Perturbation of the Morphology of the *trans*-Golgi Network following Brefeldin A Treatment: Redistribution of a TGN-specific Integral Membrane Protein, TGN38

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Abstract. Brefeldin A (BFA) has a dramatic effect on the morphology of the Golgi apparatus and induces a rapid redistribution of Golgi proteins into the ER (Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. *Cell.* 56:801–813). To date, no evidence that BFA affects the morphology of the *trans*-Golgi network (TGN) has been presented. We describe the results of experiments, using a polyclonal antiserum to a TGN specific integral membrane protein (TGN38) (Luzio, J. P., B. Brake, G. Banting, K. E. Howell, P. Braghetta, and K. K. Stanley. 1990. *Biochem. J.* 270:97–102), which demonstrate that incubation of cells with BFA does induce morphological changes to the TGN. However, rather than redistributing to the ER, the majority of the TGN collapses around the microtubule organizing center (MTOC). The effect of BFA upon the TGN is (*a*) independent of protein synthesis, (*b*) fully reversible, (*c*) microtubule dependent (as shown in nocodazole-treated cells), and (*d*) relies upon the hydrolysis of GTP (as shown by performing experiments in the presence of GTPγS). ATP depletion reduces the ability of BFA to induce a redistribution of Golgi proteins into the ER; however, it has no effect upon the BFA-induced relocalization of the TGN. These data confirm that the TGN is an organelle which is independent of the Golgi, and suggest a dynamic interaction between the TGN and microtubules which is centered around the MTOC.

THE last Golgi-associated compartment through which secretory proteins pass before reaching their final destination has been termed the *trans*-Golgi network (TGN)[1] (Griffiths and Simons, 1986). One of the unique functions of the TGN is to package proteins into different types of transport vesicles which can be directed either to a pre-lysosomal/lysosomal compartment, dense-core secretory granules or different domains of the plasma membrane (Farquhar, 1985; Griffiths and Simons, 1986). It also participates in retrieval and re-utilization of plasma membrane components internalized by endocytosis such as the mannose-6-phosphate and transferrin receptors (Duncan and Kornfeld, 1988; DeBrabander et al., 1988). The morphological attributes of this compartment reflect these specialized functions. Non-coated vesicles deliver constitutively secreted and integral membrane proteins to the plasma membrane. Clathrin-coated buds are involved in packaging lysosomal and regulated secretory proteins (Tooze and Tooze, 1986; Orci et al., 1987; Robinson, 1990). These buds are associated only with the TGN whereas non-clathrin-coated buds and vesicles are found in the other Golgi cisternae (Orci et al., 1986; Malhotra et al., 1989). The identification and description of this compartment has been facilitated by use of cells infected with vesicular stomatitis virus (VSV) in which protein export from the TGN is blocked by incubation of cells at 20°C (Matlin and Simons, 1983; Griffiths and Simons, 1986). Quantitative studies have demonstrated that ~12% of the TGN in these cells is made up of flattened cisternae whereas the rest has a tubular–vesicular structure (Griffiths et al., 1989). The enzyme responsible for addition of the terminal residues on N-linked carbohydrate moieties, sialytransferase, has been localized primarily to the TGN (Farquhar, 1985) although there are also reports of its distribution throughout the rest of the Golgi stacks (Bretz et al., 1980).

Immunocytochemical and biochemical experiments have shown that the fungal metabolite Brefeldin A (BFA) inhibits protein secretion in eukaryotic cells (Misumi et al., 1986), and causes the redistribution of *cis*-, *medial*-, and *trans*-Golgi markers into the ER (Doms et al., 1989; Lippincott-Schwartz et al., 1990). This redistribution is due to the inhibition of anterograde vesicular transport from the ER to the Golgi and between Golgi stacks combined with the concomitant formation of an extensive tubular network that connects previously separate cisternae (Lippincott-Schwartz et al., 1990; Orci et al., 1991). The end result is the mixing of ER

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1. *Abbreviations used in this paper*: BFA, Brefeldin A; MTOC, microtubule organizing center; TGN, *trans*-Golgi network; VSV, vesicular stomatitus virus; WGA, wheat germ agglutinin.

and Golgi proteins. This effect has been shown to be specific as well as energy, GTP, and microtubule dependent (Lippincott-Schwartz et al., 1990; Donaldson et al., 1990). Several lines of evidence suggest that, although the *trans*-Golgi cisternae redistribute into the ER, the TGN does not. Immunolocalization experiments using antibodies to galactosyltransferase, a *trans*-Golgi marker, demonstrated the redistribution of this enzyme into the ER following BFA treatment (Lippincott-Schwartz et al., 1990). Additional evidence for the redistribution of galactosyltransferase has been derived from experiments in which mannose-6-phosphate receptors acquired N-linked galactose in the ER of BFA-treated cells (Chege and Pfeffer, 1990). In contrast, recently published data have been interpreted to mean that the TGN does not redistribute following treatment of cells with BFA. Most analyses of the effects of BFA on the TGN have been based on functional assays assessing the ability of sialyltransferases to sialylate proteins trapped in the ER after treatment. These experiments showed that neuraminidase digestion failed to alter the molecular weight of VSV-G protein extracted from BFA-treated cells, indicating that there was no addition of the terminal sialic acid residues normally acquired by this protein in the TGN (Doms et al., 1989). If BFA induced redistribution of the TGN into the ER had occurred, then one would have expected the VSV-G protein to have been sialylated. Chege and Pfeffer (1990) showed that newly synthesized mannose-6-phosphate receptors do not acquire sialic acid after BFA treatment (as judged by their inability to bind to a sialic acid–specific lectin); however, cell surface receptors were partially sialylated after endocytosis. The results of immunolocalization experiments using wheat germ agglutinin (WGA) and a TGN-specific mAb have also been interpreted as indicating that the TGN does not redistribute following BFA treatment of cells (Lippincott-Schwartz et al., 1990).

To investigate the effects of BFA on the morphology of the TGN we have taken an immunocytochemical approach, using antibodies to an integral membrane protein of the TGN, TGN38. The cDNA encoding TGN38 was isolated using a polyclonal antiserum raised against a detergent-extracted Golgi membrane fraction (Luzio et al., 1990). Immunogold EM using anti-TGN38 antibodies showed staining only on the *trans* face of the Golgi complex in thin frozen sections. In cells infected with VSV and incubated at 20°C before fixation, VSV-G protein and TGN38 colocalized to the TGN. In this report we demonstrate a rapid and reversible redistribution of TGN38 upon BFA treatment and have characterized this effect in terms of its energy requirement and dependence on guanine nucleotides and microtubules.

