Inhibition of human cytomegalovirus maturation by brefeldin A

M. Eggers, 1 E. Bogner, 1 B. Agricola, 2 H. F. Kern 2 and K. Radsak 1*

1 Institut für Virologie der Philipps-Universität, Robert-Koch-Strasse 17 and 2 Institut für Zellbiologie und Zellpathologie, Robert-Koch-Strasse 5, 355 Marburg, Germany

Brefeldin A (BFA) was found to interfere with specific events of human cytomegalovirus (HCMV) maturation in human fibroblasts. Ultrastructural as well as biochemical studies suggested that short-term exposure of infected cultures to BFA during the late infectious cycle primarily prevented Golgi-dependent processes, e.g. envelopment of naked cytoplasmic nucleocapsids in the trans-Golgi network (TGN) and normal processing of glycoprotein B. In contrast, the nuclear phase of viral morphogenesis, e.g. transport budding at the nuclear envelope, was not impaired. These observations were compatible with the interpretation that HCMV morphogenesis may involve sequential budding events at the nuclear envelope and at cisternae of the TGN. BFA treatment during the early infectious cycle efficiently inhibited HCMV-DNA synthesis and thus late viral functions, preventing production of viral progeny. Cytotoxicity was excluded as a cause for these findings.

Introduction

There is general agreement that herpesvirus morphogenesis involves budding at the nuclear envelope (Roizman & Sears, 1990) prior to subsequent maturation in the cytoplasm. However the details of the cytoplasmic maturation events have remained a matter of controversy. Experimental evidence suggests (Campadelli-Fiume et al., 1991) on one hand that herpesviral nucleocapsids are enveloped exclusively during budding through nuclear membranes. Subsequent maturation, e.g. of viral envelope glycoproteins, is thought to take place during passage of virions through the Golgi complex. The presence of naked cytoplasmic nucleocapsids in infected cells was interpreted to represent aberrant degradation of progeny virus (Campadelli-Fiume et al., 1991). On the other hand, data obtained for varicella-zoster virus (VZV; Jones & Grose, 1988), herpes simplex virus (HSV; Cheung et al., 1991) and pseudorabies virus (PRV; Whealy et al., 1991) support the concept of two sequential events in envelopment, i.e. transient envelopment of nucleocapsids at the inner nuclear membrane and release at the outer nuclear membrane of naked nucleocapsids into the cytoplasm (Gong & Kieff, 1990; Severi et al., 1988). These receive their final envelope by enclothe with parts of trans-Golgi cisternae which are modified by fully processed viral glycoproteins. Within Golgi-derived vesicles mature viral progeny particles are transported to the plasma membrane and released by exocytosis.

Regarding the presumed involvement of the Golgi complex in herpesvirus morphogenesis, functional disintegration of this cellular organelle by the fungal metabolite brefeldin A (BFA; Chege & Pfeffer, 1990; Fujiwara et al., 1988; Lippincott-Schwartz et al., 1990; Ulmer & Palade, 1991; Orci et al., 1991) should clearly impair the late phase of virus maturation. The observations reported for the alphaherpesviruses HSV and PRV (Cheung et al., 1991; Whealy et al., 1991) support this view since infectious enveloped particles are not released from infected cells in the presence of BFA. In contrast, the preceding phase of the infectious cycle, including Golgi-independent events, e.g. nuclear synthesis of nucleocapsids and transport budding at the inner nuclear membrane, is not impeded by BFA, thus allowing the uninterrupted appearance of enveloped viral nucleocapsids in the perinuclear cisterna and rough endoplasmic reticulum (RER).

Experiments were undertaken to study the role of the Golgi apparatus in the morphogenesis of the betaherpesvirus, human cytomegalovirus (HCMV). During the course of our experiments it became apparent that, in the case of HCMV, BFA interfered not only with late but also with early events of the HCMV replication cycle. The finding that BFA addition in the early phase after infection efficiently prevented HCMV DNA replication was unexpected as the corresponding effect was not observed in other herpesvirus systems (Cheung et al., 1991; Whealy et al., 1991). In addition, ultrastructural and biochemical data will be presented showing that treatment of HCMV-infected cultures exclusively during the later phases of the infectious cycle impeded Golgi-dependent virus maturation events.
**Methods**

**Cells and virus.** All experiments were carried out with confluent human foreskin fibroblasts (HFFs) between the sixth and 15th passage. The monolayers were cultivated with Eagle's MEM (Gibco), supplemented with 10% foetal calf serum (FCS). For experimental infection the Towne strain of HCMV (Furukawa et al., 1973) and the KOS strain of herpes simplex virus (Radsak, 1978) were used at multiplicities of 3 and 1, respectively. Under these conditions viral antigen was produced in about 95% and 50% of the cells, respectively, during the first round of replication. Virus titres (infectious units, i.u.) were determined by the endpoint dilution method combined with indirect immunofluorescence using commercial monoclonal antibody (MAb) (Dupont) for the detection of early viral antigen (Kaiser & Radsak, 1987). For the determination of extra- and intracellular virus titres, culture medium of infected cells was used as the source of extracellular virus; lysates of infected cells, after three cycles of freezing and thawing in PBS (1·5 × 10⁶ cells/ml), were used as the source of intracellular virus.

