Cytosolic herpes simplex virus capsids not only require binding inner tegument protein pUL36 but also pUL37 for active transport prior to secondary envelopment

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Summary

As the inner tegument proteins pUL36 and pUL37 of alphaherpesviruses may contribute to efficient intracellular transport of viral particles, we investigated their role in cytosolic capsid motility during assembly of herpes simplex virus type 1 (HSV1).

As reported previously for pUL36, untagged pUL37 and UL37GFP bound to cytosolic capsids before these acquired outer tegument and envelope proteins. Capsids tagged with CheVP26 analysed by live cell imaging were capable of directed long-distance cytoplasmic transport during the assembly of wild-type virions, while capsids of the HSV1-ΔUL37 or HSV1-ΔUL36 deletion mutants showed only random, undirected motion. The HSV1-ΔUL37 phenotype was restored when UL37GFP had been overexpressed prior to infection. Quantitative immunoelectron microscopy revealed that capsids of HSV1-ΔUL37 still recruited pUL36, whereas pUL37 did not colocalize with capsids of HSV1-ΔUL36. Nevertheless, the cytosolic capsids of neither mutant could undergo secondary envelopment. Our data suggest that pUL36 and pUL37 are important prior to their functions in linking the inner to the outer tegument. Efficient capsid transport to the organelle of secondary envelopment requires recruitment of pUL37 onto capsids, most likely via its interaction with pUL36, while capsid-associated pUL36 alone is insufficient.

Introduction

Herpes simplex virus type 1 (HSV1; human alphaherpesvirus 1) causes life-threatening neonatal and brain infections, serious eczema and eye infections, as well as usually healing infections of the skin and oral mucous membranes (Roizman et al., 2007; Steiner et al., 2007; Koelle and Corey, 2008). HSV1 virions consist of the DNA genome packaged into an icosahedral capsid that is encased by an amorphous asymmetric tegument layer and an envelope containing many viral membrane proteins (Grünewald et al., 2003; Mettenleiter et al., 2009). Preassembled nuclear herpesvirus capsids obtain a transient envelope by budding at the inner nuclear membrane that is lost upon subsequent fusion with the outer nuclear membrane or the endoplasmic reticulum membrane. As a result of this process, the capsids gain access to the cytosol and translocate to the organelle of secondary envelopment, most likely modified membranes of the trans-Golgi network (TGN), where they acquire their final envelope (reviewed in Mettenleiter et al., 2009; Johnson and Baines, 2011; Henaff et al., 2012).

The tegument proteins of herpesviruses are important during both virus assembly and cell entry, and have additional functions in viral and host gene regulation and countering the immune response (Roizman and Campadelli-Fiume, 2007; Kelly et al., 2009; Mettenleiter et al., 2009). The tegument connects the capsid with the envelope via many diverse and multivalent protein–protein interactions, and may contain up to 26 different proteins in HSV1 (Loret et al., 2008; Kelly et al., 2009; Radtke et al., 2010). Most tegument proteins could be classified as inner or outer tegument, based on their predominant association with capsids or the viral envelope and their membrane precursors, as well as their fractionation behaviour during detergent lysis of extracellular virions (Fuchs et al., 2004; Michael et al., 2006; Wolfstein et al., 2006; Radtke et al., 2010). The structural integrity...
and the formation of the tegument layer is independent of the capsids, as during assembly so-called L-particles consisting of a viral envelope and tegument but lacking capsids can be formed (McLauchlan and Rixon, 1992). Among the herpesviruses, inner tegument proteins are more conserved than the outer ones (Mettenleiter, 2004). While virions seem to have a rather fixed and low number of the inner tegument pUL36 and pUL37, the copy number of, for example, the outer tegument VP22 can be increased by higher VP22 expression levels (Heine et al., 1974; McLauchlan, 1997; Loret et al., 2008; Radtke et al., 2010). When non-essential outer tegument proteins are deleted, the void is filled by packaging host proteins as well as other tegument proteins in higher abundance (del Rio et al., 2005; Michael et al., 2006).

The HSV1 open reading frame UL36 encodes an inner tegument protein of 3164 amino acid residues (aa) that is conserved in alpha-, beta- and gammaherpesvirinae; the corresponding proteins of other human herpesviruses are called p22 in varicella zoster virus, pUL48 in human cytomegalovirus (HCMV), pUL31 in the human herpesvirus 6 and 7, BPLF1 in Epstein–Barr virus and ORF63 in Kaposi’s sarcoma-associated herpesvirus (KSHV; Mocarski, 2007; Kelly et al., 2009). pUL36 of PrV, an alphaherpesvirus of swine, and pUL48 of HCMV are essential for viral replication, while ORF64 of KSHV is critical during primary infection and the reactivation from latency (Gregory et al., 2011). Nevertheless, deleting UL37 of PrV impairs secondary envelopment as well as nuclear targeting of parental capsids, while pUL47 of HCMV has important functions early in infection (Bechtel and Shenk, 2002; Krautwald et al., 2009).

HSV1-pUL37 harbours a nuclear export signal between aa 263 and 272, a pUL36 binding domain between aa 568 and 1123, and a TRAF6 binding domain between aa 1099 and 1104; furthermore, a pUL37 N-terminal domain of 300 aa can self-associate with the pUL37 C-terminal half of aa 568 to 1123 (Watanabe et al., 2000; Liu et al., 2008; Bucks et al., 2011). Yeast-two-hybrid screens have identified further interactions with the capsid proteins VP26 and VP19c as well as the abundant outer tegument protein VP11/12 (Vittone et al., 2005; Lee et al., 2008; Fossum et al., 2009), and with the host protein TAOK3 (Kelly et al., 2012). HSV1-pUL37 also seems to be recruited to capsids prior to secondary envelopment (Pasdeloup et al., 2010). HSV1-pUL37, HSV1-GFPUL37, PrV-pUL37 and PrV-UL37GFP remain, like pUL36, associated with parental capsids during targeting to the nuclear pores (Graznow et al., 2005; Lluxton et al., 2005; Copeland et al., 2009; Möhl et al., 2009; Antinone and Smith, 2010; Aggarwal et al., 2012; Schipke et al., 2012). The amount of capsid-associated HSV1-GFPUL37 seems to be reduced or its epitopes modified during cytoplasmic passage towards the nuclear pores, and HSV1-pUL37 is not required for capsid docking at the nuclear pore and genome release into the nucleoplasm (Roberts et al., 2009; Aggarwal et al., 2012). HSV1 and PrV mutants lacking pUL37 accumulate cytosolic progeny capsids; however, while PrV is only impaired in secondary envelopment, HSV does not form any virions in the absence of pUL37 (Desai et al., 2001; Fuchs et al., 2004; Leege et al., 2009; Roberts et al., 2009).

The HSV1 inner tegument proteins pUL36 and pUL37 can interact directly with each other, and both require each other for their mutual incorporation into L-particles (Klupp et al., 2002; Fuchs et al., 2004; Vittone et al., 2005; Lee et al., 2008; Roberts et al., 2009; Bucks et al., 2011).
Such an interaction has also been shown for the respective homologues of PrV, HCMV and KSHV (Bechtel and Shenk, 2002; Klupp et al., 2002; Fuchs et al., 2004; Rozen et al., 2008). The targeting of HSV1-pUL37 to the membranes of secondary envelopment requires HSV1-pUL36 but not capsids (Desai et al., 2008). This might be explained by the interactions of pUL37 with VP11/12, and of pUL36 with VP16 (Vittone et al., 2005; Lee et al., 2008; Svobodova et al., 2012) as these outer tegument proteins accumulate at the secondary budding site.

