culture surface between 10 and 60 $\mu E/m^2s$.

Freeze-fracturing

Thalli of *E. subintegra* grown for 15 days were scraped off the bottom of the petri dishes, immediately mounted on the depression of a 2 mm single replica gold support or on a 2 mm double replica copper support, and frozen in nitrogen slush without any prior chemical fixation. Frozen material was stored in nitrogen slush until it was fractured with a Balzers BAF 060 or BAF 400-T apparatus (for details of the method see Reiss *et al*., 1984 and Okuda *et al*., 1994).

Transmission electron microscopy

Thalli of *E. subintegra* grown for 15 days were fixed for 7 hrs at 22°C, in 5% glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer and 0.25 mol l^{-1} sucrose (pH = 7.2). Washes were done at 0°C with 0.1 mol l^{-1} cacodylate buffer plus sucrose (0.25, 0.125 and 0.0625 mol l^{-1}) and then with buffer alone. The samples were subsequently postfixed for 12 hrs at 0°C with 1% osmium tetroxide in distilled H_2O, dehydrated with acetone (10%, 30%, 50%, 70%, 90% and 100%) for 10 min each time (up to 50% acetone at 0°C, and afterwards at room temperature) and for 1 h with 100% acetone, which was gradually replaced by Spurr's resin (for details of the method see Tsekos, 1985). Samples were kept in 100% Spurr's resin overnight, embedded in 100% Spurr's resin and polymerized at 70°C for 48 hrs. Both the replicas and ultrathin sections were examined either on a Phillips EM 400 or on a Zeiss EM 9 S-2 electron microscope.

Inhibitor treatments

In all cases, the material was cultivated in the von Stosch culture medium. The inhibitor (BFA) was initially diluted in a specific solvent (dimethyl sulfoxide,

DMSO) for any case before it was added to the culture medium.

To obtain control samples for every experiment, additional cultures were inoculated only with the culture medium, without the inhibitor but with the solvent used in each case.

The concentration of the stock solution of BFA (Sigma B7651) was 100 mM in DMSO. The inhibitor was added in the culture medium during the inoculation of the material. The final concentration of BFA in the culture medium was 100 μM. For the freeze-fractured samples the applied concentration of BFA was 70 μM for 90 min.

RESULTS

As illustrated in Figs 1 and 2, untreated *E. subintegra* cells examined by transmission electron microscopy in thin-section and after freeze-fracture, typically exhibited Golgi stacks consisted of seven to ten cisternae, which can be subdivided into *cis*, medial and *trans* types based on morphological criteria (Zhang & Staehelin, 1992).

By electron microscopy it was apparent that *Erythrocladia* cells showed a variable reaction to BFA treatment. Most of the cells treated with 100 μM BFA showed extensive Golgi modifications. After

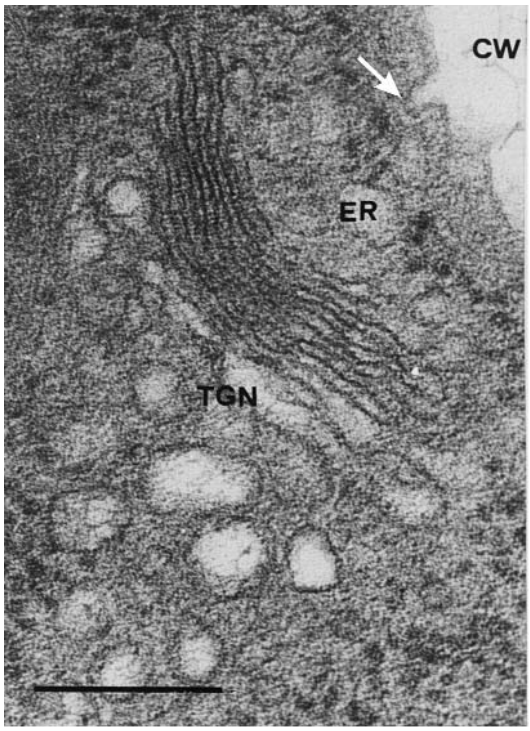

FIG. 1. Thin-section of a Golgi stack in untreated cells. The morphology of the Golgi stacks and of the associated TGN and ER is shown. The *cis* (top cisterna), medial (next two cisternae) and *trans*-Golgi cisternae (bottom three cisternae) can be distinguished. A Golgi vesicle is discharging its content (arrow) into the cell wall (CW). Scale bar = 0.2 μm.

