Characterization of the herpes simplex virus (HSV)-1 tegument protein VP1-2 during infection with the HSV temperature-sensitive mutant tsB7

F. Abaitua,1 R. N. Souto,2† H. Browne,2 T. Daikoku1† and P. O’Hare1

1Marie Curie Research Institute, The Chart, Oxted RH8 0TL, Surrey, UK
2Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

VP1-2, encoded by the UL36 gene of herpes simplex virus (HSV), is a large structural protein, conserved across the family Herpesviridae, that is assembled into the tegument and is essential for virus replication. Current evidence indicates that VP1-2 is a central component in the tegumentation and envelopment processes and that it also possesses important roles in capsid transport and entry. However, any detailed mechanistic understanding of VP1-2 function(s) remains limited. This study characterized the replication of HSV-1 tsB7, a temperature-sensitive mutant restricted at the non-permissive temperature due to a defect in VP1-2 function. A tsB7 virus expressing green fluorescent protein-fused VP16 protein was used to track the accumulation and location of a major tegument protein. After infection at the permissive temperature and shift to the non-permissive temperature, the production of infectious virus ceased. VP1-2 accumulated in altered cytosolic clusters, together with VP16 and other virion proteins. Furthermore, correlating with the results of immunofluorescence, electron microscopy demonstrated abnormal cytosolic capsid clustering and a block in envelopment. As VP1-2 encompasses a ubiquitin-specific protease domain, the occurrence of ubiquitin-conjugated proteins during tsB7 infection was also examined at the non-permissive temperature. A striking overaccumulation was observed of ubiquitin-specific conjugates in cytoplasmic clusters, overlapping and adjacent to the VP1-2 clusters. These results are discussed in relation to the possible functions of VP1-2 in the assembly pathway and the nature of the defect in tsB7.

INTRODUCTION

Formation of the herpesvirus virion requires the spatially and temporally regulated interaction of numerous virus–host and virus–virus factors, and progressive recruitment of individual components or pre-formed subassemblies. The tegument compartment, between the capsid and the envelope, is composed of a complex repertoire of proteins recruited in different stoichiometries, and comprising both essential and non-essential components (Mettenleiter, 2002; Rixon, 1993). The tegument protein VP1-2, encoded by the UL36 gene, is an essential protein and is conserved across the family Herpesviridae (Desai, 2000; Fuchs et al., 2004; Knipe et al., 1981; Lee et al., 2006; Luxton et al., 2006; Roberts et al., 2009).

VP1-2 is synthesized late during infection and is recruited into virions at a relatively low abundance of between 60 and 120 copies per virion (Heine et al., 1974; McNabb & Courtney, 1992a, b). It is classed as an inner tegument protein, required for recruitment of additional components, and, at least in pseudorabies virus (PrV), is tightly bound to the capsid during entry (Gibson & Roizman, 1972; Granzow et al., 2005; Klupp et al., 2002; Luxton et al., 2005; Michael et al., 2006). VP1-2 localizes to both the cytoplasm and the nucleus of infected cells (Abaitua & O’Hare, 2008; McNabb & Courtney, 1992b). Recent work in herpes simplex virus (HSV) has provided evidence in support of VP1-2 recruitment to type C capsids within the nucleus (Bucks et al., 2007), possibly representing initial events in the tegumentation process. On the other hand, although the protein may be associated with capsid pentons, possibly by interaction with UL25 (Trus et al., 2007; Zhou et al., 1999), in further analysis of capsid structure by cryoelectron microscopy, VP1-2 was not found to be associated with purified nuclear capsids (Trus et al., 2007). Consistent with these latter observations, immunogold analysis of PrV-infected cells failed to detect VP1-2 in primary enveloped virions in the perinuclear space (Klupp et al., 2002).
Additional studies have examined UL36 deletion mutants to elucidate the role and site of capsid recruitment. In HSV-1, a mutant lacking intact VP1-2 accumulated numerous cytosolic DNA-containing capsids that did not mature into enveloped virus (Desai, 2000), with little evidence of a significant defect in capsid nuclear egress. This phenotype was also observed in a recent analysis of an independent UL36 deletion virus (Roberts et al., 2009), and similar results were observed for a PrV UL36-negative virus (Fuchs et al., 2004). In contrast, in PrV, it has been reported that deletion of UL36 has a more profound effect on nuclear exit, with capsids largely being restricted to the nucleus, and cytoplasmic capsids seen in only a minor population of infected cells (Luxton et al., 2006).

