

# Dissociation of a 110-kD Peripheral Membrane Protein from the Golgi Apparatus Is an Early Event in Brefeldin A Action

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**Abstract.** Brefeldin A (BFA) has a profound effect on the structure of the Golgi apparatus, causing Golgi proteins to redistribute into the ER minutes after drug treatment. Here we describe the dissociation of a 110-kD cytoplasmically oriented peripheral membrane protein (Allan, V. J., and T. E. Kreis. 1986. *J. Cell Biol.* 103:2229–2239) from the Golgi apparatus as an early event in BFA action, preceding other morphologic changes. In contrast, other peripheral membrane proteins of the Golgi apparatus were not released but followed Golgi membrane into the ER during BFA treatment. The 110-kD protein remained widely dispersed throughout the cytoplasm during drug treatment, but upon removal of BFA it reassociated with membranes during reformation of the Golgi apparatus. Although a

30-s exposure to the drug was sufficient to cause the redistribution of the 110-kD protein, removal of the drug after this short exposure resulted in the reassociation of the 110-kD protein and no change in Golgi structure. If cells were exposed to BFA for 1 min or more, however, a portion of the Golgi membrane was committed to move into and out of the ER after removal of the drug. ATP depletion also caused the reversible release of the 110-kD protein, but without Golgi membrane redistribution into the ER. These findings suggest that the interaction between the 110-kD protein and the Golgi apparatus is dynamic and can be perturbed by metabolic changes or the drug BFA.

**T**HE Golgi apparatus is the central structure within the cell through which newly synthesized secretory and membrane proteins pass, become modified, and are sorted en route to their final destination inside or outside of the cell. The striking morphology of this organelle, consisting of ordered stacks of cisternae, reflects a spatially differentiated structure that is believed to correlate with distinct Golgi functions (Palade, 1975; Farquhar, 1985; Dunphy and Rothman, 1985). The central, perinuclear localization of the Golgi complex appears to involve the direct or indirect interaction of this organelle with microtubules. Indeed, this localization is lost during mitosis when depolymerization of cytoplasmic microtubules causes the breakdown of the Golgi complex into small, dispersed clusters (Lucocq and Warren, 1987; Lucocq et al., 1987). Understanding how the Golgi structure is determined and maintained, and how its structure relates to Golgi function, poses fundamental problems in cell biology. Pharmacologic perturbation of the Golgi apparatus has provided a great deal of information about these problems. Two types of perturbations have been observed. Drugs that disrupt microtubule organization such as colchicine, nocodazole, and taxol result in the fragmentation and dispersion of the Golgi apparatus (for reviews see Thyberg and Moskalewski, 1985; Kreis,

1990). Although these microtubule-perturbing agents fragment the Golgi apparatus (Rogalski and Singer, 1984; Turner and Tartakoff, 1989), they do not abrogate the identity of the Golgi apparatus as a distinct organelle (Ho et al., 1989), nor do they appreciably affect its function (Rogalski et al., 1984; Salas et al., 1986). In contrast, the ability of another drug, brefeldin A (BFA)<sup>1</sup>, to cause the loss of the Golgi complex as an identifiable organelle has added a new dimension to our ability to perturb this structure.

BFA is a heterocyclic lactone produced by several fungi (Harri et al., 1963) which dramatically alters Golgi structure (Fujiwara et al., 1988). This drug effectively halts secretion from the ER and causes the redistribution of Golgi proteins into the ER (Misumi et al., 1986; Lippincott-Schwartz et al., 1989; Doms et al., 1989). Morphological changes in the Golgi apparatus are seen within minutes after the addition of BFA. Initially, the cisternal structure is lost and replaced by swollen vacuoles that give rise to long tubulovesicular extensions through which Golgi membrane and content are directed to the ER (Lippincott-Schwartz et al., 1990). With continued presence of BFA, no recognizable Golgi structure

1. *Abbreviations used in this paper:* BFA, brefeldin A; Man II, mannosidase II; NRK, normal rat kidney.

is observed in the cell (Lippincott-Schwartz et al., 1989). This is accompanied by a cessation of anterograde transport from the ER/Golgi complex to more peripheral organelles. These dramatic effects of BFA are rapidly and completely reversed by removing the drug. We know of no other drug with a similar effect on the existence of a particular organelle. Understanding how BFA acts will likely shed light on the process of biogenesis of organelles and on the ability of eukaryotic cells to establish the highly differentiated state of membrane-bounded compartmentalization.

One potential mechanism by which BFA could act is through the disruption of underlying structural elements of the Golgi apparatus. To explore this possibility further we examined the effect of BFA on several cytoplasmically oriented proteins associated with the Golgi apparatus. These include (a) a 110-kD protein, described by Allan and Kreis (1986), that associates with the Golgi complex as a cytoplasmically oriented peripheral membrane protein and has been proposed to be a structural element that might mediate the interaction of the Golgi apparatus with microtubules since it interacts with taxol-stabilized microtubules *in vitro*; (b) a 58-kD protein, distinct from the 110-kD protein, that also localizes to the cytoplasmic face of the Golgi apparatus and binds to microtubules *in vitro* (Bloom and Brashear [1989]); and (c) two cytoplasmically oriented peripheral Golgi membrane proteins of 54 and 86 kD recognized by the mAb 6F4C5 (Chicheportiche et al., 1984).

In this report we show that the 110-kD protein dissociates from the Golgi complex within 30 s after the addition of BFA. This precedes any other morphologic change observable at the light microscopic level. Removal of the drug after these short times results in the rapid reassociation of the protein with the Golgi apparatus and the direct recovery of Golgi structure. If, however, cells are exposed to BFA for 1 min or longer, a portion of the Golgi apparatus is committed to move into the ER, even after the removal of the drug. In contrast, the 58-kD protein and the two peripheral membrane proteins recognized by 6F4C5 are not released in response to BFA. The rapid dissociation of the 110-kD protein from the Golgi apparatus upon treatment with BFA suggests this is an early event in the action of BFA preceding the disassembly of the Golgi apparatus.

## Materials and Methods

### Materials and Cell Culture

BFA was obtained from Sandoz Ltd. (Basel, Switzerland) and Epicentre Technologies (Madison, WI) and was stored at  $-20^{\circ}\text{C}$  as a stock solution of 5 mg/ml in methanol. Nocodazole was purchased from Sigma Chemical Co. (St. Louis, MO) and where indicated was used at 20  $\mu\text{g}/\text{ml}$ . Normal rat kidney (NRK) cells were maintained in RPMI supplemented with 10% FCS and 0.3% gentamycin at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Cells were plated onto 12-mm-diam glass coverslips 48 h before experiments.

### Antibodies

A rabbit IgG directed against Golgi mannosidase II (Man II) (Moremen and Touster, 1985) was generously supplied by Dr. K. Moremen (Massachusetts Institute of Technology, Boston, MA). The mouse mAb M3A5, characterized by Allan and Kreis (1986), recognizes the 110-kD Golgi peripheral membrane protein, and the mouse monoclonal antibody (clone 9) recognizes the 58-kD peripheral Golgi membrane protein (Bloom and Brashear, 1989). The mAb C6F4C, which recognizes two peripheral Golgi proteins of 54,000 and 86,000  $M_r$  (Chicheportiche et al., 1984), was kindly

provided by Dr. A. Tartakoff (Case-Western Reserve University, Cleveland, OH). Rhodamine- and fluorescein-labeled goat anti-mouse and goat anti-rabbit IgG were purchased from Organon Teknika (Westchester, PA) and used at 1:500 dilution of manufacturer's stock solution.

