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Brefeldin A Arrests the Maturation and Egress of Herpes Simplex Virus Particles during Infection

PETER CHEUNG, BRUCE W. BANFIELD, AND FRANK TUFARO*

Department of Microbiology, University of British Columbia, 300-6174 University Boulevard, Vancouver, British Columbia, Canada V6T 1W5

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Herpes simplex virus (HSV) requires the host cell secretory apparatus for transport and processing of membrane glycoproteins during the course of virus assembly. Brefeldin A (BFA) has been reported to induce retrograde movement of molecules from the Golgi to the endoplasmic reticulum and to cause disassembly of the Golgi complex. We examined the effects of BFA on propagation of HSV type 1. Release of virions into the extracellular medium was blocked by as little as 0.3 μg of BFA per ml when present from 2 h postinfection. Characterization of infected cells revealed that BFA inhibited infectious viral particle formation without affecting nucleocapsid formation. Electron microscopic analyses of BFA-treated and untreated cells (as in control cells) demonstrated that viral particles were enveloped at the inner nuclear membrane in BFA-treated cells and accumulated aberrantly in this region. Most of the progeny virus particles observed in the cytoplasm of control cells, but not that of BFA-treated cells, were enveloped and contained within membrane vesicles, whereas many unenveloped nucleocapsids were detected in the cytoplasm of BFA-treated cells. This suggests that BFA prevents the transport of enveloped particles from the perinuclear space to the cytoplasmic vesicles. These findings indicate that BFA-induced retrograde movement of molecules from the Golgi complex to the endoplasmic reticulum early in infection arrests the ability of host cells to support maturation and egress of enveloped viral particles. Furthermore, we demonstrate that the effects of BFA on HSV propagation are not fully reversible, indicating that maturation and egress of HSV type 1 particles relies on a series of events which cannot be easily reconstituted after the block to secretion is relieved.

Herpes simplex virus (HSV) requires the host cell secretory apparatus for transport and processing of membrane glycoproteins during the course of virus assembly. It has been shown that pharmacologic agents that impinge on the secretory organelles, such as monensin (9), bromovinyldeoxyuridine (22), and tunicamycin (26), interfere with formation and release of HSV from infected cells. Furthermore, mutant cell line gro29, which is defective in protein secretion, is unable to release virions when infected with HSV type 1 (HSV-1), despite normal viral gene expression. These studies suggest that the secretory pathway is critical to HSV release and that specific host cell factors facilitate viral egress from infected cells (1, 33). Other cell lines defective in a variety of enzymes required for processing of N-linked oligosaccharides have been shown to produce less infectious virus than normal when infected with HSV-1, suggesting that specific oligosaccharide moieties contribute to efficient viral maturation and egress (2, 31).

To further our understanding of the virus-host interactions that govern the processes of viral maturation and egress, we investigated the effects of the fungal metabolite brefeldin A (BFA) on the propagation of HSV-1 in culture. BFA is a macrocyclic lactone that causes rapid redistribution of Golgi into the endoplasmic reticulum (ER) (5, 8, 15). It is thought to act by inducing interaction of the Golgi complex with an intermediate recycling compartment (14), although its mechanism of action and the cellular target responsible for this activity have not been identified. Incubation of cells with BFA leaves no discernible Golgi structure and blocks transport of proteins into post-Golgi cellular compartments (17, 21). Other processes, such as endocytosis, protein synthesis, and lysosomal degradation, appear to be unaffected at the low concentrations of BFA used in these studies (17, 19). Removal of BFA results in rapid flux of Golgi components out of the ER and reorganization of the Golgi (5, 15).

In this report, we demonstrate that BFA blocks accumulation and egress of infectious virus in HSV-1-infected cells. This failure to yield infectious virus is not caused by a failure to produce nucleocapsids, which were abundant in both BFA-treated and untreated cells. Electron microscopic analyses revealed that envelopment of nucleocapsids occurred at the nuclear membrane and enveloped particles accumulated in the perinuclear space of BFA-treated cells. However, enveloped particles were not detected in the cytoplasm. This is in contrast to untreated cells, in which large numbers of enveloped particles were found in the cytoplasm enclosed in membrane vesicles. Virally encoded glycoprotein D (gD) was synthesized normally in BFA-treated cells, suggesting that the failure to produce infectious virions was not due to a paucity of these proteins during infection. However, the cellular distribution of HSV-1 gD was perturbed in BFA-treated cells in that most of the mass of gD appeared to be localized to the ER. Despite this cellular location, most of the oligosaccharide moieties on gD were resistant to digestion by endoglycosidase H (endo H), suggesting that the glycoprotein contained at least partially processed oligosaccharides. These results suggest that the presence of viral components, namely, glycoproteins and nucleocapsids, is not sufficient for assembly and egress of infectious, enveloped particles in herpesvirus-infected cells. We infer from these data that the flow of molecules from the ER to the Golgi complex that exists in normal cells is critical early in infection for formation or accumulation of infectious HSV-1 particles. Furthermore, the effects of BFA on HSV propagation are not fully reversible, indicating that maturation and

* Corresponding author.

egress of HSV-1 particles rely on a series of events which cannot be easily reconstituted after the block to secretion is relieved.

MATERIALS AND METHODS

Abbreviations. DMEM, Dulbecco modified Eagle medium; hGH, human growth hormone; p.i., postinfection; MOI, multiplicity of infection; TCA, trichloroacetic acid.

Cells and viruses. L and HSV-1 KOS cells were a kind gift from D. Coen. All cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS). BFA was a kind gift from W. Jefferies. BFA was also obtained from Bio/Can Scientific, Mississauga, Ontario, Canada. Anti-gD monoclonal antibodies were kind gifts from M. Zweig and R. Philpotts.

Treatment of cells with BFA. Monolayers of L cells were infected with HSV-1 at an MOI of 10 PFU per cell. After 1 h of infection, the inoculum was removed and DMEM containing 10% FBS was added. At 2 h p.i., the medium was removed and fresh medium containing BFA was added. The extracellular medium was sampled at 4-h intervals for 20 h. The titer of infectious particles in each sample was determined by infecting confluent monolayers of Vero cells with appropriate dilutions of virus. The inoculum was removed after 1 h, and medium containing methylcellulose was added. Plaques were counted after 3 and 5 days of infection.

Harvesting of virus. Medium was removed from infected-cell monolayers and subjected to low-speed centrifugation to pellet cell debris and high-speed centrifugation to pellet virions. Pellets were suspended in phosphate-buffered saline (PBS) and sedimented through a 5 to 40% dextran T10 gradient formed in 50 mM NaCl–10 mM Tris (pH 7.8) for 1 h at 22,000 rpm in a Beckman SW41 rotor. Gradients were fractionated from the bottom of the tube into 0.3-ml fractions. For determination of radioactivity in insoluble material, 15% of each fraction to be analyzed was added to 50 μg of bovine serum albumin, followed by 1 ml of 10% cold TCA. Insoluble material was collected on filters after 1 h, and radioactivity was measured by scintillation counting. For determination of virus titers, gradient fractions were diluted serially with medium and used to inoculate monolayers of Vero cells growing in 96-well dishes. Microtiter wells were scored and titers were calculated when a generalized cytopathic effect was noticed in control infected samples.

Determination of titers of cell-associated virions. Monolayers of L cells were infected with HSV-1 (MOI, 10), and 3 μg of BFA per ml was added at 2 h p.i. At 8 and 18 h p.i., cell monolayers were harvested with EDTA, washed thoroughly with PBS, and suspended in reticulocyte standard buffer. The cell suspension was sonicated and centrifuged to remove cellular debris. The virus titers of these samples were determined by plaque assay on Vero cell monolayers.

