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The interaction of the HSV-1 tegument proteins pUL36 and pUL37 is essential for secondary envelopment during viral egress

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ABSTRACT

The herpes simplex virus type 1 (HSV-1) tegument proteins pUL36 (VP1/2) and pUL37 are essential for viral egress. We previously defined a minimal domain in HSV-1 pUL36, residues 548–572, as important for binding pUL37. Here, we investigated the role of this region in binding to pUL37 and facilitating viral replication. We deleted residues 548–572 in frame in a virus containing a mRFP tag at the N-terminus of the capsid protein VP26 and an eGFP tag at the C-terminus of pUL37 (HSV-1pUL36Δ548-572). This mutant virus was unable to generate plaques in Vero cells, indicating that deletion of this region of pUL36 blocks viral replication. Imaging of HSV-1pUL36Δ548-572-infected Vero cells, in comparison to parental and resucant, revealed a block in secondary envelopment of cytoplasmic capsids. In addition, immunoblot analysis suggested that failure to bind pUL37 affected the stability of pUL36. This study provides further insight into the role of this essential interaction.

Introduction

Herpes simplex type 1 (HSV-1) has a linear double stranded DNA genome of approximately 152 kb, which is enclosed in an icosahedral-shaped capsid. This capsid is encased in an amorphous layer of protein termed the tegument, which is in turn enclosed in a glycoprotein-containing host cell membrane-derived envelope (Roizman et al., 2007). The HSV-1 tegument layer consists of at least 23 virally-encoded proteins (Loret et al., 2008; Mettenleiter, 2004; Roizman et al., 2007) with both structural and non-structural functions (Guo et al., 2010; Kelly et al., 2009). The inner tegument proteins pUL36 and pUL37 are conserved across all members of the *Herpesviridae* family (Kelly et al., 2009). For HSV-1 and the related Pseudorabies virus (PrV) in cell lines, deletion of the genes encoding either of these proteins blocks secondary envelopment of capsid and virion maturation (Desai et al., 2001; Desai, 2000; Fuchs et al., 2004; Klupp et al., 2001; Leege et al., 2009; Roberts et al., 2009; Sandbaumhuter et al., 2013; Schipke et al., 2012).

pUL36 has several roles in both early and late stages of the replication cycle. Essential and nonessential functional domains of PrV pUL36 have been delineated using a combination of random and directed deletion mutagenesis (Fig. 1A) (Bottcher et al., 2007, 2006; Mohl et al., 2010). pUL36 is involved in targeting of incoming capsids to the nucleus and release of the viral genome into the nucleus, where DNA transcription and replication occur (Abaitua et al., 2012; Batterson et al., 1983; Batterson and Roizman, 1983; Delboy et al., 2008; Jovasevic et al., 2008; Knipe et al., 1979; Roberts et al., 2009; Schipke et al., 2012). It is required for intracellular capsid transport during egress (Luxton et al., 2006; Shanda and Wilson, 2008) and is thought to be a receptor for molecular motors (Radtke et al., 2010; Wolfstein et al., 2006; Zaichick et al., 2013). Binding sites for both the inner tegument protein pUL37 and the major tegument protein VP16 have been identified in the N-terminal region of the protein (Klupp et al., 2002; Ko et al., 2010; Mijatov et al., 2007; Vittone et al., 2005), while the C-terminus contains two experimentally confirmed binding sites for the capsid protein pUL25 and a proposed binding site for capsid protein VP5 (Fig. 1A) (Coller et al., 2007; Pasdeloup et al., 2009; Schipke et al., 2012). Thus, pUL36 appears to form a bridge between the capsid and the remainder of the tegument (Cardone et al., 2012; Ko et al., 2010). In the case of PrV, the N-terminal region of pUL36 has also been shown to mediate

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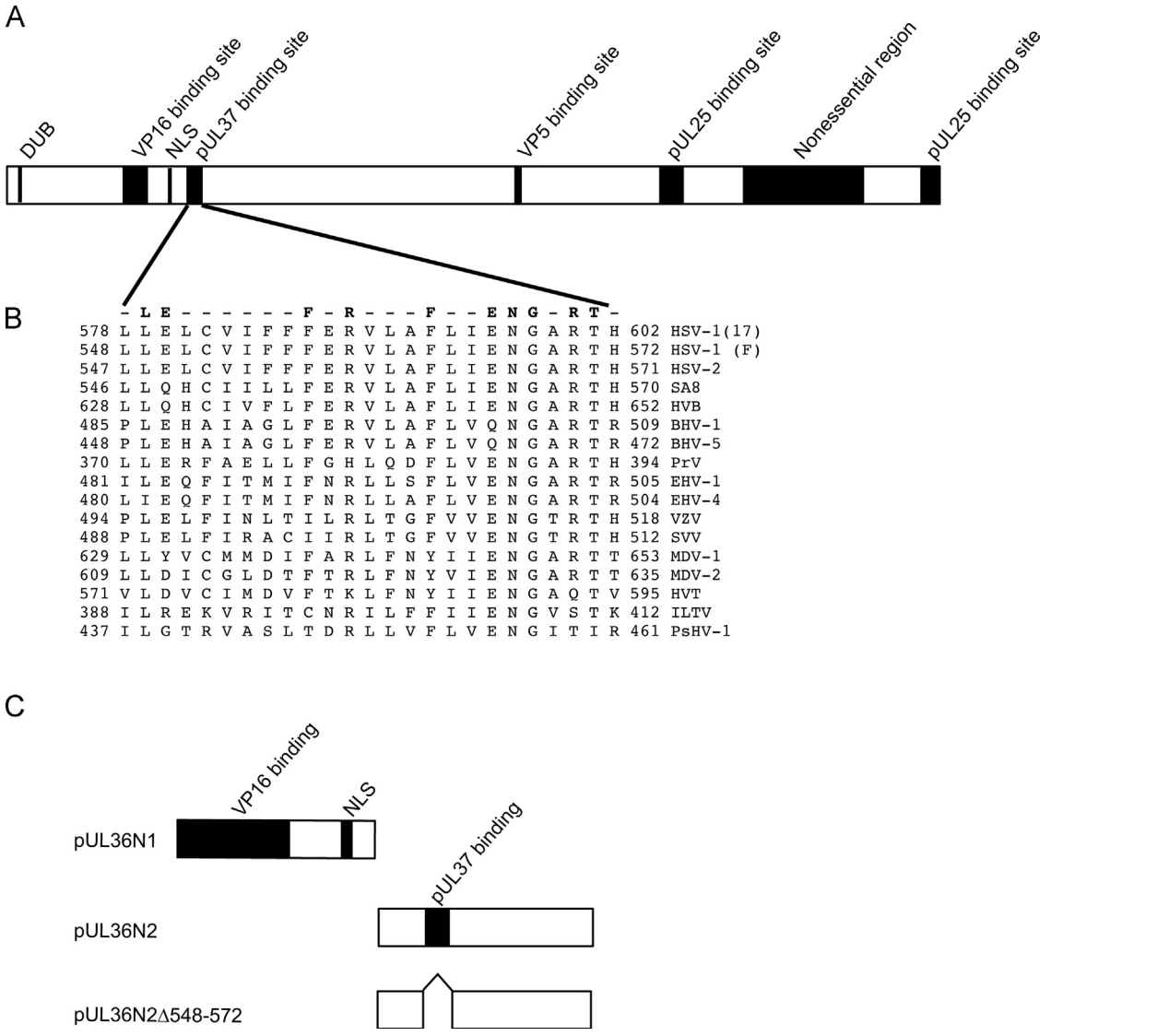

