

Brefeldin A: Insights into the Control of Membrane Traffic and Organelle Structure

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THE definition of cellular organelles has evolved over the last hundred years largely driven by morphologic observations, but more recently has been supplemented and complemented by functional and biochemical studies (Palade, 1975). Thus, organelles are now identified both by their morphology and by the set of components that comprise them. Determining how organelle identity is established and maintained and how newly synthesized protein and membrane are sorted to different organelles are the central issues of organellogenesis. Essential to the many cellular functions that take place within the central vacuolar system (which consists of the ER, Golgi apparatus, secretory vesicles, endosomes, and lysosomes) is membrane traffic which mediates the exchange of components between different organelles. There are two critical characteristics of membrane traffic. First, only certain sets of organelles exchange membrane and the patterns of this exchange define what are called membrane pathways. Second, multiple pathways intersect at specific points within the central vacuolar system. For specific components to "choose" the correct pathway at such points of crossing, mechanisms exist to impose choices on specific molecules. This process is called sorting.

The characteristics of each organelle within the central vacuolar system are likely to be intimately tied to the properties of membrane traffic. An imbalance in the magnitude of membrane input into and egress from an organelle would have profound effects on the size of that compartment. In addition, failures in sorting or aberrations in targeting pathways would be expected to profoundly affect the identity of individual organelles. Recently, the relationship between the control of membrane traffic and the maintenance of organelle structure has been investigated with the use of a remarkable drug, brefeldin A (BFA).¹ In this review we will summarize recent findings with BFA and propose some speculative models concerning the mechanism and regulation of membrane traffic within the central vacuolar system.

BFA Affects the Early Secretory Pathway

Inhibition of Protein Secretion

BFA is a macrocyclic lactone synthesized from palmitate (C₁₆) by a variety of fungi (Harri et al., 1963). Its structure, along with those of several BFA derivatives is shown in

1. *Abbreviations used in this paper:* ARF, ADP ribosylation; BFA, brefeldin A; man II, mannosidase II; TGN, *trans*-Golgi network.

Fig. 1. Although initially isolated and characterized as an antiviral antibiotic (Tamura et al., 1968), BFA's utility for cell biologists began with the recognition that in BFA-treated cells, protein secretion is inhibited at an early step in the secretory pathway (Oda et al., 1987; Takatsuki and Tamura, 1985; Misumi et al., 1986). That the block occurred in a pre-Golgi compartment was supported by immunofluorescence and electron microscope observations demonstrating that secretory and membrane proteins were retained in the ER of BFA-treated cells (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Yamashina et al., 1990). Several studies subsequently have used BFA as a specific inhibitor of membrane transport, addressing questions related to class I antigen presentation (Nuchtern et al., 1989), toxin transport (Yoshida et al., 1990; Hudson and Grillo, 1991; Sandvig et al., 1991), ER degradation (Klausner et al., 1990), and the localization of glycosaminoglycan elongation and sulfation reactions (Spiro et al., 1991).

Disassembly of the Golgi Apparatus and Its Mixing with the ER

Although newly synthesized proteins appeared to be retained in the ER of BFA-treated cells, further biochemical characterization of these proteins revealed the surprising result that these proteins become processed by Golgi enzymes (Lippincott-Schwartz et al., 1989; Doms et al., 1989). Moreover, ER resident glycoproteins themselves showed evidence of Golgi processing, becoming endo H resistant in BFA-treated cells (Lippincott-Schwartz et al., 1989; Ulmer and Palade, 1989). An explanation for these paradoxical results is that within 1 h of BFA treatment, the Golgi enzyme markers mannosidase II (man II) and thiamine pyrophosphatase appeared within both reticular and cisternal elements of the ER as well as within the nuclear envelope (Lippincott-Schwartz et al., 1989; Fujiwara et al., 1989). Indeed, no recognizable Golgi stacks were observed in the BFA-treated cells. Other markers of the Golgi apparatus including the enzyme galactosyl transferase (localized predominantly to *trans*-Golgi cisternae), a Golgi lipid marker, NBD-ceramide, and newly synthesized VSV G protein passing through the Golgi complex all were shown to redistribute into the ER in the presence of BFA (Lippincott-Schwartz et al., 1990, 1991a; Strous et al., 1991; Young et al., 1991; Doms et al., 1989; Ulmer and Palade, 1991). In contrast, components of the *trans*-Golgi network (TGN) did not redistribute into the ER (Lippincott-Schwartz et al., 1991b; Wood et al., 1991; Chege and Pfeffer, 1990).

