The Morphology but Not the Function of Endosomes and Lysosomes Is Altered by Brefeldin A

Salli A. Wood and William J. Brown

Section of Biochemistry Molecular and Cell Biology, Cornell University, Ithaca, New York 14850

Abstract. Brefeldin A (BFA) induces the formation of an extensively fused network of membranes derived from the *trans*-Golgi network (TGN) and early endosomes (EE). We describe in detail here the unaffected passage of endocytosed material through the fused TGN/EE compartments to lysosomes in BFA-treated cells. We also confirmed that BFA caused the formation of tubular lysosomes, although the kinetics and extent of tubulation varied greatly between different cell types. The BFA-induced tubular lysosomes were often seen to form simple networks. Formation of tubular lysosomes was microtubule-mediated and energy-dependent; interestingly, however, maintenance of the tubulated lysosomes only required microtubules and was insensitive to energy poisons. Upon removal of BFA, the tubular lysosomes rapidly recovered in an energy-dependent process. In most cell types examined, the extensive TGN/EE network is ephemeral, eventually collapsing into a compact cluster of tubulo-vesicular membranes in a process that precedes the formation of tubular lysosomes. However, in primary bovine testicular cells, the BFA-induced TGN/EE network was remarkably stable (for >12 h). During this time, the TGN/EE network coexisted with tubular lysosomes, however, the two compartments remained completely separate. These results show that BFA has multiple, profound effects on the morphology of various compartments of the endosome–lysosome system. In spite of these changes, endocytic traffic can continue through the altered compartments suggesting that transport occurs through noncoated vesicles or through vesicles that are insensitive to BFA.

THE fungal metabolite brefeldin A (BFA)[1] has provided a powerful tool for investigating the molecular basis of a variety of intercompartmental transport pathways (for review see Klausner et al., 1992). Intercisternal transport in the Golgi complex as well as between the ER and the Golgi complex is mediated by nonclathrin-coated vesicles (for review see Rothman and Orci, 1992). The formation of these vesicles is dependent on the recruitment of a set of proteins, the coatomer, from the cytosol to the budding membrane. BFA blocks this step, as demonstrated by the redistribution of β-COP, a component of the coat complex (coatomer), from Golgi membranes to the cytosol, within 30 s after addition of BFA (Donaldson et al., 1990; Orci et al., 1991). In the absence of vesicle formation, Golgi cisternae tend to form tubules, which are elongated along microtubules and rapidly fuse with the ER (Lippincott-Schwartz et al., 1989, 1990). Thus, enzymes normally resident in the stacked elements of the Golgi complex are redistributed to the ER and transport out of the hybrid organelle is inhibited (Lippincott-Schwartz et al., 1989).

Delivery of newly synthesized lysosomal enzymes from the *trans*-Golgi network (TGN) to the endosomal system, mediated by the mannose-6-phosphate receptor (M6PR), occurs via clathrin-coated vesicles (reviewed by Kornfeld and Mellman, 1989). Formation of these vesicles requires a distinct set of proteins, the adaptins, including γ-adaptin, as well as clathrin (reviewed by Pearse and Robinson, 1990). Analogous to its effects in the Golgi complex, BFA inhibits the association of clathrin and γ-adaptin with the membranes of the TGN, rapidly redistributing these coat components to the cytosol (Robinson and Kreis, 1992; Wong and Brodsky, 1992). By an unknown mechanism, this results in the extension of an elaborate network to tubular processes from the TGN, which elongates towards the plus end of microtubules, and rapidly fuses with early endosomes (Wood et al., 1991; Lippincott-Schwartz et al., 1991). The two novel BFA-induced hybrid organelles, the ER/Golgi and TGN/early endosomes (TGN/EE), remain separate and transport between them is inhibited.

A third vesicle-mediated transport pathway is endocytosis, which, given the fusion of early endosomes with the TGN, could conceivably be affected by BFA. Delivery of material from the plasma membrane or extracellular space to the early endosome occurs via clathrin-coated vesicles (Anderson et al., 1977). The coats of these vesicles are composed of clathrin and adaptor proteins such as α-adaptin, which are

---

homologous to the adaptins of the TGN but not functionally interchangeable (Pearse and Robinson, 1990). BFA has no effect on the distribution of either α-adaptin or clathrin at the plasma membrane (Robinson and Kreis, 1992). Thus, in the presence of BFA, receptors, including the transferrin receptor, the M6PR, and IgA receptor, as well as a variety of fluid phase markers, are internalized in clathrin-coated vesicles and delivered with normal kinetics to the fused TGN/EE (Lippincott-Schwartz, 1991; Hunziker et al., 1991; Damke et al., 1991). Despite the morphology of this hybrid organelle (an extensive tubular network at early times in BFA or a collection of tubulo-vesicular elements clustering at the microtubule organizing center after 1-2 h in BFA), certain recycling receptors, including the transferrin receptor, IgA receptor, and the M6PR, are returned to the cell surface with normal or increased kinetics (Lippincott-Schwartz, 1991; Hunziker et al., 1991; Damke et al., 1991). In fact, after BFA-induced fusion of the TGN with early endosomes, M6PRs within the TGN gain more ready access to this efficient recycling system and are diverted to the cell surface, increasing levels of surface M6PR up to fivefold (Wood et al., 1991; Damke et al., 1991).

Although the mechanism of transport beyond the early endosome has yet to be fully established, it may occur via a direct maturation of early into late endosomes (Dunn and Maxfield, 1992) or a unique population of carrier vesicles which migrate along microtubules to the centrally located late endosome or prelysosomal compartment (PLC) (Matteoni and Kreis, 1987; Scheel et al., 1990; Bomsel et al., 1990). This portion of the endocytic pathway is apparently functional in the presence of BFA (Wood et al., 1991; Lippincott-Schwartz et al., 1991). However, the fate of endocytosed material beyond this point is unclear. Misumi et al. (1986) reported that the rate of lysosomal degradation of an endocytosed substrate in BFA-treated cells was 80% that of untreated cells. While confirming this result, Lippincott-Schwartz et al. (1991) found that endocytosed material did not reach lysosomes in the presence of BFA because transport was blocked beyond the late endosome.

We sought to more closely examine BFA's effects on both the function and morphology of compartments along the endocytic pathway. We found that delivery to lysosomes can occur in the presence of BFA, despite alterations in the morphology of both early endosomes and lysosomes.

Materials and Methods

BFA, stored as a 10-mg/ml stock solution in ethanol at −20°C, nocodazole, stored as a 6 mg/ml stock solution in DMSO, 2-deoxy-D-glucose, sodium azide, Lucifer yellow (LY), saponin, and DAB hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). MEM, RPMI 1640, FBS, calf serum-supplemented, and penicillin/streptomycin were from Gibco Laboratories (Grand Island, NY).

Transformed bovine testicular cells (BTRD), primary bovine testicular cells, normal rat kidney cells (NRK), Madin Darby bovine kidney cells (MDBK), Clone 9 hepatocytes, and normal human fibroblasts were grown in MEM with 10% calf serum-supplemented and 1% penicillin/streptomycin. P338D1 mouse macrophages were maintained in RPMI 1640 with 10% FBS. All cells were grown at 37°C in a humidified atmosphere of 95% air, 5% CO_2.

Rhodamine-conjugated donkey anti-rabbit IgG and FITC-conjugated donkey anti-mouse IgG were from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Rabbit polyclonal antibodies were raised against the rat cation-independent M6PR (Brown and Farquhar, 1987) and the lysosomal enzyme cathepsin D as described (Park et al., 1991). Mouse mAbs

against tubulin were a gift from Dr. George Bloom (University of Texas, Southwestern Medical Center, Dallas, TX). Fab fragments of sheep anti-rabbit IgG conjugated with HRP were obtained from Biosys, S.A. (Compeigne, France).

Experimental Procedures

Cells were treated with 10 μg/ml BFA for various periods of time at 37°C and at the end of each experiment fixed and processed for immunofluorescence or immunoperoxidase. In other experiments, energy was depleted by incubating cells for various periods of time with 50 mM 2-deoxy-D-glucose and 0.05% sodium azide in Earle's balanced salt solution without glucose, as indicated. To disrupt microtubules, cells were treated with 6 μg/ml nocodazole. In some experiments, compartments on the endocytic pathway were labeled by uptake of the highly fluorescent fluid phase marker, LY (Swanson et al., 1985), or with 8-nm colloidal gold particles, prepared according to Handley (1989). The specific details of these experiments are given in the Results and figure legends. For double labeling IF, the monoclonal antitubulin antibodies were detected with FITC-conjugated donkey anti-mouse IgG and the rabbit anticathepsin D antibodies were detected with rhodamine-conjugated donkey anti-rabbit IgG. In cells labeled by LY uptake, rabbit antirat M6PR or rabbit antibovine cathepsin D were detected with rhodamine-conjugated donkey anti-rabbit IgG. Immunoperoxidase labeling of cathepsin D was performed as described (Brown and Farquhar, 1989).

