Influenza virus hemagglutinin trimers and monomers maintain distinct biochemical modifications and intracellular distribution in brefeldin A-treated cells

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Brefeldin A (BFA) induces the retrograde transport of proteins from the Golgi complex (GC) to the endoplasmic reticulum (ER). It is uncertain, however, whether the drug completely merges the ER with post-ER compartments, or whether some of their elements remain physically and functionally distinct. We investigated this question by the use of monoclonal antibodies specific for monomers and trimers of the influenza virus hemagglutinin (HA). In untreated influenza virus-infected cells, monomers and trimers almost exclusively partition into the ER and GC, respectively. In BFA-treated cells, both monomers and trimers are detected in the ER by immunofluorescence. Cell fractionation experiments indicate, however, that whereas HA monomers synthesized in the presence of BFA reside predominantly in vesicles with a characteristic density of the ER, HA trimers are primarily located in lighter vesicles characteristic of post-ER compartments. Biochemical experiments confirm that in BFA-treated cells, trimers are more extensively modified than monomers by GC-associated enzymes. Additional immunofluorescence experiments reveal that in BFA-treated cells, HA monomers can exist in an ER subcompartment less accessible to trimers and, conversely, that trimers are present in a vesicular compartment less accessible to monomers. These findings favor the existence of a post-ER compartment for which communication with the ER is maintained in the presence of BFA and suggest that trimers cycle between this compartment and the ER, but have access to only a portion of the ER.

Introduction

Brefeldin A (BFA)¹ is a fungal metabolite that reversibly blocks exocytosis of membrane-bound and soluble proteins (Takatsuki and Tamura, 1985). BFA specifically blocks a portion of the exocytic process itself, in as much as its effects cannot be attributed to direct interference with core glycosylation of proteins, protein folding, or assembly of oligomeric proteins. Electron microscopy of BFA-treated cells reveals that the Golgi complex (GC) is rapidly disassembled and that with time the endoplasmic reticulum (ER) becomes distended (Fujiwara *et al.*, 1988). Immunological techniques performed in conjunction with the electron or light microscope reveal that proteins synthesized in the presence of BFA remain largely, although perhaps not exclusively, localized to the ER (Misumi *et al.*, 1986; Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). When characterized biochemically, the same proteins demonstrate oligosaccharide modifications characteristic of the action of enzymes that reside in the *cis*- or medial-GC, but not the *trans*-GC (Magner and Pappiannes, 1988; Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989; Ulmer and Palade, 1989). These paradoxical findings are explained by the ability of BFA to induce the retrograde transport of both resident and itinerant proteins of the *cis*- and medial-GC to the ER (Doms *et al.*, 1989;

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¹ Abbreviations used in this paper: BFA, brefeldin A; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMN, 1-desoxymannojirimycin; endo H, endo- β -N-acetylglucosaminidase H; ER, endoplasmic reticulum; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GC, Golgi complex; HA, hemagglutinin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; mAb, monoclonal antibody; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PR8, A/Puerto Rico/8/34; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; TRITC, tetramethylrhodamine-isothiocyanate.

Lippincott-Schwartz *et al.*, 1989), including the GC-associated machinery responsible for carbohydrate maturation.

Although it is clear that BFA induces proteins in the post-ER compartments to return to the ER, the extent to which the drug removes the functional and physical distinctions between these compartments is uncertain. The findings of Lippincott-Schwartz *et al.* (1990) suggest that BFA-treated cells maintain some post-ER elements in the exocytic pathway that communicate with the ER by vesicular transport. In particular, they reported evidence consistent with the ideas that the intermediate compartment located between the ER and GC is intact in BFA-treated cells and that GC-resident proteins continuously cycle between the ER and this compartment. This compartment is believed to be involved in returning certain resident ER proteins to the ER (Schweizer *et al.*, 1988; Pelham, 1989). It has been proposed (Donaldson *et al.*, 1990; Serafini *et al.*, 1991) that BFA acts by blocking the association of vesicles derived from the intermediate compartment with a specific protein (β -COP) that permits the vesicles to fuse with the GC, the existence of which depends on these vesicles to replace membranes continuously recycled to the ER. As the GC withers, whatever signals act to retain GC resident proteins are voided and the proteins are transported to the ER. Although extremely elegant, this model has many aspects that rest on limited experimental findings. A critical part of the model, the maintenance of a post-ER compartment in communication with the ER, is based on immunolocalization studies that have yet to be corroborated by biochemical or physical evidence. It is also uncertain whether GC proteins returned to the ER have access to the entire ER or are localized to certain regions.

In the present study, we use the hemagglutinin (HA) glycoprotein from the influenza virus A/Puerto Rico/8/34 (PR8) to address these issues. The HA is a trimeric glycoprotein consisting of a globular domain atop a fibrous stem that anchors the molecule to membranes. Numerous monoclonal antibodies (mAbs) have been produced that recognize the globular domain of the HA (Caton *et al.*, 1982), including those that specifically recognize monomeric and trimeric forms. Analysis of HA biosynthesis using mAbs indicates that the globular domain of monomers folds rapidly, possibly even cotranslationally, into a conformation very similar to that of trimers (Copeland *et al.*, 1988; Yewdell *et al.*, 1988). Monomers then assemble into trimers with a half-time of ~ 5 –10 min (Gething *et al.*, 1986; Copeland *et al.*, 1988; Yewdell *et al.*, 1988). Trimerization of the PR8 HA appears to occur in a post-ER compartment (Yewdell *et al.*, 1988), either the *cis*-GC or the intermediate compartment implicated in the retrieval of ER proteins (T. Bächli, H.P. Hauri, C. Lapham, J. Bennink, and J. Yewdell, unpublished data). As a consequence, trimers are not detected in the ER of PR8-infected cells and serve as an indicator that HA has departed the ER.