The microtubule motors cytoplasmic dynein and kinesins catalyse efficient intracellular transport of host and viral structures (Döhner et al., 2005; Greber and Way, 2006; Radtke et al., 2006; Dodding and Way, 2011). Inner tegument proteins such as pUL36 and pUL37 are likely candidates to recruit microtubule motor proteins that could catalyse efficient transport of intracellular HSV1 structures, both during assembly and cell entry (Wolfstein et al., 2006; Antinone and Smith, 2010; Radtke et al., 2010; Dodding and Way, 2011). To analyse cytoplasmic HSV1 capsid transport, we therefore have generated viruses with capsids tagged at the small capsid protein VP26 with monomeric Cherry either in combination with a monomeric GFP tag on pUL37, or with a deletion of UL36 or UL37 respectively. Using these HSV1 strains, we show that pUL36 and pUL37 were required for directed transport of cytosolic capsids to the site of secondary envelopment. Immunofluorescence and quantitative immunoelectron microscopy demonstrated that an early recruitment of pUL37 to cytosolic capsids required capsid-associated pUL36, and that capsid-associated pUL37 was essential for efficient capsid transport during assembly and egress.

**Results**

*Generation of HSV1(17*)-Lox mutants*

To analyse the roles of pUL36 and pUL37, we constructed HSV1 strains tagged at VP26, and then either added another tag to pUL37 or deleted UL36 or UL37 entirely (Fig. 1A). As our first bacterial artificial chromosome (BAC) backbone pHSV1(17*)blueLox (Nagel et al., 2008; 2012; Jirmo et al., 2009; Zhang et al., 2009; Schipke et al., 2012) harboured an accidental point mutation in the gene UL44 that prevented expression of the non-essential glycoprotein C, we generated the new BAC pHSV1(17*)Lox. In HSV1(17*)Lox and all strains derived from it, gC and the thymidine kinase have been repaired and the reporter gene for β-galactosidase removed (A. Pohlmann, N. Müther, K. Döhner and B. Sodeik, to be published elsewhere).

To track HSV1 capsids in living cells, we replaced the first seven amino acids of VP26 with an autofluorescent protein as reported previously (Nagel et al., 2008; 2012). As tags with a high propensity to dimerize impair HSV1 nuclear egress and induce nuclear protein aggregates (Nagel et al., 2008; 2012), we used here monomeric Cherry (Shaner et al., 2004). Furthermore, we inserted either monomeric GFP or monomeric GFP with a stop codon (Zacharias et al., 2002) two codons upstream the stop codon of UL37 as this site can tolerate insertions (McLauchlan, 1997; Desai et al., 2008; Roberts et al., 2009). Furthermore, we deleted UL36 or UL37 in HSV1(17*)Lox or the single-colour strain HSV1(17*)Lox-CheVP26 (Fig. 1A). Adding mCherry to VP26 or mGFP to UL37 resulted in NotI fragments changing from 6.7 kb to 7.3 kb or from 4.4 kb to 5.1 kb respectively (Fig. 1B). Deleting UL37 removed fragments of 2.5, 10 and 4.4 HSV1(17*)Lox only) or 5.1 kb [HSV1(17*)Lox-CheVP26 only], respectively, and generated one of 14 kb that was slightly larger in pHSV1(17*)Lox-CheVP26-ΔUL37 than in pHsv1(17*)Lox-ΔUL37, because of the remaining 194 bp of the GFP sequence (Fig. 1B, left panel). Deletion of UL36 removed fragments of 7.3, 4.4, 2.4, 1.1 and 1 and generated one of 6.7 kb (Fig. 1B, right panel). Further restriction digest analyses of the new HSV1(17*)Lox strains with the five enzymes Ascl, BamHI, EcoRI, EcoRV and XhoI also resulted in the expected fragment patterns indicating that the mutagenesis procedures had not introduced any major unintended genomic rearrangements (not shown).

Next, we investigated single-step growth curves of HSV1(17*)Lox, -CheVP26, -UL37GFP, -CheVP26-UL37GFP, -ΔUL37 and -CheVP26-ΔUL37. In Vero cells, HSV1(17*)Lox with CheVP26, UL37GFP or both replicated with identical kinetics as their parental strain (Fig. 2Ai and Aii), whereas HSV1-ΔUL37 and -CheVP26-ΔUL37 were not amplified (Fig. 2Aiii and Aiv). In the UL37-complementing RS-80C02 cells, HSV1(17*)Lox-ΔUL37 reached similar titres as HSV1(17*)Lox in Vero, but was slightly impaired when compared to HSV1(17*)Lox replication in RS-80C02 cells (Fig. 2Aiii and Aiv). HSV1(17*)Lox-CheVP26-ΔUL37 reached only a 10-fold lower titre than HSV1(17*)Lox-ΔUL37 in RS-80C02 cells (Fig. 2Aiii and Aiv).

To analyse the expression kinetics of UL37GFP, Vero cells were infected with HSV1(17*)Lox-CheVP26, -CheVP26-UL37GFP, or for comparison with the published strain HSV1(17*)-vUL37-GFP (Saksena et al., 2006). The major capsid protein VP5 served to monitor viral infection and the host protein dynamitin as a loading control (Fig. 2B). HSV1(17*)Lox-CheVP26 initially progressed a bit faster than -CheVP26-UL37GFP or HSV1(17*)-vUL37-GFP. Expression of UL37GFP was detected at 147 kDa as early as 6 h post infection (PI); the slightly higher molecular weight of UL37GFP expressed by HSV1(17*)-vUL37-GFP was due to a linker between pUL37 and GFP. As the infection progressed, the
anti-GFP antibody detected a second band around 120 kDa; a corresponding smaller band has also been described for untagged pUL37 (Shelton et al., 1990; Desai et al., 2001). CheVP26 was expressed as early as 6 h PI with HSV1(17')Lox-CheVP26, and at 8 h with HSV1(17')Lox-CheVP26-UL37GFP, similarly as VP26 by HSV1(17')-vUL37-GFP. The expression of the neighbouring genes UL36 coding for pUL36, and UL38 coding for VP19c, another major capsid protein, was not influenced by attaching GFP to pUL37 or deleting UL37 (Fig. 2C).

These results confirm that HSV1-pUL37 is an essential protein as reported (Desai et al., 2001; Leege et al., 2009; Roberts et al., 2009), as HSV1-ΔUL37 strains were amplified only in UL37 complementing cells. Attaching GFP to the C-terminus of pUL37 and the combination of tagged pUL37 with a tagged VP26 in the dual-colour strain did not result in any growth defect. Thus, these new HSV1(17')Lox strains provide good tools to further characterize potential functions of pUL37 during the viral life cycle.

HSV1-pUL37 associates with cytosolic capsids prior to secondary envelopment

To analyse the subcellular localization of pUL37, we infected Vero cells and performed immunofluorescence microscopy. Capsids of HSV1(17')Lox, detected with an antibody against VP5, were mainly in the nucleus, but many had already left the nucleus for the cytoplasm at 10 h PI (Fig. 3A1) as reported previously (Nagel et al., 2008; Rode et al., 2011; Schipke et al., 2012). pUL37 (Fig. 3Aii) colocalized with almost all cytoplasmic but not nuclear capsids (yellow in Fig. 3Aiii). There was a weak, diffuse nuclear signal in cells infected with HSV1(17')-Lox-CheVP26-ΔUL37 and HSV1(17')-Lox-CheVP26-ΔUL36 with NotI. The expected fragment sizes were calculated based on the published sequences of HSV1(17') (GenBank accession no. NC_001806) and our own sequencing of the pHSV1(17')Lox BAC. DNA fragments and marker bands are indicated in kb. Fragments had shifted due to the insertion of mGFP (G) or mCherry (C), disappeared due to gene deletion (open arrowheads), or were generated (filled arrowheads).
HSV1(17+)Lox-ΔUL37, which was considered background (Fig. 3Bii). After infection with HSV1(17+)Lox-UL37GFP, UL37GFP (Fig. 3Cii) also colocalized with almost all cytoplasmic capsids but was not enriched on nuclear ones (yellow in Fig. 3Bii). Similar to pUL37, UL37GFP was also located in the cytoplasm independent of capsids (green in Fig. 3Cii). As the infection progressed, the cytoplasmic expression of both pUL37 and UL37GFP increased continuously (data not shown).

