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Evaluation of the primary effect of brefeldin A treatment upon herpes simplex virus assembly

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Addition of the drug brefeldin A (BFA) to cells infected by herpes simplex virus (HSV) type 1 is known to result in a complex pattern of defects in particle assembly. BFA-treated, infected cells accumulate perinuclear enveloped virions and non-enveloped ('naked') cytoplasmic capsids, and it has been difficult to interpret these data in terms of the assembly pathway of HSV and the known effects of BFA on the secretory apparatus. Since BFA is a cytotoxic drug, and earlier studies commonly examined the effects of long-term BFA incubations on infected cells, it was hypothesized that the drug could have pleiotropic and indirect effects on HSV assembly. To test this, use was made of an HSV synchronized assembly assay, in which cells are infected with the virus mutant *ts*Prot.A and maintained at 39 °C to induce reversible accumulation of a population of procapsids. By first adding BFA and then shifting these cells to 31 °C for 3 h to allow the accumulated procapsids to mature, it was possible to test the effect of short-term BFA treatment on only those HSV assembly events that are downstream of procapsid maturation. Under these conditions, it was found that procapsids matured and packaged the viral genome normally, but remained non-enveloped and failed to exit the nucleus. It is concluded that the primary effect of BFA on HSV replication is to inhibit budding at the inner nuclear membrane.

Introduction

Herpes simplex virus (HSV) assembly initiates in the nuclei of infected cells, where procapsids become packaged with the viral genome and mature into nucleocapsids. These capsids are then thought to bud into the inner nuclear membrane, generating enveloped particles in the perinuclear space (Roizman & Sears, 1993, 1996). The subsequent pathway of egress is a contentious issue (Enquist *et al.*, 1998). One view, the single envelopment model, is that the perinuclear virions traffic out of the cell via the classical secretory pathway (Di Lazzaro *et al.*, 1995; Torrisi *et al.*, 1992). An alternative view, the re-envelopment model, is that the initial envelope acquired by the virions from the inner nuclear membrane fuses with the outer nuclear membrane to release naked capsids into the cytoplasm. These capsids then traffic through the cytoplasm, bud into an organelle such as the Golgi, trans-Golgi network (TGN) or endosomes, and acquire their final envelope (Stackpole, 1969; Holland *et al.*, 1999; Miranda-Saksena *et al.*, 2000; Griffiths & Rottier, 1992). Lipid analysis of the HSV envelope (van Genderen *et al.*, 1994) and the results of targetting of HSV glycoproteins gH and gD to particular organelles (Browne *et al.*, 1996; Whiteley *et al.*, 1999) are most consistent with the re-envelopment model for egress. Furthermore, we have demonstrated recently, by fractionation of infected-cell organelles, that infectious HSV particles cannot be detected in the Golgi cisternae during egress, but reside in compartments with properties similar to those of endosomes and the TGN (Harley *et al.*, 2001). This finding is also consistent with re-envelopment of cytoplasmic capsids rather than trafficking of perinuclear virions through the classical secretory pathway.

One reagent that has been used to study HSV assembly is the drug brefeldin A (BFA). BFA is a fungal metabolite that has multiple effects upon the organelles of the secretory pathway, including inhibition of trafficking from endoplasmic reticulum (ER) to the Golgi apparatus, fusion of the *cis*, *medial* and *trans* cisternae of the Golgi with the ER (and subsequent disappearance of the Golgi) and fusion of the TGN with endosomes (Pelham, 1991; Sciaky *et al.*, 1997). In principle,