Using monomers and trimers as markers of the ER and GC, respectively, we have made observations consistent with the idea that BFA-treated cells maintain post-ER elements in communication with the ER. Additionally, we provide the initial evidence that ER subcompartments are maintained in BFA-treated cells and that proteins redistributed from the GC by BFA are denied access to one of these compartments.

Results

Immunofluorescence localization of HA monomers and trimers synthesized in the presence of BFA

To study the effects of BFA on the intracellular trafficking of HA trimers and monomers, we initially performed immunofluorescence with the use of a protocol that allowed us to simultaneously visualize Y8-10C2 (anti-monomer) and biotinylated-H17-L2 (anti-trimer) mAbs with fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Figure 1, right panels), and Texas Red-conjugated streptavidin (Figure 1, left panels), respectively. In the absence of BFA, anti-trimer mAb stains the post-ER exocytic organelles of PR8-infected cells, sparing the ER (Figure 1A, left). Staining with anti-monomer mAb reveals a pattern characteristic of the ER; i.e., staining included the nuclear membrane and a reticular network (Figure 1A, right). The validity of this double-staining technique is demonstrated by the nearly perfect colocalization observed when the anti-trimer antibody is used for both FITC and Texas Red staining (Figure 1F).

Incubation of cells with nocodazole beginning 1 h after infection had little effect on the staining of anti-monomer mAb (Figure 2B, right panel), but it dispersed the stain with anti-trimer mAb into smaller vesicular structures (Figure 2B, left panel). This is consistent with prior observations (Rogalski *et al.*, 1984; Turner and Tartakoff, 1989) that disruption of microtubules causes the fragmentation of the GC. Incubation of cells with BFA beginning at 1 h postinfection blocked the

Figure 1. Double-immunofluorescence localization of HA monomers and trimers in BFA-treated cells. L929 cells were infected with PR8 for 1 h and then incubated for an additional 4 h at 37°C without inhibitors (A), with 20 μ g/ml nocodazole (B), with 5 μ g/ml BFA (C), with 20 μ g/ml nocodazole and 5 μ g/ml BFA (D and F), or with 5 μ g/ml BFA and then for 30 min with 5 μ g/ml BFA, 20 μ g/ml nocodazole, and 25 μ g/ml anisomycin (E). Cells were then fixed, permeabilized, stained with biotinylated anti-trimer mAb detected by Texas Red streptavidin (A–F, left panel) and either anti-monomer mAb (A–E, right panel) or anti-trimer mAb (F, right panel), and detected by rabbit anti-mouse FITC.

Figure 2. HA trimers do not have access to all ER subcompartments in the presence of BFA. L929 cells were infected with PR8 for 1.5 h and incubated for an additional 3 h with BFA at 5 μ g/ml. Double fluorescence was performed on fixed and permeabilized cells. (A) anti-trimer mAb, (B) anti-monomer mAb, (C) anti-BiP mAb. Localization of ConA in the same field is shown in D–F.

surface expression of HA as determined by cytofluorographic analysis (not shown). BFA treatment did not grossly alter the staining pattern with anti-monomer mAb, which continued to stain the ER (Figure 1C, right panel). Now, however, anti-trimer mAb also stained the ER (Figure 1C, left panel), often intensely staining the nuclear membrane (this is most clearly seen in Figure 2A). Close examination of Figure 1C reveals that staining with anti-trimer and anti-monomer mAbs was largely coincident, but that a number of small vesicles in the cytoplasm were preferentially stained by anti-trimer mAb. Suspecting that this might represent a post-ER compartment, we examined the effect of the microtubule-disrupting agent nocodazole on the

distribution of monomers and trimers. Nocodazole has been found by Lippincott-Schwartz *et al.* (1990) to greatly enhance the amount of GC-resident proteins in the intermediate compartment in BFA-treated cells, presumably because transport from this compartment to the ER is microtubule dependent. Treatment of cells with BFA and nocodazole continuously beginning 1 h postinfection increased the number and size of trimer-rich punctate cytoplasmic structures (Figure 1D, left panel). A similar effect was observed by incubating BFA-treated cells for 30 min with BFA, nocodazole, and anisomycin (to prevent additional HA synthesis) (Figure 1E, left panel). These findings are consistent with the idea that cells maintain a trimer-rich,

monomer-poor compartment in the presence of BFA.

In the course of performing multiple immunofluorescence experiments, we noted in five experiments the existence of large structures that were intensely stained by anti-monomer mAb (Figure 2B) and not by anti-trimer mAb (Figure 2A). The numbers of discrete structures varied considerably between different cells, and the dimensions of the structures were distributed over a wide range. This compartment is part of the ER because its anti-monomer staining colocalized with staining by the lectin ConA (Figure 2, D–F), which, in many cell types, preferentially binds to high-mannose oligosaccharides in the ER. The ER localization of ConA in PR8-infected L929 cells was confirmed by its colocalization with a mAb specific for GRP78/BiP (Bole *et al.*, 1986), a well-defined ER-resident protein (Figure 2C). The compartment was present in non-BFA-treated cells but was generally enhanced by 4-h BFA treatment. Its appearance was not altered by the PR8 infection.