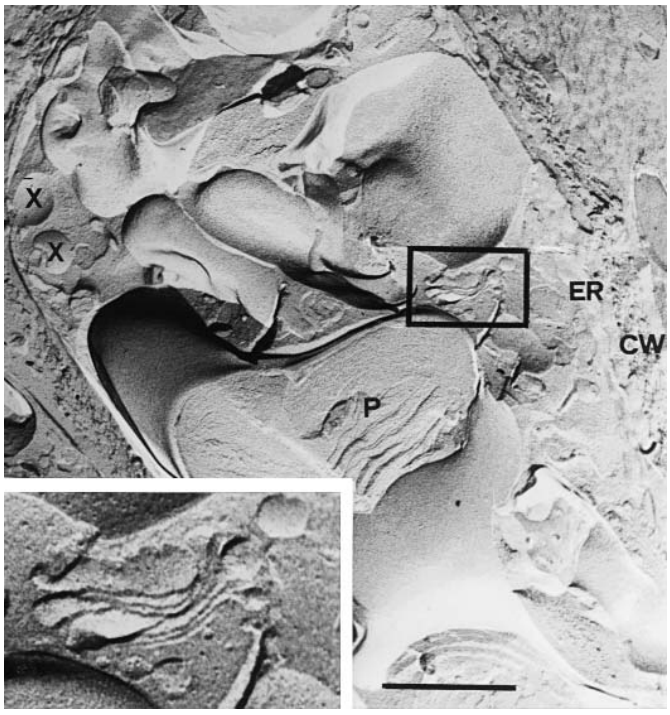

FIG. 2. Freeze-fracture image of a complete cell showing few organelles (such as plastid, P; endoplasmic reticulum, ER; vacuoles, X; cell wall, CW), among which an active dictyosome can also be seen (marked region). Scale bar = 2 μm. Inset: The dictyosome in a higher magnification with intramembrane protein particles (×54000).

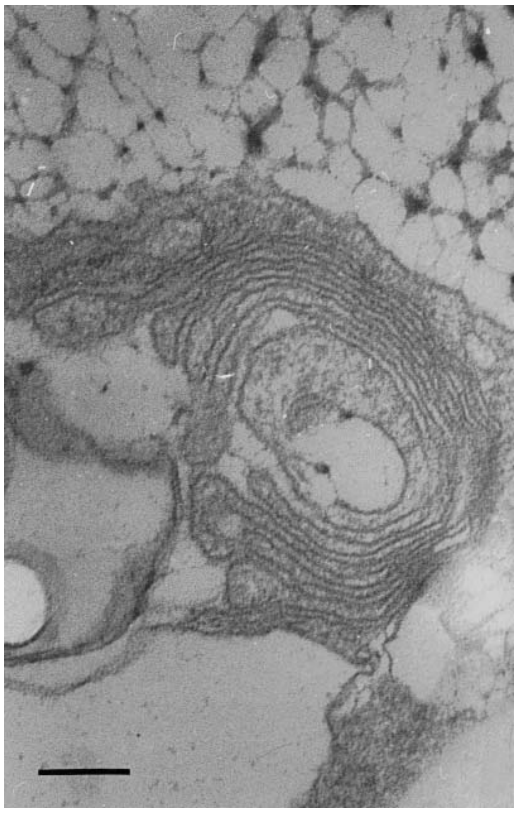

FIG. 3. Ultrathin section of a 100 μM BFA treated *E. subintegra* cell for 1 hour. The Golgi cisternae appear abnormally curved and a large vesicle is apparent in the center. Scale bar = 0.2 μm.