Materials and Methods

Materials

BFA was purchased from Cambio (Cambridge, U.K.) and Epicentre Technologies (Madison, Wisconsin), stored as a 5 mg/ml stock in methanol at −20°C and used at a final concentration of 5 μg/ml in culture medium. Nocodazole, cycloheximide, and 2-deoxy-D-glucose were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). The concentration of nocodazole was 20 μg/ml in all incubations and was kept as a 10 mg/ml stock in DMSO at −20°C. Cycloheximide (freshly prepared) was used at final concentration of 10 μg/ml. GTPγS was purchased from Boehringer Mannheim U.K. (Lewes, East Sussex, U.K.) and used at a final concentration of 1 mM.

Antibodies

The rabbit anti-rat TGN38 polyclonal antiserum has been described previously (Luzio et al., 1990). The anti-mannosidase II mAb (Burke et al., 1982; Baron and Garoff, 1990) was the kind gift of Dr. Graham Warren (Imperial Cancer Research Fund, London) and the monoclonal anti-lysosomal antibody was generously provided by Dr. Dan Cutler (Imperial College, London). The anti-tubulin antibody was purchased from Serotec (Kidlington, Oxford, U.K.). Fluorescein-labeled goat anti-rabbit IgG and fluorescein- and rhodamine-labeled goat anti-mouse IgG were obtained from Sigma Chemical Company. Rhodamine-labeled swine anti-rabbit IgG was from Dako (High Wycombe, Bucks., U.K.).

Cell Culture and Immunofluorescence Microscopy

Normal rat kidney cells were grown in McCoy's medium (GIBCO, Life Technologies Ltd., Paisley, Scotland) supplemented with 10% FCS (GIBCO, Life Technologies Ltd.), 2 mM glutamine (GIBCO, Life Technologies Ltd.), 100 μg/ml streptomycin (Evans Medical Ltd., Langhurst, Horsham, England), and 60 μg/ml penicillin (Glaxo Laboratories Ltd., Greenford, England) at 37°C in 5% CO_2. Cells were plated onto 22-mm glass coverslips 24–48 h before use (~60–70% confluency). After the appropriate drug treatments, cells were washed three times with PBS and then fixed and permeabilized by incubation in methanol at −20°C for 5 min. The coverslips were then incubated in PBS/0.2% BSA for 5 min followed by a PBS wash and incubation with primary antibody in PBS/0.2% BSA for 1 h at room temperature. After washing (4 × 2 mls) in PBS/0.2% BSA to remove excess primary antibody, the cells were incubated at room temperature with fluorescently-labeled secondary antibody for 30 min, washed, and mounted in Mowiol (Calbiochem, San Diego, CA). Epifluorescence microscopy was performed on a Universal microscope (Zeiss, Oberkochen, Germany) with a 63×, 1.4 oil immersion objective.

Cell Permeabilization

Cells grown on coverslips were permeabilized by filter stripping with nitrocellulose according to the method of Simons and Virta (1987) and as described by Donaldson et al. (1990). Briefly, a pre-soaked nitrocellulose filter (Millipore Continental Water Systems, Bedford, MA) was overlaid on top of subconfluent NRK cells on coverslips for 1 min, and then gently peeled off. Cells were then prepared for immunofluorescence microscopy as described above.

Results

Effects of BFA on the Subcellular Localization of TGN38

Previous immunocytochemical studies at the light and EM level have shown the disappearance of the Golgi cisternae upon BFA treatment and concomitant appearance of secretory proteins diffusely spread throughout the ER (Fujiwara et al., 1988). To determine the fate of the TGN after similar treatment we treated NRK cells with 5 μg/ml BFA for 3 h at 37°C and examined the subcellular localization of TGN38 by immunofluorescence microscopy (as described in Materials and Methods). As shown in Fig. 1 *a*, the pattern of staining in control cells reveals a typical perinuclear cap which is punctate in appearance and has been reported previously (Luzio et al., 1990). There is an absence of staining of the plasma membrane, cytosol, and more distal portions of the cell. After 3 h of BFA treatment, however, the pattern of staining reveals a concentration of TGN38 in the majority of cells in one distinct perinuclear spot (Fig. 1 *b*). In addition, there is a diffuse staining of the cytoplasm which consequently enables the visualization of the entire cell as opposed to just the perinuclear staining seen in control cells. Confocal microscopy allowed sampling of different planes of the cell and revealed that this diffuse pattern of staining occurred throughout the entire cytoplasm (data not shown).

