Tubulation of Golgi Membranes In Vivo and In Vitro in the Absence of Brefeldin A

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Abstract. Recent in vivo studies with the fungal metabolite, brefeldin A (BFA), have shown that in the absence of vesicle formation, membranes of the Golgi complex and the *trans*-Golgi network (TGN) are nevertheless able to extend long tubules which fuse with selected target organelles. We report here that the ability to form tubules (>7 μm long) could be reproduced in vitro by treatment of isolated, intact Golgi membranes with BFA under certain conditions. Surprisingly, an even more impressive degree of tubulation could be achieved by incubating Golgi stacks with an ATP-reduced cytosolic fraction, without any BFA at all. Similarly, tubulation of Golgi membranes in vivo occurred after treatment of cells with intermediate levels of NaN_3 and 2-deoxyglucose. The formation of tubules in vitro, either by BFA treatment or low-ATP cytosol, correlated precisely with a loss of the vesicle-associated coat protein β-COP from Golgi membranes. After removal of BFA or addition of ATP, membrane tubules served as substrates for the rebinding of β-COP and for the formation of vesicles in vitro. These results provide support for the idea that a reciprocal relationship exists between tubulation and vesiculation (Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz. 1992. *J. Cell Biol.* 116:1071–1080). Moreover, they show that tubulation is an inherent property of Golgi membranes, since it occurs without the aid of microtubules or BFA treatment. Finally the results indicate the presence of cytosolic factors, independent of vesicle-associated coat proteins, that mediate the budding/tubulation of Golgi membranes.

MEMBRANE transport between intracellular compartments of eucaryotic cells appears to be mediated by small vesicles that bud from a donor compartment and fuse with a target (for historical perspective see Palade, 1975; for a recent review see Melançon et al., 1991). Well-established examples of vesicular transport can be found at the plasma membrane and at the *trans*-Golgi network (TGN), where ~80–100 nm clathrin-coated vesicles mediate the selective endocytosis of extracellular ligands and the packaging of newly synthesized lysosomal enzymes, respectively (for review see Pearse and Robinson, 1990). Numerous studies have shown that clathrin-coated vesicles are composed of a unique set of vesicle-associated proteins, including clathrin and its associated adaptin molecules, which appear to be required for vesicle formation (Smythe et al., 1989; Lin et al., 1991; Schmid and Smythe, 1991). Different sets of adaptin complexes associate with clathrin to form plasma membrane- and TGN-specific coated vesicles (Robinson, 1987; Ahle et al., 1988).

Another well-established example of vesicular traffic has come from the work of Rothman and colleagues. Their series of elegant reconstitution experiments demonstrated that a new class of ~90-nm vesicles mediate the transport of material between cisternal elements of the Golgi complex (for reviews see Rothman and Orci, 1990; Duden et al., 1990; Rothman and Orci, 1992). Recent studies have shown that these Golgi transport vesicles are also coated, not with clathrin (Orci et al., 1986), but with a group of proteins known as COPs, which form a macromolecular complex (coatomer) surrounding nascent and free vesicles, and which appear to be required for some step in the actual formation of vesicles (Malhotra et al., 1989; Serafini et al., 1991; Waters et al., 1991). Interestingly, the COP proteins are similar to clathrin and its associated adaptin proteins in molecular weight and, in the case of β-COP and β-adaptin, primary amino acid sequence as well (Duden et al., 1991; Serafini et al., 1991). Using a genetic approach, Pryer et al. (1992) have also identified components involved in the formation of transport vesicles in yeast (for review see Pryer et al., 1992). For example, the protein product of the SEC21 gene is weakly homologous to mammalian β-COP (Pryer et al., 1992). Currently, it is thought that the COPs and clathrin (with associated proteins) assemble onto appropriate membranes, somehow participate in the formation of a vesicle, and then dissociate from the vesicle before or after docking with a target membrane. The vesicle-associated coat proteins then recycle for another round of vesicle formation and docking (Rothman and Orci, 1992; Klausner et al., 1992). Although it is now clear that coatomers and clathrin complexes serve similar functions to produce different types of vesicles, their exact role in vesicle formation has not been determined.

It is generally appreciated that vesicle formation accounts for much of the traffic between intracellular organelles; however, recent studies on the striking effects of the fungal metabolite, brefeldin A (BFA),[1] have suggested that vesicle-associated coat proteins may mask a more fundamental behavior of organellar membranes (for review see Klausner et al., 1992). BFA has been shown to inhibit secretion by blocking ER–Golgi and intercisternal Golgi transport (Misumi et al., 1986; Oda et al., 1987). In doing so, BFA dramatically altered both the structural and functional integrity of the Golgi complex by causing the rapid redistribution of resident Golgi proteins to the ER and the disappearance of pancake-like stacks of cisternae (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Doms et al., 1989). By inhibiting the forward (anterograde) pathway, BFA revealed the existence of a microtubule-dependent, retrograde pathway from the Golgi to the ER (Lippincott-Schwartz et al., 1990). An additional surprising observation was that this retrograde transport occurred by the formation of long membrane tubules which extended from Golgi membranes and fused with the ER (Lippincott-Schwartz et al., 1990).

More recently, BFA has also been found to profoundly influence the organization of various other intracellular compartments by, for example, causing extensive membrane tubule formation from the TGN, endosomes, and lysosomes (Wood et al., 1991; Lippincott-Schwartz et al., 1991; Hunziker et al., 1991). In the case of the TGN, BFA induced membrane tubules to extend out and fuse with endosomal vesicles resulting in an extensive TGN–endosomal network, in a process that is very similar to the formation of a fused ER–Golgi network. As with BFA-induced retrograde ER transport (Lippincott-Schwartz et al., 1990), formation of the TGN–endosomal network appears to be facilitated by microtubules (Wood et al., 1991).

The first detectable effect of BFA in vivo is to prevent the association of β-COP with Golgi membranes (Donaldson et al., 1990). Recently, it has been shown that BFA has a similar effect on clathrin and its γ-adaptin in the TGN, but not on the plasma membrane coated vesicles (Robinson and Kreis, 1992; Wong and Brodsky, 1992). In addition, BFA inhibits the formation of COP-coated vesicles on isolated Golgi complexes in vitro, and causes isolated Golgi cisternae to fuse into an extensive network (Orci et al., 1991). Orci et al. (1991) suggested that this network arose by the formation of fusion-competent membrane tubules. Taken together, the above studies indicate that in the absence of vesicle formation, i.e., in the presence of BFA, membranes derived separately from various organelles are nevertheless capable of forming tubular structures. However, the in vivo studies do not rule out the possibility that tubulation occurs as a secondary effect of BFA treatment by, for example, allowing abnormal associations with microtubule-based motors.

In this paper we report on experiments to test the hypothesis that tubulation is an inherent property of organellar membranes. Remarkably, tubulation could be reproduced both in vivo and in vitro by BFA treatment or by simply lowering ATP levels below that apparently needed for coated vesicle formation. Moreover, formation of tubules, and their subsequent ability to serve as substrates for vesicle formation, correlated with the dissociation and re-binding of vesicle-associated coat proteins, respectively.

1. *Abbreviations used in this paper*: BFA, brefeldin A; FLP, finger-like projection; NRK, normal rat kidney.

Materials and Methods

Materials

Young male CD rats (150–200 g) were obtained from the rat colony in the Department of Neurobiology, Cornell University (Ithaca, NY) and were fed ad libitum before sacrifice. BFA was purchased from Epicentre Technologies (Madison, WI). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Normal rat kidney (NRK) cells were obtained from E. Racker (Cornell University) and grown in MEM containing 10% Nu-Serum (Collaborative Research, Bedford, MA).