Isolation and quantitation of nucleocapsids. Monolayers of L cells were infected with HSV-1 (MOI, 10). At 2 h p.i., the medium was changed to labeling medium (methionine-free DMEM, 0.1 volume of DMEM–10% FBS, 4% dialyzed FBS, 100 μCi of [^{35}S]methionine per ml) with or without 3 μg of BFA per ml. Infected cells were labeled for 16 h, after which the medium was removed and saved. Cells were harvested by washing with cold PBS and incubated for 15 min with cold lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate). The nuclei were pelleted from this extract and suspended in fresh buffer. The nuclear and cytoplasmic extracts were sonicated, 0.5 M urea was added, and urea-insoluble debris was removed by low-speed centrifugation. The supernatants were sedimented separately through 10 to 40% sucrose gradients for 1 h at 22,000 rpm in a Beckman SW41 rotor. Fractions (0.3 ml) were collected from the bottom of the tubes. Fractions were precipitated from solution by addition of 50 μg of bovine serum albumin and 1 ml of 10% TCA. After 1 h, precipitates were collected on glass fiber filters and counted by using Ready-Safe scintillation cocktail in a scintillation counter.

Indirect immunofluorescence. Cells were grown on acid-etched glass coverslips for 2 days and infected with HSV-1 (MOI, 10). At 2 h p.i., medium containing 3 μg of BFA per ml was added to the monolayers. At 10 h p.i., the BFA-containing medium was removed from the monolayers and replaced with normal medium. Cells were rinsed with PBS and fixed with 3.2% paraformaldehyde in PBS at various times after BFA removal. Fixed cells were washed and incubated for 30 min with a 1/100 dilution of anti-gD monoclonal antibody. The primary antibody was washed off, and cells were incubated for 30 min with a 1/100 dilution of fluorescein isothiocyanate- or RITC-conjugated goat anti-mouse immunoglobulin G. Coverslips were rinsed and mounted in 50% glycerol–100 mM Tris (pH 7.8). Cells were photographed with a Zeiss microscope with epifluorescence optics.

Electron microscopy. Cells were grown on Millicell HA inserts (Millipore Corp.) for 24 h prior to infection with HSV-1 (MOI, 10). BFA (3 μg/ml) was added to the relevant monolayers at 2 h p.i. At 8, 12, and 18 h p.i., the cells were rinsed with PBS and monolayers were fixed in 2.5% glutaraldehyde–0.1 M Na cacodylate (pH 7.3) for 1 h on ice. Cells were then rinsed and postfixed in 1% OsO_4 for 1 h. These samples were rinsed, dehydrated, and embedded in plastic. Specimens were sectioned, stained, and photographed with a Zeiss EM10C transmission electron microscope.

Growth hormone assays. Plasmid pXGH5, containing the gene for hGH driven by the mouse metallothionein I promoter, was described originally by Selden et al. (29). Monolayers of L cells were transfected with plasmid pXGH5 by using DEAE-dextran, followed by a dimethyl sulfoxide shock (16). After 2 days, samples of the extracellular medium were tested for the presence of hGH by using a radioimmunoassay kit according to manufacturer instructions (Joldon Diagnostics, Scarborough, Ontario, Canada). The rate of secretion of hGH for each monolayer was determined over a period of 3 h. Monolayers displaying similar rates of secretion were chosen for further study. For BFA assays, monolayers were rinsed thoroughly to remove hGH from the medium at the start of the experiment. Samples were taken immediately to determine the residual concentration in the medium. Following this, BFA (1 or 3 μg/ml) was added to the medium of cell monolayers and medium samples were removed and tested for hGH at various times. At the completion of the experiment, cells were lysed with Triton X-100 and tested for intracellular hGH activity. Triplicate samples were taken in all cases, and the averages were plotted.

Analysis of HSV-1 gD. This procedure was adapted from that of Saraste et al. (28). Briefly, monolayers of BFA-treated and untreated infected cells were harvested by trypsinization and washed extensively with cold DMEM containing 10% FBS. All further steps were done in the cold. Cells were pelleted by centrifugation and suspended in 4 volumes of homogenization buffer (0.25 M sucrose, 10 mM Tris [pH 7.4], 10 mM KCl, 1.5 mM $MgCl_2$). After 10 min on ice, the cells were homogenized with 20 strokes of a tightly fitting Dounce homogenizer. Nuclei and cell debris were

removed from the homogenate by centrifugation. The pellet was then suspended in homogenization buffer and centrifuged, and the supernatants were combined. This extract was centrifuged at 10,000 × *g* for 20 min to remove mitochondria. The postmitochondrial supernatant was then decanted and layered on top of 5 ml of 0.33 M sucrose, which in turn was layered over 1 ml of 2 M sucrose. This was centrifuged at 25,000 rpm in an SW41 rotor for 1 h. Approximately 600 μl of the turbid band at the 2 to 0.33 M interface was removed with a syringe. This total microsome sample was made 50% in sucrose with addition of 2.4 ml of 2 M sucrose and added to an SW41 tube. The following solutions (wt/wt) were layered over the top: 1 ml of 45% sucrose; 1.5 ml each of 40, 35, 30, and 25% sucrose; and 2 ml of 20% sucrose. These step gradients were then centrifuged at 170,000 × *g* for 19 h. Following centrifugation, 1-ml fractions were collected from the bottom of the tube. A 0.3-ml volume of each fraction was diluted with 0.5 ml of distilled H_2O and centrifuged at 436,000 × *g* for 10 min. The supernatants were discarded, and the pellets containing the membranes were suspended in 40 μl of endo H buffer (50 mM sodium citrate [pH 5.5], 0.5% sodium dodecyl sulfate, 0.25% β-mercaptoethanol, 1 mM NaN_3, heated at 100°C for 5 min, and centrifuged. Each supernatant was divided into two tubes containing 20 μl of distilled H_2O. The material was then digested or mock digested, respectively, for 18 h at 37°C by addition of 1 mU of endo H (Boehringer Mannheim) or 1 μl of distilled H_2O. Samples were subjected to polyacrylamide gel electrophoresis, and HSV-1 gD was detected on Western immunoblots by using an Immuno-Select Kit (BRL, Burlington, Ontario, Canada), which employs a biotinylated intermediate antibody and a streptavidin-alkaline phosphatase detection assay.

BFA chase experiment. Monolayers of L cells were infected with HSV-1 (MOI, 10), and various concentrations of BFA were added at 2 h p.i. Samples of medium were collected at 4, 8, 12, and 18 h p.i. Medium containing BFA was replaced with normal medium at 18 h p.i., and samples were collected at hourly intervals. All samples were diluted serially and used to inoculate monolayers of Vero cells growing in 96-well dishes. Titers were determined from the highest dilution that showed a cytopathic effect.

BFA sensitivity period. Cells growing in monolayers were infected with HSV-1 (MOI, 10). At various times p.i., BFA (3 μg/ml) was added for 4-h periods. Following this treatment, monolayers were washed three times with DMEM to remove BFA and incubated with fresh DMEM containing 10% FBS. At 20 h p.i., the extracellular medium was collected and the HSV-1 titer was determined by plaque assay on Vero cells. For the 16- to 20-h samples, HSV-1 titers were determined by using the BFA-containing medium. Control cell monolayers were also prepared and analyzed in this way.