Results

Endocytic Delivery to BFA-induced Tubular Lysosomes

The BFA-induced morphological changes in early endosomes do not affect the delivery of endocytosed material to this organelle or recycling of receptors to the cell surface. To determine if BFA inhibits later transport events along the endocytic pathway that are potentially mediated by coated vesicles, early endosomes, the PLC, or lysosomes were labeled with LY, a fluorescent fluid phase tracer, in the presence of BFA. In untreated cells, LY labeled early endosomes after 5 min of uptake, and the PLC after an additional 10-min chase. In NRK cells, TGN-derived tubules, labeled with antibodies against the M6PR, first appeared after 2 min of treatment with BFA (Fig. 1 B). At that time, early endosomes, labeled by uptake with LY for 6 min, remained as peripheral vesicles distinct from the tubular TGN and appeared unaltered morphologically by BFA (Fig. 1 A). Fusion of the TGN and early endosomes was first detectable 10 min after addition of BFA in both NRK cells (data not shown) and in BTRD, as indicated by the presence of LY in TGN-derived tubules after 5 min of uptake (Fig. 2, A and B). This time course indicates that tubules originate from the TGN and not early endosomes.

With a 10-min chase in the continued presence of BFA (total time in BFA = 20 min), LY was transported out of the fused TGN/EE tubules and into small vesicles (Fig. 2, C and D). This morphology and the kinetics of delivery of LY to these vesicles suggest they are the PLC. After long term BFA treatment (3 h), the TGN/EE network collapsed into tubulovesicular structures in the juxtanuclear region (Fig. 2 E). With a 10-min pulse and 10 min chase of LY during the last 20 min of a 3-h incubation in BFA, LY was transported out of these structures and into the vesicles of the PLC (Fig. 2 F). Thus, although early endosomes were morphologically altered and still fused with the TGN after 3 h in BFA, transport through the fused TGN/EE and to the PLC continued in the presence of BFA.

To determine if LY is delivered from the morphologically unaltered PLC to lysosomes in the presence of BFA, BTRD
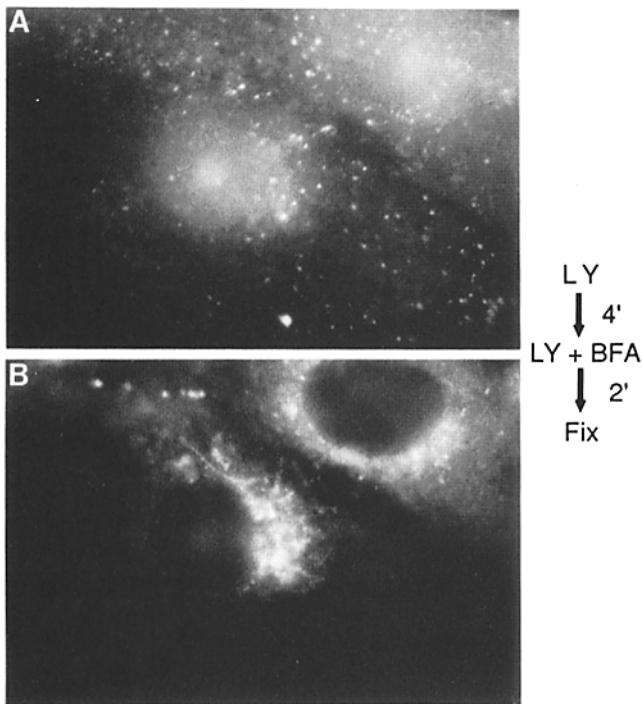
LY
↓ 4'
LY + BFA
↓ 2'
Fix

Figure 1. BFA causes tubulation of the TGN and not early endo-somes. NRK cells were incubated as shown on the right with LY for 4 min at 37°C and then with LY plus BFA (10 μg/ml) for an additional 2 min before fixing and staining for the M6PR by im-munofluorescence. (*A*) LY in peripheral early endosomal vesicles. (*B*) Same cell as in *A* but visualizing M6PR distribution which shows that after 2 min of BFA, the TGN has started to tubulate, while the early endosomes remain vesicular.

cells were pretreated with BFA for 60 min; and, in the con-tinued presence of BFA, LY was loaded and chased under conditions which label lysosomes (30 min pulse, 90 min chase). In these cells LY appeared to pass through the vesic-ular PLC and into slightly tubular structures which extended into the periphery (Fig. 2, *G* and *H*). These tubules were dis-tinct from the TGN/EE as indicated by the localization of the M6PR in juxtanuclear tubulovesicular structures (Fig. 2 *G*). These results show that in the presence of BFA, endocytosed material is delivered to a post-PLC tubular compartment, which may be a lysosome.

To address this further, the distribution of LY, under the same conditions as described above, was compared to that of a resident lysosomal enzyme, cathepsin D, in BTRD cells. Early in BFA treatment (10 min) and after 5 min of uptake, LY was delivered to the fused TGN/EE tubules which re-mained distinct from the unaltered anti-cathepsin D-labeled lysosomes (Fig. 3, *A* and *B*). With an additional 10 min of chase in BFA, LY moved into the centrally clustered PLC vesicles and did not colocalize with cathepsin D in the mor-phologically unaffected lysosomes (Fig. 3, *C* and *D*). After 3 h of pretreatment in BFA, LY was still delivered to a central cluster of vesicles with a 10-min pulse, 10-min chase in BFA (Fig. 3 *F*). However, the lysosomes in these cells formed an extended tubular network (Fig. 3 *E*). When cells were treated with BFA for a total of 3 h to produce tubular lyso-somes, LY was nevertheless chased into this tubular

lysosomal network. (Fig. 3, *G* and *H*). The LY totally filled the anti-cathepsin D-labeled tubular lysosomes, indicating that in the presence of BFA, endocytosed material is trans-ported through the endocytic pathway and delivered to lyso-somes, which were induced by BFA to form tubules. Identi-cal results were obtained using NRK cells (data not shown).

To confirm these findings, BTRD cells were treated with BFA for 60 min to initiate tubular lysosome formation, and then incubated with 8-nm colloidal gold for 30 min and chased for 120 min, all in the presence of BFA. Lysosomes were identified by immunoperoxidase staining with anti-cathepsin D antibodies. The results showed that gold parti-cles were transported through the endocytic pathway into cathepsin D-positive tubular and vacuolar lysosomes (Fig. 4), confirming the previous fluorescence experiments.

Kinetics of Formation and Recovery of BFA-induced Tubular Lysosomes

To further investigate the effects of BFA on lysosomes, the morphology and distribution of lysosomes were examined by IF during a time course of BFA treatment. In untreated BTRD cells, cathepsin D-labeled lysosomes were clustered as large vesicles in a juxtanuclear position (Fig. 5 *A*). Treat-ment of cells with BFA for 1 h caused the lysosomes to elon-gate and to migrate away from the juxtanuclear position to-wards the cell periphery (Fig. 5 *B*). These effects became more pronounced with increased time in BFA (2 h) (Fig. 5 *C*) so that some lysosomes formed short tubules radiating out from the cell center. By 4 h of BFA treatment, many lyso-somes formed apparently continuous tubules, and simple networks, or lined up in close apposition, forming beaded necklaces. The remaining vesicular lysosomes were scat-tered throughout the cytoplasm and often concentrated at the tips of cell processes (Fig. 5 *D*). Further incubation in BFA did not alter the extent of lysosomal tubulation in BTRD cells. Upon removal of BFA after 4 h of exposure to the drug, tubular lysosomes rapidly collapsed or fragmented, such that by 2 min of recovery lysosomes were scattered as small vesicles (Fig. 5 *E*). After 5 min of recovery, lysosomes reclustered in a juxtanuclear position and reattained their normal large vesicular morphology (Fig. 5 *F*).

