Human cytomegalovirus final envelopment on membranes containing both trans-Golgi network and endosomal markers

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Summary

The human cytomegalovirus (HCMV) has been shown to complete its final envelopment on cytoplasmic membranes prior to its secretion to the extracellular medium. However, the nature of these membranes has not been characterized. It is thought that HCMV acquires its final envelope from the trans-Golgi network (TGN), though we and others have previously reported a role for endocytic membranes. Here we studied the localization of cellular markers in HCMV-infected cells and in isolated viruses. Immunofluorescence staining indicated that HCMV induces the recruitment of TGN and endosomal markers to the virus factory. Immuno-gold labelling of isolated viral particles and electron microscopy demonstrated the incorporation of TGN46, endosomal markers early endosomal antigen 1, annexin I, transferrin receptor and CD63, and the cation-independent mannose 6-phosphate receptor, which traffics between the TGN and endosomes into the viral envelope. Virus immunoprecipitation assays demonstrated that virions containing TGN46 and CD63 were infectious. This study reconciles the apparent controversy regarding the nature of the HCMV assembly site and suggests that HCMV has the ability to generate a novel membrane compartment containing markers for both TGN and endosomes, or that the membranes that HCMV uses for its envelope may be vesicles in transit between the TGN and endosomes.

Introduction

As for all herpesviruses, the assembly of new human cytomegalovirus (HCMV) viral particles within productively infected cells is a complex multi-step process. Currently, the accepted model of herpesvirus assembly proposes that nucleocapsids assemble in the nucleus of an infected cell and bud across both the inner and outer nuclear membranes to be released into the cytoplasm (envelopment/de-envelopment process) (Stackpole, 1969; Skepper et al., 2001). In the cytoplasm the capsid becomes coated with tegument proteins and then acquires its final envelope by budding into vesicles. After envelopment, mature virions are transported in vacuoles to the cell surface and secreted to the extracellular medium (reviewed in Mettenleiter et al., 2006; Mocarski et al., 2007). Electron microscopy (EM) studies of HCMV-infected cells have shown that the site where viral particles acquire their final envelope – the virus factory – is located within a specific region of the cell often surrounded by a reoriented Golgi apparatus that contains numerous small membrane cisternae and tubules enwrapping tegumented nucleocapsids (Severi et al., 1988, and references therein).

Viral tegument, envelope and non-structural proteins have been localized to the virus factories (Margulies et al., 1996; Sanchez et al., 2000; Fraile-Ramos et al., 2002; Crump et al., 2003; Homman-Loudiyi et al., 2003; Pritchard et al., 2005; Seo and Britt, 2006; Das and Pellett, 2007; Krzyzaniak et al., 2007). However, little is known about the structure and cellular composition of these factories, a task complicated by marked changes in the expression levels of some cellular markers during the establishment of the HCMV virus factories (Das et al., 2007). In spite of this, visualization of cellular markers of the secretory pathway, endoplasmic reticulum-Golgi intermediate and Golgi compartments, using both antibodies and expression of cellular proteins fused to GFP, has shown the altered distribution of these secretory organelles in infected cells, which are distributed in a ring-like structure at the outer edge of the virus factories (Sanchez et al., 2000; Homman-Loudiyi et al., 2003; Seo et al., 2006; Das et al., 2007). It has been proposed that, as for alpha herpesvirus, HCMV acquires its final envelope from the trans-Golgi network (TGN) or from
TGN-derived vesicles, though these studies have shown only limited overlap of the site of HCMV envlovement with TGN markers (Sanchez et al., 2000; Homman-Loudiyi et al., 2003). Nonetheless, we and others have demonstrated that the intracellular membranes where HCMV assemblies are part of, or intersect with, the endocytic pathway (Tooze et al., 1993; Radasak et al., 1996; Fraile-Ramos et al., 2001; Fraile-Ramos et al., 2002; Seo et al., 2006; Das and Pellett, 2007; Das et al., 2007; Fraile-Ramos et al., 2007). In these studies internalized HCMV glycoprotein B, internalized HRP and the multivesicular bodies (MVBs) marker CD63 have been found incorporated into the viral envelope, and the early endosomal marker EEA1 associated to the assembly site. Some of these discrepancies may be due to the different cell types and reagents used in each study, together with the profound modifications that HCMV induces in the infected cell.

Here, we address the question of the nature of the membranes that HCMV uses for its final envelope with the use of well-defined cellular markers of the exocytic and endocytic pathways, and of the transport route between TGN and endosomes. First, we show that several endosomal markers are recruited to the assembly site. Second, we demonstrate that, unexpectedly, HCMV incorporated into its envelope markers of both the TGN and the endocytic pathway. Finally, virus immunoprecipitation assays showed that TGN46- and CD63-containing virions were infectious. These data suggest a new model for the final envelopment of HCMV that may reconcile the controversy regarding the HCMV assembly site.

Results

Distribution of markers of the exocytic and endocytic pathways in HCMV-infected cells

Human cytomegalovirus is believed to acquire its final envelope in the perinuclear region, an area of the cell that accommodates a complex array of membrane-bound structures. To gain insights into the changes induced by HCMV in the intracellular membrane systems and into the nature of the vesicles and tubules where HCMV undergoes its final envelopment, we carried out immunofluorescence studies with the use of well-established cellular markers. In BJ1 cells the Golgi marker Giantin and the TGN marker TGN46 showed ribbon-like staining patterns (Fig. 1 top panels). However, in HCMV-infected cells, the Golgi marker was seen in a ring-like structure surrounding the assembly site where HCMV glycoprotein H (gH) accumulates (overlap coefficient (R) = 0.30 ± 0.05), while TGN appeared embedded in the assembly site and showed a partial overlap with HCMV gH (R = 0.42 ± 0.10; Fig. 1, bottom panels).

We next analysed the distribution of endosomal markers in HCMV-infected cells. To label early endosomes we used antibodies against early endosome antigen-1 (EEA1), hepatocyte growth factor-regulated Tyr-kinase substrate (HRS) and annexin I proteins. As shown in Fig. 2 left panels, EEA1 labelling was present in distinct doughnut-shaped structures that are found to localize at the assembly site in HCMV-infected cells, as previously reported (Das et al., 2007). Some of these EEA1-positive doughnuts seemed to be in close contact with punctate structures labelled with HCMV chemokine receptor-like protein UL33 (R = 0.46 ± 0.04), which is consistent with our previous studies showing the localization of UL33 in endocytic compartments (Fraile-Ramos et al., 2002). HCMV viral envelope proteins gH and UL33 showed a good, although no complete, colocalization (R = 0.62 ± 0.06; Fig. S1) suggesting that part of these viral proteins may locate at distinct regions of the assembly site or that the access of the anti-UL33 antibody to its epitope – the cytoplasmic tail of the receptor – may be hindered when UL33 is incorporated into the viral envelope. HRS protein accumulated in the assembly site; however, little colocalization was observed with HCMV gH (R = 0.29 ± 0.06; Fig. 2, middle panels). Annexin I labelled punctate structures throughout the cytoplasm of BJ1 cells, while in HCMV-infected cells annexin I appeared to be recruited to the assembly site although this protein was not found to colocalize with HCMV UL33 protein (R = 0.31 ± 0.10; Fig. 2 right panels). It is worth notice that the punctate distribution of EEA1 and annexin I is distinct from the ribbon-like staining pattern of HRS (R = 0.31 ± 0.06 for EEA1 and HRS, and 0.27 ± 0.01 for annexin I and HRS; Fig S2, top panels), supporting the notion that HRS-positive structures might represent tubular-vesicular endosomal membranes associated with but distinct from early endosomes (Welsch et al., 2006). In HCMV-infected cells EEA1, annexin I and HRS are redistributed towards the assembly site (Fig. S2 bottom panels), although the degree of colocalization between EEA1 or annexin I and HRS seems not to change compared with mock infected cells (R = 0.36 ± 0.05 and 0.36 ± 0.03 respectively; compare these values with those obtained from mock infected cells). The transferrin receptor (TfR) cycles between endosomes and the plasma membrane, although the bulk of this receptor is found in recycling endosomes at steady state (Yamashiro et al., 1984). As shown in Fig. 3 left panels, in BJ1 cells TfR labelled tubular structures in the perinuclear region where recycling endosomes are expected to be located, as well as distinct puncta dispersed throughout the cytoplasm. However, in HCMV-infected cells TfR was found to accumulate in the assembly site and showed good, although incomplete, colocalization with HCMV UL33 (R = 0.87 ± 0.03). The tetraspanin CD63, a well-defined
Fig. 1. Subcellular localization of exocytic pathway markers Giantin and TGN46 in HCMV-infected cells. BJ1 cells were either mock infected (upper panels) or infected with HCMV at an moi of 0.5 (lower panels). After 4 dpi, cells were fixed, permeabilized and stained with anti-Giantin (green in left panels), anti-TGN (green in right panels) and anti-HCMV glycoprotein gH (red) antibodies. In HCMV-infected cells Giantin surrounded the virus factory while TGN46 was accumulated within it. Scale bars, 20 μm.
Fig. 2. Subcellular localization of early endosomal markers EEA1, HRS and annexin I in HCMV-infected cells. BJ1 cells were either mock infected (upper panels) or infected with HCMV at an moi of 0.5 (lower panels). After 4 dpi, cells were fixed, permeabilized and stained with anti-early endosome antigen 1, EEA1 (green in left panels), anti-hepatocyte growth factor-regulated Tyr-kinase substrate, HRS (green in middle panels), anti-annexin I (green in right panels) and anti-HCMV glycoproteins gH or UL33 (red) antibodies. In HCMV-infected cells some EEA1 vesicles seemed to be in close contact with HCMV UL33-positive structures, HRS was located within the virus factory and annexin I appeared to be recruited to this site. Scale bars, 20 μm.