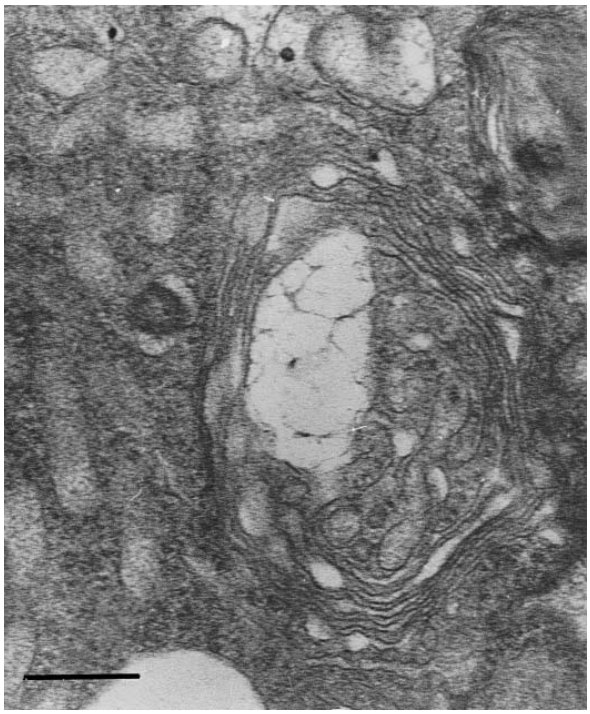

FIG. 4. Ultrathin section of an *E. subintegra* cell treated with 100 μM for 3 hours. Reduction in the number of cisternae in the Golgi stacks. The Golgi stacks tend to aggregate into circles and numerous vesicles are apparent. Scale bar = 0.2 μm.

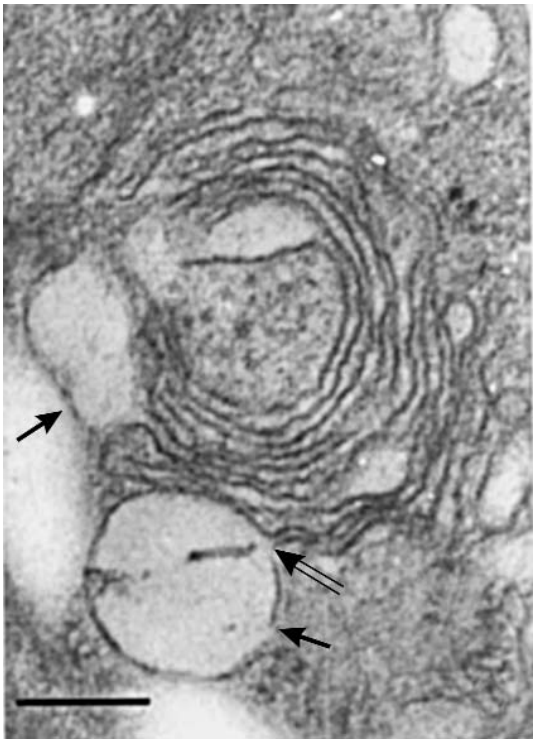

FIG. 5. Ultrathin section of an *E. subintegra* cell treated with 100 μM BFA for 1 hour. The Golgi stacks tend to aggregate into circles and the peripheral portions of the cisternae swell excessively (arrows); the continuity of the cisternae with the swollen peripheral portions is evident (double arrow). Scale bar = 0.17 μm.

FIG. 6. Freeze-fracture image of the Golgi complex during cell wall synthesis in a BFA treated cell (70 μM, 1½ h). The number of cisternae in the Golgi stacks is reduced. The Golgi cisternae (D) are straight and numerous large vesicles, endoplasmic reticulum elements (ER) and mitochondria (M) can be seen. Scale bar = 2 μm.

treatment with 100 μM BFA, Golgi cisternae tended to become curved and numerous vesicles or small vacuoles appeared surrounded by the curved cisternae (Figs 3 and 4), while isolated Golgi cisternae largely swelled at their edges (Fig. 5). Increase of the BFA incubation time results in a decrease of the number of Golgi cisternae, and in enhancement of the vesiculation process (Fig. 6). The total number of Golgi stacks per cell was greatly reduced in a long period of time (Figs 6 and 7) and in many cells, individual stacks were no longer recognizable. Instead, delimited vesiculated areas of the cytoplasm occurred