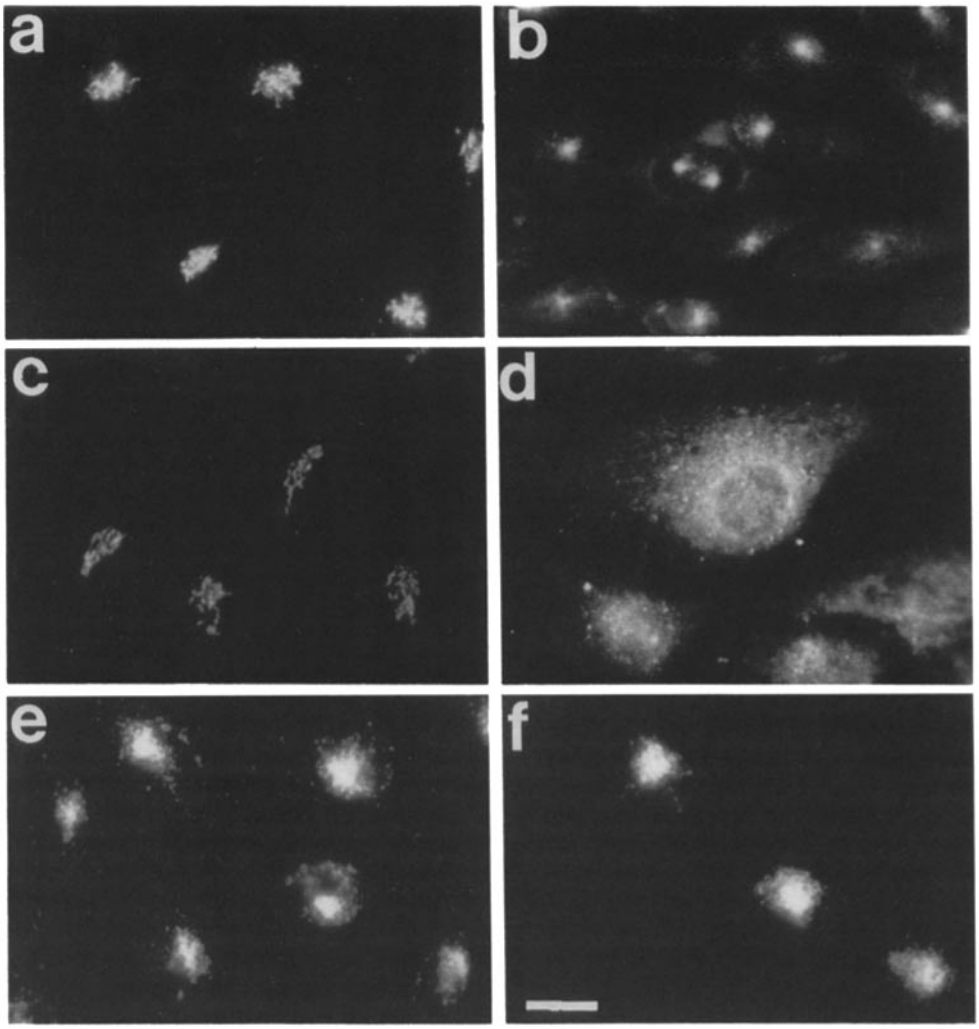

Figure 1. Effect of BFA on the subcellular localization of TGN38. Methanol-fixed NRK cells were incubated with different antibodies in the presence (*b*, *d*, and *f*) or absence (*a*, *c*, and *e*) of BFA. Anti-TGN38 antibody (*a* and *b*), anti-mannosidase II antibody (*c* and *d*), and anti-lysosomal antibody (*e* and *f*). Bar, 10 μm.

Mannosidase II, a marker of the *medial*-Golgi compartment, has been shown by immunofluorescence and immunoelectron microscopy to redistribute into the ER upon BFA treatment of cells (Lippincott-Schwartz et al., 1989). Experiments assessing the uptake and degradation of ^{125}I-asialofetuin in the presence of BFA suggested that the drug does not affect the endocytic or lysosomal pathways (Misumi et al., 1986). We have used antibodies to mannosidase II and to the lysosomal compartment to confirm that the redistribution of TGN38 is a specific effect on the TGN. In control cells, antibodies to mannosidase II exhibit a pattern of staining similar to that seen with anti-TGN38 antibodies, i.e., a distinct perinuclear localization (Fig. 1 *c*). As expected, in BFA-treated cells the anti-mannosidase II antibodies exhibit a dispersed pattern of staining throughout the cytoplasm (Fig. 1 *d*). The anti-mannosidase II antibodies do not give the concentration of juxtanuclear staining seen with anti-TGN38 antibodies on BFA-treated cells (compare Fig. 1, *b* and *d*). Anti-lysosomal antibodies revealed an evenly dispersed punctate distribution about the nucleus in the presence or absence of BFA (Fig. 1, *e* and *f*).

Microtubule Dependence of BFA-induced TGN Redistribution

The Golgi apparatus is situated at the minus ends of interphase microtubules at the site of microtubule nucleation, the microtubule-organizing center (MTOC) (Kreis, 1990; Kelly, 1990). The majority of cells treated with BFA, and immunostained for the presence of TGN38, contained only one area of concentrated immunofluorescence. However, in a minority of cells two areas were present (Fig. 1 *b*). These two patterns of fluorescence are reminiscent of those seen with interphase and mitotic cells stained with anti-MTOC antibodies (Rout and Kilmartin, 1990). To test whether there was any colocalization of TGN38 and the MTOC after BFA treatment, cells were double stained with anti-tubulin and anti-TGN38 antibodies. As shown in Fig. 2 (*a* and *c*), the arrays of tubulin emanate from a single juxtanuclear point corresponding to the MTOC. In control cells the TGN staining spreads out from the same point (Fig. 2 *b*), while in BFA-treated cells the juxtanuclear concentration of fluorescence colocalizes with the center of the tubulin array (compare Fig. 2, *c* and *d*). This colocalization suggests that, in the presence of BFA, the TGN is concentrated at the MTOC.

BFA-induced Redistribution of TGN38 is Multi-Phasic and Independent of Protein Synthesis

In BFA-treated cells, TGN38 can be seen in two distinct subcellular regions: a tight perinuclear concentration at the MTOC and diffusely throughout the cell. To assess the ki-

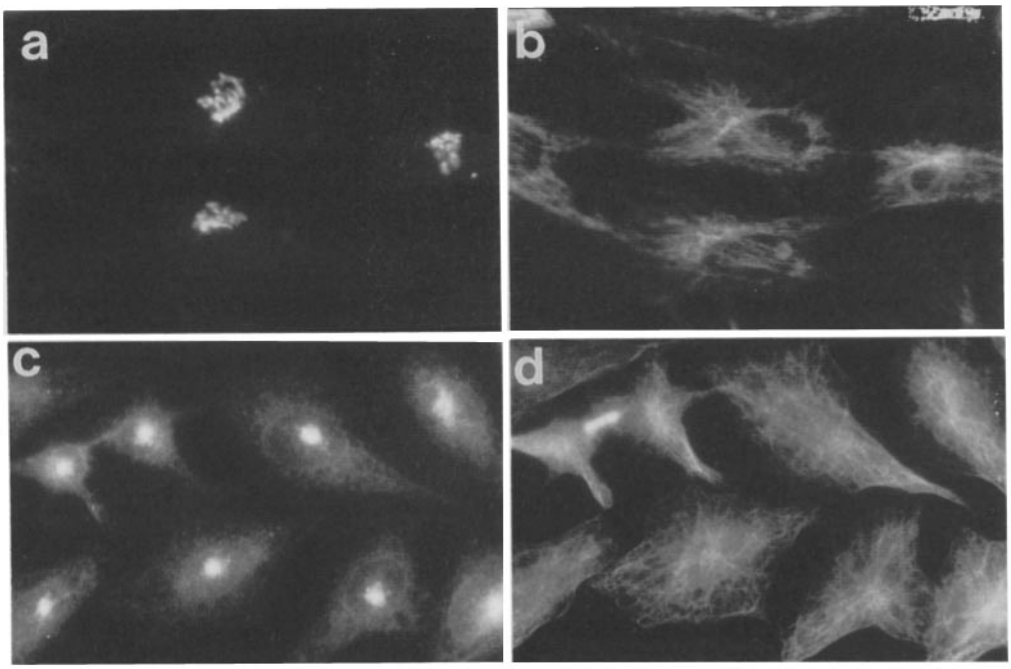

Figure 2. Colocalization of redistributed TGN with the MTOC. Methanol fixed NRK cells were incubated with murine monoclonal anti-tubulin antibodies and rabbit polyclonal antisera to TGN38 in the presence (panels *c* and *d*) or absence (panels *a* and *b*) of BFA. Binding of anti-tubulin antibody was detected using rhodamine conjugated second antibody (*b* and *d*), binding of anti-TGN38 antibody using fluorescein conjugated second antibody (*a* and *c*). Bar, 10 μm.

netics of redistribution of these two components we treated cells with 5 μg/ml BFA for varying times before immunofluorescence analysis using anti-TGN38 antibody. There were no obvious changes in subcellular distribution of the TGN at early time points (<1 min, Fig. 3, *b* and *c*). In cells treated with BFA for 5 min staining of the TGN appeared more diffuse than in control cells, but still somewhat punctate, with projections emanating outward from the typical perinuclear cap (Fig. 3 *d*). In cells which had been treated with BFA for 10 min a rapid reversal of this apparent dispersal process had occurred, and a compact center of TGN38 staining had begun to appear at the nuclear membrane (Fig. 3 *e*). Cells incubated in the presence of BFA for 30 min before immunofluorescence analysis using anti-TGN38 antibody demonstrated a clear perinuclear localization of the TGN; the diffuse staining of the cytoplasm was also apparent (Fig. 3 *g*). The distribution of the TGN seen in cells treated with BFA for 30 min had stabilized in cells treated with BFA for 1–3 h (Fig. 3, *h* and *i*). This time course was repeated multiple times with similar results.

