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Structural changes in human cytomegalovirus cytoplasmic assembly sites in the absence of UL97 kinase activity

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Abstract

Studies of human cytomegalovirus (HCMV) UL97 kinase deletion mutant (ΔUL97) indicated a multi-step role for this kinase in early and late phases of the viral life cycle, namely, in DNA replication, capsid maturation and nuclear egress. Here, we addressed its possible involvement in cytoplasmic steps of HCMV assembly. Using the ΔUL97 and the UL97 kinase inhibitor NGIC-I, we demonstrate that the absence of UL97 kinase activity results in a modified subcellular distribution of the viral structural protein assembly sites, from compact structures impacting upon the nucleus to diffuse perinuclear structures punctuated by large vacuoles. Infection by either wild type or ΔUL97 viruses induced a profound reorganization of wheat germ agglutinin (WGA)-positive Golgi-related structures. Importantly, the viral-induced Golgi remodeling along with the reorganization of the nuclear architecture was substantially altered in the absence of UL97 kinase activity. These findings suggest that UL97 kinase activity might contribute to organization of the viral cytoplasmic assembly sites.

Keywords: UL97 kinase; Cytoplasmic assembly sites; Pp28

Introduction

Human cytomegalovirus (HCMV), a betaherpesvirus, is a major cause of disease in immunocompromised individuals including AIDS patients and transplant recipients and a common cause of congenital infection leading to developmental abnormalities and hearing loss (Pass, 2001).

The HCMV virion consists of a DNA containing capsid, a tegument layer and a lipid envelope (Gibson, 1996; Mocarski and Tan Courcelle, 2001). During capsid maturation in the nucleus, large concatemeric DNA intermediates are cleaved to genome-length units and packaged into preformed capsids via the DNA packaging machinery (Gibson, 1996; Mocarski and Tan Courcelle, 2001). DNA packaging into capsid precursors displaces a scaffold structure to yield C capsids that further mature by budding through nuclear membranes (Gibson, 1996; Mocarski and Tan Courcelle, 2001). Subsequent events during nuclear egress and viral assembly have remained controversial. The current model suggests that assembled nucleocapsids bud through the inner nuclear membrane, acquiring an initial envelope layer (Mettenleiter, 2002; Mocarski and Tan Courcelle, 2001). Subsequent shuttling into the cytoplasm involves deenvelopment and budding of nucleocapsids into *trans*-Golgi-related vesicles regarded as the viral assembly sites, where final tegumentation and secondary envelopment occur (Gershon et al., 1994; Homman-Loudiyi et al., 2003; Jarvis et al., 2004; Mettenleiter, 2002; Muranyi et al., 2002; Sanchez et al., 2000a, 2000b; Skepper et al., 2001). Although several lines of evidence support this model, studies in HCMV are limited, and the viral and cellular mechanisms which govern nuclear egress, tegument trafficking and envelopment are currently unknown.

The UL97 gene product is the only well-established HCMV-encoded protein kinase (Chee et al., 1989; He et al., 1997; Michel et al., 1999; Michel and Mertens, 2004; van Zeijl et al., 1997). This unusual serine/threonine protein kinase was

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originally discovered by its unique ability to phosphorylate the major antiviral nucleoside analog ganciclovir, thus mediating its antiviral activity (Littler et al., 1992; Sullivan et al., 1992). Furthermore, mutations in the UL97 kinase domain, characterized by us and by others, lead to clinical ganciclovir resistance (Chou et al., 1995; Wolf et al., 1995). The UL97 kinase is highly conserved in all herpesviruses (Chee et al., 1989). The alphaherpesvirus homologs of UL97, the herpes simplex virus (HSV) UL13 and the varicella zoster virus (VZV) ORF47, have been shown to phosphorylate viral immediate early proteins and cellular proteins (Kawaguchi et al., 1999; Moffat et al., 1998; Ng et al., 1994; Purves et al., 1993) and, although proven non-essential for replication in cell culture, have been implicated in regulation of viral gene expression and tissue tropism (Kawaguchi et al., 1999; Moffat et al., 1998; Ng et al., 1994; Purves et al., 1993). In HCMV, the UL97 kinase plays a crucial role in viral propagation, and a UL97 deletion mutation results in a 2–3-log reduction in viral yield (Prichard et al., 1999). Moreover, the UL97 kinase has been shown to be the target of maribavir, a new potent and selective antiviral drug (Biron et al., 2002). Our previous studies of a replication deficient UL97 deletion mutant show a complex multi-step role of UL97 kinase in both early and late steps of the viral life cycle, namely, in pre-elongation step of DNA synthesis, DNA encapsidation, capsid maturation and nuclear egress (Wolf et al., 2001). Other studies implicated more specifically the involvement of UL97 in either DNA synthesis or nuclear egress (Biron et al., 2002; Krosky et al., 2003a, 2003b). Additionally, the UL97 has been shown to phosphorylate the viral DNA polymerase processivity factor UL44 (Krosky et al., 2003a, 2003b; Marschall et al., 2003) and to be involved in phosphorylation of nuclear lamins (Marschall et al., 2005), to mediate the intranuclear distribution of the tegument protein pp65 (Prichard et al., 2005) and to play some role in modification of the RNA polymerase II in vitro (Baek et al., 2004).

The multiple roles played by UL97 in viral maturation prompted us to address its possible involvement in cytoplasmic steps of HCMV tegumentation and assembly. Using a UL97 deletion mutant and specific viral inhibitors, we demonstrate that UL97 kinase activity is required for the proper subcellular distribution of viral cytoplasmic assembly sites.

Results

Modified subcellular distribution of pp28 in cells infected with the UL97 mutants (ΔUL97)

As a target for investigating the role of UL97 in HCMV maturation and tegument protein trafficking, we chose the abundant tegument protein UL99-encoded pp28, a late protein that is expressed exclusively in the cytoplasm (Kerry et al., 1997; Sanchez et al., 2000a, 2000b).

HF cells grown in 8-well slides were infected with either AD169 or ΔUL97 at a MOI of 0.5. At 96 hpi, the cells were stained with pp28 mAb, counterstained with propidium iodide (PI) and analyzed by confocal immunofluorescence microscopy (Fig. 1). Following infection of cells with wt-HCMV, pp28 was found in a compact, juxtanuclear distribution in 95% (±2.6%) of the infected cells (Fig. 1A). These findings are in agreement with the findings of Sanchez et al. (Sanchez et al., 2000a, 2000b). In contrast, following infection with each of the ΔUL97 mutants, pp28 assumed a more diffuse, perinuclear distribution observed in 86% (±7.8%) while only 14% assumed the compact juxtanuclear structure. The pp28 stained regions were abundantly punctuated by 2–8 μm wide vacuoles, not observed in cells infected with the wild type virus (see Fig. 1B, ΔUL97, arrows). Only the periphery of these vacuoles was stained by pp28, whereas their interior was negative for both pp28 and PI. These vacuoles were also clearly visible by Nomarski microscopy (Fig. 1C, arrows). Vacuoles were also observed in about 50% of the nuclei of the ΔUL97-infected cells (Fig. 1B, yellow arrow). The periphery of these vacuoles was stained with pp65 (data not shown). Similar changes were also found in the cytoplasmic localization of the UL55-encoded glycoprotein B (gB) (89% ± 1.5% compact structures in the wild type versus 10% ± 2% compact structures in the mutant; see Fig. 5B). The vacuoles were not related to lipid droplets since their interior did not stain with Nile red (not shown). In contrast to the tegument and envelope proteins, the localization of the major capsid protein was strictly nuclear in both wild type (as already described by Lai and Britt, 2003) and mutant-infected cells and was not modified in the absence of UL97 activity (data not shown).