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BFA treatment might be expected to provide some information regarding the pathway of HSV egress. The single envelopment model predicts that enveloped virions should accumulate in the perinuclear space and ER, since their export from the ER via the classical secretory pathway would be blocked. Such a phenotype is clearly seen following short-term BFA treatment of pseudorabies virus (PRV)-infected cells (Whealy *et al.*, 1991); however, the extent to which these data can be used to distinguish between the various models for PRV egress has subsequently been questioned (Enquist *et al.*, 1998). In contrast, the re-envelopment model predicts that naked capsids should accumulate in the cytoplasm, because of the effect of BFA on the organelles (Golgi/TGN/endosomes) utilized for re-envelopment. In practice, both phenotypes have been reported for BFA-treated, HSV-infected cells (Koyama & Uchida, 1994; Chatterjee & Sarkar, 1992; Cheung *et al.*, 1991). One possible explanation for this complex result is that, in many of the earlier HSV studies, cells were exposed to BFA for extended periods of time (between 12 and 24 h). BFA is a cytotoxic drug (Jensen *et al.*, 1995; Ishii *et al.*, 1989; Yan *et al.*, 1994; Argade *et al.*, 1998) and, over such time-courses, could well have pleiotropic effects upon the biology of both the virus and the infected cell.

In earlier studies, we developed a model system to dissect late events in HSV assembly. The HSV-1 strain *ts*Prot.A carries a reversible temperature-sensitive lesion in UL26, which encodes the maturational protease Pra (Rixon *et al.*, 1988; Gao *et al.*, 1994; Preston *et al.*, 1983). At the non-permissive temperature of 39 °C, *ts*Prot.A-infected cells accumulate immature nuclear procapsids (Rixon & McNab, 1999; Newcomb *et al.*, 2000). Following downshift to the permissive temperature of 31 °C, these procapsids recruit the capsid subunit VP26 (Chi & Wilson, 2000), package DNA (Preston *et al.*, 1983; Church *et al.*, 1998; Dasgupta & Wilson, 1999) and give rise to exocytosing infectious particles in a synchronous wave (Church & Wilson, 1997; Harley *et al.*, 2001). We have used this assay system to test the effect of various compounds upon HSV assembly by adding the drugs only at the time of temperature downshift (Church *et al.*, 1998; Dasgupta & Wilson, 1999; Harley *et al.*, 2001). In this way, incubations can be for short periods of time, and any observed effect must be due to the action of the drug upon assembly and not upon other processes such as DNA synthesis or gene expression.

In the current study, we used this assay system to test the primary effect of BFA upon HSV assembly. Our results confirm earlier findings that BFA blocks p.f.u. production. However, in contrast to those studies, we found the assembly defect to be at the point of formation of perinuclear enveloped virus particles. Under our conditions, packaged capsids became trapped within the nucleoplasm and failed to bud into the perinuclear space and traffic into the cytoplasm. We conclude that the immediate effect of BFA upon the assembly pathway of HSV is to block capsid envelopment at the inner nuclear membrane.

Methods

Cells and viruses. Vero cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum and 1% penicillin/streptomycin (Gibco BRL). HSV strain *ts*Prot.A was grown and titrated as described previously (Church & Wilson, 1997). In all experiments, 1 h after addition of virus to cells, unpenetrated virus was inactivated by washing with glycine-buffered saline, as described previously (Church & Wilson, 1997).

Scoring levels of infectious progeny virus and measurement of packaging and envelopment. In order to measure the number of infectious progeny, cells were scraped up and sonicated and the resulting extracts were titrated on pre-formed Vero cell monolayers. Quantification of packaged capsids and enveloped packaged capsids by DNase I protection assay was done as described previously (Harley *et al.*, 2001), except that radiolabelling of the viral DNA was achieved by incubation with 10 μCi/ml [^{3}H]thymidine (New England Nuclear). Measurement of DNA packaging by Southern blot and probing with a ^{32}P-radiolabelled SQ junction probe were done as described previously (Church *et al.*, 1998).

Preparation of nuclear extracts and post-nuclear supernatants. Infected cells were washed twice with ice-cold homogenization buffer (250 mM sucrose, 10 mM Tris–HCl, pH 7·6, 2 mM $MgCl_2$), scraped up, pelleted and resuspended in the same buffer. Cells were gently broken by repeated passage through a 25 gauge needle and the nucleus (N) and post-nuclear supernatant (PNS) were separated by centrifugation at 2000 ***g*** for 10 min.

Electron microscopy. Infected Vero cells were fixed and processed for electron microscopy as described previously (Church & Wilson, 1997).

