# Brefeldin A Redistributes Resident and Itinerant Golgi Proteins to the Endoplasmic Reticulum

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*Abstract.* Brefeldin A (BFA) has been reported to block protein transport from the ER and cause disassembly of the Golgi complex. We have examined the effects of BFA on the transport and processing of the vesicular stomatitis virus G protein, a model integral membrane protein. Delivery of G protein to the cell surface was reversibly blocked by 6 $\mu$g/ml BFA. Pulse-label experiments revealed that in the presence of BFA, G protein became completely resistant to endoglycosidase H digestion. Addition of sialic acid, a *trans*-Golgi event, was not observed. Despite processing by *cis*- and medial Golgi enzymes, G protein was localized by indirect immunofluorescence to a reticular distribution characteristic of the ER. By preventing transport of G protein from the ER with the metabolic inhibitor carbonyl cyanide m-chlorophenylhydrazone or by use of the temperature-sensitive mutant ts045, which is restricted to the ER at 40°C, we showed that processing of G protein occurred in the ER and was not due to retention of newly synthesized Golgi enzymes. Rather, redistribution of preexisting *cis* and medial Golgi enzymes to the ER occurred as soon as 2.5 min after addition of BFA, and was complete by 10–15 min. Delivery of Golgi enzymes to the ER was energy dependent and occurred only at temperatures ⩾20°C. BFA also induced retrograde transport of G protein from the medial Golgi to the ER. Golgi enzymes were completely recovered from the ER 10 min after removal of BFA. These findings demonstrate that BFA induces retrograde transport of both resident and itinerant Golgi proteins to the ER in a fully reversible manner.

PROTEINS exported to the cell surface are translocated into the ER and then sequentially transported through the *cis*-, medial, and *trans*-compartments of the Golgi complex, ultimately reaching the plasma membrane (Palade, 1975; Pfeffer and Rothman, 1987). Each compartment of the exocytic pathway has a unique complement of resident proteins, some of which catalyze specific posttranslational modifications. A central problem in cell biology is how the various subcompartments of the exocytic pathway are able to distinguish and sort resident from itinerant proteins and thereby maintain their characteristic composition.

There has been some progress in understanding the sorting of proteins in the ER. For at least some proteins, correct folding and assembly into oligomers is required for transport to the Golgi complex (for review see Rose and Doms, 1988). Improperly folded molecules retained in the ER are eventually degraded (Lippincott-Schwartz et al., 1988). A second regulatory mechanism must account for the retention of correctly folded, resident ER molecules. A number of soluble ER proteins possess the carboxy-terminal sequence KDEL, which is involved in their retention (Munro and Pelham, 1987). Pelham has proposed that a KDEL receptor in an early Golgi compartment recognizes the KDEL sequence and recycles the protein to the ER (Pelham, 1988; Pelham et al., 1988). The possibility of recycling Golgi contents to the ER is supported by the finding that fully 50% of the phospholipid in the ER is transported to the Golgi every 10 min (Wieland et al., 1987). Since this tremendous efflux of lipid is only partially compensated for by de novo synthesis, lipids must be returned to the ER from more distal compartments in the exocytic pathway, most likely the Golgi or an intermediate compartment between the Golgi and ER. If lipids are recycled in vesicular form, the potential for protein recycling is clear.

To further characterize membrane transport between the ER and Golgi complex, we have examined the effects of brefeldin A (BFA)[1] on the intracellular transport of the vesicular stomatitis virus (VSV) G protein. BFA is a fungal metabolite that has been shown to reversibly inhibit the secretion of albumin and thyroid stimulating hormone and the delivery of the VSV G protein to the plasma membrane (Takatsuki and Tamura, 1985; Misumi et al., 1986; Magner and Papagiannes, 1988; Fujiwara et al., 1988). Morphological studies have shown that BFA causes disassembly of the Golgi apparatus and, after prolonged treatment, dilation of the ER (Fujiwara et al., 1988). Retained proteins remain in high-mannose forms, consistent with a block in transport between the ER and Golgi (Takatsuki and Tamura, 1985; Misumi et al., 1986).

1. *Abbreviations used in this paper*: BFA, brefeldin A; CCCP, carbonyl cyanide m-chlorophenylhydrazone; endo D, endoglycosidase D; endo H, endoglycosidase H; TX100, Triton X-100.

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In this paper we demonstrate that resident and itinerant Golgi proteins are returned to the ER when cells are treated with BFA. We show that G protein synthesized in the presence of BFA is fully processed by *cis*- and medial Golgi carbohydrate-processing enzymes. Processing occurs in the ER and is due to a rapid redistribution of preexisting Golgi enzymes to the ER. Redistribution is temperature and energy dependent, and recovery from the effects of BFA are equally as rapid. Our results suggest that BFA may prove to be an important compound for studying membrane traffic between the ER and Golgi complex, and may reveal an underlying Golgi to ER recycling pathway.

## Materials and Methods

### Materials

The natural BFA we used in this study was kindly provided by Drs. A. Takatsuki (University of Tokyo, Tokyo, Japan) and C. R. Hutchinson (University of Wisconsin, Madison, WI), while synthetic BFA was provided by Dr. E. J. Corey (Harvard University, Cambridge, MA). BFA was kept as a 10 mg/ml stock at −20°C in MeOH. Virus stocks as well as Chinese hamster ovary (CHO) wild-type and 15B cells were provided by A. DeSilva (Yale University, New Haven, CT). Polyclonal antibody to VSV was a gift from J. K. Rose and M. A. Whitt (Yale University), and the monoclonal antibodies I1 and I14 were provided by D. Lyles (Bowman Gray School of Medicine, Wake Forest, IL). Endoglycosidase H (endo H) and endoglycosidase D (endo D) were both from Boehringer-Mannheim Diagnostics, Inc. (Houston, TX) and [$^{35}$S]methionine was purchased from ICN Biomedicals Inc. (Irvine, CA).

### Labeling of Cells

Cells were infected with the Indiana serotype of VSV or with the temperature-sensitive mutant tsO45 as previously described at 37 or 32°C, respectively (Balch et al., 1986). Labeling was preformed either with the cells adherent to the dish or while in suspension. Cells were washed once with PBS and placed in methionine-free medium lacking bicarbonate and with 20 mM Hepes pH 7.4 15 min before the addition of [$^{35}$S]methionine. Labeling was terminated by the addition of 1 mM cold methionine diluted from a 1,000× stock. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to a final concentration of 300 μM in glucose-free medium. The appropriate temperature was maintained by circulating water baths. Cells in suspension were lysed by addition of Triton X-100 (TX100) to a final concentration of 1% while adherent cells were lysed with PBS/1.0% TX100. When needed, anisomycin was added to a final concentration of 26 μg/ml.

### Trimerization Assay

VSV G protein forms noncovalently associated trimers shortly after biosynthesis that can be detected by sucrose density gradient centrifugation at pH 5.8 (Doms et al., 1987). The acid pH serves to stabilize preexisting trimers, rather than to catalyze their formation. When trimer formation was to be monitored cells were lysed at pH 5.8 with MNT buffer (20 mM 2-(*N*-morpholino) ethane sulfonic acid, 100 mM NaCl, 30 mM Tris) containing 1.0% TX100. A 200-μl aliquot was loaded onto a 5–20% (wt/vol) continuous sucrose gradient in MNT buffer, pH 5.8, with 0.1% (wt/vol) TX100. The samples were centrifuged for 16 h at 47,000 rpm in a SW55 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4°C. A total of 16 fractions were collected from the bottom of the tube with a Beckman fraction recovery system, and the bottom 12 fractions were immunoprecipitated with antibodies to G protein and analyzed by SDS-PAGE as described below.