Brefeldin A Analogues

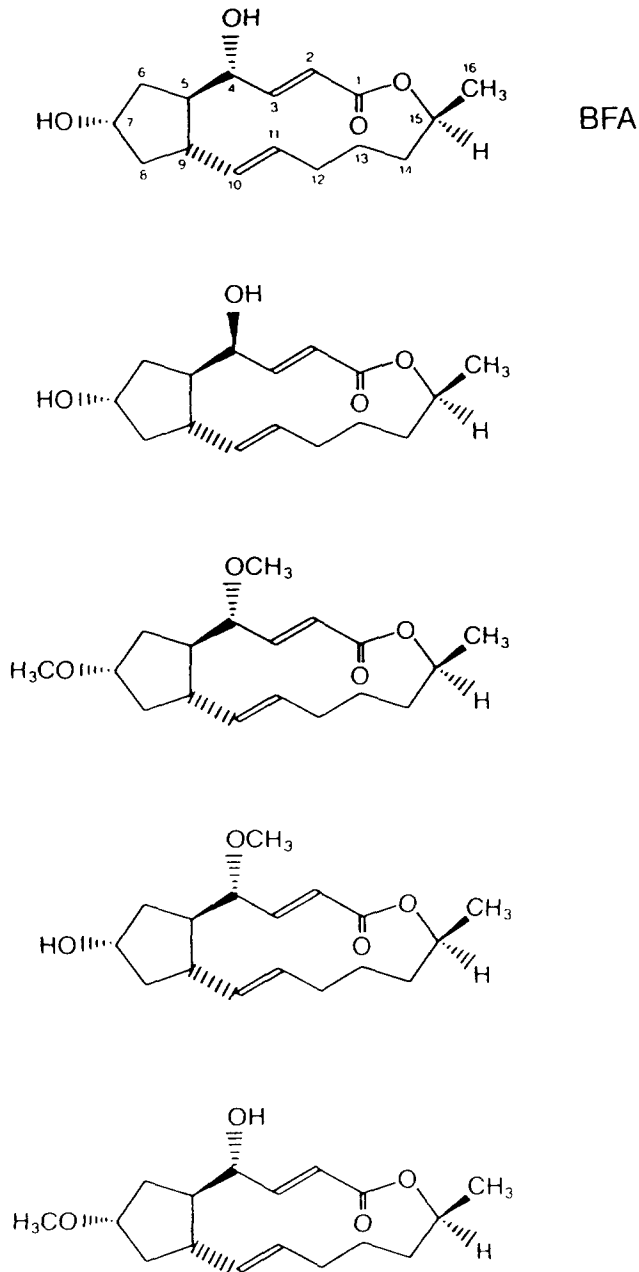


Figure 1. BFA analogues. Derivatives of BFA were synthesized by Dr. Andrew Greene (Joseph Fourier University, Grenoble, France). At concentrations between .05 and 1 $\mu\text{g/ml}$, only BFA, with its defined stereospecificity at chiral carbons 4 and 7, causes βCOP dissociation and Golgi disassembly in NRK cells.

Membrane Tubules as "Intermediates" in the Absorption of Golgi Membrane into the ER

The morphological events that characterize the breakdown of the Golgi apparatus and its redistribution into the ER in BFA-treated cells have been studied in detail, revealing a role of membrane tubules in this process (Lippincott-Schwartz et al., 1990). Within minutes of adding BFA to cells at 37°C,

Golgi markers are no longer localized by immunofluorescence microscopy to a compact, perinuclear cisternal structure. Golgi cisternae appear first to swell and then to extend long tubular processes out to the cell periphery (Fig. 2 A). Tubules budding from swollen Golgi cisternae during BFA-treatment are uniformly 90 nm in diameter with no apparent cytoplasmic "coat" material associated with them (Lippincott-Schwartz et al., 1990). That the Golgi-derived tubules extend along microtubules is supported by their absence in cells treated with drugs that depolymerize microtubules (Lippincott-Schwartz et al., 1990). After 10 min of BFA treatment at 37°C Golgi tubules are no longer visible and Golgi markers instead show a punctate-reticular distribution representing their steady-state mixing with the ER. The tubules thus appear to be intermediates in the movement of Golgi membrane into the ER.

Characterization of BFA-induced Retrograde Transport from Golgi Membrane to ER

Although BFA-induced Golgi-derived tubules appear to extend along microtubules, redistribution of Golgi into the ER during BFA treatment does not have an absolute requirement for microtubules, but the process is significantly slowed in their absence (Lippincott-Schwartz et al., 1990). Reduced temperatures and ATP depletion inhibit tubule formation and retrograde transport of Golgi membrane into the ER in BFA-treated cells. Minor modifications of the structure of BFA abrogate these cellular effects including derivatization of either of the two hydroxyl groups or changing the stereopositioning of the C4 or C7 hydroxyl (Fig. 1). Relatively low concentrations (200 nM) of BFA are sufficient for inhibiting protein secretion and disruption of the Golgi apparatus. These effects are rapidly and completely reversed by removing the drug (Lippincott-Schwartz et al., 1989, 1991a; Fujiwara et al., 1989; Ulmer and Palade, 1989). The diterpene, forskolin, has been shown to inhibit and even reverse BFA's morphologic effects on the Golgi apparatus as well as reverse BFA's block of protein secretion by a cAMP-independent mechanism (Lippincott-Schwartz et al., 1991a).

The Effects of BFA Are Not Limited to the Golgi Apparatus

Although cartoons of the central vacuolar system often display many organelles as vacuoles, careful examination has revealed that they are more aptly referred to as tubulovesicular structures (Geuze et al., 1983). One study reported that the endosomal system represents a continuous anastomosing tubular reticulum, more reminiscent of the ER than of discrete vacuoles (Hopkins et al., 1990). More recently, Tooze and Hollinshead (1991) have demonstrated tubular early endosomal networks in a variety of cells. Even classically described vacuolar lysosomes may appear as tubular structures in certain cells or under certain conditions (Swanson et al., 1987). The Golgi apparatus, likewise, has been observed to extend tubular processes (Cooper et al., 1990) and was shown by three-dimensional reconstructions to comprise tubular and vesicular elements in addition to flattened cisternae (Rambourg and Clermont, 1990). In fact, the changes in surface to volume ratios that take place as a structure transforms from a vesicle to a tubule may provide a partial explanation for the sorting of content and membrane that oc-

curs in the endocytic pathway (Geuze et al., 1983, 1984). One of the hallmarks of the morphological effects of BFA on the Golgi apparatus is the rapid and dramatic induction of tubules. Recent studies have shown that these effects of BFA are not limited to the Golgi apparatus. Membrane tubules induced by BFA have been observed for peripheral organelles including endosomes, lysosomes, and the TGN (Lippincott-Schwartz et al., 1991b; Wood et al., 1991; Hunziker et al., 1991) (Fig. 2). Electron microscopic examination of endosome tubules induced by BFA reveals long tubular processes of ~90 nm in diameter emanating from the centriole region of the cell. BFA-induced tubules derived from endosomes, lysosomes, and TGN do not co-localize with Golgi tubules within the cell (Lippincott-Schwartz et al., 1991b). Nevertheless, in many cells the same concentrations of BFA induce both Golgi tubules and tubules of the peripheral organelles. In addition, only analogues of BFA that are active on the Golgi apparatus produce the peripheral organelle tubules (Lippincott-Schwartz et al., 1991b). As with Golgi-derived tubules, tubules generated from peripheral organelles by BFA are inhibited by treatment of cells with AIF₄ (see below) or by lowering the temperature, and cannot be observed in the absence of intact microtubules (Lippincott-Schwartz et al., 1991b; Wood et al., 1991).