The extent to which cells possess this compartment is controlled by unknown factors. This variation is not due to variation in fixation or staining procedures, because cells from cultures maintained separately and processed in parallel in a single experiment displayed similar variability in possessing this compartment, as was observed between different experiments. Having identified cultures that displayed the compartment, it was consistently detected over a period of weeks. These cultures were not contaminated by mycoplasma. Although the compartment was not always detected, the facts that it was observed in a number of experiments and that monomers were preferentially detected in it suggest that, in the presence of BFA, the ER can be divided into distinct sub-compartments.

In the presence of BFA, HA trimers are modified by GC-associated enzymes

To biochemically characterize the effect of BFA on HA, we used anti-monomer and anti-trimer mAbs to immunoprecipitate HA from detergent extracts derived from cells radiolabeled with ³⁵S-methionine, and immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). When electrophoresed under nonreducing conditions, HA trimers migrate as a mixture of three species corresponding to monomers, dimers, and trimers (Selimova *et al.*, 1983) (labeled 1, 2, and 3, respectively, in Figure 3A), whereas mono-

mers migrate largely as a single species (Yewdell *et al.*, 1988). BFA added to cells at the time of pulse labeling (Figure 3A, right side) did not grossly affect the HA folding, as determined by the binding anti-monomer mAb, which recognizes a conformational determinant in the HA formed by residues present in a number of different loops of the globular domain of the HA (Gerhard *et al.*, 1981). In the experiment illustrated in Figure 3A, trimerization was not affected by BFA, as shown by the similar kinetics of trimer formation and monomer depletion in untreated and BFA-treated cells. In approximately half of other experiments, however, trimerization was partially inhibited (not shown).

In all experiments, BFA blocked two alterations in HA structure detected by SDS-PAGE. First, it prevented trimers from demonstrating the decreased mobility previously found to occur coordinately with the addition of terminal galactose moieties (Yewdell *et al.*, 1988). In Figure 3A the decreased mobility is most marked in the trimer species migrating as monomers. Second, BFA blocked the time-dependent increase in the proportion of trimers migrating in SDS-PAGE as monomers (Figure 3A; this is also apparent in Figure 8). This decrease in the tenacity of subunit interaction as the HA matures could be related to the transport-dependent reduction of HA disulfides that has been observed by Skibben *et al.* (1989).

A very close look at Figure 3A reveals that the electrophoretic mobility of trimers increases slightly within 15 min after the addition of BFA, reaching maximal values by 35 min of chase (this is most easily seen among the HA with the electrophoretic mobility of monomers). After an additional 60 min incubation in the presence of BFA, HA migrated slightly more slowly and heterogeneously. The increased trimer mobility is more easily appreciated in Figure 3B (in lanes labeled "T"), in which longer gels have been used to enhance detection. Although minor in scope, these electrophoretic differences were consistently observed in numerous (>20) experiments.

It has previously been observed that glycoproteins synthesized in the presence of BFA are trimmed by GC-associated mannosidases (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). To test whether the increased mobility of the HA was due to mannosidase activity, we treated cells with 1-desoxymannojirimycin (DMN), a specific inhibitor of Golgi mannosidase I, or swainsonine, a specific inhibitor of Golgi mannosidase II (Albein, 1987). These compounds were active in L929 cells because they

Figure 3. Effect of BFA on HA monomers and trimers. (A) PR8-infected L929 cells were labeled for 5 min with ^{35}S -methionine and immediately incubated at 0°C (0 min), or incubated for 15, 35, or 95 min at 37°C before being chilled. The right four lanes of the fluorograph represent immunoprecipitates from cells to which BFA was added during pulse labeling and maintained throughout the chase period. Detergent lysates of cells were immunoprecipitated with anti-trimer mAb (top) or anti-monomer mAb (bottom) and analyzed by SDS-PAGE under nonreducing conditions. Trimers electrophoresed without exposure to reducing agents migrate as a mixture of trimers, dimers, and monomers (labeled 3, 2, and 1). (B) PR8-infected L929 cells were continuously incubated with BFA starting 30 min before 5 min pulse labeling with ^{35}S -methionine. Cells were immediately incubated at 0°C or chased for up to 120 min at 37°C before being chilled. Detergent lysates immunoprecipitated with anti-monomer mAb (M) or anti-trimer mAb (T) were analyzed via SDS-PAGE under nonreducing conditions, because reduction of disulfides diminished the mobility increase. Only the bands corresponding to monomers are shown from the trimer immunoprecipitates because monomers were a much more sensitive indicator of the BFA-induced increased mobility than were disulfide-dependent dimers or trimers. A line orthogonal to the direction of electrophoresis is drawn over the fluorograph to aid detection of the increased electrophoretic mobility induced by BFA. (C) L929 cells were infected with PR8 for 4 h before 5 min labeling with ^{35}S -methionine and immediately incubated at 0°C or chased for up to 90 min. Cells were incubated with BFA throughout infection, labeling, and chase periods. Cells were detergent extracted and immunoprecipitated with anti-monomer mAb (M) or anti-trimer mAb (T). Immunoprecipitates were analyzed via SDS-PAGE under reducing conditions. Immunoprecipitates in the three right lanes of the fluorograph were treated with endo H before electrophoresis (+); s (sensitive) marks the mobility of HA completely deglycosylated by endo H digestion.

blocked the HA-associated oligosaccharides from becoming resistant to digestion with endo- β -*N*-acetylglucosaminidase H (endo H) in non-BFA-treated cells (Figure 4B). To examine the effect of mannosidase inhibitors in BFA-treated

cells, we treated with trypsin HA immunoprecipitated with anti-trimer mAb (which cleaves HA into 2 disulfide-linked chains termed HA1 and HA2) and analyzed the HA under reducing conditions to separate the chains. This treat-