### Immunoprecipitations and Endoglycosidase Digestions

Monoclonal or polyclonal antibodies were added to samples in sufficient quantity to insure immunoprecipitation of ⩾90% of the G protein. Antibodies were incubated with samples for either 60 min at 4°C or 30 min at 37°C. 75 μl of protein A-Sepharose (Pharmacia Inc., Piscataway, NJ) was added, and the sample gently mixed for 15–30 min. The samples were washed once by addition of 1 ml 0.1 M Tris pH 8.0, 0.5 M NaCl pH 8.0 and once by addition of 1 ml dH20. Samples were resuspended in Laemmli sample buffer with β-mercaptoethanol for SDS-PAGE. For endo H digestions, samples were resuspended in 50 μl of 0.1 M Na acetate pH 5.5 with 0.5% SDS and boiled for 5 min. The samples were then divided into two equal sized aliquots and an equivalent volume of 0.1 M Na acetate pH 5.5 without SDS and containing 0.5 mU endo H was added to one of the aliquots. Digestion was for 16 h at 37°C, after which an equal volume of 2× sample buffer containing 2% SDS was added. Endo D digestions were carried out essentially as described by Beckers et al. (1987). Cells were lysed in 10 mM Tris (pH 7.4), 200 mM NaCl, and 1% TX100. An aliquot of the lysate was digested with 2 mU endo D for 16 h at 37°C. The samples were then immunoprecipitated and prepared for SDS-PAGE as usual.

### Immunofluorescence

Immunofluorescence was performed essentially as described Timm et al. (1983). Cells were fixed with 3% paraformaldehyde and permeabilized with Triton X-100 as required. A mixture of two monoclonal antibodies to G protein (I14 and I17) were used. I17 efficiently recognizes misfolded forms of G protein while I14 reacts well with properly folded molecules (Doms et al., 1988). FITC-conjugated goat anti-mouse from Tago Inc. (Burlingame, CA) was used as the second antibody. Identical exposures were taken of all samples using a Zeiss Axiophot Photomicroscope.

## Results

### BFA Inhibits Transport of G Protein to the Cell Surface

BFA has been reported to inhibit protein transport from the ER and cause disassembly of the Golgi complex (Misumi et al., 1986; Fujiwara et al., 1988). To more fully characterize the subcellular location(s) at which BFA exerts its effects, we examined the intracellular transport of the VSV G protein by indirect immunofluorescence. G protein is an integral membrane protein that is exceptionally well characterized. It contains two N-linked oligosaccharides (Rothman and Lodish, 1977; Rose and Gallione, 1981; Machamer and Rose, 1988*a*, *b*) and forms noncovalently associated trimers before exit from the ER (Kreis and Lodish, 1986; Doms et al., 1987). G protein is then transported through the Golgi apparatus where its sugers are modified to complex forms before delivery to the plasma membrane (Kornfeld and Kornfeld, 1985; Farquhar, 1985).

BFA was added at 6 μg/ml to the culture medium of CHO cells 1 h after infection with the Indiana serotype of VSV. 4 h after infection, cells were fixed and prepared for indirect immunofluorescence. Little or no G protein was found on the surface of BFA-treated cells, in marked contrast to untreated controls. Surface expression was not observed even when cells were examined 7 h after infection, suggesting that the transport block was absolute rather than kinetic in nature. When BFA-treated cells were permeabilized with Triton X-100, the nuclear membrane and a reticular network characteristic of the ER were stained (Fig. 1 *A*). Indeed, the distribution of G protein within BFA-treated cells was indistinguishable from cells expressing the G protein of the temperature-sensitive mutant tsO45, which is restricted exclusively to the ER at nonpermissive temperature (for example, Fig. 10 C, Bergmann et al., 1981; Bergmann and Singer, 1983). Additionally, a number of small vesicles containing G protein were easily discerned.

To determine if the effects of BFA were reversible, BFA-treated cells were washed and placed in normal growth medium without BFA. Anisomycin was added 30 min before the removal of BFA to prevent additional protein synthesis,

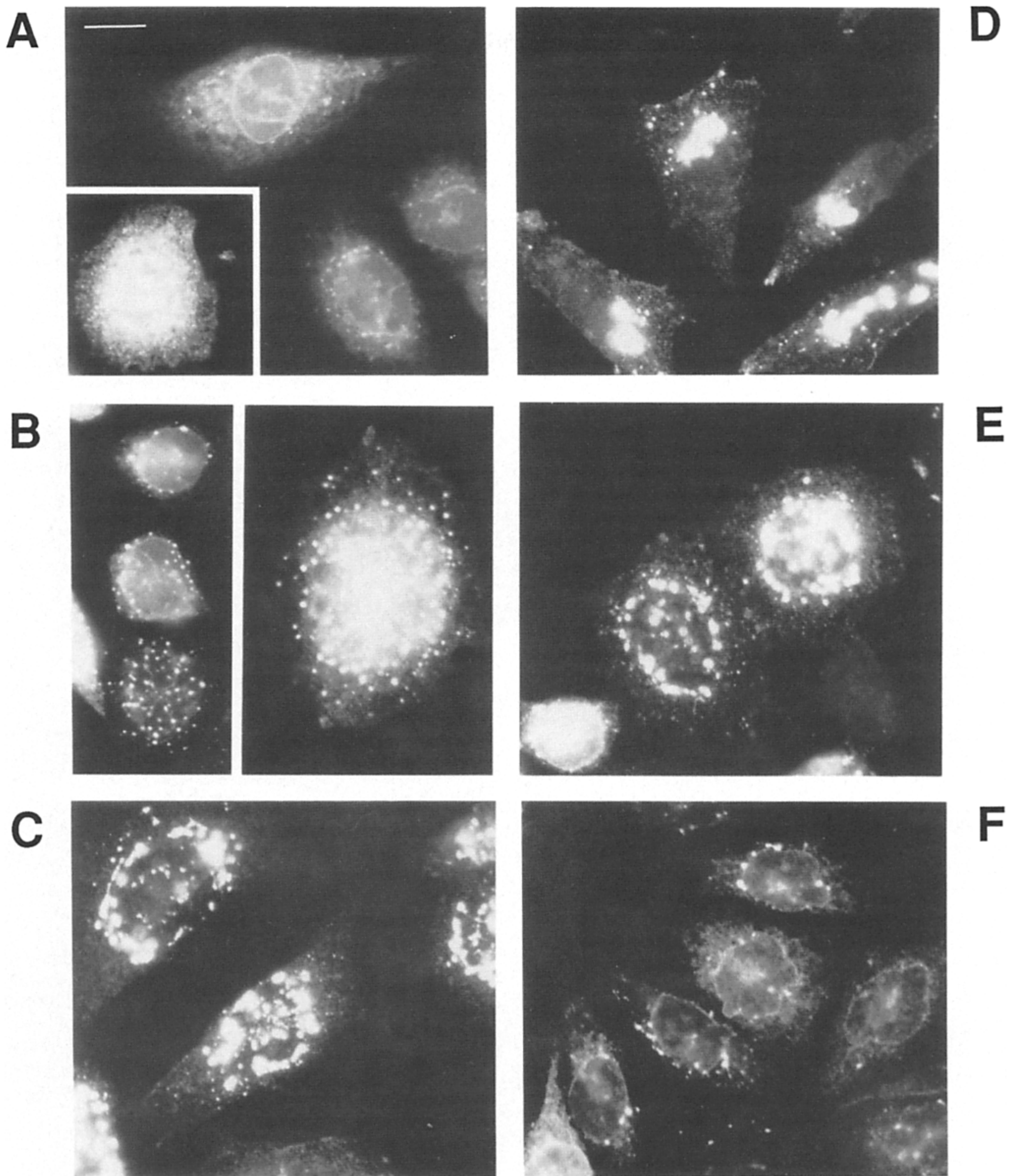


*Figure 1.* The effects of BFA on the intracellular distribution of G protein. Cells were infected with the Indiana serotype of VSV. 1 h after infection, BFA was added to a final concentration of 6 μg/ml. Anisomycin was added for 30 min and the BFA then removed for 0 (*A*), 5 (*B*), 10 (*C*), or 15 min (*D*). BFA was added again to cells for either 5 (*E*) or 10 (*F*) min after a 15-min washout period. The cells were fixed, permeabilized, and prepared for indirect immunofluorescence as described in Materials and Methods. Bar, 15 μm.