Isolation of Intact Golgi Complexes

Intact Golgi complexes were isolated from rat liver by a modification of the method used by Balch et al. (1984*a*) for cultured cells exactly as described (Cluett and Brown, 1992). Briefly, livers were excised from male CD rats, minced to a fine puree, and homogenized with a Balch/Rothman homogenizer (Balch and Rothman, 1985) equipped with a 0.2460-in. ball bearing which allowed a calculated clearance of 0.0054 in. A postnuclear supernatant (PNS) was prepared, and intact Golgi complexes were obtained by sucrose-gradient centrifugation (Cluett and Brown, 1992). The second band from the top of the gradient, representing the 0.9/1.0 M sucrose interface and containing the highest concentration of intact Golgi complexes, was harvested and used for experiments.

Preparation of Cytosol

A liver, excised from a freshly killed male CD rat, was rinsed and minced in 0.25 M sucrose, 50 mM KCl, 25 mM Tris-HCl, pH 7.4 (KST buffer). Additional KST buffer was added to make a 43% (wt/vol) suspension which was then passed 9–12 times through the ball bearing homogenizer using a 0.2460-in. ball as above. A PNS was made from the homogenate exactly as described above, and loaded into thick-walled polycarbonate tubes and centrifuged at 75,000 rpm at 4°C for 30 min in a TL 100.3 rotor which ensured an r_{min} = 150,000 *g*. The resulting high speed supernatant was passed over a G25 filtration column and eluted with 50 mM KCl and 10 mM Tris-HCl, pH 7.4. Two major protein peaks were collected and pooled separately: a first, larger peak, representing material not retained by the column, and a second, somewhat lesser peak. Only the first peak, termed "cytosol," was used fresh or frozen at −80°C.

Rat brain cytosol was prepared and used identically as above. Bovine brain cytosol, prepared essentially as described by Malhotra et al. (1989), was used in the negative staining experiments.

Incubations of Isolated Intact Golgi Complexes

In a typical experiment, 300 μl of isolated, intact Golgi complexes (fresh or freshly thawed) in ~1 M sucrose were incubated in the presence of 300 μl of rat liver or rat brain cytosol (3 mg/ml). Included in this reaction mix were 10 mM Hepes-KOH, pH 7.4, 25 mM KCl, 1 mM $MgCl_2$, 1 mM NaATP, 1 mM phosphocreatine, 7.9 U of creatine phosphokinase in a final sucrose concentration of ~0.5 M. Low-ATP cytosol was prepared by incubating the standard cytosol preparation (containing Hepes-KOH as above) with 27.5 U hexokinase (baker's yeast)/50 mM glucose for 15 min at 37°C. Subsequent studies have shown that addition of 10 μM ATP to hexokinase-treated and extensively dialyzed cytosol is sufficient to promote tubulation. To start reactions, cytosol (either ATP rich or low ATP) was added dropwise to Golgi preparations. Additional reagents and specific conditions of incubation time and temperature are described in the text and figure legends. Reactions were stopped by the addition of an equal volume glutaraldehyde (to make a final concentration of 1.5–2% glutaraldehyde) fixative as described below, and transferred to ice. The membranes were subsequently processed for EM.

Several different BFA concentrations (10–32 μg/ml) were used in these experiments which did not result in any significant differences in the final extent of membrane tubulation.

Recovery Experiments

In some experiments, we investigated if isolated Golgi complexes could re-

cover from BFA or low-ATP cytosol treatments. Two methods of recovery were used depending on whether BFA or low-ATP cytosol was used to induce tubulation. For recovery from BFA, Golgi membranes were incubated under specific conditions as described in the text, and at the end of the incubation 500 μl of this mix was added to a microfuge tube which contained a 25 μl cushion of 1.2 M sucrose, 10 mM Hepes–KOH, pH 7.4, and the tubes were then spun at 15,000 rpm for 15 min at 4°C to ensure an r_{min} = 50,000 *g*. A flocculent band representing Golgi complexes formed at the interface, was harvested with a Pipetman (volume ~50 μl), and the membranes were resuspended by gently pipetting up and down. 300 μl of fresh cytosol containing Hepes, ATP, Mg^{2+} and the ATP-regenerating system, in concentrations listed above, was added to these harvested fractions in the presence or absence of BFA as indicated in the text. Samples were reincubated for an additional 20 min at 37°C. For recovery from hexokinase-treated, low-ATP cytosol, Golgi membranes were incubated as described above to induce tubulation, and the mixture was layered on top of a step gradient in Beckman TLS 55 rotor tubes (Beckman Instruments Inc., Palo Alto, CA). The gradient consisted of 500 μl 1.2 M sucrose, 500 μl of 0.7 M sucrose, and 1 ml of Golgi reaction mix (the sucrose solutions contained 10 mM Hepes, 25 mM KCl, pH 7.4). Gradients were spun at 20,000 rpm for 15 min at 4°C in a Beckman TL-100 centrifuge. Golgi membranes (~100 μl) were harvested from the 0.7/1.2 M sucrose interface, and incubated with 400 μl of fresh cytosol containing ATP and the regenerating system as above, at 37°C for various periods of time. 50 μM GTPγS was included in the recovery steps for samples that were to be analyzed and quantitated by EM. Although not necessary for production of coated vesicles during the recovery period, GTPγS causes an accumulation of coated vesicles on Golgi membranes (Malhotra et al., 1989) which made it easier to distinguish transport vesicles from damaged or disrupted membranes. Reactions were stopped by addition of glutaraldehyde as above.

Electron Microscopy

Samples for thin-section EM were fixed by the addition of an equal volume of freshly made 4% glutaraldehyde/0.1 M Na cacodylate, pH 7.2,/0.25 M sucrose at room temperature for 1–1.5 h, centrifuged at 25,500 *g* for 60 min, and the resulting pellets were fixed in 2% glutaraldehyde/0.1 M Na cacodylate, pH 7.2/0.25 M sucrose overnight at 4°C. The following day, the pellets were postfixed in 1% OsO_4/0.1 M Na cacodylate, pH 7.2, on ice for 1 h and stained with 1% tannic acid/0.1 M Na cacodylate, pH 7.2, at room temperature in the dark for 1–1.5 h according to the protocol of Simonescieu and Simonescieu (1976). After rinsing in dH_2O, samples were stained en bloc in 2% aqueous uranyl acetate at room temperature for 1–3 h, progressively dehydrated in EtOH, and embedded in Spurr's resin. After curing, each pellet was split in half and re-embedded to expose a longitudinal cross section through the full depth of the pellet. This sections were cut, stained with lead citrate, and examined and photographed on an electron microscope (model 301; Philips Electronic Instruments Co., Mahwah, NJ). For negative staining EM, suspensions of Golgi membranes in low-ATP bovine brain cytosol were further incubated on formvar/carbon-coated EM grids for 15 min at room temperature. Grids were stained with 2% phosphotungstic acid, pH 7.2, for 0.5–1 min, rinsed in the same, and air dried for EM.