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Fig. 3. Subcellular localization of recycling endosomes (transferrin receptor), multivesicular bodies (CD63) and lysosomal (Lamp1) markers in HCMV-infected cells. BJ1 cells were either mock infected (upper panels) or infected with HCMV at an moi of 0.5 (lower panels). After 4 dpi, cells were fixed, permeabilized and stained with anti-transferrin receptor, TfR (green in left panels), anti-CD63 (green in middle panels), anti-Lamp1 (green in right panels), and anti-HCMV glycoproteins UL33 and gH (red) antibodies. In HCMV-infected cells TfR was recruited to the virus factory and showed colocalization with HCMV UL33 (yellow in merge lower panel), CD63 was recruited to the assembly site and Lamp1 was excluded from this site. Scale bars, 20 μm.
marker of late endosomes/MVBs, was located in punctate structures scattered throughout BJ1 cells (Fig. 3 top middle panel). By contrast, in HCMV-infected BJ1 cells CD63 was recruited to the assembly site though little colocalization between this marker and HCMV UL33 was observed \( (R = 0.29 \pm 0.08); \) Fig. 3, bottom middle panel). The lysosomal protein Lamp1 labelled vesicular structures distributed widely throughout the cytoplasm of BJ1 cells; nevertheless, in HCMV-infected cells Lamp1 was excluded from the assembly site and appeared to surround the area where HCMV gH accumulated \( (R = 0.33 \pm 0.03); \) Fig. 3 right panels). A similar staining pattern was observed with antibodies against the lysosomal protein Lamp2 (data not shown).

We next studied the distribution of transport vesicles between the TGN and endosomes with the use of antibodies against the two mannose 6-phosphate receptors (M6PRs), the cation-independent (CI) and cation-dependent (CD) M6PRs, involved in intracellular targeting of lysosomal enzymes (Klumperman et al., 1993). In BJ1 cells M6PR-containing puncta were located mainly in the perinuclear region (Fig. 4, top panels). The CI-M6PR accumulated at the assembly site and some colocalization was observed between this M6PR and HCMV gH \( (R = 0.49 \pm 0.05); \) Fig. 4, bottom left panel); CD-M6PR labelled small punctate structures that appeared dispersed in the assembly site and that showed some colocalization with HCMV gH \( (R = 0.59 \pm 0.10); \) Fig. 4, bottom right panel).

Together, these results indicate that HCMV induces significant changes to the intracellular membranes systems. The TGN and endosomal membranes appear to be recruited to the virus factory, while the Golgi apparatus and lysosomes are excluded and surround the assembly site. These data suggest a role for TGN and endosomal membranes in the final envelopment of HCMV.

**Expression and transcriptional regulation of cellular markers in HCMV-infected cells, and detection in purified virions**

We next examined the expression levels of cellular markers that were found associated with the assembly site and their presence in the supernatants of HCMV-infected cells by Western blot analysis (Fig. 5). The levels of early endosomal proteins EEAA1, HRS, annexin I and of the TGN to endosome shuttle vesicle marker CI-M6PR were not significantly altered by HCMV infection. However, we found a reduction of ~43%, ~38% and ~28% in the expression of TGN46, CD63 and CD-M6PR, respectively, in HCMV-infected cells, while TIR expression was increased by ~42% in infected cells (Fig. 5B). Interestingly, CI-M6PR and at a lower extent CD63 were detected in supernatants of uninfected cells, suggesting that these two proteins may be secreted to the extracellular medium. Furthermore, examination of supernatants from HCMV-infected cells showed bands of the expected molecular weight for anti-TGN46, -EEA1, -TIR, -CD63 and -CI-M6PR antibodies (Fig. 5A, lower panels). Intriguingly, anti-TGN46 serum also showed a band of a higher molecular weight (~225 kDa) in supernatants of HCMV-infected cells that may represent dimers of TGN46. Detection of these cellular proteins in supernatants of infected cells suggests that they might be incorporated into secreted virions. Nevertheless, we cannot rule out the possibility that HCMV may induce the secretion of these cellular proteins into the extracellular medium or that clarified supernatants contain residual cellular debris. Therefore, to determine whether these cellular markers were associated to virions, we purified secreted HCMV viral particles by glycerol-tartrate gradients (Talbot and Almeida, 1977). Electron microscopy examination of the virion fractions showed enveloped viral particles (Fig. 6A), some of which showed ruptured or partially detached envelopes likely due to virus purification since we did not observe as many ruptured viruses when freshly isolated HCMV particles were examined (see below). As shown in Fig. 6B, TGN46, TIR, CD63 and CI-M6PR were detected in highly purified HCMV virions, indicating their incorporation into the viral envelope. Detection of EEAA1 in supernatants of HCMV-infected cells (Fig. 5A) but not in purified virions (Fig. 6B) suggests that this endosomal marker may not be incorporated into virions or that EEAA1 protein levels in virions were under the detection limit of the Western blot assay.