Wild type and mutant viruses also had a strikingly different effect on the nuclear shape. While the nucleus of cells infected with wt-HCMV was reproducibly deformed, assuming a kidney shape (Fig. 1), no effect on the shape of the nucleus was noticed following infection with ΔUL97. Identical findings were observed following infection with the two independently isolated deletion mutants RCΔ97.08 and RCΔ97.19 (Prichard et al., 1999).

Membrane association of pp28

The pp28 membrane association dictates its cellular localization and is believed to mediate its role in secondary viral envelopment in the cytoplasm (Jones and Lee, 2004; Sanchez et al., 2000a, 2000b; Silva et al., 2003). The modified cellular localization of pp28 in cells infected with ΔUL97 virus could reflect an altered membrane association of this tegument protein. To examine the membrane association of pp28, we carried out flotation assays using CHAPS as a detergent and Nycodenz as a density medium. CHAPS is known to float many membrane-associated proteins, whereas soluble proteins not associated with membranes remain in the bottom of the gradient (Rouvinski et al., 2003). In both wild type and mutant virus-infected cells, pp28 partitioned in low density, CHAPS-insoluble membrane complexes as revealed by Western blot analysis (Fig. 2A). As controls for the flotation analysis, we used monoclonal antibodies against calnexin, a known membrane-associated protein (floating in CHAPS), and fumarase, a non-membrane-associated protein (non-floating in CHAPS) (Fig. 2A). While expression of pp28 was slightly reduced (at most a 2 to 3 fold decrease) in the absence of UL97, its relative abundance in the different flotation fractions,

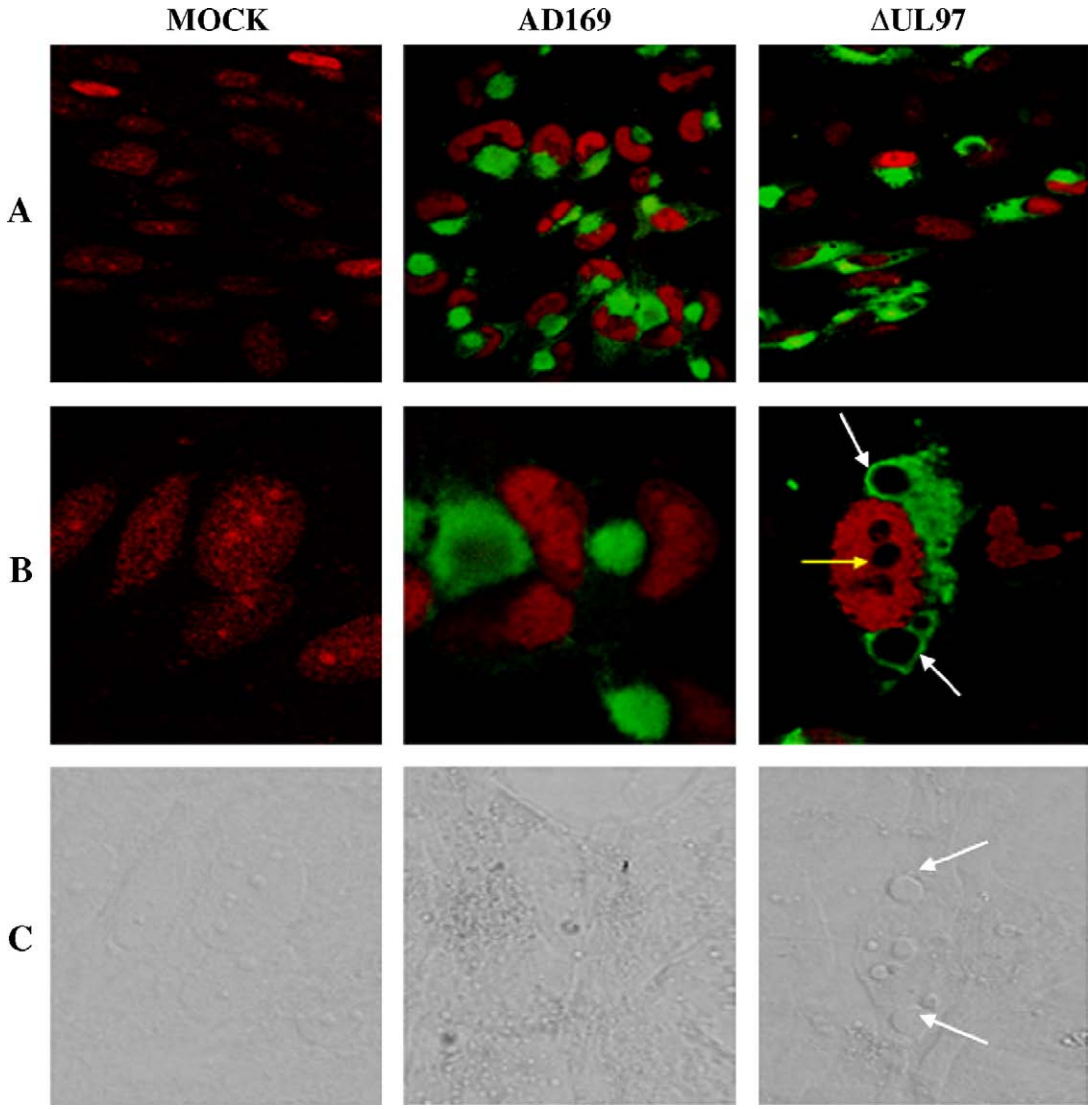

Fig. 1. Cellular distribution of pp28 in HCMV-infected cells. Human foreskin fibroblasts were infected with either wt-HCMV (AD169, A–C) or UL97 deletion mutant (ΔUL97, A–C) at an moi of 0.5 and prepared for immunofluorescence analysis at 96 hpi. The cells were stained with pp28 mAb (green), counterstained with propidium iodide (red) and analyzed by confocal microscopy. A and B rows represent low and high magnifications, respectively. The white arrows point to the cytoplasmic vacuoles in mutant-infected cells. The yellow arrow points to the nuclear vacuoles in mutant-infected cells. Nomarski images (C) are presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reflecting the proportion of membrane-associated pp28, did not differ between the wild type and the mutant virus in repeated experiments (Fig. 2B). These results demonstrate that the membrane association of pp28 is not dependent on functional UL97.

Pharmacological inhibition of UL97 kinase in cells infected with wt-HCMV results in a ΔUL97-like pp28 distribution

Our observations show that the deletion of UL97 results in an abnormal distribution of pp28 in infected cells (Fig. 1). In order to see if this effect is due to the lack of the kinase activity of the UL97 gene product, we employed the indolocarbazole NGIC-I, shown to inhibit the kinase activity of the UL97 in vitro (Marschall et al., 2001). NGIC-I (0.5 μM) was added to the growth medium following viral adsorption, and cells were further incubated in the presence of the drug for 96 h. Significantly, treatment of wt-HCMV-infected cells with NGIC-I converted the pp28 compact subcellular distribution into the ΔUL97 characteristic pattern (in 80% ± 6.7 of the infected cells, Fig. 3). In addition, the nuclei lost the typical kidney shape to assume a more round, regular shape. By contrast, NGIC-I exerted no additional effects on either the pp28 subcellular distribution or the nuclear shape in cells infected with ΔUL97 (92% ± 4.7 diffuse forms, Fig. 3). Taken together, these results strongly suggest that it is the lack of UL97 kinase activity that causes the abnormal pp28 pattern in cells infected with ΔUL97. This conclusion was further tested in a kinetic block-release assay of NGIC-I. Cells were treated for 96 h with this inhibitor and then released from the drug block and examined 24 h, 48 h, 72 h and 96 h later. Viral yield measurements indicated a two log reduction in wt-HCMV titer