Effect of BFA on Membrane Traffic

Membrane Dynamics of the Mixed ER/Golgi System of BFA-treated Cells

One of the most clear-cut effects of adding BFA to cells is the tight block of membrane traffic out of the ER. A more descriptive statement is that BFA inhibits the anterograde movement of membrane beyond the mixed ER/Golgi system. While inhibiting one path of membrane traffic (that into the Golgi apparatus), BFA appears to enhance a second pathway, the movement of Golgi membrane into the ER. A growing body of evidence supports the notion that there is a normal retrograde pathway from the Golgi apparatus (or some part of it) back to the ER (Pelham, 1988; Dean and Pelham, 1990; Lippincott-Schwartz et al., 1990; Hsu et al., 1991). Thus, it is possible that transport into the ER induced by BFA represents enhanced trafficking through this normal retrograde pathway as a result of the absorption of Golgi protein and membrane components into this pathway. We do not know whether all traffic out of the ER stops in the presence of BFA. Indeed, it has been suggested that membrane traffic within this mixed system continues in the presence of BFA with proteins not intrinsically retained within the ER being free to leave the ER, only to be rapidly returned to it via the enhanced retrograde pathway (Lippincott-Schwartz et al., 1990).

Receptor-mediated Recycling in BFA-treated Cells

Recent observations on the effects of BFA on peripheral organelles including endosomes and lysosomes indicate that membrane transport, although altered, still continues in the presence of BFA. The ability to cycle Tf and to extract its iron remains largely unperturbed in BFA-treated cells (Lippincott-Schwartz et al., 1991b), although the distribution of recycling mannose 6-phosphate receptors on the plasma membrane increases several fold (Damke et al.,

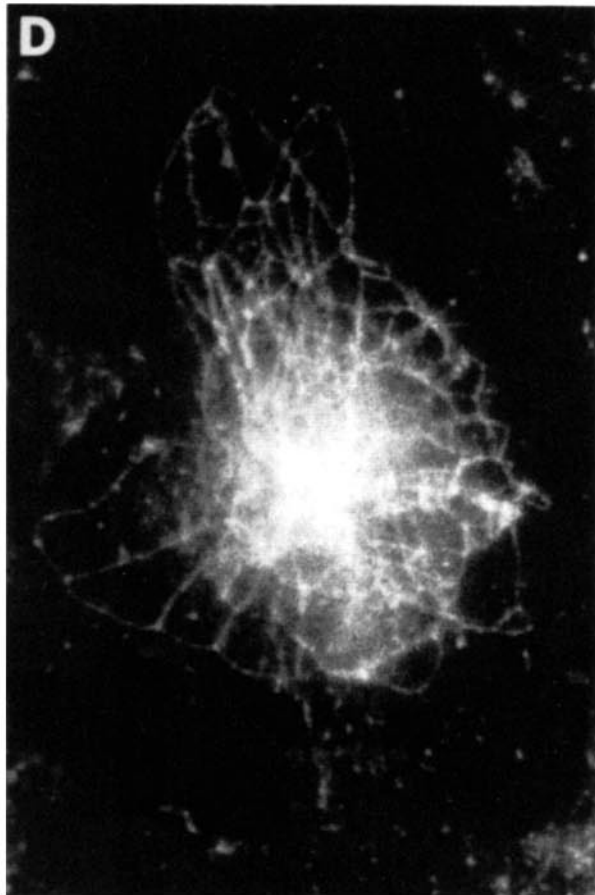
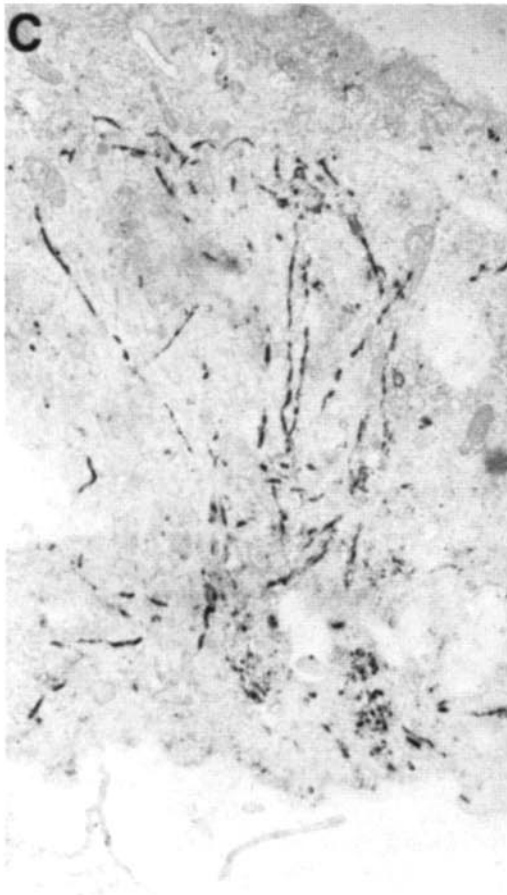
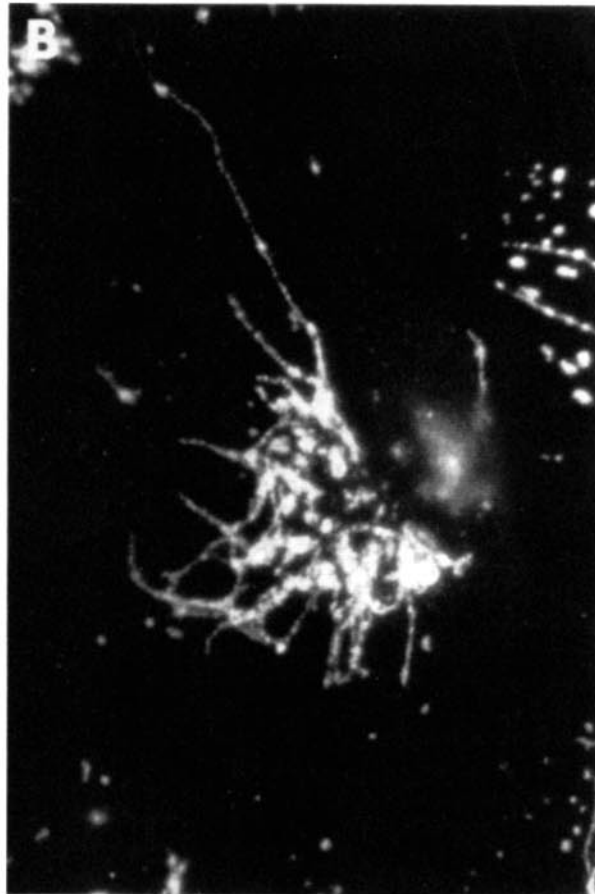
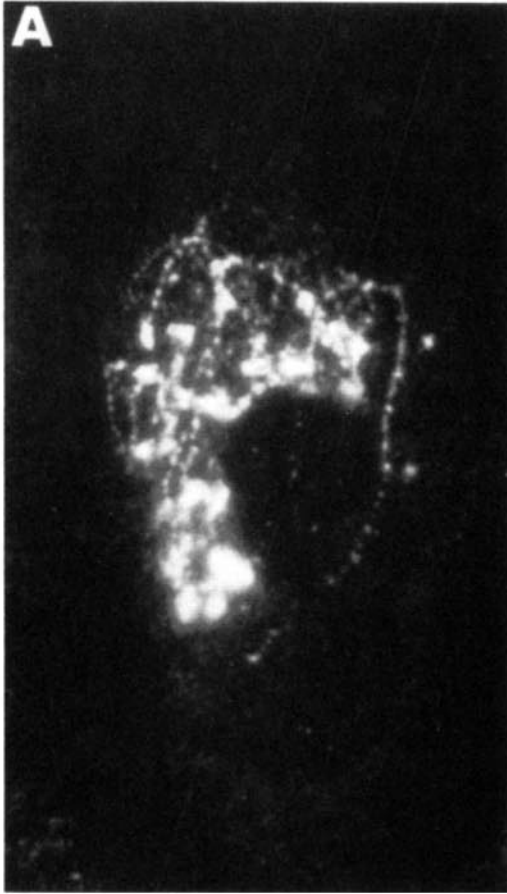
1991; Wood et al., 1991). Despite the dramatic tubulation of the early endosome system in BFA-treated cells, therefore, this is not incompatible with continued membrane traffic. As mentioned above, in the presence of BFA the early endosomal system becomes dramatically and stably tubulated into an anastomosing and possibly continuous reticulum (Lippincott-Schwartz et al., 1991b; Wood et al., 1991). Despite this morphological change, the ability to cycle transferrin and to extract its iron remains largely unperturbed (Lippincott-Schwartz et al., 1991b). The morphological changes induced by BFA are not, therefore, incompatible with continued membrane traffic through these compartments.