Figure 4. HA synthesized in the presence of BFA is modified by GC-associated mannosidases. (A) PR8-infected L929 cells were labeled with ^{35}S -methionine for 10 min and then immediately incubated at 0°C (P) or for 37 min at 37°C before detergent lysis. Lysates were treated with trypsin to cleave HA into HA1 and HA2 subunits, immunoprecipitated with anti-trimer mAb, and analyzed by SDS-PAGE under reducing conditions. Inhibitors were added to cells just before radiolabeling and maintained throughout the chase period. Cells were treated with BFA alone (B), with BFA and $10\ \mu\text{M}$ swainsonine (B/S), or with BFA and $1\ \text{mM}$ DMN (B/D). (B) PR8-infected L929 cells were labeled with ^{35}S -methionine for 5 min and then incubated for 120 min at 37°C with no inhibitors (C), with $10\ \mu\text{M}$ swainsonine (S), or with $1\ \text{mM}$ DMN (D). In lanes in right portion of the fluorograph, cells were incubated continuously with BFA beginning 30 min before radiolabeling (B). Detergent lysates were immunoprecipitated with anti-trimer mAb and treated with endo H before SDS-PAGE under reducing conditions (+). r and s denote the mobility of endo H-resistant and -sensitive species.

ment was found to further enhance detection of mobility differences induced by BFA. As seen in Figure 4A, the mobility difference between pulse-labeled HA (P) and BFA-chased HA (B) is completely abrogated by DMN, whereas swainsonine (lane labeled BD) partially blocked the mobility increase (compare BS with B and BD). This indicates that the increase in HA mobility results from the action of both Golgi mannosidases I and II.

Although the action of Golgi mannosidase II on oligosaccharides generally confers resistance to endo H digestion (Maley *et al.*, 1989), we found that, in the presence of BFA, trimers became resistant to endo H more slowly than they demonstrated swainsonine-sensitive oligosaccharide trimming. Thus, 60 min after synthesis, when the downshift in HA mobility was completed, HA was only partially resistant to digestion by endo H (Figure 3C, bottom). Even after an additional 30 min incubation, HA was still only partially resistant to endo H (Figure 3C). The intermediate mobility of HA is due to its associated oligosaccharide chains and not other

posttranslational modifications induced by BFA, because HA derived from cells treated with BFA and DMN or BFA and swainsonine was completely sensitive to digestion with endo H (Figure 4B).

HA monomers are only partially modified by GC enzymes in the presence of BFA

BFA induced a less dramatic increase in the mobility in monomers than in trimers, which was only detected when monomers were electrophoresed in longer gels (compare Figures 3A [short gel] and B [long gel]). This mobility increase is also related to oligosaccharide trimming, inasmuch as it was completely inhibited by DMN and partially inhibited by swainsonine (not shown). The difference in oligosaccharide modification between monomers and trimers was also observed on digesting immunoprecipitates from BFA-treated cells with endo H. As seen in Figure 3C, monomers from cells treated with BFA from the time of infection were completely susceptible to endo H digestion even 90

min after chasing, a time when most trimers were at least partially resistant to endo H. In additional experiments, monomers sometimes were found to display partial resistance to endo H digestion, although with slower kinetics and to a lesser extent than trimers (not shown). Thus, in the presence of BFA, trimer-associated oligosaccharides were more extensively modified than monomer-associated oligosaccharides by machinery resident in the GC of non-BFA-treated cells.

HA monomers and trimers maintain differential palmitylation in the presence of BFA

HAs of other influenza virus subtypes are known to be palmitylated (Schmidt and Lambrecht, 1985). Palmitylation probably occurs at one or more of the three cysteines in the cytosolic tail of the HA. These residues are conserved between widely divergent HA subtypes. Recent evidence suggests that palmitylation occurs in an early post-ER compartment (Bonatti *et al.*, 1989). It was therefore of interest to examine whether monomers and trimers were differentially modified in the presence of BFA.

A detailed characterization of the palmitylation of the PR8 HA will be described elsewhere (G. Russ, J. Bennink, and J. Yewdell, unpublished data). Our findings are consistent with the findings of Bonatti *et al.* (1989) that palmitylation of membrane glycoproteins occurs in a post-ER compartment. Most important for the present paper, trimers are palmitylated to a greater extent than monomers, generally more than 10-fold higher as determined by densitometry of anti-monomer mAb and anti-trimer mAb immunoprecipitates after SDS-PAGE analysis (Figure 5). BFA partially inhibited the palmitylation of both trimers and monomers (Figure 5). Critically, however, the

10-fold preferential incorporation of palmitate into trimers was maintained when cells were treated with BFA for 30 or 255 min before labeling. Thus, as with oligosaccharide modifications, BFA did not eliminate the difference between palmitylation of monomers and trimers that occurs during normal HA maturation.

HA monomers and trimers synthesized in the continuous presence of BFA have a distinct subcellular distribution

Immunocytochemical and biochemical experiments were consistent with the idea that monomers and trimers synthesized in BFA-treated cells maintained a distinct subcellular distribution. To test this conclusion, we performed subcellular fractionation experiments. In preliminary experiments we characterized the efficacy of our fractionation technique on PR8-infected L929 cells. Cells were disrupted by nitrogen cavitation, and membrane vesicles in post-nuclear/mitochondrial supernatants were fractionated by centrifugation in discontinuous sucrose gradients. The fractions with sucrose concentrations > 43% contain ER-derived vesicles because they possess virtually all the glucose-6 phosphatase activity, a marker of the ER (Figure 6, left side). In a separate experiment, we found that fractions with sucrose concentrations of 28–35% contained vesicles derived from the *trans*-GC because they contained the bulk of the galactosyl transferase activity (Figure 6, right side). Treatment of cells with BFA from the time of infection did not alter the distribution of glucose-6 phosphatase activity, but shifted the bulk of galactosyl transferase activity from less dense vesicles to vesicles of higher density (Figure 6). These preliminary experiments indicated that the fractionation technique segregated vesicles derived from the *trans*-GC and ER into the