β-COP Binding to Golgi Membranes

After various experimental conditions, the level of β-COP binding to Golgi

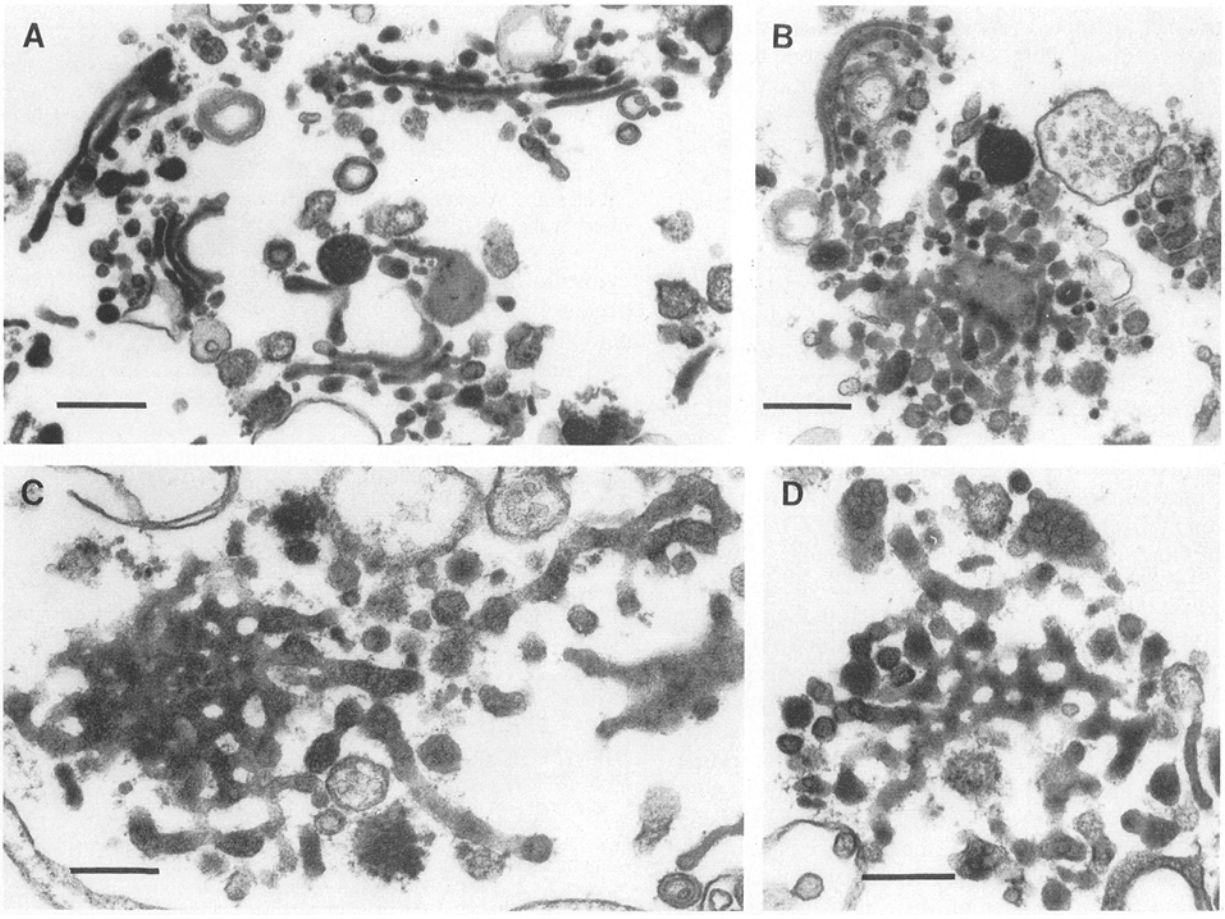

Figure 1. BFA induces that formation of tubular networks in isolated rat liver Golgi complexes under conditions that would normally promote vesicle formation. (*A*) Control Golgi complexes without added factors or reagents display stacks with separate but closely apposed cisternae and few 80–90-nm transport vesicles. (*B*) Golgi complexes incubated with cytosol plus ATP (and regenerating system) for 30 min at 32°C results in extensive vesicle formation. (*C* and *D*) Incubation of Golgi complexes with cytosol, ATP, and BFA (16 μg/ml) for 30 min at 32°C results in the formation of extensive tubular networks in which previously separate cisternae are now interconnected within the plane of the lipid bilayer. Bars: (*A* and *B*) 0.4 μm; (*C* and *D*) 0.25 μm.

membranes was determined. Membranes were incubated as described in the figure legends, centrifuged at 16,000 *g* for 15 min at 4°C. The pelleted membranes were washed two times with 25 mM KCl/10 mM Tris-HCl, pH 7.4,/0.25 M sucrose/1 mM ATP and solubilized with SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE followed by transfer to nitrocellulose paper and probing with mAbs against β-COP (diluted 1:500 from ascites fluid) (provided by Dr. T. Kreis). Immunoreactive proteins were detected with alkaline phosphatase-conjugated second antibodies and nitroblue tetrazolium cytochemistry. Bands were quantitated by scanning densitometry.

Immunofluorescence

Cells were fixed and processed using mouse mAb 10E6 which recognizes an antigen found in *cis*-Golgi cisternae (Wood et al., 1991).

Results

Limited Fusion Can Occur at the Periphery of Golgi Stacks

Recently, Orci et al., (1991) showed that BFA, in the presence of cytosol and ATP, caused the cisternae of isolated, intact Golgi complexes to form an extensively fused membranous reticulum or network. They hypothesized that inhibition of vesicle formation by BFA resulted in the formation of Golgi membrane tubules, as in vivo, which fused with adjacent cisternae. By limiting the components necessary for these phenomena, we attempted to induce intermediate stages in the formation of fused Golgi cisternae in the hope of better understanding the induction of membrane tubulation by BFA in vivo.

As seen by thin-section EM in Fig. 1 *A*, isolated, intact rat liver Golgi complexes were morphologically comparable with in vivo structures exhibiting stacks of separate, flattened cisternae and a small population of vesicles and tubules. As originally shown by Rothman and colleagues (Balch et al., 1984*b*; Orci et al., 1986), incubation of isolated Golgi stacks with cytosol and ATP (and a regenerating system) at 37°C for 30 min resulted in a substantial increase in the number of transport vesicles associated with the membranes, but the cisternae themselves remained as discrete units stacked in parallel array (Fig. 1 *B*). When BFA was added to Golgi complexes under these same conditions (cytosol plus ATP), extensive reticulum or network formation occurred identical to that seen by Orci et al. (1991) (Fig. 1, *C* and *D*). The percentage of Golgi complexes forming such tubular networks in BFA increased ~sevenfold compared with controls. However, when Golgi complexes were incubated in the presence of BFA and cytosol without exogenously added ATP or a regenerating system, then fusion occurred only at the periphery of the stacks, best seen in transverse or longitudinal profiles (Fig. 2). The central part of each cisterna remained discrete and was not fused to its neighbors. There was a large increase over controls in the percentage of Golgi stacks with fusion occurring only at the periphery of the stacks (Table I). In control samples without BFA, the presence of transport components and a low level of ATP in the cytosol caused a small increase in the number of vesicles associated with Golgi complexes (data not shown).

Formation of Tubular Projections From Golgi Complexes In Vitro and In Vivo

Because fusion of Golgi complexes into networks requires cytosolic fusogenic factors such as NEM-sensitive fusion protein (NSF) (Orci et al., 1991), we treated isolated Golgi complexes with BFA and ATP alone to identify other intermediates. Under these conditions there was a slight increase in the number of fused networks; however, the surprising observation was that when Golgi cisternae were viewed in en face profiles, there was a profusion of short, membranous tubules emanating from the rims (Fig. 3, *A* and *B*). These

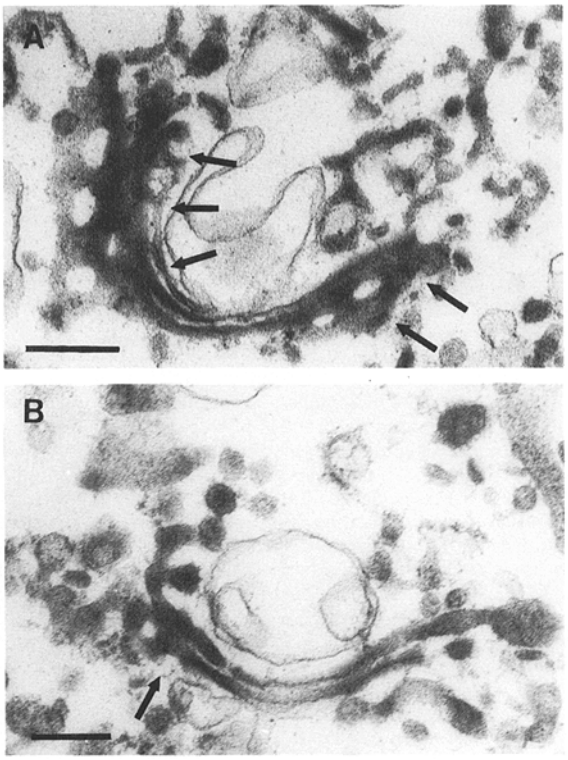

Figure 2. BFA-induced tubular networks originate at the rims of Golgi cisternae. When Golgi complexes were incubated with just cytosol and BFA (16 μg/ml) (no added ATP or regenerating system) for 20 min at 37°C, fusion occurred preferentially at the periphery of the stacks (the dilated rims) and did not spread to the interior regions of the stacks. We refer to these Golgi profiles as "Furs" for "fused rims." Arrows point to regions when apposed cisternae have fused at the rims. Bars, 0.2 μm.