Fig. 1. Domains of HSV-1 pUL36. (A) Diagram outlining identified domains of pUL36 including: a deubiquitinating (DUB) enzyme activity in the N-terminus (Kattenhorn et al., 2005); a binding site for VP16 (Mijatov et al., 2007); a binding site for pUL37 (Klupp et al., 2002; Mijatov et al., 2007; Vittone et al., 2005); a proposed binding site for the major capsid protein VP5 (Baker et al., 2006); a nonessential internal region based on a study with the related Pseudorabies virus (PrV) (Bottcher et al., 2006); and two binding sites for capsid protein pUL25 (Coller et al., 2007; Pasdeloup et al., 2009; Schipke et al., 2012). (B) Alignment of the pUL37 binding site of pUL36 homologues within the *Alphaherpesvirinae* subfamily (McGeoch et al., 2006). The majority of conserved residues were targeted for mutagenesis (highlighted in bold) in a previous study from our laboratory (Mijatov et al., 2007). Family members include HSV-1 strain 17 and F, HSV-2, Simian agent 8 (SA8), B virus (HVB), Bovine herpesvirus 1 (BHV-1), Bovine herpesvirus 5 (BHV-5), PrV, Equine herpesvirus 1 (EHV-1), Equine herpesvirus 4 (EHV-4), Varicella-zoster virus (VZV), Simian varicella virus (SVV), Marek's disease virus type 1 (MDV-1), Marek's disease virus type 2 (MDV-2), Herpesvirus of Turkey (HVT), infectious laryngotracheitis virus (ILTV) and Psittacid herpesvirus 1 (PsHV-1). The encoded pUL36 sequences were obtained from the following herpesviral genome sequence accession numbers: HSV-1 strain 17, X14112; HSV-1 strain F, GU734771; HSV-2, Z86099; SA8, AY714813; HVB, AF533768; BHV-1, AJ004801; BHV-5, AY261359; PrV, BK001744; EHV-1, AY665713; EHV-4, AF030027; VZV, X04370; SVV, AF275348; MDV-1, AF24338; MDV-2, AB049735; HVT, AF291866; ILTV, AF168792; PsHV-1, AY372243. Clustal W alignments were generated using Lasergene version 11.1 MegAlign from DNASTAR. (C) Diagram of pUL36 constructs expressed with a C-terminal GFP tag in this study.

nuclear egress (Leelawong et al., 2012). Other subdomains within the N-terminal region of the protein include a nuclear localisation signal (Abaitua et al., 2012; Abaitua and O'Hare, 2008) and a deubiquitinating activity (Fig. 1A) (Bolstad et al., 2011; Kattenhorn et al., 2005; Schlieker et al., 2005).

Like its binding partner pUL36, the 120 kDa protein pUL37 is also multifunctional in nature. In HSV-1 and PrV pUL37 increases the efficiency of transport of capsids to the nucleus, but is not essential for this process (Krautwald et al., 2009; Luxton et al., 2006). While a role in nuclear egress of HSV-1 could not be demonstrated (Leege et al., 2009; Roberts et al., 2009; Sandbaumhuter et al., 2013), it is required to direct capsids to the site of secondary envelopment (Pasdeloup et al., 2010; Sandbaumhuter et al., 2013). pUL37 coelutes with the single-stranded DNA binding protein ICP8 (Shelton et al., 1994), and in the case of HSV-2 pUL37, a functional nuclear export signal has been identified (NES) (Watanabe et al., 2000). HSV-1 pUL37 binds to a number of cellular proteins, including TNF receptor-associated factor 6 (TRAF 6), with resulting activation of NF-κB signalling pathways (Liu et al., 2008), the serine/threonine kinase thousand and one kinase 3 (TAOK3), which belongs to the Ste20 kinase superfamily (Kelly et al., 2012a; Strange et al., 2006), and dystonin, a spectraplakin protein involved in microtubule-based transport (McElwee et al., 2013; Pasdeloup et al., 2013). It is known to contain self-association domains within the N and C-terminus regions as well as a pUL36 binding domain in the C-terminal region (Bucks et al., 2011).

Binding of pUL37 is thought to occur through a direct interaction with pUL36, a feature which is conserved across the *Herpesviridae* family (Bechtel and Shenk, 2002; Klupp et al., 2002; Lee

et al., 2008; Rozen et al., 2008; Stellberger et al., 2010; To et al., 2011; Uetz et al., 2006; Vittone et al., 2005). In the case of HSV-1, residue D631 in the C-terminal pUL36 binding domain of pUL37 is essential for binding pUL36 (Kelly et al., 2012b). Deletion of *UL36* results in cytosolic capsids devoid of pUL37 (Klupp et al., 2002; Sandbaumhuter et al., 2013). The deletion of pUL36 also blocks trafficking of pUL37 to sites of secondary envelopment in a capsid independent manner, suggesting a role for the interaction between these two proteins in incorporation into the virion (Desai et al., 2008).

The pUL37-binding domain in pUL36 is located in the N-terminus of the protein (Klupp et al., 2002; Mijatov et al., 2007). A previous study on PrV pUL36 indicated that the interaction of pUL36 and pUL37 was important but not essential for secondary envelopment during virion assembly (Fuchs et al., 2004). This is not surprising since PrV pUL36 is essential for viral replication (Fuchs et al., 2004) but pUL37 is not essential (Klupp et al., 2001). In contrast, for HSV-1 both pUL36 and pUL37 are essential for replication (Desai et al., 2001; Desai, 2000; Roberts et al., 2009; Sandbaumhuter et al., 2013; Schipke et al., 2012) but to date no study has directly assessed the role of the interaction of these key tegument proteins in HSV-1 replication. In this study, we therefore sought to define the role of the interaction of pUL36 with pUL37 by examining the effects of deleting the pUL37 binding domain in pUL36 on the replication of HSV-1.

Results

Confirmation of the pUL37 binding site in pUL36

Our previous study found the binding region of pUL37 in HSV-1 pUL36 to lie within the N-terminal region of pUL36 (Fig. 1A) (Vittone et al., 2005). A similar observation has been reported for pUL36 in PrV and VZV (Klupp et al., 2002; Stellberger et al., 2010). A subsequent study by our laboratory identified a number of conserved residues within amino acid residues 548–572 (strain F numbering; equivalent to amino acids 578–602 in strain 17) that are involved in binding of pUL36 to pUL37 (Fig. 1B; (Mijatov et al., 2007)). To investigate further the role of this region in virus replication we had to confirm the requirement for this region for binding to pUL37. We deleted pUL36 amino acid residues 548–572 in a GFP-tagged fragment of pUL36 (pUL36N2-GFP; strain F amino acids 482–737) (Fig. 1C). We then tested the ability of this mutated fragment, pUL36N2Δ548-572-GFP, to bind myc-tagged pUL37 (myc-pUL37). HeLa cells were cotransfected with myc-pUL37 and pUL36N2-GFP, pUL36N2Δ548-572-GFP or pUL36N1-GFP. The pUL36N1-GFP fragment consists of a region of pUL36 (strain F amino acids 292–481) adjacent to the pUL37 binding site (Fig. 1C) and was included as a negative control. Cell lysates were harvested at 24 h post transfection and coimmunoprecipitation using anti-myc antibody was carried out. Immunoblot analysis confirmed the presence of myc-pUL37 (Fig. 2A, top panel) and of each pUL36-GFP fragment in input lysates and elutes (Fig. 2A, bottom panel)., Only pUL36N2-GFP precipitated with myc-pUL37 (Fig. 2A, bottom panel). When residues 548–572 were deleted, the GFP-tagged N2 fragment of pUL36 failed to bind myc-pUL37 (Fig. 2A, bottom panel). This confirms that the 548–572 region (Fig. 1B) of pUL36 is crucial for binding of pUL37.