### Immunofluorescence Microscopy

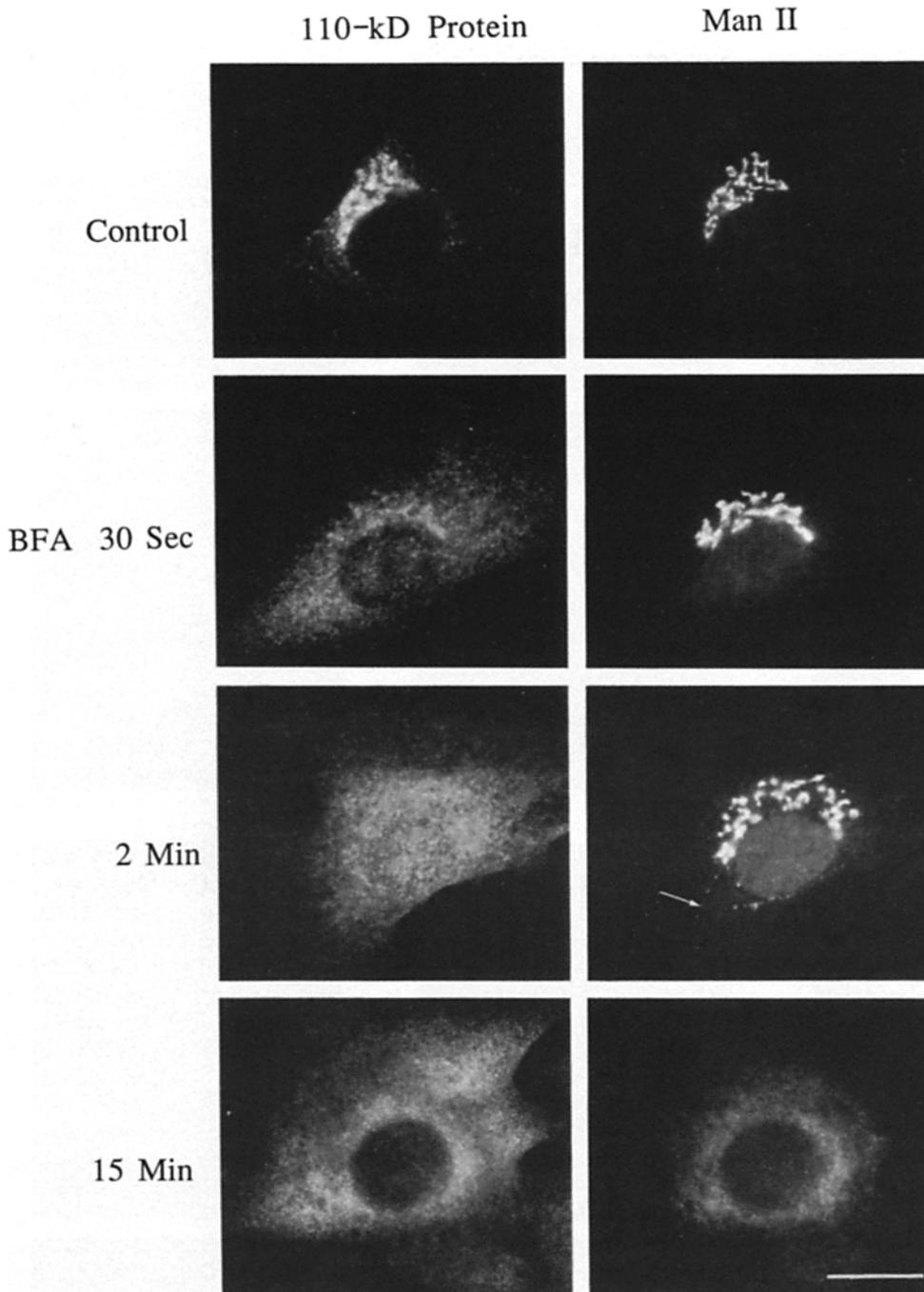
Cells on coverslips were incubated with the appropriate drugs in culture media before immunofluorescence assay. Cells were then fixed for 10 min at room temperature in 2% formaldehyde in PBS. After washing the fixed cells in PBS, the cells were permeabilized by submersion in methanol at  $0^{\circ}\text{C}$  for 1.5 min and then placed in PBS containing 10% FCS. Cells were incubated with primary antibodies in PBS/10% FCS and 0.15% saponin for 1 h, washed free of antibody, and then incubated in the fluorescently labeled second antibodies for 1 h. After washing, coverslips were mounted on slides in Fluormount G (Southern Biotechnology Associates, Birmingham, AL), and viewed with a  $63\times$  oil Planapo lens on a Zeiss microscope equipped with barrier filters to prevent crossover of fluorescein and rhodamine fluorescence.

## Results

### BFA Causes the 110-kD Protein to Dissociate from the Golgi Apparatus

We have previously shown that BFA causes the rapid redistribution of Golgi resident proteins into the ER, resulting in the loss of the Golgi apparatus as a distinct organelle (Lippincott-Schwartz et al., 1989). While the mechanism of action of BFA is far from understood, recent data suggest that the earliest effects of BFA occur at the level of the Golgi apparatus (Lippincott-Schwartz et al., 1990). To test this possibility further we examined the effect of BFA on the cytoplasmically oriented 110-kD Golgi-associated protein, believed to be a structural component of the Golgi apparatus (Allan and Kreis, 1986). As shown in Fig. 1, immunofluorescence staining of permeabilized NRK cells using an antibody to the 110-kD protein revealed labeling of a perinuclear structure typical of the Golgi complex in these cells. While this staining pattern overlapped significantly with that for the *cis*/medial-Golgi integral membrane protein, Man II, there were slight differences. For instance, whereas Man II was associated in a tightly packed structure, the 110-kD protein was more loosely associated, appearing to envelop the Golgi apparatus. In addition, the 110-kD protein was also localized to peripheral vesicles not labeled with Man II, as was observed previously (Allan and Kreis, 1986).

Addition of BFA to cells had a dramatic effect on the distribution of the 110-kD protein. Within 30 s after adding BFA, the 110-kD protein was no longer predominantly associated with the Golgi apparatus, but instead appeared widely dispersed throughout the cytoplasm. Double-labeling of these cells with antibodies to Man II, however, showed that at these early times no change in Golgi structure by light microscopy was apparent. It was not until 2 min of BFA treatment that the Man II staining pattern began to alter. The earliest changes included the extension of beaded, necklace-like tubular processes from the Golgi apparatus which carry Golgi proteins into the ER. By 15 min, there was no discernible Golgi structure. Instead, Golgi resident proteins, as visualized by Man II staining, were distributed in a fine punctate/reticular pattern that we have shown previously by immunoelectron microscopy and biochemical techniques includes their distribution within the ER (Lippincott-Schwartz et al., 1989). Throughout BFA treatment, the 110-kD protein