To aid our understanding of VP1-2 function, we examined VP1-2 in the context of the HSV temperature-sensitive (ts) virus tsB7. Analysis of this mutant virus provided the first demonstration of an essential role for VP1-2 in HSV replication (Batterson & Roizman, 1983; Batterson et al., 1983; Knipe et al., 1981). During infection with tsB7 at the non-permissive temperature, replication is blocked at a very early stage, virus gene expression does not take place and capsids apparently accumulate at the nuclear pore. A second defect in tsB7 was identified from temperature-shift experiments, where initial infection was carried out at the permissive temperature, allowing entry and gene expression, followed by a shift to the non-permissive temperature. This delayed shift to the non-permissive temperature resulted in the shut-off of late protein synthesis, although no analysis of virus replication or expression and localization of VP1-2 were reported. However, both defects were mapped to the UL36 gene by marker-rescue experiments.

The ability to manipulate the functional state of VP1-2 by temperature shift allows analysis of tsB7 infection, a potentially useful approach that might overcome some of the limitations of studies using deletion mutants and aid in our understanding of structure–function relationships. In the present work, using tsB7 infection and temperature-shift assays, combined with immunofluorescence and electron microscopy, we observed pronounced aberrant clustering of the VP1-2 protein in the cytoplasm of infected HEp-2 cells, with associated accumulation of polyubiquitinated protein species. These data are discussed in relation to the location and role of VP1-2 in assembly and egress.

**METHODS**

**Cells and viruses.** HEp-2, Vero, HS30 (Desai, 2000) and COS cells were grown in Dulbecco’s modified minimal essential medium (DMEM; Gibco) containing 10% newborn calf serum (NCS) and penicillin/streptomycin. HS30 cells contain the HSV-1 (KOS strain) full-length VP1-2 gene and complement the ts defect in tsB7 (Desai, 2000). These cells were maintained in DMEM containing 500 μg geneticin (Gibco) ml⁻¹. Mutant ts virus tsB7 and its parental strain HFEM were kind gifts of Dr A. Buchan (Knipe et al., 1981). Titrations of tsB7 were performed in Vero cells at 33°C. The UL36 deletion virus KAU36 (Desai, 2000) was propagated on the complementing cell line HS30. The UL37 deletion virus FRAUL37 was supplied by F. Rixon (Roberts et al., 2009). Infections were routinely performed at 4°C at an m.o.i. of 5. After 1 h, the monolayers were washed with DMEM and incubation continued in DMEM containing 2% NCS at 33 or 39°C.

**Construction of tsB7.GD1 recombinant virus.** To construct the tsB7 recombinant virus expressing green fluorescent protein (GFP)-fused VP16 (tsB7.GD1), as outlined in Fig. 1(a), subconfluent monolayers of COS cells were co-transfected with infectious genomic DNA from tsB7 together with the plasmid pSLA4 encoding GFP-VP16 flanked by sequences for recombination at the UL48 locus, as described previously (La Boissiere et al., 2004). Transfections were performed using the calcium phosphate precipitation technique with N,N-bis(2-hydroxy)-2-aminoethanesulfonic acid-buffered saline. Infected cells were screened 4 days later for the presence of fluorescent plaques expressing GFP-VP16. Isolated tsB7.GD1 was purified by serial dilution and three successive single plaque isolations on Vero cells. Although beyond the scope of this initial work, our long-term aim in constructing GFP-tsB7 recombinants is to examine the dynamics of localization of a series of structural components, including tegument and capsid proteins, during the temperature block and also to determine whether reversal experiments may result in a renewed productive cycle and associated transport.

**Infections and temperature-shift assays.** Prior to virus adsorption, cell monolayers were kept at 4°C for 10 min and washed with cold DMEM. The virus inoculum (m.o.i. 5) was then added in cold DMEM without serum. After 1 h at 4°C, monolayers were washed with cold DMEM, and DMEM containing 2% NCS and pre-warmed to 39°C was added. The cells were further incubated at 33 or 39°C. For temperature-shift experiments, after 10 h at 33°C, infected cultures were transferred to 39°C for further incubation. Cultures were transferred to a water bath pre-warmed to 39°C, to raise the temperature as synchronously and rapidly as possible. Cultures incubated continuously at 33 or 39°C or the shifted samples were collected at 24 h post-infection (p.i.) for analysis.