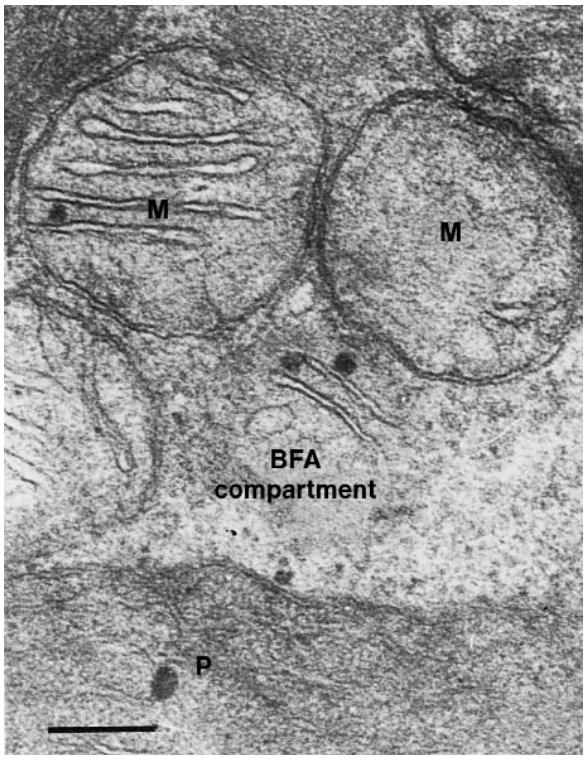

FIG. 7. Thin section of an *E. subintegra* cell treated with 100 μM BFA for 24 hours. Strong reduction in the number of cisternae in the Golgi stacks and the presence of numerous vesicles (BFA compartment) are evident. A plastid (P) and mitochondria (M) can be seen. Scale bar = 0.2 μm.

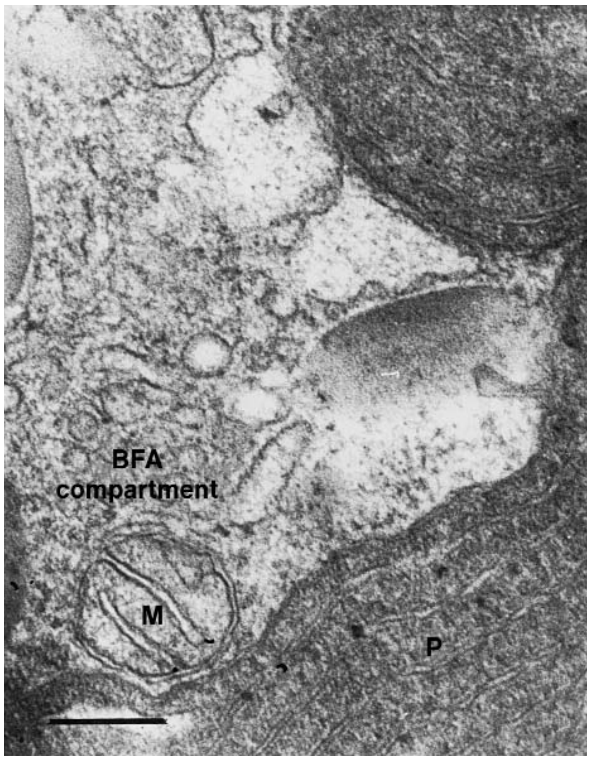

FIG. 8. Thin section of an *E. subintegra* cell treated with 100 μM BFA for 24 hours, showing formation of vesiculated areas (BFA compartment) in the cytoplasm. A mitochondrion (M) and a plastid (P) can be seen. Golgi membranes have disappeared completely. Scale bar = 0.2 μm.

as a common feature of BFA-induced modification (BFA compartment, Figs 7 and 8). Remnants of Golgi membranes were often seen at the periphery of the vesiculated areas, encircling the area after the BFA treatment.