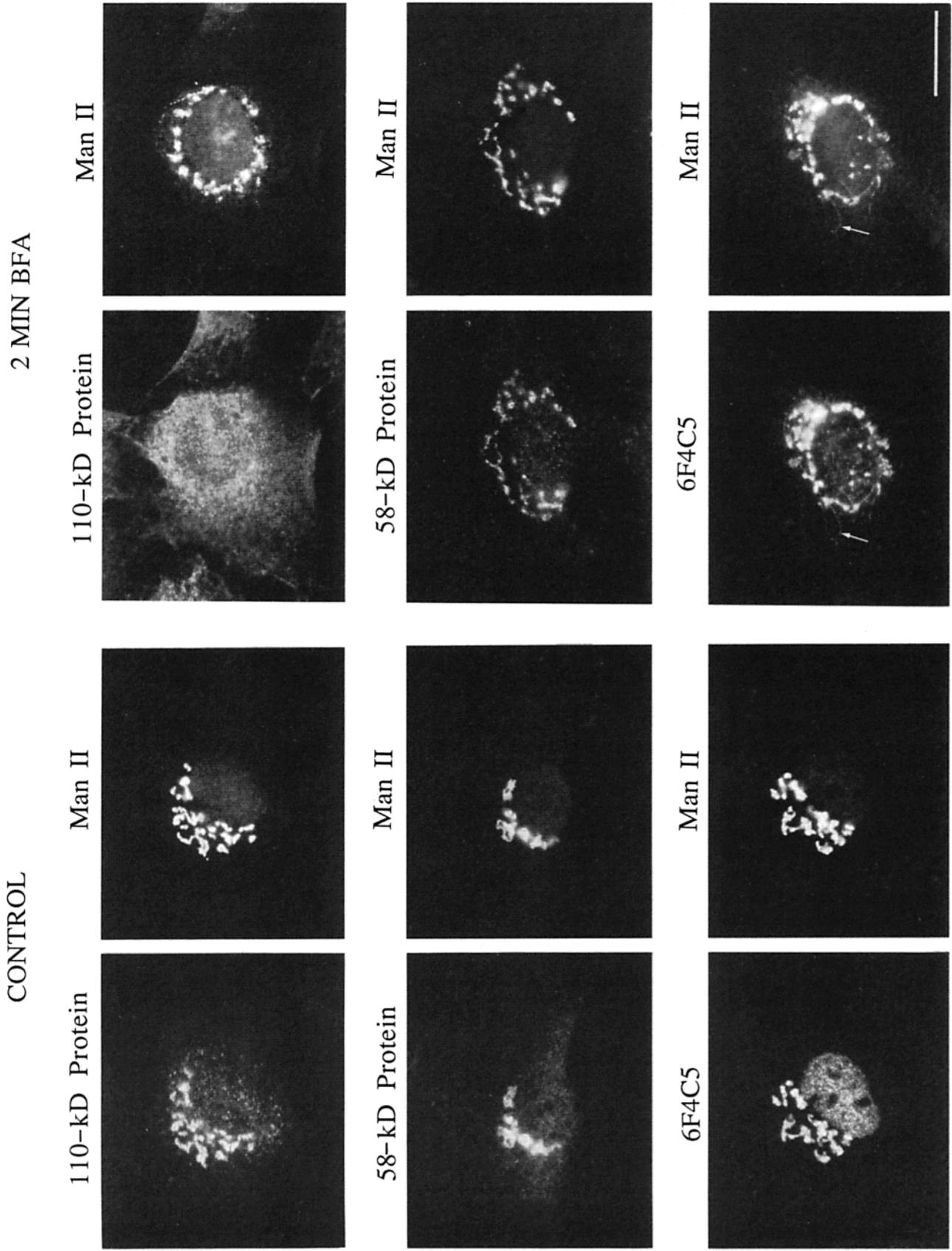
**Figure 1.** Effect of BFA on the association of the 110-kD protein with the Golgi apparatus. NRK cells were treated without (*Control*) or with 5  $\mu\text{g/ml}$  (18  $\mu\text{M}$ ) BFA at 37°C for the indicated times and then fixed, permeabilized, and stained by double-label immunofluorescence with antibodies to the 110-kD protein and Man II. In control cells Man II staining was confined to a compact perinuclear structure typical of Golgi distribution in these cells. The 110-kD protein colocalized with the Man II staining pattern enveloping the Man II-stained Golgi apparatus, with additional staining of peripheral vesicles. 30 s after the addition of BFA, the 110-kD protein was widely scattered throughout the cytoplasm, while the Golgi apparatus structure remained intact. After 2 min of treatment the Golgi apparatus, labeled with antibodies to Man II, was beginning to extrude necklace-like processes (*arrow*), evidence that the BFA-induced redistribution of Golgi proteins back to the ER had begun. By 15 min that redistribution was completed as there was no recognizable Golgi structure. Instead, Man II was distributed in a punctate/reticular pattern characteristic of ER staining. The 110-kD protein remained widely scattered throughout the cytoplasm during BFA treatment and not colocalized with Man II distribution. Bar, 10  $\mu\text{m}$ .

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remained widely dispersed throughout the cytoplasm. No obvious colocalization of the 110-kD protein and Man II could be observed after 15 min of BFA treatment. Indeed, Man II staining in BFA-treated cells was more concentrated in regions around the nucleus as was observed for an antibody to an ER resident protein (Louvard et al., 1982) (data not shown), while staining of the 110-kD protein extended out into the periphery, to the edges of the cytoplasm. During BFA treatment the 110-kD protein staining often appeared as a diffuse cytoplasmic fluorescence with regions of small punctate aggregates but at no time was it colocalized with

microtubules (data not shown). Similar effects of BFA on the 110-kD protein and Golgi structure were observed in other cell types, including HeLa and Vero cells (data not shown).

Release of the 110-kD protein could be detected as early as 20 s after BFA addition. At these early times of BFA treatment (up to 1 min) the extent of dissociation of the 110-kD protein varied somewhat from cell to cell, with many cells showing some residual 110-kD protein associated with the Golgi complex. In all cells examined, however, after 2 min of BFA treatment, no 110-kD protein remained associated with Golgi structures.



### **BFA Specifically Releases the 110-kD Protein and Not Other Known Peripheral Golgi Membrane Proteins**

To determine whether the BFA-induced dissociation of the 110-kD protein was specific for this protein or was a result of a general response of the Golgi membrane to the drug, we examined the effect of BFA on other cytoplasmically oriented peripheral Golgi membrane proteins. The effect of BFA on the distribution of two reported peripheral Golgi membrane proteins of 54,000 and 86,000  $M_r$ , recognized by the mAb 6F4C5 (Chicheportiche et al., 1984) is shown in Fig. 2. Immunofluorescence staining of one or both of these proteins was superimposable with staining of Man II fluorescence in control cells and in cells treated with BFA (Fig. 2, *bottom row*). A 58-kD Golgi-associated protein has been described with properties similar to the 110-kD protein. This protein specifically colocalizes with the Golgi apparatus by immunofluorescence and is capable of binding to polymerized microtubules in vitro (Bloom and Brashear, 1989). In contrast to the 110-kD protein, however, BFA did not cause the dissociation of the 58-kD protein from the Golgi complex. The 58-kD protein fluorescence remained associated with Man II distribution, colocalizing with Man II in Golgi structures in control cells; and during BFA treatment fluorescence remained associated with Golgi proteins (Fig. 2, *middle row*) both before and during their redistribution into the ER. Like the proteins recognized by 6F4C5, the 58-kD protein colocalized with Man II along tubular extensions of the Golgi complex after 2 min of BFA treatment. Thus the 110-kD protein, to the extent so far examined, is uniquely affected by BFA treatment.