The response of lysosomes to BFA was investigated in a number of cell types to determine if, as has been found for the Golgi complex (Ktistakis et al., 1991), the effects of BFA on lysosomes are variable. A mouse macrophage cell line, P338D1, was used because macrophage lysosomes have a propensity to tubulate under a number of conditions (Heuser, 1989; Swanson et al., 1987; Young et al., 1990). In these cells, lysosomes, as labeled by IF with the anti-cathepsin D antibody, were generally large, few in number, and clustered in the cell center (Fig. 6 *D*). However, after only 15 min of BFA treatment, the lysosomes extended long tubules that ra-diated from the juxtanuclear region (Fig. 6 *E*), and by 30 min these lysosomal tubules were long, extended into the tips of cell processes, and appeared beaded (Fig. 6 *F*). NRK cell lysosomes labeled by immunofluorescence with anti-cathep-sin D antibody, were more abundant than lysosomes in BTRD and P338D1 cells and scattered throughout the cyto-plasm (Fig. 6 *A*). The first response of lysosomes in NRK cells to BFA was apparent after 1 h of treatment, as lyso-somes clustered in the juxtanuclear region and elongated to form short radiating tubules (Fig. 6 *B*). These lysosomal tu-
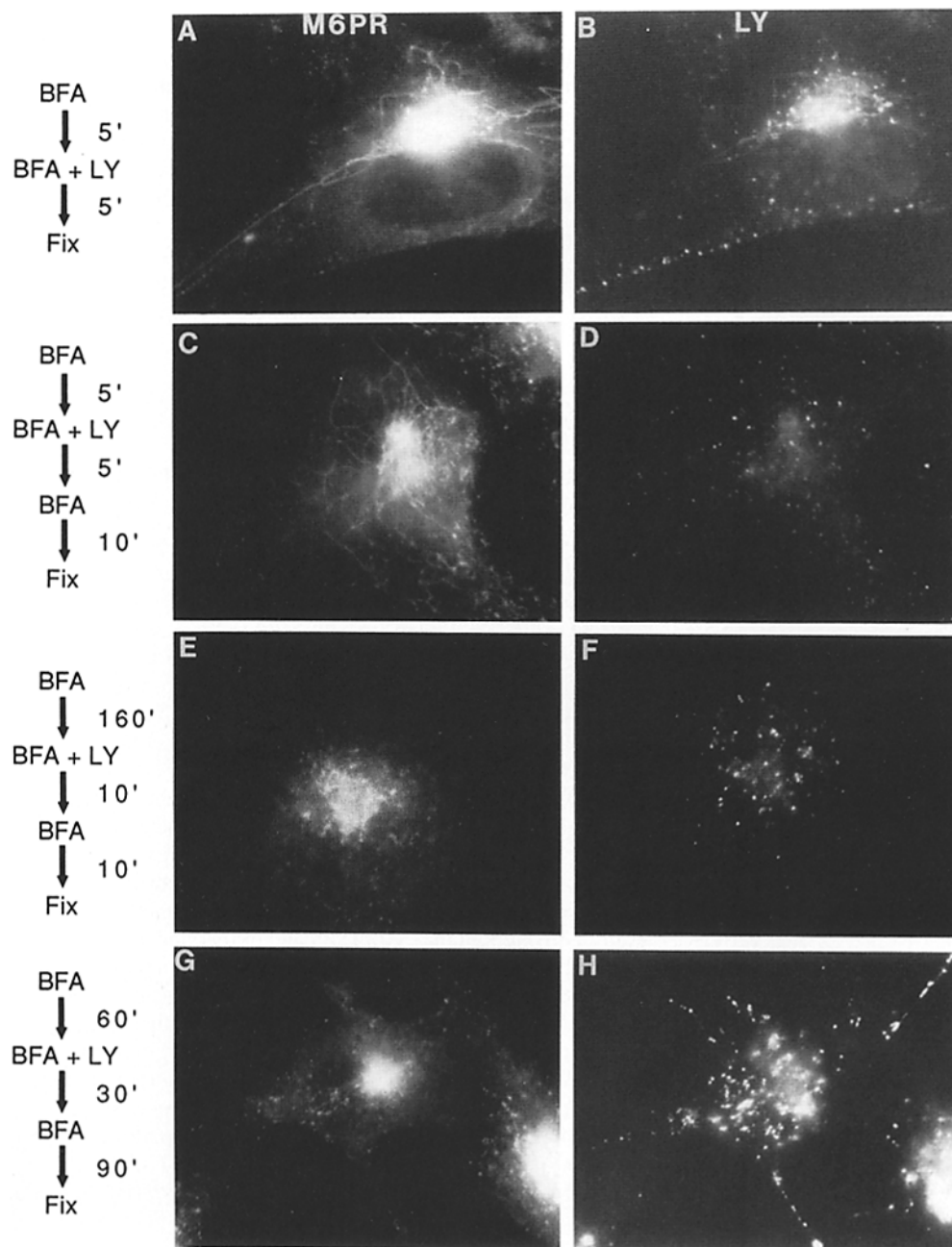
Figure 2. Endocytosed LY passes through the fused TGN/EE network to vesicular compartments located further downstream in the pathway to lysosomes. The experimental protocols for each pair of micrographs (e.g., *A* and *B*) are given on the left. Micrographs in the left column show the M6PR distribution; companion micrographs of the same BTRD cell in the right column show endocytosed LY as indicated. (*A* and *B*) By 10 min of BFA treatment, LY reaches the tubular M6PR-enriched TGN network. (*C* and *D*) LY is chased out of the M6PR-enriched network and into a vesicular PLC-like compartment. (*E* and *F*) Even after prolonged BFA treatment, by which time the TGN/EE network has collapsed into a juxtanuclear tubulo-vesicular complex, LY can be endocytosed and chased into PLC-like vesicles. (*G* and *H*) Under conditions in which LY would normally be delivered to lysosomes, LY endocytosed in the presence of BFA is located in peripheral vesicles that appear somewhat elongated.

bules increased in length to form a radiating array of tubules by 3 h of BFA treatment (Fig. 6 *C*). Lysosomes in other cell types, including MDBK cells, clone 9 hepatocytes, and normal human fibroblasts, had a less extensive response to BFA treatment; lysosomes elongated and scattered but did not form long tubules.

Formation of a Stable TGN/EE Tubular Network That Remains Distinct from Lysosomes

One of the apparent hallmarks of the BFA-induced fusion of the TGN with early endosomes is the formation of a tubular network and then the gradual collapse of this network to leave a juxtanuclear cluster of short tubulo-vesicular elements (Wood et al., 1991; Lippincott-Schwartz et al., 1991).

In most cell types examined, this process is complete long before lysosomal tubules form. To determine if lysosomal tubulation is dependent on, or influenced by, the collapse of the TGN/EE tubules, we utilized a cell type, primary bovine testicular cells (BTRS), in which the tubular TGN/EE network forms within 5 min of BFA addition but remains intact for ≥12 h in the presence of BFA (data not shown). As demonstrated in Fig. 7, even after 6 h of BFA pretreatment, LY endocytosed for 5 min was delivered to the fused TGN/EE network. Thus, as with other BFA-induced morphological changes, the stability of the TGN/early endosomal tubules is variable and cell type dependent. After 6 h of BFA treatment, lysosomes had migrated away from the juxtanuclear region and elongated forming distinct slightly discontinuous tubules (Fig. 7 *G*). The lysosomal tubules
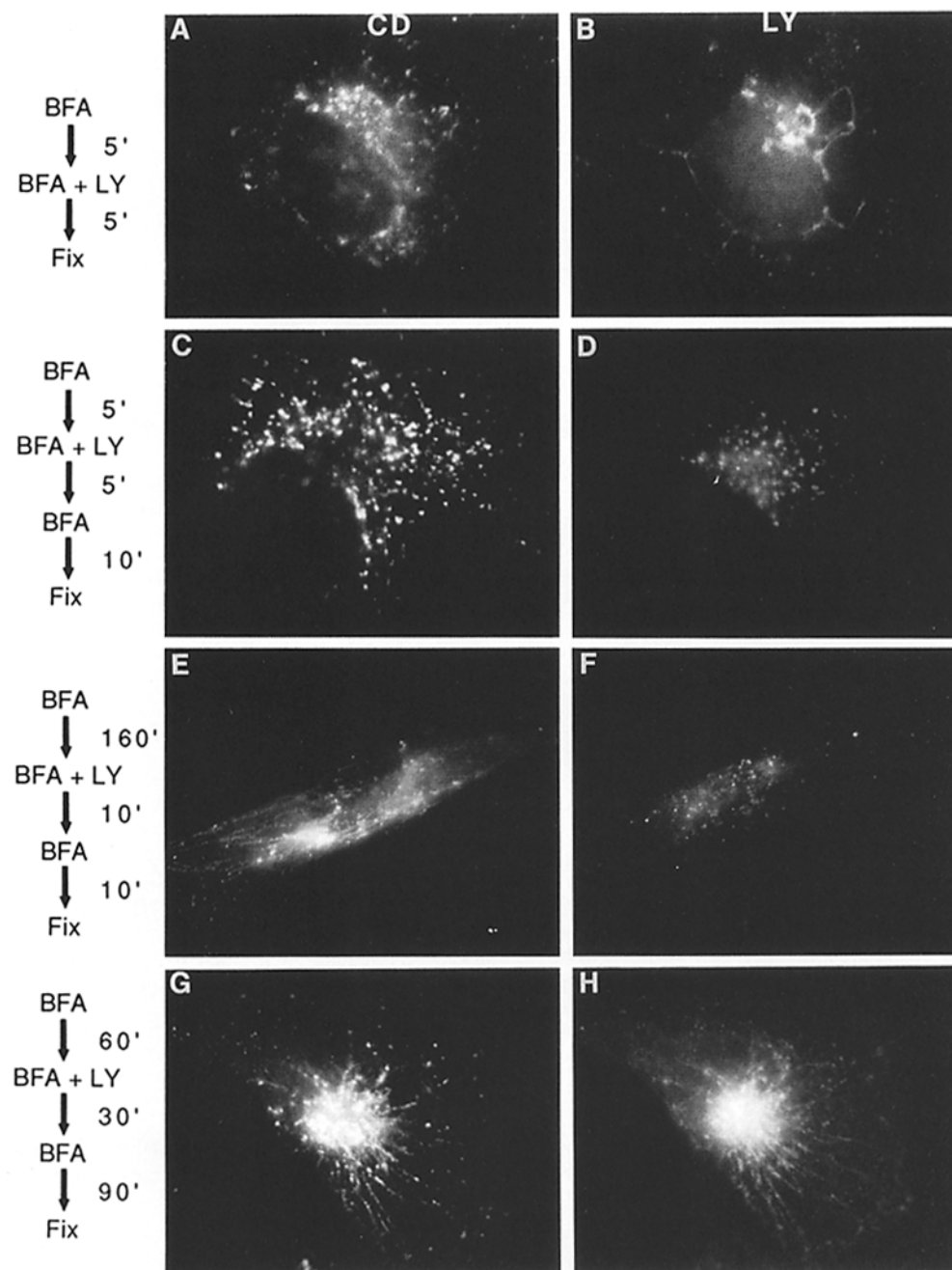
276