In untreated *Erythrocladia* cells, after freeze-fracture, randomly distributed linear protein particle aggregates (linear terminal complexes, cellulose synthase polymerases) and "membrane tetrads" were observed in the plasma membrane (Figs 9 and 10). By contrast, in *E. subintegra* cells treated with BFA (70 μM), linear terminal complexes (TCs) and "membrane tetrads" were in no case observed in the plasma membrane (Fig. 11). Moreover, globular zymogenic precursors of cellulose synthases similar to those observed by Tsekos *et al*. (1996) in normal cells of *E. subintegra* were not found in cells treated with BFA.

During cell wall synthesis in untreated *Erythrocladia* cells (Tsekos *et al*., 1996) membranes of vesicles originating from the Golgi apparatus which seemed to fuse with the plasma membrane (Fig. 1), contained large globules (the zymogenic precursor of cellulose synthase, 15-22 nm in diameter), as well as "tetrads" with a particle diameter of about 8 nm (see figures 14-17 in Tsekos *et al*., 1996).

DISCUSSION

Satiat-Jeunemaitre & Hawes (1993a, b) have reported that in plant cells the disappearance of the Golgi stacks appears to be through a cisternal vesiculation process. The pattern of Golgi disruption by BFA presented in the present study reveals that a progressive degradation of the stack begins with a curvature and vesiculation of the *trans*-face cisterna, whilst the *cis*-face one is more resistant to BFA (cf. also Satiat-Jeunemaitre & Hawes, 1993a, b; Driouich *et al*., 1997).

In plants, BFA has been shown to alter the structure of the Golgi apparatus and to inhibit secretion (Staehelin & Driouich, 1997). Treatment of sycamore suspension-cultured cells with low BFA concentrations (2.5-10 μg ml^{-1}) inhibited secretion of proteins

