BREFELDIN A ARRESTS THE MATURATION AND EGRESS OF HERPES SIMPLEX VIRUS PARTICLES DURING INFECTION

By

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ABSTRACT

Herpes Simplex Virus (HSV) requires the host cell secretory apparatus for the maturation and egress of newly synthesized viral particles. Not only do viral glycoproteins rely on the host ER and Golgi compartments for their proper processing, it is believed that enveloped particles are transported through these same organelles for their export out of the cells. Brefeldin A (BFA) is a compound that induces retrograde movement of material from the Golgi apparatus to the ER and causes the disassembly of the Golgi complex. In this study, the effects of BFA on the propagation of HSV-1 in infected cells were examined. Release of viral particles from infected cells was inhibited by as little as 1 μg/ml BFA. Further analysis revealed that BFA did not affect the normal assembly of viral nucleocapsids, but did block the movement of newly-enveloped particles from the nucleus into the cytoplasm. Naked nucleocapsids were found in the cytoplasm of infected cells treated with BFA, however, these particles were neither infectious, nor were they released from the cells. Although BFA altered the distribution of viral glycoproteins in infected cells, this alteration was reversed within 2 hours after the removal of BFA. In contrast, the BFA-induced blockage to viral release was not fully reversed after BFA was removed and cells were allowed to recover in fresh medium for 3 hours. These findings indicate that the BFA-induced retrograde movement of material from the Golgi complex to the ER early in infection arrests the ability of the host cell to support the

maturation and egress of enveloped viral particles. Furthermore, exposure of infected cells to BFA during the exponential release phase of the viral life cycle can cause irreversible damage to the egressing particles. This suggests that productive growth of HSV-1 in infected cells relies on a series of events that, once disrupted by agents such as BFA, cannot be easily reconstituted.

ABBREVIATIONS USED:

BFA	Brefeldin A
CPE	cytopathic effects
DMEM	Dulbecco modified Eagles medium
endo H	Endoglycosidase H
ER	endoplasmic reticulum
GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
h.p.i.	hours post infection
HSV-1	herpes simplex virus type 1
Man	Mannose
MOI	multiplicity of infection
PBS	phosphate buffered saline
PFU	plaque forming units
RSB	reticulocyte standard buffer
STEM	scanning transmission electron microscope
TCA	trichloroacetic acid

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INTRODUCTION

Members of the family Herpesviridae are characterised as enveloped, icosahedral viruses containing double-stranded DNA genomes. Herpesviruses are widespread in nature and are found in most animal species (Roizman and Batterson 1986). Of the 80 different types, herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), cytomegalovirus (CMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7) are the herpesviruses that infect humans. These viruses cause a variety of diseases such as cold sores, varicella (chickenpox), and infectious mononucleosis, and can pose serious threats to the health of infected individuals. In addition to the lytic phase of their replication cycle, these viruses can also establish latent infections in the hosts, and can be reactivated after a long period of dormancy. The two most recently identified human herpesviruses, HHV-6, and HHV-7, are often isolated from T-lymphocytes of patients with lymphoproliferative diseases or AIDS (Josephs, Salahuddin et al. 1986; Salahuddin, Ablashi et al. 1986; Lusso, Markham et al. 1988; Takahashi. K., Sonoda et al. 1989; Frenkel, Schirmer et al. 1990). They in turn cause secondary diseases that can be fatal in these immuno-compromised individuals. The medical significance of human herpesviruses makes them one of the most intensely studied group of viruses worldwide.

This thesis focuses on the viral-host interactions that facilitate

the maturation and egress of HSV-1 in infected animal cells. In this regard, the salient features of the structure and life cycle of HSV-1 are presented below.

I. ULTRASTRUCTURE OF HSV-1 VIRIONS:

The virion of HSV-1, like all other herpesviruses, consists of four distinct morphological components (figure 1): 1) an electron-opaque core containing the viral DNA, 2) an icosahedral capsid enclosing the core, 3) an outer envelope surrounding the capsid, and 4) a tegument between the capsid and envelope (Roizman and Batterson 1986). The HSV-1 genome consists of linear, double-stranded DNA that is about 100 x 10^6 daltons in molecular weight. It contains two covalently linked components designated as L (long) and S (short), representing 82 and 18%, respectively, of the viral DNA. The capsid, which has a diameter of approximately 100 nm, is made up of 162 capsomeres. Pentameric capsomeres are found on the vertices of the icosahedral structure, while hexameric capsomeres comprise the faces. Electron microscopy on thin sections shows that the viral envelope has a typical trilaminar appearance, and appears to be derived from patches of altered cellular membranes. Numerous glycoproteins embedded in this lipid membrane are seen as spikes ranging from 8 to 24 nm in length (Stannard, Fuller et al. 1987). While the tegument has no distinctive features in thin section electron microscopy, it does contain viral proteins that are required for the initiation of viral gene expression early in the infection cycle of HSV-1.

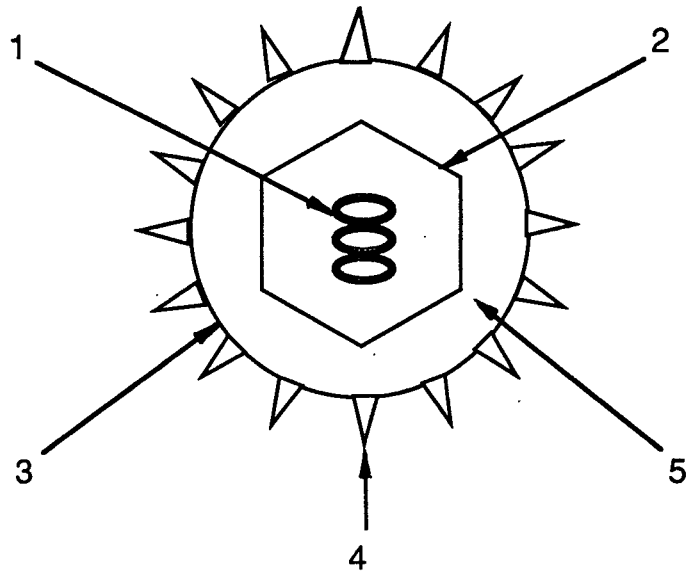


Figure 1. Schematic diagram of the ultrastructure of HSV-1.

1. viral DNA core
2. icosahedral capsid
3. viral envelope
4. glycoprotein spikes
5. tegument

II. OVERVIEW OF HSV-1 LIFE CYCLE:

The sequence of events leading to an infection starts by the attachment of the virus to the host cell and the penetration of an HSV-1 particle into the cell. Once the virus enters the cell, capsid uncoating occurs at the nucleus where viral gene transcription and DNA replication takes place. Viral particles are assembled in the nucleus, transported through the cytoplasm, and released from the infected cell. The length of the replication cycle of HSV-1 is relatively short compared to other herpesviruses such as CMV (Smith and DeHarven 1973). The entire process from the start of an infection to the first release of viral particles takes approximately 8 h for HSV-1 versus 4 days for CMV. The wide host cell range and the rapid multiplication cycle of HSV-1 makes it one of the best characterised herpesviruses.

A. Viral attachment, penetration, and uncoating:

To initiate an infection, HSV-1 particles attach to the cell surface through heparin-like cell-associated glycosaminoglycans (WuDunn and Spear 1989). After this primary binding process, stable attachment of virus to cell membrane likely occurs by interactions of viral components with specific receptors. Although such a receptor has not yet been found, ongoing research will likely identify this cell surface molecule in the near future.

After the virus is adsorbed to the cell membrane, penetration of virus is mediated by the fusion of viral envelope with host cell plasma membrane. This model of viral entry is supported by the

observation that HSV-1 envelope components are detected in the cell surface membrane immediately after viral penetration, and in the absence of viral gene expression (Para, Baucke et al. 1980). In addition, agents that raise lysosomal pH, such as ammonium ions and chloroquin, do not block the infectivity of HSV-1, suggesting that viral entry does not occur by receptor-mediated endocytosis (Holland and Person 1977).

Upon entry, the viral capsid is transported to the nuclear pores via the cellular cytoskeleton (Roizman and Batterson 1986). It is likely that the viral genome gains entry to the nucleus via the pores, although the mechanism of uncoating of the DNA has not been elucidated. One of the earliest events following uncoating is the shutoff of cellular protein synthesis (Read and Frenkel 1983; Fenwick 1984; Kwong and Frenkel 1987; Kwong, Kruper et al. 1988; Oroskar and Read 1989). This early inhibition of host protein synthesis is accomplished by a protein that is present in the virus particle. Frenkel and Kwong (Kwong and Frenkel 1987; Kwong, Kruper et al. 1988) found that a viral gene product, vhs, causes the disaggregation of cellular polyribosomes, and the degradation of host and viral mRNAs. This allows the newly-made viral mRNAs to out-compete host cell mRNAs for the available ribosomes. In addition to the virion-mediated shutoff of protein synthesis, a late secondary shutoff mechanism causes the degradation of cellular mRNA and reduces further the synthesis of host proteins (Fenwick 1984). In contrast to the early shutoff function, the delayed shutoff function is mediated by virally-encoded proteins synthesized in the infected cell. The combined effects of the early and delayed shutoff

processes inhibit completely the synthesis of host cell proteins, and favour the expression of viral genes.

B. Viral gene expression:

All herpesvirus genes are transcribed in the nucleus using host cell RNA pol II (Constanzo, Campadelli-Fiume et al. 1977), and viral mRNAs are translated in the cytoplasm by free or membrane-bound ribosomes. HSV-1 genes can be grouped into five families which are coordinately regulated (for review, see Knipe 1989). Expression of viral genes occurs in a cascade fashion and involves the sequential turning on and off of the transcription of different groups of genes. Early in the infection, the viral tegument protein, VP16, induces the expression of immediate-early (IE) genes in the absence of prior viral protein synthesis. VP16 is a major late viral phosphoprotein that is present in the tegument at approximately 400-600 molecules per particle (O'Hare and Goding 1988). At least three cellular proteins are believed to form a complex with VP16 before binding to viral DNA (Kristie, LeBowitz et al. 1989). One of these proteins has been identified as the cellular transcription factor OTF-1, which recognises the TAATGARAT (where R is a purine) sequence present on all IE genes (Kristie, LeBowitz et al. 1989). Binding of this complex to the IE promoter region allows the catalytic domain of VP16 to cis-activate the transcription of IE viral genes. Five infected cell polypeptides, ICP0, ICP4, ICP22, ICP27, and ICP47, are encoded by the IE genes, and they reach their peak rate of synthesis at about 2-4 hours post infection (h.p.i.). At least three of these proteins, ICP0, ICP4, and ICP27, are localized to the nucleus

and influence subsequent gene expression. ICP4 has also been shown to down-regulate IE gene expression by binding to viral DNA sequences and blocking transcription. Two groups of delayed-early (DE) genes are expressed under the induction of one or more IE gene products. These proteins, such as thymidine kinase, ICP8 (a DNA binding protein), and DNA polymerase, reach their peak of synthesis at about 5-7 h.p.i., and are involved principally in viral replication. Towards the end of the infection cycle, viral glycoproteins and structural proteins are made from two groups of late genes. The first group is transcribed prior to DNA replication, whereas the the second group requires DNA synthesis before it can be expressed. The expression of these five groups of viral genes is coordinately regulated such that genes belonging to the same family respond to similar regulatory mechanisms. In general, gene products serving similar functions in the HSV-1 life cycle are synthesized and regulated together, making the viral life cycle well organized and efficient.

C. Viral DNA replication, assembly and egress:

Replication of viral DNA requires many virally-encoded products and is thought to occur by a rolling circle mechanism using circular DNA templates (Roizman and Sears 1990). DNA synthesis can be detected at 3 h.p.i., and continues for another 9-12 hours (Roizman and Roane 1964; Roizman, Borman et al. 1965). When sufficient amounts of viral DNA and structural proteins are accumulated in the nucleus, capsid assembly begins by cleaving the concatameric progeny DNA, and packaging the genomes into empty

capsids (Roizman and Batterson 1986). These newly-formed nucleocapsids leave the host nucleus and journey through the cytoplasm to the cell surface for export (see Fig. 2).