**Inhibitor treatment.** BFA, prepared as a stock solution of 1 mg/ml methanol, was used at 0·2 to 6 μg/ml culture medium for various time intervals post-infection (p.i.) as indicated in Results.

**Transmission electron microscopy.** For ultrastructural analysis of untreated or BFA-treated (1 μg/ml), infected HFFs (1·5 × 10⁶ cells/ml), were harvested by scraping at various times p.i. according to the experimental schedule (see Results) after washing the monolayers with PBS. After sedimentation the cell pellet was fixed with paraformaldehyde according to Ilo & Karnovsky (1968), contrasted sequentially with osmium tetroxide and uranyl acetate prior to dehydration, embedding in Epon and ultrathin sectioning. The electron micrographs chosen for presentation in Results show typical structures of BFA-treated HCMV-infected HFFs and are representative of at least two separate experiments and more than 200 single cells examined.

**Radioactive labelling of infected cultures.** For DNA labelling [3H]thymidine (10 μCi/ml; specific activity 20 Ci/mmol) was included in the culture medium for the desired times prior to harvesting of the cultures and extraction of DNA (see below). For polypeptide labelling, complete culture medium was replaced by labelling medium consisting of MEM lacking methionine, plus [35S]methionine (20 to 50 μCi/ml; specific activity > 1000 Ci/mmol; Amersham Buchler) for appropriate times prior to cell fractionation and analysis.

**Extraction of DNA and isopycnic centrifugation in neutral CsCl.** DNA was extracted from radiolabelled cells following lysis with 50 mM-Tris-HCl pH 8, 100 mM-NaCl, 5 mM-EDTA and 1% SDS by phenol-chloroform-isooamyl alcohol treatment (Hirai & Watanabe, 1976; Kaiser & Radsak, 1987). DNA content of the samples was estimated by the method of Lowry et al. (1951). Separation of cell and viral DNA was achieved by isopycnic centrifugation in neutral CsCl (mean density 1·71 g/ml) for 65 h at 17 °C and 36000 r.p.m. in a 50Ti fixed angle rotor of a Beckman L55 centrifuge. Under these conditions HFF DNA sediments at a density of 1·696 g/ml, HCMV DNA at 1·717 g/ml and HSV DNA at 1·725 g/ml (Radsak & Weder, 1981).

**Purification of radiolabelled virus.** For isolation of extracellular radiolabelled HCMV, infected cultures (6 × 10⁷ cells) were cultivated in the presence of [35S]methionine 60 to 72 h p.i. (see above) prior to sedimentation of virus and dense bodies (100000 g for 2 h at 4 °C) from the culture medium. For isolation of intracellular virions the labelled cell monolayers were harvested by scraping and sedimentation at 1000 g for 10 min. Cell pellets were resuspended in PBS and subjected to three cycles of quick freezing and thawing to liberate the virus. Cell debris and nuclei were removed by sedimentation at 3000 g for 10 min. Virus was subsequently sedimented from the post-nuclear supernatant at 100000 g for 2 h at 4 °C. Pellets of extra- or intracellular virus were resuspended in 50 mM-Tris-HCl pH 7·5, 100 mM-NaCl. Purification and separation of virus from dense bodies was carried out by gradient centrifugation according to Talbot & Almeida (1977).

**Cell fractionation and preparation of extracts.** The method of Radsak et al. (1990) was used. Infected HFF monolayers (1·2 × 10⁶ cells) were harvested and sedimented (1000 g for 10 min) in cold PBS containing 0·5 mM-PMSF. Total extracts of cell pellets were obtained by lysis, sonication and sedimentation of insoluble material in buffer B (20 mM-Tris-HCl pH 9, 0·3 M-NaCl, 10% glycerol, 1 mM-CaCl₂, 0·5 mM-MgCl₂, 2 mM-EDTA, 0·5% NP40, 0·5 mM-PMSF). Cell fractionation for preparation of nuclei essentially free of cytoplasmic contaminants was carried out at 4 °C by the following method. Pelleted cells were swollen with occasional vortexing for 10 min in 30 volumes of hypotonic buffer A (5 mM-sodium phosphate buffer pH 7·5, 2 mM-MgCl₂, 1 mM-PMSF); sedimentation at 10000 g was followed by resuspension in 10 volumes of buffer A and disruption in a Dounce homogenizer using a tight-fitting pestle until > 90% of nuclei were liberated (approximately 10 strokes) as assessed by phase contrast microscopy. Following sedimentation at 1000 g for 10 min, nuclei resuspended in 10 volumes of buffer A were purified from residual cytoplasmic contaminants by repeated centrifugation at 1500 g for 15 min through a 3 ml cushion of 0·5 M-sucrose in buffer B. Nuclear pellets were subsequently washed and sedimented at 1000 g for 15 min in 20 volumes of buffer A containing 0·5% Triton X-100. The purified nuclei were resuspended and sonicated (three times for 15 s at maximum setting in a sonication bath of a Branson sonifier) in 0·5 ml of buffer B. Nuclear extracts were obtained after sedimentation of insoluble material at 1500 g for 15 min. For preparation of membrane extracts, membranes were sedimented from post-nuclear supernatants at 100000 g for 60 min at 4 °C followed by resuspension in 0·5 ml of buffer B, sonication as above and sedimentation of insoluble material at 1500 g for 15 min.