Immunofluorescence analysis, using anti-TGN38 antibody, was also performed on cells which had been pre-treated with 20 μg/ml cycloheximide for 1.5 h before a 3-h incubation in BFA. This was to determine whether (*a*) the diffuse pattern of staining seen throughout the cell following BFA treatment is because of dispersal of newly synthesized TGN38 from the *cis*-, *medial*-, and *trans*-Golgi cisternae into the ER and (*b*) whether any part of the BFA-induced redistribution of the TGN is dependent on protein synthesis. Cycloheximide treatment had no observable effect on BFA-induced redistribution of the TGN (data not shown).

BFA-induced Redistribution of TGN38 is Reversible

Cells grown on coverslips were incubated at 37°C in the presence of 5 μg/ml BFA for 3 h, washed, and then incubated at 37°C in the absence of BFA for varying times before immunofluorescence analysis using anti-TGN38 antibody. Within 5 min of removal of BFA from the incubation medium an elongation of the tight juxtanuclear pattern of TGN staining was observed, the first step in the reversal of its redistribution (Fig. 4 *a*). The movement of the TGN back to its original perinuclear position proceeded through a stage similar to that seen in cells which had been incubated in the presence of BFA for 5–10 min, however it was sightly slower (15 min in the absence of BFA) in reaching this distribution pattern (Fig. 4 *b*). Recovery was complete following a 1-h incubation at 37°C in the absence of BFA (Fig. 4 *d*). By this time, the immunofluorescence staining pattern seen with anti-TGN38 antibodies closely resembled that observed in control cells.

Effect of Nocodazole on BFA-induced Redistribution of the TGN

In studies using microtubule disrupting agents (Rogalski and Singer, 1984) and in studies on mitotic cells (Lucocq and Warren, 1987), it has been shown that the localization of the Golgi complex is perturbed when the microtubule network is dissociated. The retrograde transport of proteins from the Golgi stack to the ER has also been shown to be dependent on microtubules (Lippincott-Schwartz et al., 1990). The location of TGN38 in BFA-treated cells, in a tight concentration next to the nucleus, suggested that the TGN might be collapsing toward the MTOC during the course of BFA treatment. We used the ability of nocodazole to inhibit tubulin polymerization (DeBrabander et al., 1976) to assess whether microtubules are necessary for this redistribution to occur. Cells were treated with different combinations of BFA and nocodazole, fixed, permeabilized, and processed for immunofluorescence analysis using anti-TGN38 antibody. In cells which had been treated with nocodazole alone, the pattern of staining suggested a fragmentation of the TGN into tight vesicular structures dispersed throughout the cytoplasm (Fig. 5 *b*). A similar pattern has been observed when antibodies to other Golgi proteins have been used in immunofluorescence analysis of nocodazole-treated cells (Lippincott-