To gain some insights into the possible reasons resulting in the altered expression levels of some cellular proteins in HCMV-infected cells, we investigated their mRNA levels at different times post infection (Fig. 7). In the comparison with mock-infected cells, TGN46, CD63, HRS and annexin I mRNA expression levels were progressively downregulated over the time-course of infection, while EEAA1 and CI-M6PR mRNA levels seemed not to change significantly in expression after HCMV infection. TIR mRNA levels showed a transient increase at 1 day post infection (dpi), with their levels decreasing at 3 dpi and increasing at 5 dpi. CD-M6PR mRNA expression levels also transiently increased at 1 dpi, decreased until 3 dpi with little change thereafter. The decrease in mRNA levels of TGN46, CD63 and CD-M6PR may correspond to the reduction of protein expression observed by Western blot (Fig. 5), and the slightly variable expression of EEAA1 and CI-M6PR mRNA levels may correlate with the unalterable protein expression. By contrast, HRS and annexin I mRNA levels were found to be downregulated while proteins levels seemed invariable, or conversely the levels of TIR mRNA were reduced while proteins levels were increased, suggesting that regulation of these cellular proteins have been modified during infection.
Fig. 4. Subcellular localization of mannose 6-phosphate receptors in HCMV-infected cells. BJ1 cells were either mock infected (upper panels) or infected with HCMV at an moi of 0.5 (lower panels). After 4 dpi, cells were fixed, permeabilized and stained with anti-CI-M6PR (green in left panels), anti-CD-M6PR (green in right panels) and anti-HCMV glycoprotein gH (red) antibodies. In HCMV-infected cells both M6PRs were located within virus factories. Scale bars, 20 μm.
Fig. 5. Examination of cellular markers in cell lysates and supernatants of HCMV-infected cells. 
A. Equal number of BJ1 cells mock infected or HCMV infected for 4 days at an moi of 3 were lysed and viral particles pelleted by centrifugation of the clarified supernatants were mixed with non-reducing SDS-PAGE sample buffer. Lysates were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were cut and incubated, or sequentially incubated, with antibodies against cellular markers and antibodies against clathrin heavy chain or actin to check protein loading in cell lysates, and antiviral tegument protein pp28 antibodies to check HCMV viral particles secreted into the supernatants. Autoradiography films were scanned; images were cropped and assembled with Adobe Photoshop. Molecular weights in kDa are indicated. We found CD63, TGN46, EEA1, TfR and CI-M6PR in the supernatants of HCMV-infected cells. 
B. Results of cell lysates in (A) were analysed by densitometry, normalized to the expression levels of loading controls and expressed as a percentage of the levels in uninfected cells. We observed a reproducible reduction in the levels of CD63, TGN46 and CD-M6PR together with an increase in the levels of TIR by comparison with uninfected cells.

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markers by HCMV occur not only at the transcriptional level but also at the protein level. These results show that HCMV influences both transcripts and protein levels of several cellular makers, and that TGN46, EEA1, TfR, CD63 and CI-M6PR are detected in supernatants of HCMV-infected cells and in purified virions indicating that they are incorporated into the viral envelope.

**Immunogold localization of cellular markers in isolated HCMV particles**

To further investigate the incorporation of cellular markers associated with the assembly site into the viral envelope, we labelled isolated virus particles with antibodies and immuno-gold reagents and analysed them by EM. Negative staining of supernatants from infected-cells showed two classes of spherical enveloped particles: 200 nm diameter HCMV virions and larger structures corresponding to dense bodies (DBs). The ratio of virions to DBs was 1:2, although there may be some small (<200 nm) DBs, which were classified as virions. We determined the number of virions and DBs labelled with an antibody against HCMV gH and found that 97 of 137 virions (Fig. 8A) and 75 of 112 DBs were labelled with on average 7.9 ± 2.6 gold particles per virion (Table 1). The specificity of the immuno-gold labelling was assayed by omitting the primary antibody (in this case just 2 of 25 virions were labelled with one gold particle each), or using an antiviral simplex virus type 1 glycoprotein D (HSV gD, AP7) antibody as negative control (4 of 110 virions and 5 of 125 DBs were labelled with one gold particle). TGN46 was detected on 50 of 120 virions and 36 of 84 DBs with 1.4 ± 0.6 gold particles per virion, and CD63 labelled 77 of 125 virions and 73 of 110 DBs with 3.1 ± 1.5 gold particles per virion (Fig. 8B and C and Table 1). We obtained similar results when gold-conjugated secondary antibodies were used to detect anti-CD63 and anti-TGN46 antibodies, although the labelling was weaker than that obtained with bridging antibodies and PAG (data not shown). Moreover, some isolated virions were double labelled with anti-TGN46 and anti-CD63 antibodies (Fig. 8D and 40 of 120 virions and 43 of 118 DBs were labelled with 2.7 ± 1.3 10 nm gold particles per virion, marking CD63 and 1.4 ± 0.5 15 nm gold particles per virion detecting TGN46). These results indicate that HCMV envelope contains both TGN and CD63 markers.

**Fig. 6.** Examination of cellular markers in purified HCMV virions. A. Electron microscopy of purified HCMV virions negatively stained with 2% uranyl acetate. When viral particles were partially disrupted, uranyl acetate revealed the nucleocapsids. Scale bar, 200 nm.

B. 35 ng of purified virions per lane were separated by SDS-PAGE under non-reducing conditions and transferred to PVDF membranes. Membranes were cut and incubated, or sequentially incubated, with antibodies against cellular markers and antiviral tegument protein pp28 antibodies. Autoradiography films were scanned; images were cropped and assembled with Adobe Photoshop. Molecular weights in kDa are indicated. We found CD63, TGN46, TIR and CI-M6PR in purified virions.

EEA1, HRS and annexin I are membrane-bound cytosolic proteins that associate with early endosomes and these proteins might become trapped into viral particles when the virions bud into these membranes. Therefore, in order to detect these cellular proteins, enveloped particles were permeabilized with saponin before labelling. As a control for the accessibility of antibodies recognizing proteins associated with the inner leaflet of the viral envelope, a serum against the C-terminus of HCMV chemokine receptor-like protein UL33 was used. Gold particles were only found associated with permeabilized-enveloped virions, consistent with the antibody binding to the cytoplasmic C-terminal domain of UL33 (Fig. 8E and 64 of 141 virions and 72 of 151 DBs were labelled with 1.1 ± 0.3 gold particles per virion). We observed some virions and DBs stained with anti-EEA1 and anti-annexin I antibodies (Fig. 8F–G and 35 of 115 virions and 37 of 127 DBs were labelled with 1.7 ± 0.6 gold particles per virion, and 40 of 108 virions and 35 of 103 DBs were labelled with 2.5 ± 1.1 gold particles per virion respectively). However, HRS labelled very few viral particles (8 of 128 virions and 6 of 120 DBs labelled with 1 gold particle per virion). When intact virions were labelled with an antibody against the lumenal domain of the TIR, labelling was associated to numerous viral particles (Fig. 8H and 50 of 105 virions and 52 of 115 DBs were labelled with 1.9 ± 0.6 gold particles per virion). Nevertheless, labelling of permeabilized virions
Fig. 7. Transcriptional regulation of cellular markers during HCMV infection. Equal number of BJ1 cells were mock infected or infected with HCMV at an moi of 3, and RNA was extracted at the indicated times. Gene expression of cellular markers and HCMV UL83 (viral protein pp65) was measured by qPCR, normalized to 18S rRNA expression and calibrated to the levels in uninfected cells. CD63, TGN46, HRS and annexin I transcripts were downregulated over the time-course of HCMV infection, while EEA1 and CI-M6PR transcripts levels changed slightly. TIR and CD-M6PR transcripts were transiently upregulated at 1 dpi and then downregulated with the mRNA levels of TIR increasing at 5 dpi and of CD-M6PR unaltered after 3 dpi. HCMV pp65 transcripts levels increased during the infection. Data corresponding to three biological replicates were averaged, bars represent the average normalized fold increase, and error bars represent standard errors of the means.

Table 1. Staining of isolated virions and dense bodies with cellular markers.