To distinguish cytosolic capsids from capsids in the process of or after secondary envelopment, we infected Vero cells with the dual-colour strain HSV1(17+)Lox-CheVP26-UL37GFP, and labelled them with antibodies against p230, a host marker protein for TGN membranes (Gleeson et al., 1996), or the viral glycoprotein D. At 8 h PI, UL37GFP colocalized with most cytoplasmic (yellow and white in Fig. 3Div) but not with nuclear capsids (red in Fig. 3Div). Membranes containing the TGN marker p230 were enriched in cytoplasmic regions in close proximity to the nucleus (Fig. 3Diii). While most capsids did not colocalize with p230 (yellow in Fig. 3Div), there were also some in the TGN region at this early time of infection (white in Fig. 3Div). The latter were most likely in the process of secondary envelopment. An infection with HSV1(17+)Lox-CheVP26-ΔUL37 showed a clear reduction in colocalization of p230 with cytoplasmic capsids (data not shown). As fully assembled virions do not contain host TGN proteins (Loret et al., 2008; Henaff et al., 2012), we also compared the subcellular localization of cytoplasmic capsids to that of the viral membrane protein gD. There were several cytoplasmic capsids colocalizing with both UL37GFP and gD (white in Fig. 3Eiv) that most likely represented capsids in the process of secondary envelopment or complete virions. However, there were also many cytosolic capsids that had already recruited UL37GFP but not yet acquired an envelope (yellow in Fig. 3Eiv). Furthermore, UL37GFP colocalized to a large extent with gD (turquoise/blue in Fig. 3Eiv); however, the labelling for gD (Fig. 3Eiii) appeared stronger than the UL37GFP signal (Fig. 3Eiii). An infection with HSV1(17+)Lox-CheVP26-ΔUL37 also showed a clear reduction in colocalization of cytoplasmic capsids with gD (data not shown).

These experiments demonstrated that the subcellular localization of UL37GFP was very similar if not identical to that of the untagged pUL37. Like pUL36 (Schipke et al., 2012), both authentic pUL37 and UL37GFP were recruited to cytosolic capsids prior to secondary envelopment. Furthermore, the efficiency of nuclear egress was apparently not impaired by tagging pUL37 with GFP or by deleting it.

To analyse the subcellular localization of incoming pUL37, Vero cells were inoculated in the presence of cycloheximide (Fig. 4). After 15 min, the parental capsids were randomly distributed over the entire cytoplasm, and UL37GFP colocalized with capsids (Fig. 4Av, yellow) but not with gD (Fig. 4Av, green). Over time, more capsids arrived at the nucleus, and many of them colocalized with UL37GFP (Fig. 4B). These data indicate that during internalization into cells, pUL37 and the viral envelope separated and that pUL37 remained attached to a large extent on incoming HSV1 capsids until their arrival at the nuclear pores.

**Cytosolic capsids of HSV1(17+)Lox-ΔUL37 and -ΔUL37 do not acquire an envelope**

As there have been conflicting reports regarding the nuclear capsid egress of HSV1-ΔUL36 and HSV1-ΔUL37 (Desai et al., 2001; Leege et al., 2009; Roberts et al., 2009), we compared the parental strain HSV1(17+)Lox with our new deletion mutants HSV1(17+)Lox-ΔUL36 (Fig. 5) and HSV1(17+)Lox-ΔUL37 (Fig. 6) by electron microscopy. For these experiments we chose early time points, namely 10 and 14 h PI, to avoid a build-up of the HSV1 cytopathic effects. Cells infected with HSV1(17+)Lox formed (i) cytosolic capsids not associated with membranes, (ii) capsids in the process of secondary envelopment, (iii) completely enveloped virions surrounded by an electron dense layer illustrating the tegument (Fig. 5A), as well as (iv) extracellular virions (Fig. 5B).
Fig. 3. HSV1-UL37GFP behaves like pUL37 and binds to cytosolic capsids prior to secondary envelopment. Vero cells were infected with HSV1(17')Lox (A), HSV1(17')Lox-ΔUL37 (B) or HSV1(17')Lox-UL37GFP (C) at an MOI of 10 PFU cell$^{-1}$ and fixed at 10 h PI with PHEMO fixative, or Vero cells were infected with HSV1(17')Lox-CheVP26-UL37GFP and fixed at 8 h PI with PHEMO fixative (D and E). The specimens were labelled with antibodies directed against VP5 (mAb, 5C10), pUL37 (pAb against full-length GST-tagged pUL37), p230 (mAb, p230) or gD (mAb, DL-6) and analysed by fluorescence confocal microscopy. The DNA was stained with TO-PRO-3 (A–C) or Hoechst dye (D and E), and the positions of the nuclei are indicated by dashed lines. Scale bars represent 5 μm (A–C) or 2 μm (D and E).
In cells infected with HSV1(17)+Lox-D UL36 (Fig. 5C–F), there were many cytosolic capsids, but they were never attached to membranes (Fig. 5C and F). Furthermore, the cytoplasm did not contain any wrapping intermediates that may be indicative of secondary envelopment or complete virions, and there were no extracellular virions associated with the plasma membrane. Thus, HSV1(17)+Lox-D UL36 did not undergo secondary envelopment, as reported before for other strains lacking large parts of UL36 or UL36 entirely (Desai, 2000; Roberts et al., 2009; Schipke et al., 2012). As we detected cytosolic capsids as well as events of primary envelopment (Fig. 5C and E, black arrows), we presume that nuclear egress was not severely impaired in the absence of pUL36. Even if such cytosolic capsids were located close to cytoplasmic membranes they were never engulfed by them, although there were many signs of L-particle formation in close proximity (Fig. 5F). Instead of extracellular virions, there were many L-particles attached to the extracellular leaflet of the plasma membrane (Fig. 5D).

The phenotype of HSV1(17)+Lox-ΔUL36 was very similar to that of HSV1(17)+Lox-ΔUL36. Cytosplasmic capsids did not attach to membranes, and there were no wrapping intermediates in the process of secondary envelopment, or any complete virions in the cytoplasm. In contrast to HSV1(17)+Lox-ΔUL36, here cytosolic capsids were frequently detected in larger conglomerates (Fig. 6A); however, there were no apparent nuclear capsid accumulations. Nuclear egress did not seem to be impaired, as there were not only many cytosolic capsids (Fig. 6A, C and D) but also several instances of primary envelopment (Fig. 6C). These results agree with Roberts et al. (2009), who analysed a complete deletion of UL37 like us, but not with Desai et al. (2001), whose mutant HSV1 strain still expresses an N-terminal fragment of 86 aa. As reported before (Desai et al., 2001; Roberts et al., 2009), there were many extracellular L-particles but no virions bound to the plasma membrane (Fig. 6B). Taken together, these experiments show that pUL36 and pUL37 are essential for secondary envelopment but dispensable for nuclear egress.