**Immunoprecipitation.** For immunoprecipitation (Radsak et al., 1990) aliquots of nuclear and membrane extracts, respectively, of comparable protein content were pre-cleared by incubation with Protein A-Sepharose CL4B beads (Sigma). They were then incubated overnight at 4 °C with MAb 27-156 (Spaete et al., 1988) and immune complexes were absorbed for 2 h at room temperature to Protein A-Sepharose CL4B beads coated with rabbit anti-mouse IgG (Dakopatts). Following five cycles of washing the beads with PBS plus 0·1% NP40, 0·1% SDS the precipitates were subjected to SDS–PAGE, and consecutive fixation and fluorography (Bonner & Laskey, 1974) of the dried slab gels.

**Endoglycosidase H (endo H) digestion.** Digestion with endo H (Radsak et al., 1990) was performed on immunoprecipitates of gB after one additional washing cycle with buffer C (50 mM-sodium phosphate buffer pH 5·5, 0·02% SDS, 0·1%) by incubation overnight at 37 °C in 80 μl of buffer C plus 0·1 M-mercaptoethanol and 0·01 unit endo H according to the instructions of the manufacturer (Boehringer Mannheim).

**Determination of protein content.** Protein content in cellular extracts was estimated by the method of Lowry et al. (1951).

**Chemicals.** Chemicals for SDS–PAGE were purchased from Merck, and BFA from Boehringer Mannheim.

**Results**

**Inhibition of HCMV DNA synthesis by BFA**

One round of HCMV multiplication in HFFs extends over approximately 72 h. The initial period (approximately 4 to 6 h) is generally considered the immediate early
Fig. 1. Dose-dependent inhibition by BFA of HCMV DNA replication. Analysis by isopycnic centrifugation in neutral CsCl (mean density 1.71 g/ml) of DNA extracted from parallel cultures of HCMV-infected HFFs (m.o.i. approx. 3, 1 h) that were left untreated (a) or were treated with various concentrations of BFA [0.2 µg (b), 0.5 µg (c) or 1 µg BFA/ml culture medium (d) from 1 to 72 h p.i.]. Radiolabelling with [3H]thymidine (10 µCi/ml) was from 60 h until harvest at 72 h p.i. prior to extraction of DNA, centrifugation and fractionation of the gradients as described in Methods. Comparable amounts of DNA (approx. 1.5 kg) were analysed per gradient. The circles represent acid-insoluble 3H (cpm), the triangles the density (g/ml) of the corresponding fractions. The arrow indicates the position (1.680 g/ml) of 14C-labelled DNA from uninfected HFFs which was included in each gradient as an internal marker.

IE phase, and the subsequent period until onset of viral DNA synthesis at 24 to 36 h p.i. is termed early. The final phase of extensive production of viral structural components and maturation of progeny virus is termed late (Rapp, 1983; Stinski, 1990). The effect of BFA on HCMV DNA synthesis was examined by pulse-labelling of infected drug-treated cultures with [3H]thymidine during the late phase of the infection (60 to 72 h p.i.), followed by analysis of the extracted DNA by isopycnic sedimentation in CsCl. Because of its higher G + C content HCMV DNA (1.715 g/ml) is separated from cellular DNA (1.695 g/ml) by this method. The amounts of acid-precipitable radiolabel in gradient fractions of the appropriate density thus provide estimates of the rates of viral and host DNA synthesis (Fig. 1 to 3).

Inclusion of BFA in the culture medium (from 1 h p.i. until the end of the pulse) caused a dose-dependent inhibition of precursor incorporation into HCMV DNA (Fig. 1b to d). All subsequent experiments were performed at a concentration of 1 µg BFA/ml which was found to abolish precursor incorporation completely. By pulse labelling at intermediate intervals p.i. the possibility was excluded that viral DNA synthesis was transiently initiated in the presence of BFA prior to a decline during the late infectious cycle (not shown).

Subsequent experiments showed that inhibition of viral DNA synthesis was no longer observed when BFA (1 µg/ml) was added to the culture medium only during the late phase of the infectious cycle, 36 h, 48 h or 60 h p.i., i.e. after onset of HCMV DNA synthesis (Fig. 2b to f). Infected cultures were also kept in the presence of
Fig. 3. Lack of inhibition by BFA of HSV DNA replication. CsCl centrifugation (see Fig. 1 legend) of DNA extracted from parallel cultures of HSV-infected HFFs (m.o.i. approx. 1) that were left untreated (a) or were treated with 1 lag BFA/ml from 1 to 24 h p.i. (b). Radiolabelling with \(^{3}\text{H} \text{thymidine} \) (10 \(\mu\text{Ci/ml} \) was from 12 to 24 h p.i. prior to extraction of DNA, centrifugation and fractionation of the gradients as described in Methods. Comparable amounts of DNA (approx. 1-5 lag) were analysed per gradient. Symbols are as described in the legend for Fig. 1.