| Markers          | No. of labelled | % labelled |  |
|------------------|-----------------|------------|
|                  | Controla        | Virions    | DBs | No. of gold particles | Virions | DBs |
| Viral            |                 |            |     |                      |         |     |
| HCMV gH          | 2 (25)          | 97 (137)   | 75 (112) | 7.9 ± 2.6             | 62.9    | 59.3 |
| HCMV UL33        | 1 (24)          | 64 (141)   | 72 (151) | 1.1 ± 0.3             | 41.4    | 43.7 |
| HSV-1 gD         | 1 (25)          | 4 (110)    | 5 (125)  | 1.0 ± 0.0              | 0.0     | 0.0  |
| Endosomes        |                 |            |     |                      |         |     |
| EEA1             | 1 (28)          | 35 (115)   | 37 (127) | 1.7 ± 0.6             | 26.9    | 25.6 |
| HRS              | 1 (25)          | 8 (128)    | 6 (120)  | 1.0 ± 0.0              | 2.3     | 1.0  |
| Annexin I        | 1 (28)          | 40 (106)   | 35 (103) | 2.5 ± 1.1             | 33.5    | 30.5 |
| TFR (lumen)      | 1 (23)          | 50 (105)   | 52 (115) | 1.9 ± 0.6             | 43.3    | 40.9 |
| TFR (c-tail)     | 2 (30)          | 18 (120)   | 16 (122) | 1.0 ± 0.0              | 8.3     | 6.4  |
| CD63             | 1 (25)          | 77 (125)   | 73 (110) | 3.1 ± 1.5             | 58.0    | 62.4 |
| TGN              |                 |            |     |                      |         |     |
| TGN46            | 1 (25)          | 50 (120)   | 36 (84)  | 1.4 ± 0.6             | 37.7    | 38.9 |
| TGN46 + CD63b    | 2 (25)          | 40 (120)   | 43 (118) | CD63 2.7 ± 1.3         | 25.3    | 28.5 |
| TGN – Endosomes  |                 |            |     |                      |         |     |
| CI-M6PR (lumen)  | 1 (24)          | 47 (145)   | 55 (155) | 1.3 ± 0.5             | 28.3    | 31.4 |
| CI-M6PR (c-tail) | 2 (27)          | 39 (145)   | 42 (150) | 1.0 ± 0.0              | 19.5    | 20.6 |
| CD-M6PR (lumen)  | 1 (24)          | 10 (110)   | 12 (115) | 1.0 ± 0.0              | 4.9     | 6.2  |
| CD-M6PR (c-tail) | 2 (27)          | 12 (112)   | 13 (115) | 1.0 ± 0.0              | 3.3     | 4.2  |

a. Non-specific binding was assayed omitting the primary antibody. Virions and DBs were classified according to their size in multiple fields at a magnification of 20,000. The number of viral particles analysed is indicated in brackets. The number of gold particles associated to each virion and DBs was calculated, and means and the standard deviations are shown.

b. The number of double-labelled viruses and DBs, and gold particles associated were determined. To calculate the percentage of gold-labelled virions and DBs, the non-specific binding was subtracted and the number of viral particles analysed was set at 100%.

Virions and DBs were classified according to their size in multiple fields at a magnification of 20 000. The number of viral particles analysed is indicated in brackets. The number of gold particles associated to each virion and DBs was calculated, and means and the standard deviations are shown.

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with an antibody against the cytoplasmic tail of TIR showed few viral particles labelled (18 of 120 virions and 16 of 122 DBs were labelled with 1 gold particle per virion), suggesting that accessibility of the antibody to its epitope may be hindered.

We next analysed whether the M6PRs were incorporated into the viral envelope. When intact enveloped particles were labelled with a serum against the lumenal domain of the CI-M6PR, labelling was associated to the surface of 47 of 145 virions and 55 of 155 DBs with 1.3 ± 0.5 gold particles per virion (Fig. 8I). In addition, a serum against the cytoplasmic tail of the CI-M6PR marked 39 of 145 permeabilized virions and 42 of 150 permeabilized DBs with 1 gold particle per virion, confirming the incorporation of the CI-M6PR into the viral envelope. However, sera against the lumenal domain or the cytoplasmic tail of the CD-M6PR labelled very few intact and permeabilized enveloped particles respectively (10 of 110 virions and 12 of 115 DBs were labelled with 1 gold particle per virion, and 12 of 112 virions and 13 of 115 DBs were labelled with 1 gold particle per virion respectively).

Taken together, these results indicate that virions and DBs acquire their envelope from the same membranes and confirm that markers for the TGN, the endocytic pathway and the transport intermediates between TGN and endosomes are incorporated into the viral envelope.

**Virus precipitation with antibodies against cellular proteins**

Our observations suggest that HCMV acquires its final envelope from membranes derived from the TGN and the endocytic pathway. To determine whether these membranes are the source of secreted infectious virions, we precipitated viruses collected from cell culture media with antibodies directed against the ecto (lumenal) domain epitopes of CD63 or TGN46. Cell-free media from BJ1 cells infected with a recombinant strain of HCMV AD169 expressing green fluorescent protein (McSharry et al., 2001) were mixed with the respective antibody and subsequently bound to Pansorbin cells. Samples were centrifuged and the supernatants were assayed for unprecipitated infectious virus on BJ1 cells and by GFP expression and FACS analysis (Fraile-Ramos et al., 2007). As a positive control for precipitation, we used an antibody against HCMV gH, which completely eliminated infectivity in the supernatant (Fig. 9). A similar reduction in infectivity was observed with an antibody against HCMV gB (data not shown). An antibody against HSV-1 gD was used to determine non-specific virus precipitation, which was low since this antibody produced only a small (< 5%) reduction in the number of infectious particles. As shown in Fig. 9, antibodies against TGN46 and CD63...
precipitated some of the infectious viruses (~50% and ~35% respectively).

We conclude that the viral particles that underwent final envelopment in TGN46 and CD63 containing membranes are a source of infectious virions.

Discussion

We and others have reported the presence of endocytosed HRP and CD63 in the HCMV budding compartment and on free extracellular viruses, supporting the notion of a role for endosomal membranes in the final envelopment of HCMV (Tooze et al., 1993; Fraile-Ramos et al., 2007). To extend this work and to further identify the nature of the cellular membranes where HCMV acquires its final envelope, we have now analysed the expression, distribution and presence of well-defined cellular markers of the exocytic and endocytic pathways in HCMV-infected cells, in purified virions and in isolated viral particles. Our immunofluorescence studies showed that HCMV induces significant changes in the intracellular membrane systems in infected cells. In particular, endosomes, identified with markers such as EEA1, annexin I, TIR or CD63, seemed to be recruited to the assembly site where TGN, and TGN to endosomes shuttle vesicles also accumulated. By Western blot analysis of purified virions and immunolabeling of isolated viral particles followed by EM we found that TGN, endosomal markers and MP6Rs were incorporated into the viral envelope. Moreover, virus immunoprecipitation assays revealed that the TGN46- and CD63-positive viruses were infectious. Based on these results, we propose a model for the final steps in HCMV morphogenesis in which the tegumented nucleocapsids in the virus factories wrap into membrane cisternae (Fig. 10). The cisternal membranes have characteristics of both TGN and endosomes, suggesting that they may represent the transport vesicles between endosomes and the TGN. Perhaps this transport vesicle compartment is expanded in HCMV-infected cells, or it is possible that HCMV generates a novel hybrid compartment with both endosomal and TGN components. The mature HCMV particles are contained in vesicles, which can fuse with the plasma membrane to release the virions into the extracellular medium. Importantly, our findings reconcile the apparent controversy on the HCMV assembly site.

Like a number of enveloped viruses, HCMV induces the formation of virus factories – large accumulations of viral components and specific cell organelles in the perinuclear region where viral morphogenesis takes place (reviewed in Novoa et al., 2005a). In the case of HCMV this cytoplasmic virus factory has also been called ‘assembly compartment’ or ‘assembly complex’ (Sanchez et al., 2000; Homman-Loudiyi et al., 2003). Our immunofluorescence analysis with anti-Giantin antibodies revealed this Golgi protein encircling the assembly site (Fig. 2), as previously reported (Severi et al., 1988; Sanchez et al., 2000; Seo et al., 2006; Das et al., 2007). When we analysed the distribution of TGN46 in infected cells, we observed the loss of the typical tubular network of the TGN and the localization of this cellular marker with HCMV glycoprotein gH within virus factories, a finding consistent with previous studies (Sanchez et al., 2000; Homman-Loudiyi et al., 2003; Seo et al., 2006; Das et al., 2007). As observed for TGN46, early endosomal marker HRS and M6PRs are accumulated in the perinuclear region of uninfected cells and remain in this area in HCMV-infected cells. However, some endosomal markers distributed throughout the cytoplasm of uninfected cells, such as EEA1 and annexin I early endosomal proteins, the recycling endosome protein TIR and the MVB marker CD63, were recruited to the virus factories in infected cells. By contrast, we found that lysosomal markers Lamp1 and Lamp2 were excluded from the assembly site. Except for TIR, the cellular markers located within the assembly site showed little colocalization with viral envelope glycoproteins (Figs 2–5) even though TGN46, EEA1, annexin I, CD63 and CI-M6PR were found associated with viral particles in our Western blot and immuno-EM assays (Figs 5 and 6, Table 1). This low level of colocalization at the immunofluorescence level may be explained with distinct subcellular localization of the bulk of cellular proteins and the viral proteins. However, it is also possible that accessibility of the antibodies to their particular antigens may differ between cellular and viral membranes likely due to the
compact association of tegument proteins with the viral envelope or due to the high membrane curvature of the virion envelope.