Figure 3. Endocytosed LY passes through the fused TGN/EE network into tubular lysosomes in the presence of BFA. The experimental protocols for each pair of micrographs (e.g., *A* and *B*) is given on the left. Micrographs in the left column show lysosomes by cathepsin D (*CD*) immunofluorescence; companion micrographs of the same BTRD cell in the right column show endocytosed LY as indicated. (*A* and *B*) After a short pulse in BFA, LY fills the tubular TGN/EE network which is distinct from vesicular lysosomes. (*C* and *D*) After a 5-min pulse and 10-min chase in the presence of BFA, LY has passed into the vesicular PLCs which are also distinct from lysosomes. (*E* and *F*) After longer BFA treatment, lysosomes have tubulated, and LY is still able to reach the PLC after a 10-min pulse, 10-min chase. (*G* and *H*) After longer BFA treatment and a 30-min pulse, 90-min chase, LY is now found to precisely colocalize with tubular lysosomes.

were clearly separate from the stable TGN/EE network (Fig. 7 *H*). Therefore, the distinction between these two compartments is not simply a temporal separation; even in cells in which they coexist as tubular structures, the TGN/EE and lysosomes do not fuse.

Energy Is Required for the Formation of Tubular Lysosomes

The formation of tubules from the Golgi cisternae and the TGN in BFA requires energy as shown by depleting cellular ATP levels with deoxyglucose (DOG) and sodium azide (NaAz) (Lippincott-Schwartz et al., 1990; Cluett, E. B., S. A. Wood, M. Banta, and W. J. Brown, unpublished observations). To examine the energy requirements for the for-

mation of BFA-induced lysosomal tubules, BTRD cells were treated with BFA for 3 h in the presence of 50 mM DOG and 0.05% NaAz. Under these conditions, lysosomes did not form tubules but instead scattered and apparently fragmented (Fig. 8 *E*). However, if lysosomes were induced to tubulate by treatment with BFA for 3 h, and then the cells were incubated with 50 mM DOG and 0.05% NaAz for an additional 1 h while still in the presence of BFA, the tubular lysosomes were maintained (Fig. 8 *B*). The complexity and extent of lysosomal tubulation under these conditions was equal to or greater than that seen in cells treated with BFA alone for 4 h (Fig. 8 *A*). After treatment with DOG, NaAz, and BFA under these conditions, >95% of the cells were viable as determined by trypan blue dye exclusion. Moreover, after wash-
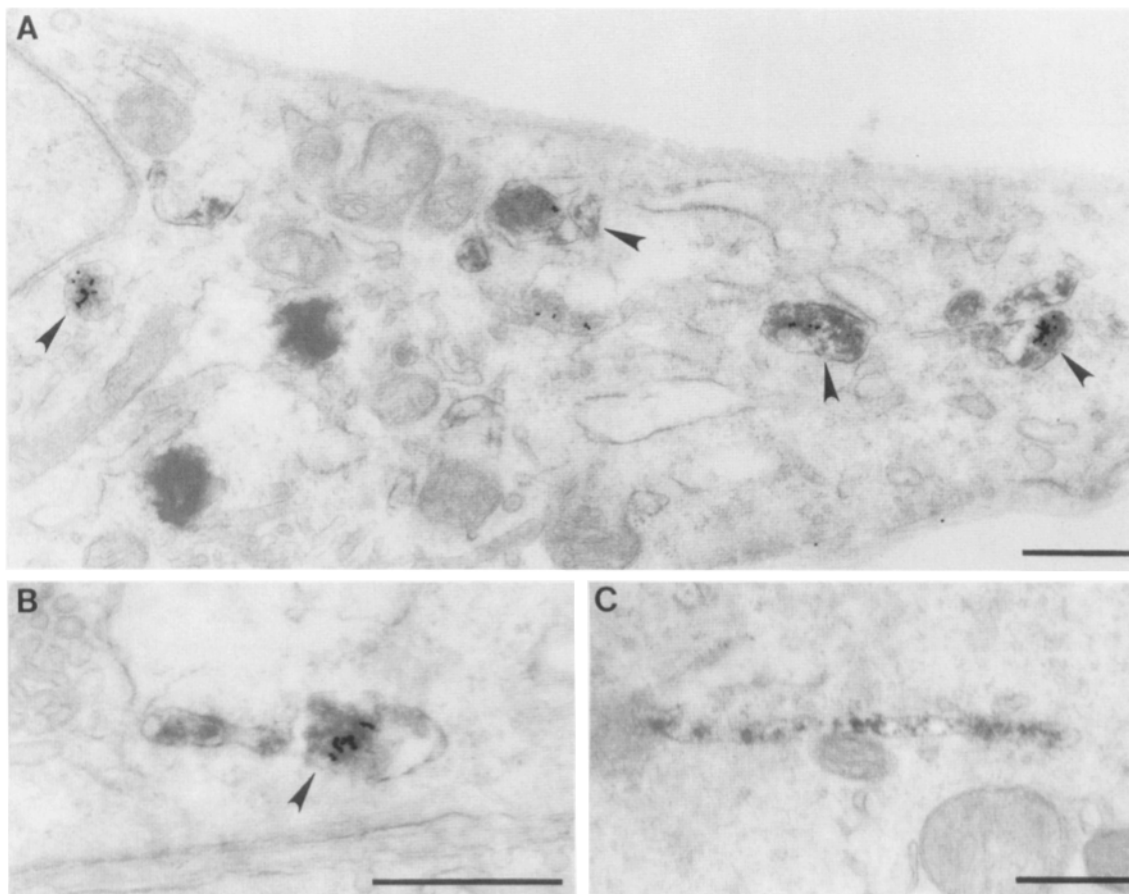
Figure 4. Endocytosed colloidal gold particles are delivered to cathepsin D–positive lysosomes in BFA-treated cells. BTRD cells were treated with BFA for 60 min, and then with 8-nm colloidal gold for 30 min followed by a chase for 120 min (all in the presence of BFA). Cells were fixed and processed for immunoperoxidase to localize cathepsin D. (*A*) A low magnification micrograph showing that many of the cathepsin D–positive lysosomes also contained endocytosed colloidal gold particles (*arrowheads*). (*B*) Cathepsin D–positive tubular lysosome containing endocytosed colloidal gold. (*C*) Cathepsin D–staining tubular lysosome. Bars, 0.5 μm.

out of DOG, NaAz, and BFA and reincubation overnight, cells remained adherent and underwent division.

To determine whether recovery from BFA treatment is energy dependent, lysosomes were induced to tubulate by incubating cells with BFA for 4 h. BFA was washed out and cells were allowed to recover either in the presence or absence of DOG and NaAz. Recovery from lysosomal tubulation in normal media was complete within 30 min of BFA washout (Fig. 8 C), while DOG and NaAz addition inhibited recovery, preventing the breakdown of lysosomal tubules (Fig. 8 D).

The Formation of Tubular Lysosomes Is Microtubule Mediated

Lysosomes in BTRD cells are clustered in the juxtanuclear region of the cell which double labeling with anti-cathepsin D and anti-tubulin antibodies revealed is the MTOC (Fig. 9, *A* and *B*). Maintenance of this juxtanuclear positioning is dependent on microtubules. Treatment of BTRD cells with nocodazole for 1 h completely disrupted the microtubule network and caused lysosomes to scatter (Fig. 9, *C* and *D*). Treatment with BFA for 4 h to induce lysosomal tubulation and double labeling with anti-cathepsin D and anti-tubulin

antibodies revealed a colocalization of lysosomal tubules and microtubules (Fig. 9, *E* and *F*). To further characterize the association of lysosomes with microtubules during BFA treatment, cells were treated with BFA for 4 h and then with nocodazole and BFA for an additional 1 h. Under these conditions, the microtubule network was disrupted, lysosomal tubules collapsed, and lysosomes formed clusters of small vesicles (Fig. 9, *G* and *H*). If cells were pretreated with nocodazole for 1 h and then treated with BFA in the continued presence of nocodazole for 4 h, lysosomes did not form tubules but instead clustered in irregular clumps of small vesicles (Fig. 9 *I*). Thus, microtubules mediate both the formation and maintenance of BFA-induced lysosomal tubules and, in their absence, BFA induces the clustering of lysosomes.