1 \(\mu\)g BFA/ml for 48 h p.i. and then released from the block. Viral DNA synthesis was resumed under these conditions within approximately another 48 h (not shown).

To examine the effect of BFA (1 \(\mu\)g/ml starting at 1 h p.i.) on viral DNA synthesis in another herpesvirus system, HSV-infected HFFs were analysed in a similar way (Fig. 3). In view of the shorter replication cycle of HSV (approximately 24 h in HFFs) radiolabelling was performed from 12 to 24 h p.i. prior to extraction of DNA, centrifugation and fractionation of the gradients as described in Methods. Comparable amounts of DNA (approx. 1-5 lag) were analysed per gradient. Symbols are as described in the legend for Fig. 1.

The continuous presence of BFA after infection, beginning 1 h p.i., reduced the production of extracellular progeny virus by several orders of magnitude (Table 1). This observation was in agreement with the described inhibitory action of BFA on viral DNA synthesis.

Morphogenesis of HCMV in the presence of BFA

For the ultrastructural studies of BFA-treated HCMV-infected cells two aspects had to be considered. First, the unusual length of the HCMV infectious cycle required extended intervals of BFA exposure in some experiments. Instability of BFA had to be excluded which would result during long-term exposure (24 to 48 h) in the loss of the inhibitory action and thus in reconstitution of the Golgi apparatus. Second, in view of the inhibitory action of the drug on viral DNA synthesis and thus also on late viral events, short intervals of BFA treatment (1 to 3 h) had to be used for analysis of Golgi-dependent virus maturation processes late p.i.

(i) Effect of short-term BFA treatment during the late HCMV infectious cycle

To study the consequences of elimination of a functional Golgi on HCMV morphogenesis, infected cultures were treated with BFA for 1 to 3 h (short-term treatment) or 24 h (intermediate treatment) prior to harvest at 72 h p.i. and processing for transmission electron microscopy. After short-term or intermediate treatment the nuclear phase of HCMV morphogenesis appeared to be unimpaired: nucleocapsids were observed within and in the vicinity of the nucleus of drug-treated infected cells (Fig. 5a). Those in the neighbourhood of the nucleus exhibited coats of fuzzy material that was not apparent on intranuclear nucleocapsids. Similarly, single virus particles in the process of transport budding or enveloped nucleocapsids were usually found at low frequency in the perinuclear space (Fig. 5b and c, 6a) as in untreated infected controls (not shown).

In contrast, short-term or intermediate treatment with BFA severely interfered with the cytoplasmic phase of HCMV morphogenesis. Prominent features of untreated HCMV-infected fibroblasts were cytoplasmic nucleocapsids and dense bodies, both of which were generally trapped in various stages of envelopment at trans-Golgi cisternae (Fig. 4a to c). Following the described schedule of BFA treatment, however, the stacked Golgi structures were absent (Fig. 5d) and replaced by an accumulation of vesicles surrounding dense bodies and cytoplasmic nucleocapsids which were devoid of envelopes (Fig. 5e and f). After intermediate treatment (24 h) large

<table>
<thead>
<tr>
<th>Time of harvest (h p.i.)</th>
<th>Untreated cells</th>
<th>BFA-treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra</td>
<td>Extra</td>
</tr>
<tr>
<td>24</td>
<td>(10^3)</td>
<td>(10^2)</td>
</tr>
<tr>
<td>48</td>
<td>(10^5)</td>
<td>(10^3)</td>
</tr>
<tr>
<td>96</td>
<td>(10^6)</td>
<td>(10^6)</td>
</tr>
</tbody>
</table>

* BFA treatment (1 \(\mu\)g/ml) was initiated at 1 h p.i. and continued until harvest of the culture at the respective times. Lysates of PBS-washed infected cells obtained after three cycles of freezing and thawing were used as the source of intracellular virus (Intra); culture medium clarified by centrifugation at 2000 g, at 4 °C, was used as the source of extracellular virus (Extra).

Table 1. Effect of BFA* on the production of intra- and extracellular HCMV
aggregates of naked nucleocapsids embedded in dense (body) material became prominent (Fig. 6d to f). It is noteworthy in this context that the latter phenomenon, i.e. enclosure of nucleocapsids by dense body-like material, was never seen in untreated infected cells. Furthermore the widened cisternae of the RER frequently contained apparently aberrant naked virus particles, an observation that contrasted with the model of HCMV maturation that predicted only enveloped nucleocapsids in the RER (Fig. 6b and c).