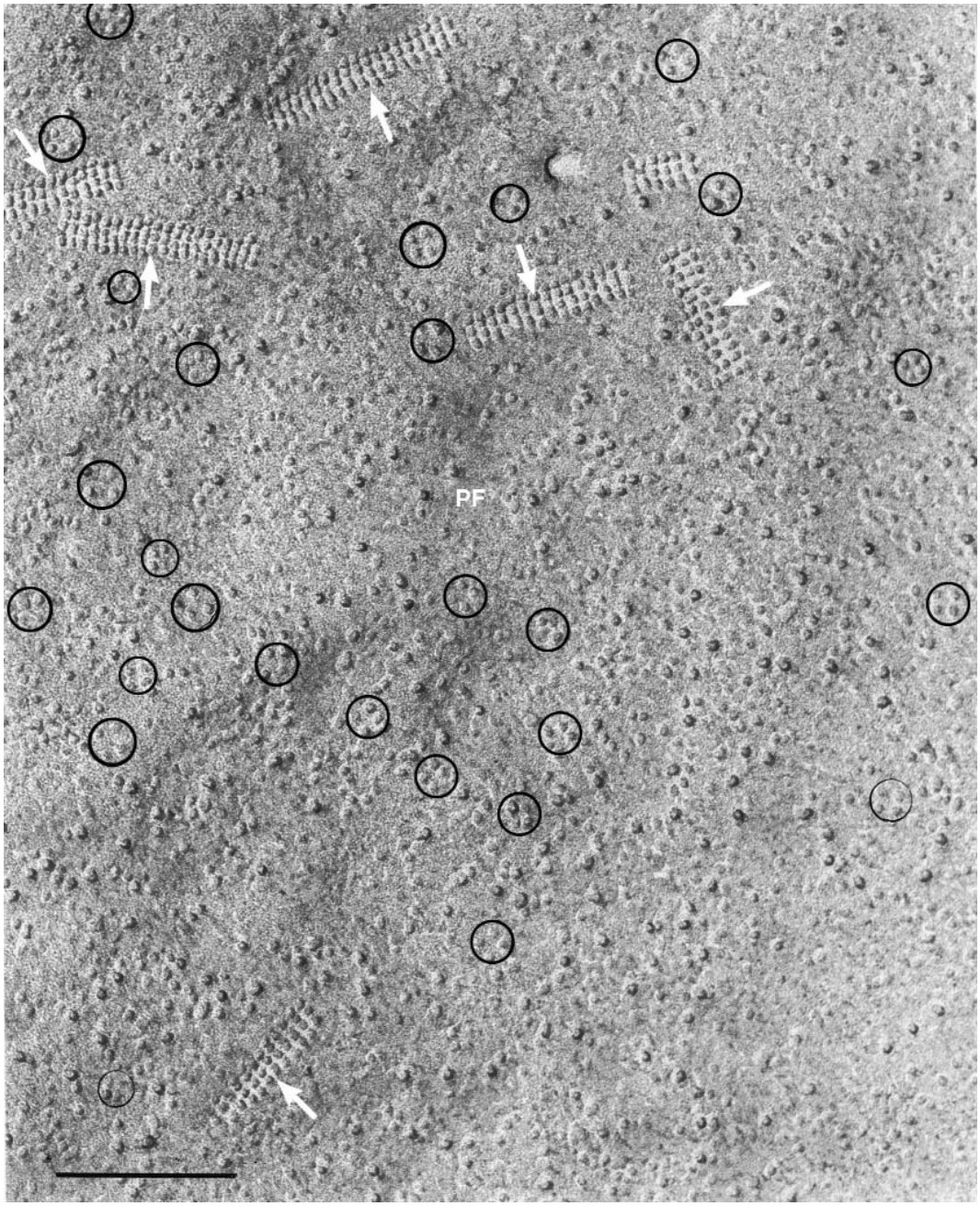

FIG. 9. Freeze-fractured control cell at the stage of cell wall synthesis showing the rotary shadowed PF face of the plasma membrane. Note the numerous tetrads (circled) and several randomly oriented TCs (arrows). Scale bar = 1.4 μm.

and caused several structural changes in the Golgi stacks without their complete disintegration (Driouich *et al*., 1993). Moreover, the plant Golgi stacks, after BFA treatment, tended to aggregate into circles and the cisternae curved inwards giving the impression of sliding apart around the curve during the vesiculation process (Satiat-Jeunemaitre & Hawes, 1992a, b).

It should be pointed out that in *E. subintegra* cells the whole stack curves inwards around the *trans*-face (Satiat-Jeunemaitre & Hawes, 1993a, b). This pattern of Golgi disruption by BFA suggests that a progressive degradation of the stack begins with curvature and vesiculation of the *trans*-face cisterna, whilst the *cis*-face one is more resistant to BFA.