Our analysis of the expression of cellular markers by Western blot and qPCR showed that HCMV regulates both transcript and protein levels. TGN46, CD63 and CD-M6PR are downregulated at 4 dpi on HCMV-infected cells. Consistent with these Western blot results, we noted a reduction in the staining for TGN46 and CD63 by immunofluorescence in some of the HCMV-infected cells that correlate with observed downregulation in the transcripts levels of these markers during HCMV infection.

Immunolabelling and EM of cryosections of HCMV-infected cells has allowed the detection of several cellular proteins and the detailed analysis of viral particles and their interactions with endomembranes in infected cells (Fraile-Ramos et al., 2002; Homman-Loudiyi et al., 2003; Fraile-Ramos et al., 2007). Thus, Homman-Loudiyi and colleagues have detected the secretory pathway markers mannosidase II and Rab3, and the early endosome marker Rab5 over the membranes of virions (Homman-Loudiyi et al., 2003), and we have previously shown the localization of viral glycoproteins (chemokine receptor-like proteins US27 and UL33, gH and gB) and of an endocytic tracer and CD63 over the membranes into which virions were wrapping, in tubules and vesicles, and in MVBs (Fraile-Ramos et al., 2002; Fraile-Ramos et al., 2007). Here, to gain further insights into the nature of the HCMV cytoplasmic envelopment compartment we carried out Western blot analysis of purified virions, and immuno-gold labelling and EM analysis of isolated viral particles. Intriguingly, despite the low labelling, the immunolocalizations results revealed the incorporation of all of the cellular markers analysed, into the virions. These data, together with the localization and recruitment of many cellular markers within the virus factories, raise questions on the specificity of how HCMV selects its membranes. Does HCMV need specific membranes, or can it more broadly use many of the organelles that aggregate around the microtubule-organizing centre? Perhaps any membrane that contains viral glycoproteins can support envelopment. In this line, we cannot rule out that the lack of staining of some viral particles with anti-HCMV gH antibodies may be due to inaccessibility by antibodies or gold particles, or that some virions fail to incorporate HCMV gH and the normal complement of viral envelope glycoproteins into their membrane, resulting in non-infectious viral particles. In support of this notion, it has been shown that the ratio of viral particles to infectious virus is unusually high for HCMV (Benyesh-Melnick et al., 1966).
Quantitative analysis indicated that TGN46 and CD63 were associated with viral particles and, more importantly, double labelling immuno-EM assays identified both proteins over the same viral envelope (Fig. 8D and Table 1). We extended the immuno-EM analysis to other well-defined endosomal makers. We found EEA1 associated with some viral particles, a result consistent with the previously described localization of EEA1 to virus factories (Das and Pellett, 2007; Das et al., 2007). Transferrin receptor, a recycling endosomal marker (Yamashiro et al., 1984), was also incorporated into the viral envelope and showed the strongest colocalization with the viral envelope protein UL33 within virus factories, suggesting a role for recycling endosomal membranes in HCMV envelopment, in agreement with results from William Britt group (M. Krzyzaniak and W. Britt, pers. comm.). By contrast, HRS, which binds phosphatidylinositol-3-phosphate and ubiquitylated receptors sorted into the degradative MVB pathway (Urbe et al., 2000; Raiborg et al., 2002), seemed to be associated with few viral particles. Nevertheless, annexin I a Ca\(^{2+}\)-regulated membrane binding protein implicated in sorting of epidermal growth factor receptor into MVBs (Futter et al., 1993) was found in some of viral particles, in agreement with the identification of this cellular protein in HCMV virions by mass spectrometry-based proteomic approaches (Varnum et al., 2004). Surprisingly, annexin I was not detected in supernatants of HCMV-infected cells and in purified virions by Western blot assays, perhaps the protein levels associated to virions might be under the limit of detection by this technique.

Because there is trafficking between TGN and endosomes, and because it is known that M6PRs shuttle between these organelles (Klumperman et al., 1993), we analysed the presence of the two M6PRs in viral particles. Surprisingly, the CI-M6PR was found incorporated into the envelope of some viral particles, a result confirmed using two sera against both the cytoplasmic tail and the lumenal domain of the receptor, while CD-M6PR sera stained few viral particles (Fig. 8I and Table 1). The mechanisms through which viral tegument proteins and viral glycoproteins are targeted to the virus factories are largely unknown. Nevertheless, several sorting signals have been identified, such as an acidic cluster motif within the cytoplasmic tails of HCMV glycoprotein gB and gM, and in the membrane-associated tegument protein pp28, as well as a tyrosine-based motif in the cytoplasmic domain of gM (Tugizov et al., 1999; Crump et al., 2003; Jones and Lee, 2004; Seo et al., 2006). The acidic cluster motif within the cytoplasmic tail of HCMV gB has been described to interact with the PACS-1 ‘connector’ molecule, a protein involved in retrograde transport from endosomes to the TGN, and depletion of PACS-1 by siRNA reduced the production of infectious HCMV (Crump et al., 2003). Trafficking of M6PRs is directed by several sorting signals that are present within the cytoplasmic tails of the receptors and a cluster of acidic residues within the cytoplasmic domain of the CI-M6PR binds PACS-1 protein (Wan et al., 1998). It is possible that HCMV gB and the CI-M6PR utilize the same trafficking pathway and that their localization in the transport vesicles from endosomes to TGN would allow their incorporation into the viral envelope. It is also possible that, as for the alphaherpesviruses HSV-1 and varicella-zoster virus (Gabel et al., 1989; Brunetti et al., 1998), HCMV contains glycoproteins that are modified by M6P, and thus are ligands for CI-M6PR. The receptor may sort M6P-complexed viral glycoproteins to the virus factories and some CI-M6PRs may be unable to return to the TGN but become trapped into the viral envelope. Taken together, these results strongly support a role for the retrograde and/or anterograde transport intermediates between TGN and endosomes in HCMV envelopment.

Interestingly, recent high quality ultrastructural studies by Mori and colleagues have demonstrated that the betaherpesvirus human herpesvirus 6 (HHV-6) buds into MVBs and at TGN-derived membranes that contain both CD63 and TGN46, and that CD63 is incorporated into virions (Mori et al., 2008). The authors suggested that HHV-6 virus-wrapping membranes have characteristics intermediate between those of the TGN and endosomes. We have previously shown that most of HCMV virus particles bud into membrane vesicles, although others were also occasionally seen within and budding into MVBs, and that CD63 is incorporated into viral envelopes (Fraile-Ramos et al., 2002; Fraile-Ramos et al., 2007). In the present study, we found TGN46 and CD63 associated with HCMV particles and demonstrated by virus precipitation assays that the CD63 and TGN46 containing virions were infectious. In summary, these data suggest that the betaherpesviruses HCMV and HHV-6 may use the same source of membranes for their final envelopment.

In conclusion, our results support the notion that HCMV induces significant changes in cellular membrane systems and indicate that the virus may have the ability to generate a novel membrane compartment that contains markers for both TGN and endosomes, or more likely that the membranes that HCMV uses for its envelope may be the transport vesicles between endosomes and TGN, thus reconciling the different hypotheses on the HCMV assembly site. The challenge now is to understand the possible function of the cellular factors identified within the viral particle in HCMV envelopment.