HSV1-pUL36 and -pUL37 are required for long-distance transport of cytosolic progeny capsids

The lack of association with membranes for capsids of HSV1-ΔUL36 or -ΔUL37 could be either due to an inability to link the capsid-associated inner tegument with the membrane, or to an inhibition of capsid transport resulting in a reduced meeting frequency with wrapping membranes, or a combination of both. We therefore infected Vero cells with HSV1(17)+Lox-CheVP26, -CheVP26-ΔUL36 or -CheVP26-ΔUL37, and recorded capsid transport in living cells. At 6 h PI, all virus strains showed a similar progression in infection, when the nuclei appeared filled with capsids, and there were several capsids that had already egressed into the cytoplasm. From 8 h PI on, there were sufficient amounts of cytosolic capsids while the cell morphology was only slightly altered. At later time points, more and more cells had rounded up, but not detached yet, and in HSV1(17)+Lox-CheVP26-ΔUL37 capsids accumulated in larger cytoplasmic build-ups as observed with conventional electron microscopy (Fig. 6). Therefore, we performed live cell imaging from 8 to 10 h PI, and recorded time-lapse of the Cherry fluorescence with a temporal resolution of five images per second.

Subsequently, single motile particles were tracked and their transport characteristics were analysed. Initial inspection of the movies revealed that about 10–30% cytosolic capsids
HSV1(17')Lox-ChexVP26 capsids moved very dynamically over long distances (Fig. 7A, Movie S1). They had a strong directionality with only a few changes in transport direction. There was long-range capsid transport towards both the cell periphery and the nucleus. However, there were also many stationary capsids (Fig. 7A, e.g. track no. 9) with little movement and no directionality at all during the recording time (48 s) of a movie. In contrast, the cytoplasmic capsids of HSV1(17')Lox-ChexVP26-DUL36 (Fig. 7B, Movie S2) or -CheVP26-DUL37 (Fig. 7C, Movie S3) showed only undirected oscillatory movements but no long-distance transport. The transport defect of HSV1(17')Lox-ChexVP26-DUL37 was rescued in cells that had been transfected with a plasmid encoding UL37GFP prior to infection with HSV1(17')-CheVP26-DUL37 (Fig. 7D); UL37GFP restored active cytoplasmic capsid transport over long distances (Fig. 7Dii, Movie S4). However, high expression levels (Fig. 7Dii) were required to rescue transport efficiently, indicating that the phenotype of HSV1(17')-CheVP26-DUL37 was solely due to the lack of pUL37.

We measured the distance that individual capsids moved in one transport step between two adjacent frames of a movie (0.2 s), and determined various parameters of the tracking profiles for the four experimental conditions. The mean square displacement (MSD) is defined as the square of the travelled distance from the starting point of

Fig. 5. HSV1 capsids lacking pUL36 do not undergo secondary envelopment. Vero cells were infected with an HSV1(17')Lox (A and B) or HSV1(17')Lox-DUL36 (C–F) at an MOI of 10 PFU cell−1, fixed at 14 h PI and analysed with conventional electron microscopy. Black arrows indicate primary virions, black arrowheads cytosolic capsids not associated with membranes, white arrowheads wrapping intermediates of secondary envelopment, and white arrows virions after complete secondary envelopment. Scale bars: 500 nm (C), 200 nm (A, B, D–F).
the track, which is calculated and plotted against the time. The slope of such a curve equals the MSD exponent and defines whether a particle underwent active transport (>1) or was moved by diffusion (≤1; Saxton, 1994; Ewers et al., 2005; Vrljic et al., 2007). The HSV1(17\textsuperscript{+})Lox-CheVP26 capsids had been actively transported as indicated by an average MSD exponent of around 2 (Fig. 8A, WT). In contrast, the MSD exponents of -CheVP26-ΔUL36 or -CheVP26-ΔUL37 averaged around 1, a typical value for diffusion (Fig. 8A). We defined the relative displacement exponent by calculating a MSD exponent in respect to the centre of the nucleus as a reference point, which then determines the overall transport direction. The broad distribution of the relative displacement exponent demonstrated that HSV1(17\textsuperscript{+})Lox-CheVP26 capsids had a net transport in both directions (Fig. 8B) towards the nucleus (expressed in negative values) and towards the cell periphery (expressed in positive values). As capsids of both HSV1(17\textsuperscript{+})Lox-CheVP26-ΔUL36 and -ΔUL37 had not been transported in either direction, their relative displacement exponents were close to zero. The mean velocity is the average step velocity derived from all steps of one track, but excluding the steps with zero velocity. The cytoplastic capsids of HSV1(17\textsuperscript{+})Lox-CheVP26 had a significantly higher mean velocity when compared to -CheVP26-ΔUL36 or to -CheVP26-ΔUL37 (Fig. 8C). Finally, the relative displacement angle describes changes in direction between two motion steps; a value of zero has been defined for a capsid with no change in transport direction. Both deletion mutants showed much larger changes in transport direction than the parental virus (Fig. 8D) that is typical for diffusion. Overexpression of UL37GFP prior to infection with HSV1(17\textsuperscript{+})Lox-CheVP26-ΔUL37 restored all transport parameters almost to the levels of HSV1(17\textsuperscript{+})Lox-CheVP26. Thus, in summary, cytosolic HSV1 capsids lacking either pUL36 or pUL37 did not show any active transport after nuclear egress.

**Fig. 6.** HSV1 capsids lacking pUL37 do not undergo secondary envelopment. Vero cells were infected with HSV1(17\textsuperscript{+})Lox-ΔUL37 at an MOI of 10 PFU cell\textsuperscript{-1}, fixed at 14 h PI and analysed with conventional electron microscopy. The black arrow indicates a primary virion and the black arrowheads cytosolic capsids not associated with membranes. Scale bars: 500 nm (A), 200 nm (B–D).

**Protein composition of HSV1(17\textsuperscript{+})Lox-ΔUL36 and HSV1(17\textsuperscript{+})Lox-ΔUL37 capsids**

As HSV1-ΔUL36 and -ΔUL37 were impaired in cytosolic transport, we asked which proteins these capsids exposed
towards the cytosol. Immunofluorescence microscopy experiments showed that pUL37 was not recruited to cytosolic capsids after infection with HSV1(17+Lox-DUL36) (Fig. 9B and C). In contrast, irrespective of the presence or absence of pUL37, pUL36 colocalized with cytosolic capsids of both HSV1(17+Lox and HSV1(17+ΔUL36) (Fig. 9D–F). As expected, pUL36 or pUL37 were not detected after infection with HSV1(17+Lox-ΔUL36 or HSV1(17+Lox-ΔUL37 respectively.

To identify cytosolic capsids unambiguously, we also analysed the deletion mutants by immunoelectron microscopy using thawed cryosections (Griffiths, 1993). This technique uses a light fixation, no dehydration, and no heavy metals before immunolabelling, and therefore preserves antigens and membranes much better than a conventional plastic embedding. However, the samples also appear in a different contrast that is solely based on uranyl acetate but omits osmium and lead. Therefore, we first characterized the appearance of cytoplasmic capsids in cells infected with HSV1(17+Lox with an antibody against the small capsid protein VP26 that labelled characteristic symmetric structures of dark to moderate contrast of an average diameter of 120 nm (Fig. 10A). As they looked strikingly similar to incoming capsids analysed by this method (Sodeik et al., 1997), and as uninfected cells did not contain such structures, the structures represented cytoplasmic capsids in various stages of assembly: namely cytosolic capsids, wrapping intermediates indicative of secondary envelopment, and fully assembled cytoplasmic virions (Fig. 10A).