Results

Effect of BFA on production of infectious virion progeny

Our general experimental design is shown in Fig. 1(*a*). Vero cells were infected with HSV strain *ts*Prot.A at a multiplicity of 10 and incubated at 39 °C to accumulate procapsids. BFA was added after 6·5 h (to a final concentration of 5 μg/ml in all experiments) and, after a further 0·5 h, the incubation temperature was lowered to 31 °C in order to allow procapsid maturation to proceed. Samples were collected at the time of shift in order to determine the background levels of HSV maturation and after 3 h at 31 °C, when maturation of the accumulated procapsids into infectious particles was complete (Church & Wilson, 1997). Fig. 1(*b*) shows the numbers of p.f.u. in cell extracts harvested at these time-points. After 7 h of incubation at 39 °C, there were $1{\cdot}2 \times 10^4$ p.f.u. present in our extracts. As shown previously, this is derived from surviving input virus (Church & Wilson, 1997). After 3 h incubation at 31 °C, the number of p.f.u. increased by approximately 4 logs in untreated cells, while, in the presence of BFA, this increase was 1000-fold less. Note that, since these assays were performed on total cell extracts and not on secreted virus from the medium, the loss of progeny p.f.u. was not due merely to inhibition of egress from the cell. The decrease in p.f.u. was also

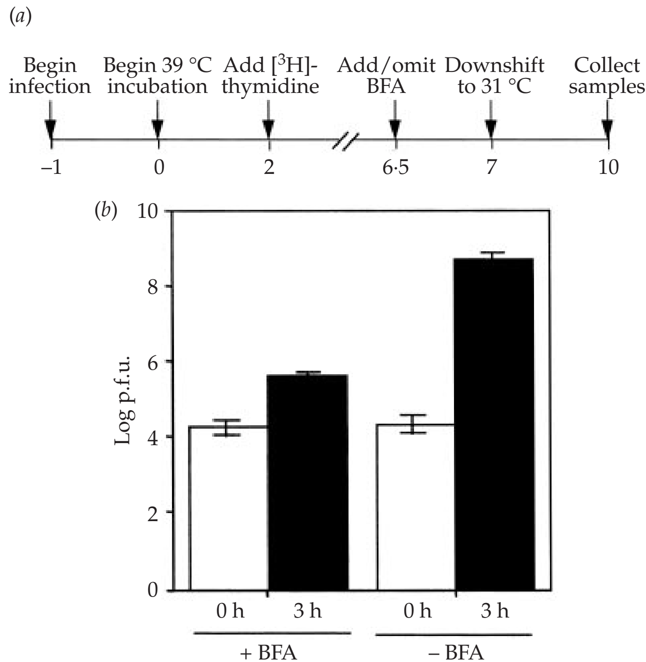

Fig. 1. Effect of BFA on production of infectious virus. (*a*) General experimental design. Numbers below the line correspond to the time (in h) after completion of initial infection. Vero cells were infected with HSV strain *ts*Prot.A at a multiplicity of 10. After 6·5 h of incubation at the non-permissive temperature, BFA was added to a concentration of 5 µg/ml, or omitted, and cells were incubated for a further 0·5 h before being shifted to 31 °C. Three h after downshift to the permissive temperature, infected cells were collected and extracts were prepared. (*b*) P.f.u. yields in cell extracts, represented on the logarithmic vertical axis. Virus yields at 0 h are derived from residual input virus. Plotted values are the mean of four independent determinations and error bars indicate the range from the mean.

not due to subsequent interference with plaque formation by trace levels of BFA present in the cell extracts, since no reduction in numbers of p.f.u. resulted when extracts from BFA-treated, infected cells were mixed with extracts from untreated infected cells (data not shown). We conclude that BFA inhibits p.f.u. production under our conditions of synchronized assembly. Interestingly, the inhibition was not complete; even in the presence of BFA, there was a reproducible 10-fold increase in p.f.u. over background during the 3 h incubation at 31 °C. This partial inhibition of HSV replication contrasts with the more dramatic effects reported in earlier studies. This could reflect the fact that, in our assay system, BFA is given the opportunity to inhibit only HSV assembly and not other steps in the HSV life-cycle. Alternatively, this population may have arisen via a BFA-resistant pathway, as suggested previously (Koyama & Uchida, 1994).