enabling us to monitor the distribution of G protein molecules that had accumulated at the BFA block. As shown in Fig. 1 *B*, the distribution of G protein changed from a reticular to a punctate pattern 5 min after removal of BFA, with vacuolar staining evident throughout the cytoplasm. By 10 min the vacuoles had begun to concentrate around the nu-

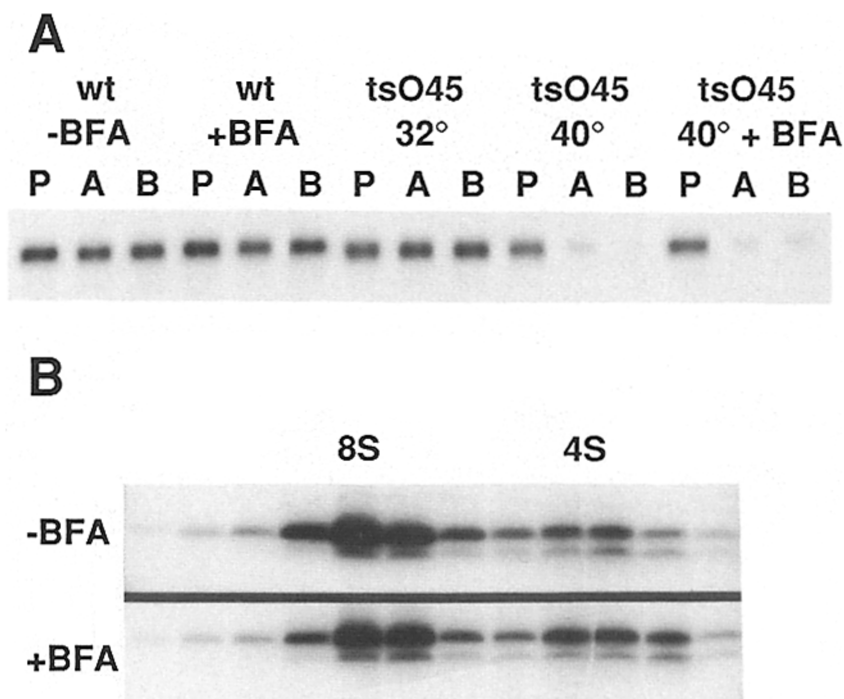


*Figure 2.* BFA does not affect folding or assembly of G protein. (*A*) CHO cells infected with wild-type or ts045 VSV were labeled with [$^{35}$S]methionine for 30 min at 37°(for wild type) or at either 32 or 40°C for ts045. The cells were then chased in the presence of excess cold methionine at the indicated temperature for 30 min, lysed in TX100, and aliquots immunoprecipitated with a polyclonal antibody to VSV (*P*) or with monoclonal antibodies I1 (*A*) or I14 (*B*). The immunoprecipitations were quantitative judging by the failure to precipitate additional G protein after the initial round. The samples were analyzed by SDS-PAGE. BFA was present for 30 min before the pulse-label as well as during the pulse and the chase periods where indicated. (*B*) Cells infected with wild-type VSV were labeled for 10 min, chased for 10 min, and then lysed with 1% TX100 in MNT buffer at pH 5.8. BFA was added to cells 30 min before labeling where indicated. Aliquots were centrifuged on continuous sucrose gradients to separate 8 S trimers from 4 S monomers. The gradients were fractionated and each fraction immunoprecipitated and analyzed by SDS-PAGE. The band migrating immediately beneath G protein is $G_s$, a water-soluble, truncated form of G protein secreted during the course of infection (Little and Huang, 1978).

cleus (Fig. 1 *C*) and 15 min after BFA removal the vacuoles had coalesced to a juxtanuclear location typical of the Golgi complex (Fig. 1 *D*). After an additional 15 min, G protein could be detected on the cell surface. Thus, the BFA block was rapidly overcome upon removal of the drug, in agreement with previous results (Misumi et al., 1986; Takatsuki and Tamura, 1985).

### *BFA Does Not Affect Folding or Assembly of G Protein*

We first determined if the effects of BFA on transport were due to alterations in the folding or assembly of G protein. Newly synthesized G protein monomers fold and acquire disulfide bonds before the formation of noncovalently associated trimers in the ER (Kreis and Lodish, 1986; Doms et al., 1987, 1988). Only properly folded trimers are transported to the cell surface. To monitor folding, we used a pulse-chase radiolabeling protocol followed by detergent extraction and immunoprecipitation with the conformation specific monoclonal antibodies I1 and I14. The epitopes defined by these antibodies are acquired 1–2 min after synthesis (LeFrancois and Lyles, 1982; Doms et al., 1988). G protein synthesized in the presence of BFA was immunologically indistinguishable from protein synthesized in its absence (Fig. 2 *A*). By contrast, G protein from the temperature-sensitive mutant, ts045, which folds abnormally at elevated temperatures (Kreis and Lodish, 1986; Doms et al., 1987, 1988), reacted poorly with the antibodies (Fig. 2 *A*). To monitor trimerization of G protein, detergent extracts of pulse-labeled cells were centrifuged on continuous sucrose gradients designed to differentiate 4 S monomers and 8 S trimers (Doms et al., 1987). BFA did not affect the efficiency or rate of trimer formation (Fig. 2 *B*, for example). These findings indicate that the effects of BFA on the transport of G protein cannot be ascribed to alterations in its folding or assembly.

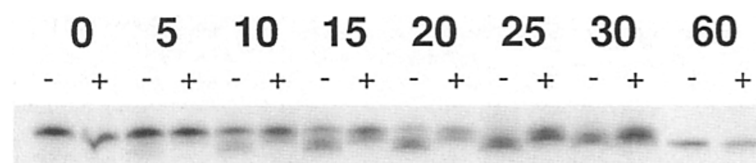


*Figure 3.* Trimming of G protein by *cis*-Golgi mannosidase. CHO15B cells incubated with or without BFA were pulse-labeled for 5 min and then chased for various times. Cells were lysed and the lysates incubated with 2.5 mU endo D for 16 h at 37°C. The samples were then immunoprecipitated with a polyclonal antibody to G protein, and the samples analyzed by SDS-PAGE. In the first lane, all of the G protein is resistant to digestion with endo D, indicating it has not yet been trimmed to the $Man_5GlcNAc_2$ form. In contrast, three bands are seen in the seventh lane, representing G protein molecules with 0, 1, or 2 trimmed chains.