**Immunofluorescence studies.** Immunofluorescence analysis was performed as described previously (Abaitua & O’Hare, 2008) using the following antibodies: anti-VP5 (diluted 1:1000; East Coast Bio), anti-VP1-2, (zVP1-2NT1r, 1:250; Abaitua & O’Hare, 2008), anti-VP16 (LP1, 1:400; McLean et al., 1982), anti-VP26 (1:200; a kind gift of P. Desai, Johns Hopkins Medical School, MD, USA), anti-polyubiquitin (FK2, 1:200; Biomas International), anti-TGN46 (1:200; Serotec) and anti-β-COP (1:100; ABR). After incubation with the primary antibody diluted in PBS with 10% NCS (45 min at room temperature), coverslips were washed with PBS and incubated with fluorochrome-conjugated (Alexa Fluor 488, Alexa Fluor 543 or Pierce DyLight 549) secondary antibodies as appropriate. Images were routinely acquired using a Plan-Apochromat ×63 oil-immersion objective lens, NA 1.4 and zoom factors ranging from 3 to 8 of the LSM410 acquisition software.

**Live-cell microscopy analysis.** Cells for live analysis of GFP expression were plated into two-well coverglass chambers (Quadrachem Laboratories). Mock or infected cells were examined using a Zeiss LSM410 inverted confocal microscope and images were collected at different times after infection.

**Electron microscopy.** Cells for electron microscopy were prepared as follows. At 24 h p.i., 100 mm dishes of infected and mock cells were washed with PBS and fixed in 4% formaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. The monolayers were then scraped into 1 ml fixative and centrifuged at 11 330 g for 15 min. The fixative was carefully removed and the pellet washed three times in 0.1 M phosphate buffer,
followed by cryoprotection, freezing, freeze substitution and low-temperature embedding as described by Skepper et al. (2001). Sections were examined using a Philips CM100 transmission electron microscope.

**SDS-PAGE and Western blotting.** Electrophoresis on gradient gels and immunodetection were performed exactly as described previously (Abaitua & O’Hare, 2008). Antibodies for Western blotting were used as follows: anti-VP16 (LP1, 1:2000), anti-VP5 (East Coast Bio, 1:3000), anti-VP1-2 (1:250; Abaitua & O’Hare, 2008), anti-polyubiquitin antibody (FK2, 1:1000; Biomol International) and anti-GFP (Abcam, 1:2000).

**RESULTS**

**Construction of a recombinant mutant tsB7 virus expressing GFP–VP16**

To aid in the analysis of the defect in tsB7, we generated a version of this virus encoding VP16 as a candidate tegument protein, fused to GFP (Fig. 1a). Purified tsB7 genomic DNA was co-transfected with plasmid pSL44 (La Boissiere et al., 2004) and GFP–VP16-expressing plaque-purified stock was isolated at 33 °C as described in Methods. The recombinant virus, tsB7.GD1, expressed GFP–VP16 at similar levels to VP16 expression from the parental tsB7 virus at 33 °C (Fig. 1b). The ts defect measured by the plaque-forming ratio in HEp-2 cells at 39 °C (open columns) or 39 °C (filled columns) are shown. (d) Upper panels: live-cell images of HEp-2 cells infected with tsB7.GD1 at 33 °C (i, ii) or 39 °C (iii, iv), at 10 and 24 h p.i. as indicated. Lower panels: fluorescent plaque formation by tsB7.GD1 on Vero cells and the complementing HS30 cell line at 33 °C (v, vi) and 39 °C (vii, viii).