Figure 5. Preferential palmitylation of trimers is maintained in the presence of BFA. PR8-infected L929 cells were labeled for 15 min with [9,10-³H]palmitic acid, extracted with detergent, and immunoprecipitated using anti-monomer mAb (monomer) or anti-trimer mAb (trimer). Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions. BFA was added to cells 255 or 30 min before radiolabeling and maintained throughout the labeling period. Densitometry readings of fluorographs were as follows (left to right): monomer; 0.21, 0.54, 0.78; trimer; 2.87, 5.25, 9.98.

Figure 6. Characterization of cell fractionation technique. PR8-infected L929 cells were disrupted and fractionated as detailed in Materials and methods. ●, fractions derived from cells treated with BFA; ○, fractions from untreated cells. In separate experiments, the glucose-6 phosphatase (left panel) and galactosyl transferase activities (right panel) were determined for each gradient fraction.

least dense and most dense fractions, respectively. Although the markers we used did not allow us to identify the membrane vesicles sedimenting in the middle density regions, the localization of HA trimers to this region (see below) strongly suggests that these vesicles are derived from the GC.

Using this fractionation scheme, we determined the intracellular distribution of HA monomers and trimers after 5-min pulse-labeling with ^{35}S -methionine and after a 30-min chase. Determination of the sucrose concentration and amount of acid-precipitable ^{35}S in the fractions revealed that the fractionation technique was highly reproducible between different samples (Figure 7). This also revealed some variation in the total amount of counts that were recovered from each gradient (see Figure 7 legends). Although this resulted in differences between the absolute amount of ^{35}S -methionine-labeled HA immunoprecipitated, these differences do not affect the interpretation of the results, because the critical parameter is the monomer-to-trimer ratio.

Fractions derived from the top (fraction 3), middle (fractions 7–9), and bottom (fractions 9 and 10) of the gradients were detergent treated and immunoprecipitated with mAbs; the non-reduced immunoprecipitates were analyzed by SDS-PAGE (Figure 8). In cells not exposed to BFA, HA monomers were located primarily in the bottom fractions, both immediately after pulse labeling and after 30 min further incubation at 37°C . By contrast, immediately after pulse labeling, trimers were more heavily represented in the top and middle fractions. After 30 min chase, trimers were even more heavily represented in the top and middle gradient fractions. Note that the monomeric electrophoretic species of trimers predominates in the top fraction, whereas the trimeric species predominates in the middle fraction. Because this modification in trimer resistance to dissociation is time dependent (Figures 3 and 8), it suggests that the vesicles in the top and middle fractions are enriched for the more distal elements of the secretory pathway, further supporting the validity of the fractionation technique.

Figure 7. Characterization of fractions from radiolabeled cells used for immunoprecipitation in Figure 8. Cells were infected with PR8 for 4.5 h before 2.5 min labeling with ^{35}S -methionine. Cells were then either incubated on ice or chased for 30 min at 37°C . Cells were disrupted and fractionated on discontinuous sucrose gradients. The sucrose concentration and acid-precipitable ^{35}S was determined for each fraction. (A) cells were not exposed to BFA during infection and labeling. (B) cells were incubated with BFA throughout this period. $\circ$, pulse-labeled cells; $\bullet$, cells chased for 30 min; $\triangle$, cells incubated with BFA during the chase period. Total counts recovered are as follows: (A) pulse 17 069 cpm; chase 58 557 cpm; chase with BFA, 68 808. (B) pulse 29 372; chase 29 017; chase with BFA, 28 568.

Incubation of cells with BFA, either during the chase period only (Figure 8, last 3 lanes of left half of fluorograph) or throughout the infection and labeling and chase periods (Figure 8, first 6 lanes of right half of fluorograph), did not affect the distribution of monomers, which remained largely in the bottom fraction. It did, however, increase the proportion of trimer recovered from the middle fraction and also increased the resistance of trimers in all fractions to dissociation after boiling in SDS-PAGE sample buffer. The effect of BFA on trimer localization was partially reversed by removing BFA from cells before pulse labeling, while monomers remain localized in the bottom fraction (Figure 8, last 3 lanes). These findings, in conjunction with the differences in immunofluorescence localization and posttranslational modifications of monomers and trimers, indicate that trimers and monomers are present in differing proportions in the various compartments of the early secretory

apparatus that are maintained in the presence of BFA.

Discussion

BFA as a tool for discriminating ER versus GC events in normal cells

A vexing problem in studying the assembly and posttranslational modifications of proteins exported from the ER is determining whether a given event occurs in the ER or in a post-ER compartment. There was considerable hope when the effect of BFA on retaining proteins in the ER was first reported (Takatsuki and Tamura, 1985; Misumi *et al.*, 1986) that BFA would be a simple and powerful tool for solving this type of problem. Indeed, we initially obtained BFA to determine whether HA trimerizes in the ER or in a post-ER compartment, a question that has been difficult to answer unequivocally (Gething *et al.*, 1986; Copeland *et al.*, 1988; Yewdell *et al.*, 1988).