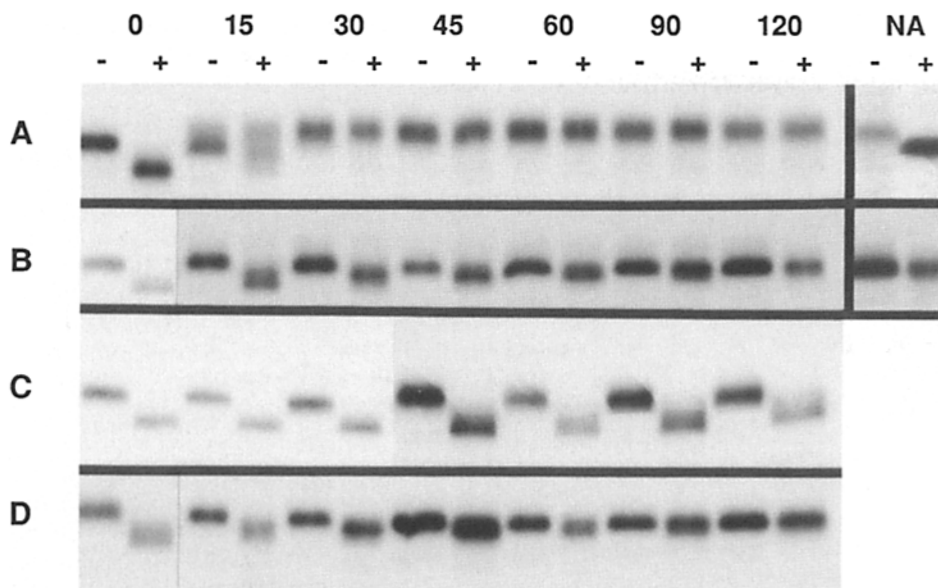


*Figure 4.* G protein becomes endo H resistant in the presence of BFA. Cells were labeled with [$^{35}$S]methionine for 10 min and then chased for the indicated periods of time before lysis with TX100. G protein was immunoprecipitated and incubated with (+) or without (−) endo H at 37°C overnight. (*A*) Cells without BFA and CCCP. An increase in molecular weight due to addition of sialic acid is seen by 15 min, and was complete by 30 min. Neuraminidase digestion confirmed that the decrease in mobility was due to sialic acid addition (*far right*). (*B*) Cells were treated with BFA 30 min before labeling, as well as during the label and chase periods. Neuraminidase digestion confirmed that sialic acid was not added to the G protein. (*C*) CCCP was added to a final concentration of 10 μg/ml immediately after the 10-min labeling period. (*D*) Cells were treated with BFA (5 μg/ml) for 30 min before the 10-min label. CCCP was added immediately after the labeling period, and the BFA was removed 5 min later. The cells were then incubated with CCCP alone for the remainder of the chase period.

### *Carbohydrate Processing by cis-Golgi Enzymes Occurs in the Presence of BFA*

To more precisely determine the site at which transport of G protein to the cell surface was blocked, we monitored processing of G protein's two N-linked oligosaccharides. By using SDS-PAGE and several endoglycosidases, it is possible to determine if N-linked carbohydrate side chains have been modified by enzymes associated with either the *cis*-, medial, or *trans*-Golgi (Kornfeld and Kornfeld, 1985; Farquhar, 1985; Dunphy and Rothman, 1985). Initially, N-linked oligosaccharides are trimmed from the high-mannose forms found in the ER to $Man_5GlcNAc_2$ by alpha-1, 2-mannosidase I in the *cis*-Golgi. Normally, GlcNAc residues are rapidly added to $Man_5GlcNAc_2$ oligosaccharides (Kornfeld and Kornfeld, 1985; Farquhar, 1985). In CHO 15B cells, however, the medial Golgi processing enzyme, *N*-acetylglucosamine transferase, is absent. Consequently, G protein accumulates in the electrophoretically distinct $Man_5GlcNAc_2$ form (Schlesinger et al., 1976). This down-shift in molecular weight can be enhanced by digestion with endo D, which removes only $Man_5GlcNAc_2$ oligosaccharides (Tai et al., 1975; Mizuochi et al., 1984; Beckers et al., 1987). The combination of CHO 15B cells and endo D digestion thus provides a sensitive and specific assay for exposure of G protein to a *cis*-Golgi enzyme (Beckers et al., 1987).

BFA was added to infected CHO 15B cells 30 min before a 5-min pulse-label with [$^{35}$S]methionine, after which cells were chased in the presence of excess unlabeled methionine for various periods of time. Cell extracts were incubated with endo D, and the G protein immunoprecipitated and analyzed by SDS-PAGE. As shown in Fig. 3, approximately half of the

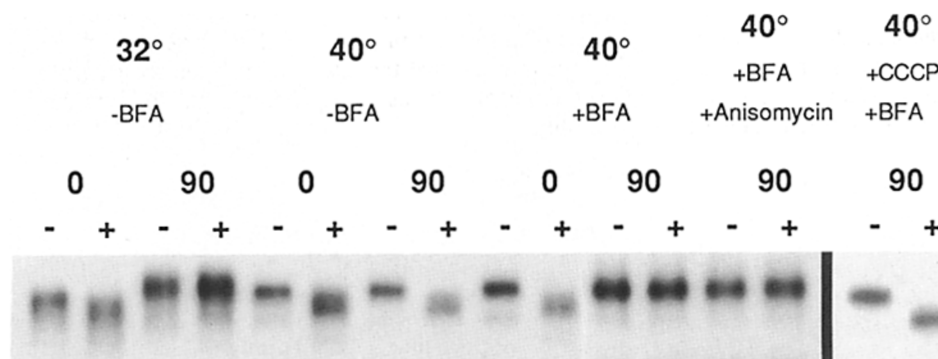


*Figure 5.* tsO45 becomes endo H resistant at nonpermissive temperature. Cells infected with tsO45 were labeled for 10 min at the indicated temperature and then chased for 0 or 90 min. The cells were lysed and the G protein immunoprecipitated and incubated overnight with (+) or without (−) endo H. BFA was added immediately after the pulse-label where indicated. To show that processing at 40°C was not due to retention of newly synthesized Golgi enzymes, anisomycin was added immediately after the labeling period for 60 min to inhibit further protein synthesis. BFA was then added for an additional 90 min, also in the presence of anisomycin, before the cells were lysed. Finally, CCCP was added to cells labeled at 40°C immediately after the labeling period. BFA was added 5 min later and the cells incubated for an additional 85 min at 40°C. The G protein remained endo H sensitive, demonstrating that redistribution was energy dependent.

G protein molecules were trimmed after a 10-min chase in the absence of BFA. Three distinct bands were observed (e.g., Fig. 3, seventh lane) representing molecules with 0, 1, or 2 trimmed N-linked oligosaccharides. In contrast, G protein was trimmed much more slowly in the presence of BFA, with 50% trimming not observed until after 25 min. By 60 min, trimming of both chains was ≥90% complete.

### *Processing by Medial Golgi Enzymes Occurs in the Presence of BFA*

To determine if G protein was also exposed to medial Golgi processing enzymes during the BFA transport block, we monitored endo H resistance in CHO wild-type cells. Infected wild-type CHO cells were treated continuously with BFA for a 30-min prelabeling period, a 10-min pulse label, and for various times of chase. As shown in Fig. 4 *A*, VSV G protein acquired endo H resistance in untreated cells with a $t_{1/2}$ of ~15-20 min. A further increase in molecular weight due to the addition of sialic acid (confirmed by neuraminidase digestion, see Fig. 4 *A*) in the *trans*-Golgi was observed shortly after the acquisition of endo H resistance. In the presence of BFA, G protein became endo H resistant but, as with trimming, the process was slowed (Fig. 4 *B*). All of the G protein became partially resistant to endo H by 45 min of chase (Fig. 4 *B*). This intermediate sensitivity was infrequently observed in the absence of BFA, and is consistent with the asynchrony observed in trimming to the $Man_5GlcNAc_2$ form (Fig. 3). This is likely due to differential processing of the two N-linked chains. By 90 min of chase, G protein was ≥90% endo H resistant. However, addition of sialic acid was not observed, even after 4 h of chase (Fig. 4 *B*). Thus, G protein is exposed to medial but not *trans*-Golgi processing enzymes when its transport is blocked by BFA.

### *Processing Occurs in the ER*

The acquisition of endo H resistance by a protein that by indirect immunofluorescence appeared to be restricted to the ER was surprising. A number of possibilities could account for this observation. First, G protein could be transported to the Golgi, but in the presence of BFA the Golgi apparatus might vesiculate and thereby give rise to a reticular staining pattern. Alternatively, BFA could block the exit of G protein from the ER, and processing in the ER might be due to retention of newly synthesized Golgi enzymes or by redistribution of preexisting enzymes from the Golgi complex to the ER. Finally, G protein might rapidly cycle between the ER and Golgi during BFA treatment.