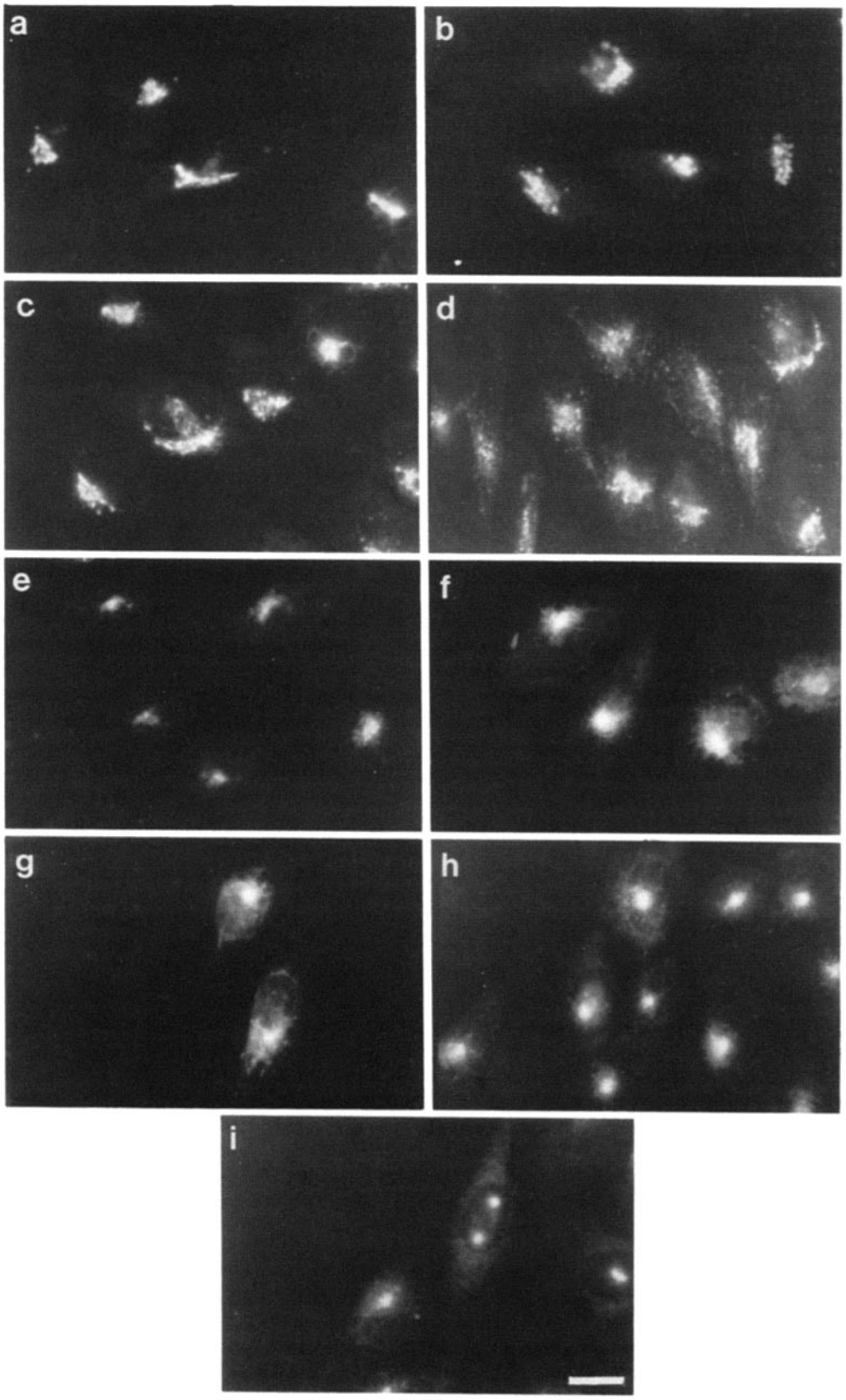

Figure 3. Time course of BFA-induced redistribution of the TGN. NRK cells were incubated at 37°C in the presence of 5 μg/ml BFA for the indicated times before processing for immunofluorescence analysis using anti-TGN38 antibody (*a* = 0 min, *b* = 0.5 min, *c* = 1 min, d = 5 min, e = 10 min, f = 15 min, *g* = 30 min, *h* = 60 min, *i* = 180 min). Bar, 10 μm.

Schwartz et al., 1990). Cells pre-treated with nocodazole for 2 h before addition of BFA for 1 h lack the tight perinuclear dot seen in cells incubated with BFA alone (compare Fig. 5, *c* with *a*). However, small vesicular structures are stained throughout most of the cell and there is very diffuse staining of the entire cytoplasm (Fig. 5 *c*). Cells treated with BFA for 1 h before nocodazole treatment for 2 h also contain punctate vesicular structures in addition to the diffuse cytoplasmic

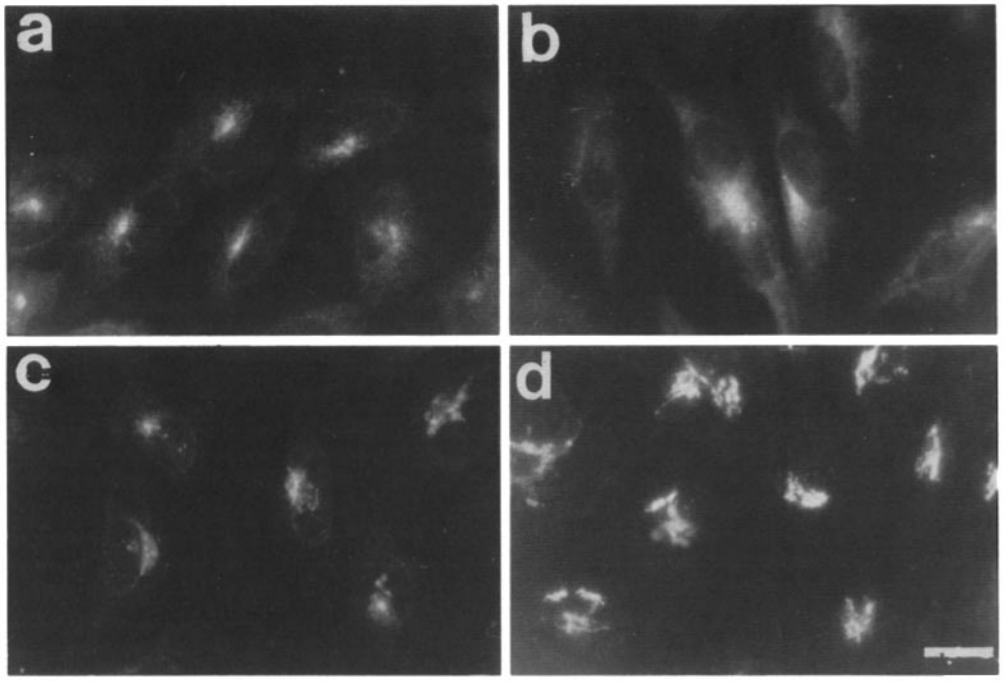

Figure 4. NRK cells were incubated at 37°C in the presence of 5 μg/ml BFA for 3 h, then at 37°C in the absence of BFA for 5 min (*a*), 15 min (*b*), 30 min (*c*), or 60 min (*d*) before processing for immunofluorescence analysis using anti-TGN38 antibody. Bar, 10 μm.

staining; however, these vesicles are concentrated in a perinuclear region rather than being dispersed throughout the cell (Fig. 5 *d*). These results support the hypothesis that microtubules are essential for both (*a*) the initial collapse of the TGN towards the nucleus and (*b*) maintaining this position once attained.

Energy Requirement for BFA-induced Redistribution of the TGN

To determine whether TGN redistribution was energy dependent we lowered cellular ATP levels by incubating NRK cells with 50 mM 2-deoxy-D-glucose and 3 mM sodium azide for 3 min at 37°C before addition of 5 μg/ml BFA. Cells were then incubated at 37°C for a further 30 min before processing for immunofluorescence analysis (Lippincott-Schwartz et al., 1989). ATP depletion inhibited the BFA-induced redistribution of mannosidase II (Fig. 6, *b* and *c*) (Lippincott-Schwartz et al., 1989). However, it had no effect on the BFA-induced redistribution of TGN38 (Fig. 6, *e* and *f*). This result demonstrates that the effect of BFA on the TGN is less sensitive to energy-depletion protocols than the BFA-induced redistribution of mannosidase II to the ER. Similar results were obtained using the anti-TGN38 antibody and a more stringent protocol for reducing ATP levels. This consisted of