Figure 8. HA trimers and monomers have a distinct distribution in membrane-derived vesicles separated by equilibrium centrifugation. PR8-infected cells were labeled with ^{35}S -methionine for 2.5 min and immediately incubated on ice (pulse) or incubated for 30 min at 37°C before being chilled. In the right half of the fluorograph, BFA was added to cells at the time of infection and was either maintained throughout the chase period (30'/BFA) or removed after radiolabeling (30'). In the last three lanes in the left half of the fluorograph, cells were incubated with BFA during the 30-min chase (30'/bfa). After nitrogen cavitation, postnuclear and mitochondrial supernatants were fractionated on discontinuous sucrose gradients. Fractions from the top (1, fraction 3), middle (2, fractions 7–9), and bottom (3, fractions 9 and 10) of the gradients were detergent treated, immunoprecipitated with anti-trimer or anti-monomer mAb, and analyzed by SDS-PAGE under nonreducing conditions.

Unfortunately, the mechanism by which BFA retains proteins in the ER makes it unsuitable for solving problems of this nature. First, on the basis of our present results and those of Lippincott-Schwartz *et al.* (1990), it appears, that in the presence of BFA, proteins such as HA trimers that are predominantly located in the GC of untreated cells are not actually arrested in the ER but cycle between ER and post-ER compartments. Thus, we cannot distinguish whether HA trimers present in the ER were assembled there or were transported from a post-ER compartment where assembly occurred. Second, even if HA trimers are formed in the ER in the presence of BFA, we could not eliminate the possibility that this is due to the retrograde transport into the ER of factors present in post-ER compartments needed for trimerization.

The ambiguity in the site of trimerization in BFA-treated cells leaves two plausible explanations

for our finding that trimers are the predominant species of HA in a vesicular compartment of lower density than ER-derived vesicles. Trimerization might be required for HA to leave the ER and enter this compartment. Alternatively, monomers could be exported from the ER but not reach similar steady-state levels in the compartment as trimers, either because trimerization occurs rapidly or because monomers are returned to the ER more rapidly than trimers. In either of these models, inefficiencies introduced in the process by alterations in cellular physiology could account for the variable effects of BFA on trimerization that we noted among different experiments.

Trimer-rich compartment is maintained in BFA-treated cells

The major conclusion of the present paper is that monomers and trimers maintain a distinct

intracellular distribution in BFA-treated cells. This conclusion is supported by three lines of evidence. First, trimers synthesized in the presence of BFA were biochemically modified to a greater extent than monomers by enzymes that normally reside in the *cis*- and medial GC. Second, immunofluorescence experiments indicate that monomers and trimers can exist in physically distinct regions in the presence of BFA. Third, subcellular fractionation experiments indicate that monomers synthesized in the presence of BFA were located almost exclusively in vesicles with a density of the ER, whereas trimers were predominantly located in less dense vesicles.

The identity of these trimer-rich vesicles remains to be established. Their deficiency in glucose 6-phosphatase activity and HA monomers suggests they are derived from a post-ER compartment. On the basis of the findings of Lippincott-Schwartz *et al.* (1990), the nocodazole-mediated enhancement of trimer-rich, monomer-poor structures we found in immunofluorescence experiments suggests that these vesicles are derived from the intermediate compartment. It is also possible, however, that the post-ER compartment we detect in cell fractionation experiments represents vesicular carriers returning proteins from the intermediate compartment, remnants of the GC that persist in BFA-treated cells (as suggested by Yamashina *et al.* [1990]), or perhaps the subcompartment of the ER described by Vertel *et al.* (1989). The findings of Lodish *et al.* (1987), that vesicles derived from the intermediate compartment of a human hepatoma cell line are of lower density than GC-derived vesicles, would favor the idea that the trimer-rich post-ER compartment we detect in cell fractionation experiments represents something other than the intermediate compartment. This is an isolated report, however, and it is uncertain whether these findings would apply to L929 cells.

There are at least three mechanisms that could, individually or in concert, account for the preferential modification of trimers by GC-associated enzymes in BFA-treated cells. First, carbohydrate groups or cytosolic cysteines on trimers might be more accessible than the corresponding groups on monomers to the enzymatic machinery responsible for carbohydrate maturation and palmitylation. The conformational similarities between the globular domains of monomers and trimers argues against this possibility and, indeed, are the major attraction for the use of HA to study the effects of BFA on cells. This possibility cannot, however, be

dismissed, particularly because monomers may exist in the ER not as independent species, but in a loose complex with polypeptide chain-binding proteins such as BiP (Gething *et al.*, 1986; Hurtley *et al.*, 1989). Second, although GC enzymes returned to the ER appear to be active to some extent in the environment of the ER, because they modify resident ER proteins, their activity might be severely compromised (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). In this case, much of the GC-associated modifications in BFA-treated cells might still occur in post-ER compartments, in which trimers are present in greater quantities than monomers. The relative inefficiency of carbohydrate modification and palmitylation in BFA-treated cells could reflect either the suboptimal performance of the enzymes in the remaining post-ER compartment or a limitation in the flow of trimers through this compartment. Third, monomers might be sequestered in a region of the ER devoid of trimers that is also inaccessible to GC enzymes returned to the ER by BFA. Our detection of a trimer-poor ER compartment is consistent with this possibility. If monomers were retained in this compartment for long times, this could explain the ability of BFA to partially inhibit trimerization in some experiments.

Is the ER subcompartmentalized?

The immunofluorescence detection of the monomer-rich, trimer-poor ER compartment in BFA-treated cells suggests that the ER can be compartmentalized in the presence of BFA. Inasmuch as trimers appear to be deficient in this compartment in BFA-treated cells, there seems to be selectivity in transport between it and the portions of the ER forming the nuclear membrane and reticular regions that clearly contain trimers returned to the ER by BFA. Although L929 cells would not be expected to contain an extensive smooth ER, the trimer-poor subcompartment bears a striking resemblance to immunofluorescence images of the smooth ER of UT-1 cells, which, like the trimer-poor ER subcompartment of L929 cells, was intensely stained by ConA and the GRP78/BiP-specific mAb (Bergman and Fusco, 1990).