### **Effect of BFA Dose on the Kinetics of 110-kD Protein Dissociation and Movement of Man II into the ER**

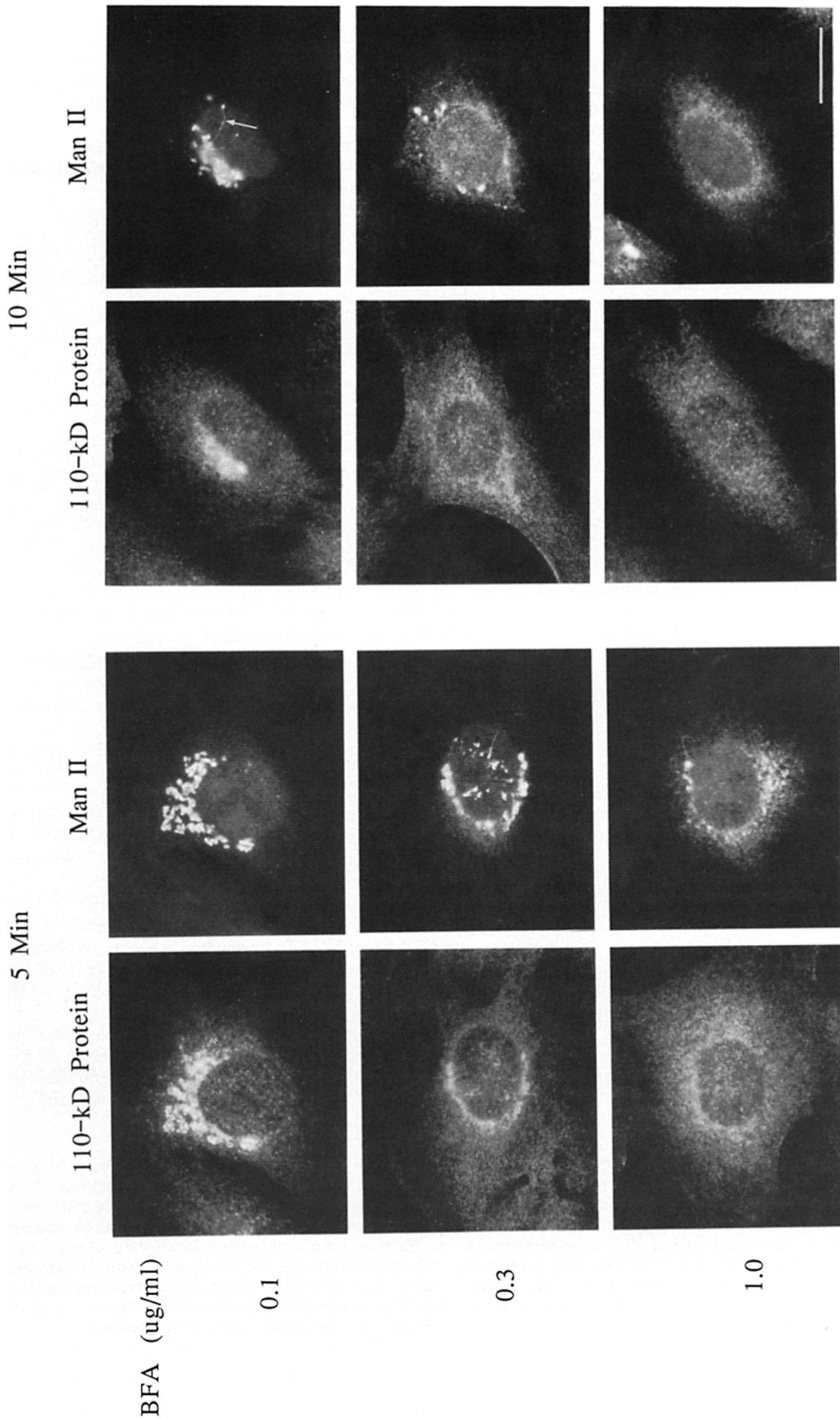
In the above experiments we used a relatively high concentration of BFA (5  $\mu\text{g/ml}$ ) to demonstrate that the dissociation of the 110-kD protein preceded the redistribution of Man II into the ER. To determine whether lower concentrations of BFA were capable of inducing a complete BFA response (i.e., dissociation of the 110-kD protein and redistribution of Golgi components to the ER), we looked at the dose dependency of this process. As shown in Fig. 3, after 5 min of incubation with the low dose of 0.1  $\mu\text{g/ml}$  of BFA, there was no observable change in either Golgi structure, as visualized by Man II or in the association of the 110-kD protein, compared with control cells (compare Figs. 1 and 3). It was not until after a 10-min incubation at this dose, when tubulovesicular Golgi processes appeared (*arrow*), that a partial dissociation of the 110-kD protein from the Golgi apparatus was observed. The 110-kD protein was never observed to be associated with these tubular Golgi extensions, however. In contrast, incubation of cells for 5 min in 1.0  $\mu\text{g/ml}$  BFA

resulted in complete dispersal of the 110-kD protein throughout the cytoplasm, even though Man II labeling of remnant Golgi structures could still be observed. By 10 min, all of the Man II staining appeared in a diffuse punctate network with no Golgi structures visible. The rate of these changes in Golgi structure induced by 1.0  $\mu\text{g/ml}$  BFA was similar to that observed with 5  $\mu\text{g/ml}$  (compare Figs. 1 and 3). Thus, at BFA concentrations  $\geq 1$   $\mu\text{g/ml}$ , the kinetics of dissociation of the 110-kD protein from the Golgi complex, and the redistribution of Golgi membrane into the ER appeared to be maximized. Intermediate doses of BFA (0.3  $\mu\text{g/ml}$ ) resulted in intermediate kinetics for the rate of dissociation of the 110-kD protein and redistribution of Golgi complex membrane. The overall pattern demonstrated that lowering the dose of BFA results in the slowing of both the release of the 110-kD protein and of the Golgi protein redistribution into the ER. Extending the period of incubation with BFA showed that the times required to totally redistribute Man II into the ER, defined by the absence of any Golgi structure, were 5–10 min for 1.0  $\mu\text{g/ml}$ , 15 min for 0.3  $\mu\text{g/ml}$ , and 25–30 min for 0.1  $\mu\text{g/ml}$  BFA. Importantly, regardless of the time required to translocate Golgi complex proteins into the ER, the final phenotype in each case resembled that seen with high doses of BFA; i.e., Man II in a punctate distribution characteristic of ER and the 110 kD-protein widely scattered throughout the cytoplasm (Fig. 1).

### **The 110-kD Protein Reassociates with Golgi Membrane upon Removal of BFA**

While the 110-kD protein appears to remain dissociated from the Golgi membrane in the presence of BFA, once BFA is removed, the 110-kD protein rapidly reassociates with Golgi membrane as this membrane reforms a Golgi apparatus. This is shown in Fig. 4 where NRK cells that had been treated with BFA for 30 min were rinsed twice with culture media to remove BFA, and then incubated at 37°C before double immunolabeling with antibodies to Man II and the 110-kD protein. Movement of the 110-kD protein from the periphery in toward the location of Golgi membrane proteins could be detected within minutes after drug removal, and the extent of reassociation of the 110-kD protein with the Golgi apparatus increased with the time of recovery. By 5 min, Man II staining began to accumulate in discrete vesicles that weakly colocalized with the 110-kD protein (data not shown). By 10 min these structures had enlarged and tubular-like structures were also stained both with the antibodies to the 110-kD protein and Man II. After 30 min these structures and the associated 110-kD protein had condensed to form recognizable Golgi elements which by 60 min had become more compact, resembling the Golgi apparatus in untreated NRK cells.

**Figure 2.** The BFA-induced dissociation is specific to the 110-kD Golgi protein. Cells were incubated at 37°C in the absence (*CONTROL*) or presence of 5  $\mu\text{g/ml}$  BFA for 2 min before fixation, permeabilization, and preparation for indirect immunofluorescence. Cells were double labeled with antibodies to Man II and either the 110- or the 58-kD protein, or 6F4C5, a mAb that recognizes two (a 54- and 86-kD) peripheral membrane Golgi proteins. In control cells all of the Golgi-associated proteins (i.e., the 110- and 58-kD proteins and 6F4C5) colocalized with Man II staining. After 2 min of BFA treatment, however, only the 110-kD protein was released from the Golgi apparatus and found in a widely scattered diffuse distribution. The 58-kD protein remained associated with Golgi elements during BFA treatment. The 54- and 86-kD proteins recognized by 6F4C5 also remained with Golgi elements during BFA treatment, and were observed colocalized with Man II-stained Golgi and tubular extensions (*arrows*) of the Golgi complex. Bar, 10  $\mu\text{m}$ .



### Commitment of Golgi Proteins to Enter the ER after Short BFA Treatment

Dissociation of the 110-kD protein from the Golgi apparatus is the earliest event so far detected during BFA treatment, occurring before any noticeable redistribution of Golgi membrane into the ER. Since we know that the 110-kD protein can rapidly reassociate with Golgi membrane once BFA is removed, we examined the fate of the Golgi apparatus using the marker Man II and the 110-kD protein in cells treated with BFA for 1 min and then washed free of the drug. As shown in Fig. 5, after 1 min of BFA treatment, Man II staining of the Golgi apparatus appeared similar to untreated cells, even though the 110-kD protein was widely scattered throughout the cytoplasm. 5 min after removing BFA, Man II was no longer restricted to its original morphology or location but appeared to be present to a significant amount in punctate/reticular pattern consistent with its presence in the ER. The 110-kD protein colocalized with these Man II-stained structures. These results suggest that brief exposure to BFA is sufficient to commit Man II and other Golgi proteins to follow the pathway into the ER after the BFA is removed. Then, 15 min after the removal of BFA, Man II staining was again associated with large, perinuclear, cisternal-like structures. This rapid recovery into enlarged Golgi-like structures after 1 min BFA exposure is in contrast to recovery after longer exposures to BFA (as in Fig. 4).