To determine if processing did in fact occur in the ER, we prevented transport of G protein from this organelle using CCCP. Protein transport is critically dependent upon cellular energy levels (Jamieson and Palade, 1968; Balch et al., 1986; Balch and Keller, 1986; Persson et al., 1988). CCCP rapidly lowers cellular ATP levels, and presumably prevents transport from the ER on this basis (Tartakoff and Vassalli, 1977; Farquhar, 1985). When CCCP was added to infected cells immediately after a 10-min pulse, G protein remained endo H sensitive throughout a 2-h chase (Fig. 4 *C*). Likewise, no processing was observed if BFA was added in addition to CCCP (Fig. 6, 11th and 12th lanes). However, if cells were pretreated with BFA for 30 min before pulse labeling and CCCP added immediately after the labeling period, G protein became completely endo H resistant (Fig. 4 *D*). Removal of BFA 5 min after addition of CCCP still resulted in the acquisition of endo H resistance. Thus, to acquire endo H resistance in the presence of CCCP, it was necessary to preincubate cells with BFA.

To further test the idea that processing occurred in the ER, we used the temperature-sensitive mutant tsO45. At the nonpermissive temperature of 40°C, tsO45 G protein is restricted exclusively to the ER where it is retained in a misfolded, aggregated state (Lafay, 1974; Zilberstein et al., 1980; Bergmann et al., 1981; Lodish and Kong, 1983; Balch et al., 1986; Doms et al., 1987, 1988). The distribution of tsO45 G at 40°C as detected by indirect immunofluorescence was unaffected by the addition of BFA (not shown), and the protein remained misfolded (Fig. 2 *A*) and aggregated. Nevertheless, tsO45 became endo H resistant at 40°C after a 90-min chase when BFA was added at the end of a 10-min pulse-label (Fig. 5). As noted for the wild-type protein, sialic acid was not added. Addition of CCCP immediately after the pulse-label prevented processing unless, as described above, cells were pretreated with BFA before labeling and the energy block (Fig. 5). Thus, a well-characterized ER marker became completely endo H resistant in the presence of BFA.

### *Processing Is due to Redistribution of Golgi Enzymes*

Having shown that processing occurs in the ER in the presence of BFA, we had to differentiate between processing by retention of newly synthesized Golgi enzymes in the ER versus redistribution of preexisting enzymes from the Golgi complex. We considered processing by newly synthesized enzymes to be unlikely given the fact that G protein remained completely endo H sensitive when retained in the ER by CCCP treatment, presumably along with newly synthesized processing enzymes. Further, we expect that little if any processing enzymes are synthesized by these cells since VSV shuts off synthesis of most host cell proteins during infection (Weck et al., 1979). Nevertheless, to test this hypothesis more fully, cells expressing tsO45 G protein were labeled for 10 min at 40°C. Anisomycin was then added for 60 min to prevent additional protein synthesis and to allow newly synthesized Golgi enzymes to be cleared from the ER. Anisomycin reduced incorporation of [$^{35}$S]methionine by 98%. Under these conditions, tsO45 G protein restricted to the ER remained endo H sensitive. However, complete endo H resistance was acquired by 90 min if BFA was added after the 60-min anisomycin pretreatment period (Fig. 5). Thus, G protein restricted to the ER at 40°C was processed in the presence of BFA and in the absence of protein synthesis. We concluded that BFA induces redistribution of Golgi proteins into the ER.

### *Kinetics of Enzyme Redistribution*

Having shown that BFA induces redistribution of enzymes from the Golgi apparatus to the ER and that this process could be completely inhibited by CCCP, we were able to determine the rate at which redistribution occurred. Cells were treated with BFA for different periods of time both before and during a 10-min pulse-label. CCCP was added immediately after the labeling period to prevent further redistribution and the cells incubated for an additional 90 min before extrac-

tion, immunoprecipitation, and endo H digestion. After 15 min of BFA treatment, ER-restricted G protein became completely resistant to endo H after a 90-min chase, and longer treatment times did not affect the rate of this process (Fig. 6). Intermediate sensitivity was observed at other times of BFA treatment. For example, detectable amounts of partially endo H-sensitive G protein were first observed when BFA was added at the beginning of the 10-min pulse-label. Only 60% of the G protein became resistant to endo H when BFA was added midway (5 min) through the pulse-label, and ~20% became resistant if BFA was present only during the final 2.5 min of the pulse-label. Thus, redistribution of Golgi enzymes could be detected as early as 2.5 min after BFA addition.

The intermediate sensitivity of G protein to endo H observed after 2.5–10.0-min treatments with BFA followed by a 90-min chase in the presence of CCCP suggested that enzyme redistribution was incomplete when BFA was present for ≤10 min. If so, then a rate-limiting step in endo H resistance might be related to the concentration of Golgi enzymes redistributed to the ER. We therefore determined the kinetics with which G protein became resistant to endo H after different BFA treatment periods. As shown in Fig. 6 (lanes 1–4) and Fig. 4 *B*, G protein became >90% resistant to endo H when cells were treated with BFA for ≥15 min. We found that G protein became >90% resistant to endo H after 120 and 180 min when cells were treated with BFA for 10 and 5 min, respectively. G protein became only partially resistant to endo H when cells were treated with BFA for 2.5 min regardless of chase time. Complete processing to the endo H–resistant form might have been prevented due to the cytopathic effects associated with long term incubation with CCCP. From these results we concluded that redistribution to the ER begins as early as 2.5 min after addition of BFA, and was completed between 10 and 15 min.

### *Temperature Dependence of Redistribution*

Transport between the ER and Golgi is blocked at 15°C (Saraste and Kuismanen, 1984; Balch et al., 1986; Balch and Keller, 1986; Tartakoff, 1986) while transport from the *trans*-Golgi to the plasma membrane can be blocked at 20°C (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Fuller et al., 1985; Griffiths et al., 1985). It was therefore of interest to examine the temperature dependence of BFA-induced redistribution of Golgi enzymes to the ER. Cells were labeled with [$^{35}$S]methionine for 10 min, after which they were chased at 37, 20, 15, or 0°C in the presence of BFA for 90 or 180 min. As shown in Fig. 7, G protein became endo H resistant at 37 and 20°C, but not at 15 or 0°C, suggesting that redistribution occurred only at the higher temperatures. Alternatively, redistribution at 15°C might have remained undetected if processing itself was directly inhibited. To rule at this possibility, cells were pretreated with BFA for 30 min at 37°C, labeled for 10 min at 37°C, and then chased at 15°C. Under these conditions, when redistribution was allowed to occur 37°C but processing had to occur at 15°C, the G protein became endo H resistant after a 180-min chase. Thus, had redistribution occurred at 15°C, G protein would have been processed. Taken together, these experiments show that redistribution of Golgi enzymes only occurred at temperatures >15°C.