We noted that in our input lysates (Fig. 2A, bottom panel) the level of pUL36N2-GFP detected was higher than that of pUL36N2Δ548-572-GFP when each was cotransfected with myc-pUL37, although there were still adequate expression levels of pUL36N2Δ548-572-GFP to establish it no longer binds pUL37 (Fig. 2A, bottom panel). Our previous study, which defined pUL37 amino acid D631 to be critical for binding of pUL36, reported reduced levels of both pUL36N2-GFP and full-length pUL36 when pUL37 was absent or when binding of pUL37 to pUL36 was disrupted (Kelly et al., 2012b). It appeared that a similar effect was being seen here. To confirm this, we carried out another cotransfection experiment. HeLa cells were cotransfected with pUL36N2-GFP, pUL36N2Δ548-572-GFP, or pUL36N1-GFP and myc-pUL37 or myc-Filamin (chosen as a negative control since it has a similar size to pUL37). Immunoblot analysis of transfected cell lysates using anti-myc antibody showed similar levels of myc-pUL37 and myc-Filamin expressed in each sample (Fig. 2B, top panel). Detection with an anti-GFP antibody showed that higher levels of pUL36N2-GFP were detected when this construct was cotransfected with myc-pUL37 compared with cotransfection with myc-Filamin, while levels of pUL36N2Δ548-572-GFP remained similar when contransfected with either myc-pUL37 or myc-Filamin (Fig. 2B, bottom panel). When bands were quantified and the results normalised to myc-pUL37 levels, the amount of pUL36N2-GFP was 2.6 fold higher than that of pUL36N2Δ548-572-GFP when myc-pUL37 was present. This further confirms our finding that binding of pUL37 to this region of pUL36 stabilises pUL36.

Construction and initial characterisation of recombinant HSV-1 viruses

To investigate the importance of residues 548–572 in the context of viral replication, the region of *UL36* encoding residues 548–572 was deleted in a HSV-1F strain virus with an mRFP tag at the N-terminus of capsid protein pUL35 (VP26) and an eGFP tag at the C-terminus of pUL37 (Fig. 3A). All modifications were introduced using BAC recombination as previously described (de Oliveira et al., 2008). The resulting mutant virus was termed HSV-1FpUL36Δ548-572, while the parental virus expressing the fluorescent tags was designated HSV-1FR3537G. A rescuant virus was constructed by homologous recombination restoring the pUL36 coding region to the wild type sequence. The rescuant was termed HSV-1FpUL36Δ548-572R.

The kinetics of virus production by HSV-1F, HSV-1FR3537G, HSV-1FpUL36Δ548-572 and HSV-1FpUL36Δ548-572R was examined in Vero cells. Cells were infected at an MOI of 10 pfu/cell. Extracellular and cell associated virus titres were analysed separately at 4, 8, 16 and 24 h p.i. For both extracellular and cell associated virus, titres of HSV-1FR3537G and HSV-1FpUL36Δ548-572R were approximately 1 log lower than that of the wild type, HSV-1F (Fig. 3B). This is similar to what has been found previously for fluorescently tagged HSV-1 F-strain (de Oliveira et al., 2008) and is most likely due to fusion of the RFP tag to VP26 which has previously been reported to result in formation of nuclear aggregates of VP26 and to subsequently impair capsid nuclear egress (Nagel et al., 2012). Titres of HSV-1FpUL36Δ548-572 were significantly reduced compared to all three other viruses (Fig. 3B), indicating a similar attenuation to that previously reported for HSV-1 pUL37 deletion mutants (Desai et al., 2001; Leege et al., 2009; Roberts et al., 2009; Sandbaumhuter et al., 2013). The magnitude of the defect in HSV-1FpUL36Δ548-572 attributable to the deletion in pUL36 may be less than it appears due to the presence of the fluorescent tags.

Plaque morphology of the mutant virus was compared to that of the parental and the rescuant. Both Vero cells and pUL36 complementing cells (HAUL36-1) on coverslips were infected with HSV-1FR3537G, HSV-1FpUL36Δ548-572 or HSV-1FpUL36Δ548-572R. Cells were fixed after 3 days and plaque morphology was observed by fluorescent microscopy. While HSV-1FR3537G and HSV-1FpUL36Δ548-572R formed similar sized plaques in both cell lines (Fig. 3C), HSV-1FpUL36Δ548-572 only formed plaques in the complementing cell line (Fig. 3C). While individual Vero cells

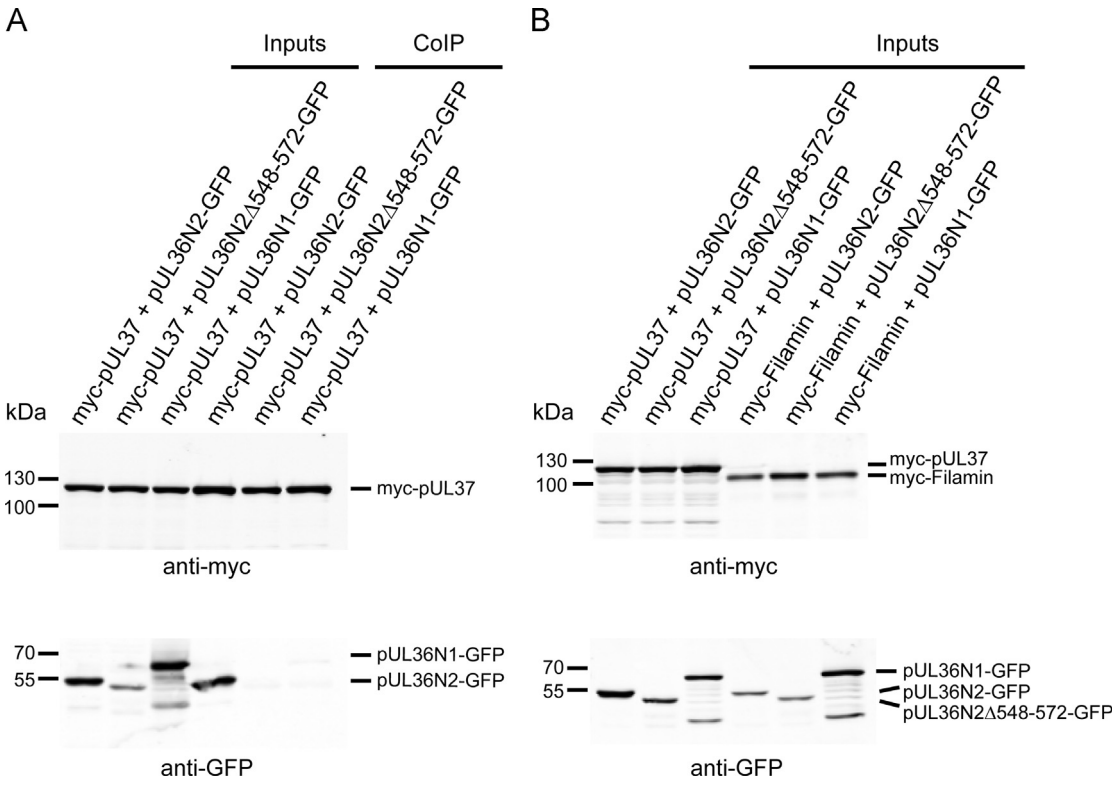

Fig. 2. Amino acid residues 548–572 of pUL36 are required for binding of pUL37. (A) HeLa cells were cotransfected with the indicated constructs and harvested at 24 h post transfection. A coimmunoprecipitation assay was carried out. Mouse IgG whole molecule agarose was incubated with mouse anti-myc antibody before incubation with cell lysates. Input lysates and immunoprecipitated proteins were analysed by 12% SDS-PAGE and immunoblotting using rabbit anti-myc antibody (top panel) and rabbit anti-GFP antibody (bottom panel). (B) HeLa cells were cotransfected with the indicated constructs to further assess stabilisation of pUL36 by bound pUL37. Cell lysates were harvested at 24 h post transfection and analysed by 10% SDS-PAGE and immunoblot using anti-myc (top panel) and anti-GFP (bottom panel) antibodies.

expressing fluorescently tagged viral proteins were observed for HSV-1FpUL36Δ548-572 infections, the virus was unable to spread from the initially infected cells (Fig. 3C). Together these observations suggest that the deletion of pUL36 residues 548–572 blocks spread of the virus from one cell to the next. The fact that the rescuant virus exhibited similar growth kinetics and plaque morphology to that of the parental virus, confirms that the phenotype observed for HSV-1FpUL36Δ548-572 in non-complementing cells is solely due to the Δ548–572 mutation and not to a mutation present somewhere else in the genome.