(ii) Effect of long-term BFA treatment
Prevention of HCMV DNA synthesis by BFA would be expected also to preclude consecutive viral morphogenetic events, e.g. formation of nucleocapsids. To confirm this conjecture, BFA was added to infected cultures for the final 48 h of the infectious cycle, i.e. 24 h p.i. prior to the onset of significant viral DNA synthesis. At the end of the infectious cycle (72 h p.i.) cultures were harvested and prepared for transmission electron microscopy. Examination of the samples allowed the following conclusions. (i) Compared to the untreated infected control (Fig. 7a), long-term BFA treatment (24 to 72 h p.i.) drastically reduced the number of progeny nucleocapsids in nucleolar areas of infected nuclei (Fig. 7b). On the other hand, virus-induced morphological alterations, e.g. nuclear enlargement, fragmentation of nucleoli and replacement of heterochromatin by euchromatin, apparent in untreated infected cells at 72 h p.i. (Fig. 7b), were not prevented. (ii) The cytoplasm of BFA-treated infected cells was essentially devoid of nucleocapsids and dense bodies (Fig. 7c). In addition, it was obvious that in the HFF system used here, a single BFA dose of 1 µg/ml was sufficient to destroy the normal Golgi structure and prevent its reconstitution in infected cells during the extended treatment interval of 48 h (Fig. 7c). Separate experiments with infected cultures showed that removal of BFA after 48 h treatment resulted in reformation of
Fig. 5. Effect of short-term BFA treatment on HCMV morphogenesis during the late infectious cycle. HCMV-infected HFFs (m.o.i. 3) were treated with BFA (1 μg/ml) for 3 h prior to harvest at 72 h p.i. and processing for transmission electron microscopy. (a to c) Nuclear regions with unaltered nucleocapsids within and adjacent (arrows in a) to the nucleus (N) as well as transport budding at the nuclear envelope (b and c) in the presence of BFA. (d to f) Alterations in the cytoplasm of a BFA-treated infected fibroblast with fragmentation of the Golgi complex and aggregates of smooth vesicles (d, arrows) that surround dense bodies [db in e and f and nucleocapsids (arrows)]. Nucleocapsids are observed only occasionally in the normal process of envelopment [arrow in (e)]. Bar markers represent 1 μm (a), 100 nm (b), 100 nm (c), 1 μm (d), 250 nm (e) and 200 nm (f).
Fig. 6. Effect on HCMV morphogenesis of BFA treatment for intermediate time intervals during the late infectious cycle. HCMV-infected HFFs (m.o.i. 3) were treated with BFA (1 µg/ml) for 24 h prior to harvest at 72 h p.i. and processing for transmission electron microscopy. (a) Nucleocapsids (arrows) and dense body (db) material adjacent to the nucleus (N) as well as unaltered transport budding (arrowheads) in the presence of BFA. (b and c) Aberrant localization of nucleocapsids in the widened cisternae of the RER. (d to f) Cytoplasmic areas with aggregates (arrows in d and e) of naked nucleocapsids (arrows in e) embedded in amorphous dense body-like (db in e and f) material and in association with smooth vesicles (V in e). Bar markers represent 200 nm (a), 1 µm (b), 200 nm (c), 1 µm (d), 200 nm (e) and 250 nm (f).
Fig. 7. Effect of long-term BFA treatment on HCMV morphogenesis. HCMV-infected HFFs (m.o.i. 3) were treated with BFA (1 μg/ml) from 24 h p.i. until harvest at 72 h p.i. prior to processing for transmission electron microscopy. (a) Abundant aggregates of nucleocapsids (arrows), many of them with dense cores (see inset) in the nucleus of an untreated infected fibroblast. (b) Single empty nucleocapsids (arrows and inset) in the nucleus of a BFA-treated infected fibroblast. (c to e) Cytoplasmic areas show extensive enlargement of RER cisternae, persistent fragmentation of the Golgi complex (arrows in c) and absence of virus particles and dense bodies after extended BFA treatment. Bar markers represent 1 μm (a) and (b), 200 nm (insets a, b), 1 μm (c), 100 nm (d) and 250 nm (e).
the Golgi apparatus within 2 h (not shown). These latter observations again support the notion that BFA is stable in human fibroblasts and that the BFA effect is reversible. Another obvious alteration after extended BFA treatment besides persistent vesiculation of the Golgi was an extreme enlargement and widening of RER cisternae (Fig. 7c to e).

Processing and transport of glycoprotein B (gB) of HCMV in the presence of BFA

(i) Effect of BFA on proteolytic cleavage of gB

Of the HCMV-specific envelope glycoproteins so far described (Chang et al., 1989; Cranage et al., 1986; Lehner et al., 1989; Mach et al., 1986; Rasmussen et al., 1988), gB is made in large quantities and is also apparently dominant in eliciting neutralizing antibodies (Britt, 1984; Britt & Auger, 1986; Britt & Vugler, 1989; Britt et al., 1990; Kniess et al., 1991; Utz et al., 1989). The reported data are consistent in that a high Mr precursor of gB is synthesized in the RER and cotranslationally N-glycosylated to produce molecules of approximately 150K. The precursor is subsequently processed during transport through the Golgi apparatus into a 160K intermediate and slowly (within hours) cleaved, presumably by a cellular protease(s), into disulphide-linked products of about 100K to 110K and 58K (Britt & Auger, 1986; Britt & Vugler, 1989; Spaete et al., 1988; Fig. 8, lanes 2 and 3). Both components are thought to become virion-associated during maturational budding of nucleocapsids into trans-Golgi vesicles. Cellular as well as virion-associated gB-related products are isolated as high Mr complexes of 160K, 190K and <300K under non-reducing conditions (gcI; Gretch et al., 1988; Karl et al., 1986) suggesting that oligomerization occurs during processing to the mature form.