RESULTS AND DISCUSSION

Inhibition of HSV-1 propagation by BFA. To determine whether BFA inhibits HSV-1 propagation, monolayers of mouse L cells were infected with HSV-1 and BFA was added at 2 h p.i. Growth media from BFA-treated and untreated cells were sampled at 4-h intervals, and virus titers were determined. The results of this analysis (Fig. 1A) revealed that HSV-1 propagation was sensitive to as little as 0.3 μg of BFA per ml in the culture medium, resulting in a delay in the appearance of infectious virions in the extracellular medium. At this concentration, virus titers reached

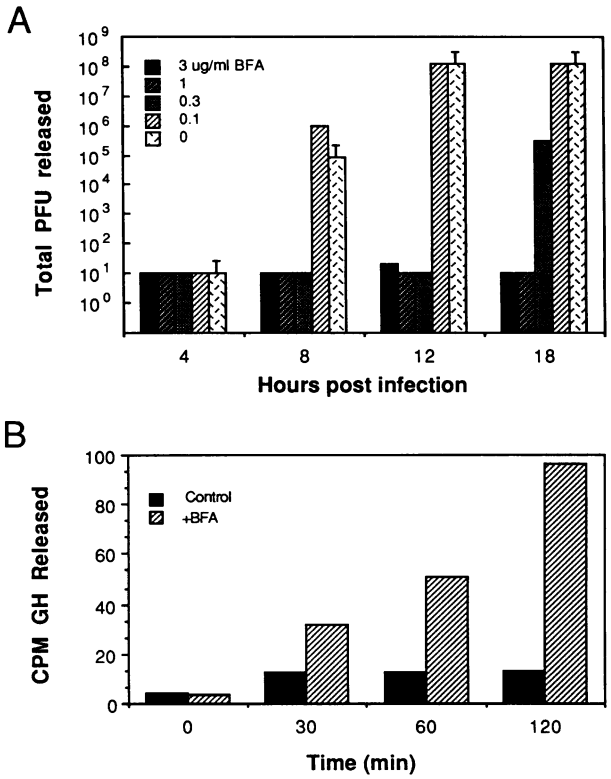


FIG. 1. Effects of BFA on secretion. (A) Monolayers of L cells were infected with HSV-1 (MOI, 10) and incubated from 2 h p.i. with various concentrations of BFA, as indicated. The extracellular medium was sampled to determine the number of PFU released in the presence of BFA. The titers were measured by limiting dilution and plaque assay. The error bars are shown once per time point but pertain to each member in the set. (B) Monolayers of L cells were transfected with pXGH5, which contains the gene for hGH driven by the mouse metallothionein I promoter. After 2 days, the monolayers were rinsed three times with medium to remove hGH and fresh medium was added. BFA (1 μg/ml) was added as indicated, and the extracellular medium was taken at the times shown. The amount of hGH in each sample was determined from triplicate samples, and the average was plotted. CPM, 10^2 counts per minute.

0.1% of the normal yield of untreated controls by 18 h p.i. The delayed appearance of virus in the medium could be explained if BFA was degraded during incubation to concentrations that were no longer effective at inhibiting viral propagation. Treatment with 1 or 3 μg of BFA per ml resulted in a 7-order-of-magnitude decrease in virus yield. This is a minimum estimation of inhibition, because the BFA-treated samples did not increase above the background during the experiment.

To test whether the low concentrations of BFA used in this study were sufficient to block cellular protein secretion, cell monolayers were transfected with a plasmid containing the gene for hGH driven by the mouse metallothionein I promoter (29). hGH is a nonglycosylated secretory protein and is useful for measuring the effects of BFA on secretion apart from its effects on glycoprotein processing. Release of hGH into the growth medium was monitored by radioimmunoassay at 2 days posttransfection. The results (Fig. 1B) indicate that 1 μg of BFA per ml blocked hGH secretion from transfected cell monolayers within 30 min after addition, and no further hGH was released during the 2 h of

sampling. The results obtained with 3 μg of BFA per ml were identical (data not shown). This reduction in secretion was not caused by a failure of BFA-treated cells to synthesize hGH, since hGH accumulated intracellularly during BFA treatment. The results shown in Fig. 1B were obtained with uninfected cells but were similar to data obtained with HSV-infected cells. In infected cells, hGH release from both BFA-treated and untreated cells was diminished by 40% compared with that of uninfected cells, likely because of the inhibitory effects of viral infection on host cell macromolecular synthesis. We conclude that the low concentrations of BFA used in this study were effective at blocking protein secretion.

To evaluate the effects of BFA on intracellular infectious particle production in infected cells, extracts were prepared from BFA-treated and untreated cells at 8 and 18 h p.i. and the virus titer of each sample was determined by using a plaque assay on Vero cells. At 8 h p.i., untreated cells accumulated 6 PFU per cell whereas cells treated with 3 μg of BFA per ml contained 6×10^{-4} PFU per cell. By 18 h p.i., untreated cells contained 270 PFU per cell while BFA-treated cells contained 1.2×10^{-3} PFU per cell, which represents a greater than 5-order-of-magnitude reduction in the intracellular titer compared with untreated control cells. More importantly, the infectious virus concentration inside BFA-treated cells did not increase substantially during the 8- to 18-h interval. We conclude from these results that BFA prevents accumulation of infectious particles in HSV-infected cells.

Inhibition of viral egress by BFA. Previous studies have shown that perturbations in the secretory pathway inhibit HSV egress (1, 9). On the basis of these considerations, we anticipated that the reduction in protein transport from the ER to the Golgi complex caused by BFA would lead to a marked reduction in the release of viral particles from infected cells. To test this hypothesis, cell monolayers were infected with HSV-1 and treated with 3 μg of BFA per ml at 2 h p.i. Both BFA-treated and untreated cells were incubated with 50 μCi of [^{35}S]methionine per ml from 2 to 18 h p.i. to radiolabel the virions assembled during infection. The culture medium was harvested at 18 h p.i. and subjected to centrifugation on a 5 to 40% dextran gradient to isolate virions. Gradients were fractionated, and the radioactivity in TCA-insoluble material was measured. There was a peak of radioactivity in the control cell gradient (Fig. 2, No BFA, fraction 21) which corresponded to the peak of infectivity (data not shown). We conclude that these fractions contained the virions released into the medium during infection. By contrast, the medium from BFA-treated samples (Fig. 2, +BFA) did not contain a corresponding peak of radioactivity, indicating that release particle viral from infected cells was greatly reduced in BFA-treated cells.

Normalcy of nucleocapsid assembly in BFA-treated cells. Several possibilities could account for the inability of BFA-treated cells to release viral particles. It may be that nucleocapsids or other viral components were not synthesized correctly in BFA-treated cells, thus inhibiting viral particle assembly. Alternatively, if viral assembly was normal in BFA-treated cells, the block to egress could result from failure to transport the particles out of the cells. This would imply that vectorial transfer of viral particles from their site of envelopment in the nuclear membrane to the cell surface requires a mechanism that is sensitive to the action of BFA. Experiments were carried out to distinguish between these possibilities.

To determine whether nucleocapsids assemble in BFA-

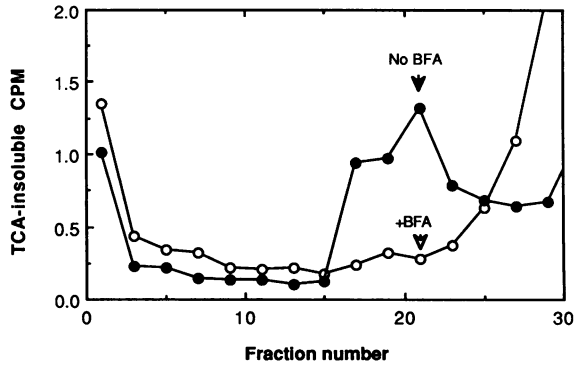


FIG. 2. Effects of BFA on viral egress. Monolayers of infected cells (MOI, 10) were treated with 3 μg of BFA per ml at 2 h p.i. and incubated along with untreated controls with 50 μCi of [^{35}S]methionine per ml until 18 h p.i. The extracellular medium was harvested and centrifuged to pellet virions and cell debris. Pellets were suspended in PBS and centrifuged on a 5 to 40% dextran gradient to separate virions from other radioactive material. The gradients were fractionated from the bottom, and the amount of TCA-insoluble radioactivity in each fraction was determined by scintillation counting (see Materials and Methods). CPM, 10^2 counts per minute.