Such capsids were also labelled by antibodies directed against the inner tegument proteins pUL36 (Fig. 10B) or pUL37 (Fig. 11A). A quantification of these experiments showed that the labelling intensities were significantly higher for pUL36 or pUL37 on HSV1(17+Lox when compared to the corresponding deletion mutants lacking either pUL36 (Fig. 11C) or pUL37 (Fig. 11D). We next analysed capsids of HSV1(17+ΔUL37 for the presence of pUL36 (Fig. 10C) and capsids of HSV1(17+ΔUL36 for the presence of pUL37 (Fig. 11B). While the capsids of HSV1(17+ΔUL37 showed the same labelling intensity for pUL36 as capsids of the parental strain (Fig. 11C), the capsids of HSV1(17+ΔUL36 had the same very low labelling intensity as the capsids of HSV1(17+ΔUL37 (Fig. 11D). The immunoelectron and immunofluorescence microscopy experiments demonstrated that the deletion mutants HSV1(17+ΔUL36 or ΔUL37 generated two different types of cytosolic capsids: the capsids lacked both inner tegument proteins pUL36 and pUL37 in the absence of pUL36, while in the absence of pUL37, pUL36 was still recruited to cytosolic capsids. These data suggest that after nuclear egress cytosolic capsids first recruited pUL36, which then allowed pUL37 binding.

**Discussion**

Neurotropic alphaherpesviruses undergo long axonal transport during cell entry, assembly and egress, and intracellular microtubule transport of viral particles is essential for their pathogenesis (Enquist et al., 1998; Döhner et al., 2005; Diefenbach et al., 2008; Salinas et al., 2010; Smith, 2012). The inner tegument proteins pUL36 and pUL37 might be responsible for recruiting the microtubule motors dynein, kinesin-1 and kinesin-2 as...
Fig. 8. HSV1-pUL36 and HSV1-pUL37 are required for active transport of cytosolic capsids. The tracking profiles of HSV1(17')Lox-CheVP26 (WT), HSV1(17')Lox-CheVP26-ΔUL36, HSV1(17')Lox-CheVP26-ΔUL37 or HSV1(17')Lox-CheVP26-ΔUL37 after expression of UL37GFP (ΔUL37 + pUL37GFP) were analysed. Sixty-five tracks of parental capsids (WT), 48 of ΔUL36, 48 of ΔUL37, 36 of ΔUL37 overexpressing UL37GFP were analysed. Each dot represents one track. The mean values are indicated by horizontal lines.

A. The mean square displacement exponent.
B. Relative displacement exponent using the centre of the nucleus as a reference point. Negative values indicate motion towards the nucleus, positive values to the cell periphery.
C. The mean step velocity.
D. The relative displacement angle expressed as radian (rad).

The statistical significance calculated with unpaired t-test is indicated as *** < 0.0001, ** < 0.001, * < 0.01, ns > 0.01 (not significant).
Fig. 9. HSV1-pUL37 requires pUL36 to bind to cytosolic capsids but not vice versa. Vero cells were infected with HSV1(17')Lox (A and D), HSV1(17')Lox-ΔUL36 (B and C) or HSV1(17')Lox-ΔUL37 (E and F) at an MOI of 10 PFU cell$^{-1}$, fixed at 10 h PI with 3% PFA and permeabilized with TX-100. The specimens were labelled with antibodies directed against VP5 (mAb, 5C10), pUL36 (pAb, #147) or pUL37 (pAb against full-length GST-tagged pUL37) and analysed by fluorescence confocal microscopy. The position of the nuclei was determined using the DNA stain TO-PRO-3, and the positions of the nuclei were indicated by dashed lines. Scale bar represents 5 μm.
well as the dynein cofactor dynactin onto cytosolic capsids (Wolfstein et al., 2006; Pasdeloup et al., 2010; Radtke et al., 2010; Dodding and Way, 2011). Furthermore, such inner tegument proteins may be bound to the cytosolic surface of transport vesicles that may contain viral glycoproteins with associated outer tegument proteins on their limiting membranes. These inner tegument proteins exposed to the cytosol may also recruit microtubule motors to these transport vesicles containing fully assembled alphaherpesviruses in their lumen (Shanda and Wilson, 2008; Smith, 2012). We therefore compared the roles of HSV1-pUL36 or HSV1-pUL37 for active, microtubule-mediated intracellular transport during assembly and egress.

Characterization of HSV1-UL37GFP, HSV1-ΔUL36 and HSV1-ΔUL37 strains

As reported for other HSV1 strains (McLauchlan et al., 1994; Desai et al., 2008; Roberts et al., 2009), tagging pUL37 with GFP did not delay or impair viral replication, and the subcellular localization of UL37GFP was very similar to that of pUL37. Furthermore, HSV1-pUL37 and UL37GFP were distributed throughout the entire cytoplasm. This non-capsid localization most likely indicates an association of pUL37 with membranes that also harbour viral membrane and associated outer tegument proteins (Vittone et al., 2005; Lee et al., 2008; Fossum et al., 2009; Ko et al., 2010), and therefore could provide binding sites for inner tegument proteins.

The phenotypes of HSV1(17+)-Lox-ΔUL36 and HSV1(17+)-Lox-CheVP26-ΔUL36 were similar to that of HSV1(17+)-blueLox-ΔUL36 (Schipke et al., 2012). Surprisingly, HSV1(17+)-Lox-CheVP26-ΔUL37 produced about 10 times less infectious virions than HSV1(17+)-Lox-ΔUL37 in the UL37 complementing cell line RS-80C02. This result may indicate that this complementing cell line did not provide the same amount, or the same form of pUL37 as the parental virus. Thus, while the sole deletion of UL37 could be compensated well, deleting UL37 in combination...
with the fluorescent tag on VP26 apparently resulted in a synthetic phenotype as suggested by Nagel et al. (2012). It is formally also possible that HSV1(17)+Lox-CheVP26-DUL37 had acquired additional debilitating or HSV1(17)+Lox-DUL37 further enabling mutations. However, either scenario seems rather unlikely as we performed all experiments with viral stocks of passage 2 after transfection of the BACs into the complementing cell line, yielding too few generations for a DNA virus to accumulate compensatory mutations. For both authentic pUL37 (McLauchlan, 1997; Desai et al., 2008) and UL37GFP, a full-length and a truncated form were synthesized, but it is unclear whether this was due to degradation or a second start codon. This truncation occurred from the N-terminus, as both forms were detected with an antibody directed against C-terminal GFP.

**Function of HSV1-pUL36 and -pUL37 in cytosolic capsid transport**

As neither pUL36 (Schipke et al., 2012), pUL37 or UL37GFP were associated with nuclear capsids by immunofluorescence microscopy, and as HSV1-ΔUL36 and HSV1-ΔUL37 did not show any prominent accumulation of nuclear capsids, we consider it unlikely that either pUL36 or pUL37 directly affect nuclear capsid egress. Furthermore, inconsistent with Luxton et al. (2006) and Desai et al. (2001) but consistent with Desai (2000), Leege et al. (2009) and Roberts et al. (2009), our electron microscopy analysis did not reveal any indication for capsid aggregation in the nucleus in the absence of pUL36 or pUL37. However, capsids of HSV1-ΔUL36 or HSV1-ΔUL37 did not associate with cytoplasmic
membranes, and there was no secondary envelopment, as reported previously (Desai, 2000; Desai et al., 2001; Leege et al., 2009; Roberts et al., 2009). Interestingly, capsids of PrV-ΔUL37 are also impaired in secondary envelopment and virion morphogenesis, although virus release is not completely blocked (Leege et al., 2009). Thus, HSV1-pUL37 is more crucial than PrV-pUL37, whose functions might be compensated by other PrV proteins.