The commitment of Golgi membrane to move into the ER required at least 1 min of BFA treatment. A striking contrast was seen with shorter treatments of BFA. When cells were treated with 5  $\mu\text{g}/\text{ml}$  BFA for 30 s the 110-kD protein was widely distributed throughout the cytoplasm as observed in Fig. 1. However, when BFA was removed after the 30-s incubation, no detectable Man II appeared to cycle through the ER. 5 min after removal of BFA, the staining patterns of the 110-kD protein and Man II colocalized, resembling that observed in control cells. At no time, during recovery times of up to 3 h after 30 s of BFA treatment, was Man II observed in tubular structures of the Golgi apparatus or in a diffuse punctate pattern characteristic of the ER (data not shown). Thus, despite significant release of the 110-kD protein after 30 s of BFA, Golgi membrane was not irreversibly committed to move into the ER before reforming the Golgi apparatus. The length of BFA treatment required to commit the Golgi proteins to move into the ER during recovery was correlated with dose. For example, while 1 min of exposure was sufficient for doses of BFA between 1 and 5  $\mu\text{g}/\text{ml}$ , 5 min of exposure was necessary at 0.3  $\mu\text{g}/\text{ml}$  BFA (data not shown).

ATP was required during these short BFA treatments in order to commit Golgi membrane to move into the ER. When

cells were pretreated with ATP-depleting media, containing 50 mM deoxyglucose and 0.05% sodium azide, for 5 min before addition of BFA for 1 or 2 min, and then washed and incubated in drug-free complete media, no movement of Golgi membrane into the ER was observed (data not shown).

### Low Temperature and Microtubule Disruption Inhibit the BFA-induced Redistribution of Man II But Not the Dissociation of the 110-kD Protein

The BFA-induced transport of Golgi proteins into the ER can be inhibited by low temperature and agents that disrupt microtubules (Lippincott-Schwartz et al., 1990). To further distinguish the dissociation of the 110-kD protein from subsequent BFA-induced morphologic events we tested the effect of reduced temperatures and microtubule inhibitors on the BFA-induced dissociation of the 110-kD protein (Fig. 6). After 1 h at 16°C in the absence of BFA the 110-kD protein staining remained associated with the Golgi apparatus and appeared identical to cells at 37°C. But after adding BFA to these cells at 16°C the 110-kD protein was released from the Man II-stained Golgi complex which remained intact except for a few tubular extension structures similar to what was observed after 2 min BFA at 37°C. Thus the reduced temperature did not block the BFA-induced release of the 110-kD protein but did inhibit Man II redistribution into the ER. Removal of BFA from these cells at 16°C, followed by raising the temperature to 37°C, demonstrated that the Golgi proteins had been committed to follow the recycling pathway back to the ER and then out again to reform the Golgi apparatus during postdrug incubation (data not shown) as was observed in Fig. 5.

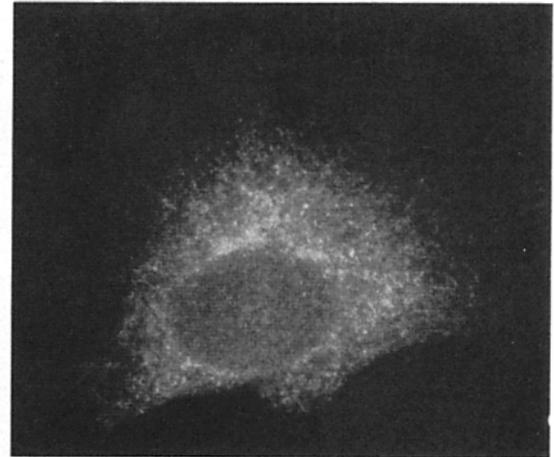
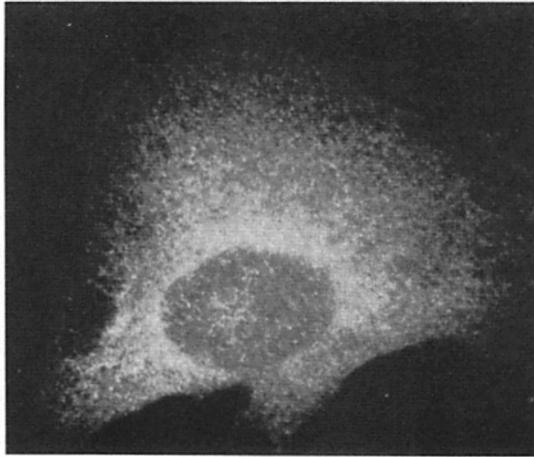
Two microtubule-disrupting agents, nocodazole and griseofulvin, were used to study the effect of microtubules on the association of the 110-kD protein with the Golgi apparatus. Nocodazole treatment resulted in the expected fragmentation of the Golgi complex but the maintenance of the association of the 110-kD protein with the Golgi fragments, as had been reported by Allan and Kreis (1986). The addition of BFA to nocodazole-treated cells failed to result in the redistribution to the ER of Man II which remained in the fragmented Golgi structures. However, nocodazole did not inhibit the release of the 110-kD protein induced by BFA as it was found diffusely throughout the cytoplasm (Fig. 6). The use of griseofulvin in place of nocodazole resulted in the same observations. Recovery of such BFA-treated cells into nocodazole-containing media resulted in the rapid (5–10 min) reassociation of the 110-kD protein with the Golgi fragments; incubation in media alone, which would allow for the reformation of microtubules, resulted in Golgi reformation, but it was difficult to determine whether the Golgi apparatus

**Figure 3.** Effect of BFA concentration on the 110-kD protein dissociation and movement of Man II into the ER. NRK cells were treated with BFA at 0.1, 0.3, or 1.0  $\mu\text{g}/\text{ml}$  (0.36, 1.07, and 3.57  $\mu\text{M}$ , respectively) for either 5 or 10 min before fixation, permeabilization, and staining for immunofluorescence. At the low dose of 0.1  $\mu\text{g}/\text{ml}$ , the labeling of the 110-kD protein colocalized with the Man II-stained Golgi structure after 5 min, while after 10 min at this dose there was a partial release of the 110-kD protein from the Golgi complex, and tubular extensions from the Golgi complex carrying Golgi proteins into the ER were now evident (arrow). At 1.0  $\mu\text{g}/\text{ml}$  the time course of the 110-kD protein dissociation and BFA-induced Man II redistribution paralleled that seen in Fig. 1 with 5  $\mu\text{g}/\text{ml}$ . By 5 min of incubation all of the 110-kD protein was widely dispersed throughout the cytoplasm while most of the Man II labeling was distributed in an ER pattern, although some remnant Golgi structure and tubular processes remained. By 10 min at this dose, however, all of the Man II labeling was distributed in a punctate/reticular pattern consistent with an ER location while the 110-kD protein remained widely scattered to the far edges of the cell. Bar, 10  $\mu\text{m}$ .