treated cells, cell monolayers were infected with HSV-1 and treated with BFA from 2 to 18 h p.i. During this time, cells were labeled with 100 μCi of [^{35}S]methionine per ml to allow detection of nucleocapsids. Cells were then lysed in buffer containing low concentrations of Nonidet P-40 and Na-deoxycholate, which solubilizes cytoplasmic components while leaving nuclei largely intact (32a). The nuclei were pelleted from this extract and suspended in fresh buffer. These nucleus- and cytoplasm-enriched extracts were sonicated and centrifuged separately on 10 to 40% sucrose gradients to isolate nucleocapsids. The TCA-insoluble counts in gradient fractions of BFA-treated and untreated cells are shown in Fig. 3. Peaks of radioactivity in nucleocapsids were visible for both the cytoplasm-enriched (Fig. 3A, fraction 13, 10-ml gradient, 0.3-ml fractions) and nucleus-enriched (Fig. 3B, fraction 5, 8-ml gradient, 0.5-ml fractions) samples. On the basis of radioactivity, the nucleus-enriched fraction contained approximately 20% of the total nucleocapsids in the cell. There was no significant difference between the numbers of cytoplasmic nucleocapsids isolated from BFA-treated and untreated cells (Fig. 3A). Similarly, the number of nucleocapsids was reduced only slightly in the nuclei of infected cells treated with BFA (Fig. 3B). Because the extracts were treated with detergents prior to isolation of the nucleocapsids, it is likely that the cytoplasmic fraction would include nucleocapsids that were present in the perinuclear space. Experiments in which whole-cell extracts were analyzed were also performed. In these studies, BFA-treated and untreated cells contained similar numbers of nucleocapsids, as assessed by radioactivity (data not shown). To confirm that these isolation procedures yielded samples enriched in nucleocapsids, cell extracts were analyzed by negative staining in an electron microscope (data not shown). No substantial differences were observed in the gross morphology or the concentration of nucleocapsids isolated from BFA-treated and untreated cells.

Accumulation of enveloped viral particles in the perinuclear space of BFA-treated cells. One explanation to account for the paucity of infectious particles in the BFA-treated cells could be that BFA interfered with nucleocapsid envelop-

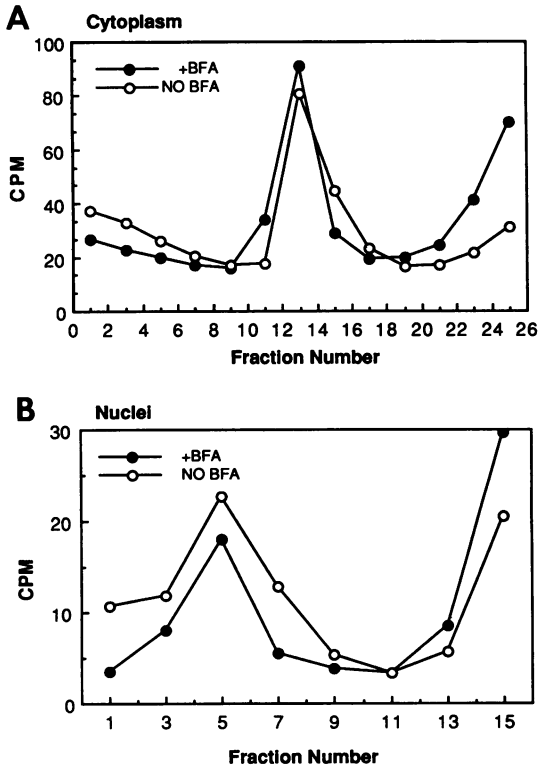


FIG. 3. Effects of BFA on nucleocapsid assembly. Monolayers of infected cells (MOI, 10) were treated with 3 μg of BFA per ml at 2 h p.i. and incubated along with untreated controls with 100 μCi of [^{35}S]methionine per ml until 20 h p.i. The monolayers were washed extensively and incubated with Nonidet P-40 lysis buffer (see Materials and Methods). After 15 min on ice, the nuclei were pelleted. Postnuclear supernatants and nuclear fractions were sonicated, and urea was added to 0.5 M to free nucleocapsids from cellular structures. Samples were centrifuged through a 10 to 40% sucrose gradient, and fractions were collected from the bottoms of the tubes. The amount of TCA-insoluble radioactivity in each fraction was determined by scintillation counting. The data for nucleocapsids isolated from cytoplasm-enriched fractions (A) and nucleus-enriched fractions (B) are shown. CPM, 10^2 counts per minute.

ment within the cells. To investigate this, monolayers of BFA-treated and untreated infected cells were prepared for electron microscopy at 8, 12, and 18 h p.i. Several hundred cells were analyzed in two separate experiments. At 8 h p.i., control L cells contained numerous enveloped HSV-1 particles enclosed in membranous vesicles in the cytoplasm (Fig. 4A). This was also true of untreated cells at 12 and 18 h p.i. (data not shown). By contrast, BFA-treated cells failed to accumulate enveloped particles in the cytoplasm at any of the times analyzed. Movement of particles to the cytoplasm of BFA-treated cells was not blocked, however, and nonenveloped nucleocapsids were clearly visible in this region of the cell (Fig. 4B). We conclude that BFA strongly inhibits the appearance of enveloped particles in the cytoplasm during infection. We infer from these data that this accounts for the failure to detect large numbers of infectious particles in BFA-treated cells at 8 or 18 h p.i.

Further analysis of the nuclei of BFA-treated cells revealed that the failure to detect enveloped particles in the cell cytoplasm was not due to a block in the mechanism of envelopment at the inner nuclear membrane. Enveloped particles were abundant in BFA-treated cells and accumulated principally in an intranuclear region likely to be the perinuclear space of infected cells (Fig. 4D to F). The particles occupied a region of the cell which lacked ribosomes, suggesting that the cellular compartment enclosing these particles was not a pocket of cytoplasm invaginating into the nucleus. This pattern of intranuclear accumulation was only rarely observed in untreated cells (Fig. 4C). Moreover, it cannot be determined from this study whether the particles shown in Fig. 4C and D were confined to the same cellular compartment.

One explanation for the aberrant accumulation of enveloped particles in the perinuclear space could be that the normal route of transport for newly enveloped particles is from the ER to the Golgi complex and that this pathway is blocked by BFA. In this regard, large regions of the cytoplasm that were contiguous with the perinuclear space were mostly devoid of enveloped or nonenveloped viral particles (Fig. 4F). From these considerations, it appears that BFA blocks the movement of newly enveloped particles from a position proximal to the nucleus in the perinuclear space to a more distal location in the ER membranes. In many BFA-treated cells, an unusually high concentration of nonenveloped particles was found immediately adjacent to the pockets of enveloped particles in the perinuclear space (Fig. 4E). This phenomenon was detected in most of the cells which contained enveloped particles. Our interpretation of Fig. 4E is that newly enveloped particles accumulated in the perinuclear space and then fused with the outer nuclear membrane to enter the cytoplasm. This pathway into the cytoplasm would result in loss of intracellular infectivity and is consistent with a mechanism wherein BFA induces passage of newly enveloped particles into the cytoplasm of infected cells. BFA-treated cells at 12 and 18 h p.i. were indistinguishable from those at 8 h p.i., except that at 18 h p.i. the cytoplasm of BFA-treated cells contained extensive regions of distended cytoplasmic membranes which were devoid of viral particles (data not shown).