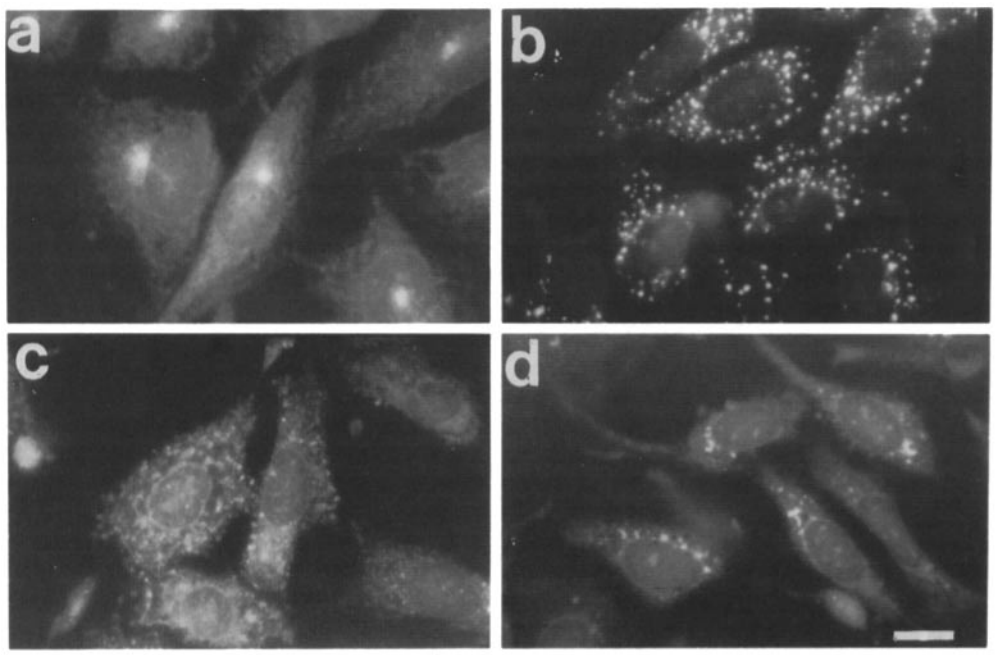

Figure 5. NRK cells were subjected to the following treatments before processing for immunofluorescence analysis using anti-TGN38 antibody. Incubation for 2 h at 37°C in the presence (*b*) or absence (*a*) of 20 μg/ml nocodazole alone, or in the presence of 20 μg/ml nocodazole for 2 h followed by 5 μg/ml BFA for 1 h (*c*), or in the presence of 5 μg/ml BFA for 1 h followed by 20 μg/ml nocodazole for 2 h (*d*). Bar, 10 μm.

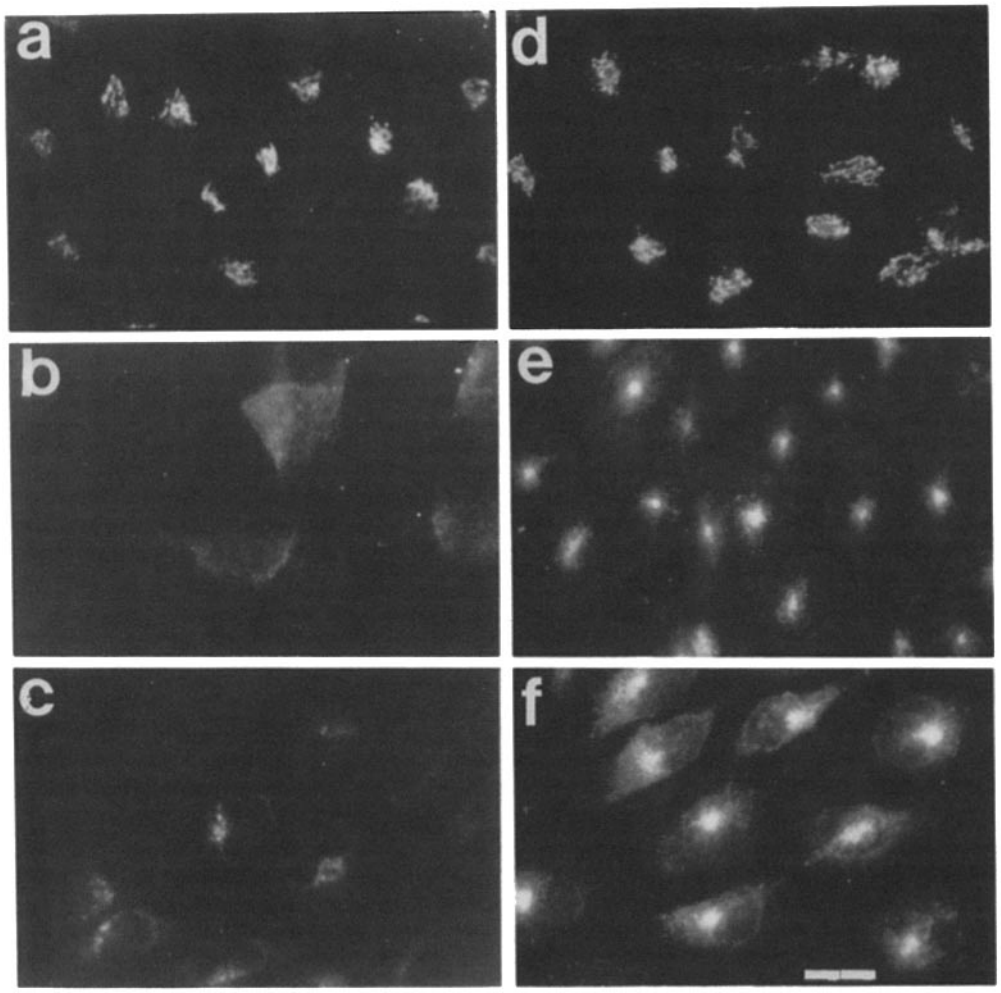

Figure 6. NRK cells were subjected to the following treatments before processing for immunofluorescence analysis using anti-Mannosidase II antibody (*a–c*) or anti-TGN38 antibody (*d–f*). Incubation for 3 min at 37°C in the presence (*b* and *e*) or absence (*a* and *d*) of 50 mM 2-deoxy-D-glucose and 3 mM sodium azide alone; incubation for 3 min at 37°C in the presence of 50 mM 2-deoxy-D-glucose and 3 mM sodium azide followed by 30 min at 37°C in the presence of 5 μg/ml BFA (*c* and *f*). Bar, 10 μm.

treating cells with 1 mM 2-deoxy-D-glucose and 10 mM sodium azide for 30 min in serum-free medium at 37°C (Turner and Tartakoff, 1989) before incubation with BFA for an additional 30 min (data not shown).

GTP Requirement for BFA-induced Redistribution of the TGN

GTP has been shown to be involved in the uncoating of non-clathrin-coated Golgi vesicles (Melancon et al., 1987) and has been implicated in the regulation of the association and disassociation of a peripheral membrane protein, β-COP, from the Golgi stacks (Donaldson et al., 1990). β-COP has been localized not only to the transitional elements and the Golgi stacks but also to the TGN (Duden et al., 1991). To test whether any component of the redistribution of the TGN is GTP dependent we permeabilized cells grown on coverslips by filter stripping with nitrocellulose according to the method of Simons and Virta (1987) and as described by Donaldson et al. (1990). Before processing for immunofluorescence analysis with anti-TGN38 antibody, the permeabilized cells were incubated at 37°C for 1 h under the following conditions: (*a*) with incubation buffer alone, (*b*) with incubation buffer plus 5 μg/ml BFA, (*c*) with incubation buffer plus 1 mM GTPγS, or (*d*) with incubation buffer plus 1 mM GTPγS followed by addition of BFA to 5 μg/ml and incubation for a further 1 h at 37°C. There was no difference in the pattern of TGN38 staining between non-permeabilized cells and permeabilized cells incubated in buffer alone or in buffer plus GTPγS (compare Figs. 6 *a* and 7, *a* and *c*). BFA treatment had the same effect on permeabilized cells as it did on intact cells (compare Fig. 7 *b* and 6 *b*). However, in permeabilized cells incubated with GTPγS prior to BFA treatment the pattern of TGN38 staining is reminiscent of that seen in non-BFA-treated cells (compare Fig. 7, *a* and *d*). This result implies that hydrolysis of GTP is required during the BFA-induced redistribution of the TGN.