Membrane Traffic Pathways That Are Inhibited by BFA

Just as transport of newly synthesized proteins into the Golgi apparatus is inhibited by BFA, not all traffic pathways using the endosomal system remain functional in the presence of BFA. This has been most clearly demonstrated by the studies of Low et al. (1991) and Hunziker et al. (1991) who examined the ability of a polarized epithelial cell to carry out secretion and transcytosis. Low et al. (1991) demonstrated that in MDCK cells, where the Golgi apparatus but not the endosomal system is resistant to low concentrations of BFA, protein secretion at the apical surface is inhibited by BFA while basolateral secretion is enhanced. Using MDCK cells in culture, Hunziker et al. (1991) demonstrated that IgA can be taken up from the basolateral surface in a normal fashion in the presence of BFA, but its release at the apical surface is inhibited. Internalized IgA was recycled to the basolateral surface through a tubulated organellar system in BFA-treated cells. Analysis of the location of the BFA block of transcytosis suggested a failure to specifically sort out of and leave the basolateral, recycling endosomal system (Hunziker et al., 1991).

One pathway out of the early endosomal system leads to lysosomes. It has been shown that BFA can cause lysosomes to extend long tubules in a microtubule-dependent fashion (Heuser, J. 1989. *J. Cell Biol.* 109:238a.; Lippincott-Schwartz et al., 1991b). When the fate of fluid phase markers taken up by endocytosis was followed morphologically in BFA-treated cells, a significant inhibition in the delivery of these markers to lysosomes was observed (Lippincott-Schwartz et al., 1991b). Endocytosed proteins, destined for lysosomes, can be biochemically characterized by the fact that they are degraded by lysosomal hydrolases. Since BFA only slightly inhibits the degradation of these proteins (Mitsumi et al., 1986; Lippincott-Schwartz et al., 1991) the BFA block between the endosomal and lysosomal systems may be only partial and/or the degradation might represent the action of lysosomal hydrolases in prelysosomal structures.

The effect of BFA on the distribution of two different components of the TGN, a 38-kD glycoprotein (Luzio et al., 1990) and the mannose 6 phosphate receptor has also been examined (Lippincott-Schwartz et al., 1991b; Wood et al., 1991). In the presence of BFA, both proteins enter membrane tubules before establishing a new steady-state distribution that is either mixed into or communicates with the early endosomal system. This latter conclusion is based upon (a) the ability to accumulate TGN 38 antibody intracellularly into regions of the cell which co-distribute with antigen in the presence, but not the absence of BFA (Lippincott-Schwartz et al., 1991b) and (b) co-localization of mannose



6 phosphate-containing tubules induced by BFA with lucifer yellow internalized by cells for 5 min at 37°C (Wood et al., 1991).