BFA-altered processing of HSV-1 glycoproteins in infected cells. It is well established that BFA induces alterations in glycoprotein processing and transport in mammalian cells (5, 7, 10, 11, 15, 17, 24, 25, 34). It is also known that changes in glycosylation can have a profound effect on HSV propagation (2, 9, 22, 23, 26, 31). To study the effects of BFA on viral glycoprotein processing in HSV-1-infected cells, cytoplasmic membranes were isolated and the HSV-1 gD contained in those membranes was incubated with endo H and analyzed on Western immunoblots. Because endo H cleaves high-mannose chains but not complex oligosaccharides, it serves as a useful probe for N-linked glycoprotein processing (13). HSV-1 gD contains three sites for N-linked oligosaccharides (3) and two O-linked chains (30). Moreover, the precursor forms of gD contain high-mannose oligosaccharides sensitive to endo H that become resistant to endo H as the oligosaccharide moieties are processed to more complex forms in the Golgi complex. Monolayers of infected cells were treated with 3 μg of BFA per ml from 2 h p.i. and harvested at 13 h p.i. Total microsomes were isolated from the cells and centrifuged in discontinuous sucrose gradients to separate heavy from light membrane fractions. Samples of membranes were collected from the gradients, and the proteins contained in the fractions were subjected to digestion with endo H. Following polyacrylamide gel electrophoresis of digested and mock-digested samples, the polypeptides were transferred to nitrocellulose and HSV-1 gD was detected by using an anti-gD monoclonal antibody. Figure 5

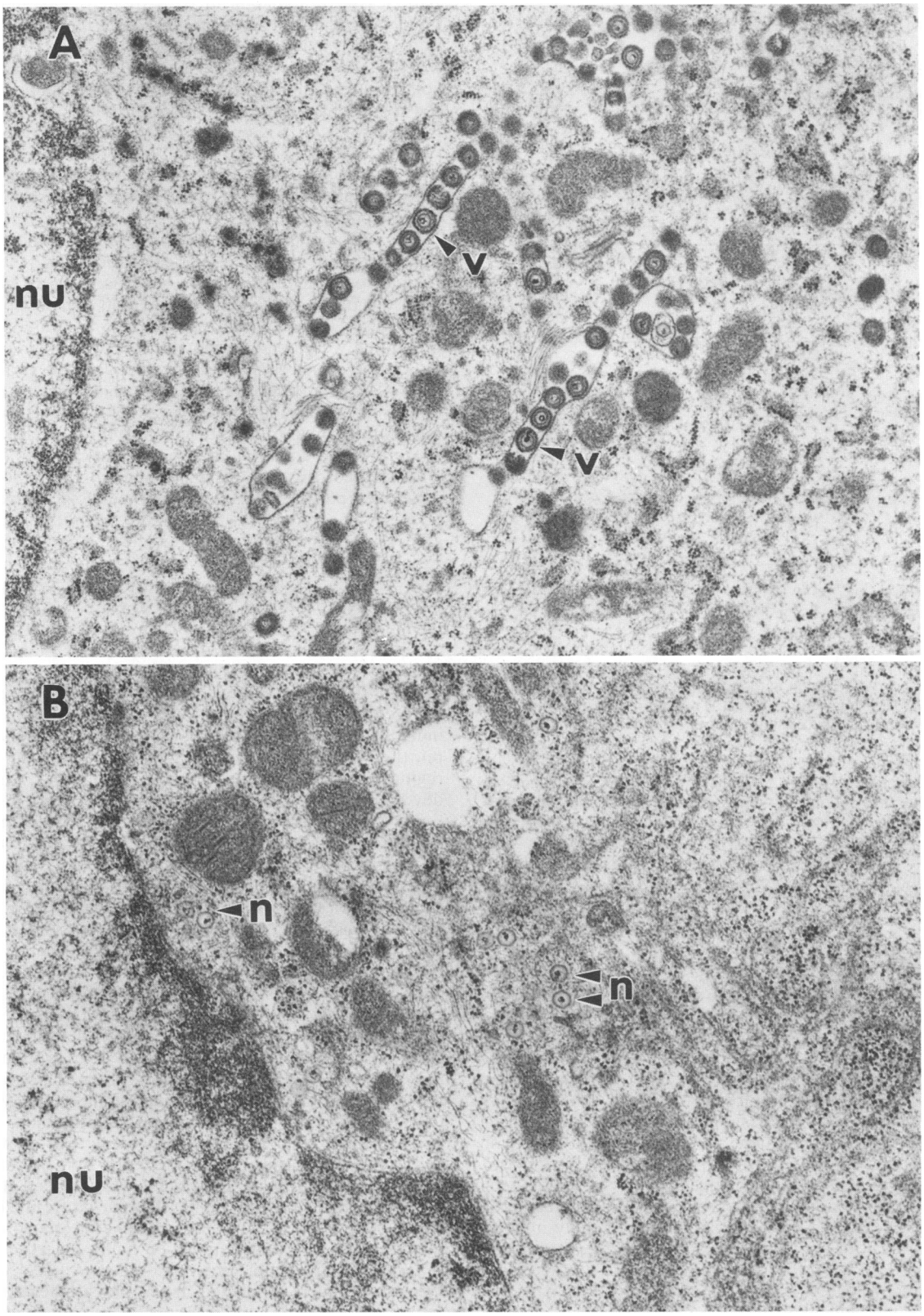
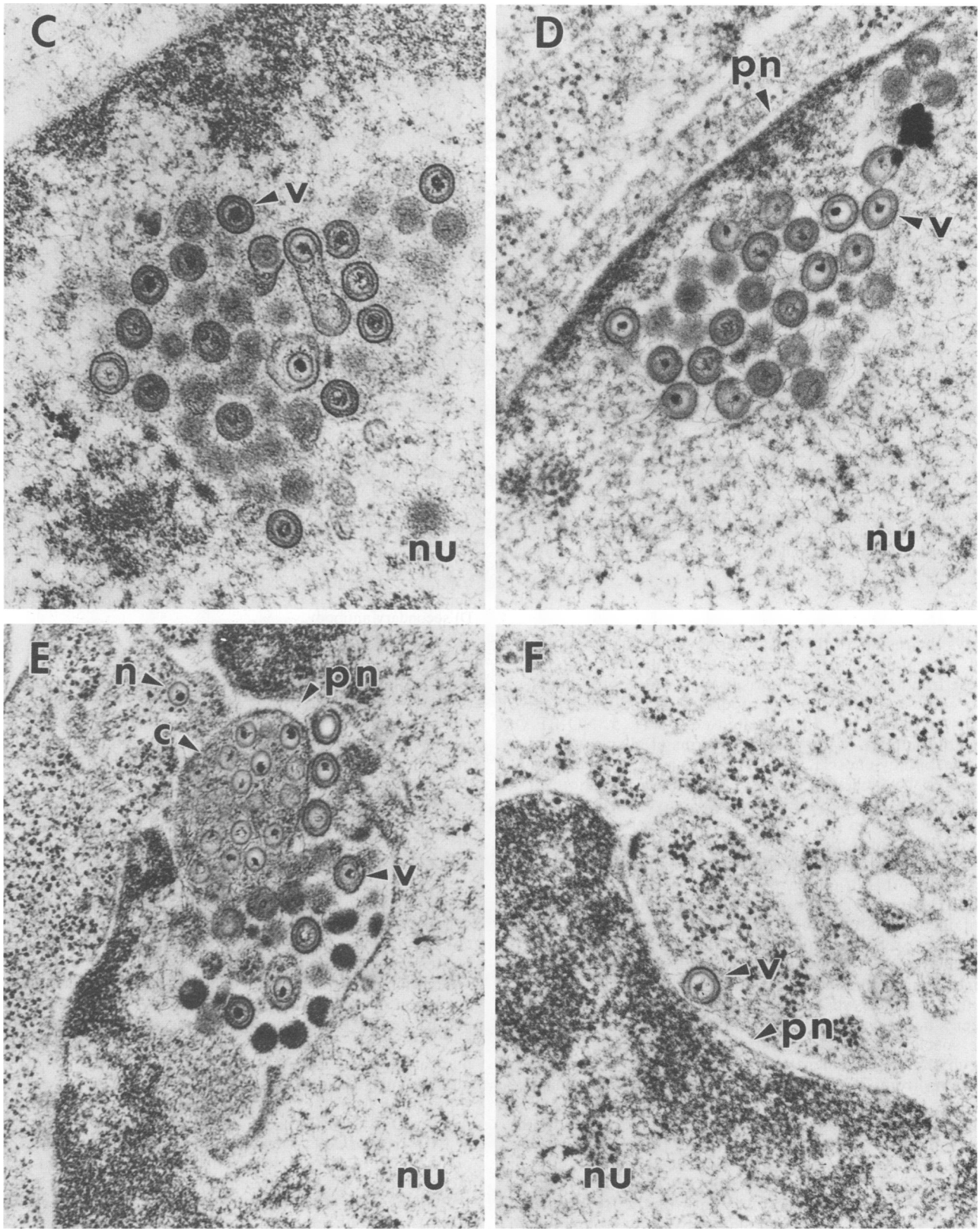