Next we looked at levels of pUL37 and pUL36 in virus-infected cell lysates. We infected Vero cells with HSV-1FR3537G or HSV-1FpUL36Δ548-572 at an MOI of 10 pfu/cell and harvested infected cell lysates at 24 h p.i. Immunoblot analysis was carried out using antibodies against GFP and pUL36 (Fig. 4). The pUL37-GFP protein was detected in both HSV-1R3537G- and HSV-1FpUL36Δ548-572-infected cell lysates (Fig. 4, top panel), however, we observed essentially no full-length pUL36 in HSV-1FpUL36Δ548-572-infected cell lysates when compared with HSV-1FR3537G-infected lysates (Fig. 4, bottom panel). This is based on a predicted molecular mass for full-length pUL36 of 332.9 kDa for WT compared to 330 kDa for Δ548–572. The absence of full-length pUL36 was not a result of deletion of the epitope for pUL36 in HSV-1FpUL36Δ548-572 as the polyclonal anti-pUL36 antibody was raised against an expression product corresponding to amino acids 1845 to 2001 (strain F numbering) of HSV-1 pUL36 (Thomas Mettenleiter, personal communication). Our inability to detect the full length protein may instead be due to its inherent instability in the absence of pUL37 binding, as seen above for the GFP-tagged pUL36 fragments and in our previous study (Kelly et al., 2012b). For both HSV-1FR3537G and HSV-1FpUL36Δ548-572 we observed major proteolytic fragments of pUL36 at ~270 kDa and ~60 kDa (Fig. 4, bottom panel). Proteolysis of the N-terminus of pUL36 has been previously documented (Bolstad et al., 2011; Jovasevic et al., 2008; Leelawong et al., 2012; Schipke et al., 2012). Therefore, the ~270 kDa pUL36 fragment most likely corresponds to proteolysis of the N-terminus which would be sufficient to remove the pUL37 binding domain.

Electron microscopy

To determine the point at which replication of the mutant virus was being blocked, we infected Vero cells with HSV-1FR3537G, HSV-1FpUL36Δ548-572 or HSV-1FpUL36Δ548-572R, fixed cells at 23 h p.i., and analysed samples by electron microscopy. For both HSV-1FR3537G (Fig. 5A–C) and HSV-1FpUL36Δ548-572R (Fig. 5D–E) virion maturation occurred as normal. Cytosolic capsids and all stages of secondary envelopment of particles in the cytoplasm were observed in each case (Fig. 5A, B and D), as were fully mature extracellular virions (Fig. 5C and E). For cells infected with HSV-1FpUL36Δ548-572 (Fig. 6), capsids successfully exited the nucleus by primary envelopment (Fig. 6B) and de-envelopment, or they may have gained access to the cytoplasm via impaired nuclear pores (Leuzinger et al., 2005). However, once in the cytoplasm, capsids appeared to remain there with no evidence of secondary envelopment (Fig. 6A, C and D). Instead of extracellular mature virions, capsidless membrane-bound electron dense particles were observed (Fig. 6D and E) which were recognised as L-particles since they were not found in uninfected cells (not shown). This is comparable to the phenotype of pUL36 and pUL37 deletion mutants (Desai et al., 2001; Desai, 2000; Fuchs et al., 2004; Klupp et al., 2001; Leege et al., 2009; Roberts et al., 2009; Sandbaumhuter et al., 2013), suggesting that if pUL37 does not