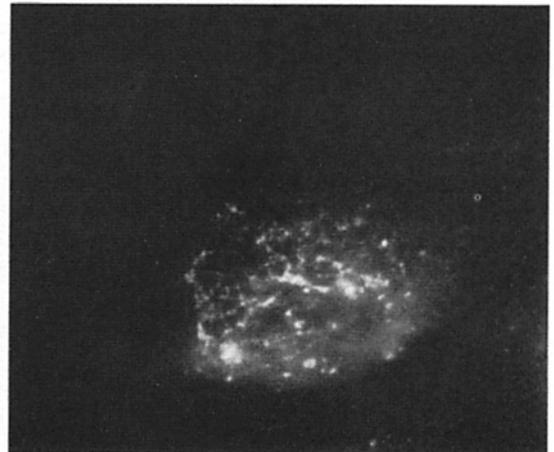
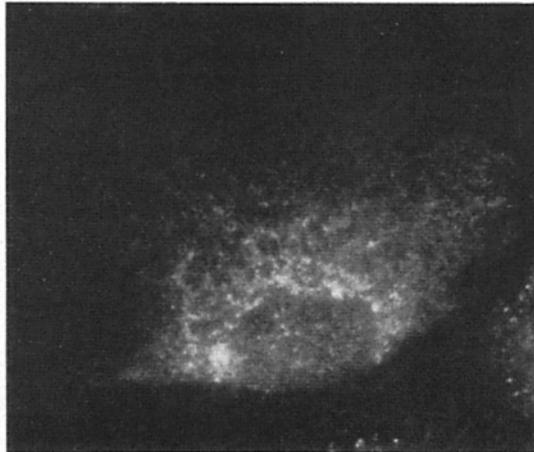
## 110-kD Protein

## Man II

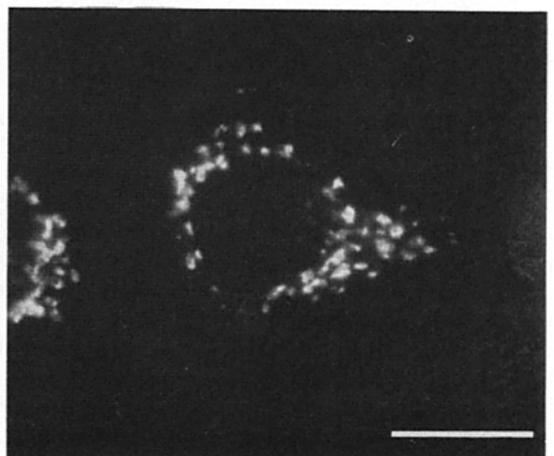
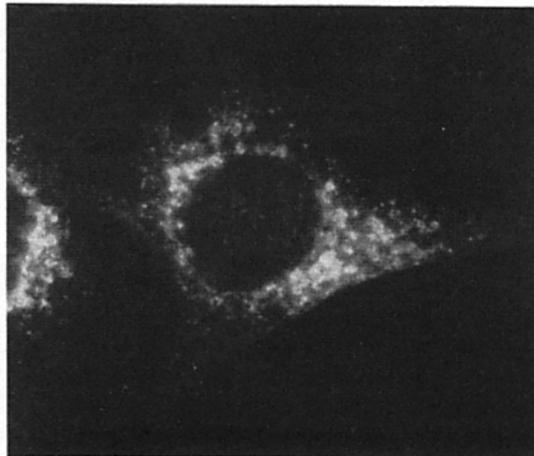
0 Min