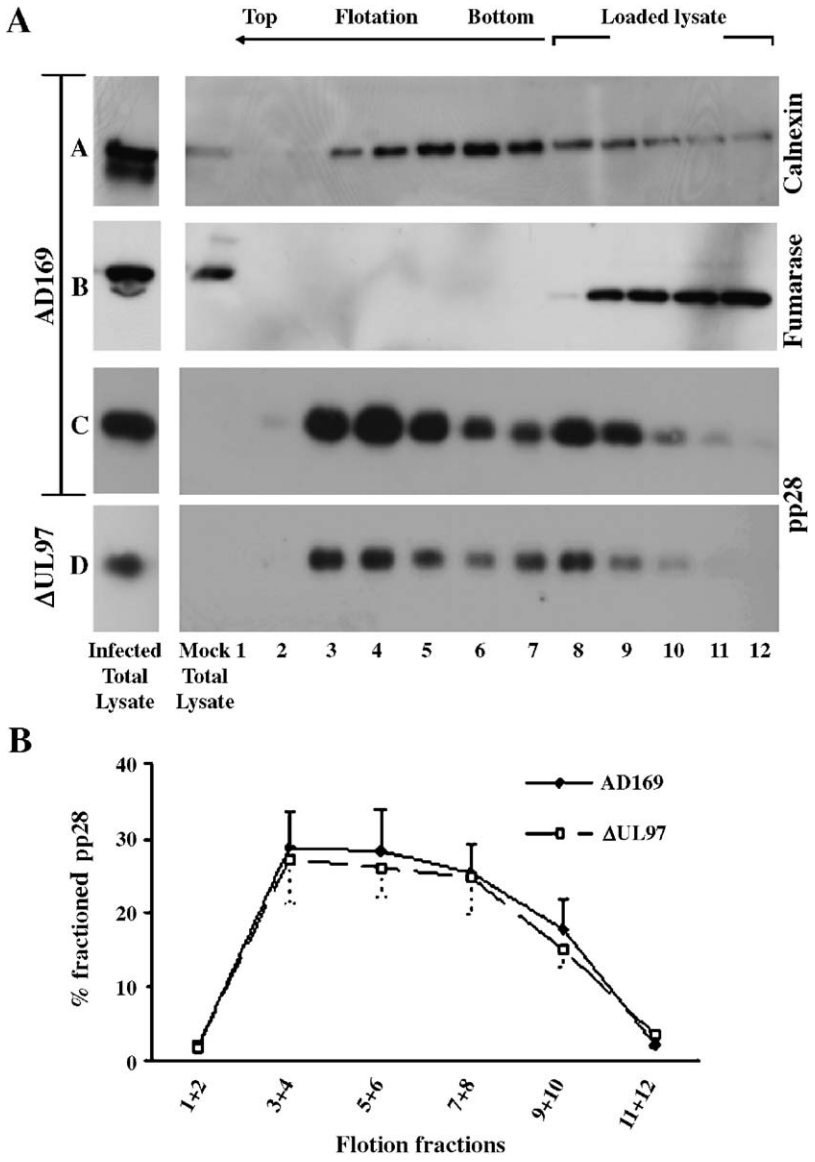

Fig. 2. Membrane association analysis of pp28. (A) Flotation fractions (numbers at the bottom) of CHAPS extracts of cells infected with either wt-HCMV (AD169) or with the mutant (ΔUL97) were collected from the top of Nycodenz gradient (1st—low density, 12th—high density) and subjected to Western blot analysis using pp28 mAb (C and D). Flotation fractions of AD169 infected cells were also subjected to Western blot analysis using calnexin mAb (A) and fumarase mAb (B). (B) Calculated percentage of pp28 in the different flotation fractions (1–12) of AD169 and ΔUL97-infected cells. The results were derived from 3 independent experiments.

following 96 h of drug treatment relative to non-treated infected cells (10^3 versus 10^5 PFU/ml), while no effect of the treatment was observed on the ΔUL97 viral yield (which ranged between 5×10^2 and 10^3 PFU/ml in both untreated and NGIC-I treated cells). Although NGIC-I is known to inhibit other protein kinases, this further demonstrated a specific inhibition of UL97 kinase by NGIC-I, under the indicated concentration. Drug release of wt-HCMV-infected cells resulted in a gradual reversal of pp28 subcellular distribution to a wild-type-like phenotype, from 14% during block to 36% and 54% at 24 h and 72 h after release, respectively (Fig. 3), with resumption of wild type viral yield levels (1.5×10^5 PFU/ml) at 72 h following release. While the proportion of compact forms observed at 72 h following release was lower that that observed in untreated wild-type-infected cells, it appeared sufficient for resumption of normal viral yield. The slow morphological reversion could result from accumulation of non-reversible "end products" in the absence of UL97 or from delayed downstream effect of the release of an earlier block.

Tegument protein pp28 co-localizes with wheat germ agglutinin (WGA) ligands in wild-type- and ΔUL97-infected cells

Previous studies have localized pp28 to Golgi-related structures of the secretory pathway (Sanchez et al., 2000a, 2000b). We thus examined whether the disparate pp28 patterns in cells infected with wt-HCMV and ΔUL97 might reflect a different organization of cellular organelles. To this end, we probed cells with FITC-conjugated WGA, which binds to

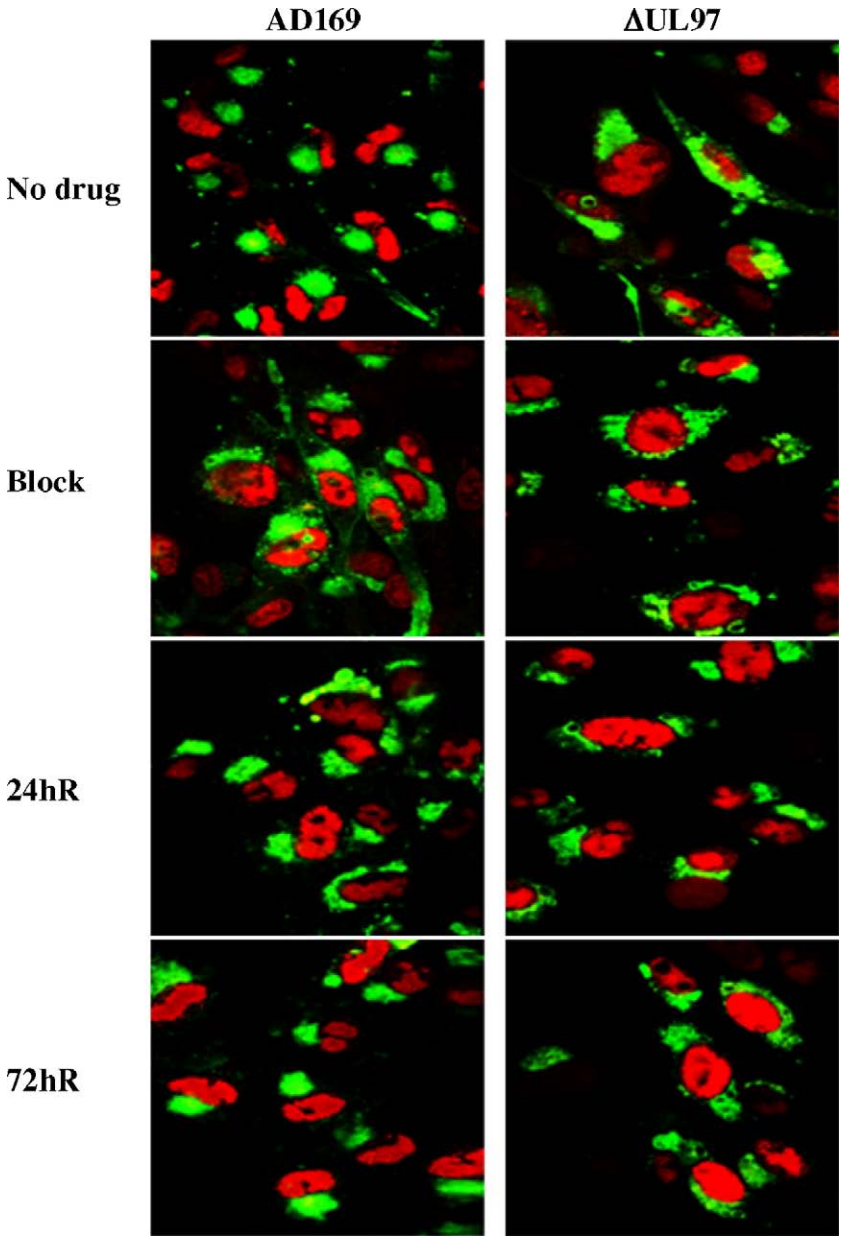

Fig. 3. Cellular distribution of HCMV pp28 during NGIC-I block and following NGIC-I block-release. NGIC-I (0.5 μM) was added to AD169 or ΔUL97-infected cells for 96 hpi (block). NGIC-I was washed out, and cells were analyzed at 24 h (24 hR) and 72 h (72 hR) following NGIC-I release. The cells were prepared and analyzed as described in the legend to Fig. 1.