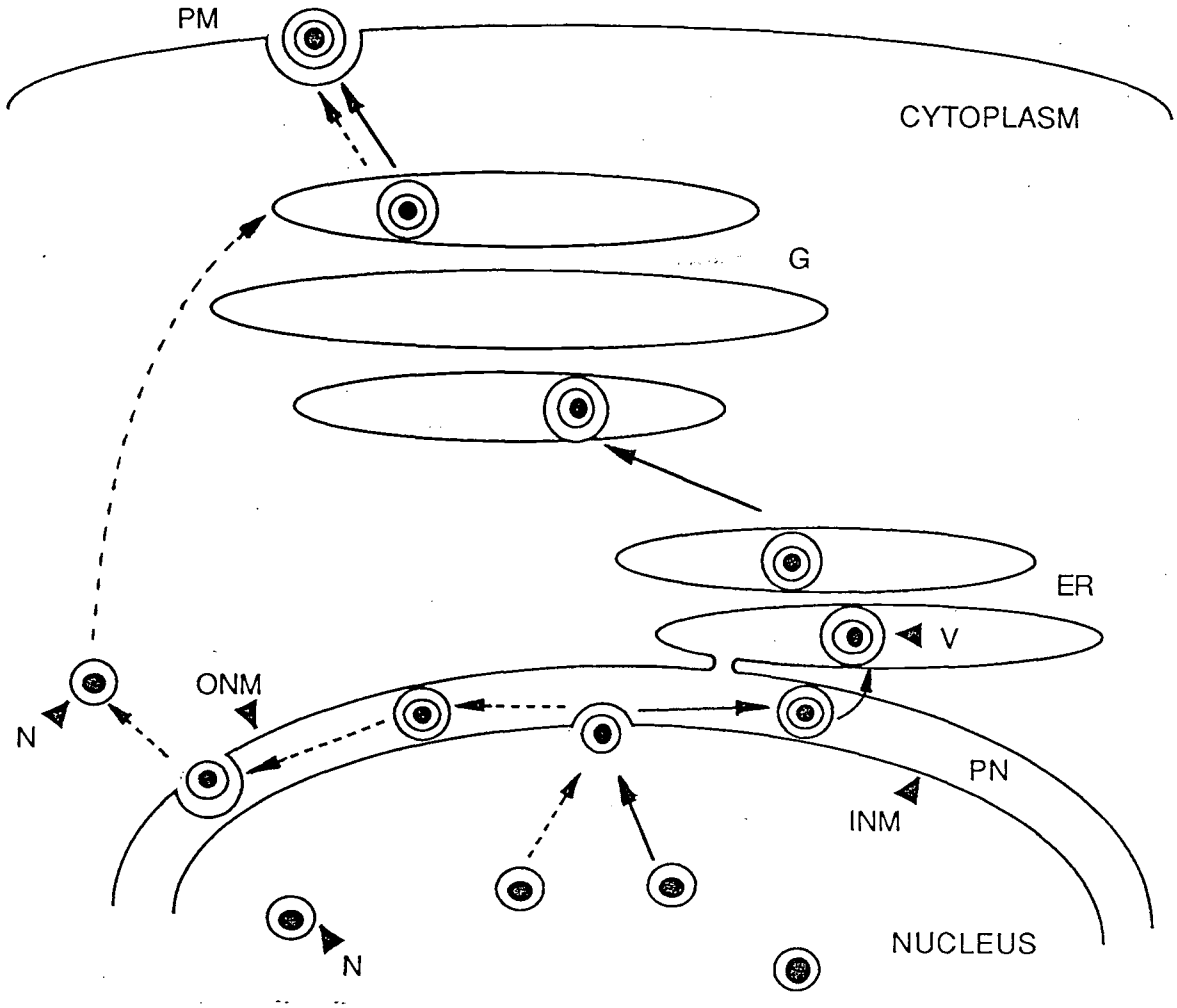
The assembled nucleocapsids accumulating in the nucleus exit this organelle by budding through the inner nuclear membrane into the perinuclear space (Nii, Morgan et al. 1969). Electron microscopy studies reveal that viral envelopes are derived from the inner lamellae of the nuclear membrane. The most widely accepted model of egress hypothesizes that enveloped particles in the perinuclear region are transported through the host ER and Golgi complex *en route* to the cell surface for release (Nii, Morgan et al. 1969; Johnson and Spear 1982). Enveloped particles in the nucleus contain immature glycoproteins on their membranes (Morgan, Rose et al. 1959), and these glycoproteins are processed as the particles traverse the host secretory system. An alternative model suggests that enveloped viral particles in the perinuclear space do not move to the ER. Instead, they de-envelope by fusing with the outer nuclear membrane and enter the cytoplasm as naked nucleocapsids (Stackpole 1969; Komuro, Tajima et al. 1989). Free viral glycoproteins are processed by the host ER and Golgi apparatus, and accumulate at the *trans* Golgi membranes where envelopment of the cytoplasmic nucleocapsids occur. Although the precise mechanism of egress is not clear, both models imply that the host cell secretory pathway plays a major role in the maturation of viral glycoproteins and the release of HSV-1 particles.

Figure 2. Schematic diagram of the egress pathway of HSV-1.

Abbreviations:	PM	plasma membrane
	G	Golgi apparatus
	ER	endoplasmic reticulum
	PN	perinuclear space
	INM	inner nuclear membrane
	ONM	outer nuclear membrane
	N	nucleocapsids
	V	enveloped virion

Pathway 1: (solid arrows) Nucleocapsids in the nucleus are enveloped as they bud through the inner nuclear membrane into the perinuclear space. Enveloped particles are then transported through the host ER and Golgi appartus for viral glycoprotein processing. Mature viral particles are released at the plasma membrane.

Pathway 2: (dashed arrows) Nucleocapsids are also enveloped at the inner nuclear membrane. However, these particles in the perinuclear space de-envelope when they fuse with the outer nuclear membrane, and enter the cytoplasm as naked capsids. Free viral glycoproteins are processed as they traverse the host ER and Golgi complex. Mature glycoproteins then accumulate at the *trans* Golgi membranes where cytoplasmic envelopment of nucleocapsids occur. Enveloped virions are released at the plasma membrane.


PM
CYTOPLASM
G
ER
V
ONM
N
PN
INM
N
NUCLEUS

D. Function of viral glycoproteins:

HSV-1 encodes at least seven glycoproteins, designated gB, gC, gD, gE, gG, gH, and gI (Spear 1985; Kuhn, Kramer et al. 1990; Raviprakash, Rasile et al. 1990). All of these glycoproteins contain *N*-linked oligosaccharides, and several, such as gC and gD, contain *O*-linked sugars as well (Spear 1985). *N*-linked glycosylation is initiated by the *en bloc* transfer of Glc_3-Man_9-$GlcNAc_2$ glycans from dolicol phosphate lipid carriers to the nascent polypeptide chains (for review, see Kornfeld and Kornfeld 1985). After this transfer, the glycan chains are processed by ER and Golgi resident enzymes to yield high-mannose, complex, or hybrid forms of oligosaccharide side chains. The assembly of *O*-linked glycans starts with the transfer of GalNAc residues to the polypeptide chain, followed by the sequential addition of sugars to the growing glycan chain (Campadelli-Fiume and Serafini-Cessi 1985). Maturation of *O*-linked oligosaccharides likely occurs in the Golgi complex (Johnson and Spear 1983), although the process is not as well characterised as is *N*-linked glycosylation.

HSV-1 glycoproteins found on the surface of viral envelopes are involved in functions that mediate viral infectivity such as virus binding, penetration, and egress. Nucleocapsids without envelopes are not infectious because they lack these viral glycoproteins and therefore cannot interact with host membranes (Stein, Todd et al. 1970). Studies on viral mutants that have deletions in the genes for individual glycoproteins show that gC, gE, gG, and gI are dispensable for viral replication (Longnecker, Chatterjee et al. 1987; Longnecker and Roizman 1987; Weber, Levine et al. 1987), whereas gB, gD, and

gH are required for virion infectivity (Cai, Person et al. 1987; Desai, Schaffer et al. 1988; Ligas and Johnson 1988). Except for gG and gI, which can associate to form immunoglobulin G Fc receptors (Johnson, Frame et al. 1988), no interactions of the other glycoproteins have been documented. gC, gB, gD, and gH have all been implicated to function in viral attachment or penetration (Little, Jofre et al. 1981; Gompels and Minson 1986; Fuller and Spear 1987; Kuhn, Kramer et al. 1990), but there is no clear model of their mode(s) of action. By studying the formation of complexes by HSV virion proteins with biotinylated cell membrane components, Kuhn *et al* (Kuhn, Kramer et al. 1990) found that gC, gB, and gD bind to surface components of various cell lines. Because it has been shown that gC is not essential for infectivity, it is likely that gC is only one of several viral glycoproteins that can mediate viral attachment to the host cell.

Several pieces of evidence suggest that gB, gD, and gH are involved in the process of viral penetration. It has been shown that monoclonal antibodies directed against any one of these glycoproteins can block fusion of the virus with the host cell plasma membrane without having any significant effect on viral attachment (Fuller and Spear 1987; Highlander, Sutherland et al. 1987; Highlander, Cai et al. 1988; Fuller, Santos et al. 1989). In addition, virions lacking any one of these three glycoproteins fail to gain entry into the host cells to start an infection, despite the observation that they bind to cells efficiently. (Sarmiento, Haffey et al. 1979; Little, Jofre et al. 1981; Cai, Gu et al. 1988; Desai, Schaffer et al. 1988; Ligas and Johnson 1988). Johnson has shown that the

initial adsorption of HSV-1 to cells apparently involves numerous sites and is difficult to saturate (Johnson and Ligas 1988). However, soluble gD can block virus entry (Johnson, Burke et al. 1990), suggesting that gD interacts with a smaller subset of cell surface molecules. It has been postulated that after the initial binding of the virion to heparan sulfate moieties of cell surface proteoglycans, gD binds tightly to specific receptors on the cell surface. The close apposition of viral and cellular membranes that ensues after attachment allows gH and gB to mediate fusion of the viral and host cell membranes (Campadelli-Fiume, Arsenakis et al. 1988; Johnson and Spear 1989; WuDunn and Spear 1989).

Recently, Hajjar *et al* reported identifying the basic fibroblast growth factor (FGF) receptor as the cell surface receptor for HSV-1 recognition (Baird, Florkiewicz et al. 1990; Kaner, Baird et al. 1990). They found that antibodies directed against FGF inhibit the entry of HSV-1 into target cells. In addition, previously non-permissive cell lines can be rendered susceptible to HSV-1 infection by transfecting FGF receptors into these cells. This receptor is not the putative gD receptor because it does not appear to interact with gD. Instead, Hajjar's data suggest that FGF molecules are associated with the virion and facilitate the recognition and internalization of HSV-1 by the FGF receptor. It is possible that HSV-1 enters host cells via several independent mechanisms. The multiple models of virus entry and release underscores the complexity of the life cycle of HSV-1.

III. IMPORTANCE OF THE HOST CELL GLYCOSYLATION MACHINERY TO HSV-1 INFECTION:

A. Chemical inhibitors of glycosylation affect HSV-1 propagation:

Glycosylation of viral proteins requires a large number of host enzymes to process the growing oligosaccharide chains attached to the protein backbone (for review, see Kornfeld and Kornfeld 1985). By studying the effects of inhibitors that interfere at different steps of this process on the growth of HSV-1, it has been clearly demonstrated that the acquisition of infectivity and egress of newly-synthesized viral particles are dependent on the normal functioning of the host secretory system.

One of the first glycosylation inhibitors tested for its effect on HSV-1 growth was tunicamycin (Pizer, Cohen et al. 1980; Peake, Nystrom et al. 1982). This drug acts as an analogue of UDP-*N*-acetyl glucosamine, and disrupts the formation of the dolicol phosphate intermediate required in the initial step of *N*-linked glycosylation. HSV-1 infected cells treated with tunicamycin fail to yield infectious progeny particles. As expected, only severely underglycosylated forms of viral glycoproteins accumulate in these cells. Electron microscopy revealed that in tunicamycin-treated cells, only a small number of enveloped particles are detected on the cell surface, suggesting that there is a defect in the process of envelopment of the virion.

Another reagent that interferes with the propagation of HSV-1 in infected cells was studied by Campadelli-Fiume *et al* (Campadelli-Fiume, Sinibaldi-Vallebona et al. 1980). Benzhydrazone (BH) is a derivative of bis-amidinohydrazone which

prevents the addition of high-mannose oligosaccharides to proteins. It causes an early block in the *N*-glycosylation process similar to the effect induced by tunicamycin. Once again, infected cells treated with BH do not accumulate mature viral glycoproteins, nor are infectious particles produced.