*Table I. Formation of Tubular Intermediate Structures from Isolated Golgi Membranes**

Treatments	% FURs	% Tubular FLPs	*n*
Cytosol + ATP	1	5	178
Cytosol + BFA	24.5	28	192
ATP + BFA	4	42	462
Hexo/glu-cytosol	3	33.5	188
BFA	1	13	273

In these experiments, the standard incubation conditions were 35–37°C for 20 min. Electron micrographs were randomly taken through the entire depth of Golgi membrane pellets and profiles were counted and placed in one of two categories. *% FURs* refers almost exclusively to transverse profiles which showed only "fusion at the rims" of cisternae (on the periphery of stacks). *% Tubular FLPs* refers to Golgi profiles with both long and short tubular "finger-like projections" seen in en face and certain transverse profiles. Only those profiles with ≥3 tubular FLPs were included in this category. The results are expressed as a percentage of total Golgi profiles with the remaining exhibiting normal stacked morphology.

* Buffers, volumes, and concentrations for each of the treatments are described in Materials and Methods.

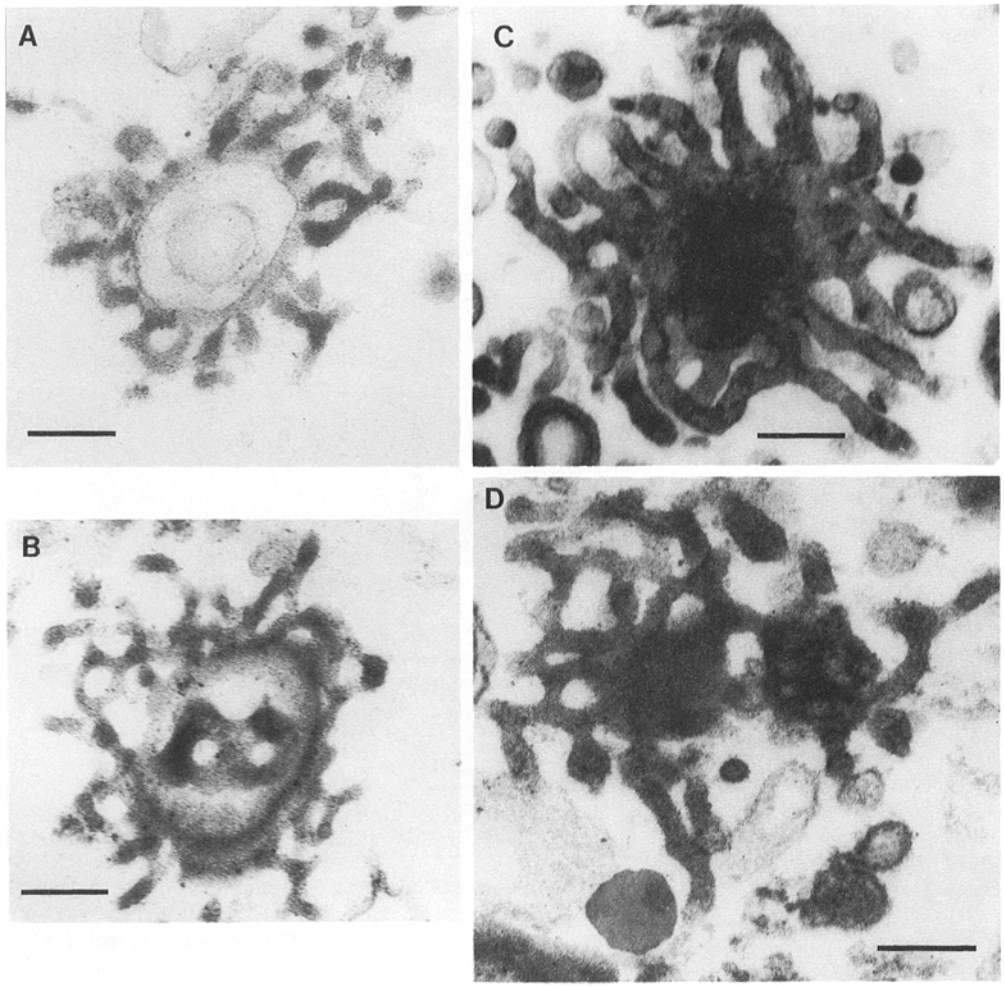

Figure 3. Tubulation of isolated Golgi membranes with and without BFA. (*A* and *B*) Golgi complexes incubated in BFA (10 μg/ml) plus 1 mM ATP for 20 min at 37°C exhibit short tubules which emanate from central regions of cisternae. These tubular FLPs are best seen in en face profiles shown here. Under these conditions, many fewer vesicles and fused networks are seen when compared with incubation with cytosol, ATP ± BFA (as in Fig. 1). (*C* and *D*) Tubulation of Golgi membranes occurs by simply lowering the ATP levels in cytosol. Incubation of Golgi membranes with hexokinase/glucose-treated (low ATP) cytosol for 30 min at 35°C results in the formation of long tubular FLPs. Low-ATP cytosol produced tubular FLPs that were generally longer than those produced by BFA and ATP (in *A* and *B*). The diameter of the tubular extensions produced by either treatment was 60–80 nm. Bars, 0.2 μm.

tubular "finger-like" projections (tubular FLPs), had a diameter of ~60–70 nm and were generally short, ranging from 0.2–0.3 μm, although occasionally longer tubules (up to 0.8 μm) were seen. In en face profiles of Golgi complexes treated with BFA and ATP alone, short tubular FLPs were the predominant structural feature. Quantitation of these results showed a significant increase in the number of Golgi profiles exhibiting tubular FLPs when compared with Golgi complexes treated with ATP alone (not shown) or ATP and cytosol (Table I).

We next wanted to investigate the role that cytosolic factors might play in this tubulation process. However, since the cytosol preparation used in these experiments contains a residual amount of ATP (which leads to network formation in the presence of BFA), we incubated isolated Golgi complexes in cytosol that had been pretreated with hexokinase–glucose to reduce ATP levels (henceforth called "low-ATP cytosol"). We were surprised to find that when Golgi complexes were incubated with low-ATP cytosol alone, extensive tubular FLPs extended from cisternae, both in en face and transverse profiles (Fig. 3, *C* and *D*). These tubular FLPs were longer than the FLPs seen during ATP plus BFA treatment, with some being at least 1-μm long. However, the width of these tubular FLPs was essentially the same as those produced by BFA. Isolated Golgi complexes treated with low-ATP cytosol and BFA also formed tubular FLPs although not to the same extent as with low-ATP cytosol alone (Table I). Of particular significance, Golgi complexes treated with low-ATP cytosol had very few associated vesicles as compared with Golgi complexes treated with cytosol and an ATP regenerating system (Fig. 3). These results show that by merely lowering the ATP levels below that apparently needed for coated-vesicle formation, Golgi membranes have the capacity to form extensive tubular projections in vitro.

Because membrane tubules could extend out of the plane of the thin section, it was difficult to precisely know their true lengths. Therefore, we visualized Golgi profiles by whole mount, negative staining EM (Fig. 4). By these methods, an even more impressive degree of Golgi tubulation was observed. Golgi membranes treated with BFA- or low-ATP cytosol were found to generate membrane tubules >7-μm long, lengths that match those seen in BFA-treated cells.