clustered terminal *N*-acetylneurarninic acid residues and to *N*-acetylglucosamine-containing oligosaccharides on proteins (Bhavanandan and Katlic, 1979) and decorates distal Golgi cisternae, the *trans*-Golgi network and the cell surface (Tartakoff and Vassalli, 1983; Virtanen et al., 1980).

In uninfected fibroblasts, WGA stained perinuclear structures that were reminiscent of the classical Golgi organization (Fig. 4). The WGA staining was strikingly modified by viral infection. First, the WGA staining intensity in both wt-HCMV and ΔUL97-infected cells was much stronger than in uninfected fibroblasts. Most interestingly, the WGA pattern assumed either compact or diffuse shapes (Fig. 4) in wt-HCMV (94% ± 1.5% compact) and ΔUL97-infected cells (91% ± 2.5%) diffuse), respectively, similar to the patterns observed for pp28 (Fig. 1). In particular, with ΔUL97, the WGA-positive structures were punctuated by vacuoles of non-reactive material similar to those found with pp28 (Fig. 1). These observations suggested that (i) viral infection induces a profound reorganization of WGA-positive structures (presumably including Golgi membranes), and (ii) pp28 might co-localize with these cellular structures.

To examine the latter point, we performed double staining experiments (Fig. 5). Cells were infected with either virus for 96 h and then co-stained with WGA-FITC and with the mAb to pp28 followed by an Alexa red 633-conjugated secondary Ab (Fig. 5A). With both viruses, the two antigens significantly co-localized. Strikingly, with ΔUL97 (RCΔ97.08 and RCΔ97.19 isolates), both pp28 and WGA distributions were punctuated by

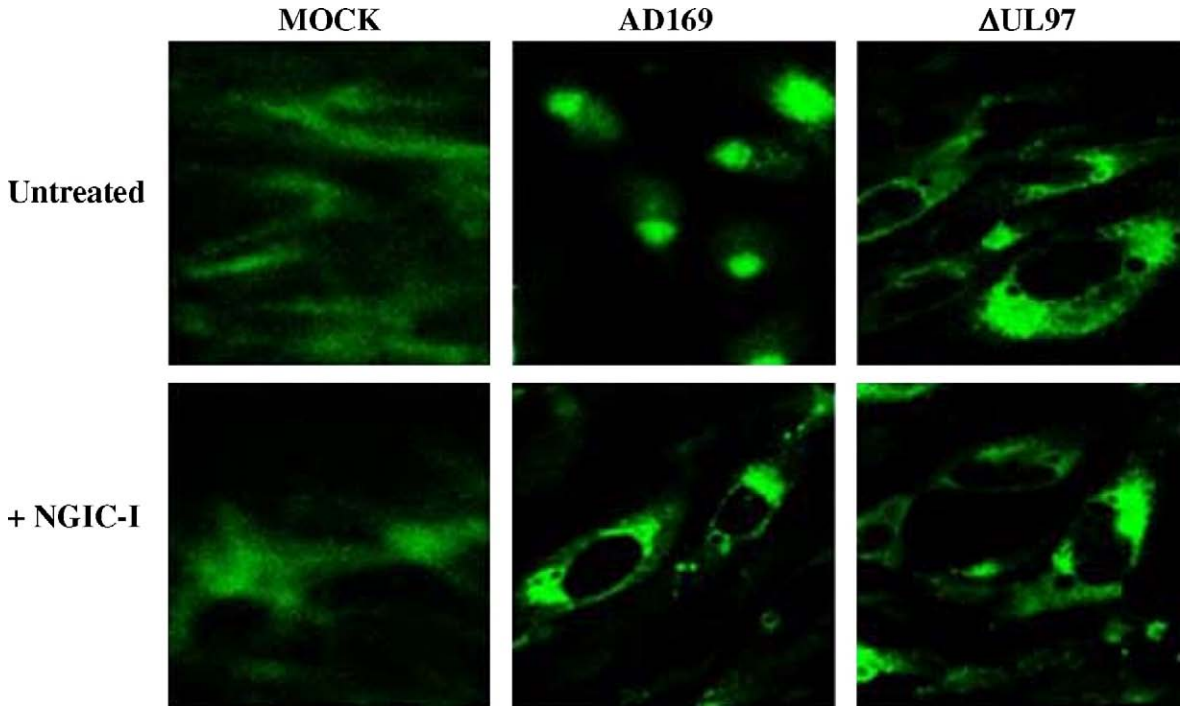

Fig. 4. Distribution of WGA in HCMV-infected HF cells. Cells infected with AD169 or ΔUL97 viruses were examined at 96 hpi by confocal microscopy following immunofluorescence staining with FITC-conjugated WGA. The upper and lower rows show untreated cells or cells treated with NGIC-I (0.5 μM) throughout the infection period, respectively. Mock-infected cells served as a control. The white arrows point to the cytoplasmic vacuoles in NGIC-I treated AD169-infected cells.

the same round vacuoles. Similar findings were observed in co-localization studies with pp28 and mannosidase II antibodies (data not shown). These results are consistent with the idea that: (i) pp28 positive Golgi structures are remodeled in the infected cells and (ii) UL97 affects this Golgi redistribution.

To determine if this effect was due to the loss of UL97 kinase activity in ΔUL97, infected cells were treated with NGIC-I. In cells infected with wt-HCMV, the WGA staining assumed the readily recognizable diffuse pattern with vacuoles, characteristic of the ΔUL97 mutant (91 ± 2% diffuse forms; Fig. 4). In contrast, NGIC-I had no effect on the WGA pattern in cells infected with the mutant strain (90% diffuse forms) nor did it alter the general appearance of the WGA ligands in mock-infected cells (Fig. 4). Thus, although NGIC-1 may nonspecifically inhibit cellular kinases, the lack of effect in mutant-infected and mock-infected cells supports the specific association of UL97 kinase inhibition with the observed phenotype.

Taken together, these results confirm that (i) there is a Golgi remodeling in HCMV-infected cells and that (ii) the lack of viral UL97 kinase activity is the primary cause for the characteristic ΔUL97 Golgi pattern.

Subcellular distribution of the HCMV major glycoprotein and its correlation to pp28

Previous studies have shown that the major viral glycoprotein gB localizes to perinuclear Golgi structures in human fibroblasts infected with wt-HCMV (Sanchez et al., 2000a, 2000b). To examine if the virus-induced reorganization of Golgi membranes is also reflected in the localization of gB, cells infected with either viral strain were co-stained with a gB mAb and with WGA-FITC (Fig. 5B). In most cells, these two antigens co-localized almost completely. In particular, gB stained the rims of the abundant vacuoles seen in cells infected with ΔUL97.