To examine the role of the Golgi in these gB processing events, HCMV-infected fibroblasts in the late phase of the infectious cycle (72 h p.i.) were subjected to pulse-labelling with [35S]methionine (1 h) and a chase (6 h), including BFA in the culture medium during pulse and/or chase (Fig. 8, lanes 4 to 6). Cell extracts were prepared, used for immunoprecipitations with a gB-specific MAb (MAb 27-156; Spaete et al., 1988; Radsak et al., 1990) and the precipitates were analysed by SDS-PAGE under reducing conditions and fluorography. The presence of BFA throughout pulse and chase efficiently prevented proteolytic cleavage of the gB precursor (Fig. 8, lane 4). An identical result was obtained when BFA was present exclusively during the chase period (Fig. 8, lane 5). Presence of BFA during the pulse allowed only proteolytic cleavage to resume during the subsequent chase interval (Fig. 8, lane 6). The precursor molecules accumulating in BFA-treated samples appeared less homogeneous in size, the main radioactive band migrating clearly faster than that of the untreated control (Fig. 8, HCMV, lanes 2 and 3).

No specific precipitates were obtained with MAb 27-156 using extracts from uninfected pulse-labelled cells (Fig. 8, lane 1). A non-specific coprecipitation by MAb 27-156 of a 65K viral polypeptide. M, standards are shown on the left: 170K, α2-macroglobulin; 97K, phosphorylase b; 68K, BSA; 55K, glutamate dehydrogenase; 36K, lactate dehydrogenase.

(ii) Cellular compartmentation and endo H sensitivity of gB synthesized in the presence of BFA

Previously we have shown that uncleaved precursor forms of gB are predominantly found in the nuclear
M. Eggers and others

Fig. 9. Endo H sensitivity of HCMV gB from BFA-treated HFFs. Analysis of gB-specific immunoprecipitates from nuclear (lanes 1 to 4) and membrane (lanes 5 to 8) extracts of untreated (lanes 1, 2, 5 and 6) or BFA-treated (lanes 3, 4, 7 and 8) (BFA, 1 μg/ml) HCMV-infected (m.o.i. 3) HFF cultures (2 x 10^7 cells each) labelled with [35S]methionine (20 μCi/ml) from 60 to 72 h p.i. BFA was added to the cultures concomitantly with the radiolabel. Harvesting of the cultures was followed by cell fractionation, preparation of extracts and immunoprecipitation with MAb 27-156. Half of the individual immunoprecipitates was left untreated (lanes 1, 3, 5 and 7) to serve as a control, the other half was digested with endo H (lanes 2, 4, 6 and 8). Subsequently the samples were subjected to SDS-PAGE (8% polyacrylamide) under reducing conditions and fluorography. The large arrowheads on the right and in the middle indicate the positions of undigested gB precursor of 160K and cleavage product of 55K, the small arrowheads those of gB-specific endo H digests. Mr standards are shown to the left (see Fig. 8).

(compartment of infected fibroblasts, whereas the cleaved forms were recovered mainly from the membrane fraction (Radsak et al., 1990). Immunoprecipitations with MAb 27-156 were prepared using nuclear or membrane extracts of infected cultures labelled with [35S]methionine late p.i. and concomitantly treated with BFA. For identically treated controls without BFA treatment the previous observations were confirmed (Fig. 9, lanes 1, 2, 5 and 6). Analysis of the immunoprecipitates from BFA-treated samples using SDS–PAGE under reducing conditions and fluorography revealed exclusively uncleaved gB-specific forms that again migrated somewhat faster and less homogeneously (Fig. 9, lanes 3 and 7) than their counterparts from untreated cells (Fig. 9, lanes 1 and 5). It is also demonstrated in Fig. 9 that all immunoprecipitates exhibit complete sensivity to endo H; however, endo H-treated gB precursors obtained from BFA-treated cells migrated more slowly (Fig. 9, lanes 4 and 8) than those of the untreated infected control (Fig. 9, lanes 2 and 6).

(iii) Virion association of uncleaved gB precursor
The observation that firstly, enveloped virus particles were present in the perinuclear cisternae of infected cells following BFA treatment late p.i., and that secondly, BFA prevented cleavage of the gB precursor, suggested that the uncleaved gB precursor might become virion-associated. Intra- as well as extracellular virions were therefore purified by gradient centrifugation (see Methods) from infected cultures which had been labelled with [35S]methionine (84 to 96 h p.i.) and simultaneously treated with BFA. Virus preparations from untreated infected cultures served as the control. Immunoprecipitates were prepared from viral extracts using MAb 27-156 and subjected to SDS–PAGE under reducing conditions and fluorography. Both uncleaved gB precursor and the cleavage products were found to be associated with intracellular virions from untreated infected cells (Fig. 10, lane 1), whereas extracellular virus evidently contained only the cleavage products (Fig. 10, lane 2). In contrast, only uncleaved gB precursor of lower Mr was recovered from intracellular virions of BFA-treated cultures. This implies that the (aberrant) uncleaved gB precursor indeed becomes virion-associated (Fig. 10, lane 3). Such virions presumably originated from the nuclear cisterna as cytoplasmic envelopment did not occur in the presence of BFA (see above). Furthermore, radiolabelled gB-specific polypeptides were not present in extracellular virions from cultures treated with BFA during the radiolabelling interval (Fig. 10, lane 4).