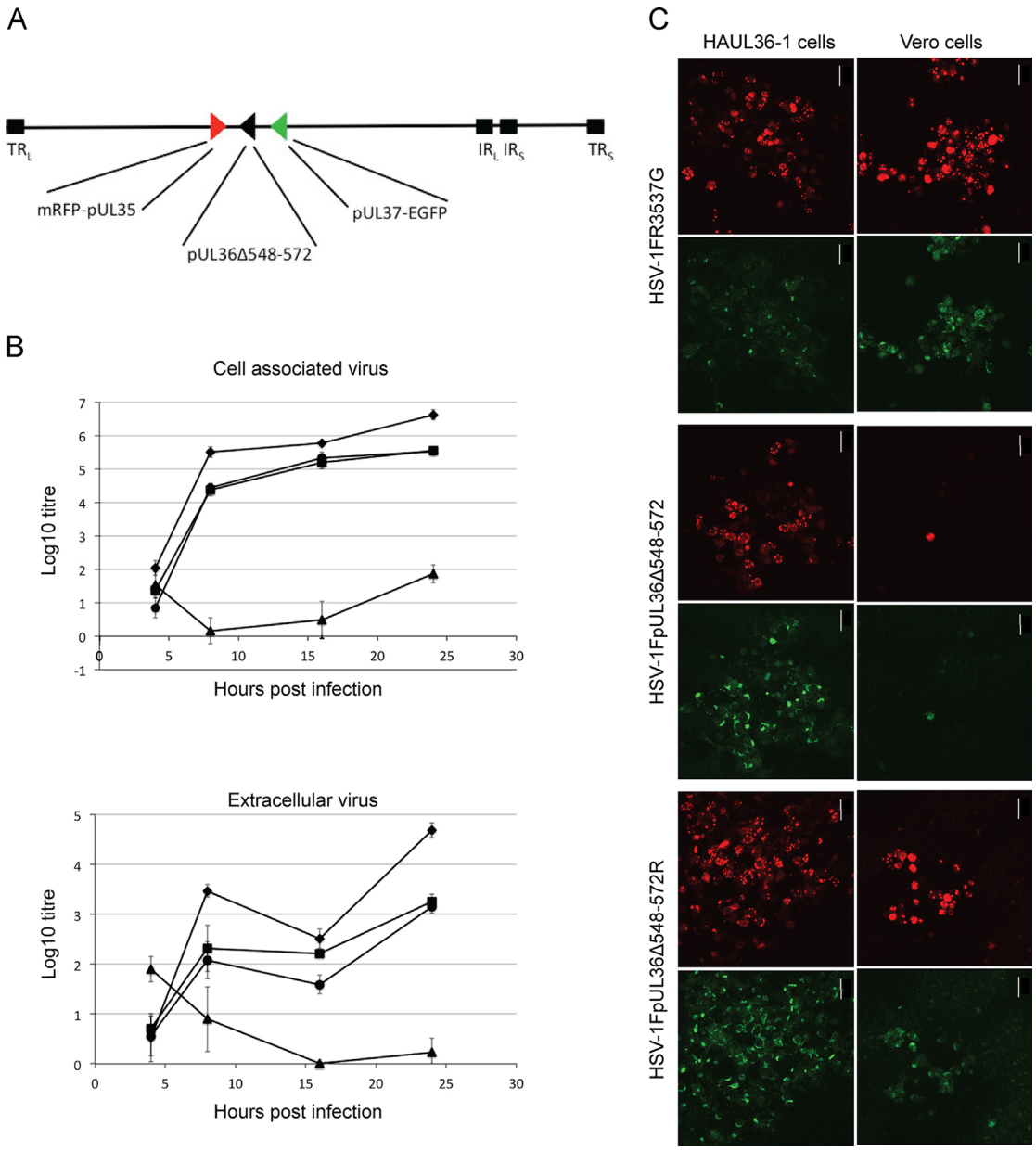

Fig. 3. Characterisation of recombinant viruses. (A) Diagram indicating the modifications introduced into HSV-1 F strain genome. The parental virus, HSV-1FR3537G, produced by BAC recombination has an mRFP tag at the N-terminus of capsid protein VP26 (pUL35) and a eGFP tag at the C-terminus of tegument protein pUL37. In this background, a deletion of residues 548–572 in pUL36 (Δ) was engineered by BAC recombination to produce HSV-1FpUL36Δ548-572. A rescuant, HSV-1FpUL36Δ548-572R, was subsequently generated from HSV-1FpUL36Δ548-572 using standard homologous recombination. The direction of the arrowhead indicates the direction of gene transcription for the gene in question. TR_L, terminal repeat of the long segment; IR_L, internal repeat of the long segment; IR_S, internal repeat of the short segment; TR_S, terminal repeat of the short segment. (B) For growth kinetics, Vero cells were infected at an MOI of 10 pfu/cell with HSV-1F (◆), HSV-1FR3537G (■), HSV-1FpUL36Δ548-572 (▲) or HSV-1FpUL36Δ548-572R (●). Cell associated virus and extracellular virus was harvested at the indicated timepoints and viral titres were estimated by plaque assay. (C) For plaque morphology, HAUL36-1 cells (left panel) or Vero cells (right panel) on coverslips were infected with serial dilutions of the indicated viruses. After infection with virus, cells were overlaid with a semi solid overlay and incubated for 3 days before being fixed and viewed using a confocal microscope. Green fluorescence represents pUL37-GFP, while red fluorescence represents mRFP-VP26. Scale bars equal 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bind to pUL36 the effect is similar to the absence of pUL37 i.e. a block in secondary envelopment.

Confocal microscopy

The subcellular localisation of pUL37 during viral egress was analysed by fluorescent microscopy (Fig. 7). Vero cells were infected with the parental HSV1-1FR3537G, the mutant strain HSV-1FpUL36Δ548-572, or the recuant HSV-1FpUL36Δ548-572R, fixed at 12 or 23 h p.i., and analysed by confocal laser scanning microscopy. At the earlier time point, 12 h p.i., only about 10% of the cells had mRFP-VP26 in the nucleus, indicating virus production. We restricted our analysis to those cells. In all analysed cells infected with the parental HSV1-1FR3537G (Fig. 7A) or the rescuant HSV-1FpUL36Δ548-572R (Fig. 7C) we identified capsids in the cytoplasm by their mRFP-VP26 signals which colocalised with pUL37-GFP. This suggests that pUL37 is bound to capsids, presumably via an interaction with pUL36, which has previously been shown to be required for incorporation of pUL37 into capsids (Ko et al., 2010; Sandbaumhuter et al., 2013). In contrast the majority of cytoplasmic capsids in cells infected with the deletion mutant HSV-1FpUL36Δ548-572 did not colocalise with pUL37-

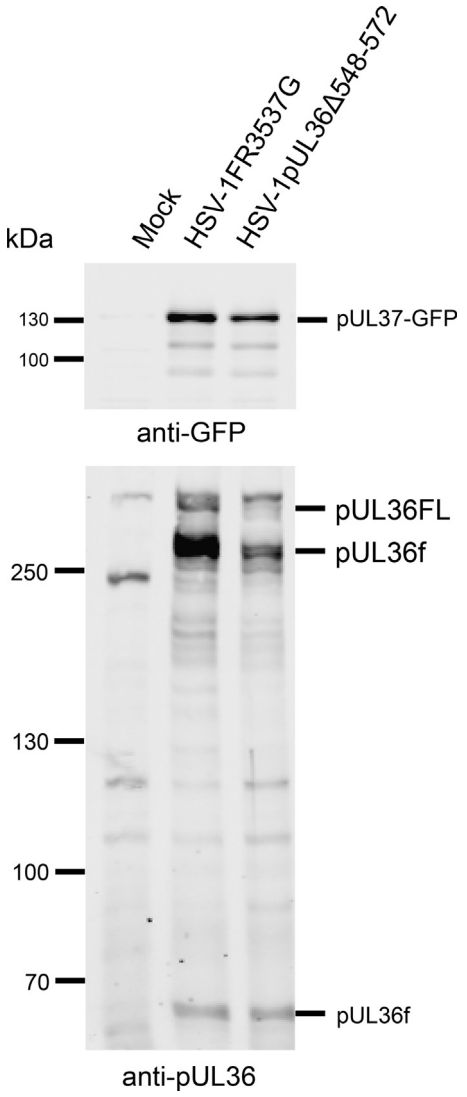

Fig. 4. Increased proteolysis and reduced expression levels of pUL36 in the absence of bound pUL37. Vero cells were infected with the indicated viruses. Viral lysates were harvested at 24 h p.i. and analysed by SDS-PAGE and immunoblot using antibodies against GFP (top panel) and pUL36 (bottom panel). Full-length (pUL36FL) and predominant proteolytic fragments (pUL36f) of pUL36 are indicated.

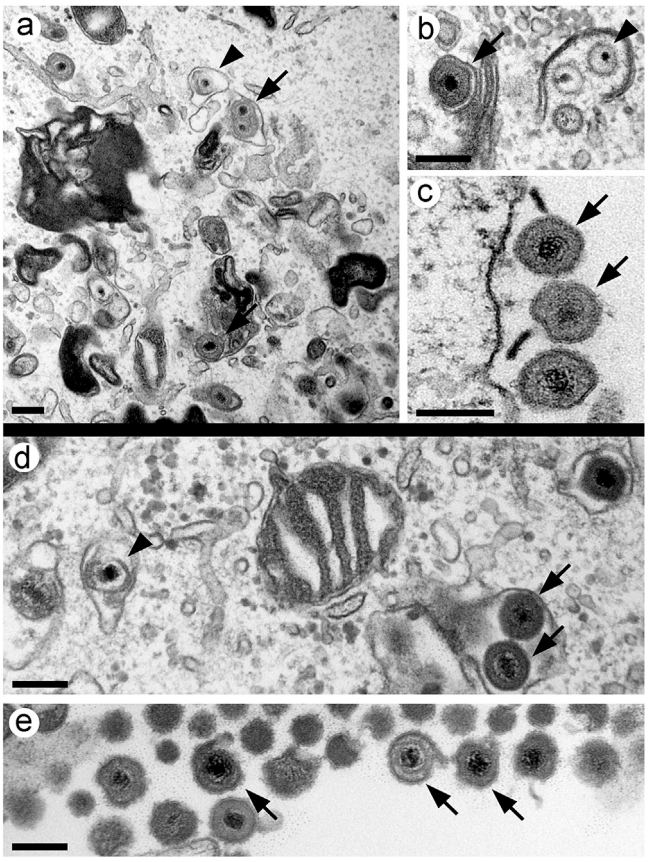

Fig. 5. HSV-1FR3537G and HSV-1FpUL36Δ548-572R capsids undergo secondary envelopment. Vero cells were infected with the parental strain HSV-1FR3537G (a–c) or HSV-1FpUL36Δ548-572R (d–e), fixed at 23 h p.i., and analysed by electron microscopy. Black arrows (a, b, and d) indicate cytoplasmic virions after complete secondary envelopment or extracellular virions (c and e). Black arrowheads indicate wrapping intermediates of secondary envelopment (a, b, and d). Scale bars equal 200 nm.

GFP (Fig. 7B). At 23 h p.i. more cells displayed virus production. Capsid and pUL37 signals accumulated in a juxtanuclear location in addition to individual capsids outside this juxtanuclear location (data not shown). Similar to the 12 h p.i. condition the latter did colocalise with pUL37-GFP in the parental and rescuant strains while in the mutant strain many of these capsid signals did not colocalise with the signals for pUL37. This observation, like our EM findings, indicates a block in secondary envelopment, causing a build up of capsids and sequestering of tegument proteins at sites where this process would normally take place.