**Experimental procedures**

**Reagents and antibodies**

Tissue culture reagents and Nunc tissue culture plastic were from LabClinics S.A. (Barcelona, Spain), and chemicals were from Sigma Aldrich (Madrid, Spain), unless otherwise indicated.
Details of the antibodies used in this study are shown in Table 2 and were as follows: anti-annexin I (Isacke et al., 1989) was provided by Dr Claire Isacke (CRUK, London, UK); anti-EEA1 and anti-CHC were purchased from BD Bioscience (Palo Alto, CA, USA); anti-TGN46 were provided by Dr Claire Isacke (CRUK, London, UK); anti-actin serum and anti-

Table 2. Antibodies used in the study.

| Antigen                        | Antibodies name | Species and isotype | Source                        |
|--------------------------------|-----------------|---------------------|-------------------------------|
| HCMV glycoprotein H            | HCMV 16         | Mouse IgG1          | Helena Browne                 |
| HCMV glycoprotein B            | HCMV 37         | Mouse IgG2a         | Helena Browne                 |
| HCMV UL33                      | Anti-HCMV UL33  | Rabbit polyclonal   | Wade Gibson                   |
| HCMV pp28                      | Anti-HCMV pp28  | Mouse IgG1          | Thomas Shenk                  |
| HSV-1 glycoprotein D           | AP7             | Mouse IgG2a         | Helena Browne                 |
| CD63 (lumenal domain)          | Anti-CD63, 1B5  | Mouse IgG2b         | Mark Marsh                    |
| TGN46                          | Anti-TGN46      | Sheep polyclonal    | AbD-Seronotec                 |
| Annexin I                      | Anti-annexin I  | Mouse IgG1          | Claire Isacke                 |
| Early endosome antigen 1       | Anti-EEA1       | Rabbit polyclonal   | BD Biosciences                |
| Hepatocyte growth factor-regulated | Anti-HRS 958/3  | Mouse IgG1          | Sylvie Urbé                   |
| Tyr-kinase substrate           |                 |                     |                               |
| Transferrin receptor (lumenal domain) | B3/25           | Mouse IgG1          | Santa Cruz Biotechnology      |
| CD-M6PR (lumenal domain)       | H68.4           | Mouse IgG1          | Zymed Laboratories            |
| CD-M6PR (cytoplasmic tail)     | Anti-MPR300 G VII-3 | Rabbit polyclonal | Kurt von Figura               |
| CD-M6PR (lumenal domain)       | Anti-MPR300 H-CI-II | Rabbit polyclonal  | Kurt von Figura               |
| CD-M6PR (cytoplasmic tail)     | Anti-MPR46 MCI  | Rabbit polyclonal   | Kurt von Figura               |
| CD-M6PR                        | Anti-MPR46 22d4 | Mouse IgG1          | DSHB                          |
| β-actin                        | Anti-β-actin AC-15 | Rabbit polyclonal | Sigma Aldrich                 |
| Actin                          | Anti-actin      | Rabbit polyclonal   | Sigma Aldrich                 |
| Clathrin heavy chain           | Anti-CHC 23     | Mouse IgG1          | BD Biosciences                |

Human foreskin fibroblasts (BJ1) cells were purchased from Clontech (California, USA) and maintained in 4:1 ratio Dulbecco’s modified Eagle’s medium (DMEM): medium 199 containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 4 mM L-glutamine, 100 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin. The Towne strain of HCMV and a recombinant strain of HCMV AD169 expressing GFP (McSharry et al., 2001), RCMV288, were propagated on BJ1 cells and titered by immunofluorescence detection of HCMV pp65 expression and GFP visualization respectively.

Extracellular HCMV particles were purified essentially as previously described (Talbot and Almeida, 1977; Irimieri and Gibson, 1983; Blankenship and Shenk, 2002) with some modifications. BJ1 cells were infected with HCMV Towne stocks at a multiplicity of infection (moi) of three infectious particles per cell. When ~50% of the cells were detached from the plate, normally at 6 dpi, medium was collected and cleared of cells and cell debris by centrifugation at 1200 r.p.m. for 10 min at 4°C. The clarified medium was layered over a sorbitol cushion (20% D-sorbitol, 50 mM Tris pH 7.4, 1 mM MgCl₂) and viral particles were pelleted by centrifugation (20 000 r.p.m. for 90 min at 4°C, Beckman SW28 rotor). Crude viral particles were resuspended in buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl and protease inhibitors (Roche), sonicated, layered onto a glycerol-tartarate gradient, and subjected to centrifugation (40 000 r.p.m. for 15 min at 4°C, Beckman SW41 rotor with slow acceleration and braking). Three fractions were removed from the gradient (non-infectious enveloped particles, virions and DBs), and virions were further purified by an additional rate-velocity sedimentation step (as above). Particles extracted from the gradients were diluted sevenfold in buffer (50 mM Tris pH 7.4, 100 mM NaCl), pelleted by centrifugation (21 000 r.p.m. for 1 h at 4°C, Beckman SW41 rotor), and resuspended in the same buffer supplemented with protease inhibitors. Purity was confirmed by negative staining with 2% uranyl acetate and electron microscopy, and the protein content of purified virions was assayed by a micro BCA assay (Thermo Scientific).

**Immunofluorescence microscopy**

BJ1 cells growing on glass coverslips were mock infected, or infected with RCMV288 or the Towne strain of HCMV at an moi of...
0.5 infectious particles per cell. At 4 dpi, cells were first fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS), quenched with NH₄Cl, permeabilized and stained with appropriate antibodies in the presence of 0.05% saponin and 10% human serum, essentially as described (Fraile-Ramos et al., 2001). Cell nuclei were stained with 5 μg ml⁻¹ DAPI (Sigma Aldrich) in PBS. Following staining, coverslips were mounted in ProLong Gold anti-fade reagent (Invitrogen) and analysed using a Leica DMi6000 microscope equipped with a Leica TCS-SP5 multispectral confocal laser scanning system. Series of optical sections were taken through the depth of the cells at intervals of 0.4 μm. Quantitative colocalization analysis was performed by calculating the Manders’ overlap coefficient (ranging from 0, minimum colocalization degree, to 1, maximum colocalization degree; (Manders et al., 1993)), with the use of the Leica Application Suite Advanced Fluorescence software (Leica Microsystems). At least 10 different cells were analysed (3 sections for mock infected and a minimum of 5 sections for HCMV-infected cells), and the overlap coefficient (means ± SE) was then calculated. To prepare the figures, digital images were transferred to Adobe Photoshop and adjusted so that intensity values extended over the full measurable range (0–255 grey levels).

**Western blotting**

For the analysis of cell lysates, equal numbers of BJ1 cells mock infected or infected with the Towne strain of HCMV for 4 days at an moi of 3 infectious particles per cell were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl supplemented with 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) containing protease inhibitors. After removal of the nuclei and cell debris by centrifugation at 14 000 r.p.m. for 10 min at 4°C, the lysates were loaded on 10% SDS-polyacrylamide gels under non-reducing conditions. For the analysis of viral particles secreted into the supernatants, 100% confluent BJ1 cells growing on 175 cm² tissue culture flasks were mock infected or infected with HCMV at an moi of 3 infectious particles per cell. At 4 dpi media were harvested and removed of cellular debris by low speed centrifugation as above. A volume of 1.5 ml of clarified media was subjected to centrifugation at 10 000 r.p.m. for 10 min at 4°C, supernatants were removed and viral particles were lysed in 10 μl of non-reducing SDS-PAGE sample buffer and separated on 10% SDS-PAGE gels. For the analysis of purified viral particles, samples containing 35 ng of purified virions in 10 μl of non-reducing SDS-PAGE sample buffer were separated as above. After electrophoresis, proteins were transferred to PVDF membranes (Millipore) and blots were analysed as described (Fraile-Ramos et al., 2001). Cell lysates blots were developed with the use of SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), while supernatants and purified virions blots were developed with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) that allows higher sensitivity. Autoradiography films were scanned and the bands were quantified with the use of the public domain ImageJ 1.33 program (developed at the US National Institute of Health and available on the Internet at http://rsb.info.nih.gov/ij/). For the cell lysates analysis, protein loading was normalized to either clathrin heavy chain or actin expression levels. For the supernatants and purified virions analysis, HCMV viral particles were assayed with antibodies against the viral tegument protein pp28. Images were cropped and assembled with Adobe Photoshop.