Live-cell analysis (Fig. 1d) of GFP–VP16 expression in tsB7.GD1-infected HEp-2 cells at the permissive temperature [Fig. 1d(i) and (ii)] showed a similar pattern of distribution to that reported previously in the context of wild-type HSV strain 17 (La Boissiere et al., 2004). GFP–VP16 was initially observed in a diffuse nuclear pattern (6 h p.i., data not shown), with subsequent accumulation in foci on the periphery of nuclear replication.
compartments and the appearance of more intense cytoplasmic accumulations (Fig. 1d, 10 and 24 h p.i.). As expected, cells infected at the non-permissive temperature showed no newly synthesized GFP pattern [Fig. 1d(iii) and (iv)]. The fluorescent puncta seen at the non-permissive temperature represent progressive uptake of residual virus or non-infectious material from the inoculum, but there was no productive infection. Growth of tsB7.GD1 at the restrictive temperature was complemented in the VP1-2-containing cell line HS30 [Fig. 1d(viii)], as demonstrated by fluorescent plaque formation after low-multiplicity infection at the non-permissive temperature, in contrast to control cells [Fig. 1d(vii)].

**Altered localization of GFP–VP16 during tsB7.GD1 temperature-shift assays**

No previous data have been reported on the effect of the ts mutation in VP1-2 on the localization of tegument proteins, or on actual yields of infectious virus when infection is initiated at 33°C, to allow entry, followed by incubation at 39°C to cause VP1-2 dysfunction. We wished to pursue this by performing experiments in which infection was initiated and allowed to proceed at 33°C to intermediate times (10 h p.i.), followed by a shift to 39°C, the non-permissive temperature, and continued incubation to 24 h p.i. We first examined the effect of this temperature-shift regime on the yield of infectious virus. The results (Fig. 2a) demonstrated that, in contrast to the increasing yield during continual incubation at 33°C (Fig. 2a, filled circles), a temperature shift to 39°C at 10 h p.i. (shaded circles) resulted in no further increase and significant loss in infectious virus. To examine whether overall levels of viral proteins were affected by the temperature-shift regime compared with continual incubation at the permissive temperature, extracts were made from parallel cultures and analysed for the accumulation of VP5, VP16 and VP1-2 (Fig. 2b). The results demonstrated similar levels of these proteins compared with infection at 33°C, although some reduction was observed for VP1-2 itself. In contrast, there was no expression during continual infection at 39°C, as expected from the original characterization of tsB7.

We next examined the effect of the temperature shift on GFP–VP16 location in live cells. At 10 h p.i. at 33°C, VP16 was observed in a nuclear location in replication compartments, together with a diffuse cytoplasmic population and the beginnings of small vesicular clusters [Fig. 2c(ii)]. By 24 h p.i. at 33°C, GFP–VP16 had accumulated in more cytoplasmic clusters and also exhibited a relatively homogeneous nuclear pattern of distribution with some nuclear dots [Fig. 2c(ii), arrowhead].

The localization changed dramatically when infection was shifted to the non-permissive temperature. A highly prominent feature was GFP–VP16 accumulation in a single perinuclear cluster, frequently at the indent of a kidney-shaped nucleus [Fig. 2c(iii), arrows; see also Figs 3–5].

Whilst cytosolic vesicular-type GFP–VP16 was routinely observed during progressive infection, this feature of a large singular aggregation was not observed during infection at elevated temperatures with the parental strain HFEM (Fig. 2d). It should be noted that, in initial experiments, we also examined the characteristics of altered localization in Vero cells. Vero cells at the normal permissive temperature showed a distinct pattern of GFP–VP16 accumulation in foci around the periphery of nuclear replication compartments, which became even more intense following the temperature shift (data not shown). We do not presently understand the reason for the difference in localization in the two cell types, but we found that the temperature-shift regime resulted in a more pronounced breakdown of VP1-2 in Vero cells, making subsequent interpretation of localization difficult. As a result, we confined our analysis in this work to HEp-2 cells only.

**Altered VP1-2 localization during temperature shift**

To examine whether the altered localization of GFP–VP16 reflected altered localization of VP1-2 itself, and whether representative components of the virion, such as the VP5 major capsid protein, were also altered, we followed the same regime of infection and temperature shift used above, and examined localization of these components by immunofluorescence.