### *Kinetics of Enzyme Recovery After Removal of BFA*

As shown in Fig. 1, the BFA-induced transport block of G protein was rapidly overcome upon removal of BFA. We next determined the kinetics with which Golgi enzymes were recovered from the ER and returned to the Golgi apparatus once BFA was removed. To monitor enzyme recovery, cells were treated with BFA for 30 min to induce complete redistribution of enzymes to the ER. BFA was then removed for various periods of time before a 5-min label. Further membrane transport was then inhibited by addition of CCCP. Thus, by determining the period of time cells had to remain in BFA-free medium before G protein restricted to the ER by CCCP remained endo H sensitive, we were able to monitor the rate at which enzymes were recovered from the ER. As seen in Fig. 8, the kinetics of recovery were similar to the kinetics of redistribution. A BrA-free period of 10 min was sufficiently long to maintain G protein in an endo H-sensitive state. Shorter recovery periods gave intermediate results. Thus, the kinetics of enzyme recovery from the ER measured by this assay were consistent with the recovery of G protein previously observed by immunofluorescence. Recovery of enzymes from the ER was also energy dependent. If CCCP was added to cells and BFA removed immediately after a pulse-label, G protein became endo H resistant, sug-

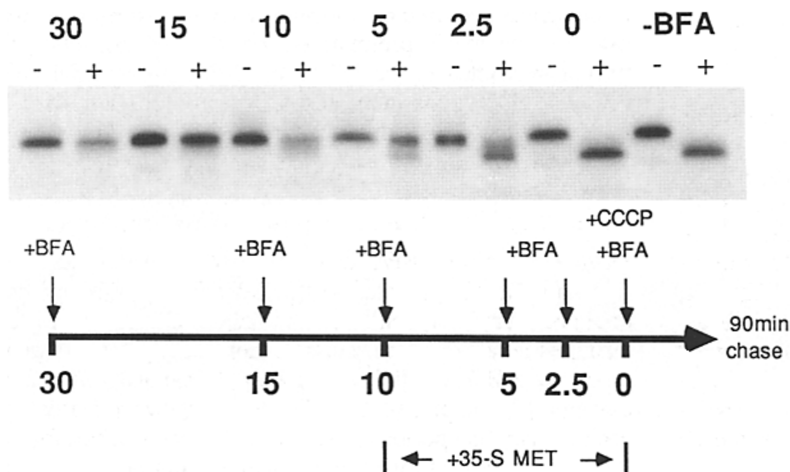


*Figure 6.* Kinetics of enzyme redistribution. BFA was added to cells for the indicated periods of time after which CCCP was added to prevent further redistribution. The cells were incubated for an additional 90 min before lysis, immunoprecipitation, and endo H digestion (+). The labeling period was for 10 min. Thus, for the 2.5-min treatment period, BFA was added for the final 2.5 min of the labeling period before CCCP addition. BFA was removed from cells 5 min after the addition of CCCP, and the cells incubated with CCCP alone for the remaining 85 min. However, for the 0-min time-point, the BFA and CCCP were added immediately after the labeling period and the BFA was not removed, showing that CCCP blocked BFA-induced redistribution of Golgi enzymes.

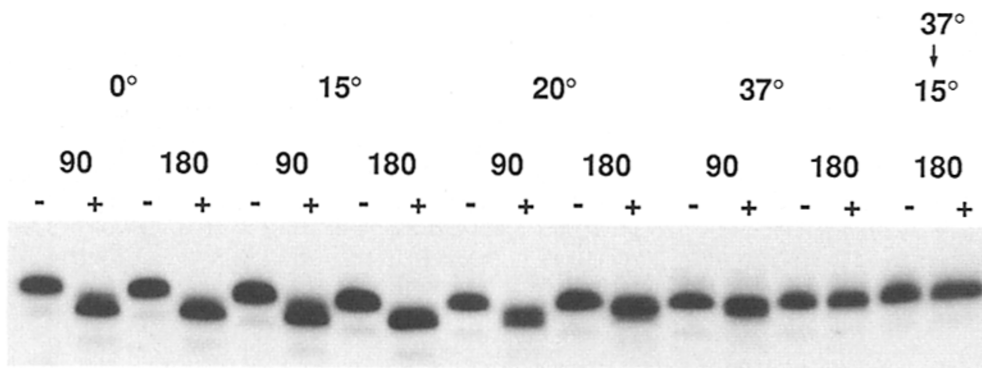


*Figure 7.* Temperature dependence of enzyme redistribution. Cells were labeled for 10 min at 37°C and then chased in the presence of BFA at 37, 20, 15, or 0°C as indicated for 90 or 180 min. The cells were lysed, the G protein immunoprecipitated, and incubated overnight with (+) or without (−) endo H. To show that processing itself was not inhibited at 15°C, cells were treated with BFA for 30 min and labeled for 10 min at 37°C, and then incubated at 15°C for 180 min. G protein was endo H sensitive immediately after the labeling period (not shown) but became fully resistant to endo H after 3 h at 15°C (*far right lanes*).

gesting that enzyme recovery was prevented or at least greatly inhibited (Fig. 4 *D*). Finally, we found that enzyme recovery occurred at 20°C (not shown).

### *Kinetics of G Protein Transport after Removal of BFA*

Since G became endo H resistant in the ER in the presence of BFA, we had to rely on a different processing step to monitor G protein transport to the Golgi apparatus. Addition of sialic acid to G protein, a *trans*-Golgi event (Kornfeld and Kornfeld, 1985; Farquhar, 1985), is easily detected by SDS-PAGE (see Fig. 4 *A*) and does not occur in the presence of BFA (see Fig. 4 *B*). Cells were continuously treated with BFA starting with a 30-min pretreatment before a 10-min pulse-label and 30-min chase. Cells were then placed in BFA-free medium for different periods of time. As shown in Fig. 9, G protein became completely endo H resistant 30 min after BFA removal, and addition of sialic acid was largely complete by 45 min. Since G protein acquires a juxtanuclear position by 15 min after BFA removal (Fig. 1 *D*), it was somewhat surprising that an additional 30 min was required before sialic acid addition was complete. This discrepancy may indicate that additional time is required before functional reassembly of the Golgi complex is complete.

### *Transport of G Protein from the Medial Golgi to the ER*

Having shown that BFA induced the return of resident Golgi proteins to the ER, it was important to determine whether G protein was similarly effected. We first used indirect immunofluorescence. Cells in which G protein was restricted to the ER by BFA treatment were washed and incubated for 15 min at 37°C in the absence of BFA. As previously shown in Fig. 1, *B–D*, G protein rapidly acquired a juxtanuclear distribution after BFA removal. If BFA was added again for 5 min after a 15-min washout period, G protein was found in perinuclear vesicles (Fig. 1 *E*). After an additional 5 min in the presence of BFA, a reticular staining pattern resulted (Fig. 1 *F*). Thus, it appeared that the BFA block could be reimposed even after G protein had reached the Golgi apparatus, and that the G protein was returned to the ER.

For more direct evidence that G protein was recycled to the ER, we used tsO45. As mentioned above, tsO45 G protein is arrested in the ER at 40°C. At the permissive temperature of 32°C, tsO45 G protein is exported from the ER and transported to the cell surface normally (Balch et al., 1986). Importantly, once tsO45 G protein leaves the ER its transport is no longer temperature sensitive (Balch et al., 1986). Thus, tsO45 chased into the *cis*-Golgi at 32°C is transported to the cell surface normally even if the cells are shifted back to 40°C (Balch et al., 1986; Balch and Keller, 1986). We used this useful characteristic to determine if G protein, once delivered to the Golgi complex, is returned to the ER upon BFA treatment.

Cells infected with tsO45 were treated with 10 $\mu$M monensin at 32°C. In the presence of monensin, transport of viral proteins from the medial to *trans*-Golgi is inhibited (Griffiths et al., 1983). 3 h after infection, cells were treated anisomycin to inhibit protein synthesis and incubated for an additional 30 min to allow all of the G protein to accumulate at the monensin block. As expected, the G protein was resistant to endo H but unsiaylated (not shown). When fixed permeabilized cells were examined by indirect immunofluorescence, juxtanuclear staining characteristic of the Golgi ap-

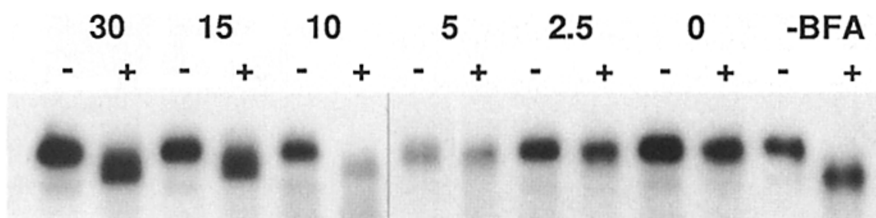


*Figure 8.* Kinetics of enzyme recovery. Cells were treated with BFA for 30 min to induce complete redistribution of Golgi enzymes to the ER. The cells were then incubated without BFA for the indicated periods of time before addition of CCCP to prevent further membrane transport. The cells were labeled for 5 min immediately before the addition of CCCP. For the 2.5-min washout period, cells were treated with BFA for 27.5 min. [$^{35}$S]Methionine was then added along with the BFA for 2.5 min. The medium was then replaced with methionine-free medium containing [$^{35}$S]methionine but without BFA for a final 2.5 min before addition of CCCP.