These results suggest that pp28 and gB proteins in both wild type and mutant HCMV-infected cells localize to modified compartments of the secretory pathway which may include the Golgi apparatus as well as related structures. UL97 kinase appears to be required for the viral-induced modification of the cellular secretory pathway.

The modified subcellular distribution of pp28 and WGA in ΔUL97-infected cells does not result from impaired DNA synthesis or encapsidation

We have previously reported that ΔUL97 mutant synthesizes 4 to 6 times less viral DNA than the wild type virus with several fold reduction in late protein accumulation and exhibits a defect in DNA encapsidation (Wolf et al., 2001). To examine whether any of these effects (on DNA synthesis and encapsidation and late protein expression) could nonspecifically lead to the assembly sites phenotype observed in the absence of UL97, infected cells were treated with either the DNA polymerase inhibitor PFA or the cleavage-packaging inhibitor BDCRB (20 μM) (Fig 6). The PFA concentration for the comparative experiments was selected following incubation of wild-type-infected cells with different PFA concentrations ranging from 1 to 300 μg/ml. The selected concentration (30 μg/ml) was the one which reduced wild type DNA synthesis by 3- to 5-fold to a

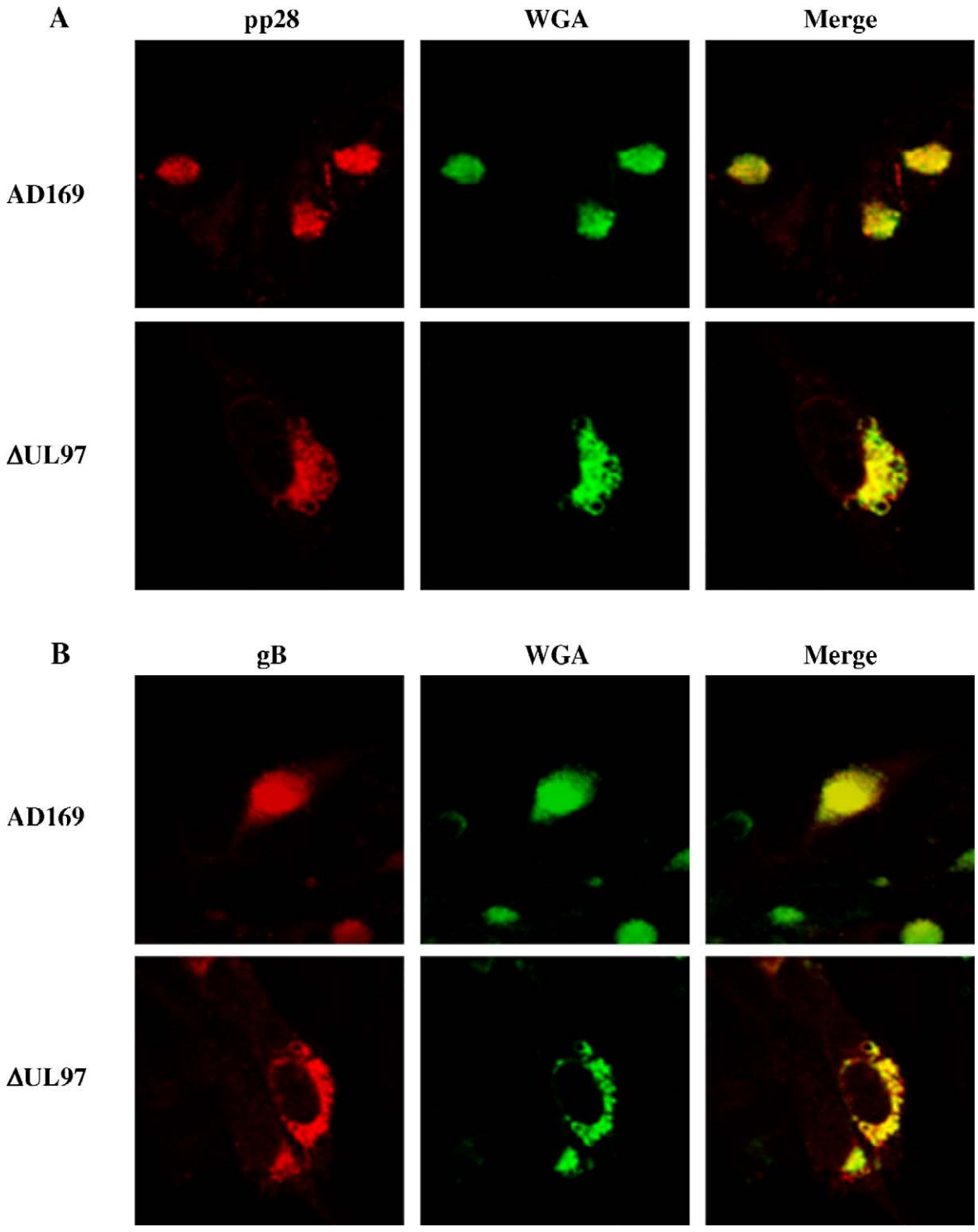

Fig. 5. Co-localization of viral structural proteins with WGA. HF cells were infected with either AD169 or ΔUL97 viruses. At 96 hpi, cells were fixed, stained with mAbs to either pp28 (A) or gB (B), both shown in red, co-stained with WGA-FITC (green) and analyzed by confocal microscopy.

similar level observed in the untreated mutant in parallel experiments, as measured by real time PCR of infected cells. When mutant-infected cells were treated with similar PFA concentration, DNA accumulation was reduced by 2-fold. As expected, an early block in viral replication by PFA reduced the synthesis of pp28 to a varying degree in different experiments. pp28 distribution assumed a weak compact or partially diffuse pattern in treated wild-type-infected cells (Fig 6). This pattern was completely different from that observed upon inhibition of UL97, despite comparable levels of DNA and protein expression. Of note, in the presence of PFA, the nuclei maintained the spherical shape, observed in uninfected cells.