Alternatively, the existence of the trimer-poor compartment may indicate that the rough ER itself is compartmentalized. Two types of compartmentalization are conceivable. First, discrete regions of the ER could be specialized to perform certain functions, and entrance into this region would require a specialized signal. The

proposed ER degradation of unassembled or defective proteins (Lippincott-Schwartz *et al.*, 1988; Bonafacino *et al.*, 1990) may occur in such a compartment. Second, compartmentalization might be more general in nature. For example, the ER might be organized such that nascent membrane proteins move vectorially from their sites of synthesis to regions where packaging for vesicular transport to the GC occurs. In this model, the trimer-poor compartment would represent elements of the ER distant from the transport regions. Trimers would be unable to reach these regions because they are formed in post-ER elements and because they are present only in those regions of the ER that communicate bidirectionally with regions of the ER that fuse with vesicles shuttling between the ER and the intermediate compartment. Indirect support for this model comes from the findings of Ceriotti and Colman (1988) that diffusion of HA and other membrane glycoproteins in the ER of *Xenopus* oocytes can be limited, even 20 h after their synthesis. More directly, Vertel *et al.* (1989) have described a smooth ER compartment in chondrocytes that excludes resident ER proteins but contains precursors of chondroitin sulfate proteoglycan that are exported from the ER to the GC.

The trimer-poor compartment poses additional critical questions. Does the compartment exist in cells not exposed to BFA? Why is its detection by immunofluorescence variable? Does this indicate variation in its existence or simply in its appearance? The answers to these questions, as well as the basic problem of determining the physiological relevance of the compartment, require further investigation.

Materials and methods

Materials

Natural BFA was obtained from Dr. Takatsuki (University of Tokyo, Tokyo, Japan) and Dr. C.R. Hutchinson (University of Wisconsin, Madison, WI). Synthetic BFA was purchased from Dr. E. Corey (Harvard University, Cambridge, MA). Aquasol, ³⁵S-methionine, and [9,10-³H]palmitic acid were purchased from Dupont-New England Nuclear (Boston, MA). Amplify was purchased from Amersham (Arlington Heights, IL). Swainsonine, endo H, and DMN were purchased from Boehringer Mannheim (Indianapolis, IN). Bovine serum albumin (BSA); 1,4 diazabicyclo[2.2.2]octane; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES); DNAase type I; *α*-biotin; ovalbumin; and phenylmethylsulfonyl fluoride were purchased from Sigma (St. Louis, MO). FITC-conjugated ConA was purchased from Vector (Burlingame, CA). Virus-free chicken eggs were obtained from SPAFAS (Boston, MA). Autopow Eagle's minimum essential medium (MEM) was obtained from GIBCO (Grand Island, NY). FITC- and TRITC-conjugated rabbit anti-mouse IgGs were purchased from Dako (Carpinteria, CA). Triton X-100 was purchased from Pierce (Rockford, IL).

Cells, virus, and BFA treatment

L929 cells were maintained in antibiotic-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% (vol/vol) fetal bovine serum (FBS). Cells were passaged weekly by trypsinization. PR8 virus was grown in the allantoic cavity of embryonated chicken eggs and stored as infectious allantoic fluid at -70°C. For L929 cell infection, cells were trypsinized, washed twice with phosphate-buffered saline (PBS), and suspended at 10⁷ cells/ml in Autopow MEM supplemented with 0.1% (wt/vol) BSA, 20 mM HEPES, and adjusted with HCl to pH 6.8. One milliliter infectious PR8 allantoic fluid (~4000 hemagglutinating units) was added per 10⁷ cells, and cells were incubated for 1 h at 37°C while being slowly rotated to prevent settling. DMEM supplemented with 7.5% FBS and 20 mM HEPES, pH 7.0, was then added to yield a concentration of 2 × 10⁶ cells/ml, and the rotating incubation was continued for another 3–4 h at 37°C. Preliminary experiments established that the effects of BFA on exocytosis or posttranslational modification of HA in L929 cells were not altered by a 10-fold increase in dose. On the other hand, BFA at 1 μg/ml only partially inhibited HA exocytosis.

Metabolic labeling

For labeling with ³⁵S-methionine, cells were incubated in serum-free, methionine-free DMEM for 30 min at 37°C and then incubated at 1–2 × 10⁶ cells/ml in 200 μl of the same media supplemented with ³⁵S-L-methionine at 1 mCi/ml. After 2–10 min at 37°C, cells were pelleted and resuspended at 2 × 10⁶ cells/ml in DMEM supplemented with 7 mM L-methionine. For labeling with [9,10-³H]palmitic acid, cells were incubated at 2 × 10⁶ cells/ml for 15 min at 37°C in serum-free DMEM containing 100 μCi/ml [9,10-³H]palmitic acid. For all radiolabeling experiments, cells were shifted to 0°C after appropriate incubation periods until detergent extraction, or trypsin-treatment followed by detergent extraction, was performed.