Discussion

Although several intraorganellar transport events are blocked in the presence of BFA, and while the morphology of the compartments on the endocytic pathway is altered by BFA, we found that endocytosis and the delivery of endocytosed
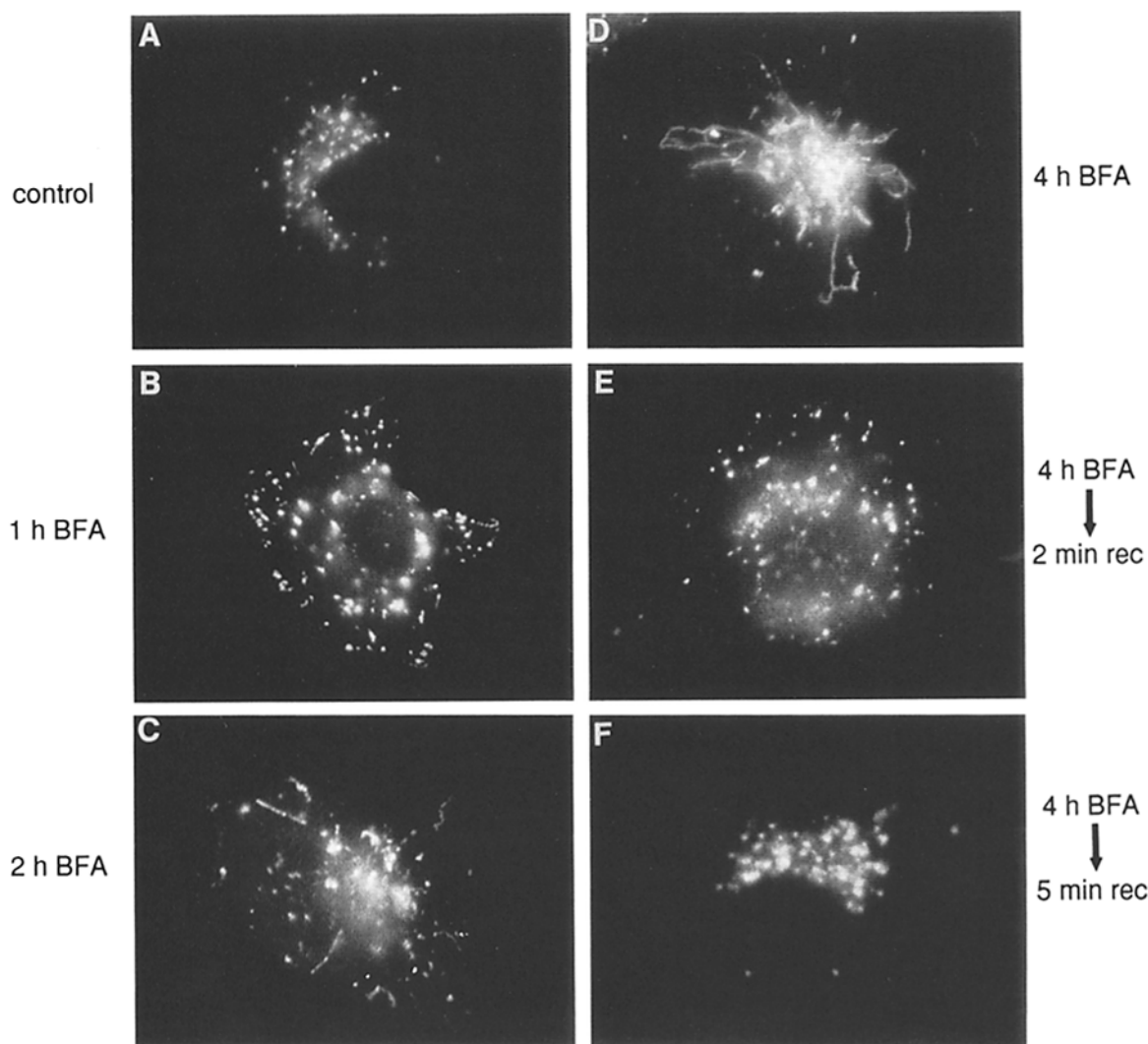
278

Figure 5. Time course of tubular lysosome formation and recovery. BTRD cells were incubated for various periods of time with 10 µg/ml BFA, and stained by immunofluorescence to visualize cathepsin D–positive lysosomes. By 4 h of treatment, lysosomes were extensively tubulated. Recovery from the tubulated state is very rapid; by 2 min of recovery (*2 min rec*), lysosomes have resumed a vesicular morphology, and by 5 min (*5 min rec*) they have relocated back to the central region of the cell.

material to lysosomes in several cell types was unaffected. Early in a time course of BFA treatment, LY was endocytosed and delivered to the tubular, fused TGN/early endosome as previously reported (Wood et al., 1991; Lippincott-Schwartz et al., 1991). Within 10 min, LY was transported out of this compartment and reached the physically distinct and apparently morphologically unaltered PLC. Even during prolonged pretreatment with BFA, endocytosed LY was still transported through the fused TGN/early endosome and to the late endosome. Delivery of LY and colloidal gold particles to tubular lysosomes occurred with normal kinetics even though the morphology and distribution of these organelles had been altered. These results differ from those of Lippincott-Schwartz et al. (1991), who found an inhibition of transport of the endocytic tracer rhodamine-ovalbumin to lysosomes labeled with antibodies to lep100 in chick embryo fibroblasts. While the use of different markers could explain

this discrepancy, it may in fact be simply another example of cell type–specific differences in the response of organelles to BFA (Ktistakis et al., 1991). On the other hand, our results are more consistent with those of Misumi et al. (1986) who reported that BFA treatment had little effect on the lysosomal degradation of endocytosed asialo-fetuin.

Although the time course of their development varies, the induction of lysosomal tubules by BFA in a number of cell types is generally slower than the tubulation of the Golgi complex and TGN, occurring after fusion of the Golgi complex and ER and fusion of the TGN and early endosomes are complete. However, as with other organelles, lysosomes rapidly recover their untreated morphology upon removal of BFA. Tubular lysosomes at times appeared discontinuous, as if individual lysosomal vesicles are closely apposed but not fused. Thus, either lysosomes are less fusogenic in the presence of BFA than either the Golgi cisternae or the TGN, or
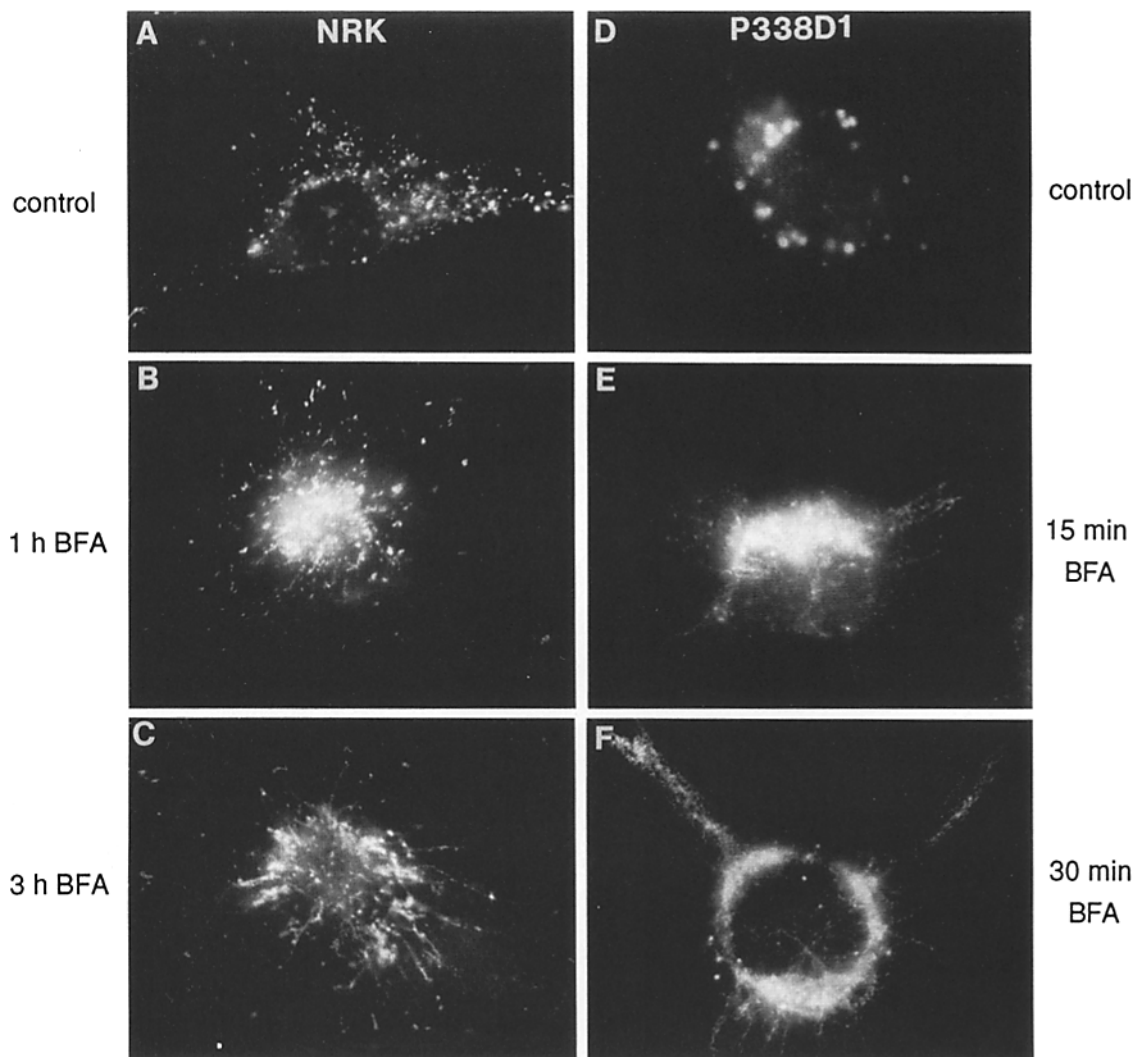
Figure 6. Kinetics of tubular lysosome formation vary in different cells. Times of BFA treatment are indicated next to each micrograph. NRK cells respond slowly to BFA, as lysosomal tubulation only becomes apparent after 1 h and is maximal at 4 h. P388D₁ macrophage-like cells respond more rapidly as tubular lysosomes were abundant by 15 min.