Since the above results demonstrate that tubulation of isolated Golgi membranes could occur without BFA treatment if ATP levels in the cytosol preparation were reduced, we tested to see if this were true in living cells as well. To do this, NRK cells were treated with various concentrations of NaN_3, plus 50 mM 2-deoxy-D-glucose (DOG), to reduce intracellular ATP levels, and the cells were fixed and stained for immunofluorescence using mAb 10E6 which recognizes a *cis*-Golgi antigen (Wood et al., 1991). In untreated cells,

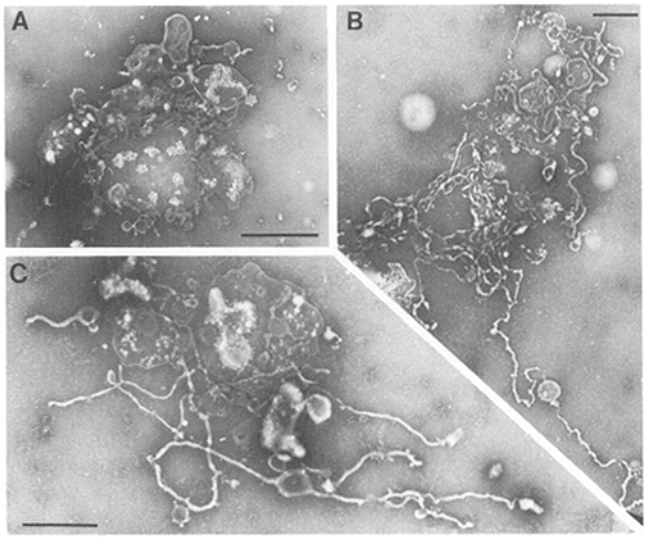

Figure 4. Visualization of Golgi membrane tubulation by negative staining of whole mounts. (*A*) Whole mount of control Golgi complex incubated with 5 mg/ml BSA and ATP for 30 min at 37°C. A few vesicles and short tubules can be seen associated with the pile of Golgi cisternae. (*B*) Golgi membranes incubated with low-ATP cytosol (5 mg protein/ml) for 30 min at 37°C. (*C*) Golgi membranes incubated with BFA, cytosol and ATP for 30 min at 37°C. The representative images in *B* and *C* reveal an even more impressive degree of tubulation as the number and length of tubules is greater than seen by EM of thin sections. Note that the tubulated Golgi membranes in *B* was printed at lower magnification to accommodate all the tubules. In *B* and *C*, many tubules induced by low-ATP cytosol and BFA were found to be >7 μm long. Bars, 1 μm.

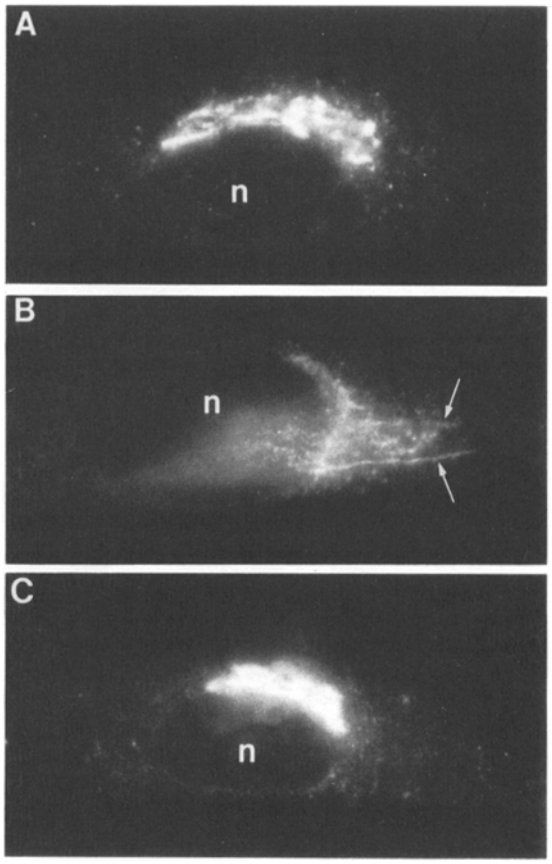

Figure 5. Lowering cellular ATP levels causes Golgi membrane tubulation in vivo as revealed by immunofluorescence staining with mAb 10E6, a *cis*-Golgi marker. (*A*) In control, untreated NRK cells, 10E6 reveals a typical juxtanuclear Golgi staining pattern. (*B*) Treatment of cells with an intermediate level of NaN_3 (0.02%), plus 50 mM 2-deoxyglucose (DOG), for 10 min at 37°C induced the formation of 10E6-enriched tubules (*arrows*) which emanated from the Golgi region. (*C*) In NRK cells treated with higher amounts of NaN_3 (0.05% plus 50 mM DOG for 10 min at 37°C), no tubulation of Golgi membranes was observed. In these experiments, NaN_3 and DOG were added to cells in MEM. *n*, nucleus.

10E6 stains the fairly compact, juxtanuclear Golgi complex (Fig. 5 *A*). However, in cells treated with 0.02% NaN_3 and 50 mM DOG for 10 min, thin membrane tubules were found emanating from the juxtanuclear region and extending into the cytoplasm (Fig. 5 *B*). These tubules are similar to those seen extending from the Golgi complex (Lippincott-Schwartz et al., 1989) and the TGN after BFA treatment (Wood et al., 1991). The tubules produced by lowering cellular ATP levels were less extensive and slower to form than when cells were treated with BFA; however, they could reach nearly the same lengths. This tubulation is, however, energy dependent because treatment with higher concentrations of NaN_3 (0.05% plus 50 mM DOG for 10 min) prevented the formation of 10E6-enriched tubules (Fig. 5 *C*). The effects produced by intermediate levels of NaN_3 and DOG were reversible after washing out and reincubation in normal media (data not shown).

Golgi Membrane Tubules Are Competent to Form Vesicles—Recovery In Vitro

Since membranes of the Golgi complex appear to tubulate when vesicle formation is inhibited, we wanted to know if the process was reversible. That is, can tubular membranes of isolated Golgi complexes serve as substrates for vesicle formation? First, fused Golgi networks (formed by BFA, ATP, and cytosol) were recovered by centrifugation onto a sucrose cushion and resuspension in fresh cytosol and ATP (Fig. 6, *C–F*). After this recovery procedure, the BFA-induced tubular reticulum had disappeared, and the Golgi complexes resembled more typical stacks with closely apposed cisternae and few intercisternal connections (compare Fig. 6 *C*, the recovered preparation, with Fig. 1 *A*, untreated controls). As would be expected since the recovery conditions should promote transport, numerous vesicles, often with smooth or spiked cytoplasmic coats, were seen budding from both the ends and middle regions of cisternae (Fig. 6, *D–F*). In mock recovery controls, i.e., continued incubation in BFA/cytosol/ATP throughout the recovery period, the tubular networks remained intact and few vesicles were seen (Fig. 6, *A* and *B, arrowheads*).