Effect of BFA on packaging and envelopment of HSV

We have recently developed assays that permit quantification of the relative amounts of naked and enveloped HSV capsids in infected-cell extracts (Harley *et al.*, 2001). We used these assays to determine the step at which BFA blocks virus maturation. [^{3}H]Thymidine-labelled infected cells were taken through the time-course shown in Fig. 1(*a*) and then harvested and sonicated to make extracts. The extracts were divided into two. One half was treated with DNase I for 2 h to destroy all unpackaged high molecular mass DNA, while the other half was incubated with proteinase K for 2 h prior to DNase I treatment to destroy both unpackaged DNA and packaged DNA present in naked capsids, lacking envelopes. Extracts were then precipitated with TCA, washed and subjected to scintillation counting. Fig. 2(*a*) shows that BFA treatment had little effect on DNA packaging; however, there was a drop of about 3-fold in the number of enveloped capsids in the presence of BFA. In order to verify independently that BFA had no effect on packaging of the viral genome, we carried out a Southern blot analysis of DNase I-treated cell extracts with a cleavage junction-specific, ^{32}P-labelled DNA probe. Fig. 2(*b*) shows that there were similar amounts of packaged DNA in BFA-treated and untreated cells (compare lanes 3 and 4). As expected, at 0 h after downshift, the viral genome was not yet packaged and hence was completely sensitive to DNase I treatment (lanes 1 and 2). Thus, it appears that the block in HSV-1 replication by BFA is after formation of normal packaged nuclear capsids, but before their envelopment.

BFA treatment results in capsids being retained in the nucleus

If nuclear capsids are really enveloping less efficiently at the inner nuclear membrane, this predicts that fewer capsids should be present in the cytoplasm of BFA-treated cells. To test this, we prepared nuclear pellets and PNS from infected cells and performed packaging assays to score capsid yields in each fraction. Table 1 shows the results of four such independent experiments. We observed that, upon BFA treatment, there was a decrease in the number of packaged capsids in the cytoplasm of infected cells relative to the number of capsids in the nuclear fraction. While there was some variation in the ratio of nuclear to post-nuclear counts for each sample (N/PNS columns), addition of BFA consistently resulted in an approximately 3-fold increase in the N/PNS capsid ratio. These results are consistent with failure of capsids to bud at the inner nuclear membrane.

Short-term BFA treatment does not result in the accumulation of perinuclear enveloped virions

Our results suggest that capsids accumulate in the nucleus upon BFA treatment. By biochemical assay, these capsids appear to be unenveloped; hence, they cannot be accumulating in the perinuclear space, but would be expected to reside in the nucleoplasm. This is in contrast to previous findings, where perinuclear enveloped capsids were readily apparent following long-term BFA treatment (see Introduction). In order to test