We performed time-lapse live cell imaging of cells infected with HSV1-CheVP26 mutants lacking either pUL36 or pUL37 at early times PI when there were mostly single cytosolic capsids and few signs of cytoplasmic capsid accumulation and included only single particles in our tracking analysis. These cytosolic capsids of HSV1-CheVP26-ΔUL36 or -ΔUL37 could undergo only random and undirected movements but no long-distance transport. In PrV, the absence of pUL36 results in a complete loss of cytosolic capsid transport similarly to nocodazole treatment, while the deletion of UL37 only reduces the overall transport kinetics, although directed capsid transport still occurs (Luxton et al., 2006). During early infection stages of PrV, pUL37 is required for efficient nuclear targeting (Krautwald et al., 2009) also pointing to a role of PrV-pUL37 for cytosolic capsid transport.

**Tegument formation in the absence of HSV1-pUL36 or HSV1-pUL37**

The cytosolic capsids of HSV1-ΔUL36 lacked both pUL36 and pUL37, while capsids of HSV1-ΔUL37 obviously lacked pUL37, but still had recruited pUL36. Earlier studies on HSV1-ΔUL37 (Pasdeloup et al., 2010) and PrV-ΔUL37 (Klupp et al., 2002) also reported the formation of large cytosolic capsid conglomerations very late in infection that contain pUL36. Thus, HSV1-pUL36 binding to cytosolic capsids did not require pUL37, but pUL36 alone does not suffice to mediate secondary envelopment and virion assembly, although it can interact with the major tegument protein VP16 (Vittone et al., 2005; Roberts et al., 2009; Ko et al., 2010). Similarly, the potential association of HSV1-pUL37 with the capsid proteins VP26 and VP19c was not strong enough to recruit pUL37 onto capsids in the absence of pUL36. Instead, cytoplasmic HSV1-pUL37 might have associated with membranes that contain HSV1 glycoproteins and associated outer tegument proteins such as the pUL37 binding partner VP11/12 (Lee et al., 2008). However, besides binding to capsids, pUL36 also promotes the association of pUL37 with membranes that may recruit pUL36 via its interaction with VP16 (Desai et al., 2008). Strikingly, both pUL36 and pUL37 require the other inner tegument protein for their incorporation into L-particles, and thus for their stable association with the other tegument proteins in the absence of any potentially stabilizing interactions between the capsid and the inner tegument layer (Roberts et al., 2009). These results do not address whether pUL36 and pUL37 might form a cytosolic complex prior to their binding to cytosolic capsids. Alternatively, pUL37 may only bind stably to capsid-associated pUL36 after its binding to capsid-associated pUL25 has induced a conformational change in pUL36 resulting in a higher affinity for pUL37 (Coller et al., 2007; Pasdeloup et al., 2009; Schipke et al., 2012).

The motility analysis of the HSV1 deletion mutants tagged with CheVP26 demonstrated that directed transport of cytosolic progeny capsids required both pUL36 and pUL37. These results may be explained by the notion that the transport defect was solely due to a lack of pUL37 on capsids. The inability of HSV1-ΔUL36 or PrV-ΔUL37 particles to utilize active transport along microtubules (Luxton et al., 2006; Shanda and Wilson, 2008) might therefore be due to the concomitant lack of pUL37. pUL37 may either directly interact with microtubule motor proteins, or it may be required to stabilize motor binding to capsids, or to activate motor activity, both of which would influence active transport along the microtubules. However, these data do not exclude a contribution of pUL36, as binding to pUL37 might change the conformations of both proteins, and thus mediate the recruitment of microtubule motors or the regulation of their activity. Nevertheless, capsid-associated HSV1-pUL36 is unable to enlist microtubule motor activity in the absence of pUL37. It is possible that pUL37 recruits another tegument protein that in turn binds to or activates microtubule motors for intracellular capsid transport. Extensive quantitative mass spectroscopy and immunoelectron microscopy studies have shown that HSV1 capsids that recruit microtubule motors and translocate along microtubules in vitro contain several tegument proteins in addition to pUL36 and pUL37 (Radtke et al., 2006; Wolfstein et al., 2006).

In summary, our studies have identified a new function for HSV1-pUL37 during viral assembly in addition to its function during secondary envelopment and the formation of virions. Is efficient intracellular transport of cytosolic capsids a mandatory prerequisite prior to the association of the inner with the outer tegument during secondary envelopment? The present data on the HSV1-ΔUL36 and -ΔUL37 deletion mutants do not answer this question as they already have defects in the first function, namely transport. Based on their potential interactions with other tegument proteins, pUL36 with VP16 and pUL37 with VP11/12, it seems likely that they both also contribute substantially to connecting capsid-associated tegument proteins with glycoprotein-associated tegument proteins. Therefore, we need to expand on experimental approaches to, for example, biochemical assays (Wolf-
stein et al., 2006; Radtke et al., 2010), to further elucidate transport and envelopment requirements independently of each other to fully characterize the viral components and protein domains mediating intracellular transport of viral structures, as well as the association of inner with outer tegument during assembly and egress.

**Experimental procedures**

**Cells and viruses**

Vero cells (ATCC CCL-81) were cultured in MEM supplemented with 7.5% (v/v) fetal calf serum (FCS; Life Technologies Gibco, Darmstadt, Germany), and BHK-21 cells (ATCC CCL-10) in MEM supplemented with 10% (v/v) FCS. The UL36 complementing Vero cell line HS30 was cultured in MEM containing 7.5% FCS, and 500 μg ml⁻¹ G418 (PAA Laboratories GmbH, Pasching, Austria) were added in every fifth passage (Desai, 2000). The UL37 complementing rabbit skin cell line RS-80C02 was propagated in DMEM containing 10% (v/v) FCS and non-essential amino acids (Life Technologies Gibco), and was propagated in BHK, Vero, HS30 or RS-80C02 cells as described previously (Sodeik et al., 1997; Döhner et al., 2006). For most experiments, extracellular viruses sedimented from the supernatant of infected cells were used for infection (Nagel et al., 2008), while for the cell entry experiments (Fig. 4) the virions were further purified on linear nycodenz gradients (Döhner et al., 2006).

**Antibodies**

We used a rabbit polyclonal antibody (pAb) raised against full-length GST-tagged pUL37 (Leege et al., 2009), rabbit pAb #147 against a recombinant fragment (aa 1408 to 2112) of pUL36 (Wolfstein et al., 2006), rabbit pAb anti-VP26 against aa 95 to 112 of VP26 (Desai et al., 1998), the mouse monoclonal antibodies (mAb) SC10 (Trus et al., 1992) and H1.4 (Meridian Life Science, Saco, ME, USA) against VP5, the rabbit pAb NC2 raised against VP19c (Cohen et al., 1980) and the mouse mAb DL-6 raised against gD (Eisenberg et al., 1985). To visualize cellular proteins we used the mouse mAb antibody 3F2.3 against dynactin GST-Cap23 (Schafer et al., 1996; obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Iowa City, USA), the mouse mAb JL-8 raised against GFP (Clontech, San Jose, CA, USA), a mouse mAb against the dynactin subunit dynamitin p50 (BD Biosciences, Laboratories, Lexington, KY, USA) and a mouse mAb raised against the TGN protein p230 (BD Biosciences).