FIG. 4. Electron microscopy of BFA-treated and untreated cells. Monolayers of L cells were grown on Millicell HA inserts (Millipore) for 24 h prior to infection with HSV-1 (MOI, 10). Following inoculation, fresh DMEM containing 10% FBS was added and the infection was allowed to proceed for 8, 12, or 18 h. For treated samples, BFA (3 μg/ml) was added at 2 h p.i. and remained in the medium for the duration of the incubation. Cell monolayers were then rinsed, fixed in glutaraldehyde, embedded, and sectioned. All images shown are for infected cells harvested at 8 h p.i. (A) Cytoplasm of HSV-1-infected L cells containing enveloped viral particles. (B) Cytoplasm of HSV-1-infected L cells



treated with BFA containing nonenveloped particles. (C) Nucleus of an HSV-1 infected L cell containing enveloped particles in an intra- or perinuclear region. (D) Nucleus of an HSV-1-infected L cell treated with BFA containing enveloped particles in the perinuclear space. (E) Perinuclear space of a BFA-treated cell containing enveloped particles. (F) Viral particle in the perinuclear space of a BFA-treated cell. nu, Nucleus; v, enveloped virus particle; n, nucleocapsid; pn, perinuclear space; c, cytoplasmic invagination.

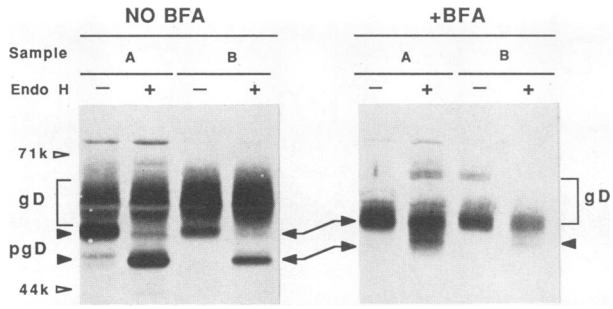


FIG. 5. Effects of BFA on HSV-1 gD processing during infection. Cells were infected with HSV-1 (MOI, 10), incubated without BFA (NO BFA) or with 3 μg of BFA per ml (+BFA), and harvested at 13 h p.i. Total microsomes were prepared and fractionated further into ER- and Golgi-enriched samples on sucrose gradients as described in Materials and Methods. Fractions were collected from the bottom of the tube, and samples of each fraction were subjected to digestion with endo H and analyzed by polyacrylamide gel electrophoresis and Western immunoblotting. HSV-1 gD was detected by using a monoclonal antibody and an avidin-biotin alkaline phosphatase detection system. The two fractions shown (A and B) contained most of the mass of the protein and had the highest activity of the ER marker NADPH cytochrome *c* reductase (data not shown). These fractions also had the largest proportion of endo H-sensitive forms. The positions of several molecular mass markers representing 71- and 44-kilodalton proteins are shown. The immature (pgD) and mature forms of gD are indicated. The positions of the major endo H-sensitive and -resistant forms are indicated by the arrowheads.

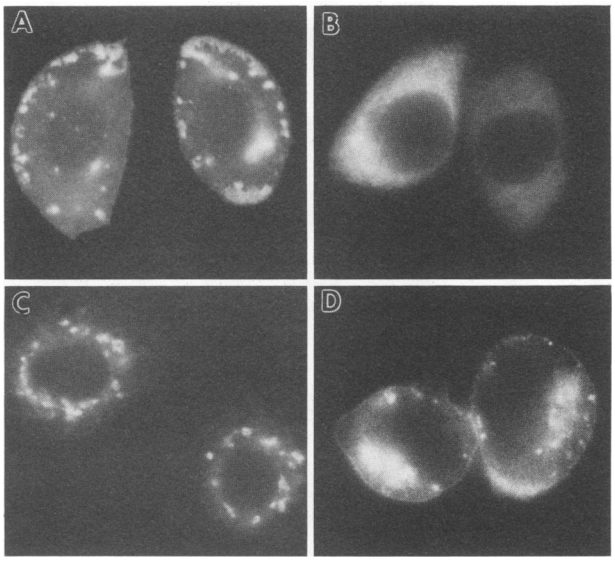


FIG. 6. Immunofluorescence analysis of HSV-1 gD in infected cells treated with BFA. Cells were grown on glass coverslips for 3 days to allow good adherence. Monolayers were infected with HSV-1 (MOI, 10). At 2 h p.i., 3 μg of BFA per ml was added to the medium (B, C, and D) or omitted for controls (A). At 13 h p.i., the medium was replaced with fresh medium without BFA. Sample monolayers were incubated at 37°C for 0 min (B), 30 min (C), or 3 h (D) and fixed with formaldehyde. Control cells (A) were fixed at 16 h p.i. gD was detected by using a monoclonal antibody and a fluorescein-conjugated (A and C) or rhodamine-conjugated (B and D) secondary antibody.

shows the results of this assay for several peak fractions (A and B) obtained from BFA-treated and untreated cells. These fractions, which comprised most of the protein mass, also contained the highest proportion of endo H-sensitive forms of HSV-1 gD. In untreated cells (Fig. 5, NO BFA), most of the mass of the precursor forms of gD was sensitive to digestion by endo H. There was also a substantial amount of material which was resistant to endo H digestion (gD), representing polypeptides that had traversed the medial Golgi cisternae en route to the plasma membrane. Fraction B, which included membranes of lower density than those in fraction A, also contained a mixture of endo H-resistant and -sensitive forms of gD.

In BFA-treated cells, by contrast, both fractions (A and B) contained partially processed forms of this glycoprotein, as judged by relative mobilities. The major species of gD exhibited a lower relative mobility than did the endo H-sensitive forms accumulating in untreated cells. A small amount of this material was endo H sensitive, and the digested species had lower relative mobilities than the endo H-sensitive species detectable in untreated cells. Although we did not characterize further the oligosaccharide structures in the fractions from BFA-treated cells, the observation that gD was largely resistant to endo H digestion indicates that most of the N-linked oligosaccharides on the HSV-1 glycoproteins were processed to intermediate forms by 13 h p.i. These results could be explained if BFA induced redistribution of Golgi processing enzymes into the ER of HSV-1-infected cells. This interpretation is consistent with the BFA-induced perturbations that have been shown to occur in uninfected cells as well (5, 15).