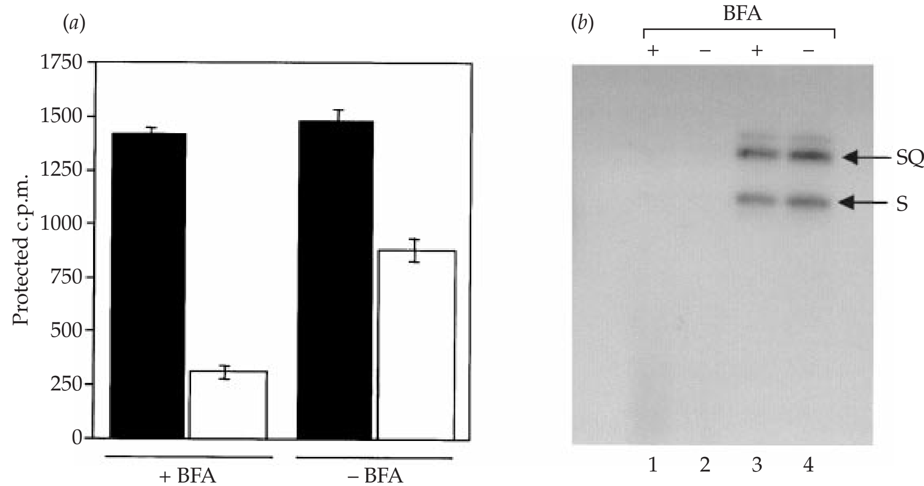

Fig. 2. BFA affects HSV-1 envelopment but not DNA packaging. (*a*) Vero cells were infected and treated with BFA (+BFA) or mock treated (−BFA) as in Fig. 1 (*a*). Cells were harvested at the time of downshift or after 3 h at 31 °C and extracts were prepared. Extracts were subjected to packaging (filled bars) or envelopment (open bars) assays as shown. Background levels of packaging and envelopment at the 0 h time-point (201 c.p.m. or less in each case) were subtracted from the signals at the 3 h time-point and the differences were plotted. (*b*) In order to verify independently that DNA packaging and cleavage are not affected by BFA, cells were infected as described in (*a*) and treated with BFA (lanes 1 and 3) or mock treated (lanes 2 and 4). Cells were then harvested either at the 0 h time-point (lanes 1 and 2) or after 3 h at 31 °C (lanes 3 and 4) and extracts were digested with DNase I. Protected DNA was purified by phenol–chloroform extraction and ethanol precipitation and then digested with *Bam*HI, electrophoresed on a 1·0% agarose gel, blotted to a nylon membrane and hybridized with a ^{32}P-labelled probe corresponding to the SQ cleavage junction. The positions of the uncleaved internal SQ junction sequence and the S cleavage product are indicated at the right of the gel. The Q band reacts weakly with the probe and is not visible in this exposure.

Table 1. Quantification of the effect of BFA on the intracellular distribution of packaged capsids

Nuclear pellets (N) and post-nuclear supernatants (PNS) were scored for c.p.m. of DNase I-resistant, [^{3}H]thymidine-labelled DNA and the mean of three independent assays was determined for each of four independent experiments. The N/PNS ratio for BFA-treated samples was divided by the N/PNS ratio for untreated samples to generate the values in the right-most column.

Treatment	Mean c.p.m. N	Mean c.p.m. PNS	N/PNS ratio	Fold effect of BFA on N/PNS
Experiment 1*				
BFA-treated	822	198	4·1	3·5
Untreated	663	560	1·2	
Experiment 2				
BFA-treated	2548	1303	1·95	2·9
Untreated	1304	1927	0·67	
Experiment 3				
BFA-treated	1156	1424	0·8	2·7
Untreated	1047	3476	0·3	
Experiment 4				
BFA-treated	1088	1425	0·76	3·2
Untreated	828	3441	0·24	

* Fivefold fewer cells were used than in experiments 2–4.