Studies on the effects of tunicamycin and BH on HSV-1 infected cells suggest that glycosylation is a requirement for infectivity of HSV-1. However, other studies have shown that full maturation of viral glycoproteins is not essential for infectivity. Monensin is a monovalent cation ionophore which blocks the transit of membrane vesicles from the Golgi apparatus to the cell surface (Tartakoff 1983). Johnson and Spear demonstrated that monensin can inhibit the full processing and transport of HSV-1 glycoproteins as well as reducing the egress of virions from infected cells (Johnson and Spear 1982). They found that although viral glycoproteins are not fully processed in the presence of monensin, some infectious virions accumulate in the cytoplasm of monensin-treated cells. The studies highlighted so far demonstrate that while the absence of *N*-linked glycans precludes the envelopment and hence the yield of infectious HSV-1, the presence of fully-processed, complex-type glycans in not an obligatory requirement for herpesvirus infectivity.

B. Cell lines defective in glycoprotein processing also affect growth of HSV-1:

Cell lines that are defective in glycoprotein processing have also been shown to interfere with HSV-1 growth. Serafini-Cessi and Campadelli-Fiume found that two mutant baby hamster kidney

(BHK) cell lines that are resistant to killing by the galactose-binding lectin ricin, also interfered with the propagation of HSV-1 when infected with this virus (Campadelli-Fiume, Poletti et al. 1982; Serafini-Cessi, Dall'Olio et al. 1983). Ricin is a plant lectin that binds to cell surface oligosaccharides containing galactose (Vischer and Hughes 1981). Most mature *N*-linked glycoproteins have galactose residues near the end of their oligosaccharide chains (Kornfeld and Kornfeld 1985). The two cell lines, Ric14 and Ric21, were found to be defective in *N*-acetylglucosaminyl transferase I and *N*-acetylglucosaminyl transferase II respectively (Vischer and Hughes 1981). The lack of these two enzymes prevents the addition of *N*-acetylglucosamine to the growing glycan chain. This in turn prevents the incorporation of galactose, and results in the ricin-resistant phenotype. Campadelli-Fiume found that the release of virions from infected ricin-resistant cells is significantly reduced compared with infected wild type cells. However, fully infectious particles are found in the cell cytoplasm in spite of the lack of complete processing of viral glycoproteins in the mutant cells.

Another mutant cell line defective in the release of HSV-1 particles was isolated by Tufaro *et al* (Tufaro, Snider et al. 1987; Banfield and Tufaro 1990). This cell line, termed gro29, was shown to be defective in the transport and processing of glycoproteins through the Golgi complex. Although some infectious particles are formed, they remain trapped in the cell cytoplasm. All of these studies emphasize the dependence of HSV-1 on host secretory and glycoprotein processing functions for their normal propagation and egress.

IV. EXPERIMENTAL RATIONALE :

To further the understanding of the virus-host interactions governing the processes of viral maturation and egress, this study set out to investigate the effects of the fungal metabolite Brefeldin A (BFA) on the propagation of HSV-1 in infected cells. Research done in the last few years showed that BFA blocks the movement of proteins from the ER to the Golgi complex (Takatsuki and Tamura 1985; Misumi, Miki et al. 1986; Oda, Hirose et al. 1987). Although its mechanism of action has not been identified, Lippincott-Schwartz *et al* proposed that BFA acts by inducing the fusion of early Golgi compartments with the intermediate recycling compartment located between the ER and the Golgi (Lippincott, Donaldson et al. 1990). The intermediate compartment appears to be involved in the sorting of secreted and ER-resident proteins (Saraste and Kuismanen 1984; Lodish, Kong et al. 1987; Schweizer, Fransen et al. 1988). ER resident proteins are sequestered in the intermediate compartment and returned to the ER in membrane vesicles via a microtubule-dependent transport mechanism. Because it has been established that the movement of proteins between the intermediate compartment and the Golgi complex is facilitated by a microtuble-independent process (Armstrong and Warren 1990), it can be concluded that Golgi proteins are not recycled to the ER directly from the Golgi complex.

In the presence of BFA, the intermediate compartment is induced to fuse with the Golgi apparatus. This allows proteins in the Golgi complex to gain access to the microtubule-dependent

pathway to the ER. The anterograde movement of material from the ER to the Golgi complex is slower than the BFA-induced retrograde flow of membranes, resulting in the disappearance and redistribution of *cis* and *medial* Golgi into the ER. The net effect of BFA is to block the movement of proteins through the secretory organelles. It has been shown that the treatment of cells with low concentrations of BFA has no effect on endocytosis, protein synthesis and lysosomal degradation (Misumi, Miki et al. 1986; Nuchtern, Bonifacino et al. 1989), despite the observation that the maturation of oligosaccharide moieties on newly-synthesized glycoproteins is inhibited (Doms, Russ et al. 1989).

Analysis of protein transport in BFA-treated cells using VSV G protein shows that G protein is not transported to the plasma membrane as it normally is in untreated cells (Doms, Russ et al. 1989). Modification of G protein by *cis* and *medial* Golgi enzymes in BFA-treated cells is demonstrated by their partial resistance to endoglycosidase H digestion. However, addition of sialic acid, which is a *trans* Golgi event, is not observed. These results implicate that as the early Golgi compartments recycle into the ER, the contents of the Golgi, including its resident enzymes, are redistributed to the expanded ER. Removal of BFA results in the rapid flux of Golgi components out of the ER and the reorganization of the Golgi complex. Doms *et al* (Doms, Russ et al. 1989) reported that Golgi enzymes are completely recovered from the ER to the reformed Golgi by 10 min post BFA removal. Functional transport of proteins through the reorganising secretory organelles may take longer, but it is still a rapid process. G protein from recovering cells becomes

completely endo H-resistant in 30 min after BFA removal, and the addition of sialic acid is completed in 45 min.

This report demonstrates that BFA blocks the accumulation and egress of infectious HSV-1 in infected cells. This failure to yield infectious particles is not due to any disruption of the assembly or envelopment of nucleocapsids. Instead, there appears to be a blockage in the movement of enveloped particles into the cytoplasm. Although the cellular distribution of viral glycoproteins is perturbed by BFA, this alteration is reversed within 2 h after the removal of BFA from the growth medium. In contrast, the BFA-induced impediment to the release of viral particles is not reversible. These effects of BFA that have been observed are distinct from the effects of other glycosylation inhibitors on the propagation of HSV-1 in infected cells. BFA appears to affect the movement of viral particles at an early stage of the egress pathway, and prevents the acquisition of full infectivity. Further understanding of the viral-host interactions disrupted by BFA may serve to clarify the precise mechanism of egress and the requirements for infectivity of the newly-synthesized viral particles. The results of this study have been published in the Journal of Virology, 1991 (Cheung, Banfield et al. 1991).

MATERIALS AND METHODS

Cells and viruses.

L cells and HSV-1 KOS were obtained from D. Coen. All cells were grown at 37° C in DMEM supplemented with 10% FBS in a 5% CO_2 atmosphere. Brefeldin A was a gift from W. Jefferies. BFA was also obtained from Bio/Can Scientific, Ontario, Canada. Anti-gD monoclonal antibodies were obtained 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/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 in various concentrations was added. The extracellular medium was sampled at 2 h intervals for 20 h. The titres of infectious particles in each sample were determined by infecting confluent monolayers of Vero cells with appropriated dilutions of virus. The inoculum was removed after 1 h and medium containing methocell was added. Plaques were counted after 3 and 5 d of infection.

Harvesting of virus.

Monolayers of L cells were infected with HSV-1 (MOI=10). At 2 h.p.i., the medium was changed to labeling medium made up of methionine-free DMEM, 1/10 volume of DMEM with 10% FBS, 4%

dialysed FBS, [^{35}S]methionine (50 μCi/ml), and BFA (3 μg/ml). Infected cells were labelled for 16 h, after which the medium was removed and subjected to low-speed centrifugation to pellet cell debris. The supernatant was sedimented through a 5-40% 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 into 0.3 ml fractions. For determination of radioactivity in insoluble material, 15% of each fraction to be analyzed was added to 50 μg BSA followed by 1 ml of 10% cold TCA. Insoluble material was collected onto glass fibre filters after 1 h and radioactivity was determined by scintillation counting.

Isolation and quantitation of nucleocapsids.

Monolayers of L cells were infected, treated with or without BFA, and labelled with [^{35}S]methionine (100 μCi/ml) as described before. At 18 h.p.i., cells were harvested by washing with cold PBS and incubated for 15 min with cold lysis buffer (10mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate). Lysed cells were either sonicated or frozen and thawed three times. 0.5M urea was added and the cellular debris was pelleted by low speed centrifugation. The supernatant containing the nucleocapsids was sedimented through a 10-40% sucrose gradient for 1 h at 22K rpm using a Beckman SW 41 rotor. 0.3 ml or 0.5 ml fractions were collected, precipitated with 10% cold TCA, and counted in a scintillation counter as described earlier.

Determination of the titre of cell-associated virions.

Monolayers of L cells were infected and 3 μg/ml of BFA was added at 2 h.p.i.. At 8 or 18 h.p.i., cells were washed thoroughly with PBS and were lifted with PBS containing 5 mM EDTA. Harvested cells were pelleted by low speed centrifugation and were suspended in RSB. The cell suspension was sonicated and centrifuged to remove cellular debris. The titres of virus in these samples were determined by plaque assay on Vero cell monolayers.

Isolation of intracellular viral particles for Electron Microscopy.

Infected monolayers of L cells were treated or mock-treated with BFA (3 μg/ml) from 2 to 18 h.p.i.. At 18 h.p.i., cells were washed with PBS and were lifted with PBS containing 5 mM EDTA. Harvested cells were pelleted at low speeds (600 rpm for 10 min) and were suspended in ice cold RSB. Cells were broken up by dounce homogenization and intact nuclei were removed by centrifugation (1000 rpm for 10 min). Cytoplasmic extracts were then sonicated and large debris were removed by medium speed centrifugation (4200 rpm for 5 min). Clarified extracts were layered over 20% sucrose and viral particles were pelleted by ultracentrifugation for 30 minutes at 75K rpm using a Beckman TLA 100.2 rotor. Sediments were washed in PBS, repelleted, and were suspended in 20 μl of distilled water. Concentrated viral particles were negatively stained with 2% phosphotungstic acid (pH 7.4) on formvar coated 400 mesh copper grids and were examined using a Zeiss 10C STEM.

Thin Section Electron microscopy.

L cells were grown on Millicell HA inserts (Millipore Corporation) overnight prior to infection with HSV (MOI of 10). At 2 h.p.i., fresh medium with or without BFA (3 μg/ml) was added. At 8, 12, or 18 h.p.i., cells were rinsed with PBS, and fixed with 2.5% glutaraldehyde in 0.1 M NaCaC (pH 7.3). After 1 h of fixation, cells were rinsed with 0.1 M NaCaC and were post fixed in 1% OsO_4/ 0.1 M NaCaC for 1 h. Samples were rinsed, dehydrated in increasing concentrations of ethanol and then in 100% propylene oxide, and were embedded in Epon Araldite. Thin sectioning was done on a Reichert-Jung ultracut E. Sections were stained with uranyl acetate then with lead citrate, and were examined and photographed using a Zeiss 10C STEM.

Indirect immunofluorescence.

Cells were grown on acid-etched glass coverslips for 2 d and infected with HSV-1 (MOI=10). At 2 h.p.i., medium containing 3 μg/ml of BFA was added to the monolayers. At 10 h.p.i., the BFA-containing medium was removed and the monolayers were further incubated in normal medium for 0, 30, 60, or 90 minutes. Cells ready to be stained were first rinsed with PBS and fixed with 3.2% paraformaldehyde in PBS. Fixed cells were washed and incubated for 30 min with a 1/100 dilution of anti-gD monoclonal antibody. Primary antibody was washed off and cells were incubated for 30 min with a 1/100 dilution of FITC- or RITC-conjugated goat anti-mouse IgG. Coverslips were rinsed and mounted in 50% glycerol, 100 mM Tris, pH 7.8. Cells were photographed using a Zeiss

microscope with epifluorescence optics.

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 then replaced with normal medium at 18 h.p.i.. Further samples were collected at 30, 60, 120, and 180 minutes after BFA removal. All samples were diluted serially and used to inoculate monolayers of Vero cells growing in 96-well dishes. Titres were determined from the highest dilution that showed cytopathic effect.

BFA sensitive period.

Parallel dishes of HSV-1 infected cells were treated with or without 3 μg/ml of BFA for 4 h between 0-4, 4-8, 8-12, 12-16, and 16-20 h.p.i.. At the end of each BFA treatment, monolayers were washed extensively and normal medium was added. Release of infectious viral particles post BFA treatment was determined by harvesting the extracellular medium at 20 h.p.i. for all dishes and determining their titres by plaque assays on Vero cells.

RESULTS

BFA inhibits HSV-1 propagation.