Biochemical Changes Induced by BFA: Towards a Model of BFA Action

Release of the Peripheral 110-kD Protein/ β COP from Golgi Membranes by BFA

The inability to maintain the structure and identity of the Golgi apparatus in the presence of BFA raised the question of whether BFA interferes with the assembly and/or function of structural elements of the Golgi apparatus. To further characterize proximal changes in the Golgi apparatus induced by BFA, Donaldson et al. (1990) tested by immunofluorescence whether any cytosolic proteins which are specifically associated with the Golgi apparatus are released upon addition of BFA. One protein of 110-kD protein (Allan and Kreis, 1986), now known as β -COP (Duden et al., 1991; Serafini et al., 1991b), was found to rapidly redistribute from a Golgi-like pattern into a diffuse, cytosolic pattern upon BFA treatment. Redistribution is detectable as early as 20 s after BFA addition at 37°C and is complete by 1 to 2 min, thus preceding the movement of Golgi membrane into the ER. Upon removal of BFA, β -COP rapidly reassociates with Golgi membrane. Furthermore, conditions that inhibit movement of Golgi membrane into the ER during BFA treatment, namely lowered temperature and microtubule depolymerization, do not inhibit the rapid cytosolic redistribution of β -COP.

Association of β -COP with Golgi Membranes Is Regulated by Guanine Nucleotides

Many studies have supported the involvement of GTP-binding proteins in membrane traffic (for reviews see Balch, 1990; and Bourne, 1988). The effects of guanine nucleotides on the BFA-induced β -COP redistribution were examined in filter perforated, semi-intact cells (Donaldson et al., 1991b). As in intact cells, treatment of semi-intact cells with BFA alone causes the rapid release of β -COP and movement of Golgi membrane into the ER. Pretreatment of the cells with GTP γ S, a nonhydrolyzable analog of GTP, inhibits these effects. If, on the other hand, BFA is added first, subsequent addition of GTP γ S cannot inhibit BFA's effects. GTP γ S treatment by itself has little effect on the immunofluorescence distribution of β -COP. The ability of GTP γ S to inhibit BFA action can be abrogated by adding GTP and is mimicked by the addition of AlF₄⁻ in place of GTP γ S. While GTP γ S requires cell permeabilization to be effective, AlF₄⁻, an activator of heterotrimeric G proteins (Gilman, 1987), is also effective in intact cells (Melançon et al., 1987; Donaldson et al., 1991b).

A model was proposed for the regulated cycling of β -COP between the membrane and the cytosol whereby β -COP rapidly associates/dissociates with Golgi membrane, requiring GTP to bind to the membrane and GTP hydrolysis to be

released from the membrane. The order of addition experiments (described above) appear most compatible with the site of BFA sensitivity being the association step. The membrane/cytosol cycle of β -COP characterized in these immunofluorescence studies using antibodies to the 110-kD protein was remarkably similar to that previously proposed by Rothman, Orci and colleagues for the interaction of non-clathrin Golgi-associated coat proteins with the Golgi apparatus (Orci et al., 1989). This was the basis, therefore, for comparing the sequence of the 110-kD protein recognized by the antibody of Kreis (Allan and Kreis, 1986; Duden et al., 1991) with that derived independently from the 110-kD subunit of the purified Golgi coat (Serafini et al., 1991b). The two sequences proved to be identical, so the 110-kD protein is now referred to as β -COP for the β subunit of the non-clathrin Golgi coat protein complex (Duden et al., 1991; Serafini et al., 1991b). Recently, the association of β -COP with Golgi membranes was investigated with partially purified CHO Golgi membranes and cytosol (Orci et al., 1991; Donaldson et al., 1991a). Association of β -COP with Golgi membranes is dependent upon the addition of ATP, is enhanced with GTP γ S and AlF₄⁻ (Donaldson, 1991a) and is inhibited by BFA, but not by inactive BFA analogues (Orci et al., 1991; Donaldson, 1991a). Thus, the in vitro system mimics the effects of both GTP γ S and the "order of addition" experiments in intact and semi-intact cells.

BFA Also Inhibits the Association of ADP Ribosylation Factor with Golgi Membranes

ADP ribosylation factors (ARF) are a family of low molecular weight GTP binding proteins distinct from the ras, rab, and rho families of proteins (Kahn et al., 1991). Studies in yeast with *Saccharomyces cerevisiae* have demonstrated the importance of ARF in secretion (Stearns et al., 1990a) and ARF, like β -COP, is primarily localized to the Golgi apparatus in mammalian cells (Stearns et al., 1990b) and recently has been identified as a component of Golgi-derived non-clathrin-coated vesicles (Serafini et al., 1991a). Like β -COP, the addition of BFA to cells results in the rapid redistribution of ARF to a cytosolic pattern, which can be reversed by the removal of the drug. Using the in vitro binding assay (Donaldson et al., 1991a), binding of ARF to Golgi membranes is enhanced in the presence of GTP γ S, inhibited by BFA and when given in combination, the order of addition determines the response. Unlike β -COP, however, ARF binding is not enhanced by AlF₄⁻ (Donaldson et al., 1991a).

Possible Role of Trimeric G Proteins in Regulating Membrane Assembly of BFA-sensitive Coat Proteins

A possible explanation for the differential effects of AlF₄⁻ and GTP γ S on ARF and β -COP binding was suggested by recent experiments which demonstrated that, whereas all tested trimeric G proteins are sensitive to AlF₄⁻, including G_s, G_q, and G_i (Gilman, 1987), none of the examined low molecular weight GTP binding proteins, including ARF, are

Figure 2. Membrane tubulation is a hallmark of BFA action on Golgi, lysosomes, and endosomes. Immunofluorescent staining of the Golgi apparatus (using antibodies to man II) (A), lysosomes (using antibodies to LGP120) (B), and endosomes (using internalized Tf) (D), in cells treated with BFA for 5 min (A) or 30 min (B and D). In (C), Tf-loaded cells treated with BFA for 30 min were immunoperoxidase labeled and examined by EM. The electron micrograph was kindly provided by Lydia Yuan.