Ultrastructural analysis of the BFA-treated *E.*

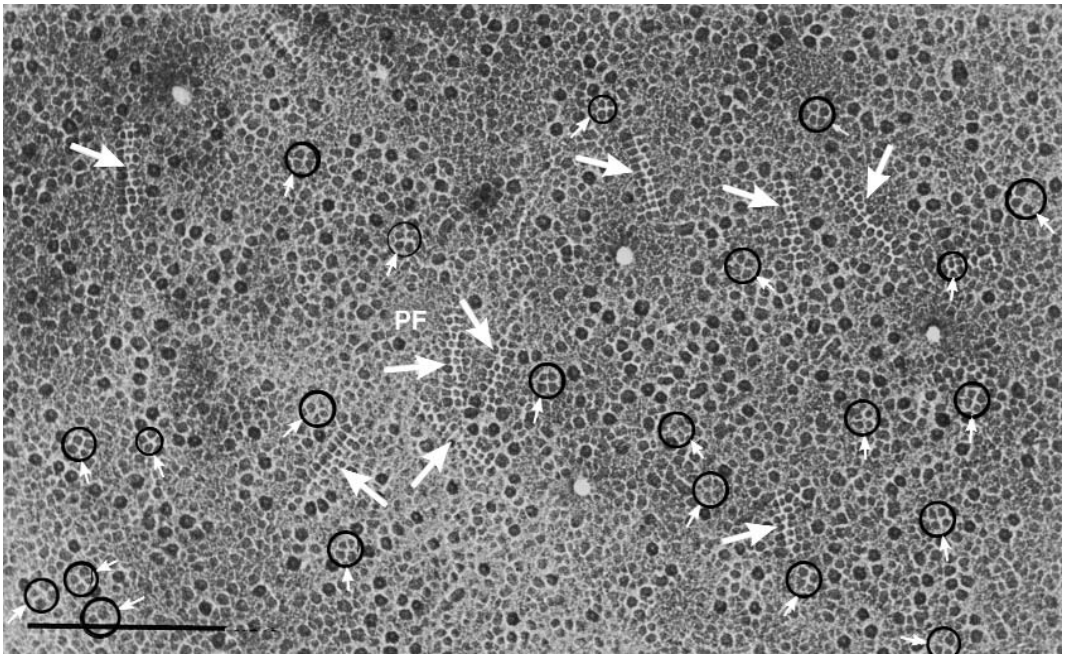

FIG. 10. Freeze-fractured control cell at the stage of cell wall synthesis showing the rotary shadowed PF face of the plasma membrane. Note the numerous tetrads (small arrows) and nine randomly oriented TCs (large arrows). The large electron dense protein particles (15-22 nm in diameter) are the zymogenic precursors, the globules (Tsekos *et al*., 1996), from which the linear terminal complexes (TCs) seem to be assembled. Scale bar = 0.3 μm.

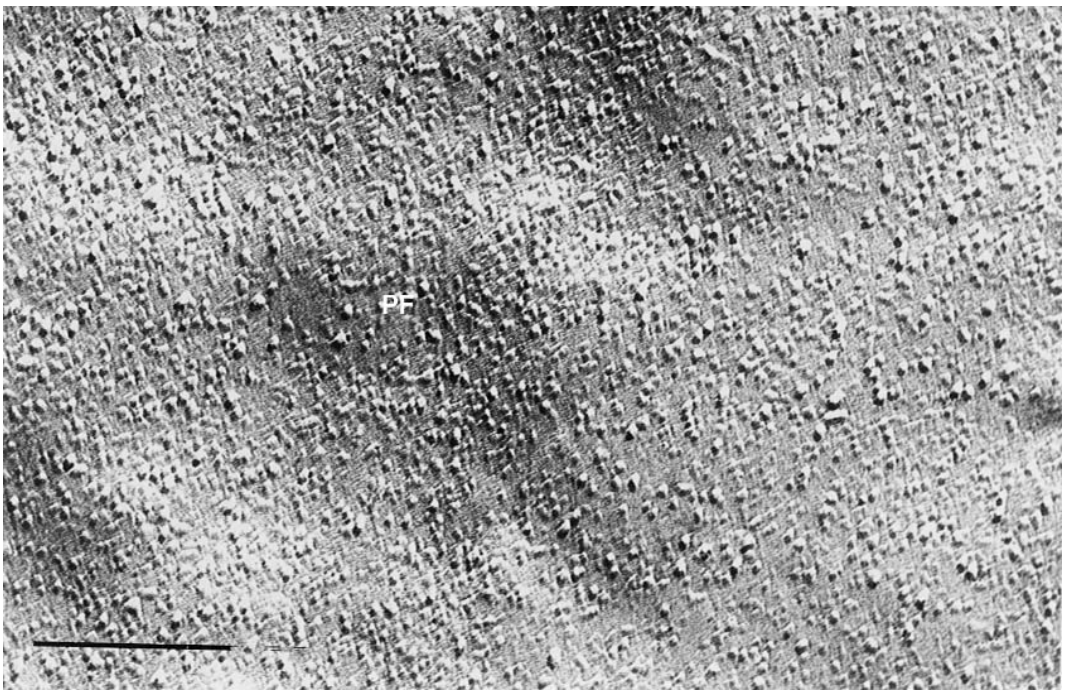

FIG. 11. Fracture through a cell treated with 70 μM BFA for 1½ hour showing the unidirectionally shadowed PF face of the plasma membrane. The presence of many intramembrane particles and the entire absence of linear terminal complexes (TCs) and "tetrads" should be noted. Scale bar = 0.2 μm.

subintegra cells revealed that the Golgi cisternae become gradually diminished and finally disappeared during BFA treatment, as in the case of the BY-2 tobacco cells (*Nicotiana tabacum* var. *Bright Yellow* 2) (Ritzenthaler *et al*., 2002). This cisternal loss may be caused by the continuous maturation of Golgi cisternae combined with a lack of formation of new ones (Nebenführ *et al*., 1999, 2002; Ritzenthaler *et al*., 2002).