To determine the effect of BFA on the propagation of HSV-1, monolayers of L cells were infected with HSV-1 and were exposed to various concentrations of BFA from 2 to 20 h.p.i.. To measure the release of infectious viral particles, growth medium of these monolayers were sampled every 2 h and their viral titres were determined on Vero cells. Initial results, shown in Fig 3, indicate that 1 μg/ml of BFA was able to effectively block the release of viral particles from infected cells. This resulted in a seven order of magnitude decrease in virus yield as compared to non-BFA treated cells. Subsequent studies reveal that even at 0.3 μg/ml, BFA can affect the release of viral particles by delaying the exponential release of HSV into the extracellular medium by at least 8 h (Fig. 3).

BFA inhibits viral egress.

Since the lack of infectious viral particles in the extracellular medium of BFA treated infected cells may not be due to a block in viral egress, but that viral particles released are defective in their infectivity, we also examined the export of particles into the growth medium using radiolabelled virions. Cell monolayers were infected with HSV-1 and 0 or 3 μg/ml of BFA was added at 2 h.p.i.. Both BFA-treated and untreated cells were incubated with 50 μCi/ml of [^{35}S]methionine from 2-18 h.p.i. to radiolabel virions assembled during infection. The culture medium was harvested at the end of

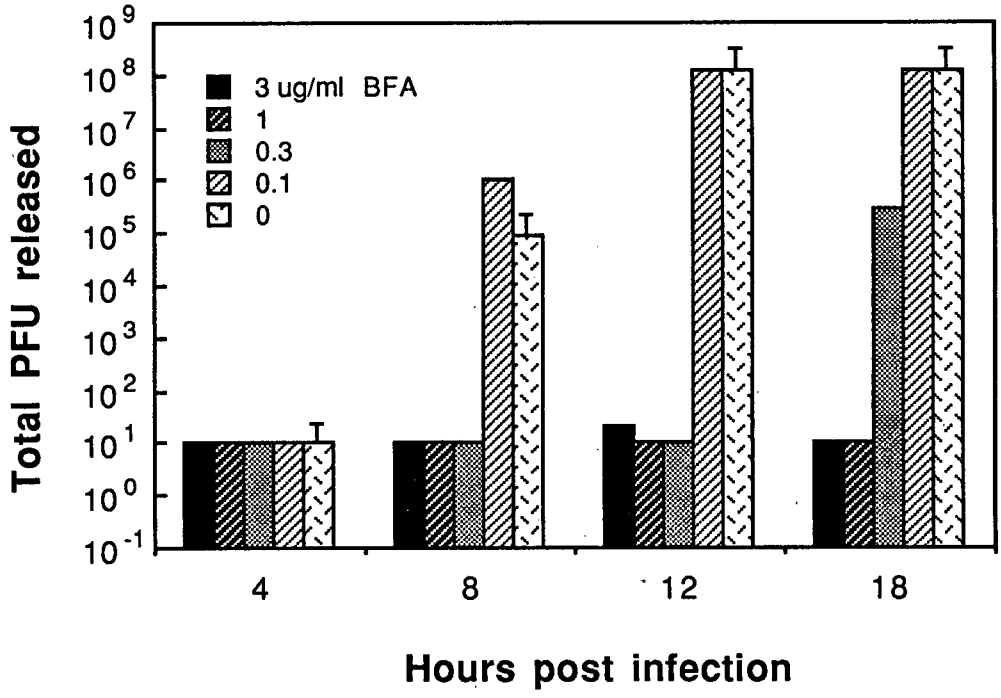


Figure 3. Effects of BFA on release of PFU into growth medium.

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 PFU released in the presence of BFA. The titres were measured by limiting dilution and plaque assay.

the labelling period, and subjected to centrifugation on 5-40% dextran gradients to isolate virions. The gradients were fractionated and the amount of radioactivity in the TCA insoluble material of each fraction was measured. As shown in Fig. 4, a peak of radioactivity representing the released virions was seen in the control cell gradient but not in the BFA treated sample. This observation suggests that treatment of infected cells with BFA does result in a block of viral release.

Assembly of nucleocapsids is normal in BFA-treated cells.

To determine whether nucleocapsids form in BFA-treated cells, monolayers were infected with HSV-1 and treated with BFA (3 μg/ml) from 2-18 h.p.i.. During this time, cells were labelled with 100 μCi/ml [35S]methionine to allow detection of nucleocapsid proteins. Cells were then lysed in buffer containing NP40 and Na-deoxycholate, which keeps nuclei intact while solubilizing other cellular components. The nuclei were pelleted and suspended in the same buffer. The nuclear and cytoplasmic extracts were sonicated and centrifuged separately on 10-40% sucrose gradients to isolate nucleocapsids. The amount of TCA-insoluble radioactivity in gradient fractions of BFA-treated and untreated cells are shown in Fig 5. Peaks of radioactivity representing nucleocapsids were visible for both cytoplasmic (fraction 13, 10 ml gradient, 0.3 ml fractions) and nuclear enriched samples (fraction 5, 8 ml gradient, 0.5 ml fractions). Based on the amount of radioactivity, the nuclei contained approximately 20% of the total nucleocapsids in the cell. There was no significant difference in the number of cytoplasmic

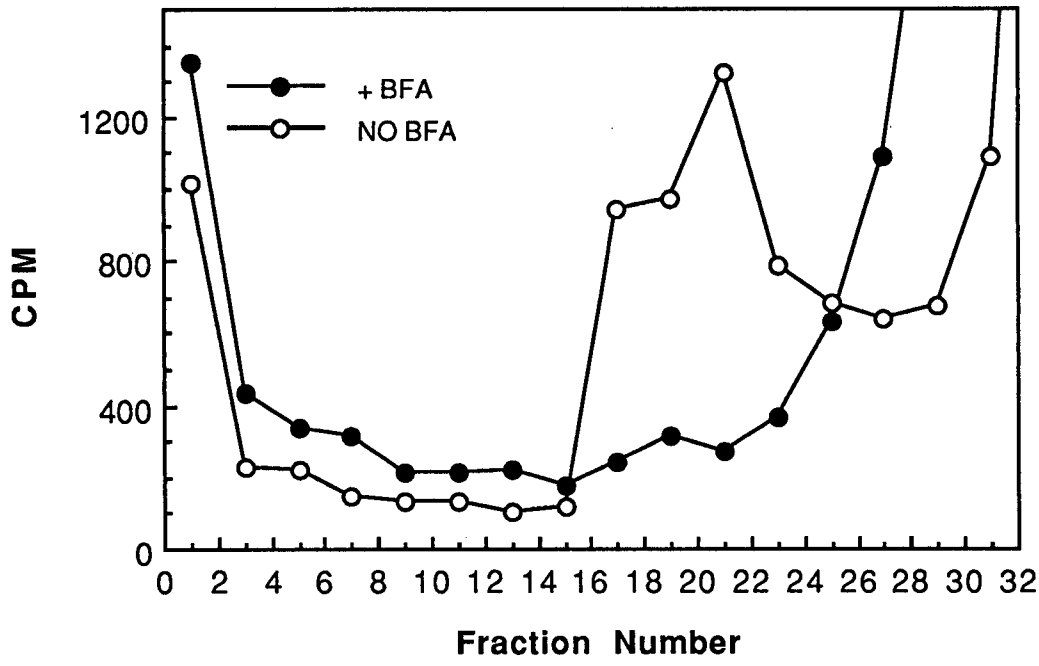


Figure 4. Effects of BFA on viral release.

Monolayers of infected cells (MOI=10) were treated with 3 μg/ml of BFA at 2 h.p.i. and incubated along with untreated controls with 50 μCi/ml of [^{35}S]methionine 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 5-40% dextran gradient to separate virions from other radioactive material. The gradients were fractionated from the bottom and amount of TCA-insoluble radioactivity in each fraction was determined by scintillation counting (see Materials and Methods).

CPM=CPM x 10^{-2}

nucleocapsids isolated from BFA-treated and untreated cells (Fig. 5A), indicating that BFA did not impede nucleocapsid assembly, nor their movement to the cytoplasm. Similarly, the number of nucleocapsids was reduced only slightly in the nuclei of infected cells treated with BFA (Fig. 5B). In experiments where whole cell extracts were analyzed on sucrose gradients (Fig. 5C), BFA-treated and untreated cells contained similar peaks of radioactivity representing labelled nucleocapsids, whereas the control gradient did not.

BFA also affects the accumulation of cell associated virions.

To evaluate the effects of BFA on the accumulation of intracellular infectious particles in infected cells, extracts were prepared from BFA-treated and untreated cells, sonicated, and the viral titres were measured by plaque assays on Vero cells. Titres of cell extracts harvested at 8 h.p.i. were approximately 0.0006, and 6 PFU/cell for BFA treated and non-treated cells. Cell extracts harvested at 18 h.p.i. yielded titres of approximately 0.0012, and 270 PFU/cell for BFA treated and non-treated cells. The magnitude of reduction in intracellular HSV seen in the 18 h.p.i. samples was similar to the reduction in the extracellular titres noted before. It appears from these results that BFA prevents the formation or accumulation of infectious particles in HSV-infected cells.

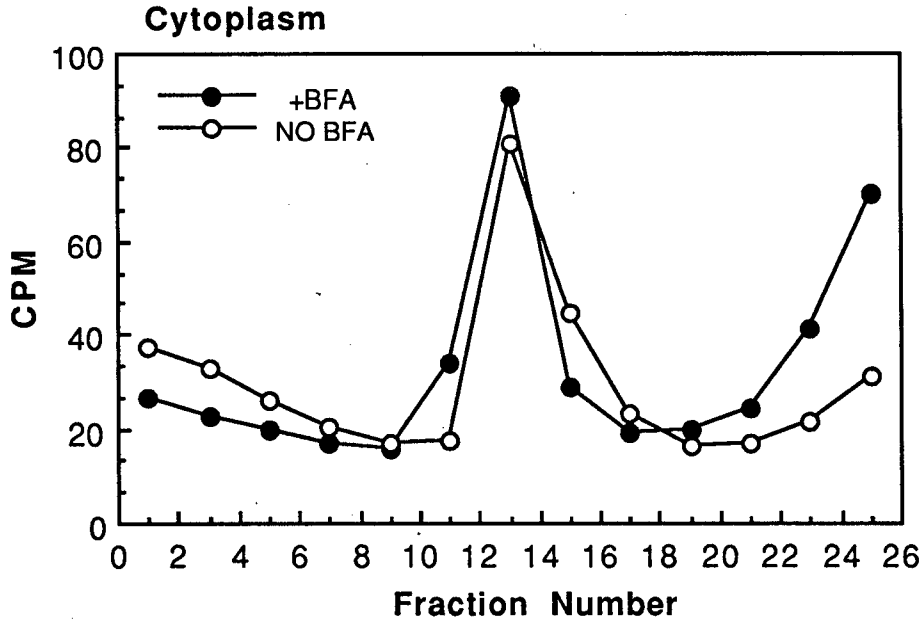
The lack of intracellular infectious virions in BFA treated cells is due to the lack of enveloped particles in cells.