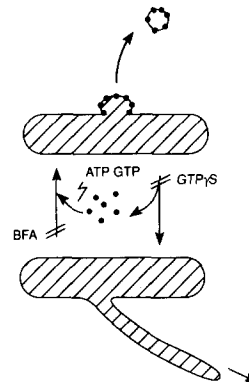
sensitive to this agent (Kahn, 1991). These observations suggest that AlF_4^- may be a useful discriminator between heterotrimeric G proteins and low molecular weight GTP binding proteins. Most effects of trimeric G proteins are mediated via the free $G\alpha$ subunits and are inhibited by the association of $G\alpha$ with $\beta\gamma$ subunits (Gilman, 1987). This has been the basis for the ability to specifically inhibit G protein mediated effector functions by the addition of purified exogenous $G_{\beta\gamma}$ subunits (Northup et al., 1983; Okabe et al., 1990). Addition of purified $\beta\gamma$ subunits of G proteins to the *in vitro* binding assay inhibits both $GTP\gamma S$ - and AlF_4^- -induced binding of β -COP (Donaldson et al., 1991a). Addition of $G_{\beta\gamma}$ also inhibits the $GTP\gamma S$ -induced binding of ARF to the membranes. The extent of the inhibition of binding of ARF and β -COP observed with $G_{\beta\gamma}$ was similar to that observed with BFA. The implied role of a heterotrimeric G protein in regulating intracellular traffic is intriguing. The localization of a specific G protein, $G_{\alpha 3}$, to the Golgi apparatus has been reported (Ercolani et al., 1990), and Stow et al. (1991) have shown that overexpression of $G_{\alpha 3}$ results in an inhibition of the secretion of a proteoglycan. Current data suggest that the assembly of cytosolic proteins on and off Golgi membrane is a process controlled by one or more membrane-bound trimeric G proteins. One possible site, consistent with the observations, is that BFA inhibits either the activation of the trimeric G protein or the coupling of G protein to effector, thus preventing the association of ARF and β -COP with Golgi membrane.

Based on both kinetic and pharmacologic data, the inhibition of binding of coat proteins to Golgi membranes seems to be the most proximal effect of BFA on this organelle. It is of significance that the Golgi apparatus in some cells appears to be resistant to the effects of BFA (Kistakis et al., 1991; Hunziker et al., 1991; Low et al., 1991; Sandvig et al., 1991). In both PtK1 cells (Kistakis et al., 1991) and MDCK cells (Hunziker et al., 1991) the association of β -COP with the Golgi apparatus is not changed with BFA treatment. How the Golgi apparatus of these cells is made resistant to the effects of BFA is an open question. Kistakis et al. (1991) showed using permeabilized cells and cell fusion experiments that the PtK1 resistance phenotype is a property of Golgi membranes rather than of a cytosolic factor.

BFA Does Not Inhibit In Vitro Golgi Transport Assays

The ability of BFA to inhibit the assembly of cytosolic coat proteins onto membranes has been examined in the *in vitro* inter-Golgi transport assay developed by Rothman and colleagues (Orci et al., 1991). In the absence of BFA, the ability to transfer membrane between two distinct sets of Golgi stacks requires ATP and cytosol and is inhibited by $GTP\gamma S$ (Balch et al., 1984; Melançon et al., 1987). In the presence of BFA, inter-Golgi membrane transfer occurs, but is no longer inhibited by subsequent addition of $GTP\gamma S$, while the addition of these two drugs in the opposite order abrogates this transfer (Orci et al., 1991). By both biochemical and morphologic measurements, the addition of BFA to Golgi-enriched membranes inhibits the association of non-clathrin coats (Orci et al., 1991; Donaldson et al., 1991a). A potential explanation for the *in vitro* "transport" under BFA conditions was suggested by ultrastructural examination of the BFA-treated membranes (Orci et al., 1991). Incubation of

Alternative Mechanisms for Membrane Transport



were first bound irreversibly with $GTP\gamma S$. Release of coat structures by BFA would be associated with tubule formation. Controlled binding of BFA-sensitive cytosolic coat proteins would regulate the balance between coated vesicle- and tubule-mediated transport.

these membranes with cytosol and an ATP-generating system, in the presence of BFA, induces a striking morphologic change. Stacks of cisternae are replaced by an anastomosing tubular network. The most reasonable explanation of this is that distinct Golgi cisternae mix via the formation of this network. As expected, no coats are seen on the membranes of the network. Extended tubules, as observed in intact or semi-permeabilized cells, are not seen, but this would not be expected in the absence of microtubules.

These results suggest that in the presence of BFA, where membrane binding of non-clathrin coats is inhibited, exchange of membrane between two organelles occurs, although its biochemical and morphologic characteristics change. Are these effects only seen in the presence of BFA? Probably not. When such *in vitro* Golgi transport assays are performed using lower concentrations of cytosol, in the absence of BFA, transport is insensitive to $GTP\gamma S$ (Melançon et al., 1987). The inhibitory effect of $GTP\gamma S$ is only attained as the concentration of cytosol is increased, suggesting that cytosol contains inhibitory factor(s) required for $GTP\gamma S$ sensitivity. The nature of this inhibitory factor in cytosol and the morphologic characteristics of these membranes have not been reported.

Membrane Budding in the Absence of Coats

The *in vitro* studies with BFA described above suggest two alternative models for membrane traffic (Fig. 3). One is initiated by the process of coat protein assembly and requires coat disassembly which is inhibited by $GTP\gamma S$. The other, observed with BFA treatment, uses uncoated, fusion-competent membrane buds and is not inhibited by subsequent addition of $GTP\gamma S$. Since formation of the Golgi reticulum with BFA in the *in vitro* assay system shared similar pharmacologic properties to those described for BFA-induced Golgi tubules in intact and semi-intact cells (Orci et al., 1991; Donaldson et al., 1991b), it is likely that the morphological change induced by BFA observed *in vitro* is the counterpart of that seen in living cells treated with BFA.