lysosomal tubules are less stable and fragment under identical fixation conditions (Robinson et al., 1986; Swanson et al., 1987). The formation of tubular lysosomes is energy dependent, as is the formation of tubules from Golgi cisternae and the TGN (Lippincott-Schwartz et al., 1990; Cluett, E. B., S. A. Wood, M. Banta, and W. J. Brown, manuscript submitted for publication). Surprisingly, however, energy is not required for the maintenance of pre-formed lysosomal tubules, but energy depletion prevents the collapse of lysosomal tubules upon removal of BFA. This suggests that an active process is required for the recovery of lysosomes back to the vesicular state.

Tubular lysosomes identical to those induced by BFA treatment have been noted previously in thioglycolate-elicited peritoneal macrophages (Swanson et al., 1987), in phorbol ester-stimulated, cultured macrophage-like cells (Young et al., 1990), and in macrophages recovering from cytoplasmic acidification (Heuser, 1989). It is therefore interesting to note that of the cell types examined, macrophage

lysosomes are the most responsive to BFA. The elongation of lysosomes in activated macrophages is microtubule-dependent and is mediated by the plus end–directed, microtubule-associated motor protein, kinesin (Swanson et al., 1987; Hollenbeck and Swanson, 1990). Likewise, the elongation of lysosomal tubules induced by BFA towards the cell periphery is microtubule enhanced, and may require kinesin or a similar plus end–directed motor. Tubulation of Golgi and TGN membranes is also apparently mediated by microtubules (Lippincott-Schwartz et al., 1990; Wood et al., 1991). We should note, however, that recently we have been able to induce the tubulation of Golgi membranes in vitro when no microtubules, or BFA for that matter, are present (Cluett, E. B., S. A. Wood, M. Banta, and W. J. Brown, manuscript submitted for publication). Therefore, in vivo tubulation may only be enhanced by microtubules which serve to guide membrane tubules to the periphery of the cell. Lysosomes in the untreated cells examined are concentrated in the juxtanuclear region at the microtubule organizing center.
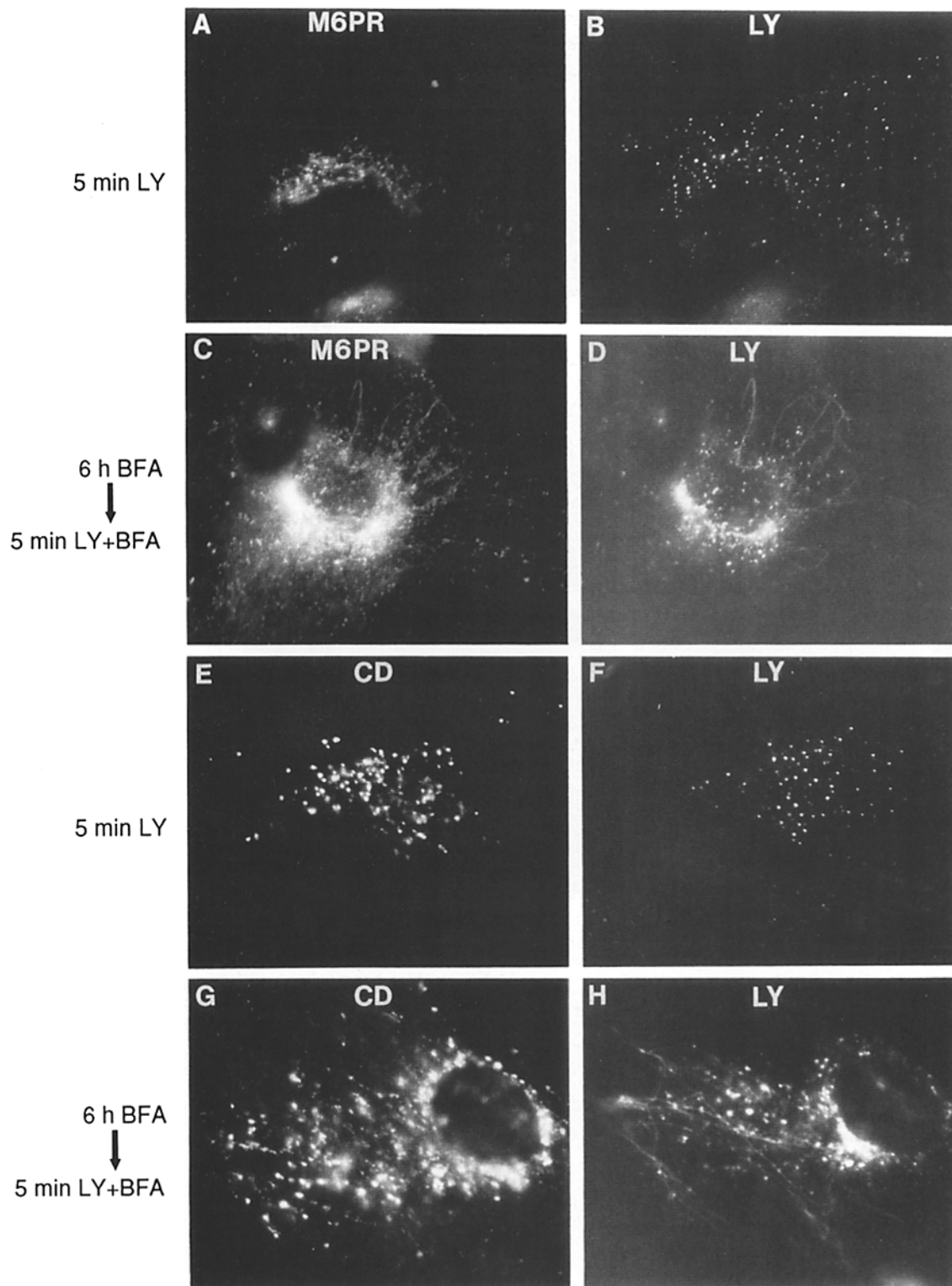
Figure 7. Formation of a stable TGN/EE network in primary bovine testicular cells that remains distinct from tubular lysosomes. Each of the pairs of micrographs show the distribution of M6PRs or cathepsin D (*CD*), and LY (as indicated at the top of each micrograph) in the same cells after various treatments with BFA and pulse-chase labeling conditions with LY (as shown to the left of each pair). (*A* and *B*) LY uptake for 5 min in untreated cells labels peripheral early endosomal vesicles but not M6PR-enriched organelles. (*C* and *D*) Even after 6 h in BFA, M6PR tubules can be labeled with LY after only 5 min of uptake. (*E* and *F*) Cells labeled by IF to localize cathepsin D after 5 min of LY uptake. (*G* and *H*) After 6 h in BFA, LY is delivered within 5 min to the TGN/EE which remains distinct from the cathepsin D–labeled lysosomes. In primarily bovine testicular cells, lysosomes do not tubulate as extensively as in other cells.
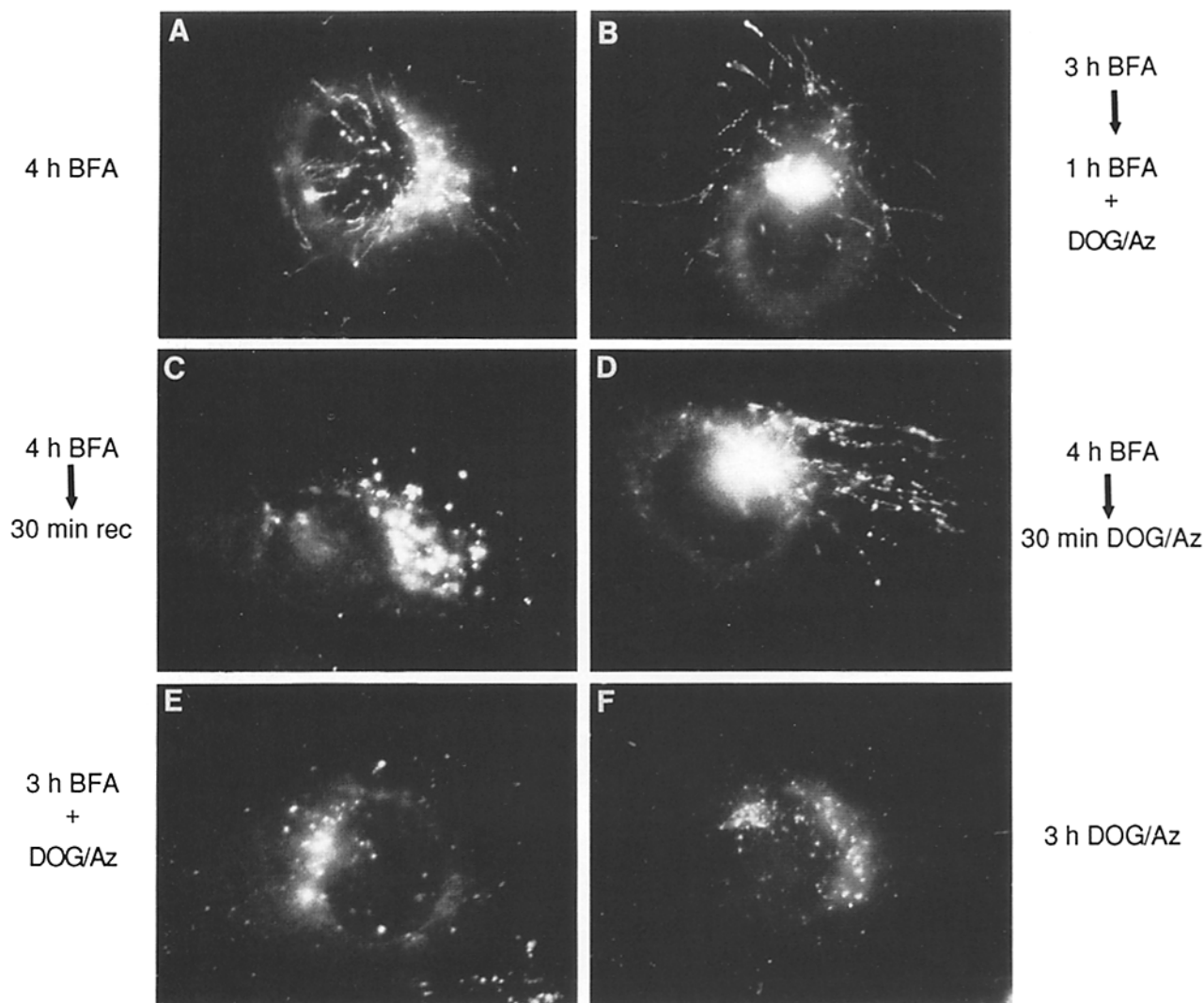
Figure 8. Lysosomal tubulation by BFA and recovery to vesicular morphology are energy dependent. Lysosomes were visualized in BTRD cells by IF of cathepsin D. Experimental protocols are outlined next to each cell. (*A*) Treatment with BFA for 4 h produces tubular lysosomes. (*B*) Treatment with BFA for 3 h, followed by incubation with 50 mM DOG/0.05% NaN₃ while still in the presence of BFA for 1 h, had little effect on the tubular morphology of lysosomes. (*C*) Cells were treated with BFA for 4 h and then washed free of the drug and incubated in normal medium for 30 min resulting in the restoration of vesicular lysosomes. (*D*) Recovery as in *C* but with the addition of DOG/NaN₃ as above during the recovery period; lysosomes remain primarily tubular. (*E*) Incubation of cells with BFA and DOG/NaN₃ prevents the formation of tubular lysosomes. (*F*) Control cells treated with DOG/NaN₃ do not show tubulated lysosomes.