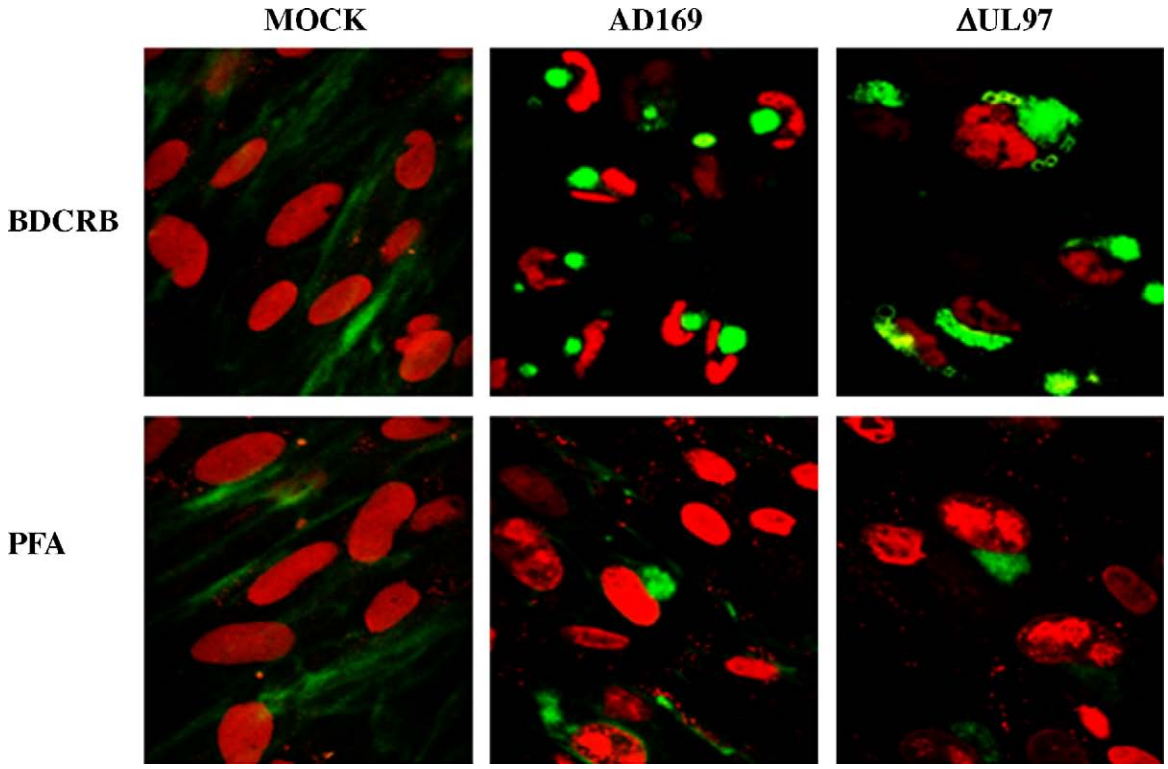

Fig. 6. Cellular distribution of HCMV pp28 in BDCRB and PFA treated infected cells. Either BDCRB (20 μM) or PFA (30 μg/ml) was added to AD169 or ΔUL97-infected cells or to mock-infected cells for 96 hpi. Drug-treated infected cells were stained with pp28 mAb, while drug-treated mock-infected cells (shown in the Mock panels) were stained with FITC-conjugated WGA. Cells were counterstained with propidium iodide (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BDCRB block resulted in inhibition of DNA cleavage and encapsidation with abrogation of virus replication in both wild-type- and mutant-infected cells, without inhibition of virus DNA synthesis or late protein expression (data not shown) (Wolf et al., 2001). No effect of BDCRB on pp28 subcellular distribution was observed following infection with either wild type or mutant virus (Fig. 6). These results suggest that the unique morphology of viral cytoplasmic assembly sites formed in the absence of UL97 is not simply a consequence of early or late block in the replication cycle but is rather more specifically related to a deficiency of UL97 kinase activity.

Discussion

Previous studies have shown that the HCMV UL97 kinase plays a major role in early and late nuclear phases of the viral life cycle including DNA replication and encapsidation and nuclear egress (Biron et al., 2002; Krosky et al., 2003a, 2003b; Wolf et al., 2001). In this study, we reveal a novel involvement of UL97 kinase in the distribution of the viral cytoplasmic assembly sites and in the remodeling of Golgi structures during HCMV infection.

The distribution of viral cytoplasmic assembly sites is modified in the absence of UL97 kinase activity

In cells infected with wt-HCMV, several structural proteins including pp28 congregate into compact, juxtanuclear structures that appear to impact on the nucleus (Fig. 1). Our results suggest that the UL97 kinase activity is required for the formation of these compact assembly sites. When this activity was abrogated either by deleting the UL97 gene or by using the kinase inhibitor NGIC-I, the pp28-containing compact structures were replaced by more diffuse perinuclear structures. Importantly, this altered distribution was punctuated by large cytoplasmic vacuoles, with peripheral pp28 staining. Similar changes were found in the cellular localization of the tegument protein pp65, which also stained the rims of intranuclear vacuoles (data not shown) and of the envelope glycoprotein gB (Fig. 5), thus reflecting a general modification in the assembly sites of viral structural proteins in the absence of active UL97 kinase. Interestingly, Prichard et al. have recently characterized the formation of similar nuclear vacuoles in the absence of UL97 kinase as aberrant aggregates of viral structural proteins, composed predominantly of pp65 (Prichard et al., 2005). Together, the combined findings argue for a defect in nuclear and cytoplasmic steps of tegument trafficking and assembly upon inhibition of UL97 function.

pp28 membrane association is not affected by UL97

The proper intracellular trafficking of pp28 and its membrane association (via a myristic acid moiety) are required for HCMV secondary envelopment (Jones and Lee, 2004; Sanchez et al., 2000a, 2000b; Silva et al., 2003). Although the

mechanisms involved are not yet known, the membrane association of pp28 could promote the budding of partially tegumented nucleocapsids into cytoplasmic vacuoles or serve in the bridging of the tegument to membrane-associated glycolipids during assembly. The anomalous cellular localization of pp28 observed with the ΔUL97 mutant initially suggested that UL97 could affect the membrane association of pp28. However, our CHAPS flotation results indicate that membrane association of pp28 does not depend on functional UL97 (Fig. 2).

Deficiency of UL97 kinase activity affects the remodeling of cellular structures during HCMV infection

Infection of cells with wt-HCMV significantly alters the morphology of the cellular secretory pathway, as reflected by the modified distribution of WGA ligands (which label primarily the *trans*-most sites of the Golgi (Tartakoff and Vassalli, 1983; Virtanen et al., 1980) (Fig. 4). Most noticeably, Golgi structures and viral assembly sites are compacted together at the centrosomal region by microtubule-dependent mechanisms (Sanchez et al., 2000a, 2000b). The importance of this redistribution for the viral life cycle, and the mechanisms that lead to it, has not yet been established. However, this finding is consistent with the utilization of a Golgi-derived compartment by HCMV for final envelopment (Homman-Loudiyi et al., 2003; Mettenleiter, 2002; Sanchez et al., 2000a, 2000b). One of the most dramatic effects of UL97 kinase abrogation, observed here, is that the viral-induced Golgi pattern is markedly different from that induced by the wild type virus. Importantly, the viral structural proteins pp28 and gB still co-localized with the Golgi in cells infected with the ΔUL97 mutant (Fig. 5). We conclude that the UL97 kinase is required for the proper reorganization of the Golgi by HCMV. By contrast, it is not required for the association of the viral assembly sites with Golgi elements.

An effect of UL97 kinase deletion on the shape of the nuclei in infected cells was also observed. In cells infected with wt-HCMV, the nuclei assume a kidney shape and seem to be impacted by the compact Golgi-related viral assembly site. This can be seen in Fig. 1, as well as in the work of other investigators (Sanchez et al., 2000a, 2000b). In the absence of UL97 activity, the nuclei maintained their spherical shape (Figs. 1 and 3). This failure to rearrange the nucleus could indirectly result from inhibition of early replication steps, similar to the effect observed with PFA treatment (Fig. 6), however, it could also be related to the recently reported role of UL97 in phosphorylation and redistribution of nuclear lamina components (Marschall et al., 2005). As has been shown for murine CMV, reduced phosphorylation and dissolution of the nuclear lamina could account for the impairment in nuclear cytoplasmic egress found in the ΔUL97 mutant (Muranyi et al., 2002; Krosky et al., 2003a).

How could the UL97 kinase affect the organization of cytoplasmic assembly sites?

UL97 kinase has been shown to mediate several events during the nuclear phase of viral replication. The observed cytoplasmic structural changes could potentially result from the nuclear effects, including the failure of the ΔUL97 mutants to remodel the nuclear lamina and rearrange the nucleus, combined with reduced assembly and export of nuclear particles to the cytoplasmic sites. However, antiviral pharmacologic inhibition of replication and assembly did not exert the assembly site pattern observed in the absence of UL97 kinase. While these experiments have not ruled out that the observed cytoplasmic effects are due to the role of UL97 in nuclear egress, our findings link the phenotype to specific UL97 kinase activity and raise the question of whether UL97 kinase might contribute directly to organization of the cytoplasmic assembly sites. In cells infected with wt-HCMV, Golgi elements accumulate strictly at the centrosome (Sanchez et al., 2000a). This suggests that viral infection either exaggerates the coupling of the Golgi with microtubuli or alters the microtubule cytoskeleton. UL97 kinase could act directly on cellular targets to induce these events. Direct alteration of Golgi morphology by UL97 could be exerted by phosphorylation of one or more of its structural component/s. Phosphorylation of Golgi structural proteins has been linked to morphological changes that take place during Golgi disassembly at mitosis (Lowe et al., 2000) and may occur during viral infection and enhance cytoplasmic transport.