**Quantitative real-time PCR**

BJ1 cells growing in 60 mm tissue culture dishes were mock infected or infected with the Towne strain of HCMV at an moi of 3 infectious particles per cell. Total RNA was extracted at 1, 2, 3, 4 and 5 dpi using the RNeasy mini kit (Quiagen, supplied by Izasa S.A., Barcelona, Spain). Dnase I (Quiagen) digestion was carried out directly in spin column. For each time point three tissue culture dishes were used. The analysis of the relative gene expression of TGN46, EEA1, annexin I, transferrin receptor, CD63, cation-independent and cation-dependent mannose 6-phosphate receptor and HCMV UL83 by quantitative real-time PCR (qPCR) was carried out at the Genomics Core Facility at the Centro de Biología Molecular ‘Severo Ochoa’ CSIC-UAM, Madrid. DNA contamination was assessed by amplification of non reverse-transcribed samples. Amplification was either negative or quantitatively weak enough even with the non-intron-spanning assays [see reverse-transcription (RT) information below]. RNA was quantified in a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and samples showed 260/280 ratio values of ~2 and yield ranges between 2.1 and 3 μg ml⁻¹. RNA integrity was tested with the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA Integrity Number values were between 8.80 and 10 showing excellent integrity. RT reactions were performed using the High Capacity RNA-to-cDNA Master Mix with No RT Control (Applied Biosystems S.A., Madrid, Spain) following manufacturer’s instructions. cDNAs were stored at ~20°C. For each cellular gene, intron-spanning assays were designed using Probe Finder software (Roche Applied Science, http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000), and when several transcripts have been described one primer pair common to all the transcripts was used. Probe Finder was also used to designed assays for HCMV UL83 gene. qPCR reactions were performed in triplicates in a 10 μl final volume with the cDNA amount equivalent to 5 ng of total RNA, 250 nM of each primer and 5 μl of Power SYBR Green PCR Master Mix (Applied Biosystems) using MicroAmp Optical 384-well reaction plates with barcode (Applied Biosystems) with the ABI 7900HT (Applied Biosystems). The cycling conditions comprised an initial denaturation step of 10 min at 95°C followed by 40 two-step cycles (95°C for 15 s and 60°C for 60 s). A dissociation curve from 60°C to 95°C (2% ramp) was performed after each run to eliminate primer-dimer formation and other non-specific amplification. PCR amplification efficiency and linear dynamic range were determined for each assay (Table S1). Data analysis was performed with SDS 2.2.1 (Applied Biosystems) and 18S rRNA was used for normalization.

**Immuno-gold labelling of isolated viral particles**

Localization of viral and cellular proteins on the envelope of viral particles was carried out by immuno-gold labelling and electron microscopy with the use of antibodies against viral envelope proteins and cellular markers, and PAG (10 or 15 nm; from the EM Laboratory, Utrecht University, the Netherlands). HCMV glycoproteins gp68 and gp34 bind the Fc domain of some immunoglobulins (Ig) among them human and rabbit IgG (Atalay et al., 2002, and references therein). Therefore, to be able to use rabbit antibodies during immunolabelling assays we blocked viral Fc receptors with human serum and 5 nm PAG. Viral suspensions were fixed for 15 min with a solution of 4% paraformaldehyde.
(PFA) in PBS, spun in an A-100/18 rotor of a Beckman Airfuge (Beckman Coulter, Palo Alto, USA) at 30 psi (pounds per square inch, ~120 000 g) for 3 min at room temperature, supernatans were removed and viral particles were resuspended in PBS, sonicated and kept at 4°C until labelling was carried out. Viral particles were allowed to adsorb for 3 min onto electron microscopy grids coated with Formvar and carbon (glow discharged). Excess liquid was absorbed with filter paper, and immunolabeling was conducted by placing the grids on drops of different solutions as described previously (Novoa et al., 2005b). When antibodies directed against the cytoplasmic epitopes of transmembrane proteins, or against proteins associated with the inner leaflet of the viral envelope were used, viral particles were first permeabilized with 0.05% saponin for 3 min. Otherwise, the first step was a 7 min incubation with 30 mM Tris-HCl, pH = 8.2, 150 mM NaCl and 10% human serum to block viral Fc receptors followed by a 7 min incubation with TBG (30 mMTris-HCl, pH = 8.2, 150 mM NaCl, 0.1% bovine serum albumin and 0.1% gelatin) containing 5 nm PAG (diluted 1:10) to occupy the protein A binding site of the human IgG Fc region. On lightly contrasted viral preparations, we were able to quantify 2.1 ± 1.5 5 nm gold particles per virion. Following this treatment, the PAG-5 labelled samples were stabilized by a 5 min fixation with 4% PFA in PBS and residual fixative was inactivated by a 5 min incubation in quenching buffer (0.2 M NH4Cl in PBS). Grids were then transferred to drops of primary antibodies diluted in TBG for 7 min. Viral particles stained with mouse monoclonal and sheep polyclonal antibodies were incubated with rabbit anti-mouse or anti-Goat bridging antibodies, respectively, before labelling with 10 nm or 15 nm PAG. For double-labelling assays, viral particles were first stained with an anti-CD63 mouse monoclonal antibody, a rabbit anti-mouse bridging antibody and 10 nm PAG, and were fixed with 4% PFA before quenching and staining with an anti-TGN46 sheep serum, a rabbit anti-goat bridging antibody and 15 nm PAG. Grids were then floated on 3 drops of PBS and 5 more drops of dH2O before staining with a solution of 2% uranyl acetate for 30 s. Samples were examined with a transmission electron microscope (model JEOL 1011). Virions and DBs were classified according to the size: ≤200 nm in diameter spherical enveloped particles as virions and larger structures as DBs. For each staining non-specific binding was quantified omitting the primary antibody. At least 20 viral particles were analysed for the control samples, while a minimum of 100 virions and 80 DBs were examined and the number of associated gold particles was calculated. To prepare the figure, images were recorded onto electron image film (SO-163; Kodak). Negatives were scanned and the images were transferred to Adobe Photoshop and adjusted as above.

**Virus immunoprecipitation assays**

For virus immunoprecipitation we adapted a procedure described previously (Esser et al., 2001). RCMV288 virus samples diluted 1 x 10^6 infectious units ml^-1 in PBS containing 3% BSA were mixed with antibodies against CD63, TGN46, HCMV gH, HCMV gB and HSV-1 gD at a concentration of 10 μg ml^-1 in a final volume of 100 μl and incubated overnight at 4°C. Pansorbin cells (formalin-fixed *Staphylococcus aureus*; Calbiochem) were washed, blocked for 1 h at room temperature in PBS containing 3% BSA and added to the virus/antibody mixture. After incubation for 30 min at room temperature, Pansorbin cells-captured viruses were sedimented by centrifugation at 6000 r.p.m., for 1 min. Supernatants were added to BJ1 cells to assay for unprecipitated infectious virus by GFP expression and FACS analysis.

**Acknowledgements**

We thank colleagues who have supported this work, in particular, Dr C. Risco, Dr A. Pelchen-Matthews and Dr M. Marsh for advice and comments on the manuscript. Dr H. Browne, Dr W. Gibson, Dr T. Shenk, Dr M. Marsh, Dr C. Isacke, Dr S. Urbé and Dr K. von Figura for providing reagents, the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, USA, for the anti-CD-M6PR, 22d4 antibody developed by Dr Donald Messner, and Dr G. Wilkinson and Dr V. Emery for providing the recombinant HCMV AD169 expressing GFP and the HCMV Towne strain respectively. We specially thank for technical assistance to S. Gutierrez and J. Coll with the confocal microscope and FACS analysis, to F. Carrasco and Dr L. Tabera with the qPCR analysis, and for discussion and advice to Dr M. Rejas and Dr C. Risco with the immunolabelling EM Studies and to Dr T. Shenk and S. Liu with the purification of HCMV virions. A.F.-R. was supported by a Ramon y Cajal contract and by Grants BFU2006-14379/BMC from the MICINN of Spain and 200620M034 from the Comunidad Autonoma de Madrid/CSIC. V.C. was the recipient of a pre-doctoral fellowship from the MICINN of Spain.