Discussion

Previous work carried out in our laboratory identified an interaction between HSV-1 pUL36 and pUL37 and mapped the binding site for this interaction to an N-terminal region of pUL36 (Vittone et al., 2005). We subsequently defined a number of conserved residues within this region that are critical to this interaction and postulated that the minimal binding domain for pUL37 resides within pUL36 residues 578–602 in HSV-1 strain 17 (residues 548–572 in HSV-1 strain F; Fig. 1B) (Mijatov et al., 2007). In this report we initially confirmed that the binding site of pUL37 in pUL36 does indeed reside within this region of conserved residues. We also noted that there was disparity in the levels of pUL36N2-GFP and pUL36N2Δ548-572-GFP detected when each was cotransfected with myc-pUL37. When the 548–572 region was present and the pUL36N2-GFP fragment bound to myc-pUL37, more of this fragment was detected in lysates compared to pUL36N2Δ548-572-GFP, which was shown by coimmunoprecipitation not to bind to myc-pUL37. This effect was confirmed by cotransfecting myc-pUL37 or a myc-Filamin control with pUL36N2-GFP, pUL36N2Δ548-572-GFP or pUL36N1(residues 292–481)–GFP. Thus, when this fragment of pUL36 is prevented from binding to pUL37 it appears to be degraded, suggesting that binding of pUL37 to pUL36 plays a role in stabilising the protein.

The role of pUL36 residues 548–572 in the context of viral replication was then ascertained. When virus infected cell lysates were analysed by immunoblot, no full-length pUL36 was detected for the mutant virus, HSV-1FpUL36Δ548-572 which had the pUL37 binding site deleted, compared to the parental virus, HSV-1FR3537G. We also observed a reduction of pUL36 in our previous study with a single amino acid mutant of the pUL36 binding site in pUL37 (D631A) (Kelly et al., 2012b), indicating that the phenomenon is not specific to the BAC derived HSV-1F strain mutant, HSV-1FpUL36Δ548-572, used here. A similar effect has been reported for the pUL36 homologue in HCMV, pUL48 (Bechtel and

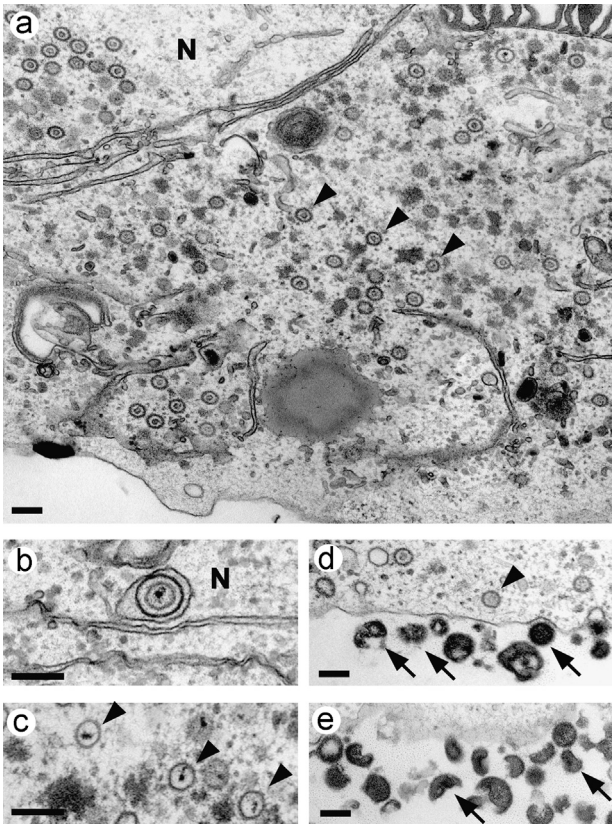

Fig. 6. HSV-1FpUL36Δ548-572 mutant capsids do not undergo secondary envelopment. Vero cells were infected with HSV-1FpUL36Δ548-602, fixed at 23 h p.i., and analysed by electron microscopy. Black arrowheads indicate cytosolic capsids (a–d) that appeared to have left the nucleus (N) by primary envelopment (b) and de-envelopment. No cytoplasmic capsids associated with membrane, or extracellular virions, were observed. Instead many capsidless L-particles were detected (d–e, black arrows). Scale bars equal 200 nm.

Shenk, 2002). Lower levels of pUL48 protein were detected in cells infected with a virus that did not express the pUL37 homologue pUL47, compared with levels in wild type infected cells. The steady state level of pUL48 RNA was shown to be unaffected by the mutation in pUL47, suggesting the level of pUL48 is reduced in the mutant virus at a translational or posttranslational level (Bechtel and Shenk, 2002). A study to characterise the deubiquitinating activity within the N-terminus of HSV-1 pUL36 found that transfected N-terminal variants of pUL36, in particular fragments encompassing the pUL37 binding site, were dependent upon the pUL36 deubiquitinating enzyme activity for accumulation (Bolstad et al., 2011). Unstable variants were stabilised not only by proteasome inhibition, but also by virus infection, indicating a possible role for interaction of protein partners in masking ubiquitination sites or altering protein folding, thus stabilising pUL36 (Bolstad et al., 2011). Our observations here suggest that the binding of pUL37 may play such a role in stabilising pUL36 and in the absence of bound pUL37 there is increased proteolysis of the N-terminal domain of pUL36. Future studies with proteasomal inhibitors such as MG132 will provide further insight into the role of pUL37 in stabilizing pUL36 during HSV-1 replication.

Analysis of growth kinetics, plaque morphology and the use of electron microscopy indicated a block in virion secondary envelopment with an accumulation of cytosolic capsids when the pUL36Δ548-572 mutation was present. The phenotype was similar to that of pUL36 and pUL37 deletion mutants in both HSV-1 and PrV, (Desai et al., 2001; Desai, 2000; Fuchs et al., 2004; Klupp et al., 2001; Leege et al., 2009; Roberts et al., 2009; Sandbaumhuter et al., 2013). Fluorescent microscopy revealed that many individual cytosolic capsids of the mutant virus did not colocalize with pUL37 most likely because the mutant pUL36 no longer bound pUL37. With the progression of infection juxtanuclear regions accumulated high levels of capsid and pUL37, suggesting that the block in secondary envelopment leads to a build up of capsid and tegument proteins at sites where this process should take place. Recruitment of pUL37 to sites of secondary envelopment is known to be independent of capsid but to require pUL36 (Desai et al., 2008). Future studies examining whether capsid and pUL37 colocalize with cellular markers to intracellular membranes will ultimately confirm that the phenotype observed for HSV-1FpUL36Δ548-572 is similar to previous studies with pUL36/pUL37 deletion mutants. Once at the site of secondary envelopment the interaction of pUL36 with pUL37 is essential to drive acquisition of the outer tegument and secondary envelope. In summary, this report defines the region of pUL36 required for binding of its inner tegument protein pUL37 and demonstrates that interaction of these two proteins is essential for secondary envelopment to occur.

Materials and methods

Cell culture and viruses

Vero cells and HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% (v/v) foetal bovine serum (FBS) (JRH Bioscience). The pUL36 complementing cell line (HAUL36-1; kindly provided by Frazer Rixon, MRC Virology unit, University of Glasgow, Glasgow, United Kingdom; (Roberts et al., 2009)) was maintained in DMEM supplemented with 10% FBS, 1x non-essential amino acids (NEAAs) (Invitrogen), and 0.5 mg/ml of Geneticin (Invitrogen). All cells were grown at 37 °C (5% CO_2). The HSV-1F virus (wild type strain F), the parental virus HSV-1FR3537G and the rescuant virus HSV-1FpUL36Δ548-572R were propagated in Vero cells. The pUL36 mutant virus HSV-1FpUL36Δ548-572 was propagated in HAUL36-1 cells. In this study we use HSV-1F strain pUL36 amino acid numbering (Szpara et al., 2010) (total length 3134 amino acids) when referring to our recombinant viruses since they were made in an F strain background. The deleted region of pUL36 (amino acid residues 548–572) is equivalent to the previously determined pUL37 binding site in pUL36 (HSV-1 strain 17, amino acid residues 578–602) (Fig. 1B) (Mijatov et al., 2007). For virus infections, virus inocula were prepared in DMEM supplemented with 2% FBS and 1% penicillin and streptomycin (P/S), with or without NEAA and geneticin, depending on the cell line, and plated onto cells in monolayers for 1 h at 37 °C, unless otherwise specified. After this time fresh DMEM, supplemented as for inocula, was added. For transient transfections, HeLa cells were grown to 80% confluency in 6 well plates. Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