Immunoprecipitation, endoglycosidase treatment, and SDS-PAGE

Metabolically labeled cells were extracted with Triton X-100 and immunoprecipitated essentially as described (Yewdell *et al.*, 1988), with the exception that detergent lysates were incubated with protein A sepharose preloaded with mAbs at a ratio of ~200 μg antibody/ml of packed gel. Control experiments established that virtually all HA was immunoprecipitated from samples under these conditions. For endo H digestion, antibodies and antigens were eluted from 25 μl protein A sepharose gel by heating to 100°C for 10 min in 10 μl of 5 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 1% (wt/vol) SDS, 2% (vol/vol) β-mercaptoethanol, that was adjusted to pH 6.9 with HCl, and incubated overnight at 31°C with 1 mU endo H in 20 μl 0.1 M sodium acetate, 5 mM EDTA, 0.5 mM PMSF, adjusted to pH 6.0. Samples were analyzed by SDS-PAGE using the buffer system of Laemmli (1970) as previously described (Yewdell *et al.*, 1988). Fixed gels were incubated in Amplify, dried at 80°C, and exposed to preflashed films at -70°C to improve the linearity of their response to low levels of radioactivity (Bonner and Laskey, 1974). Fluorographs were developed by the use of an automated film processor. Quantitation of bands in fluorographs was performed with the use of a model 620 video densitometer (BioRad, Richmond, CA).

Immunofluorescence

L929 cells were plated onto acid-cleaned glass coverslips in 24-well plates at a concentration of 5 × 10⁵ cells/well in

DMEM supplemented with 7.5% FBS. After 16 h incubation at 37°C, cells were washed three times with PBS, and incubated for 60 min with infectious allantoic fluid diluted 1/10 in 500 μ l Autopow MEM supplemented with 0.1% BSA and 20 mM HEPES, adjusted to pH 6.8. After addition of 1 ml of DMEM supplemented with 7.5% FBS, cells were incubated for another 3–5 h at 37°C with inhibitors as described in the text and figure legends. Cells were fixed by 20 min incubation at 20°C with PBS containing 3% paraformaldehyde, and permeabilized by 2 min incubation at 20°C with PBS containing 1% Triton X-100. Single-antibody immunofluorescence was performed as described (Bachi *et al.*, 1985) with the exception that ConA-conjugated FITC at 50 μ g/ml was included during the final incubation with TRITC-conjugated rabbit anti-mouse IgG. Double-antibody immunofluorescence was performed by incubating cover slips for 2 h at 20°C with PBS containing 25% (vol/vol) hybridoma tissue culture supernatant. After washing with PBS, coverslips were incubated for 2 h at 20°C with PBS containing 1% (vol/vol) FITC-conjugated rabbit anti-mouse IgG and 10 μ g/ml avidin. Coverslips were then washed and incubated for 20 min at 20°C with PBS containing 1% (vol/vol) of ascites fluid containing 10 μ g/ml biotin and a Sendai virus-specific mAb of (to block unoccupied γ 2a binding sites on the FITC-rabbit anti-mouse IgG). Anti-trimer mAb conjugated to biotin by standard procedures was added to a concentration of 50 μ g/ml, and incubated for a further 2 h. After washing, coverslips were incubated for 1 h with Texas Red-conjugated streptavidin at a concentration of 2 μ g/ml, washed, and mounted in Mowiol containing 5% (wt/vol) 1,4-diazabicyclo[2.2.2]octane to retard fading. Cells were examined with a Zeiss Axiophot photomicroscope (Thornwood, NY) equipped with appropriate filters to gate red and green fluorescence. Images obtained with a Hamamatsu C2400-08 SIT video camera (Hamamatsu City, Japan) were digitally processed with a Hamamatsu DVS-3000 image processor and either printed on high-resolution thermal paper with a Sony UPS 930 printer (Tokyo, Japan) (Figure 2), or imported into a Macintosh II computer (Figure 1). Using the Adobe Photoshop program, we further processed the image and printed it with a Sony UP 5000 printer.

Subcellular fractionation

PR8-infected, 35 S-methionine-labeled cells were suspended in 0.25 M sucrose, 2 mM MgCl_2 , 2 mM CaCl_2 , 10 mM KCl, 5 mM Tris-HCl, pH 7.4, and cavitated after 15 min incubation at 54 atmospheres of N_2 . After centrifugation at $2000 \times g$ for 15 min to remove nuclei, supernatants were centrifuged at $10\,000 \times g$ for 5 min to remove mitochondria and then adjusted to 55% (wt/vol) sucrose and overlaid with 45, 40, and 20% sucrose. After 15 h centrifugation at 29 000 RPM with a Beckman SW41 rotor (Fullerton, CA), fractions were collected and, based on sucrose concentration and trichloroacetic acid (TCA)-precipitable 35 S-methionine dpm, pooled into three fractions representing the bottom, middle, and top of the gradient. Fractions were then incubated with Triton X-100 and immunoprecipitated as above. A similar scheme was used to examine the distribution of galactosyl transferase activity in PR8-infected cells, except that, before fractionation on 20–45% discontinuous gradients, the postmitochondrial supernatant was overlaid on layers of 10% sucrose and 60% sucrose and membranes were collected at the 10–60% interface after 3 h centrifugation at 27 000 RPM in a Beckman SW28 rotor. The location of glucose-6-phosphatase in membrane fractions was determined by standard assay procedures (Aronson and Touster, 1974). The location of galactosyl transferase activity was determined by mixing samples with an equal volume of 100 mM

Tris-HCl pH 7.0, 10 mM MgCl_2 , 14 mg/ml ovalbumin, 20 mg/ml CHAPS, 0.5 mg/ml UDP-galactose, and 0.28 μ Ci UDP- ^3H -galactose. Samples were incubated at 37°C for 60 min, and the reaction was terminated by adding TCA to a final concentration of 10% (wt/vol). Precipitated material was collected on nitrocellulose filters that were washed with 10% TCA and the radioactivity determined by scintillation counting using Aquasol.

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