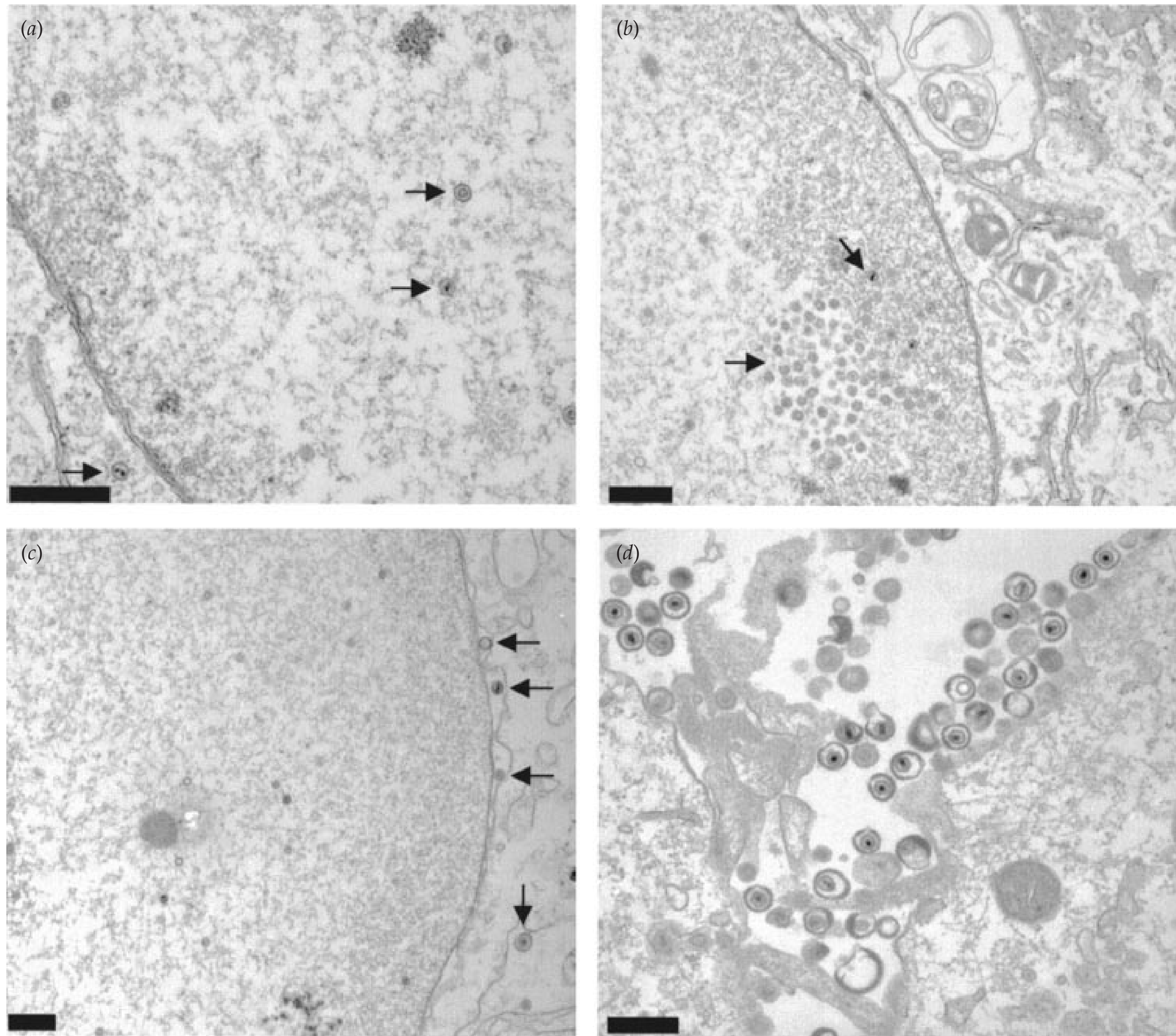

Fig. 3. Ultrastructural analysis of *ts*Prot.A-infected cells. (*a*)–(*b*) Vero cells were infected with *ts*Prot.A and taken through the complete time-course shown in Fig. 1 (*a*), with BFA added at 6·5 h post-infection. Arrows indicate capsids in nuclei or a naked cytoplasmic capsid (*a*, lower left), rarely seen in BFA-treated cells under these conditions. The left-hand arrow in (*b*) indicates a capsid cluster, as observed previously (Church & Wilson, 1997). (*c*)–(*d*) Vero cells were infected with *ts*Prot.A at a multiplicity of 10 but then incubated for 16 h in the presence (*c*) or absence (*d*) of BFA. In (*c*), arrows indicate perinuclear enveloped virions and (lower right) an enveloped particle inside a cytoplasmic vacuole (possibly ER). In (*d*), note the abundant extracellular enveloped particles, never seen in the presence of BFA. Bars, 500 nm.

our conclusion further, we performed electron microscope studies. Fig. 3 shows that, when cells were taken through the time-course shown in Fig. 1 (*a*) and then fixed and processed for electron microscopy after 3·5 h in the presence of BFA, there was no accumulation of enveloped particles in the perinuclear space (Fig. 3 *a*, *b*). The effect of BFA on envelopment was due to our synchronized assembly conditions and short-term drug treatment, rather than to some other unusual property of the *ts*Prot.A strain, since infection of cells with *ts*Prot.A at the permissive temperature for 16 h in the presence of BFA led to accumulation of enveloped virions in the perinuclear space (Fig. 3 *c*) rather than at the cell surface, as seen when BFA was absent (Fig. 3 *d*).