To quantitatively establish that vesicles are produced from tubules, a time course of recovery was conducted. Golgi membranes induced to tubulate with low-ATP cytosol were recovered as above, and the number of tubules and coated vesicles per Golgi stack profile was determined at various

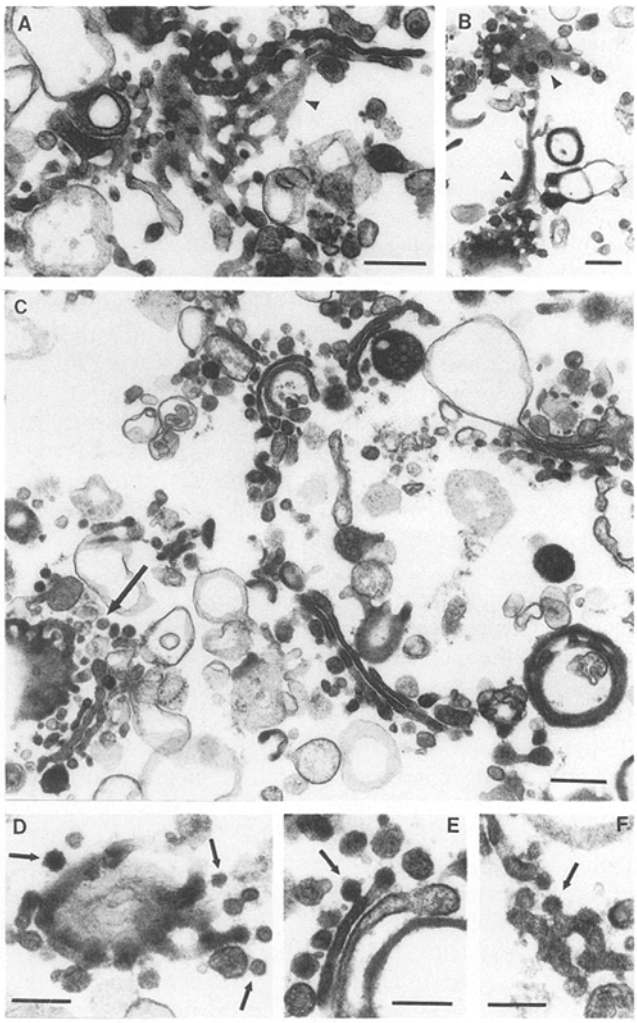

Figure 6. Vesicles form from tubules upon recovery from BFA-treated Golgi membranes in vitro. (*A* and *B*) Mock recovery: Golgi membranes treated with BFA (32 μg/ml), cytosol, and ATP for 20 min at 37°C, then centrifuged onto a sucrose cushion, and reincubated with BFA, cytosol, and ATP for 20 min at 37°C, retain the network of fused cisternae (*arrows*) and show few associated vesicles. (*C–F*) Recovery from BFA: Golgi membranes were treated with BFA as in *A* but then reisolated and incubated with fresh cytosol and ATP without BFA for 20 min at 37°C. 50 μM GTP-γ-S was included in the recovery reactions to distinguish bona fide transport vesicles from disrupted membranes. In transverse profiles, the Golgi membranes were clearly restored to a stacked morphology with closely apposed cisternae and no apparent membrane connections as seen in BFA-treated stacks. As seen in the en face profile (*arrow* in *C*), numerous vesicles were found to bud from the periphery of the flattened cisterna. Higher magnification images of similar profiles revealed vesicles often with smooth and spike-like coats budding from both the rims and central regions of cisternae (*arrows* in *D–F*). Bars: (*A–C*) 0.4 μm; (*D–F*) 0.2 μm.

times during recovery (Fig. 7 *A*). The results clearly showed that there was a rapid increase in the number of coated vesicles and a concomitant loss of tubules during the recovery period. An even more direct relationship between tubules and vesicles was established by finding that there was a rapid increase in the percentage of tubules exhibiting ≥1 budding coated vesicle during recovery (Fig. 7 *B*).

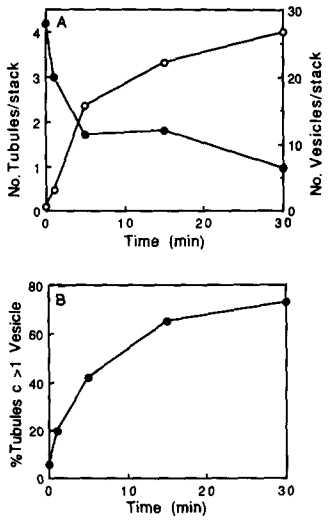

Figure 7. Time course of recovery from tubulation. Golgi membranes were induced to tubulate by treatment with low-ATP cytosol. They were separated from the low-ATP cytosol by centrifugation through a sucrose step gradient and harvested for recovery in fresh cytosol plus ATP and GTP-γ-S (to distinguish transport vesicles from broken tubules). At the times indicated, the recovery reaction was stopped by addition of glutaraldehyde, and samples were prepared for thin-section EM. (*A*) Graph showing the average number of tubules (●) and vesicles (○) per Golgi stack at various times of recovery. Each time point represents an average of 95 Golgi profiles counted. The results show a rapid decrease in the number of tubules and a concomitant increase in vesicles/Golgi stack during the recovery period. (*B*) Graph showing the percentage of Golgi membrane tubules with ≥1 associated budding coated vesicle at various times of recovery. An average of 224 tubules were counted at each time point.

Amount of β-COP on Golgi Membranes Correlates with Tubule and Vesicle Formation

The amount of β-COP on Golgi membranes after various treatments was determined by Western blotting. After incubation with cytosol ± ATP, Golgi membranes contained substantial amounts of β-COP (Fig. 8 *A*). However, incubation under conditions that inhibit vesiculation but promote fused networks (i.e., cytosol, ATP, BFA), resulted in significant loss of β-COP. An even greater loss (>85%) of β-COP occurred under conditions that induced tubulation, i.e., low ATP-cytosol or BFA plus ATP. The amount of β-COP on Golgi membranes was also measured after recovery from treatments that result in tubulation and network formation (Fig. 8 *B*). Recovery experiments were conducted exactly as above for the morphological observations of vesicle reformation. After recovery from either BFA treatments, or low-ATP cytosol, there was ~2–4-fold increase in the amount of β-COP of Golgi membranes. The results of these experiments show that β-COP is lost from Golgi membranes under conditions that promote tubular FLP or cisternal network formation, and rebinds under conditions that allow vesicle reformation.

Discussion

Tubule Formation Is a Basic Property of Organelle Membranes

A central tenet of eucaryotic cell biology holds that transport between membrane-bound organelles occurs by discrete vesicles which bud from one compartment and fuse with a target. These vesicles are uniformly sized, contain characteristic sets of cytoplasmic coat proteins, and fall into two major classes: clathrin-coated and COP-coated vesicles

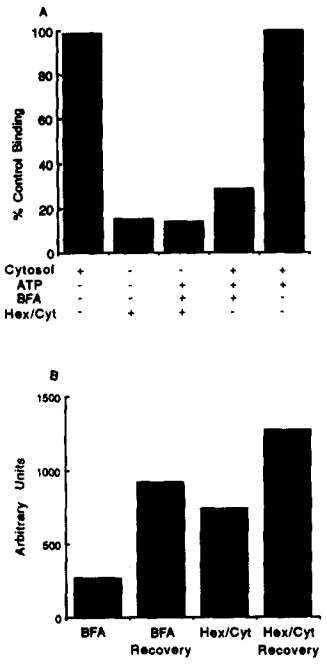

Figure 8. The presence or absence of β-COP correlates with vesiculation or tubulation, respectively. The amount of β-COP on membranes was determined by Western blotting with anti-β-COP mAb and scanning densitometry. (*A*) Loss of β-COP from Golgi membranes after incubation with BFA or hexokinase/glucose-treated (low ATP) cytosol (Hex/Cyt on graph). Specific conditions of incubations are listed below the graph and detailed in Materials and Methods. The results are normalized to the amount of β-COP found on Golgi membranes after incubation with cytosol alone (100%). Incubation with BFA plus ATP or hexokinase/glucose-treated cytosol, both of which induce tubulation but not vesiculation, resulted in a significant loss of β-COP. In other control samples, the amount of β-COP pelleted after incubation with cytosol and ATP but no Golgi membranes ranged from being undetectable to 1.9% of that recovered with membranes, cytosol, and ATP. Also, Golgi membranes alone (without cytosol or ATP) contained ~25% of the amount of β-COP found on Golgi membrane after incubation with cytosol and ATP. (*B*) Reassociation of β-COP with Golgi membranes after removal of BFA or addition of cytosol plus ATP. Experimental conditions are indicated below each bar. (*BFA*) Golgi membranes incubated with BFA plus cytosol and ATP; (*BFA Recovery*) Golgi membranes recovered from BFA, cytosol and ATP treatment as in Fig. 6, and reincubated with fresh cytosol containing ATP and a regenerating system; (*Hex/Cyt*) Golgi membranes incubated with hexokinase/glucose treated (low ATP) cytosol; and (*Hex/Cyt Recovery*) Golgi membranes recovered from treatment with low-ATP cytosol and reincubated with fresh cytosol containing ATP and a regenerating system. In this case, the data were expressed as arbitrary transmittance units to show the relative increases in β-COP binding after removal of BFA or addition of ATP-enriched cytosol.