Yet, the gradual reversion of the assembly site distribution following NGIC-I block-release appears more consistent with earlier biosynthetic inhibitions, having downstream effects on the cytoplasmic remodeling events. In this regard, we favor the possibility that the gross morphological changes recently found in the distribution of tegument proteins in the nucleus upon reduction of UL97 kinase activity (Prichard et al., 2005) could be interlinked with the reorganization of the cytoplasmic assembly sites of viral structural proteins.

Despite the critical functions ascribed to the UL97 kinase in nuclear events of virion assembly and egress, its absence results in partial replication defect, and its major impact on specific viral replication steps varies in different cellular systems and growth conditions (Biron et al., 2002; Krosky et al., 2003a; Wolf et al., 2001), suggesting dynamic interaction with cellular components and complementation by host cell kinases. Here, we propose that UL97 kinase may also be required for later cytoplasmic steps of viral replication, involving the formation of assembly sites. Thus far, the sequence of tegument protein acquisition and trafficking during virion assembly has remained unknown. The studies of the UL97 kinase function could identify connections between nuclear and cytoplasmic steps of tegumentation and shed light on protein interactions mediating the organization of the host cell secretory pathway during infection.

Materials and methods

Cells, viruses, antibodies and drugs

HCMV strain AD169 (wt-HCMV; American Type Culture Collection) and the UL97 deletion mutants RCΔ97.08 and RCΔ97.19 (ΔUL97; Prichard et al., 1999) were propagated in

human foreskin fibroblasts (HF) as described previously (Wolf et al., 2001). Primary mouse monoclonal antibodies (mAbs) against HCMV pp28, pp65 and glycoprotein B (gB) were purchased from Rumbaugh-Goodwin Institute (Plantation, Florida, USA). The secondary antibodies were Alexa Fluor 633 goat anti-mouse IgG (Molecular probes, OR, USA) and fluorescein anti-mouse IgG (Vector, Burlingame, CA, USA). All secondary antibodies are highly cross-absorbed. Wheat germ agglutinin (WGA) coupled to fluorescein isothiocyanate (FITC) was obtained from Sigma (St. Louis, Missouri, USA). The antiviral drugs BDCRB (20 μM, a kind gift from John Drach, University of Michigan) or NGIC-I (0.5 μM, Calbiochem, Germany) or phosphonoformate (PFA) (30 μg/ml; Sigma) were added after a 1 h period of virus adsorption to cells that were incubated in the presence of the drug for 96 h post infection (pi). For block-release experiments, cultures were washed five times with PBS and then further incubated for the indicated times post drug release in drug-free medium.

Immunofluorescence assay

Cells grown on 8-well glass slides (Labtek chamber slide w/ cover, #177402, Nalge Nunc International, Naperville, IL 60563) were infected at a multiplicity of infection (moi) of 0.5 PFU/cell, unless otherwise indicated. Supernatant was removed at 96 h pi, and cells were washed 3 times with PBS and fixed with 3.7% formaldehyde (Sigma Aldrich) for 30 min at room temperature. Cells were washed 5 times with PBS + 1% NH_4Cl, permeabilized by 0.1% TX100 for 5 min and washed 5 times with PBS + 1% NH_4Cl. After 1 h of blocking using 1% BSA in PBS, the indicated primary antibody was added to the cells in appropriate dilution in 0.5% BSA in PBS and incubated either at room temperature for 2 h or overnight at 4 °C. The secondary antibody was added to the cells in appropriate dilution in PBS containing 0.5% BSA for 30 min at room temperature and washed by PBS. For co-localization studies, WGA-FITC was added with the secondary antibody. Following mounting in 5% *n*-propyl gallate, 100 mM Tris–HCL, pH 9, 70% glycerol (Giloh and Sedat, 1982), the cells were analyzed by confocal microscopy (Zeiss Axiovert 135 M, Germany). Results were monitored on at least 3 independent experiments, scoring between 300 and 400 cells.

CHAPS flotation assay

HF (5×10^6 cells) infected with either AD169 or ΔUL97 viruses at an moi of 0.05 were harvested at 6 days pi, washed and incubated for 10 min at 4 °C in cold PBS. Cells were then scraped and pelleted for 10 min at 4 °C, 3000 rpm, 1000 gav. The cell pellet was re-suspended in 550 μl TNE-CHAPS lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA + 1% CHAPS) and incubated for 30 min at 4 °C (on ice) followed by 5 min sedimentation at 4 °C, 3000 rpm. The supernatant (450 μl) was transferred to pre-cooled 2 ml Beckman ultracentrifuge tube and mixed gently with 450 μl cold 70% Nycodenz in TNE. The gradient was then built by loading the tubes with 200 μl of decreasing Nycodenz concentrations: 25, 22.5, 20, 18, 15, 12 and 8%. Following centrifugation in TLS55 Beckman rotor for 3 h, 55,000 rpm, 260,000 gav, 4 °C, without break, twelve 180 μl fractions were removed, starting from the top of the gradient, and analyzed by Western blot using the ECL detection method.

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References

Baek, M.C., Krosky, P.M., Pearson, A., Coen, D.M., 2004. Phosphorylation of the RNA polymerase II carboxyl-terminal domain in human cytomegalovirus-infected cells and in vitro by the viral UL97 protein kinase. Virology 324 (1), 184–193.

Bhavanandan, V.P., Katlic, A.W., 1979. The interaction of wheat germ agglutinin with sialoglycoproteins. The role of sialic acid. J. Biol. Chem. 254 (10), 4000–4008.

Biron, K.K., Harvey, R.J., Chamberlain, S.C., Good, S.S., Smith III, A.A., Davis, M.G., Talarico, C.L., Miller, W.H., Ferris, R., Dornsife, R.E., Stanat, S.C., Drach, J.C., Townsend, L.B., Koszalka, G.W., 2002. Potent and selective inhibition of human cytomegalovirus replication by 1263W94, a benzimidazole L-riboside with a unique mode of action. Antimicrob. Agents Chemother. 46 (8), 2365–2372.

Chee, M.S., Lawrence, G.L., Barrell, B.G., 1989. Alpha-, beta- and gammaherpesviruses encode a putative phosphotransferase. J. Gen. Virol. 70 (Pt5), 1151–1160.

Chou, S., Erice, A., Jordan, M.C., Vercellotti, G.M., Michels, K.R., Talarico, C.L., Stanat, S.C., Biron, K.K., 1995. Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J. Infect. Dis. 171 (3), 576–583.

Gershon, A.A., Sherman, D.L., Zhu, Z., Gabel, C.A., Ambron, R.T., Gershon, M.D., 1994. Intracellular transport of newly synthesized varicella-zoster virus: final envelopment in the *trans*-Golgi network. J. Virol. 68 (10), 6372–6390.

Gibson, W., 1996. Structure and assembly of the virion. Intervirology 39 (5–6), 389–400.

Giloh, H., Sedat, J.W., 1982. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propyl gallate. Science 217 (4566), 1252–1255.

He, Z., He, Y.S., Kim, Y., Chu, L., Ohmstede, C., Biron, K.K., Coen, D.M., 1997. The human cytomegalovirus UL97 protein is a protein kinase that autophosphorylates on serines and threonines. J. Virol. 71 (1), 405–411.

Homman-Loudiyi, M., Hultenby, K., Britt, W., Soderberg-Naucler, C., 2003. Envelopment of human cytomegalovirus occurs by budding into Golgi-derived vacuole compartments positive for gB, Rab 3, *trans*-Golgi network 46, and mannosidase II. J. Virol. 77 (5), 3191–3203.