To investigate the cause of the apparent lack of infectious

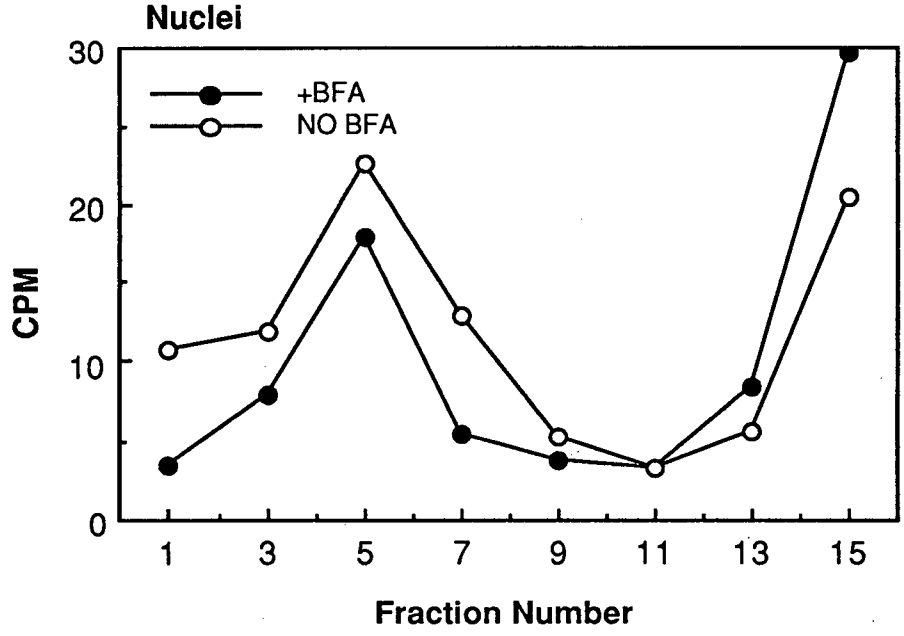
Figure 5. Effects of BFA on nucleocapsid assembly

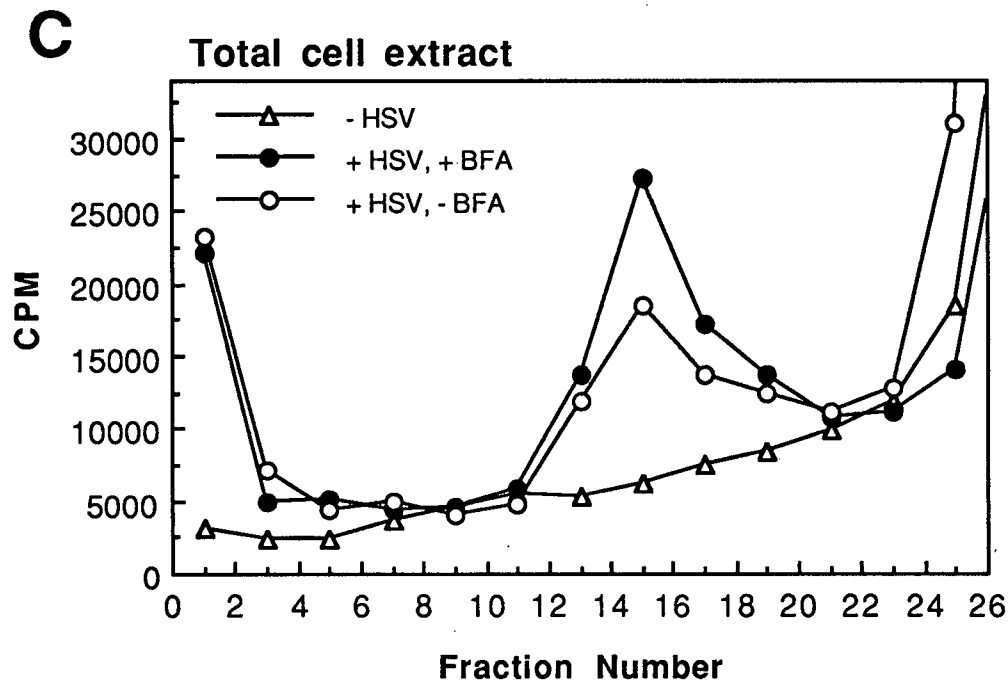
Monolayers of infected cells (MOI=10) were treated with 3 μg/ml of BFA at 2 h.p.i. and incubated along with untreated controls with 100 μCi/ml of [^{35}S]methionine until 20 h.p.i.. Monolayers were washed extensively and incubated with NP-40 lysis buffer (see Materials and Methods). After 15 min on ice, the nuclei were pelleted. Post-nuclear 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 10-40% sucrose gradient and fractions were collected from the bottom of the tubes. The amount of TCA-insoluble radioactivity in each fraction was determined by scintillation counting. The data for nucleocapsids isolated from (A) cytoplasm-enriched fractions, (B) nuclear-enriched, and (C) total cell extracts are shown.

A


Cytoplasm
+BFA
NO BFA
CPM
Fraction Number
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26

B


Nuclei
+BFA
NO BFA
CPM
Fraction Number
0
10
20
30
1 3 5 7 9 11 13 15

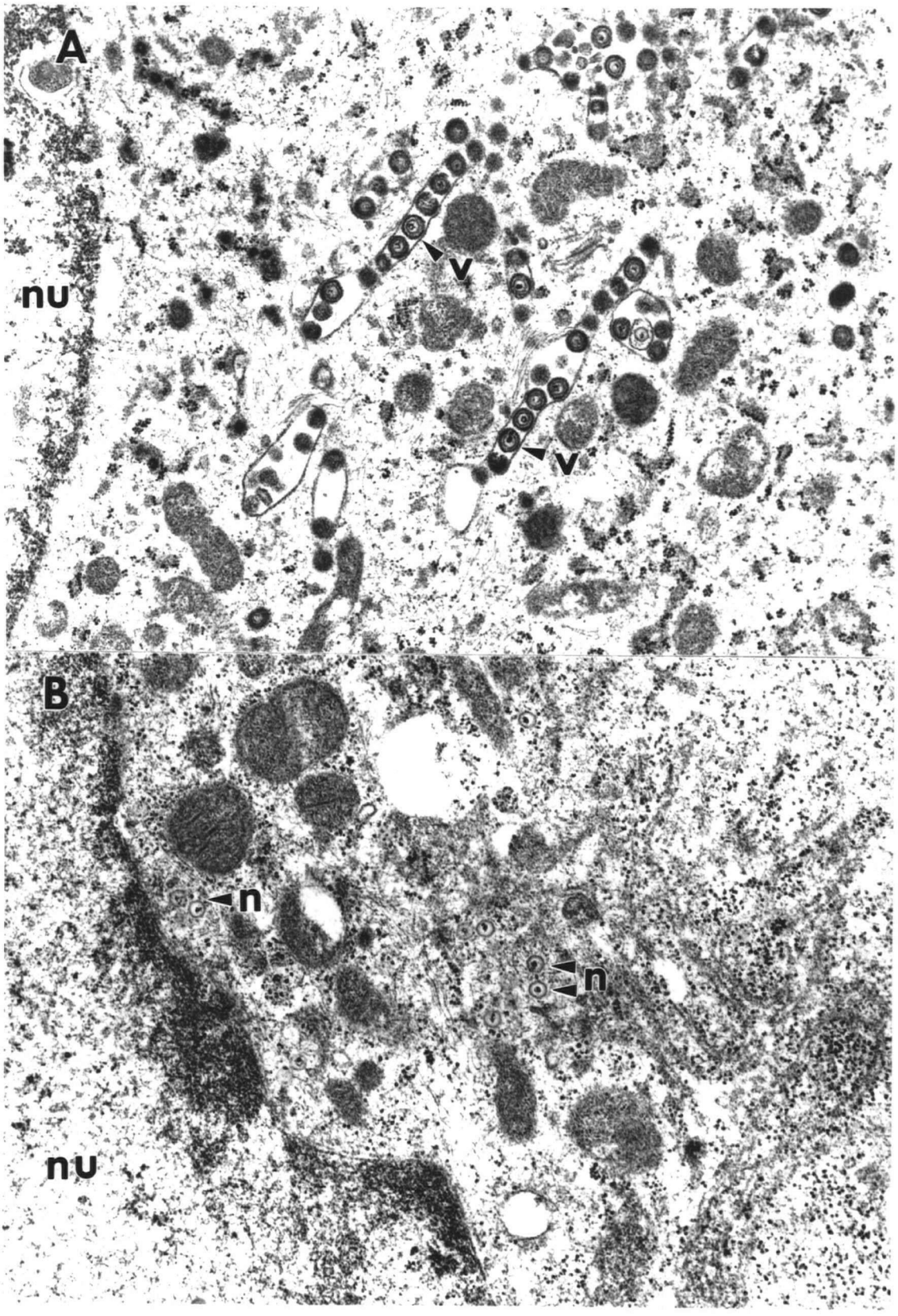

C
Total cell extract
- HSV
+ HSV, + BFA
+ HSV, - BFA
CPM
30000
25000
20000
15000
10000
5000
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Fraction Number

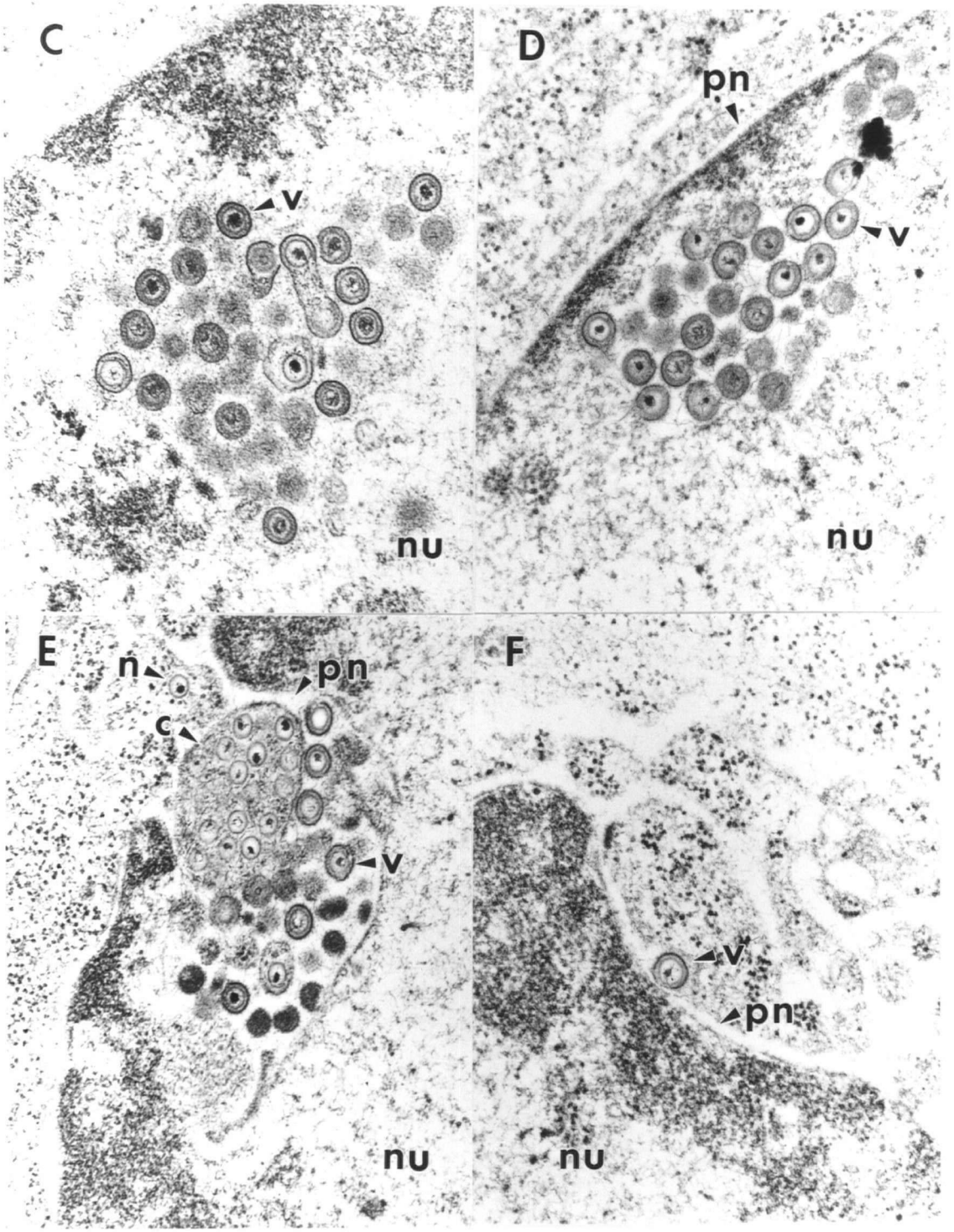
particles in BFA-treated cells, electron microscopy techniques were employed to study the status of the viral particles present in BFA-treated cells. More specifically, we tested the possibility that this lack of intracellular infectious particles was due to the absence of enveloped virions in the cell cytoplasm. Cytoplasmic extracts of BFA-treated and non-treated cells were collected, concentrated over sucrose pads and were negative stained for direct examination under the electron microscope. Repeated trials of this experiment showed that whereas naked nucleocapsids were seen in cytoplasmic extracts from BFA-treated cells, no enveloped particles were observed. Unfortunately, it was difficult to concentrate intracellular virus particles from both BFA-treated and non-treated cell extracts possibly due to the low amounts of viruses in infected cells. Also, there were some difficulties in identifying positively enveloped particles using negative staining techniques. To overcome these shortcomings, infected cells that were treated or mock-treated with BFA were thin sectioned for transmission electron microscopy examination. Figure 6A, and B represents typical images observed in BFA-treated or non-treated cells harvested at 8 h.p.i.. The cytoplasm of non BFA-treated cells contained many enveloped particles accumulated in membrane vacuoles. In contrast, there was a complete lack of enveloped particles in the cytoplasm of BFA-treated cells even though naked nucleocapsids were seen. When enveloped particles were observed in BFA-treated cells, these particles were enclosed in membranous pockets located inside the nucleus as shown in Fig. 6D. In wild-type cells, similar pockets of enveloped particles inside the nucleus were occasionally observed

Figure 6. 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 (3μg/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 non-enveloped particles. (C) Nucleus of HSV-1 infected L cells containing enveloped particles in an intra- or peri-nuclear region. (D) Nucleus of HSV-1 infected L cells treated with BFA containing enveloped particles in the perinuclear space. (E) Perinuclear space of BFA-treated cells containing enveloped particles. (F) Viral particle in the perinuclear space of BFA-treated cells.

nu, nucleus; v, enveloped virus particles; n, nucleocapsid; pn, perinuclear space; c, cytoplasmic invagination.