**References**

Atalay, R., Zimmermann, A., Wagner, M., Borst, E., Benz, C., Messerle, M., and Hengel, H. (2002) Identification and expression of human cytomegalovirus transcription units coding for two distinct Fc gamma receptor homologs. *J Virol* 76: 8596–8608.

Benyesh-Melnick, M., Probstmeyer, F., McCombs, R., Brunschwig, J.P., and Vonka, V. (1966) Correlation between infectivity and physical virus particles in human cytomegalovirus. *J Bacteriol* 92: 1555–1561.

Blankenship, C.R., and Shenk, T. (2002) Mutant human cytomegalovirus lacking the immediate-early TRS1 coding region exhibits a late defect. *J Virol* 76: 12290–12299.

Brunetti, C.R., Dingwell, K.S., Wale, C., Graham, F.L., and Johnson, D.C. (1998) Herpes simplex virus gD and virions accumulate in endosomes by mannose 6-phosphate-dependent and – independent mechanisms. *J Virol* 72: 3330–3339.

Cranage, M.P., Kouzarides, T., Bankier, A.T., Satchwell, S., Weston, K., Tomlinson, P., *et al.* (1986) Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. *EMBO J* 5: 3057–3063.

Cranage, M.P., Smith, G.L., Bell, S.E., Hart, H., Brown, C., Bankier, A.T., *et al.* (1988) Identification and expression of a human cytomegalovirus glycoprotein with homology to the Epstein-Barr virus BXL2F2 product, varicella-zoster virus gplll, and herpes simplex virus type 1 glycoprotein H. *J Virol* 62: 1416–1422.

Crump, C.M., Hung, C.H., Thomas, L., Wan, L., and Thomas, G. (2003) Role of PACS-1 in trafficking of human cytomegalovirus glycoprotein B and virus production. *J Virol* 77: 11105–11113.
Das, S., and Pellett, P.E. (2007) Members of the HCMV US12 family of predicted heptamerspanning membrane proteins have unique intracellular distributions, including association with the cyttoplasmic virion assembly complex. *Virology* 361: 263–273.

Das, S., Vasanji, A., and Pellett, P.E. (2007) Three-dimensional structure of the human cytomegalovirus cyttoplasmic virion assembly complex includes a reoriented secretory apparatus. *J Virol* 81: 11861–11869.

Esser, M.T., Graham, D.R., Coren, L.V., Trubey, C.M., Bess, Isacke, C.M., Lindberg, R.A., and Hunter, T. (1989) Synthesis of a cytoplasmic tail of glycoprotein M (gpUL100) expresses trafficking signals required for human cytomegalovirus assembly and replication. *J Virol* 63: 4264–4276.

Futter, C.E., Felder, S., Schlessinger, J., Ullrich, A., and Gibson, W. (1983) Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. *Virology* 130: 118–133.

Gabel, C.A., Dubey, L., Steinberg, S.P., Sherman, D., Gershon, M.D., and Gershon, A.A. (1989) Varicella-zoster virus glycoprotein oligosaccharides are phosphorylated during posttranslational maturation. *J Virol* 63: 4264–4276.

Gabel, C.A., Dubey, L., Steinberg, S.P., Sherman, D., Gershon, M.D., and Gershon, A.A. (2002) Localization of HCMV US33 and US27 in Endocytic Compartment and Viral Membranes. *Traffic* 3: 218–232.

Gabel, C.A., Dubey, L., Steinberg, S.P., Sherman, D., Gershon, M.D., and Gershon, A.A. (1989) Varicella-zoster virus glycoprotein oligosaccharides are phosphorylated during posttranslational maturation. *J Virol* 63: 4264–4276.

Homman-Loudiyi, M., Hultenby, K., Britt, W., and 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: 3191–3203.

Irmiere, A., and Gibson, W. (1983) Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. *Virology* 130: 118–133.

Isacke, C.M., Lindberg, R.A., and Hunter, T. (1989) Synthesis of p36 and p35 is increased when U-937 cells differentiate in culture but expression is not inducible by glucocorticoids. *Mol Cell Biol* 9: 232–240.

Jones, T.R., and Lee, S.W. (2004) An acidic cluster of human cytomegalovirus UL99 tegument protein is required for trafficking and function. *J Virol* 78: 1486–1502.

Komada, M., and Kitamura, N. (1995) Growth factor-induced tyrosine phosphorylation of Hrs, a novel 115-kilodalton protein with a structurally conserved putative zinc finger domain. *Mol Cell Biol* 15: 6213–6221.
Sanchez, V., Greis, K.D., Sztul, E., and Britt, W.J. (2000) 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**: 975–986.

Seo, J.Y., and Britt, W.J. (2006) Sequence requirements for localization of human cytomegalovirus tegument protein pp28 to the virus assembly compartment and for assembly of infectious virus. *J Virol* **80**: 5611–5626.

Severi, B., Landini, M.P., and Govoni, E. (1988) Human cytomegalovirus morphogenesis: an ultrastructural study of the late cytoplasmic phases. *Arch Virol* **98**: 51–64.

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

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

Stackpole, C.W. (1969) Herpes-type virus of the frog renal adenocarcinoma. I. Virus development in tumor transplants maintained at low temperature. *J Virol* **4**: 75–93.

Talbot, P., and Almeida, J.D. (1977) Human cytomegalovirus: purification of enveloped virions and dense bodies. *J Gen Virol* **36**: 345–349.

Tooze, J., Hollinshead, M., Reis, B., Radsak, K., and Kern, H. (1993) Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *Eur J Cell Biol* **60**: 163–178.

Tugizov, S., Maidji, E., Xiao, J., and Pereira, L. (1999) An acidic cluster in the cytosolic domain of human cytomegalovirus glycoprotein B is a signal for endocytosis from the plasma membrane. *J Virol* **73**: 8677–8688.

Urbe, S., Mills, I.G., Stenmark, H., Kitamura, N., and Clague, M.J. (2000) Endosomal localization and receptor dynamics determine tyrosine phosphorylation of hepatocyte growth factor-regulated tyrosine kinase substrate. *Mol Cell Biol* **20**: 7685–7692.

Varnum, S.M., Streblow, D.N., Monroe, M.E., Smith, P., Auberry, K.J., Pasa-Tolic, L., *et al.* (2004) Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. *J Virol* **78**: 10960–10966.

Wan, L., Molloy, S.S., Thomas, L., Liu, G., Xiang, Y., Rybak, S.L., and Thomas, G. (1998) PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization. *Cell* **94**: 205–216.

Welsch, S., Habermann, A., Jager, S., Muller, B., Krijnse-Locker, J., and Krausslich, H.G. (2006) Ultrastructural Analysis of ESCRT Proteins Suggests a Role for Endosome-Associated Tubular-Vesicular Membranes in ESCRT Function. *Traffic* **7**: 1551–1566.

Yamashiro, D.J., Tycko, B., Fluss, S.R., and Maxfield, F.R. (1984) Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell* **37**: 789–800.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Subcellular localization of viral envelope proteins UL33 and glycoprotein H in HCMV-infected cells. BJ1 cells were infected with HCMV at an moi of 0.5. After 4 dpi, cells were fixed, permeabilized and stained with anti-HCMV glycoproteins UL33 (green) and gH (red) antibodies. Structures positive for both viral proteins are seen in yellow on the right, merged panel. Scale bar, 20 μm.

**Fig. S2.** Subcellular localization of early endosomal markers EEA1, HRS and annexin I in HCMV-infected cells. BJ1 cells were either mock infected (upper panels) or infected with a recombinant strain of HCMV expressing GFP at an moi of 0.5 (lower panels). After 4 dpi, cells were fixed, permeabilized and stained with anti-HCMV glycoproteins UL33 (green) and gH (red) antibodies. Structures positive for both viral proteins are seen in yellow on the right, merged panel. Scale bar, 20 μm.

**Table S1.** Details of quantitative real-time PCR assays carried out in this study.

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