(Pearse and Robinson, 1990; Rothman and Orci, 1992; Pryer et al., 1992). With good reason, it has been assumed that vesiculation describes the most fundamental behavior of transporting membranes. However, recent studies showing that BFA inhibits vesicle formation while concomitantly promoting a microtubule-enhanced tubulation of a variety of membranous organelles, raise questions about this assumption (Lippincott-Schwartz et al., 1990; Wood et al., 1991; Lippincott-Schwartz et al., 1991; Hunziker et al., 1991). We show here that this tubulation response to BFA can be reconstituted in vitro with isolated Golgi membranes without the aid of microtubules. Remarkably, Golgi membranes could also be induced to tubulate without BFA at all, both in vitro and in vivo, respectively, by simply lowering ATP levels in organelle-free cytosol preparations or in cells. In addition, our in vitro results indicate that cytosolic factor(s) exist that promote tubulation in the absence of vesiculation. Importantly, these tubules served as substrates for both the rebinding of vesicle-associated coat proteins and vesiculation itself. We conclude from our studies that vesiculation masks a more fundamental behavior of organellar membranes, i.e., tubulation.

Tubulation as a Precursor to Vesiculation

Work by Orci et al., (1991), and confirmed here, showed that isolated Golgi complexes incubated in BFA, cytosol, and ATP form tubular networks instead of COP-coated vesicles. By limiting components required for this BFA-induced Golgi network, we have been able to identify alternative structures that are likely to be important for understanding the mechanisms of membrane trafficking. We believe that the data provided here and elsewhere are most consistent with the model of Golgi membrane behavior shown in Fig. 9.

Normally, transport within the Golgi complex is mediated by membrane vesicles whose formation requires ATP and a host of cytosolic factors including the COP proteins that form a coatomer complex surrounding nascent vesicles (Melançon et al., 1987; Orci et al., 1986, 1989, 1991; Malhotra et al., 1989). After vesicle formation and docking, the coatomer complex is removed from the membrane in a GTP-dependent manner and probably recycles for multiple rounds of vesicle formation (Melançon et al., 1987).

It has recently been shown that BFA inhibits the association of β-COP with Golgi membranes (Donaldson et al., 1990) and the formation of Golgi COP-coated vesicles (Orci et al., 1991). The consequences of this inhibition on isolated Golgi complexes vary depending on the presence or absence of other factors. If BFA is added with cytosol and ATP, vesiculation is inhibited, but tubules form and rapidly fuse with adjacent cisternae, resulting in an extensive network. However, by limiting ATP (i.e., adding cytosol without an ATP regenerating system), we found that BFA caused fusion primarily at the edges of Golgi stacks. We do not know at this time if these stacks with peripherally fused membranes would eventually become extensively fused networks upon addition of more ATP. Addition of BFA alone to isolated Golgi membranes had no effect (Table I). However, when BFA was added with ATP (and no cytosol), short tubular membrane extensions formed but little if any fusion occurred due to the absence of cytosolic fusion machinery (Wilson et al., 1991). Even more remarkably, extensive tubulation of isolated Golgi membranes occurred in the presence of just low-ATP cytosol (no BFA added at all). We reason that under these conditions, ATP levels presumably fall below that needed for vesicle formation and membrane fusion, and the cytosol supplies extra "tubulation factors." In vivo, BFA-induced tubulation is inhibited by depleting cellular ATP with DOG/NaN_3 (Lippincott-Schwartz et al., 1991; our data not shown). Surprisingly, we found that tubulation of Golgi membranes could also occur in vivo by simply using intermediate levels of DOG/NaN_3, without any added BFA. Consistent with our results, Persson et al. (1992) found that lowering cellular ATP levels inhibited secretory traffic in hepatocytes and caused Golgi-processed secretory products to accumulate in flattened cisternae. Thus, we conclude from these experiments that tubulation is an energy-dependent process; however, it probably requires much less ATP than membrane vesiculation or fusion.

In a recent review, Klausner et al. (1992) proposed a model in which a reciprocal relationship exists between vesiculation and tubulation for any particular organelle. That is,

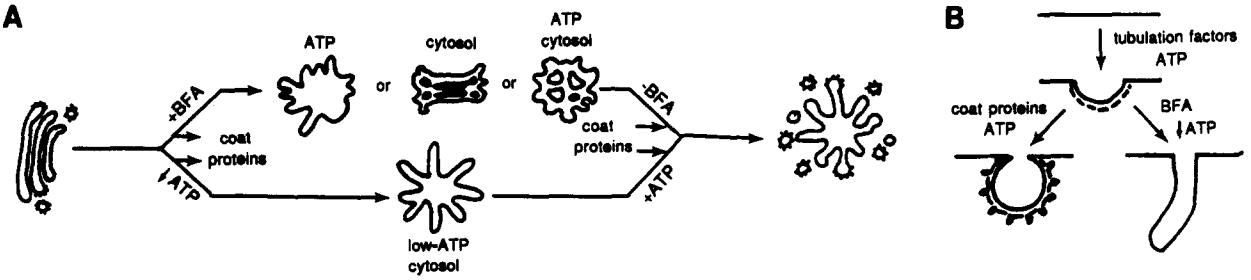

Figure 9. Summary of the behavior of organellar membranes in response to conditions that promote vesiculation or tubulation. The upper pathway in *A* shows conditions under which BFA produces alternative structures to extensively fused networks of Golgi complex membranes. After loss of coat proteins and consequent cessation of vesiculation in BFA and ATP alone, short tubular projections form and do not fuse together (*ATP*). In just cytosol alone, cisternal membranes fuse only at the periphery of the stack (*cytosol*). If ATP and cytoplasmic fusion factors are present, eventually the entire stack will become an extensively fused network (*ATP/cytosol*). As shown in the lower pathway in *A*, low-ATP cytosol also causes a loss of coat proteins and even more extensively tubulated Golgi membranes. Removal of BFA or restoration of ATP levels allows coat proteins to reassociate with Golgi membranes leading to the formation of Golgi vesicles (and restoration of stacks). (*B*) Diagrammatically shows that ATP-dependent "tubulation factors" precede the action of coat proteins. If coat proteins are present or supplied subsequent to tubule formation, then vesicles form. However, if coat proteins are prevented from binding, either by BFA or low ATP conditions, then tubulation continues unabated.

membrane tubulation is suppressed when conditions favor vesiculation, and vice versa. We believe that our data on the recovery from tubulation of isolated Golgi membranes is entirely consistent with this model, at least for this organelle. Our results are also consistent with the idea that a precursor–product relationship exists between the tubules and vesicles (Klausner et al., 1992). Because BFA- or low-ATP–induced membrane tubules could serve as substrates for vesicle formation, we view these tubules as "exaggerated intermediates" in the normal process of vesiculation. In other words, if for any reason (or at least the ones shown here) the coatomer complex of vesicle-associated proteins cannot bind to membranes, nascent buds continue to grow unabated into tubules. Our data also indicates that addition of coat proteins reverses the tubulated state to favor vesiculation as predicted (Klausner et al., 1992).

Several questions arise from these findings and conclusions. First, what is the source of membranes for the growing tubules, especially those emanating from isolated Golgi stacks? Are the tubules forming from a fixed number of budding sites or are entire cisternae being transformed into tubules? With regard to the source of membrane for in vitro Golgi tubulation, it is our impression that the central cisternal regions shrink in size during extensive tubulation (see Fig. 4), suggesting that membrane from the flattened cisternae is recruited by lateral diffusion into nascent tubules, however, this remains to be definitely shown. This membrane could be recruited into either a fixed number of budding sites and/or new budding sites could be generated during tubulation in vitro. It will be difficult to answer this question until it becomes possible to quantify the number of budding sites on a normal Golgi cisternae.