A
nu
v
v
B
n
n
nu


C
v
nu
D
pn
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nu
E
n
pn
c
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nu

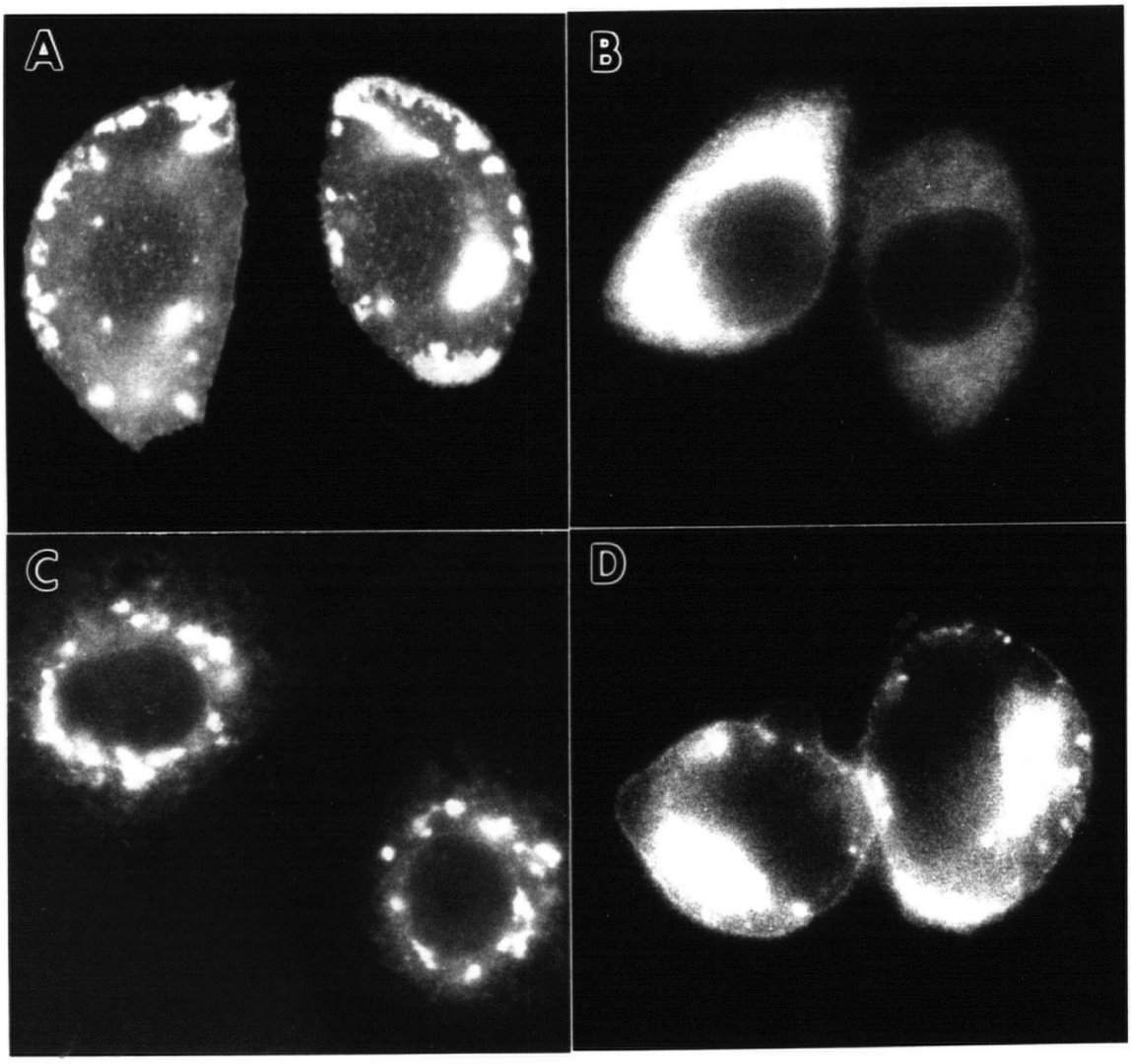
(Fig. 6C), however, their frequency was rare compared to cells treated with BFA. The conclusion drawn from these electron microscopy studies suggests that absence of infectious particles within BFA treated cells are due to the lack of enveloped virions accumulating in the cytoplasm of infected cells. It is possible that the pockets of enveloped particles seen in both BFA-treated and non-treated cells represent accumulation of enveloped virions in the distended perinuclear space of infected cell nuclei. Whether these virions are infective remains unclear. The apparent higher incidence of intranuclear pockets of enveloped particles observed in BFA-treated cells may be a reflection of the blockage to normal viral traffic in the cell cytoplasm induced by BFA.

BFA alters the distribution of viral glycoproteins.

To study the effect of BFA on the distribution of HSV-1 glycoproteins, indirect immunofluorescence was done on infected cells using monoclonal antibodies directed against HSV-1 gD. Cell monolayers were infected and treated with BFA (3 μg/ml) from 2 h.p.i. onward. At 10 h.p.i., BFA was removed and cells were incubated in BFA-free medium for various lengths of time (see Fig. 7). To detect gD, cells were fixed and subsequently stained with an anti-HSV-1 gD monoclonal antibody. Examination of control cells (Fig. 7A) 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. The perimeter staining likely represents virion-containing vacuoles that

Figure 7. Immunofluorescence analysis of HSV-1 gD in infected cells treated with BFA.

Cells were grown on glass coverslips for 3 days to allow for good adherence. Monolayers were infected with HSV-1 (MOI=10). At 2 h.p.i., 3 μg/ml of BFA 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 37^0C for 0 min (B), 30 min (C), or 3 h (D) and then fixed with formaldehyde. Control cells (A) were fixed at 16 h.p.i.. gD was detected using a monoclonal antibody and a fluorescein-conjugated (A,C) or rhodamine-conjugated (B,D) secondary antibody.


A
B
C
D

were *en route* to the plasma membrane.

Treatment of infected cells with BFA induced a drastic alteration in the distribution of gD (Fig. 7B). In contrast to control cells, gD staining was homogeneous throughout the cell cytoplasm. Furthermore, gD was not detectable in the nuclear or plasma membranes. Instead, a fine, evenly-distributed reticular staining characteristic of the ER was evident. The Golgi complex was not evident, suggesting that it was dispersed in BFA-treated infected cells or that gD was not present in Golgi membranes.

Many studies have concluded that the effects of BFA on protein secretion and dissolution of the Golgi complex are reversible within minutes after removal of BFA (see introduction). Evidence that some of the effects of BFA are reversible in HSV-1 infected cells can be found in Fig 7C and 7D. By 30 min after removal of BFA (Fig. 7C), regions of discrete staining were visible in a roughly perinuclear ring. This suggests that gD which was previously recycled into the ER was sorted and re-distributed into the reorganising Golgi membranes. Some ER staining was also evident as indicated by the reticular staining surrounding the nucleus. By 3 h after removal of BFA (Fig. 7D) juxtanuclear staining was evident in a pattern characteristic of the Golgi complex. Small regions of punctate staining were also present at the cell periphery, suggesting that the pathway of virion egress was functioning to some extent. The plasma membrane was also clearly stained by this time, indicating that gD was transported to this membrane.

The effects of BFA on HSV-1 propagation are not fully reversible.

To investigate whether the ability to secrete infectious 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 amount of infectious virions released over the next 3 h into the extracellular medium was determined. The results of this analysis (Fig. 8) revealed that cells treated with non-inhibitory concentrations of BFA (0.03 and 0.1 μg/ml) released large quantities of infectious particles into the medium within 30 min after the removal of BFA. Controls with no BFA were indistinguishable from 0.03 μg/ml BFA. However, this viral production was not matched in the monolayers treated with higher concentrations of BFA. In these cells, there was a lag in the appearance of PFU in the extracellular medium. Even though we observed a gradual increase in extracellular PFU during the 3 h after BFA removal in the 1 μg/ml samples, only 0.1% of the normal amount of extracellular PFU released into the medium. The data shown indicate the most virus that we have detected during the 3 h release from the effects of 1 μg/ml BFA. Other experiments have yielded as few as 10^4 PFU during this period. These results suggest that unlike the rapid recovery of cellular protein secretion post BFA removal (Doms, Russ et al. 1989), recovery of virion release to normal levels, if it does occur, requires a very long time.

A BFA sensitive period exists.

To examine the possibility of a critical time period during the replication cycle that is most sensitive to the effects of BFA, BFA

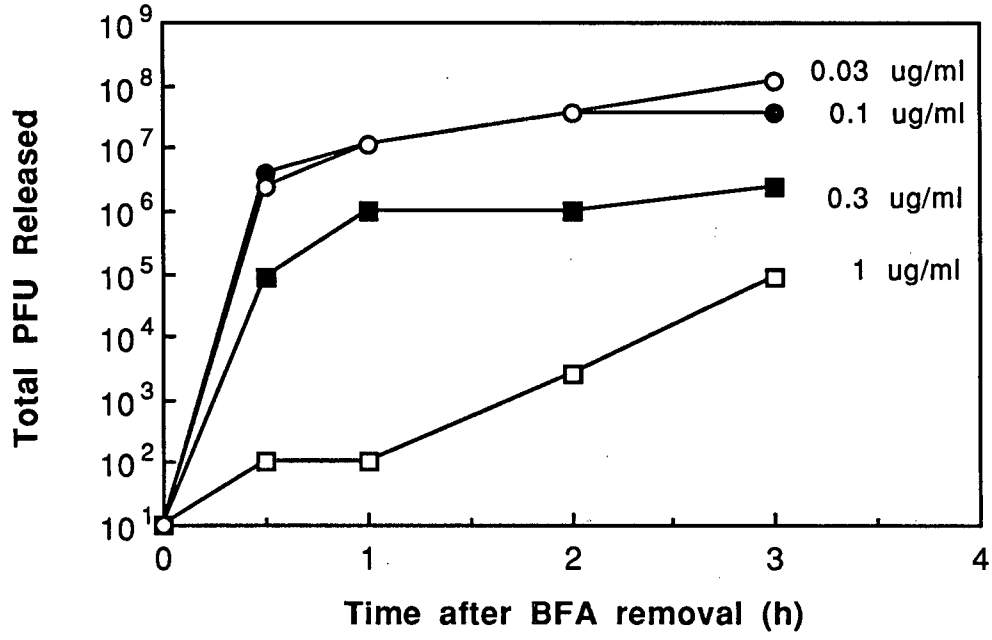


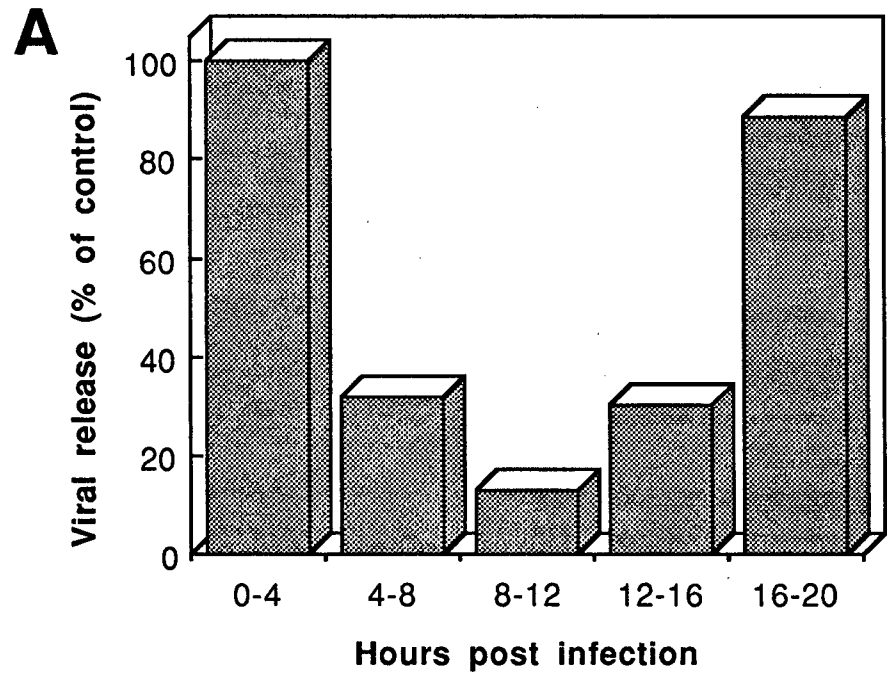
Figure 8. Determination of the rate of viral release following BFA removal.