BFA-altered distribution of gD in HSV-infected cells. The results of these cell fractionation experiments suggest that gD distribution was perturbed in cells treated with BFA. To analyze this further, we examined the intracellular location of gD by indirect immunofluorescence. Cell monolayers were infected and treated with BFA from 2 h p.i. At 13 h p.i., BFA was removed and the cells were incubated in BFA-free medium for the times indicated in the legend to Fig. 6. To detect gD, the cells were fixed and subsequently stained with an anti-HSV-1 gD monoclonal antibody (Fig. 6). Examination of control cells (Fig. 6A) revealed punctate staining of gD at the periphery of the infected cells. Juxtanuclear staining, characteristic of the Golgi complex, was also evident. The plasma membrane was well defined in these cells, indicating that gD was present in this membrane as well. The staining at the periphery of the cells likely represents virion-containing vacuoles that were en route to the plasma membrane or plasma-membrane-associated gD.

Treatment of infected cells with BFA induced drastic alteration in the distribution of gD (Fig. 6B). In contrast to control cells, gD staining was homogeneous throughout the cell cytoplasm. Furthermore, gD was not detectable in the nuclear or plasma membrane. Instead, fine, evenly distributed reticular staining, characteristic of the ER, was evident. It appeared from these results that BFA induced accumulation of gD in the ER. The Golgi complex was not detectable in BFA-treated cells, suggesting that the membranes of this organelle were dispersed or that gD was not present in the membranes of the Golgi complex.

Evidence that some of the effects of BFA are reversible in HSV-1-infected cells can be found in the results of the experiment described in Fig. 6C, in which removal of BFA led to rapid redistribution of gD in the cells. By 30 min after removal of BFA (Fig. 6C), regions of discrete staining were visible in a perinuclear ring, suggesting that the contents of

the ER were transported to another cellular compartment during this time. This likely represents gD that was transported to the Golgi membranes. Some ER staining was also evident, as indicated by the reticular staining surrounding the nucleus. By 3 h after BFA removal, juxtanuclear staining was evident in a pattern characteristic of the Golgi complex (Fig. 6D). Small regions of punctate staining were also present at the cell periphery, suggesting that the pathway of virion egress did function to some extent. The plasma membrane was also clearly evident by this time, indicating that gD was transported to this membrane.

Lack of full reversibility of the effects of BFA on HSV-1 propagation. The results obtained so far indicated that nucleocapsids were assembled and enveloped in BFA-treated cells and that gD was processed to endo H-resistant forms during infection, despite being confined largely to the membranes of the ER. From these considerations, it is likely that all of the HSV-1 glycoproteins were synthesized and processed to some extent in BFA-treated cells. If the presence of these viral components is sufficient for mature virion formation, the effects of BFA on HSV-1 propagation should be reversible after the block to secretion is relieved. In this regard, many studies have concluded that the effects of BFA on secretion and dissolution of the Golgi complex are reversible within minutes after removal of BFA (see references 5, 14, 15, and 20 for examples). To investigate whether the ability to secrete infectious viral particles was regained after BFA was removed, cells were infected, treated with BFA at 2 h p.i., and incubated for a further 16 h. At this time, BFA was washed out and the number of infectious virions released into the extracellular medium during the next 3 h was determined. The results of this analysis (Fig. 7A) revealed that cells treated with noninhibitory concentrations of BFA (0.03 and 0.1 μg/ml) released large quantities of infectious particles into the medium within 30 min after BFA removal. Controls with no BFA were indistinguishable from those treated with 0.03 μg of BFA per ml (data not shown). This rate of virus release was not matched in the monolayers treated with higher concentrations of BFA, however. In these cells, there was a lag in the appearance of PFU in the extracellular medium which likely reflected the time taken for the Golgi to reorganize. By 3 h after removal of BFA from the 1-μg/ml samples, 0.1% of the expected PFU was present in the medium, although a gradual increase in extracellular PFU was observed during the 3 h of the experiment. This was the highest virus yield observed after treatment with 1 μg of BFA per ml. Other experiments have yielded as few as 10^4 PFU during this period (data not shown).

hGH secretion was analyzed next to determine whether the effects of BFA on this process were reversible. To do this, monolayers of infected L cells transfected with hGH expression plasmid pXGH5 were treated with BFA from 10 to 18 h p.i., which completely blocked hGH release from the cells. Following this incubation, BFA was washed out and the rate of hGH secretion was monitored by radioimmunoassay. In this experiment, the ability to secrete hGH was recovered within 30 min (Fig. 7B). By 60 min, more hGH had been released from BFA-treated cells than from untreated cells, suggesting that a pool of hGH had built up intracellularly prior to BFA release. This was confirmed by measuring the intracellular hGH concentration at the start of the experiment. These data indicate that the block to protein secretion was reversed within 30 min after removal of BFA.

BFA-sensitive period of herpesvirus infection. Given the dramatic effects of BFA on viral propagation, we wanted to determine whether HSV-1 was sensitive to the action of BFA at all times postinfection or if there was a BFA-sensitive period of virus production. Cell monolayers were infected and treated with 3 μg of BFA per ml for 4-h periods from 0 to 20 h p.i. After removal of BFA from the monolayers, the medium was replaced and collected at 20 h p.i. to determine the effects of BFA treatment on virus release during the 20 h of the experiment. The results of these studies revealed that infected cells were most sensitive to BFA during the period of exponential virus release that occurred between 8 and 12 h p.i. (Fig. 8A and B). Treatment of the cells with BFA during this interval led to 88% reduction in the virus yield by 20 h p.i. By contrast, treatment of cells between 0 and 4 and 16 and 20 h p.i. had little effect on the ultimate virus yield.

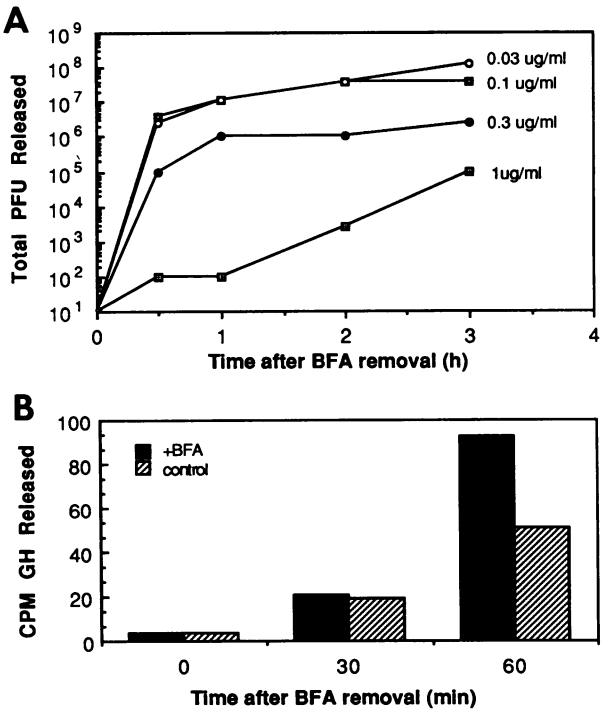


FIG. 7. Determination of the rate of virus release following BFA removal. (A) Cells were infected and treated as described in the legend to Fig. 1. At 18 h p.i., cell monolayers were washed thoroughly to remove BFA. Samples of the extracellular medium were taken at various times, and the HSV-1 titers were determined. (B) Cell monolayers secreting hGH after transfection with pXGH5 were infected with HSV-1 and treated with 1 μg of BFA per ml from 10 to 18 h p.i. Monolayers were then washed to remove BFA, and samples of the medium were removed at various times. hGH was detected by radioimmunoassay. CPM, 10^2 counts per minute.