As demonstrated by the scattering of lysosomes in nocodazole-treated cells, this positioning is dependent on intact microtubules, as previously shown (Matteoni and Kreis, 1987). Interestingly, BFA caused the clustering of lysosomes into large aggregates in cells with disrupted microtubules, indicating that the drug has effects on the associations between lysosomes as well as between lysosomes and microtubules.

A preponderance of evidence suggests that BFA blocks intracellular transport events by preventing the binding of coat proteins with nascent vesicles (Klausner et al., 1992). This results in the inability of both COP- and clathrin-coated transport vesicles to pinch-off from the Golgi stack and TGN, respectively (Orci et al., 1991; Robinson and Kreis, 1992). That BFA does not inhibit movement through the en-

Figure 9. The formation of lysosomal tubules is microtubule dependent. BTRD cells were double labeled for IF using anti-cathepsin D (*CD*) antibodies to visualize lysosomes (*left*) and anti-tubulin antibodies to visualize microtubules (*right*). (*A* and *B*) Lysosomes in untreated cells cluster at the MTOC. (*C* and *D*) Treatment with 6 µg/ml nocodazole disrupts microtubules and causes scattering of lysosomes. (*E* and *F*) Treatment with BFA for 4 h induces the formation of lysosomal tubules which closely associate with microtubules (*arrows*). (*G* and *H*) Cells were treated with BFA for 4 h and then with BFA and nocodazole for 1 h. The lysosomal tubules collapse and form tight clusters of vesicles in the absence of microtubules. (*I* and *J*) Pretreatment with nocodazole for 1 h to disrupt microtubules prevents formation of lysosomal tubules during a subsequent 4-h incubation in nocodazole and BFA. However, the lysosomes form tight clusters of vesicles.

282
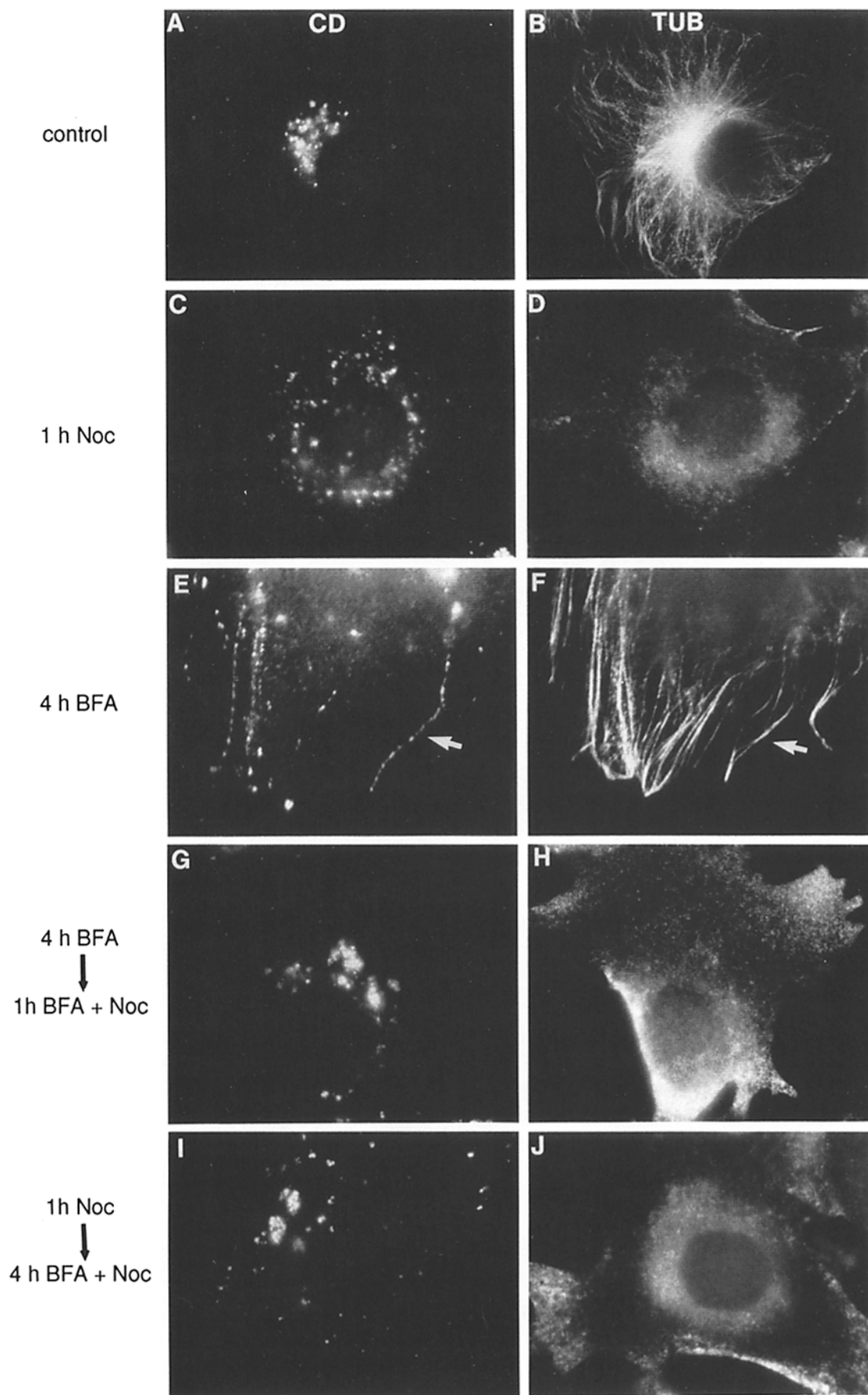
docytic compartments to lysosomes suggests (a) that transport events between compartments of the endosome–lysosome system do not use coated vesicles; and/or (b) coated vesicular transport steps between these organelles are resistant to BFA. In fact, both reasons are probably correct. It is well-established that the first step in receptor-mediated endocytosis occurs via cell surface clathrin-coated vesicles, and it has recently been shown that formation of these vesicles is not inhibited by BFA (Robinson and Kreis, 1992; Wong and Brodsky, 1992). Currently, there is no explanation for the finding that TGN- and plasma membrane-derived clathrin-coated vesicles are differentially sensitive to BFA, except to note that each have a different set of associated adaptin molecules (Pearse and Robinson, 1990).