(iv) Effect of BFA on the formation of gcI
Regarding the obvious interference of BFA with gB processing events, formation of gcI complexes in drug-treated infected cells was examined. For this purpose immunoprecipitations were performed with extracts from infected cells labelled with [35S]methionine (72 to 84 h p.i.) and analysed by SDS–PAGE under non-reducing conditions. As shown in Fig. 11 (Fig. 8, lane 3), the presence of inhibitor during the labelling period did not prevent formation of radiolabelled high Mr complexes of about 160K and 300K which again migrated somewhat faster than those of the untreated control (Fig. 11, lane 2).
BFA effect on cytomegalovirus

Discussion

HCMV DNA replication has previously been shown to be sensitive to inhibitors of glycosylation, such as 2-deoxyglucose or tunicamycin (Radsak & Weder, 1981). In addition monensin, which is thought to block primarily intracellular transport at the level of the distal Golgi (Saito et al., 1984), also interferes efficiently with HCMV DNA replication (Kaiser & Radsak, 1987). As a possible interpretation for this general effect of the diverse inhibitors it was speculated that regulation of HCMV DNA synthesis might involve an immediate early or early glycosylated viral (or alternatively a virus-induced cellular) product; furthermore, vectorial Golgi-dependent transport into the nuclear compartment might be required for the function of such a regulatory product (Kaiser & Radsak, 1987). The data presented here extend these findings to BFA which inhibits the anterograde transport from the RER to the Golgi and leads to morphological and functional disintegration of the Golgi apparatus (Lippincott-Schwartz et al., 1990; Ulmer & Palade, 1991). The relatively low concentration of BFA used efficiently prevented precursor incorporation into HCMV DNA. This particular effect was supported at the ultrastructural level (see below). A cytotoxic effect on cellular metabolism as a possible cause of this effect was largely excluded by appropriate control experiments: the inhibitory effect of BFA was reversible as demonstrated by recovery of viral DNA synthesis as well as by ultrastructural observations; BFA was ineffective when added after onset of HCMV DNA replication; BFA did not impair HSV DNA synthesis in the same cell culture system.

Our biochemical findings concerning HCMV DNA synthesis were fully supported by the fine structural studies. Following BFA treatment for 48 h starting at 24 h p.i. (Fig. 7), few virus particles, mostly of the non-infectious type (without dense cores), were observed in the nucleus, and were completely absent from the cisternal space of the RER or the cytoplasm. The Golgi structure was disintegrated, with only small aggregates of vesicles and tubules remaining in the vicinity of the RER (Fig. 7c). Concomitantly the cisternal space of the RER was greatly distended.

Because of this inhibitory action of BFA on early functions of HCMV, fine structural studies concerning the drug effect on virion assembly, virus envelopment and egress, and on glycoprotein processing had to be limited to the late phase of the infectious cycle. Preliminary experiments on uninfected HFFs showed that BFA treatment resulted in disruption of the stacked Golgi cisternae within 30 min and that morphological recovery occurred within 120 min after removal of the inhibitor.

Exposure to BFA of infected cells for 1 to 3 h late p.i. revealed a normal egress of nucleocapsids from the nucleus by budding through the inner (Fig. 5 b and c) and outer nuclear membranes. Numerous unenveloped

Fig. 10. Effect of BFA on virion-associated HCMV gB. Analysis of gB-specific immunoprecipitates from intracellular (lanes 1 and 3) and extracellular (lanes 2 and 4) virions from untreated (lanes 1 and 2) or BFA-treated (lanes 3 and 4) (BFA; 1 µg/ml) HCMV-infected (m.o.i. 3) HFF cultures (4 x 10⁷ cells each) labelled with [³⁵S]methionine (50 µCi/ml) from 84 to 96 h p.i. BFA was added to the culture medium concomitantly with the radiolabel. Virion extracts were immunoprecipitated with MAb 27-156 and the precipitates subjected to SDS-PAGE (8% polyacrylamide) under reducing conditions and fluorography. The large arrowheads on the right indicate the positions of the gB precursor of 160K and cleavage product of 55K, the small arrowhead that of the gB-specific precipitate from BFA virions. Mr standards are shown to the left (see Fig. 8).