The results (Fig. 7 and 8) demonstrated that the effects of BFA on virus propagation were not fully reversible, despite the observation that the secretory pathway for hGH functioned normally within 30 min after BFA removal. One possibility to account for the poor recovery is that the components required for virus maturation were no longer available after treatment with BFA. In this regard, we have shown that the distribution and processing of the HSV-1 glycoproteins were grossly altered during BFA treatment (Fig. 5 and 6). Considering the events of viral assembly and egress, the distribution of HSV-1 glycoproteins is likely to be

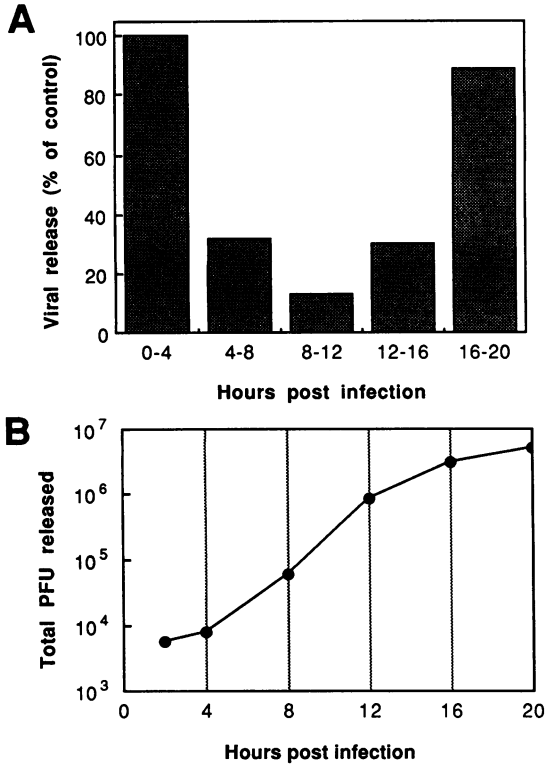


FIG. 8. Determination of BFA sensitivity during viral propagation. Monolayers of cells were infected with HSV-1 (MOI, 10) and treated with BFA (3 μg/ml) for 4-h intervals from 0 to 20 h p.i. Control monolayers were also analyzed in parallel experiments. Following treatment with BFA, the monolayers were washed and the medium was replaced with fresh DMEM containing 10% FBS. Samples of the medium were taken at 20 h p.i., and the HSV-1 titer was determined. (A) Virus yield from BFA-treated cells expressed as a percentage of the yield from untreated control cells. Duplicate samples were analyzed. (B) Virus yield from untreated control cells treated in the same manner as in part A. Virus titers from samples harvested at each time point were added together to reflect the cumulative yield during the 20 h of the experiment.

important for efficient assembly of progeny virions. It has been proposed that HSV-1 virions begin assembly in the nucleus, become enveloped as they exit the nucleus, and are carried to the ER and Golgi apparatus and then to the plasma membrane in vesicles similar to those which carry newly made membrane glycoproteins and secreted proteins (9). Budding occurs at the inner nuclear membrane, where immature glycoproteins are more prevalent than the processed mature forms (4). Because virions released from infected cells contain mature glycoproteins, it is likely that the envelope glycoproteins are modified in the Golgi apparatus while resident in the virus membrane (9, 32). A second model proposes that cytoplasmic vacuoles or the cisternae of the *trans* Golgi, and not the nuclear membrane, are the predominant sites of envelopment within infected cells (12, 18, 27). Both models predict that perturbations in the distribution of viral glycoproteins or other components of the secretory apparatus could hinder viral assembly. In this regard, it has been shown previously that BFA does not grossly interfere with the appearance of proteins in the nuclear membrane (6), and our electron micrographs (Fig. 4) are consistent with the conclusion that viral budding at the nuclear membrane is not prevented by the action of BFA on the host cell.

Another explanation for the poor recovery of viral egress after BFA treatment is that the redistribution of Golgi enzymes into the ER induced by BFA causes newly enveloped viral particles to be mistargeted in the cell. This could occur if specific targeting signals existed for HSV transport through the secretory pathway en route out of the cell. Alternatively, the BFA-induced redistribution of Golgi proteins into the ER that was demonstrated (Fig. 5 and 6) may cause viral particles to come into contact with proteins that they do not usually encounter. Both of these effects could render viral particles noninfectious by preventing them from migrating from the perinuclear space into the host cell secretory pathway or by causing them to pass directly into the cell cytoplasm. Moreover, both of these phenomena would be expected to be irreversible in infected cells. Consideration must also be given to the possibility that uncharacterized properties of BFA induce all or part of the phenotype that we have observed. The likelihood of this cannot be addressed until the mechanism of action and cellular targets of BFA are defined.

What can account for the observation that nucleocapsids are present in the cytoplasm of BFA-treated cells? It is clear that viral particles are able to bud into the perinuclear space which is contiguous with the membranes of the ER. If enveloped particles normally traverse the ER-to-Golgi pathway, it may be that BFA blocks this movement. Our data are consistent with the possible existence of a minor BFA-insensitive pathway wherein nucleocapsids bud into the cytoplasm from the perinuclear space, thus bypassing the ER-to-Golgi route. Evidence for such a BFA-resistant pathway also derives from a recent study of the transport of cholesterol and vesicular stomatitis virus G protein through the secretory apparatus (34). Cells containing both vesicular stomatitis virus G protein and cholesterol in the ER were treated with BFA, and the transport of these molecules was monitored. BFA did not impede cholesterol transport to the plasma membranes whereas it strongly inhibited vesicular stomatitis virus G protein transport. It was proposed that the cholesterol-containing vesicles bypassed the Golgi apparatus and proceeded to the plasma membrane, whereas the G protein-containing vesicles followed the Golgi route to the cell surface (34). It may be that newly budded viral particles in the perinuclear space are shunted into a nonproductive alternative pathway in HSV-1-infected cells when secretion is blocked. Although this could account for the presence of nonenveloped particles in the cytoplasm, we have already demonstrated that these particles are neither infectious, enveloped, nor capable of exiting from host cells.

The effects of BFA that we observed are distinct from those caused by treatment of cells with the ionophore monensin (9), which also interferes with productive HSV-1 infection. Monensin has been shown to interfere with processing of N-linked oligosaccharides, addition of O-linked sugars to HSV-1 glycoproteins, transport of HSV-1 glycoproteins from the Golgi apparatus to the plasma membrane, and virion egress (9). Monensin induces reduction of nucleocapsid assembly in infected cells, although infectious virus accumulates intracellularly to levels roughly 5 to 10% of those in untreated cells. These studies demonstrate that virions mature to enveloped, infectious particles in monensin-treated cells, despite monensin-induced alterations in the processing of HSV-1 glycoproteins and the reduction in the transport of glycoproteins and progeny virus to the cell surface (9). The most striking phenomenon observed in

monensin-treated cells is accumulation of enveloped particles in cytoplasmic vacuoles, and it has been proposed that these vacuoles may be derived from normal intermediates in the pathway of virion egress (9). Similar vacuoles containing HSV-1 have also been found in the cytoplasm of secretory mutant gro29 at late times p.i. (1). By contrast, we have shown that BFA blocks viral maturation and egress at an earlier point in the viral life cycle by preventing transport of enveloped particles to any peripheral cytoplasmic location. These considerations suggest that BFA interferes with a host cell component that is distinct from the component(s) affected by monensin or defective in gro29 mutant cells.

Considering the known action of BFA, the results of our investigations suggest that HSV-1 requires net anterograde movement of molecules from the ER to the Golgi complex during infection for efficient viral propagation. The observation that the effects of BFA on viral propagation are not fully reversible in HSV-1-infected cells suggests that viral particles require a functional secretory pathway when they assemble to enter the cytoplasm of a host cell in an enveloped form. The effects of BFA are unique with regard to HSV-1 propagation, and it should continue to be a useful reagent for defining the host cell functions that facilitate production and release of mature viral particles during herpesvirus infection.

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