Discussion

The data presented demonstrate that BFA induces a redistribution of the TGN in NRK cells. The initial effects of BFA are fairly rapid, with observable alterations to the morphology of the TGN within 5 min of addition of the drug. Approximately 1 h after addition of BFA the TGN has stabilized in its new position, predominantly juxtanuclear (colocalized with the MTOC), but with significant amounts distributed throughout the cell. Previous work has shown that the BFA-induced redistribution of components of the Golgi stack to the ER is energy, GTP, and microtubule dependent as well as being reversible (Lippincott-Schwartz et al., 1989; Lippincott-Schwartz et al., 1990; Donaldson et al., 1990; Donald-

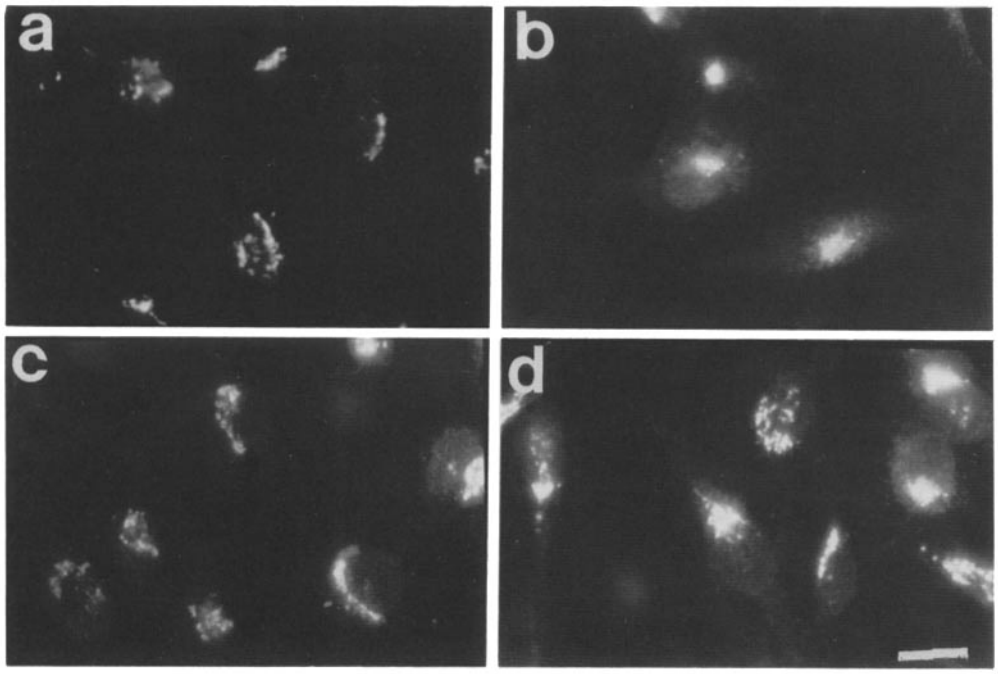

Figure 7. Filter-stripped NRK cells were subjected to the following treatments before processing for immunofluorescence analysis using anti-TGN38 antibody. Incubation for 1 h in incubation buffer (*a*), incubation for 1 h at 37°C in incubation buffer plus 5 μg/ml BFA (*b*), incubation for 1 h at 37°C in incubation buffer plus 1 mM GTPγS (*c*), incubation for 1 h at 37°C in incubation buffer plus 1 mM GTPγS followed by incubation for 1 h at 37°C in incubation buffer plus 5 μg/ml BFA (*d*). Bar, 10 μm.

son et al., 1991). We have characterized BFA-induced redistribution of the TGN with respect to each of these parameters, and found that it too is GTP and microtubule dependent as well as being reversible. Significantly, however, BFA-induced redistribution of the TGN is not energy dependent.

EM studies have shown that the cisternal Golgi stacks disappear following BFA treatment of cells (Misumi et al., 1986; Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989). This disappearance of the Golgi stacks is paralleled by the appearance of clusters of small vesicles associated with the centrioles. The number of vesicles observed is insufficient to account for all the membrane previously found in the Golgi cisternae (Ulmer and Palade, 1989). Recent work would suggest that, in BFA-treated cells, the majority of Golgi-derived membrane would not be in vesicular structures but colinear with the ER membrane (Lippincott-Schwartz et al., 1989; Orci et al., 1991). In light of the data we have presented, it is probable that the clusters of small, centriole-associated vesicles observed in BFA-treated cells by Ulmer and Palade (1989) are derived from the TGN.

Previous studies to have addressed the question of whether BFA treatment affects the TGN have assayed the degree of sialylation of proteins trapped in the ER after collapse of the *cis*-, *medial*- and *trans*-Golgi compartments into the ER. In BFA-treated cells, VSV-G protein became endoglycosidase H resistant. This indicated that it had been processed by enzymes, normally resident in *cis*- and *medial*-Golgi cisternae, which had redistributed to the ER. Addition of sialic acid to VSV-G protein did not occur, implying that the contents of the TGN had not been affected in the same way as those of the *cis*- and *medial*-Golgi cisternae (Doms et al., 1989). Addition of sialic acid to hemagglutinin was markedly inhibited in BFA-treated cells as was transport of the protein to the cell surface (Nuchtern et al., 1989). Chege and Pfeffer (1990) reported that, in cells incubated in BFA for 8 h, <10% of mannose-6-phosphate receptors acquired sialic acid. In these experiments some sialylation of receptors recycled from the cell surface was detected. By contrast, in BFA-treated murine erythroleukemia cells, glycophorin was eventually sialylated (as judged by its ability to bind WGA) (Ulmer and Palade, 1989). It has been proposed that the slightly different results obtained by the two groups may be due to different degrees of disruption of the Golgi complex in the two cell types. It has also been suggested that the sialylation of O-linked carbohydrate on glycophorin might be due to an enzyme which is distinct from that required for the addition of sialic acid residues to N-linked sugars on mannose-6-phosphate receptors, and that this enzyme is normally located in either the *medial*- or *trans*-Golgi cisternae rather than the TGN (Chege and Pfeffer, 1990). Taken together these data suggest that BFA does not cause redistribution of resident TGN enzymes into the ER and that, even in the presence of BFA, it may retain some of its functions. They do not address the question of whether or not BFA induces redistribution of the TGN to sites other than the ER. Others have performed limited immunocytochemical studies using a TGN-specific mAb and WGA as markers of the TGN (Lippincott-Schwartz et al., 1989). These experiments have been interpreted as showing that the TGN is morphologically unaffected by BFA. However, while WGA does bind the terminal *N*-acetylneuraminic acid residues one would expect to find predominantly in the TGN and thereafter in the secretory pathway (Tartakoff and Vassalli, 1983), it also binds other carbohydrate structures (Goldstein and Hayes, 1978), thus limiting its validity as a specific marker of the TGN. The WGA-associated fluorescence staining presented by Lippincott-Schwartz et al. (1990) is quite intense; our data show that the distribution of the TGN in control and BFA-treated cells is subtly different, the intensity of the WGA-associated fluorescence may well have masked this difference. Intensity of staining might also explain why the mAb used by Lippincott-Schwartz et al. (1990) failed to discriminate between the morphology of the TGN in BFA treated and control cells. We have repeated our immunofluorescence assays using the fixation and permeabili-