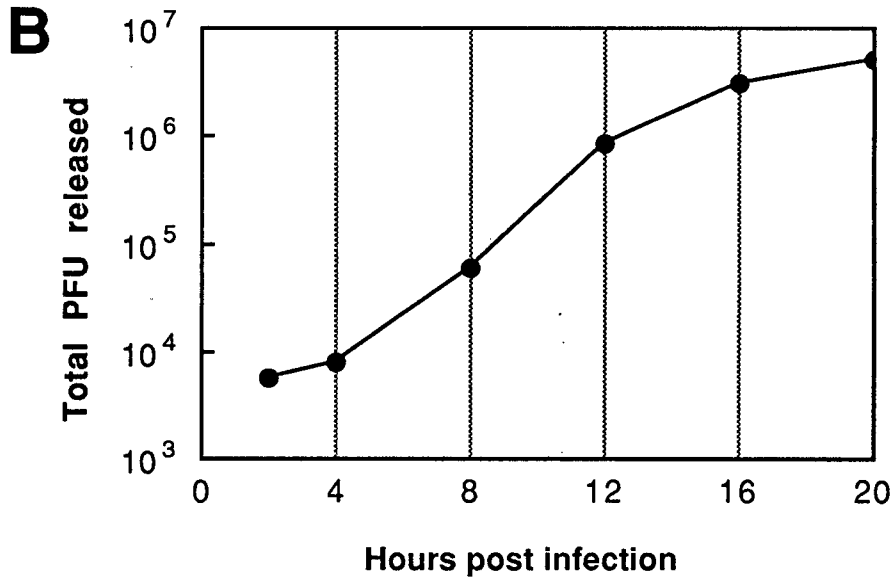
Cells were infected and treated as described in the legend to Fig. 3. At 18 h.p.i., cell monolayers were washed thoroughly to remove BFA. Samples of the extracellular medium were taken at various times and the HSV-1 titres were determined by limiting dilution.

was applied to infected cells in 4 h blocks at different times of the infection cycle. Recovery from the effects of these 4 h BFA exposures were measured at 20 h.p.i. by comparing the cumulative release of infectious virions post BFA treatment of treated and mock-treated cells. Viral titres of the extracellular medium collected at 20 h.p.i. of the test and control cells were determined and were expressed as percentages of viral titre of the mock-treated control cells. Figure 9A shows the result obtained in a representative experiment. Although the actual percentages of viral titre fluctuated from experiment to experiment, the overall trend was identical to that shown in the representative experiment. Exposures of infected cells to BFA from 0-4 or 16-20 h.p.i. did not seem to significantly alter the release of viral particles from cells. Application of BFA to infected cells at 4-8, 8-12, or 12-16 h.p.i. appeared to reduce the subsequent release of virions by at least 60% of control cell viral release experiments. Moreover, the 8-12 h.p.i. BFA treatment time period was repeatedly shown to be the time period most affected by BFA. This period corresponds to the exponential virus release phase of a typical infection, thus further emphasizing that BFA affects the egress mechanism of the viral life cycle.

Figure 9. Determination of BFA-sensitivity during viral propagation.

Monolayers of cells were infected with HSV-1 (MOI=10) and treated with BFA (3μg/ml) for 4 h intervals from 0 to 20 h.p.i.. Control monolayers were also analysed 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 titre was determined by plaque assay. (A) Virus yield from BFA-treated cells expressed as a percentage of the yield from untreated control cells. Duplicate samples were analysed. (B) Virus yield from untreated control cells treated in the same manner as in part A. Virus titres from samples harvested at each time point were added together to reflect the cumulative yield during the 20 h of the experiment.


A
Viral release (% of control)
100
80
60
40
20
0
0-4
4-8
8-12
12-16
16-20
Hours post infection


B
Total PFU released
10^7
10^6
10^5
10^4
10^3
0
4
8
12
16
20
Hours post infection

DISCUSSION

It has long been appreciated that herpes simplex virus (HSV) requires the host secretory apparatus for the maturation and egress of newly-synthesized viral particles (Campadelli-Fiume and Serafini-Cessi 1985). Not only do viral glycoproteins depend on the host cell ER and Golgi compartments for their processing, it is likely that enveloped virions are transported through these same organelles for their export out of the cell (Johnson and Spear 1982). In this report, the effects of Brefeldin A (BFA) on the propagation of HSV-1 in infected cells were investigated. BFA is a drug that inhibits the cellular transport of proteins from the ER to the Golgi complex. By examining how the BFA-induced blockage to host cell secretion affects the growth of HSV-1, we hope to further define the interactions between virus and host components that are required for the efficient propagation of this virus.

Results from this study demonstrated that treatment of infected cells with BFA leads to a reduction in extracellular virus yields as compared to untreated control cells (Fig. 3). Treatment of infected cells with as little as 0.3 μg/ml of BFA delayed the appearance of infectious particles into the extracellular medium by at least 8 hours. Furthermore, higher concentrations of BFA such as 1 μg/ml or above led to a blockage of the release of infectious virions. This is illustrated by the seven order of magnitude reduction in the cumulative PFU released from BFA-treated cells as compared to control cells at 18 h.p.i. (Fig. 3). The transient

inhibitory effect seen with 0.3 μg/ml of BFA is likely a reflection of BFA being metabolized by cells over long incubation periods to concentrations that are no longer effective in inhibiting viral propagation. To ensure that sufficient BFA was present during the long experimental procedures, all subsequent experiments were performed using 3 μg/ml of BFA.

By labelling progeny viral particles assembled during HSV-1 infection with [^{35}S]methionine, it was demonstrated that the lack of plaque forming units in the extracellular medium was not due to the release of non-infectious viruses. Instead, there was a blockage to particle egress in BFA-treated cells (Fig. 4). Further investigations on the cause of this inhibition revealed that BFA caused a major reduction in the accumulation of infectious particles inside the infected cells (see results). It appears that BFA interferes with the formation of mature viral particles in host cells, and hence few viral particles are exported into the extracellular medium.

The interference of the formation of infectious particles can occur at different parts of the virus life cycle. It is unlikely that BFA disrupts the assembly of viral nucleocapsids in the nucleus since similar amounts of assembled nucleocapsids were found in total cell extracts from BFA-treated and non-treated cells (Fig. 5). Furthermore, when cell extracts from BFA-treated cells were separated into fractions enriched for nuclei or cytoplasm, assembled nucleocapsids were found in both fractions. This suggests that BFA does not affect the assembly of nucleocapsids in the infected cell nucleus, nor does it impede the transport of these nucleocapsids into the cytoplasm.

Another explanation to account for the lack of cell-associated infectious particles is that BFA interferes with the envelopment of nucleocapsids. It has already been shown that glycosylation inhibitors such as tunicamycin disrupts the envelopment of viral capsids (Peake, Nystrom et al. 1982). When cytoplasmic extracts from infected cells were negatively stained and examined by electron microscopy, naked nucleocapsids, but not enveloped particles, were observed in BFA-treated cells. To obtain more evidence that BFA-treated cells lack enveloped particles in their cytoplasm, transmission electron microscopy was employed to study the viral particles in thin-sectioned cells. Monolayers of BFA-treated and untreated cells were infected, and prepared for electron microscopy at 8, 12, and 18 h.p.i.. In contrast to the abundance of enveloped particles enclosed in cytoplasmic vesicles seen in control cells, no enveloped virions were present in the cytoplasm of BFA-treated cells at any of the analysed time points (Fig. 6A and B). Similar amounts of non-enveloped nucleocapsids were observed in the cytoplasm of both BFA-treated and control cells, and this is consistent with the results obtained in the radiolabelled nucleocapsid study.

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 frequently found in BFA-treated cells, but they accumulate exclusively in membranous compartments within the nuclei (Fig. 6D-F). These intranuclear pockets of virions, which are often located adjacent to

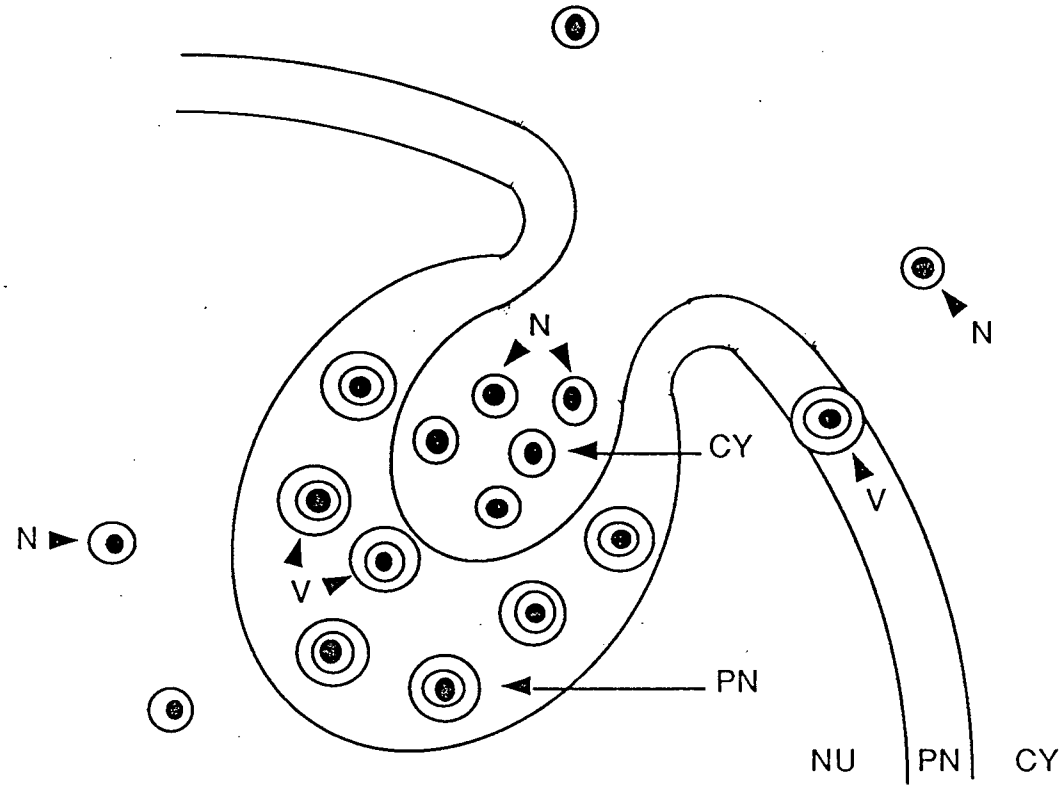
nuclear membranes, are not likely to be invaginated cytoplasm because they do not contain ribosomes. Instead, they probably represent newly-enveloped particles accumulating in the distended perinuclear space. It has been reported that HSV-1 infection induces the duplication of the nuclear membranes for the envelopment of newly made particles to occur (Poliquin, Levine et al. 1985). Also, the presence of intranuclear compartments containing enveloped particles has been documented for HSV infected cells (Dargan 1986). When infected control cells were examined by transmission electron microscopy, enveloped particles were seen in intranuclear structures (Fig. 6C), albeit their rare occurrence. There is little doubt that virions are normally enveloped at the inner nuclear membrane as they bud into the perinuclear space (Darlington and Moss 1969; Nii, Morgan et al. 1969). These newly enveloped particles accumulate rarely at this cellular location in wild type cells because they are immediately transported to their next destination, most likely to be the ER. In BFA-treated cells, since the normal transport of viral particles from the ER to Golgi is blocked, this may cause an aberrant accumulation of enveloped particles in the perinuclear space.

Upon closer examination of the nuclei of BFA-treated cells, a high concentration of naked nucleocapsids in a separate compartment adjacent to the pocket of enveloped virions was often observed (Fig 6E). These structures are interpreted as enveloped particles in the perinuclear space surrounding an area of invaginated cytoplasm where naked nucleocapsids and ribosomes are seen (see Fig. 10). As mentioned before, enveloped particles

Figure 10. Schematic diagram of an intranuclear pocket of enveloped particles seen in BFA-treated cells.

Abbreviations:	N	nucleocapsid
	V	enveloped virion
	NU	nucleus
	PN	perinuclear space
	CY	cytoplasm or cytoplasmic invagination

The pockets of enveloped particles seen in the nucleus of BFA-treated infected cells are likely viral particles accumulating in the distended perinuclear space. Naked nucleocapsids are often seen in separate membranous compartments adjacent to the enveloped particles. They may represent enveloped viral particles that fused with the outer nuclear membrane and are located in the invaginated cytoplasm as non-enveloped capsids.