Expression constructs

The HSV-1 full length UL37 ORF encoding amino acids 1–1123 cloned into pCMVmyc has been previously described (Vittone et al., 2005). The pUL36 fragments corresponding to amino acids 317–511 and 512–767 (HSV-1 strain 17 amino acid numbering (McGeoch et al., 1988)) cloned into pEGFP-N1 have been previously described (pUL36N1-GFP and pUL36N2-GFP, respectively; (Kelly et al., 2012b)). The pUL36 N1 and N2 constructs correspond to HSV-1 strain F pUL36 residues 292–481 and 482–737 respectively. An in-frame deletion of pUL36 residues 548–572 (strain F numbering; corresponding to strain 17 amino acids 578–602) was introduced

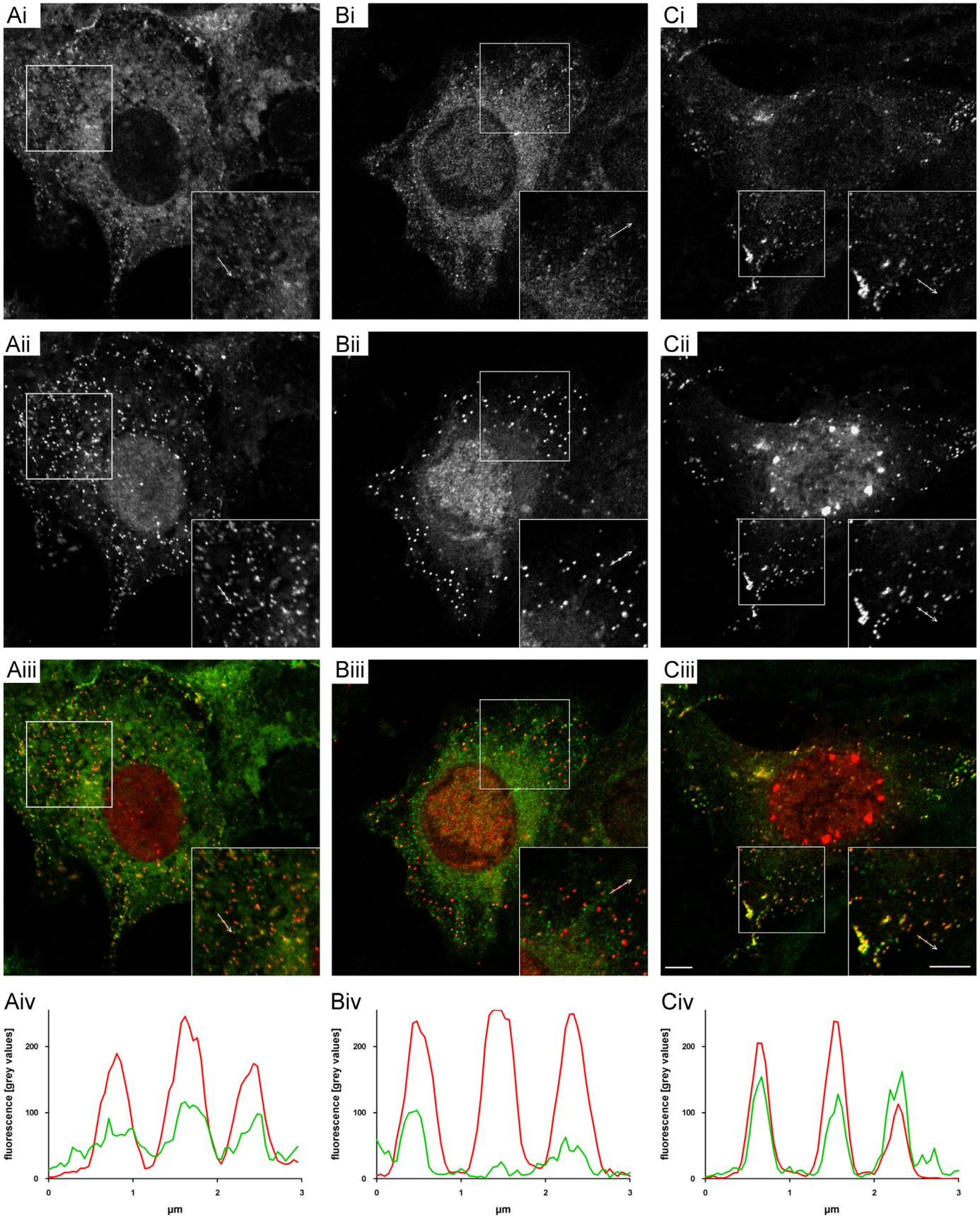

Fig. 7. HSV-1FpUL36Δ548-572 capsids do not colocalise with cytosolic pUL37-GFP. Vero cells were infected with the parental HSV1-1FR3537G (A), the mutant HSV-1FpUL36Δ548-572 (B), or the recuant HSV-1FpUL36Δ548-572R (C), fixed at 12 h p.i., and analysed by confocal laser scanning microscopy. The pUL37-GFP channel is shown in (Ai) to (Ci), the mRFP-VP26 is shown in (Aii) to (Cii), and the merge of both channels is shown in (Aiii) to (Ciii). Insets represent the indicated areas scanned at higher magnification and pixel resolution. Scale bars equal 5 μm. To evaluate the degree of colocalisation (Aiv–Civ) fluorescence was measured along a line highlighted by the arrows and plotted as grey values (green=pUL37-GFP, red=mRFP-VP26) against the length of the line in μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

into pUL36N2-GFP using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) to produce pUL36N2Δ548-572-GFP. This region of pUL36 encompasses the pUL37 binding site (Fig. 1B) (Mijatov et al., 2007). A fragment corresponding to amino acids 1779 to 2647 of Filamin A was cloned into the *Bam*HI site of pCMVmyc. All generated inserts in each expression construct were confirmed by sequencing.

Coimmunoprecipitation

Coimmunoprecipitations were performed as previously described (Kelly et al., 2012b). Briefly, cells were harvested at 24 h post transfection. Mouse anti-IgG (whole molecule) agarose (Sigma) was incubated overnight with 5 μg of mouse anti-myc antibody (Santa Cruz) at 4 °C with gentle rotation. The following day, agarose beads were washed five times with PBS 0.1% (v/v) Triton X-100. Cell lysates were added to the agarose beads and incubated for 3 h at 4 °C with gentle rotation. Agarose beads were harvested by spinning at 500*g* for 5 min and then washed 5 times with 500 μl of wash buffer (PBS containing 0.1% (v/v) Triton X-100). Protein complexes were then eluted into 50 μl of 2X reducing SDS-PAGE sample buffer (Sigma) by heating at 95 °C for 5 min.

Analysis of protein complexes

Proteins were separated by SDS-PAGE and immunoblots were processed as previously described (Kelly et al., 2012b; Vittone et al., 2005). For immunoblots of pUL36, 6% SDS-PAGE was used. Detection was performed with an Odyssey Infrared Imaging system (Licor-Biosciences). Primary antibodies used included mouse monoclonals against GFP (1:1000 dilution; Santa Cruz) and c-myc (1:1000 dilution; Santa Cruz); and rabbit polyclonals against HSV-1 pUL36 (1:1000 dilution) and HSV-1 pUL37 (1:15,000 dilution) (both kindly provided by Thomas Mettenleiter, Institute of Molecular Biology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany; (Leege et al., 2009)). Secondary antibodies for the Odyssey system included goat anti-mouse IRDye 680-conjugated IgG (1:12,000 dilution; Licor) and goat anti-rabbit IRDye 800-conjugated IgG (1:12,000 dilution; Licor).