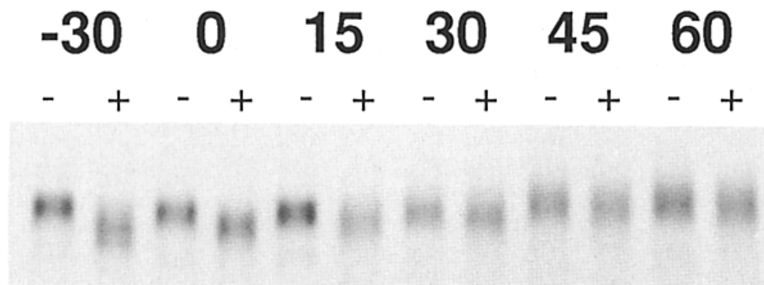


*Figure 9.* Kinetics of G protein transport after BFA removal. Cells were treated with BFA for 30 min before labeling with [$^{35}$S]methionine for 10 min. Cells were lysed at this time ($t$ = −30) or incubated for a further 30 min in the presence of BFA and excess cold methionine. The BFA was then removed for 0, 15, 30, 45, or 60 min before cells were lysed. G protein was immunoprecipitated and digested with endo H as indicated (−/+).

paratus was observed (Fig. 10 *A*). G protein was not detected on the cell surface. BFA was then added to cells for 15 min, after which monensin was removed, and the cells maintained in BFA. By 30 min, a reticular pattern characteristic of ER was observed (Fig. 10 *B*). To determine if this pattern truly represented ER, the cells were shifted to 40°C and the BFA removed for 60 min. Transport of G protein to the Golgi apparatus or cell surface monitored by immunofluorescence or addition of sialic acid was not observed. We also found that the protein lost reactivity with antibody I14, which does not efficiently immunoprecipitate ER-restricted tsO45 at 40°C (see Fig. 2 *A*), and ~40–50% of the protein sedimented in aggregated form on sucrose gradients (not shown). These results indicated that all or at least most of the protein had in fact been returned to the ER (Fig. 10, *C* and *D*) and that it reacquired a conformation characteristic of ER-restricted tsO45.

Identical results were obtained when tsO45 G protein was accumulated at a site between the ER and *cis*-Golgi by prolonged incubation at 15°C (Balch et al., 1986; Balch and Keller, 1986). tsO45 G protein is transported to the surface from this intermediate compartment normally when cells are returned to 40°C (Balch et al., 1986; Balch and Keller, 1986). However, if BFA was added to cells when they were shifted from 15 to 40°C, G protein acquired instead a reticular distribution identical to that shown in Fig. 10, *B* and *C* and was not transported to the cell surface, indicating that it had been returned to the ER (not shown).

## Discussion

Previous studies have examined the effects of BFA on exocytosis in several cell types. BFA reversibly inhibits the secretion of soluble proteins by hepatocytes and pituitary cells (Misumi et al., 1986; Magner and Papagiannes, 1988), as well as delivery of VSV G protein to the cell surface (Takatsuki and Tamura, 1985). Ultrastructural examination of BFA-treated hepatocytes revealed that albumin accumulated in the ER, which became dilated upon prolonged incubation with BFA (Fujiwara et al., 1988). Most interestingly, a morphologically identifiable Golgi complex disappeared within 15 min of adding BFA to these cells. In addition, studies have

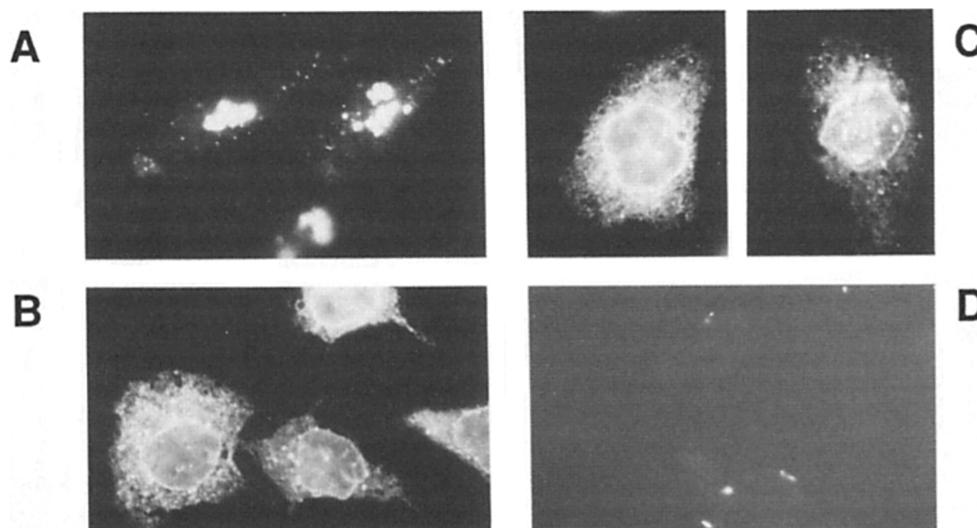


*Figure 10.* Redistribution of G protein from the medial Golgi to the ER. Cells infected with ts045 were incubated in the presence of 10 μM monensin at 32°C for 3 h. Anisomycin was added for 30 min (as well as for the remainder of the experiment) to prevent further synthesis of G protein and to allow existing molecules to accumulate in the medial Golgi. Cells were fixed and prepared for indirect immunofluorescence using monoclonal antibodies to G protein. *A* shows permeabilized cells at the end of the monensin incubation. BFA was then added for a 15-min overlap period with monensin, after which the monensin was removed and the cells incubated with BFA alone for an additional 30 min (*B*). The cells were then shifted to 40°C and the BFA removed for 1 h *C*, + TX100; *D*, − TX100). Bar, 15 μm.

found that glycoproteins synthesized in the presence of BFA retain high-mannose, endo H-sensitive oligosaccharides, which supported the conclusion that BFA acted by inhibiting ER to Golgi transport (Takatsuki and Tamura, 1985; Misumi et al., 1986).

Our results were consistent with the reported effects of BFA but with an important exception: G protein was first trimmed to $Man_5GlcNAc_2$ after which it acquired endo H resistance. Clearly, G protein was exposed to and processed by both *cis*- and medial Golgi enzymes in BFA-treated cells. Addition of sialic acid, a *trans*-Golgi event, was not observed. Our results are in disagreement with those of Takatsuki and Tamura (1985) who found that G protein remained endo H sensitive in the presence of BFA. The reasons for this discrepancy are not clear, but may be related to cell type, concentration of BFA used, or length of treatment. By retaining G protein in the ER via depletion of cellular ATP or by use of tsO45, which is restricted solely to the ER at 40°C, we were able to show that processing occurred in the ER and was not due to coretention of newly synthesized processing enzymes, an unlikely prospect to begin with in VSV-infected cells. Thus, processing had to arise from the actions of preexisting Golgi enzymes. Since ER-restricted G protein was processed, Golgi enzymes must be redistributed to the ER. The relatively slow rate of processing in the presence of BFA might be accounted for by dilution of Golgi enzymes in the larger surface area of the ER (Griffiths et al., 1984), nonsequential prosentation of G protein to processing enzymes, or by a difference in the ER environment relative to the Golgi complex. Our results are in agreement with those of Lippincott-Schwartz et al. (1989), who have recently shown that Mannosidase II was redistributed to the ER in BFA-treated cells by immunoelectron microscopy and that the *trans*-Golgi was largely unaffected.