**HSV1 strains and modification of the genes UL36 or UL37**

All newly generated viruses (see Fig. 1) were based on HSV1(17′)-Lox, which was derived from pHHSV1(17′)-blue (Nagel et al., 2008). The HSV1(17′)-Lox strains contain a BAC cassette located between the viral genes UL22 and UL23, harbouring genes for replication and maintenance in *Escherichia coli*, the resistance gene chloramphenicol, a Cre recombinase gene with an intron under the control of a eukaryotic promoter, a single fipase (Fip) recognition target (FRT) site and LoxP sites at both ends (A. Pohlmann, N. Müther, K. Döhner and B. Sodeik, to be published elsewhere). The Cre recombinase is only expressed in eukaryotic cells and excises the BAC cassette and forms a virus that lacks the OriL as reported previously for the other HSV1-BACs (Nagel et al., 2008).

The generations of all mutations were based on the BACs pHHSV1(17′)-Lox or pHHSV1(17′)-Lox-CheVP26 (Fig. 1). In pHHSV1(17′)-Lox-CheVP26 the seven first N-terminal amino acids of VP26 have been replaced by mCherry as reported previously for pHHSV1(17′)-blueLox-mRFPVP26 (Nagel et al., 2012). Then, the coding sequence for mGFP alone or mGFP and a stop codon were inserted into the UL37 gene of pHHSV1(17′)-Lox or pHHSV1(17′)-Lox-CheVP26 before the last three codons as described previously (McLauchlan, 1997; Desai et al., 2008; Roberts et al., 2009). In case of HSV1(17′)-Lox-UL36 and HSV1(17′)-Lox-CheVP26-UL36 the full UL36 coding sequence (aa 1–3164) was deleted as described previously for HSV1(17′)-blueLox-UL36 (Schipke et al., 2012; L. Ivanova et al., in preparation). The UL37 deletion mutants were generated using the BACs HSV1(17′)-Lox and HSV1(17′)-Lox-CheVP26-UL37GFP respectively. A sequence starting at base pair (bp) 58 upstream of the UL37 start codon containing the 3363 bp of the UL37 gene region corresponding to the N-terminal 1121 of the 1223 aa was deleted. Additionally, in pHSV1(17′)-Lox-CheVP26-UL37GFP, 526 bp of the GFP sequence were additionally deleted leaving 194 bp behind and resulting in pHHSV1(17′)-Lox-CheVP26-UL37. Each UL37 deletion mutant contained a single FRT site at the original gene position (Fig. 1A). The desired sequence modifications were verified by sequencing (data not shown) and restriction digest analysis.

For every sequence insertion or deletion we used an en-passant mutagenesis technique (Tischer et al., 2006). A single amino acid substitution (A206K) in pEP-EGFP-in (Tischer et al., 2006) was performed with the QuickChange Site-Directed mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA, USA) generating mGFP (Zacharias et al., 2002). The sequences of fluorescent proteins were amplified from pEP-mGFP-in and pEP-mCherry-in, while for the deletion mutants pEP-Kan-S2 was used as a template (Tischer et al., 2006). Recombinant PCR fragments were amplified and transformed for homologous recombination into *E. coli* GS1783 (Tischer et al., 2006; Nagel et al., 2008). BAC modifications were analysed by six enzyme digest and in house sequencing of UL36/UL37 and the regions around.

For the preparation of BAC DNA, 500 ml of overnight *E. coli* cultures were harvested and BAC plasmids were purified with the NucleoBond™ BAC100 kit (Macherey and Nagel, Düren, Germany). The BAC DNA was precipitated and resuspended in 50 μl of buffer (10 mM Tris-HCl, pH 8, 50 μg ml⁻¹ RNase A). Twenty-five micrograms of DNA was digested with 35 U of restriction enzymes NotI (Fermentas, St.Leon-Rot, Germany) for 3.5 h, and loaded with 1× loading dye (Fermentas) on a 6% (w/v) agarose gel in 0.5× TBE buffer (0.44 M Tris-HCl, 0.44 boric acid, 10 mM EDTA). Fragments were separated by gel electrophoresis at 66 mA for 17 h (Peqlab system, Erlangen, Germany). We calculated the expected restriction digest fragments based on...
published sequences of HSV1(17+) (GenBank accession no. NC_001806) and our own sequencing of the BAC phHSV1(17+)Lox (A. Pohlmann, M. Nüther, K. Döhner and B. Sodeik, to be published elsewhere).

After this quality control, 10 µg of DNAs of specific BACs were transfected (MBS mammalian transfection kit) in a 60 mm dish with subconfluent Vero, Vero HS30 or RS-80C02 cells. After the development of cytopathic effects, the cells and medium were collected. Samples were freeze-thawed three times and used to amplify viral stocks.

**Growth curves**

Subconfluent Vero or RS-80C02 cells were inoculated with a multiplicity of infection (MOI) of 5 plaque-forming units (PFU) cell⁻¹, and after 6, 12, 18 or 24 h PI, the supernatants and the cells were harvested. Cells were scraped into hot sample buffer [50 mM Tris-HCl, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 5% (v/v) glycerol, 0.001% (w/v) bromphenol blue] containing protease inhibitors AEL (aprotinin, E-64, leupeptin, Sigma), ABP (antipain, bestatin, pepstatin, Sigma) and PMSF (phenylmethylsulfonylfluorid in isopropanol, Sigma-Aldrich, Schnelldorf, Germany) to neutralize extracellular virus. After 3 days cells were fixed with ice-cold methanol and labelled with 0.1% (w/v) crystal violet.

**Immunoblot**

Vero cells were inoculated with CO₂-independent medium with 0.1% (w/v) cell-culture-grade BSA at an MOI of 10 PFU cell⁻¹ and incubated for 1 h at room temperature on a rocking platform. The cells were washed twice with CO₂-independent medium and were incubated for 1 h at 37°C and 5% CO₂. Prior to harvesting, the cells were washed with PBS and then scraped into hot sample buffer [50 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 5% (v/v) glycerol, 0.001% (w/v) bromphenol blue] containing protease inhibitors AEL (aprotinin, E-64, leupeptin, Sigma), ABP (antipain, bestatin, pepstatin, Sigma) and PMSF (phenylmethylsulfonylfluorid in isopropanol, Roth, Karlsruhe, Germany). Cell lysates were boiled at 95°C for 5 min and passed through a needle about 50 times before loading. After this quality control, 10 µg of DNAs of specific BACs were transfected (MBS mammalian transfection kit) in a 60 mm dish with subconfluent Vero, Vero HS30 or RS-80C02 cells. After the development of cytopathic effects, the cells and medium were collected. Samples were freeze-thawed three times and used to amplify viral stocks.

**Immunofluorescence microscopy**

The light microscopy experiments were performed as described before (Sodeik et al., 1997; Döhner et al., 2002; Nagel et al., 2008; Rode et al., 2011; Schipke et al., 2012). Vero cells were seeded onto coverslips 1 day prior to infection. For synchronous infections, the cells were pre-cooled for 20 min on ice, and inoculated with an MOI of 10 PFU cell⁻¹ or mock-treated as control in CO₂-independent medium complemented with 0.1% (w/v) BSA for 2 h on ice on a rocking platform. The cells were then shifted to regular growth medium at 37°C and 5% CO₂ for 1 h. Non-internalized virus was inactivated by a short acid wash for 3 min (40 mM citrate, 135 mM NaCl, 10 mM KCl, pH 3), and the cells were transferred back to regular growth medium. To prevent synthesis of new viral proteins, 0.5 mM cycloheximide was added for the cell entry experiments.