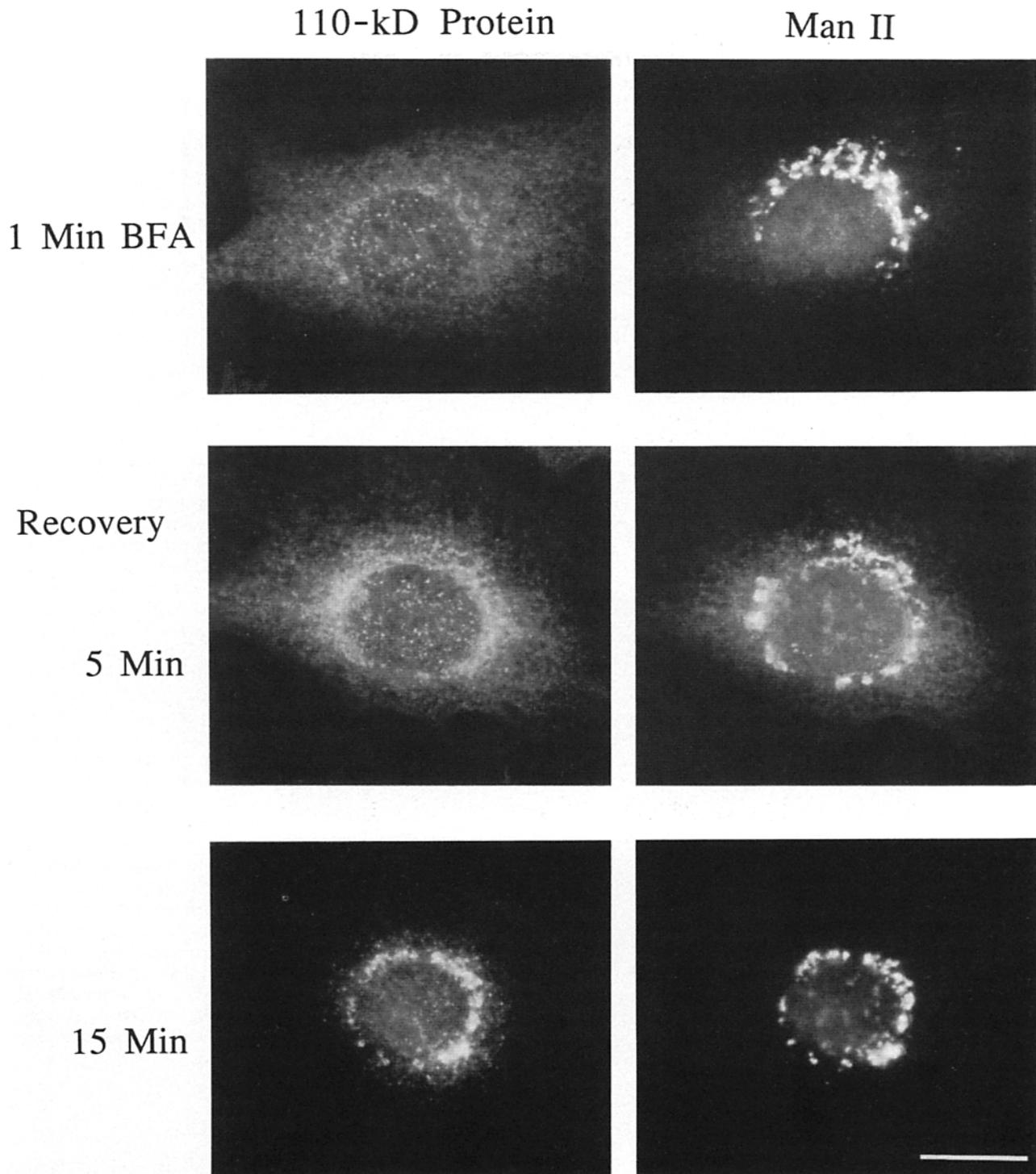
10 Min



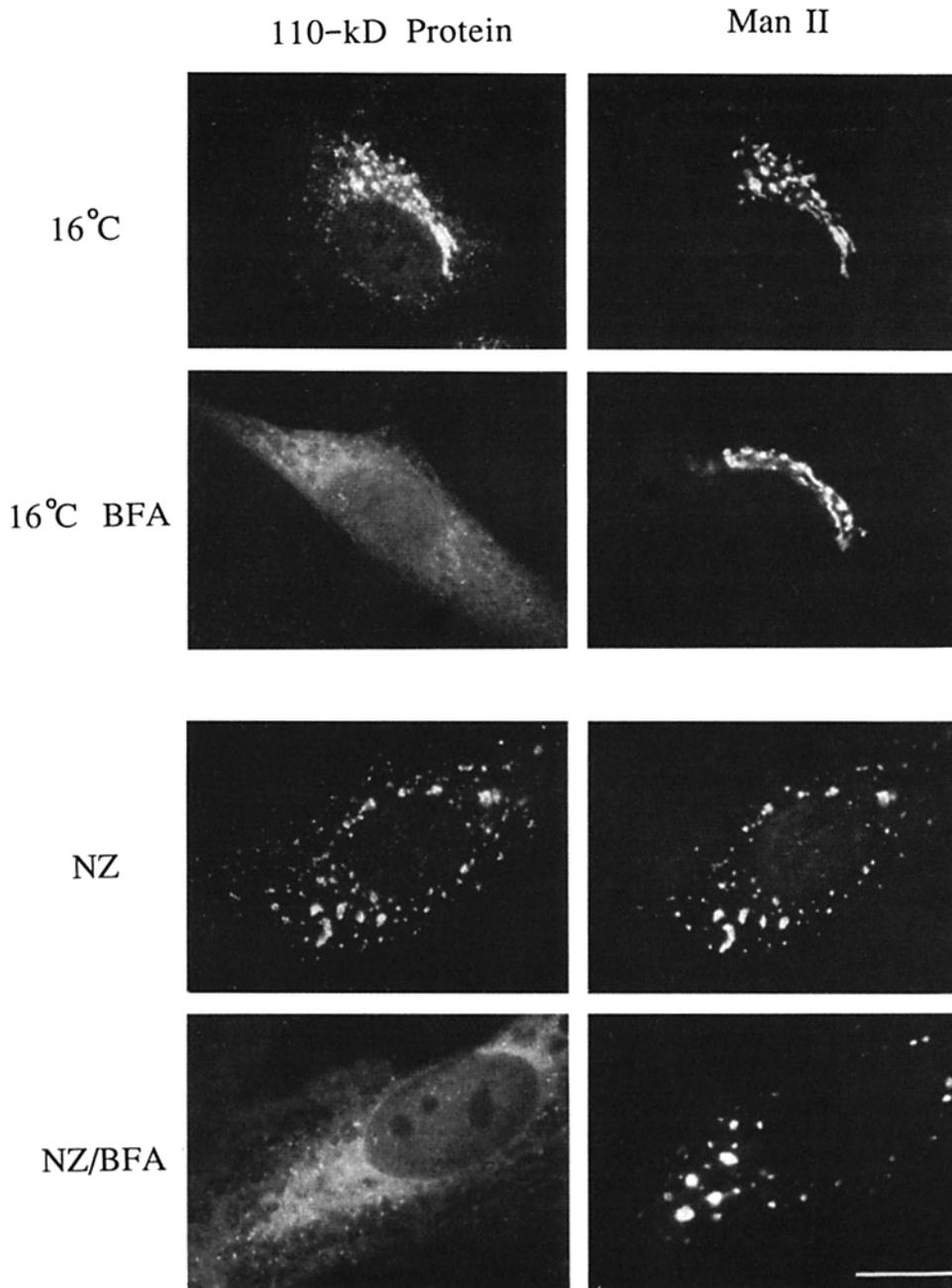
30 Min



**Figure 4.** Reassociation of the 110-kD protein with the Golgi membrane during recovery after BFA. NRK cells were first treated with 5  $\mu\text{g}/\text{ml}$  (18  $\mu\text{M}$ ) BFA for 30 min at 37°C, then rinsed at 37°C with three 1-ml changes of media before incubation in 1 ml of fresh media at 37°C for 0, 10, or 30 min. By 10 min of recovery the 110-kD protein colocalized with nearly all of the Man II-labeled enlarged structure and associated interconnecting strands located outside of the ER. By 30 min of recovery Man II localized to condensing Golgi elements surrounding the nucleus that colocalized with the 110-kD protein. Bar, 10  $\mu\text{m}$ .



**Figure 5.** Golgi proteins are committed to enter the ER after 1 min of BFA treatment. NRK cells were treated with BFA (5  $\mu\text{g}/\text{ml}$ ) for 1 min at 37°C, rinsed with three 1-ml changes of media and then placed in 1 ml of fresh media at 37°C for the indicated recovery times. Cells were then fixed, permeabilized, and prepared for indirect immunofluorescence with antibodies to Man II and the 110-kD protein. After 1 min of BFA treatment, the 110-kD protein had dissociated from the Golgi apparatus and was distributed throughout the cytoplasm, while no obvious change in Golgi morphology, labeled by Man II, was evident. But after removal of BFA and 5 min of recovery in the absence of drug, Man II was now found distributed both in an ER pattern and in residual Golgi-like structures. The 110-kD protein shifted from a widely dispersed distribution after the 1-min BFA treatment to a pattern that appeared to colocalize with the Man II staining. By 15 min of recovery the Man II staining appeared again within a Golgi structure that colocalized with the 110-kD protein. Bar, 10  $\mu\text{m}$ .



**Figure 6.** Effect of 16°C and nocodazole treatment on the BFA-induced dissociation of the 110-kD protein. NRK cells were either treated at 16°C in the absence or presence of BFA (5  $\mu\text{g}/\text{ml}$ ) for 30 min (first and second rows) or at 37°C were preincubated with nocodazole (NZ) 20  $\mu\text{g}/\text{ml}$  for 2 h and then incubated with nocodazole, in the absence or presence of BFA (5  $\mu\text{g}/\text{ml}$ ) for 30 min (third and fourth rows) before preparation for immunofluorescence. At 16°C Man II-labeled Golgi structures and its colocalization with the 110-kD protein staining pattern was similar to that observed in 37°C control cells. The addition of BFA to these cells at 16°C, however, resulted in the release of the 110-kD protein from the Golgi complex, while Man II was inhibited from moving beyond short tubulovesicular structures into the ER. Nocodazole treatment (third and fourth rows) resulted in the fragmentation and dispersion of Man II-labeled Golgi elements but the 110-kD protein remained colocalized with each Golgi fragment. Subsequent treatment of these cells with BFA resulted in the release of the 110-kD protein but Man II staining remained as fragmented Golgi structures. Bar, 10  $\mu\text{m}$ .

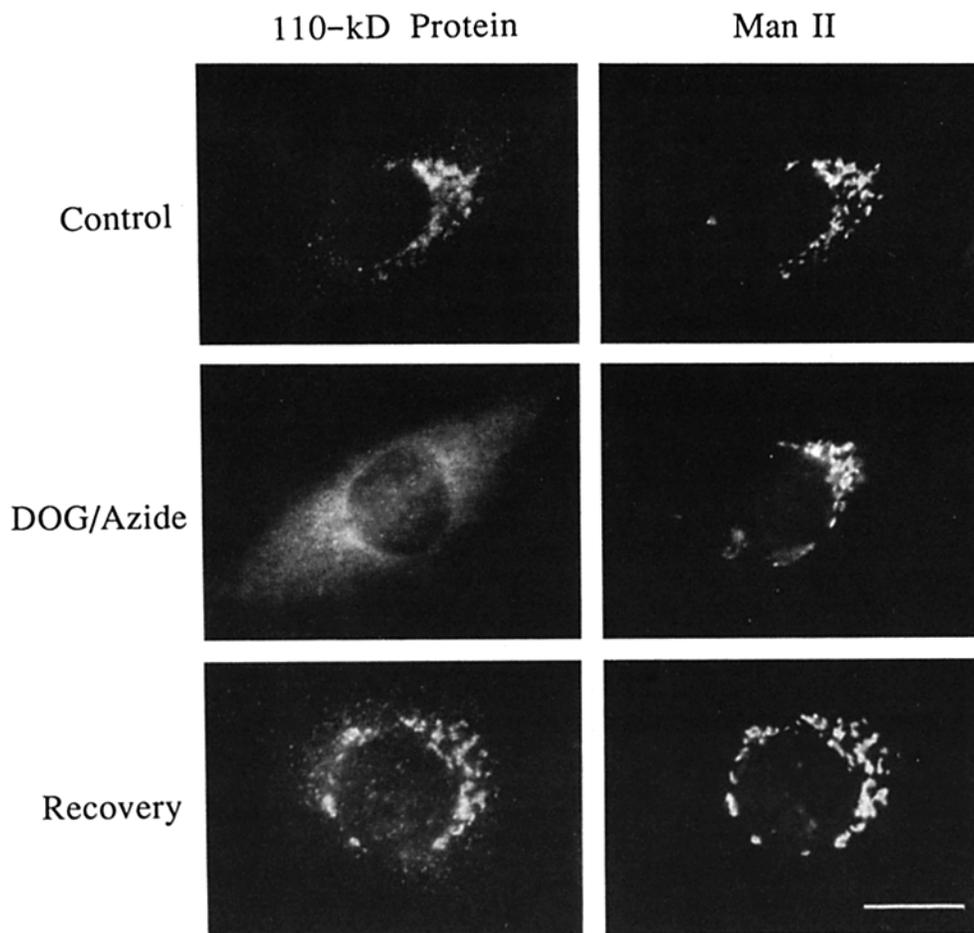
reformed directly from the fragments themselves or, in part, required the Golgi apparatus to enter the ER before reformation.