N
N
N
CY
V
V
PN
NU
PN
CY

appear to be detained abnormally in the perinuclear space of BFA-treated cells. These particles may fuse with the outer nuclear membrane, de-envelope, and localise in the invaginated cytoplasm as nucleocapsids. Such an alternative method of egress from the nucleus may provide a logical explanation for the presence of naked nucleocapsids and the lack of enveloped particles in the cytoplasm of BFA-treated cells.

There are currently two models of viral egress proposed for HSV (see Fig. 2). The first and most widely accepted model was proposed by Morgan's group based on electron microscopy studies (Nii, Morgan et al. 1969). This model suggests that newly-assembled nucleocapsids acquire their viral envelopes as they bud at the modified inner nuclear membrane into the perinuclear space. Virions accumulated in this region exit from the cell via a process of "reverse phagocytosis". Enveloped particles first move to the ER by virtue of the contiguity of the nuclear membrane and ER, or they are transported specifically to the ER by membrane vesicles. Immature glycoproteins present on the viral envelopes are processed and modified as the particles traverse the ER-Golgi pathway *en route* to the cell surface. This model is strongly supported by the monensin study done by Johnson and Spear (Johnson and Spear 1982). Monensin is known to block the transport of proteins from the Golgi stacks to the cell surface by disrupting ionic gradients between cellular organelles. Infected cells treated with this drug not only inhibits the egress of virions, infectious particles are found in cytoplasmic vacuoles that are probably derived from Golgi membranes.

The second model of viral egress, proposed by Stackpole (Stackpole 1969), agrees that newly-assembled nucleocapsid bud at the inner nuclear membrane to acquire an envelope around the viral capsid. However, it predicts that enveloped particles in the perinuclear space quickly fuses with the outer nuclear membrane, and are released as naked nucleocapsids into the cell cytoplasm. Free viral glycoproteins are made, transported into the ER, and processed as they traverse the ER and Golgi compartments. Mature viral glycoproteins then accumulate and modify the *trans* Golgi membranes where envelopment of the cytoplasmic naked nucleocapsids occurs. Proponents of this model have shown by transmission electron microscopy that one can find structures resembling Golgi membranes wrapping around naked capsids in the cytoplasm (Komuro, Tajima et al. 1989). These structures however, are more frequently found very late in the infection cycle (16 to 48 h.p.i.). Since enveloped particles are first released at about 8 h.p.i. (Smith and DeHarven 1973), this model of egress may represent a secondary pathway for virion release.

The present study does not distinguish one model of viral egress from another because both models imply that the ER-Golgi pathway plays an important role in the propagation of HSV particles. The BFA-induced recycling of cis/medial Golgi into the ER can block the movement of enveloped particles as well as immature viral glycoproteins to the *trans* Golgi. The net effect of both situations can lead to the inhibition of viral egress. In agreement with both models, enveloped particles were observed at the perinuclear space of infected cells. If these particles were to follow

the second model of egress, one would expect to see an abnormally high concentration of naked nucleocapsids in the cytoplasm since BFA should not affect the de-envelopment process of viral particles at the outer nuclear membrane. This, however, was not observed in the electron microscopy analysis. Instead, high concentrations of enveloped particles seem to accumulate aberrantly in the perinuclear space of BFA-treated cells. It is possible that enveloped virions mainly travel through the ER and Golgi for export. Newly-enveloped particles that are to be transported to the ER may be blocked by the BFA-induced retrograde movement of material from the Golgi complex to the ER. Net anterograde movement of material from the nucleus to the ER to the Golgi may be required for these viral particles to enter the host secretory pathway. As hypothesized earlier in this discussion, enveloped particles detained in the nucleus may fuse with the outer nuclear membrane and be released as nucleocapsids into the cytoplasm similar to the situation predicted by the second model of viral egress. This seems to further support the idea that both mechanisms of viral release are employed in normal infected cells, and that the blockage of one pathway shunts the egressing particles into the other route.

Although no cytoplasmic enveloped particles were present in BFA-treated cells, viral glycoproteins were abundant in the cytoplasm of these cells. The processing and distribution of HSV-1 gD was investigated as a representative of all viral glycoproteins since gD is one of the most important and well studied glycoproteins of HSV-1. By using Western immunoblot techniques, processing of HSV-1 gD in infected cells treated with BFA was found to occur even

though full maturation of this glycoprotein was not achieved (Cheung, Banfield et al. 1991). In addition, some of the gD isolated from BFA-treated cells were found to be processed to endo H-resistant forms. Resistance of glycoproteins to endo H digestion is a marker for the processing of glycoproteins by cis/medial Golgi resident enzymes. The presence of endo H-resistant forms of gD in BFA-treated cells suggests that the cis/medial Golgi and their resident enzymes were recycled into ER in the presence of BFA. The lack of mature gD in BFA-treated cells indicates that no glycoproteins were transported past the combined ER/Golgi compartment to the *trans* Golgi membranes. These results are in agreement with observations made on the processing of VSV G proteins in BFA-treated cells by Doms *et al* (Doms, Russ et al. 1989).

In addition to the alteration of glycoprotein processing, BFA also affected the distribution of viral glycoproteins in cells. Immunofluorescence studies revealed that BFA blocked the transport of gD to the plasma membrane (Fig. 7A,B). Unlike gD in control cells which localised to Golgi-like compartments (Fig. 7A), gD in BFA-treated cells was spread over the cytoplasm in a fine reticular distribution characteristic of ER structures (Fig. 7B). This again supports the hypothesis that BFA induces the Golgi and its contents to flow back to the ER compartment. gD that is in transit in the host cell secretory system is dispersed into the enlarged ER and is not able to move beyond this blockage. It is in this combined compartment where viral glycoproteins are processed by the mixture of ER and Golgi enzymes.

One of the interesting characteristics of BFA is the rapid

recovery of protein secretion upon removal of BFA. This property was examined using immunofluorescence to follow the redistribution of HSV-1 gD at various times post BFA removal. Within 30 minutes, the evenly dispersed gD began to relocalise to discrete perinuclear regions, suggesting that gD was transported to the reorganising Golgi structure (Fig. 7C). After 2 to 3 h of recovery from the effects of BFA, the distribution of gD was identical to the untreated control cells (Fig. 7D). gD was evident in Golgi-like compartments and on the plasma membrane, indicating that the transport of viral glycoproteins had also recovered. These results are in complete accordance with the results obtained by Doms *et al* on the redistribution of VSV G proteins post BFA removal (Doms, Russ et al. 1989). Together, these results suggest that the recovery of viral glycoprotein distribution and secretion, at least at a morphological level, is rapid upon the removal of BFA.

Although the immunofluorescence data showed that the effects of BFA on the distribution of viral glycoproteins were reversed within 2 to 3 h, the ability to release infectious virions was found to be not as easily reconstituted. In following the release of infectious particles from infected cells recovering from 16 h of exposure to BFA, it was observed that only a small amount (<0.1%) of viral particles were released by 3 h post BFA removal as compared to the mock BFA-treated cells (Fig. 8). In contrast, a similar experiment using the non-glycosylated human growth hormone (hGH) showed that the secretion of hGH in BFA-treated cells was recovered within 30 min post BFA removal (Cheung, Banfield et al. 1991). It is likely that a long recovery period is

required before large amounts of virus are released because enveloped particles are not accumulated in the BFA-treated cell cytoplasm. An extended period of time may be needed for the recovering cells to process and transport newly-synthesized particles through the reorganising secretory pathway.

It is also possible that infected cells treated with BFA for long periods of time may never fully regain their potential output of progeny viruses. There may be cellular functions that are critical to viral propagation at particular times of the HSV-1 infection cycle. If these functions are disrupted by BFA, fully productive viral infection will not occur. Regarding this issue, this study demonstrated that infected cells exposed to BFA for 4 h periods at different parts of the infection cycle are affected differently as measured by the cumulative amount of virus released at 20 h.p.i.. Exposure to BFA at 4-8, 8-12, and 12-16 h.p.i. resulted in 60-75% reduction of viral output as compared to mock BFA-treated cells (Fig. 9). In contrast, this effect was not observed when BFA was applied to infected cells at 0-4, and 16-20 h.p.i..

To understand these results, it is important to know about the chronological order of viral events in an HSV-1 replication cycle. Using electron microscopy studies, Smith and DeHarven reported that the assembly of viral capsids first start at about 4 to 5 h.p.i. (Smith and DeHarven 1973). Envelopment of particles at the nuclear membrane starts at 6 h.p.i., and the release of infectious particles into the extracellular medium can first be detected at about 8 h.p.i.. As noted before, BFA does not alter viral protein synthesis and DNA replication. Since viral assembly does not occur

until 4 to 5 h.p.i., it is not surprising that exposure of infected cells to BFA at 0-4 h.p.i. does not affect the subsequent egress of viral particles. When BFA was applied to infected cells at 16-20 h.p.i., a small inhibitory effect on viral release was seen. The continued release of virions in the presence of BFA observed in this case suggests that matured viruses are accumulated in cellular locations not sensitive to BFA prior to egress from cells, and that BFA does not affect the actual viral release events.

The most interesting results of this experiment are the reductions of viral release when BFA was added at 4-8, 8-12, and 12-16 h.p.i.. BFA added at 4-8 h.p.i. would probably affect the exit of enveloped particles from the infected nucleus since envelopment of particles at the nucleus occurs at about 6 h.p.i.. Even though these cells had 12 hours of recovery time from BFA, there was still a 60% reduction in the cumulative viral output at 20 h.p.i.. This suggests that enveloped particles that are blocked at the nucleus are not able to egress from the infected cells even after the effects of BFA on the secretory pathway is reversed. As suggested earlier, enveloped particles blocked at the nucleus may de-envelope and enter into the cytoplasm as naked nucleocapsids. In this case, their infectivity will not be regained even after BFA is removed from the host cells.

BFA applied at 8-12, and 12-16 h.p.i. affects a different part of the viral life cycle as assembled and presumably enveloped particles are already in transit through the secretory pathway when BFA was added. Particles in the Golgi complex may be recycled into the ER and be trapped there until BFA was removed and the

secretory system has recovered. In a study reported at the 15th International Herpesviruses Workshop, BFA was found to inhibit the egress of pseudorabies virus (PRV), which is another member of the herpesvirus family (Whealy, Robbins et al. 1990). Electron micrographs of PRV infected cells treated with BFA for short periods of time showed that enveloped particles were accumulated in the ER. Assuming that BFA applied to HSV-1 infected cells at 8-12 and 12-16 h.p.i. also leads to the accumulation of enveloped particles in the ER, one important question raised is that whether these particles resume their course of egress after BFA is removed or are they functionally dead and are never released from the cells? The lack of viral output measured at 20 h.p.i. seems to suggest that particles that are temporarily trapped are not able to regain their ability to egress from the infected cell. These detained particles may be degraded in the cytoplasm or they may be detoured to somewhere else and are unable to re-enter the normal route of egress. To fully address these possibilities, further studies determining the fate of these trapped particles are required. It is possible to use Bromodeoxyuridine (Budr), which is a base analogue of thymidine, to pulse label newly synthesized viral DNA in infected cells. Using commercially available antibodies directed against Budr, one may be able to do immuno-labeling electron microscopy and follow the egress of the Budr labelled progeny HSV particles. Applying this technique on infected cells exposed to brief periods of BFA at different time points of the virus life cycle may lead to results that will uncover the fate of these trapped viral particles.

Given the importance of the host secretory pathway to the

HSV-1 life cycle, it is not surprising to find that drugs such as BFA, which interfere with the secretion of proteins, also disturb the propagation of this virus. Nevertheless, this study did yield some unexpected results. Since BFA blocks the movement of proteins from the ER to the Golgi complex, one would expect to see herpes particles accumulating at the ER of BFA-treated cells. This, however, is not the case when BFA is present early in the infection. There appears to be a complete lack of enveloped particles in the cell cytoplasm, and viral particles accumulate aberrantly in the nucleus instead. This suggests that the mechanism of movement of virions from the nucleus to the ER is also sensitive to the effects of BFA. Another interesting result is the persistent inhibition of the release of viral particles after BFA is removed. Egressing viral particles affected by the action of BFA may never be released from the cell even after the host secretory pathway recovers from the effects of BFA. This suggests that the egress of HSV-1 progeny particles can be redirected to non-productive pathways. Further research on the cellular target of BFA and the fate of the re-routed viral particles may yield important information on the complex mechanism of egress of HSV-1. Such an understanding of this mechanism may lead to antiviral strategies that can shift the normal viral release to self-destructive pathways.

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