Construction of recombinant viruses

The two recombinant HSV-1 viruses, HSV-1FR3537G and HSV-1FpUL36Δ548-572, were generated by homologous recombination in *Escherichia coli* SW102 cells, and *galK* selection/counter-selection (Warming et al., 2005) using a bacterial artificial chromosome (BAC)-cloned HSV-1 parental strain F genome (YEbac102; kindly provided by Yasushi Kawaguchi, Tokyo Medical and Dental University, Japan; (Tanaka et al., 2003)). Introduction of the mRFP tag at the N-terminus of capsid protein VP26, where codons 1 to 225 of mRFP are fused to codons 5 to 108 of VP26, has been previously described (de Oliveira et al., 2008). The introduction of a eGFP tag at the C-terminus of tegument protein pUL37 was carried out in a similar manner. Oligonucleotide primers UL37galKF, UL37galKR, UL37GF and UL37GR were used to prepare first and second stage targeting cassettes for this modification (Table 1). To delete the fragment of *UL36* encoding amino acids 548–572 oligonucleotide primers UL36Δ548-572galKF and UL36Δ548-572galKR (Table 1) were used to prepare the first stage targeting cassette. These primers consisted of *galK* homology flanked by 50 bases homologous to either side of the desired deletion in *UL36*, so that in the first stage of recombination *galK* replaced bases 78653–78727 of the pUL36 coding sequence. The second stage targeting cassette consisted of the 100 bases homologous to the HSV-1 strain F genome, either side of where *galK* was inserted. These 100 bp single stranded oligonucleotides, UL36Δ548-572s and UL36Δ548-572as (Table 1), were heated to 100 °C and allowed to cool to room temperature (RT) for 30 min to anneal. The DNA was ethanol precipitated and quantified by running a sample on a 1% (w/v) agarose gel, alongside a known molecular weight standard. For each electroporation, at least 200 ng of targeting cassette was used. Clones which had successfully replaced *galK* with the desired modification were identified by restriction enzyme analysis using *Bsp*119I digestion.

To release the viral genome from the BAC backbone, modified HSV-1 BAC DNA was cotransfected with a Cre recombinase-expressing plasmid into Vero cells. In the case of HSV-1FpUL36Δ548-572, cotransfections were carried out in the pUL36-complementing HAUL36-1 cell line to compensate for any effect the deletion may have on production of infectious virus. Once the HSV-1FpUL36Δ548-572 mutant had been constructed a rescuant virus was prepared as previously described (Roberts et al., 2009). Briefly, oligonucleotide primers RescUL36F and RescUL36R (Table 1), containing *Eco*RI restriction enzyme sites, were used to PCR amplify a 2.7 kb region spanning bases 77663–80388 of the wild type F strain pUL36 coding region, which encompassed the region deleted in HSV-1FpUL36Δ548-572. The resulting fragment was cloned into pUC18 plasmid to produce the transfer vector pUC18-UL36resc. Vero cells were transfected with pUC18-UL36resc before being infected with the mutant HSV-1FpUL36Δ548-572 at a multiplicity of infection (MOI) of 1 pfu/cell. Resulting virus was harvested from both cells and culture medium and plaque purified on Vero cells to isolate rescued virus which produced wild type plaques on this non-complementing cell line. Once the mutant and rescuant had been prepared, viral DNA was purified and used to sequence the pUL36 region of interest to confirm the presence or absence of the Δ548–572 deletion.

Growth kinetics

To assess the growth kinetics of each virus, Vero cells were infected at an MOI of 10 pfu/cell. After 1 h incubation at 37 °C, virus inocula were removed and cells were incubated at RT for 1 min with citric acid buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3.0]) to inactivate any remaining, non-penetrated virus. Citric acid buffer was removed, cells were washed twice with PBS and fresh medium was added. Virus was harvested at 4, 8, 16 and 24 h p.i. Supernatant containing extracellular virus was removed and sonicated (60 s) before titration by plaque assay. Infected cell monolayers were washed twice with PBS before addition of 1 ml of fresh medium. Cells were scraped into medium and sonicated 3×60 s before titration by plaque assay.

Plaque morphology

Vero cells and HAUL36-1 cells were seeded on coverslips in 24 well plates. Serial dilutions of virus were prepared and plated. After incubation for 2 h, virus inocula were removed and cells were overlaid with a 1:1 solution of carboxymethylcellulose (CMC; 3.2%):DMEM supplemented with 2% FBS and 1% P/S. Cells were fixed at 72 h p.i. with 4% paraformaldehyde and coverslips were mounted onto slides using Slowfade® Gold antifade reagent (Invitrogen). Plaque morphology was analysed using an Olympus confocal laser scanning microscope. Image processing was carried out using FV10-ASW (Olympus) and Adobe Photoshop software.

Electron microscopy

Vero cells were seeded onto coverslips 1 day prior to infection. The cells were pre-cooled for 20 min on ice, and incubated with a virus dose of 1 pfu/cell in CO_2-independent medium containing

Table 1
Oligonucleotides used to generate recombinant viruses. (Homology arms for BAC recombination are underlined. EcoRI restriction sites are shown in italics.)

Name	Sequence
UL37galKF	accataacaccgagaacaacacacgggggtgggcgtaacataataaagctcctgttgacaattaatcatcggca
UL37galKR	cggaggacaccgtcgcgccacccacggacttgccgttaactagttaccaatcagcactgtcctgctcctt
UL37GF	accataacaccgagaacaacacacgggggtgggcgtaacataataaagctttacttgtacagctcgtccatgcc
UL37GR	cggaggacaccgtcgcgccacccacggacttgccgttaactagttaccaagtgagcaagggcgaggagctgttc
UL36Δ548-572galKF	agggtaaactccagcagggcggcggccgggccggccaccccggcctgggtcctgttgacaattaatcatcggca
UL36Δ548-572galKR	cgcggctcaccatcagcgccttacggtcgccgtatccggcttctccggggtcagcactgtcctgctcctt
UL36Δ548-572s	agggtaaactccagcagggcggcggccgggccggccaccccggcctgggtccccggagaagccggatacggcgaccgtaaggcgctgatggtgagccgcg
UL36Δ548-572as	cgcggctcaccatcagcgccttacggtcgccgtatccggcttctccggggacccaggccggggtggccggcccggccgccgccctgctggagtttaccct
RescUL36F	attcag*aattc*gtccagcggcttgcgttc
RescUL36R	agctag*aattc*cctctggccagtagggcac

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% CO_2 for 1 h. Non-internalised 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. After 22 h, the cells were fixed with 2% (w/v) glutaraldehyde in 130 mM cacodylate buffer at pH 7.4 containing 2 mM $CaCl_2$ and 10 mM $MgCl_2$ for 1 h at room temperature. Subsequently the cells were washed and postfixed for 1 h with 1% (w/v) OsO_4 in 165 mM cacodylate buffer at pH 7.4 containing 1.5% (w/v) $K_3[Fe(CN)_6]$, 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 an Eagle 4 k camera at a Tecnai G2 electron microscope at 200 kV (FEI, Eindhoven, the Netherlands).

Confocal laser scanning microscopy

Vero cells were infected with the parental HSV1-1FR3537G, the mutant HSV-1FpUL36Δ548-572, or the recuant HSV-1FpUL36Δ548-572R as described for electron microscopy. At 12 or 23 h p.i. cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature and embedded with Prolong Antifade (Invitrogen). Fluorescence microscopy was performed with a Zeiss LSM510 confocal laser scanning microscope and a 63x objective, NA 1.4; a pinhole aperture of 1 Airy unit. Lower magnifications were taken with a pixel size of 70 × 70 nm, higher magnifications (insets in Fig. 7) with a pixel size of 50 × 50 nm. Fluorescence was measured at the 8 bit images of the high magnification along a 1 pixel thick and 3 μm long line using the "plot profile" tool of the software ImageJ (vers. 1.48K).

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