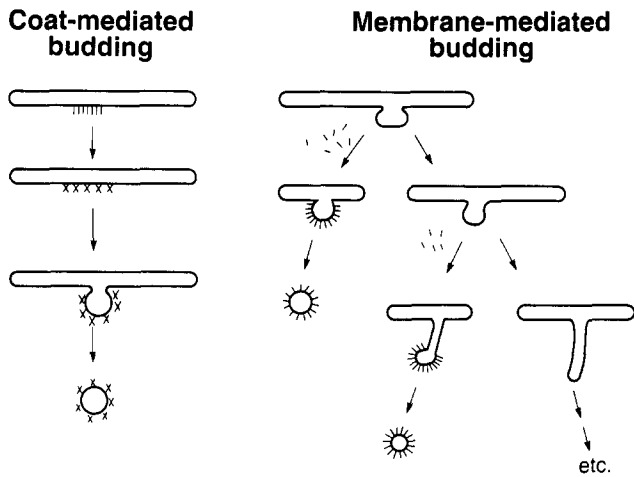


Figure 4. Coat assembly and membrane budding. On the left is shown the coat-mediated budding model in which cytosolic coat proteins assemble onto the membrane and undergo a structural transition which provides the mechanochemical force to induce budding. In contrast, membrane-mediated budding (shown on the right) occurs in the absence of assembly of cytosolic coat proteins. The bud can serve as the site of assembly of coat proteins and/or continue to grow as an uncoated tubule. We propose that the tip, at all points, may be the assembly site for coat proteins which both caps the bud/tubule and can transform the enclosed membrane into a coated vesicle.

Relationship between Coated Vesicle Formation and Uncoated Tubule Budding

A growing body of biochemical data supports the conclusion that one effect of BFA (and perhaps its only physiologically relevant effect) is the inhibition of the assembly of cytosolic proteins onto their target membranes. Can we explain the multiple effects of BFA in terms of this site of action? The regulated assembly/disassembly of cytosolic proteins onto specific membranes may perform multiple functions including the control of tubulation, the assembly of transport vesicles, the retention of resident proteins, and determining the morphology and localization of the organelle. The failure to assemble such proteins results in the stereotyped changes described in this review. Perhaps the most prominent morphologic change is the extension of uncoated tubules. If we assume that the extension of 90-nm tubules results from membrane budding, then such budding must take place in the absence of coat protein assembly. Uncoated tubule budding would seem, at first glance, to represent a process that is fundamentally different from coat-mediated budding. In the latter process, it is proposed that the structural transition from a flat membrane to a bud is induced by the mechanical forces imposed by coat assembly and rearrangement. Are these two budding processes, one leading to uncoated tubules and the other to coated vesicles necessarily different? An alternative view is shown in Fig. 4. This model proposes that the assembly of coat proteins is not required for budding but rather is superimposed upon a more generalized budding process to serve a variety of structural and regulatory roles (Fig. 4). Two regulatory roles of these coats can be envisioned. First of all, the binding of coat proteins at the sites of budding may slow or inhibit membrane budding and tubule growth. The growth of tubules probably requires the attachment of motor proteins to both the membrane and

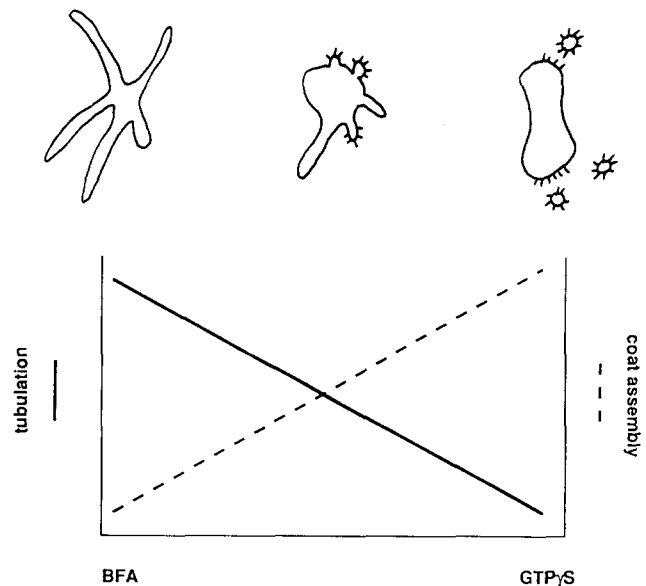


Figure 5. Coat protein "tone" and organelle tubulation. The rate of assembly/disassembly of cytosolic coat proteins onto target membranes can alter. At one extreme, BFA inhibits coat assembly while at the other extreme, $GTP\gamma S$ locks coat proteins onto the membrane. The extent of tubulation of an organelle is inversely related to the level of coat assembly. At the BFA extreme is an uncoated highly tubulated structure. Extensive coat assembly and failure to disassemble in the presence of $GTP\gamma S$ results in the absence of tubules and the profusion of coated structures. We assume that BFA sensitive organelles exist between these two pharmacologic extremes, demonstrating both tubulation and coat assembly. By restricting the sites of budding/coat assembly, we restrict the regions of coating and tubule extension.

cytoskeletal elements. Coat binding might serve to cap the tubules and prevent their further growth. Fission of these capped buds or tubules would result in the formation of coated vesicles. The rate and extent of assembly of these proteins would thereby determine the balance between tubule formation/extension and coated vesicle production. A second regulatory role would be to provide specificity to the content of the budding vesicles. This of course is a statement of one of the critical functions of the clathrin-based coats (Pearse and Robinson, 1990). It is likely that selective accumulation of specific membrane components into buds is the basis for sorting steps within the central vacuolar system. The essence of this alternative budding model shown in Fig. 4 is that the budding process, per se, does not require the assembly of exogenous coat proteins but may rather be largely or uniquely driven by intrinsic components of the organelle membranes.