Cells were either fixed with 3% (w/v) paraformaldehyde (PFA) for 20 min, followed by 50 mM NH₄Cl for 10 min and permeabilization with 0.1% TX-100 for 5 min, or for 10 min at 37°C with PHEMO fix [3.7% PFA, 0.05% glutaraldehyde, 0.5% Triton X-100 in PHEMO buffer (68 mM PIPES, 25 mM HEPES, pH 6.9, 15 mM EGTA, 3 mM MgCl₂, 10% dimethyl sulfoxide)] followed also by NH₄Cl treatment. The HSV1 Fc-receptor that is formed by a complex of the viral glycoproteins E and I and unspecific protein binding sites were blocked with a mixture of 10% human serum (v/v) of a HSV1-seronegative healthy volunteer and 0.5% BSA (w/v). Samples were labelled with primary and secondary antibodies [goat-anti mouse coupled either with rhodamine X (RedX), carbocyanine 5 (Cy5), or goat-anti rabbit coupled with FITC (Dianova, Hamburg, Germany); or goat-anti rabbit coupled with Alexa488 (Invitrogen; Karlsruhe, Germany), all antibodies were preabsorbed to prevent cross-reactivity with primary antibodies from other species]. DNA was stained using Hoechst or TO-PRO-3 dyes (Invitrogen), and the specimens were embedded in Mowiol containing 2.5% (w/v) 1,4-diazabicyclo-[2.2.2]octane. HSV1 assembly experiments were analysed with confocal fluorescence microscopes (LSM 510 Meta, software LSM 510, version 4, ZEISS, Göttingen, Germany; FV1000, Olympus, Hamburg, Germany), and the HSV1 cell entry with a fluorescence wide-field microscope with a plan-apochromat 63× objective, a mercury lamp and appropriate filter sets (AxioObserver Z1, Axiocam HRm camera, software Axiovision, version 4.8.3.0 SP1; ZEISS).

**Cloning and transfection of pcDNA3–UL37GFP**

UL37GFP open reading frame was cloned from pHSV1(17+)Lox-CheVP26-UL37GFP into the multiple cloning site of pORiR6K-zeo (from Z. Ruzsics, LMU, Munich, Germany) using the primers 5'-G CAT CGA GGT ACC CGG CGC GTC GGT ATACAC A-3' and 5'-G ACC ATG ACC CCT GGC ACA CAA TAA CAC CGA GA-3', following KpnI restriction sites (underlined) to generate pORiR6K-zeo_UL37GFP. From that, the UL37GFP sequence was amplified using the primers 5'-G CAT CGA GGT ACC GTC GGT ATACAC A-3' and 5'-G ACC ATG ACC CCT GGC ACA CAA TAA CAC CGA GA-3', via flanking KpnI restriction sites (underlined) to generate pORiR6K-zeo_UL37GFP. From that, the UL37GFP sequence was amplified using the primers 5'-G CAT CGA GGT ACC GTC GGT ATG GCA GA-3' and 5'-G ACC GCA GTATAC ACC ACC ATACAC CCG AGA-3', via flanking KpnI and EcoRV restriction sites (underlined) into pcDNA3 (Invitrogen) generating pcDNA3-UL37GFP. pORiR6K-zeo was propagated in E. coli PIR1 (Invitrogen) and pcDNA3 in XL1 blue (Stratagene, La Jolla, CA, USA). Eukaryotic cells were transfected with GeneJuice (Merck, Darmstadt, Germany) and 1 µg of pcDNA3-UL37GFP per chamber.

**Conventional and immunoelectron microscopy**

For conventional electron microscopy, Vero cells were infected synchronously at an MOI of 10 PFU cell⁻¹ as described for
fluorescence microscopy. After 10 or 14 h, the cells were fixed with 2% (w/v) glutaraldehyde in 130 mM cacodylate buffer at pH 7.4 containing 2 mM CaCl₂ and 10 mM MgCl₂. After 1 h, the cells were washed and contrasted for 1 h with 1% (w/v) OsO₄ in 165 mM cacodylate buffer at pH 7.4 containing 1.5% (w/v) K₃[Fe(CN)₆] followed by 0.5% (w/v) uranyl acetate in 50% (v/v) ethanol overnight. The cells were embedded in Epon, and 50 nm ultrathin sections were cut parallel to the substrate. Images were taken with a Tecnai G2 electron microscope at 200 kV and equipped with an Eagle 4k camera (FEI, Eindhoven, the Netherlands).

For immunoelectron microscopy, Vero cells were also infected synchronously at an MOI of 10 PFU cell⁻¹. After 16 h, the cells were fixed, embedded, frozen and sectioned following the protocol of Peters et al. (2006). Sections were labelled using specific antibodies and protein A gold of 10 nm (Cell Microscopy Centre, Utrecht School of Medicine, the Netherlands; Griffiths, 1993; Sodeik et al., 1993; Peters et al., 2006). Sections were contrasted using 0.5% (w/v) uranyl acetate in 2% methylcellulose (Merck), and analysed with a Tecnai G2 at 200 kV and equipped with an Eagle 4k camera. Gold labelling was quantified by counting each gold particle within a radius of 100 nm around the centre of a cytoplasmic capsid.

Live cell imaging and particle tracking

Vero cells in two-well chambers (LabTek, #155380, #1.0 German borosilicate cover glass) were synchronously infected as described above. Movies were recorded with five images per second and a pixel size of 79 × 79 nm using a confocal laser scanning microscope Zeiss LSM510 at 37°C. Particles were only further analysed, if they had moved at least 3 pixels within 0.2 s for five consecutive time frames. Manual tracking was performed with the ImageJ plugin MTrackJ (Meijering et al., 2012) using its local cursor snapping tool during tracking (snap feature: max. intensity; snap range: 15 × 15 pixels). The nuclei were identified by the confined nuclear capsid mobility and were marked as a reference point. Sixty-five capsid tracks of HSV1(17 Lox, 48 of -UL36, 48 of -UL37 and 36 of -UL37 overexpressing UL37GFP were analysed. The mean velocity was calculated as the average of all step velocities excluding those steps where no movement was detected. For the MSD coefficient data from the column ‘distance to reference point’ (D2R) were plotted against lg D² and the slope was calculated. Positive values represent transport to the cell periphery and negative ones to the nucleus. The relative displacement angle was calculated as the average of all angles measured between the vectors of two subsequent transport steps and expressed as radian (rad). Significance was calculated using an unpaired t-test of the software Prism (version 5.02, Graphpad, San Diego, CA, USA).

Image processing

All digital images of blots, light or electron microscopy were further processed with LSM Image Browser (version 3.8; ZEISS, Jena, Germany) or Adobe Photoshop CS (version 6.0; Adobe systems, San Jose, CA, USA).

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**Supporting information**

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

**Movies S1–S4.** Vero cells were infected at an MOI of 10 PFU cell⁻¹ with different HSV strains whose capsids had been tagged with CheVP26. Live cell imaging was performed between 8 and 10 h PI using fluorescence confocal microscopy and collecting five images per second for a total time period of 48 s. The mCherry channel was recorded and movie images were inverted. Individual capsids were identified by the intensity of the fluorescence signal and manually tracked. The quantification of the tracks is shown in Fig. 8. The upper panel presents the mCherry channel and the lower panel the mCherry channel overlaid with representative capsid tracks.

**Movie S1.** Cells infected with HSV1(17a)Lox-CheVP26.

**Movie S2.** Cells infected with HSV1(17a)Lox-CheVP26-ΔUL36.

**Movie S3.** Cells infected with HSV1(17a)Lox-CheVP26-ΔUL37.

**Movie S4.** For the rescue experiment cells were transfected with a plasmid expressing pUL37GFP 2 h prior to infection with HSV1(17a)Lox-CheVP26-ΔUL37.