Jarvis, M.A., Jones, T.R., Drummond, D.D., Smith, P.P., Britt, W.J., Nelson, J.A., Baldick, C.J., 2004. Phosphorylation of human cytomegalovirus glycoprotein B (gB) at the acidic cluster casein kinase 2 site (Ser900) is required for localization of gB to the *trans*-Golgi network and efficient virus replication. J. Virol. 78 (1), 285–293.

Jones, T.R., Lee, S.W., 2004. An acidic cluster of human cytomegalovirus UL99 tegument protein is required for trafficking and function. J. Virol. 78 (3), 1488–1502.

Kawaguchi, Y., Matsumura, T., Roizman, B., Hirai, K., 1999. Cellular elongation factor 1delta is modified in cells infected with representative alpha-, beta-, or gammaherpesviruses. J. Virol. 73 (5), 4456–4460.

Kerry, J.A., Priddy, M.A., Kohler, C.P., Staley, T.L., Weber, D., Jones, T.R., Stenberg, R.M., 1997. Translational regulation of the human cytomegalovirus pp28 (UL99) late gene. J. Virol. 71 (2), 981–987.

Krosky, P.M., Baek, M.C., Coen, D.M., 2003a. The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress. J. Virol. 77 (2), 905–914.

Krosky, P.M., Baek, M.C., Jahng, W.J., Barrera, I., Harvey, R.J., Biron, K.K., Coen, D.M., Sethna, P.B., 2003b. The human cytomegalovirus UL44 protein is a substrate for the UL97 protein kinase. J. Virol. 77 (14), 7720–7727.

Lai, L., Britt, W.J., 2003. The interaction between the major capsid protein and the smallest capsid protein of human cytomegalovirus is dependent on two linear sequences in the smallest capsid protein. J. Virol. 77 (4), 2730–2735.

Littler, E., Stuart, A.D., Chee, M.S., 1992. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. Nature 358 (6382), 160–162.

Lowe, M., Gonatas, N.K., Warren, G., 2000. The mitotic phosphorylation cycle of the *cis*-Golgi matrix protein GM130. J. Cell Biol. 149 (2), 341–356.

Marschall, M., Stein-Gerlach, M., Freitag, M., Kupfer, R., van Den Bogaard, M., Stamminger, T., 2001. Inhibitors of human cytomegalovirus replication drastically reduce the activity of the viral protein kinase pUL97. J. Gen. Virol. 82 (Pt6), 1439–1450.

Marschall, M., Freitag, M., Suchy, P., Romaker, D., Kupfer, R., Hanke, M., Stamminger, T., 2003. The protein kinase pUL97 of human cytomegalovirus interacts with and phosphorylates the DNA polymerase processivity factor pUL44. Virology 311 (1), 60–71.

Marschall, M., Marzi, A., aus dem Siepen, P., Jochmann, R., Kalmer, M., Auerochs, S., Lischka, P., Leis, M., Stamminger, T., 2005. Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina. J. Biol. Chem. 280 (39), 33357–33367.

Mettenleiter, T.C., 2002. Herpesvirus assembly and egress. J. Virol. 76 (4), 1537–1547.

Michel, D., Mertens, T., 2004. The UL97 protein kinase of human cytomegalovirus and homologues in other herpesviruses: impact on virus and host. Biochim. Biophys. Acta 1697 (1–2), 169–180.

Michel, D., Kramer, S., Hohn, S., Schaarschmidt, P., Wunderlich, K., Mertens, T., 1999. Amino acids of conserved kinase motifs of cytomegalovirus protein UL97 are essential for autophosphorylation. J. Virol. 73 (10), 8898–8901.

Mocarski Jr., E.S., Tan Courcelle, C., 2001. Cytomegaloviruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott Williams & Wilkens, Philadelphia, pp. 2629–2673.

Moffat, J.F., Zerboni, L., Sommer, M.H., Heineman, T.C., Cohen, J.I., Kaneshima, H., Arvin, A.M., 1998. The ORF47 and ORF66 putative protein kinases of varicella-zoster virus determine tropism for human T cells and skin in the SCID-hu mouse. Proc. Natl. Acad. Sci. U. S. A. 95 (20), 11969–11974.

Muranyi, W., Haas, J., Wagner, M., Krohne, G., Koszinowski, U.H., 2002. Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina. Science 297 (5582), 854–857.

Ng, T.I., Keenan, L., Kinchington, P.R., Grose, C., 1994. Phosphorylation of varicella-zoster virus open reading frame (ORF) 62 regulatory product by viral ORF 47-associated protein kinase. J. Virol. 68 (3), 1350–1359.

Pass, R.F., 2001. Cytomegaloviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott Williams & Wilkens, Philadelphia, pp. 2675–2705.

Prichard, M.N., Gao, N., Jairath, S., Mulamba, G., Krosky, P., Coen, D.M., Parker, B.O., Pari, G.S., 1999. A recombinant human cytomegalovirus with a large deletion in UL97 has a severe replication deficiency. J. Virol. 73 (7), 5663–5670.

Prichard, M.N., Britt, W.J., Daily, S.L., Hartline, C.B., Kern, E.R., 2005. Human cytomegalovirus UL97 kinase is required for the normal intranuclear distribution of pp65 and virion morphogenesis. J. Virol. 79 (24), 15494–15502.

Purves, F.C., Ogle, W.O., Roizman, B., 1993. Processing of the herpes simplex virus regulatory protein alpha 22 mediated by the UL13 protein kinase determines the accumulation of a subset of alpha and gamma mRNAs and proteins in infected cells. Proc. Natl. Acad. Sci. U. S. A. 90 (14), 6701–6705.

Rouvinski, A., Gahali-Sass, I., Stav, I., Metzer, E., Atlan, H., Taraboulos, A., 2003. Both raft- and non-raft proteins associate with CHAPS-insoluble complexes: some APP in large complexes. Biochem. Biophys. Res. Commun. 308 (4), 750–758.

Sanchez, V., Greis, K.D., Sztul, E., Britt, W.J., 2000a. Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly. J. Virol. 74 (2), 975–986.

Sanchez, V., Sztul, E., Britt, W.J., 2000b. Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum–Golgi–intermediate compartment. J. Virol. 74 (8), 3842–3851.

Silva, M.C., Yu, Q.C., Enquist, L., Shenk, T., 2003. Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids. J. Virol. 77 (19), 10594–10605.

Skepper, J.N., Whiteley, A., Browne, H., Minson, A., 2001. Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment → de-envelopment → reenvelopment pathway. J. Virol. 75 (12), 5697–5702.

Sullivan, V., Talarico, C.L., Stanat, S.C., Davis, M., Coen, D.M., Biron, K.K., 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature 358 (6382), 162–164.

Tartakoff, A.M., Vassalli, P., 1983. Lectin-binding sites as markers of Golgi subcompartments: proximal-to-distal maturation of oligosaccharides. J. Cell Biol. 97 (4), 1243–1248.

van Zeijl, M., Fairhurst, J., Baum, E.Z., Sun, L., Jones, T.R., 1997. The human cytomegalovirus UL97 protein is phosphorylated and a component of virions. Virology 231 (1), 72–80.

Virtanen, I., Ekblom, P., Laurila, P., Nordling, S., Raivio, K.O., Aula, P., 1980. Characterization of storage material in cultured fibroblasts by specific lectin binding in lysosomal storage diseases. Pediatr. Res. 14 (11), 1199–1203.

Wolf, D.G., Smith, I.L., Lee, D.J., Freeman, W.R., Flores-Aguilar, M., Spector, S.A., 1995. Mutations in human cytomegalovirus UL97 gene confer clinical resistance to ganciclovir and can be detected directly in patient plasma. J. Clin. Invest. 95 (1), 257–263.

Wolf, D.G., Courcelle, C.T., Prichard, M.N., Mocarski, E.S., 2001. Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation. Proc. Natl. Acad. Sci. U. S. A. 98 (4), 1895–1900.