zation procedures used by Lippincott-Schwartz et al. (1990) and obtained the same results as those presented in this paper.

Disruption of microtubules by nocodazole ablates the BFA-induced concentration of TGN38 at the MTOC, but has little effect on the diffuse cytoplasmic staining. The localization and stability of the Golgi complex as a whole has been shown to be microtubule dependent (reviewed in Kelly, 1990; Kreis, 1990). In addition it has been proposed that a 110-kD protein, β-COP, may play a role in linking Golgi membranes to a "scaffold" or skeletal framework based on microtubules which originate at the MTOC (Duden et al., 1991). β-COP associates not only with the more proximal Golgi stacks but also with the TGN and dissociates from membranes within minutes of BFA treatment (Duden et al., 1991; Donaldson et al., 1990). β-COP may, therefore, also be tethering the TGN to this scaffold. In vitro experiments by Orci et al. (1991) have suggested that there is a dynamic balance governing anterograde (vesicular) and retrograde (tubular) transport between the Golgi stacks. This balance is tipped in favor of retrograde transport in the presence of BFA since the release of β-COP from membranes inhibits vesicle formation but does not affect the formation of retrograde tubules. The predominance of retrograde transport rapidly redistributes components of late Golgi stacks into early Golgi stacks, and eventually into the ER (Lippincott-Schwartz et al., 1989). The BFA-induced release of β-COP from the TGN membrane does not appear to result in the same redistribution as that observed for the Golgi stacks (Nuchtern et al., 1989; Chege and Pfeffer, 1990; and this paper). This would imply that the balance between anterograde and retrograde transport found between the Golgi cisternae is not found between the Golgi and the TGN and suggests a lack of tubular retrograde transport from the TGN to the *trans*-Golgi. Thus, if β-COP does anchor the TGN to a scaffold, BFA treatment would dissociate the TGN from that scaffold and also from the Golgi stacks. This would allow the TGN to migrate to the MTOC rather than redistributing into the ER, and is the effect we have observed. The initial distribution of the TGN towards the ER may be due to a difference in kinetics between uncoupling of the TGN from the scaffold and uncoupling from the rest of the cisternae.

GTPγS has been shown to block vesicular transport between the Golgi stacks by inhibiting vesicle uncoating; this leads to an accumulation of non-clathrin-coated vesicles (Melancon et al., 1987; Orci et al., 1989). Duden et al. (1991) demonstrated that this effect of GTPγS is correlated with an increase in the amount of β-COP associated with non-clathrin-coated vesicles, implying that GTP hydrolysis is required to release β-COP from the coat as a prerequisite of vesicle uncoating. Pretreatment of cells with GTPγS prevents both the BFA-induced release of β-COP from Golgi membranes and the movement of Golgi membrane into the ER (Donaldson et al., 1991). By analogy with these findings, one would expect (*a*) GTP hydrolysis to release β-COP from an interaction between a scaffold, β-COP, and the TGN membrane, thus leading to a redistribution of the TGN, and (*b*) pretreatment with GTPγS to inhibit BFA-induced redistribution of the TGN. Data presented in this paper are in accord with this model, since GTPγS alone has no effect on the subcellular distribution of the TGN while pretreatment of cells with GTPγS prevents the BFA-induced redistribution of the TGN.

The diffuse TGN38 staining seen throughout the cytoplasm in BFA-treated cells is reminiscent of the pattern of ER staining seen with anti-mannosidase II antibodies in BFA-treated cells. This may indeed represent a BFA-induced redistribution to the ER of a proportion of the contents of the TGN and might serve to explain the observation of Chege and Pfeffer (1990) that there is some sialylation of mannose-6-phosphate receptors in BFA-treated cells. However, two pieces of evidence argue against this being redistribution to the ER. Firstly, the BFA-induced dispersal of mannosidase II into the ER can be inhibited following depletion of ATP, whereas ATP depletion has no effect on the BFA-induced redistribution of TGN38. Secondly, mannosidase II appears throughout the ER within 10 min of addition of BFA to cells (Lippincott-Schwartz et al., 1990), whereas the diffuse pattern of TGN38 staining does not begin to appear until 15 min after addition of BFA. The precise nature of the diffuse structures recognized by anti-TGN38 antibodies in BFA-treated cells remains unclear.

The TGN is morphologically distinct from the Golgi cisternae in that it has more of a tubular–vesicular structure (Griffiths et al., 1989) in keeping with its function as a sorting site for proteins destined for different locations within the cell. Other compartments with a similar configuration include the ER, the early endosomal network, and the tubular lysosomes of macrophages. A number of studies have suggested that the development of tubular extensions from these compartments are microtubule dependent (Cooper et al., 1990; Lee and Chen, 1988; Hollenbeck and Swanson, 1990). Introduction of anti-kinesin antibodies into macrophages results in the centripetal collapse of the arrays of tubular lysosomes into a perinuclear mass suggesting that kinesin is responsible for maintaining the extensions of this organelle (Hollenbeck and Swanson, 1990). If a similar situation exists for the TGN, and BFA not only inhibits the association of β-COP with membrane but also dissociates a kinesin-like motor from the TGN, one would predict that BFA treatment would result in the collapse of the TGN into the cell center. This is what we have observed.

The morphology and subcellular localization of the TGN are clearly affected by BFA in a manner which is both reversible and microtubule and guanine nucleotide dependent. It remains to be seen whether these effects are due solely to direct interactions between BFA and β-COP, or whether there are also direct or indirect interactions between BFA and kinesin-like motors.

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