At this time, there is no consensus on the exact number of physically separate endocytic compartments following the coated vesicle stage (Kornfeld and Mellman, 1989; Griffiths and Gruenberg, 1991; Murphy, 1991). Minimally, these compartments include the peripheral early endosome, the more centrally located late endosome or PLC that is enriched in M6PRs, and finally lysosomes (Griffiths and Gruenberg, 1991; Murphy, 1991). Two mechanisms have been postulated to explain the transport through the endocytic pathway: the maturation model in which early endosomes are modified to become late endosomes, and the vesicular model in which transport occurs between preexisting early and late endosomes via shuttle vesicles (Helenius et al., 1983). Evidence in favor of the preexisting model (Griffiths and Gruenberg, 1991) and the maturation model (Murphy, 1991) have recently been discussed. The preexisting model holds that transport between early and late endocytic compartments occurs via large, 0.5-μm vesicles, which are quite unlike either COP- or clathrin-coated vesicles (Gruenberg et al., 1989). Alternatively, several other groups have recently marshalled convincing evidence in favor of the maturation of early endosomes into late endosomes (Stoorvogel et al., 1991; Dunn and Maxfield, 1992). The mechanisms of transport from the late endosome/PLC to lysosomes are even less well characterized but evidence consistent with another maturation process has been presented (Roederer et al., 1990). In any case, each model obviates the need for COP- or clathrin-coated vesicles, and therefore would explain why in our studies, transport to lysosomes was unimpeded in the presence of BFA.

Interestingly, although transport through the endocytic pathway was unaffected by BFA, lysosomes tubulated in response to BFA. Drawing on the results from several studies, Klausner et al. (1992) have proposed that an equilibrium exists between vesiculation and tubulation. In the absence of coat protein binding, i.e., in the presence of BFA, the equilibrium is shifted toward tubulation. In other words, tubulation results from the cessation of vesicular traffic. The question that arises then is, what vesicular traffic pathway from lysosomes has been disrupted by BFA? Lysosomes are commonly perceived to be an intracellular dead-end into which material is delivered for terminal degradation. However, Lippincott-Schwartz and Fambrough (1987) have shown that a resident protein of lysosomal membranes, LEP100, regularly escapes lysosomes by cycling to the cell surface and back. Similarly, a recent study by Harding et al. (1992) demonstrated that some liposome-encapsulated antigens are processed in lysosomes and then recycled to the plasma mem-

brane for presentation. In neither of these cases, however, has the exact pathway from lysosomes to the cell surface been determined. It may involve transport directly to the cell surface or recycling through an intermediate compartment such as endosomes. In any case, the tubulation of lysosomes in response to BFA is consistent with these studies and suggests that there is, in fact, some vesicular transport out of lysosomes that is inhibited by BFA.

References

Anderson, R. G. W., J. L. Goldstein, and M. S. Brown. 1977. A mutation that impairs the ability of lipoprotein receptors to localize in coated pits on the cell surface of human fibroblasts. *Nature (Lond.).* 270:695–699.

Bomsel, M., R. Parton, S. Kuznetsov, T. A. Schroer, and J. Gruenberg. 1990. Microtubule and motor-dependent fusion in vitro between apical and basolateral endocytic vesicles from MDCK cells. *Cell.* 62:719–731.

Brown, W. J., and M. G. Farquhar. 1987. The distribution of 215-kilodalton mannose 6-phosphate receptors within cis (heavy) and trans (light) Golgi subfractions varies in different cell types. *Proc. Natl. Acad. Sci. USA.* 84:9001–9005.

Brown, W. J., and M. G. Farquhar. 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. *Methods Cell Biol.* 31:553–569.

Damke, H., J. Klumperman, K. von Figura, and T. Braulke. 1991. Effects of brefeldin A on the endocytic route. Redistribution of mannose 6-phosphate/insulin-like growth factor II receptors to the cell surface. *J. Biol. Chem.* 266:24829–24833.

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.

Dunn, K. W., and F. R. Maxfield. 1992. Delivery of ligands from sorting endosomes to late endosomes occurs by maturation of sorting endosomes. *J. Cell Biol.* 117:301–310.

Griffiths, G., and J. Gruenberg. 1991. The arguments for pre-existing early and late endosomes. *Trends Cell Biol.* 1:5–9.

Handley, D. A. 1989. Colloidal Gold: Principles, Methods, and Applications, M. A. Hayat, editor. Academic Press, New York. 13–32.

Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 8:245–250.

Heuser, J. 1980. Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell Biol.* 84:560–583.

Hollenbeck, P. J., and J. A. Swanson. 1990. Radial extension of macrophage tubular lysosomes supported by kinesin. *Nature (Lond.).* 346:864–866.

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.

Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483–525.

Ktistakis, N. T., M. G. Roth, and G. S. Bloom. 1991. PtK1 cells contain a non-diffusible, dominant factor that makes the Golgi apparatus resistant to brefeldin A. *J. Cell Biol.* 113:1009–1023.

Lippincott-Schwartz, J., and D. M. Fambrough. 1986. Lysosomal membrane dynamics: structure and interorganellar movement of a major lysosomal membrane glycoprotein. *J. Cell Biol.* 102:1593–1605.

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, H.-P. 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 mechanism for regulating organelle structure and membrane traffic. *Cell.* 67:601–617.

Matteoni, R., and T. E. Kreis. 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. *J. Cell Biol.* 105:1253–1265.

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.

Murphy, R. 1991. Maturation models for endosome and lysosome biogenesis. *Trends Cell Biol.* 1:77-82.

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.

Park, J. E., R. K. Draper, and W. J. Brown. 1991. Biosynthesis of lysosomal enzymes in cells of the End3 complementation group conditionally defective in endosomal acidification. *Somatic Cell Mol. Genet.* 17:137-150.

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

Robinson, J. M., T. Okada, J. J. Castellot, and M. J. Karnovsky. 1986. Unusual lysosomes in aortic smooth muscle cells: presence in living and rapidly frozen cells. *J. Cell Biol.* 102:1615-1622.

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-proteins. *Cell.* 69:129-138.

Roederer, M., J. R. Barry, R. B. Wilson, and R. F. Murphy. 1990. Endosomes can undergo an ATP-dependent density increase in the absence of dense lysosomes. *Eur. J. Cell Biol.* 51:229-234.

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

Scheel, J., R. Matteoni, T. Ludwig, B. Hoflack, and T. E. Kreis. 1990. Microtubule depolymerization inhibits transport of cathepsin D from the Golgi apparatus to lysosomes. *J. Cell Sci.* 96:711-720.

Stoorvogel, W., G. J. Strous, H. J. Geuze, V. Oorschot, and A. L. Schwartz. 1991. Late endosomes derive form early endosomes by maturation. *Cell.* 65:417-427.

Swanson, J., B. D. Yirinec, and S. C. Silverstein. 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. *J. Cell Biol.* 100:851-859.

Swanson, J., A. Bushnell, and S. C. Silverstein. 1987. Tubular lysosome morphology and distribution within macrophages depend on the integrity of cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA.* 84:1921-1925.

Wong, D. H., and F. M. Brodsky. 1992. 100-kD proteins of Golgi- and trans-Golgi network-associated coated vesicles have related but distinct binding properties. *J. Cell Biol.* 117:1171-1180.

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.

Young, M. R., A. H. Gordon, and P. D'Arcy Hart. 1990. Tubular lysosomes and their drug reactivity in cultured macrophages and in cell-free medium. *Exp. Cell Res.* 190:283-289.