Tubule Formation: Implications for Organelle Structure and Dynamics

The stereotyped morphologic transformation of organelles in response to BFA suggests that these organelles themselves exist at a fine balance maintained by the assembly of cytosolic proteins. BFA releases the organelles from the tonic constraints of coat assembly and the organelles transform into dynamic tubular systems. Since BFA-sensitive coat proteins reversibly bind to membranes, it is likely that uncoated buds and tubules are constant features of organelles. This

would explain the presence of tubules to lesser and greater extents in all of the organelles of the central vacuolar system including the ER, CGN, Golgi stacks, TGN, endosomes, and lysosomes. The extent of tubulation within any compartment, by extrapolation from the effects of BFA, can be viewed as a reflection of the "tone" of coat protein assembly/disassembly. We have two pharmacologic manipulations that set this tone at either of two extremes: BFA minimizes coat assembly and GTP γ S (or AIF₄) maximizes coat assembly. Thus, the extent of tubulation varies with the level of coat assembly (Fig. 5). Each organelle may have not only a characteristic set of coat proteins but a characteristic tone. Thus, the peripheral ER looks much like a BFA-treated organelle, while the vacuolar lysosomes lie at the other end of the spectrum. The coat protein assembly/disassembly tone can change, resulting in altered levels of tubulation.

It is obvious that if such a biochemical formulation for the basis of BFA action is correct, specific regulatory coat proteins that assemble onto endosomes, lysosomes, and the TGN, in addition to the Golgi stack will be found. These coat proteins would be predicted to be, like the non-clathrin Golgi coats, unable to assemble with their target membranes in the presence of BFA. Consistent with this prediction, rapid disassembly of the clathrin coats and γ -adaptin molecules found on the TGN is observed upon addition of BFA to cells (M. Robinson, personal communication). We suspect that this phenomenon provides the basis for the alterations in TGN-based membrane trafficking observed in BFA-treated cells. Interestingly, plasma membrane clathrin coats are not affected by either BFA or drugs that alter G proteins (K. Beck, personal communication).

The difference between tubulation in the absence of coats and coat-mediated transport provides us with a simple biochemical distinction between homotypic (takes place in the presence of BFA) and heterotypic (fails to take place in the presence of BFA) membrane traffic. Homotypic traffic can occur in the absence of assembly of these types of coat proteins. This form of transport uses the budding of non-coated membranes which, in the continued presence of BFA, tend to form extensive membrane networks. The targets of fusion for these tubules depends upon and is predicted by the site of origin of the tubule. Thus, Golgi tubules uniquely find the ER while endosomal tubules mix with the plasma membrane as well as with components of the TGN. Tubules may primarily mediate lateral mixing of organelles (ER-ER, Golgi-Golgi, endosome-endosome, etc.) and post sorting transport intermediates (endosome-plasma membrane, etc). Heterotypic traffic, by contrast, involves the ability to "jump" from one homotypic system to another and requires assembly of the cytosolic coat proteins. Thus, in addition to being able to select cargo, coats may be required to assemble selective membrane-targeting proteins required for heterotypic organelle "jumps." A homotypic system can be biochemically defined as sharing a membrane targeting machinery that does not require the assembly of BFA-sensitive cytosolic proteins. This definition of homotypic organelles does not preclude the structural and functional differentiation of organelles within a given system (i.e., ER and Golgi). Recent studies using cell fusion assays support this concept of homotypic systems. Thus, fusion between early endosomes, ER to Golgi fusion and inter-Golgi fusion is easy, while early-late endosome fusion is more difficult (Gruenberg and Howell, 1989; Melancon et al., 1991). We would predict that the function

of homotypic targeting molecules is normally regulated, perhaps by the binding of coat proteins in a manner that allows their controlled use to establish both polarity and vectorial traffic, even within a single homotypic system.

The difference between traffic via membrane tubules and coated vesicles is that the former appears to involve continuous and therefore mixing connections between organelles while the latter provides discontinuous vesicular structures, which could shuttle between organelles without mixing them. However, a variety of possibilities for the production of discontinuities exist in addition to the geometric closure of the coat lattices, including the assembly of other molecules that may be essential for pinching off membranes or the intrinsic instability of long tubules. Perhaps dynamin-like molecules help pinch off vesicles, either coated or not, as has been proposed to explain the Shibile mutant of *Drosophila* (Van der Blik and Meyerwitz, 1991; Chen et al., 1991).

Conclusions

This review has attempted to provide a synthesis of the growing number of observations on the effects of BFA on intracellular organelles of eukaryotic cells. While the dramatic morphologic effects of BFA are impressive, it has been the confluence of BFA-based studies with the extraordinary progress in elucidating the biochemical components of membrane traffic that has been most significant. To summarize the major points of this review: (a) The ability of BFA to dramatically alter the morphology and even the apparent existence of some organelles of the central vacuolar system is consistent with these compartments representing steady-state structures which are significantly affected by the extent of membrane input and outflow; (b) the effects of BFA are proposed to be explicable on the basis of its ability to inhibit binding of regulatory coat proteins to their target organelles. In this way, BFA dysregulates membrane traffic throughout the central vacuolar system. Analogous families of regulatory coat proteins to the non-clathrin coats of the Golgi stack are predicted to be found for endosomes, lysosomes and the TGN; (c) organelle-specific cytosolic coat proteins function to regulate membrane traffic into and out of any given organelle in terms of both the quantity of membrane flow as well as the specificity of membrane traffic. This specificity is exerted at the level of content, resulting in the ability to sort components through the central vacuolar system, and at the level of membrane targeting; and (d) the structural transformation that underlies membrane traffic, budding, need not be the consequence of the assembly of clathrin-like coat structures. Although we cannot rule out the role of cytosolic proteins in this budding process, it may be driven largely by intrinsic membrane components.

Studies with BFA have raised more questions about organelle biology and membrane traffic than they have answered. As we learn more about the effects of BFA on cells our ability to manipulate membrane traffic and organelle structure will improve. At the very least, this drug will continue to stir the imagination of cell biologists.

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