### *Characteristics of Retrograde Transport and Recovery*

Certainly one of the most impressive features of BFA-induced redistribution of Golgi enzymes to the ER was the speed with which it occurred. Partial processing of ER-restricted G protein was detected as soon as 2.5 min after addition of BFA. The rate at which ER-restricted G protein became endo H resistant correlated with the length of BFA treatment up to 15 min. Longer incubation times did not increase the rate of processing. We interpreted these results to mean that treatment times of <15 min resulted in incomplete redistribution of *cis*- and medial Golgi enzymes to the ER, and that the concentration of these enzymes in the ER was rate limiting for processing. We concluded that redistribution was completed sometime between 10 and 15 min after addition of BFA. Furthermore, it seems likely that the entire *cis*- and medial Golgi are delivered to the ER since: (*a*) tsO45 G protein localized to the medial Golgi by incubation with monensin was quantitatively returned to the ER by BFA, and (*b*) virtually no *cis*- and medial Golgi cisternae can be detected in BFA-treated cells (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989).

Depletion of cellular ATP by CCCP effectively abolished redistribution. Since transport steps are differentially sensitive to ATP depletion, it will be interesting to more carefully titrate the effects of reduced ATP levels on BFA-induced redistribution (Persson et al., 1988). Redistribution was also blocked by incubation at 15°C, a temperature at which proteins in transit from the ER to the Golgi complex have been found to accumulate in an intermediate compartment whose structure and function is poorly characterized (Saraste and Kuismanen, 1984; Tartakoff, 1986). The fact that redistribution was inhibited at 15 but not 20°C may indicate involvement of this compartment in the redistribution process.

Recovery of Golgi proteins from the ER occurred under the same general parameters as those for redistribution. If BFA was removed from cells for as little as 10 min, processing of ER-restricted G protein could no longer be detected. Recovery was energy dependent and occurred at 20°C. tsO45 G protein was retained in the ER at 40°C after removal of BFA, showing that the quality control mechanisms that retain misfolded proteins were still operational. While retrieval of Golgi enzymes from the ER was rapid, transport of G protein from the ER to the *trans*-Golgi occurred more slowly than in untreated controls. Though G protein acquired a juxtanuclear distribution characteristic of the Golgi complex 15 min after removal of BFA, an additional 30 min was required before sialic acid was added. Apparently, it may take additional time for the Golgi apparatus to functionally reassemble.

### *Possible Mechanisms of Action*

Certainly the most intriguing mechanism to account for BFAs effects would be if the compound enhances or reveals a constitutive Golgi to ER recycling pathway by blocking transport from the ER. If BFA selectively blocks transport from the ER, then recycling from the "donor" compartment(s) would continue unopposed. Since proteins from the intermediate compartment and both the *cis*- and medial Golgi are redistributed to the ER, recycling may occur directly from all three sites. Alternatively, any one of these organelles could be the primary donor, and other organelles with which it communicates closely might also be affected. Unopposed recycling from the *cis*-Golgi, for example, might eventually deplete the medial Golgi and intermediate compartment as well. A careful kinetic analysis of the rates at which *cis*- and medial Golgi markers are returned to the ER may differentiate between these possibilities. We note that the *trans*-Golgi, which has been implicated in other recycling pathways, seems to be excluded from this circuit (Farquhar, 1985; Snider and Rogers, 1985; Griffiths and Simons, 1986).

The delivery of resident and itinerant Golgi proteins to the ER indicates that at least one component of a constitutive recycling pathway must be vesicular in nature. Returning vesicles would help replace lipid lost from the ER in the form of transport vesicles. However, could a vesicular recycling pathway by itself return all of the necessary lipid to the ER? Wieland et al. (1987) have estimated that half of the phospholipids in the ER are transported to the Golgi apparatus every 10 min. De novo synthesis could compensate for only a fraction of this, so the remainder must be returned by recycling, probably from the Golgi complex and/or intermediate compartment. Given the relative surface areas of the ER and Golgi complex, Wieland et al. (1987) proposed that Golgi lipids have a half-time of only 1–2 min before being recycled. The half-time for redistribution of the *cis*- and medial Golgi to the ER in BFA-treated cells appears to be on the order of 6–7 min. If these estimates are correct, then an additional nonvesicular recycling pathway may exist solely for the return of lipid to the ER. Such a pathway would also serve to

concentrate membrane and secretory proteins and help insure vectorial transport (Wieland et al., 1987). While evidence for a nonvesicular recycling pathway is lacking, the presence of a vesicular mechanism does not necessarily rule it out given these considerations.

A constituitive, vesicular recycling pathway might also retrieve "escaped" ER proteins. Pelham has proposed that soluble ER proteins containing the COOH-terminal KDEL retention signal might not be strictly retained due to the fact that a receptor present in sufficient quantity to bind all KDEL proteins has not been identified (Munro and Pelham, 1987; Pelham, 1988) and KDEL proteins seem to be able to diffuse freely in the ER (Ceriotti and Colman, 1988). Rather, some fraction of soluble ER proteins might escape only to be recovered by a salvage pathway, in which a KDEL receptor in a post-ER compartment recognizes and returns the errant protein (Pelham, 1988; Warren, 1987). Since we have shown that Golgi enzymes are functional in the ER environment and resident ER glycoproteins are generally not processed (Lewis et al., 1985; Liscum et al., 1983; Rosenfeld et al., 1984; Brands et al., 1985), a vesicular recycling pathway must be relatively selective in order to prevent significant return of Golgi proteins to the ER. Proteins such as a putative KDEL receptor might selectively be incorporated into recycling vesicles while other proteins are excluded or incorporated at a low bulk-flow rate. A relatively low rate of reflux might go undetected for several reasons, including recycling from the intermediate compartment, delivery to a subdomain of the ER, and a mechanism to repair or turnover inappropriately modified ER proteins. In the presence of BFA, selectivity is apparently lost. BFA might either disrupt mechanisms that exclude Golgi proteins or unopposed recycling might concentrate resident and intinerant molecules to the point where they are incorporated into recycling vesicles at an abnormally high bulk rate.

An alternative mechanism for the effects of BFA would invoke the *cis*- and medial Golgi as the target organs for its action. If BFA causes the Golgi to vesiculate and fuse inappropriately with the ER, then the apparent "block" in transport from the ER might simply be due to the disappearance of the Golgi apparatus. This proposed mechanism might bear some analogy with mitotic cells (Fujiwara et al., 1988), in which the Golgi apparatus vesiculates and transport between the ER and Golgi is blocked though the Golgi apparatus retains its integrity (Featherstone et al., 1985). In BFA-treated cells, ER to Golgi transport is blocked and the Golgi vesiculates, but fusion with the ER also occurs. If BFA in some way overrides the mechanisms that prevent inappropriate fusion events, then the observed effects may have little to do with a Golgi to ER recycling pathway.

## Summary

We have shown that both resident and itinerant proteins are transported from the intermediate compartment and both the *cis*- and medial Golgi to the ER when cells are incubated with BFA. The *trans*-Golgi was unaffected. Whether this reflects an underlying Golgi to ER recycling pathway or represents artifactually induced fusion between the organelles remains to be determined. It is obvious and perhaps likely that BFA, like other compounds that inhibit membrane transport, may affect multiple cellular processes. The two scenerios discussed above are perhaps the simplest that can account for the observed results. More detailed studies will have to be performed to clarify the effects of BFA and to explain its actions.

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