Implications for Membrane Vesicular and Tubular(?) Traffic

The tubules formed from Golgi complexes in vitro by BFA or low-ATP cytosol treatment were ~60–70 nm in diameter, virtually identical to the diameter of vesicles (minus their coats) which would normally form (Orci et al., 1986, 1991), and similar to those tubules found in BFA-treated cells (Lippincott-Schwartz et al., 1990; Wood et al., 1991). Although difficult to quantify, we noticed that the length and number of tubules produced by low-ATP cytosol were very often greater than those made in BFA plus ATP. It seems likely therefore, that the cytosol provides components that help drive the formation of buds which, in the absence of vesiculation, grow into tubules. In preliminary studies we have found that this tubulation activity is nondialyzable, heat labile, and proteinaceous (unpublished data). Although the exact physiological function of this "factor" is not clear, one possibility is that normally tubulation factors and vesicle-associated coat proteins are closely linked, resulting in tight coupling between budding/tubulation and the formation of a spherical vesicle. In the presence of BFA or low ATP, the action of cytosolic tubulation factors and coat proteins become uncoupled leading to cessation of vesiculation and uncontrolled budding/tubulation.

Although we view the BFA- and low-ATP cytosol–induced tubulation as an exaggerated form of budding, it is possible that some intracellular transport pathways are mediated by tubules rather than vesicles. Klausner and colleagues have suggested that retrograde transport from the Golgi complex to the ER may be tubule mediated (Klausner et al., 1992). Another candidate for this type of tubule-mediated transport would be the recycling early endosome that serves to return endocytic receptors to the cell surface (for review see Gruenberg and Howell, 1989). Thus, it is tempting to speculate that Golgi tubulation factors may also work on other organelles or that a host of related tubulation factors function in an organelle-specific manner.

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References

Ahle, S., A. Mann, U. Eichelsbacher, and E. Ungewickell. 1988. Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:919–929.

Balch, W. E., and J. E. Rothman. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system. *Arch. Biochem. Biophys.* 240:413–425.

Balch, W. E., W. G. Dunphy, W. A. Braell, and J. E. Rothman. 1984*a*. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of *N*-acetylglucosamine. *Cell.* 39:405–416.

Balch, W. E., B. S. Glick, and J. E. Rothman. 1984*b*. Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell.* 39:525–536.

Cluett, E. B., and W. J. Brown. 1992. Adhesion of Golgi cisternae by proteinaceous interactions: intercisternal bridges as putative adhesive structures. *J. Cell Sci.* In press.

Doms, R. W., G. Russ, and J. W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* 109:61–72.

Donaldson, J. G., J. Lippincott-Schwartz, G. S. Bloom, T. E. Kreis, and R. D. Klausner. 1990. Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in Brefeldin A action. *J. Cell Biol.* 111: 2295–2306.

Duden, R., V. Allan, and T. Kreis. 1990. Involvement of β-COP in membrane traffic through the Golgi complex. *Trends in Cell Biol.* 1:14–19.

Duden, R., G. Griffiths, R. Frank, P. Argos, and T. E. Kreis. 1991. β-COP, a 110kD protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell.* 64:649–665.

Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara. 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* 269:18545–18552.

Gruenberg, J., and K. Howell. 1989. Membrane traffic in endocytosis: insights from cell-free assays. *Annu. Rev. Cell Biol.* 5:453–481.

Hunziker, W., J. A. Whitney, and I. Mellman. 1991. Selective inhibition of transcytosis by Brefeldin A in MDCK cells. *Cell.* 67:617–627.

Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:1071–1080.

Lin, H. C., M. S. Moore, D. A. Sanan, and R. G. W. Anderson. 1991. Reconstitution of clathrin-coated pit budding from plasma membranes. *J. Cell Biol.* 114:881–891.

Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane recycling from the Golgi to ER. *Cell.* 56:801–813.

Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H.-P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.

Lippincott-Schwartz, J., L. C. Yuan, C. Tipper, M. Amherdt, L. Orci, and R. D. Klausner. 1991. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanisms for regulating organelle structure and membrane traffic. *Cell.* 67:601–617.

Malhotra, V., T. Serafini, L. Orci, J. C. Shephard, and J. E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* 58:329–336.

Malançon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Serafini, M. L. Gleason, L. Orci, and J. E. Rothman. 1987. Involvement of the GTP-binding "G" proteins in transport through the Golgi stack. *Cell.* 51:1053–1062.

Melançon, P., A. Franzusoff, and K. E. Howell. 1991. Vesicle budding: insights from cell-free assays. *Trends in Cell Biol.* 1:165–171.

Misumi, Y., K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara. 1986. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* 261:11398–11403.

Oda, K., S. Hirose, N. Takami, A. Misumi, A. Takatsuki, and Y. Ikehara. 1987. Brefeldin A arrests the intracellular transport of a precursor of complement C3 before its conversion site in rat hepatocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 214:135–138.

Orci, L., B. S. Glick, and J. E. Rothman. 1986. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell.* 46:171–184.

Orci, L., V. Malhotra, M. Amherdt, T. Serafini, and J. E. Rothman. 1989. Dissection of a single round of vesicular transport: sequential intermediates for intercisternal movement in the Golgi stack. *Cell.* 56:357–368.

Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J. G. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, and J. E. Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell.* 64:1183–1195.

Palade, G. E. 1975. Intracellular aspects of the process of protein synthesis. *Science (Wash. DC).* 189:347–358.

Pearse, B. M. F., and Robinson, M. S. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151–171.

Persson, R., C. R. Schnell, L. A. Hakan Borg, and E. Fries. 1992. Accumulation of Golgi-processed secretory proteins in an organelle of high density upon reduction of ATP concentration in rat hepatocytes. *J. Biol. Chem.* 267:2760–2766.

Pryer, N., L. J. Wuestehube, and R. Schekman. 1992. Vesicle-mediated protein sorting. *Annu. Rev. Biochem.* 61:471–516.

Robinson, M. S. 1987. 100-kD coated vesicle proteins: molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. *J. Cell Biol.* 107:833–842.

Robinson, M. S., and T. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of Brefeldin A and G-protein activators. *Cell.* 69:129–138.

Rothman, J. E., and L. Orci. 1990. Movement of proteins through the Golgi stack: a molecular dissection of vesicular transport. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:1460–1468.

Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature (Lond.).* 355:409–415.

Schmid, S. L., and E. Smythe. 1991. Stage-specific assays for coated pit formation and coated vesicle budding in vitro. *J. Cell Biol.* 114:869–880.

Serafini, T., G. Stenbeck, A. Brecht, F. Lottspeich, L. Orci, J. E. Rothman, and F. T. Wieland. 1991. A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein β-adaptin. *Nature (Lond.).* 349:215–220.

Simonescieu, N., and M. Simonescieu. 1976. Galloylglucoses of low molecular weight as mordant in electron microscopy. *J. Cell Biol.* 70:608–621.

Smythe, E., M. Pypaert, J. Lucocq, and G. Warren. 1989. Formation of coated vesicles from coated pits in broken A431 cells. *J. Cell Biol.* 108:843–853.

Waters, M. G., T. Serafini, and J. E. Rothman. 1991. 'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature (Lond.).* 349:248–251.

Wilson, D. W., S. W. Whiteheart, L. Orci, and J. E. Rothman. 1991. Intracellular membrane fusion. *Trends Biochem. Sci.* 16:334–337.

Wong, D. H., and F. M. Brodsky. 1992. 100-kD proteins of Golgi and trans-Golgi network-associated vesicles have related but distinct membrane binding proteins. *J. Cell Biol.* 117:1171–1179.

Wood, S. A., J. E. Park, and W. J. Brown. 1991. Brefeldin A causes a microtubule-mediated fusion of the *trans* Golgi network and early endosomes. *Cell.* 67:591–600.