Examination of *E. subintegra* cells treated with different BFA concentrations and incubation times showed that the dissociation of the Golgi apparatus induced by BFA is related at least with an inhibition of the cellulose synthases. This is in agreement with the view that in animal cells, BFA blocks protein secretion and therefore has been widely used in the study of vesicle trafficking (Klausner *et al*., 1992; Nebenführ *et al*., 2002; Ritzenthatler *et al*., 2002).

During cell wall synthesis in red algae the non-crystalline matrix polysaccharides and other molecules, such as wall proteins, are delivered to the plasma membrane by Golgi derived vesicles (Ramus, 1972; Evans *et al*., 1974; Tsekos, 1981, 1985; Tsekos & Reiss, 1988; Tsekos *et al*., 1996), and the crystalline polysaccharides, such as cellulose, are synthesized at the plasma membrane. In red algae non-crystalline cell wall material is also produced and transported via mucilage sacs (Pueschel, 1979; Tsekos, 1981, 1985; Tsekos & Reiss, 1993). Thus, the observation that "tetrads" are found in membranes of the Golgi apparatus and in mucilage sacs (Tsekos *et al*., 1996; Tsekos & Reiss, 1988, 1993) supports earlier suggestions that they are probably involved in matrix polysaccharide synthesis.

In *E. subintegra* cellulose is the crystalline phase of the cell wall (Tsekos, 1996, 1999). With the exception of some algae, which produce cellulose scales (Brown *et al*., 1970), cellulose synthesis is an exclusively plasma membrane-associated process in most other cell types (Delmer, 1987; Northcote, 1991). Wall microfibrils, as well as their putative synthesizing complexes in the plasma membrane, the TCs, show no particular orientation in *E. subintegra* (Tsekos & Reiss, 1992; Tsekos *et al*., 1993; Okuda *et al*., 1994; Tsekos, 1999). The TCs probably represent multi-enzyme complexes involved in both steps of cellulose synthesis, glucan chain polymerization and their crystallization (Brown & Montezinos, 1976; Giddings *et al*., 1980; Herth, 1983; Hotchkiss & Brown, 1987; Itoh, 1990; Quader, 1991; Okuda *et al*., 1994; Tsekos & Dimopoulou, 2006).

The absence of the cellulose synthases (namely the linear terminal complexes, TCs) and of "tetrads" in the plasma membrane after treatment with BFA shows that protein secretion (cellulose synthase) and secretion of amorphous matrix polysaccharides (cf. also Driouich *et al*., 1993) are blocked, a fact being in agreement with the behaviour of higher plant and animal cells (Klausner *et al*., 1992 and the literature cited therein; Satiat-Jeunemaitre & Hawes, 1994; Nebenführ *et al*., 2002; Ritzenthaler *et al*., 2002). The inhibition of the transport pathway from the Golgi to the cell surface in *E. subintegra* cells explains why BFA causes a major reduction of wall deposition in red algae (Garbary & Phillips, 1993). Our experimental results indicate that in *E. subintegra* cells, BFA seems to exert its primary effects through perturbations of vesicular transport in the secretory pathway (cf. also Staehelin & Driouich, 1997).

Our freeze-fracture experiments have clearly demonstrated that the effect of BFA on the biosynthesis of cell wall polysaccharides is not caused by the interaction of the drug with the topological organization of the synthase complexes in the membranes, as claimed by Lanubile *et al*. (1997), but by the absolute absence of synthase complexes (TCs and tetrads) in the plasma membranes.

In conclusion, our studies have shown that in *Erythrocladia*, as in most plant and animal cells, (Klausner *et al*., 1992; Satiat-Jeunemaitre & Hawes, 1994; Staehelin & Driouich, 1997; Nebenführ *et al*., 2002; Ritzenthaler *et al*., 2002), BFA inhibits the transport pathway from the Golgi to the cell surface.

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