Fig. 11. Effect of BFA on formation of gCl. Analysis of gB-specific immunoprecipitates of total cell extracts from parallel cultures (5 x 10⁶ cells each) of uninfected (lane 1), HCMV-infected (m.o.i. 3) HFF left untreated (lane 2) or treated with BFA (lane 3) and labelled with [³⁵S]- methionine (20 µCi/ml) from 60 to 72 h p.i. BFA was added to the cultures concomitantly with the radiolabel. Harvesting was followed by preparation of extracts, immunoprecipitation with MAb 27-156, SDS-PAGE (6% polyacrylamide) of the precipitates under non-reducing conditions and fluorography. The large arrowheads on the right indicate the positions of the main components of gCl at <300K and approximately 160K, the small arrowheads those of the faster migrating components of gCl from BFA-treated HFFs. The arrowheads on the left mark the positions of α-2-macroglobulin under non-reducing conditions at 340K and phosphorylase b at 97K.
nucleocapsids and dense body material accumulated in the zone of the Golgi complex which was completely vesiculated (Fig. 5d). Both nucleocapsids and dense bodies were aggregated and surrounded by smooth vesicles (Fig. 5e) and were, in the case of dense bodies, sometimes in close apposition to parts of the RER cisternae (Fig. 5f). This accumulation of naked cytoplasmic nucleocapsids in the presence of BFA favours the concept that morphogenesis of HCMV, in addition to transient budding at the nuclear membranes, in a second envelopment in the area of the trans-Golgi network (TGN; Fig. 4b and c). Disintegration of the cisternal structure of the Golgi complex and fragmentation into vesicles by BFA apparently prevents this step of nucleocapsid or dense body encapsulation, in spite of the fact that, under the experimental conditions used, viral glycoproteins must have been inserted into the TGN membranes before treatment with BFA was initiated. This process of nucleocapsid envelopment possibly requires a specific interaction of extended domains of TGN membranes and additional adaptor-like proteins which bind the tegument (fuzzy coat) of the nucleocapsid to these membranes. BFA could remove such adaptors, as shown recently for the Golgi structure in general (Duden et al., 1991; Orci et al., 1991).

The accumulation of dense body material and naked nucleocapsids (presumably with tegument) was even more pronounced after 24 h BFA treatment late in the infectious cycle. Budding from the inner nuclear membrane remained unimpaired, but egress from the cisternal space of the RER was inhibited and virus particles accumulated in the RER (Fig. 6b and c). This effect was, however, less pronounced when compared to results for alphaherpesviruses (Cheung et al., 1991; Whealy et al., 1991).

Interestingly, after 24 h exposure of infected cells to BFA late p.i. (72 h p.i.) the large amount of extracellular enveloped virions and dense bodies contrasted with the situation in the cytoplasm which appeared devoid of enveloped virus particles. This may suggest firstly that the process of final exocytosis of already enveloped nucleocapsids and dense bodies was not affected by BFA. Secondly, the supply of nucleocapsids from the nuclear compartment to the cytoplasm in the presence of BFA should be a slow process which takes longer than 3 h but less than 24 h.

Processing of the main glycoprotein of HCMV, gB, has been shown to involve sequential steps of cotranslational N-glycosylation of the 105K polypeptide to produce a mannose-rich, endo H-sensitive 150K intermediate which is consecutively trimmed and modified to a 160K, partially endo H-resistant form by addition of complex oligosaccharides (Britt & Vugler, 1989). Subsequent proteolytic cleavage of this latter form is thought to occur in the distal Golgi complex prior to envelopment of cytoplasmic nucleocapsids in the TGN, as mature virions contain predominantly fully processed cleavage products of 58K and 100K (Britt & Vugler, 1989). In contrast to the 160K form, the 150K intermediate appears to be prominent and comparatively stable and is still seen after an extended chase period of 24 h (Britt & Auger, 1986). Previous findings (Radsak et al., 1990) and results shown here suggest that part of the 150K form of gB observed in total cell extracts may represent the uncleaved endo H-sensitive gB form that is confined to the nuclear compartment. Our observations regarding the processing of HCMV gB in BFA-treated HFFs further establish that proteolytic cleavage of the gB precursor depends on intact Golgi functions. Regarding the normal processing pathway of gB (Britt & Vugler, 1989) there is no direct plausible explanation for the accumulation of the uncleaved gB form of apparent lower Mr in BFA-treated infected cultures; it may, however, represent a so far unidentified gB processing intermediate. Analysis of this gB intermediate after pulse labelling with radiolabelled sugars might help to elucidate more precisely the exact action of BFA on HCMV glycoprotein maturation. The reduced migration of this gB form following endo H treatment is in agreement with a previous report on the biosynthesis of gIII in the PRV system (Whealy et al., 1991) and may indicate aberrant O-glycosylation in the presence of BFA.

Inhibition of proteolytic cleavage in the presence of BFA neither prevented uncleaved gB precursor from forming disulphide-linked complexes nor becoming virion-associated. Based on our ultrastructural observations, intracellular virions from BFA-treated cultures should originate from the perinuclear cisterna. The presence of gB in such virions supports the concept that HCMV gB is inserted into the inner nuclear membrane where it may introduce a signal for the exit of viral nucleocapsids from the nucleus, i.e. transport budding at the nuclear envelope. According to this concept uncleaved gB precursor should also be present in virions of the nuclear cisterna of untreated infected cells (Radsak et al., 1990). This assumption has to remain speculative, however, because methods to separate enveloped nucleocapsids reliably from the different compartments of infected cells are not available at present.

The authors are indebted to B. Becker and V. Kramer for the photographic reproduction. This investigation was supported by the Deutsche Forschungsgemeinschaft [Sonderforschungsbereich 286, Teilprojekte A3 (K.R.) and B2 (H.F.K.)]. The MAb used in these studies was generously provided by Dr W. Britt, Birmingham, Ala., U.S.A.
References


(Received 30 January 1992; Accepted 24 June 1992)