Discussion

The effect of BFA treatment on HSV assembly and egress has been difficult to interpret. One possible explanation for the complex results obtained in earlier studies is that infected cells were often treated with this cytotoxic drug for long periods of time (approximately 12–24 h) and with conditions under which DNA replication, gene transcription and translation could all be affected. In the current study, we used the reversible temperature-sensitive mutant *ts*Prot.A to permit viral DNA synthesis and gene expression to occur normally in the absence of the drug, and then added BFA only during maturation of accumulated procapsids. Our results indicate

that, while the number of packaged capsids was essentially unaltered, the ratio of nuclear capsids to cytoplasmic capsids increased 3-fold in the presence of BFA. This failure to exit from the nucleus was due to a defect in envelopment at the inner nuclear membrane, since envelopment was reduced to one-third of that occurring in untreated cells. This result was confirmed by electron microscopy: as expected, we failed to see any accumulation of enveloped virions in the perinuclear space. Therefore, unlike earlier studies, BFA treatment did not result in the accumulation of enveloped virions in the perinuclear space and naked capsids in the cytoplasm. When BFA was present for a period of 16 h in cells infected with *ts*Prot.A at the permissive temperature, perinuclear virions did accumulate, showing that this strain can demonstrate the previously reported phenotype under the appropriate conditions. Thus, the effects of short-term treatment with BFA are different from those of long-term treatment, the former appearing to block envelopment at the inner nuclear membrane.

It has been demonstrated that, upon treatment with BFA, the Golgi apparatus fuses with the ER (Sciaky *et al*., 1997). Once this occurs, the nuclear membranes, which are contiguous with the ER, become flooded with Golgi proteins and lipids. We speculate that this dramatic alteration in the biochemical composition of the inner nuclear membrane could render it less suitable for budding of the HSV capsid, thereby resulting in accumulation of unenveloped nuclear capsids. To test this possibility, it will be necessary to examine the kinetics with which the lipid composition of the inner nuclear membrane changes during BFA treatment and to compare this with the time-point at which capsids begin to accumulate in the nucleoplasm. Unfortunately, such an experiment is impossible at present, since no techniques exist to isolate inner nuclear membrane lipids in the absence of the outer nuclear membrane and ER.

Why should short-term BFA treatment lead to accumulation of non-enveloped HSV in the nucleoplasm and of enveloped PRV in the perinuclear space and ER, as reported by Whealy *et al*. (1991)? One possible explanation is that the PRV study used asynchronous infections, wherein cells contain virions at every stage of assembly. Under these conditions, even if BFA addition blocked PRV capsid envelopment at the inner nuclear membrane, one would expect there already to be enveloped virions in the perinuclear space, formed prior to addition of the drug (which would be absent in our studies, where assembly is blocked at the procapsid stage). If, in addition to blocking envelopment at the inner membrane, BFA blocked de-envelopment at the outer membrane or export from the ER, these enveloped particles would be expected to accumulate, as observed by Whealy *et al*. (1991). Alternatively, whatever the reason for inhibition of HSV envelopment at the inner nuclear membrane, there is no reason to assume that the effect on PRV would be as severe. In this context, it is worth noting that, even for HSV, perinuclear virions do accumulate upon long-term BFA treatment, indicating that the envelopment process is only slowed, not blocked completely. There are also reasons to believe that HSV and PRV may differ in the details of their envelopment apparatus (Enquist *et al*., 1998). The PRV gene UL3.5, for example, has been suggested to play an essential role in cytoplasmic envelopment (Fuchs *et al*., 1996), but has no homologue in HSV. Similarly, mutations in UL20 cause HSV to accumulate in the perinuclear space (Baines *et al*., 1991) but cause PRV to accumulate in cytoplasmic vesicles (Fuchs *et al*., 1997).

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