#### **ATP Depletion Disrupts the 110-kD Protein-Golgi Apparatus Association**

Other agents known to inhibit BFA-induced redistribution of Golgi apparatus into the ER are metabolic poisons (Lippincott-Schwartz et al., 1989). In our attempt to determine if these agents could inhibit the dissociation of the 110-kD protein from the Golgi apparatus in BFA-treated cells, we found that ATP depletion alone caused the release of the 110-kD protein. Within 5 min after treating cells with 2-deoxy-

glucose and sodium azide to lower cellular ATP levels, the 110-kD protein was released and found diffusely distributed throughout the cytoplasm while the Golgi apparatus structure remained intact (Fig. 7). Removal of 2-deoxyglucose and sodium azide resulted in the reassociation of the 110-kD protein with the Golgi apparatus. At no time during ATP depletion or subsequent recovery was there any evidence of Golgi proteins redistributing to the ER.

This effect of ATP depletion further distinguishes the 110-kD protein from the other known Golgi peripheral membrane proteins shown in Fig. 2. While the association of the 110-kD protein with the Golgi apparatus was dependent upon the presence of ATP, the Golgi staining pattern produced by the antibodies directed against either the 58-kD



**Figure 7.** ATP is required to maintain the association of the 110-kD protein with the Golgi apparatus. Cells were untreated (*Control*), incubated for 5 min at 37°C in the presence of 50 mM 2-deoxyglucose, and 0.05% sodium azide (*DOG/Azide*) to deplete cellular ATP, or incubated for 5 min in *DOG/Azide* followed by 5 min recovery in fresh media not containing *DOG/Azide* (*Recovery*), and then prepared for indirect immunofluorescence. 5 min of incubation in ATP-depleting media caused the complete dissociation of the 110-kD protein from the Golgi apparatus (*DOG/Azide*), while Golgi positioning within the cell remained unchanged. Once the ATP-depleting media was removed (*Recovery*), the 110-kD protein was once again observed colocalized with Man II staining in an intact Golgi structure with no evidence of any Man II within the ER. Bar, 10  $\mu$ m.

protein or the 54- and 86-kD proteins was not affected by incubation in 2-deoxyglucose and sodium azide (data not shown).

### Discussion

The drug BFA has remarkable effects on the structure and identity of the Golgi apparatus. Current data suggest that the effects of this drug are relatively specific to this organelle. Thus, BFA does not alter the morphology of other organelles and does not inhibit a variety of organellar processes including endocytosis (Misumi et al., 1986) and delivery of endocytosed material to lysosomes (Misumi et al., 1986), cell-mediated cytotoxicity (Nuchtern et al., 1989), and biosynthesis of proteins (Misumi et al., 1986; Ulmer and Palade, 1989). The efficacy of BFA at concentrations of  $10^{-7}$ – $10^{-8}$  M suggests it is binding to a high affinity target site (our unpublished observations). Numerous sites of action of the drug could be proposed to explain the phenotypic changes to the Golgi apparatus. However, we favor the Golgi apparatus itself as a likely target of action.

In the absence of any direct evidence as to its molecular target, we asked whether any structural alterations at the Golgi apparatus could be detected that were rapid and preceded the morphological changes previously observed. The interaction of the Golgi apparatus with organelle-specific structural proteins provided an attractive starting point. Two known candidates are the 110- and 58-kD

microtubule-binding proteins described by Allan and Kreis (1986) and Bloom and Brashear (1989), respectively. However, only the 110-kD protein proved to be affected by BFA. Within 30 s of the addition of the drug to the medium, this protein loses its association with the Golgi apparatus and is found diffusely distributed throughout the cells. This effect is rapidly reversed upon removal of BFA. Several lines of evidence point to this as a proximal event to the disassembly of the Golgi complex. First, it occurs before any morphological changes in Golgi structure can be appreciated by immunofluorescence. Second, manipulations that inhibit the disassembly of the Golgi apparatus such as lowered temperature, nocodazole, and griseofulvin all appear to act either subsequent to or independent of the ability of BFA to induce the 110-kD protein dissociation.

Brief incubation with BFA (<1 min) results in the dissociation of at least a significant portion of the 110-kD protein, as defined by immunofluorescence. Removal of BFA within this time results in the reassociation of the 110-kD protein with the Golgi apparatus without movement of Golgi proteins into the ER. In contrast, after longer times of treatment with BFA, upon removal of the drug, movement of at least some Golgi proteins into the ER is observed before the Golgi complex appears normal, as assessed by immunofluorescence. The 110-kD protein reassociates with Golgi membrane almost immediately upon removal of BFA. This reassociation is not limited to Golgi-like structures but will occur wherever Golgi proteins are distributed, including the ER.

This suggests that the 110-kD protein either directly or indirectly binds to Golgi membrane components and that BFA prevents this association. Consistent with this, the 110-kD protein does not appear to form a scaffold around the microtubule-organizing center upon which the Golgi complex is rebuilt, but rather appears to bind to Golgi proteins themselves first.

Several lines of evidence suggest that the interaction of the 110-kD protein with the Golgi apparatus may be regulated by factors other than BFA. First, lowering cellular ATP levels results in the release of the protein. This sensitivity to ATP suggests that the 110-kD protein interaction with the Golgi apparatus requires an ATP-binding event and/or phosphorylation. Second, we have observed diffuse, non-Golgi staining of the 110-kD protein in normal cells that are undergoing mitosis (data not shown) suggesting that the dissociation of this protein may participate in the cell cycle-dependent changes in Golgi structure. Despite the apparently similar effects of BFA and ATP depletion on the localization of the 110-kD protein, ATP depletion does not result in redistribution of Golgi membrane into the ER. Furthermore, ATP depletion itself blocks the ability of BFA to induce the redistribution of Golgi proteins into the ER. Therefore, it is most likely that ATP is required by the cell to undergo the membrane transport events subsequent to the 110-kD dissociation.

It will be important to determine the biochemical basis of how BFA interferes with the interaction of the 110 kD-protein with the Golgi apparatus. If we accept the possibility that this interaction is a dynamic one, then it is reasonable to ask whether BFA is indeed causing the release of the 110-kD protein or inhibiting its reassociation. If the latter were the case then the time course of the effect of BFA would indicate a rapid cycle of association/dissociation of the 110-kD protein with the Golgi apparatus. The association/dissociation of protein components between the Golgi apparatus and the cytosol has been proposed as part of the vesicular transport cycle by Orci et al. (1989). BFA may affect the association of the 110-kD protein with the Golgi apparatus by directly interacting with the protein or its binding site on the Golgi apparatus. Alternatively, BFA may alter the biochemistry of the 110-kD protein or proteins with which it interacts; e.g., by altering phosphorylation patterns. Whatever the details, these studies suggest that in BFA-treated cells specific biochemical changes at the level of the Golgi apparatus result in abrogation of anterograde transport and the disassembly of the Golgi apparatus.

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