In infected HEp-2 cells undergoing a temperature shift (Fig. 3), the singular GFP–VP16 cytosolic accumulations [Fig. 3a(vii), arrows] clearly co-localized with pronounced accumulation of VP1-2 [Fig. 3a(viii) and (ix), arrows]. This altered localization of VP1-2 was particularly prominent, shifting from the vesicular cytoplasmic pattern seen at the permissive temperature (Fig. 3a, v) to a striking single large cytoplasmic focus at the non-permissive temperature [Fig. 3a(viii)]. We also noted a minor population of VP1-2 in small punctate foci on the periphery of replication compartments (marked by GFP–VP16 distribution) after the temperature shift [Fig. 3a(viii), arrowheads]. The congregation of VP1-2 with co-localizing GFP–VP16 is shown at a higher magnification in Fig. 3(b), where virtually all of the population of VP1-2 accumulated in a singular cluster. In similar experiments, we examined localization between VP1-2, GFP–VP16 and the capsid protein VP5 during the temperature-shift regime (Fig. 3c). The pronounced clusters containing VP1-2 and GFP–VP16 also contained accumulated VP5 [Fig. 3c(i–iii), arrows]. In control infections at the permissive temperature, cytoplasmic accumulation of VP5 in such a cluster was rarely observed (data not shown).

We wished to examine whether the singular prominent cluster contained or represented candidate components of the secretory pathway, particularly the Golgi compartment (Fig. 4). However as reported previously (Ward et al., 1998), we routinely observed dispersal or loss of Golgi
Altered localization and function of the tsB7 VP1-2

Fig. 2. Defective replication and abnormal localization of GFP–VP16. (a) Monolayers of HEP-2 cells were infected with tsB7.GD1 at 4 °C. After adsorption, the cells were incubated at 39 or 33 °C. A set of samples infected at 33 °C was shifted to 39 °C at 10 h p.i. At different times, medium from infected cultures of each temperature regimes was harvested and the yield of extracellular virus was determined by plaque titration at 33 °C: yield after infection at 33 °C (filled circles), 39 °C (open circles) or 33 °C followed by a temperature shift to 39 °C (shaded circles). (b) Accumulation of the viral proteins VP5, VP16 and VP1-2, determined by Western blot analysis. (c) Live-cell images acquired at 10 and 24 h p.i. (i, ii) from HEP-2 cells infected with tsB7.GD1 at 33 °C or after a temperature shift (iii). Arrows indicate the cytosolic accumulation of GFP–VP16 detected after the temperature shift (iii), whilst the arrowheads (ii) indicate the nuclear foci of GFP–VP16 observed at the permissive temperature. (d) Control experiments showing the localization of VP16 by immunofluorescence after infection with the parental strain HFEM at 33 or 39 °C. No cytoplasmic aggregation was observed.

markers, making co-localization studies difficult [e.g. compare Fig. 4(i) and (ii)]. Nevertheless, using TGN46 as a representative marker of the trans-Golgi, we observed some overlap with GFP–VP16 localization during infection at 33 °C [Fig. 4(ii) and (iii), arrows] and co-localization of at least a subpopulation of TGN46 into the large cluster observed during the temperature-shift regime [Fig. 4(v) and (vi), arrows]. This was seen more readily in co-localization of the VP1-2 cluster and TGN46 [Fig. 4(viii) and (ix), arrow]. For certain other markers, it was difficult to make definitive conclusions due to significant loss during infection, whilst other markers, such as the coat protein β-COP, clearly showed no recruitment to the cluster (data not shown). The results indicated that the coalescing clusters formed by malfunction of VP1-2 may be associated with the disrupted trans-Golgi network, or at least with certain trans-Golgi network components.
Ultrastructural characterization of the tsB7 defect

The simplest interpretation of the immunofluorescence studies is that the ts defect in VP1-2 resulted in its singular juxta-nuclear accumulation, with the resultant co-accumulation of tegument and capsid proteins and a block in the normal assembly pathway.

To further pursue characterization of the ts defect, we next performed ultrastructural analysis by electron microscopy. At the permissive temperature, we observed extracellular virions, as expected, together with numerous single particles in the cytoplasm and occasional small clusters of particles around electron-dense material (Fig. 5a). In contrast, in cells shifted to the non-permissive temperature, no extracellular virions were detected, and a large cytosolic accumulation containing non-enveloped capsids was readily seen in individual cells, a typical example of which is shown in Fig. 5(b). We observed no significant difference in capsid assembly within the nucleus in HEp-2 cells at the non-permissive temperature compared with the permissive temperature.

Alteration of polyubiquitinated proteins

The nature of the ts defect in tsB7 could reflect misfolding of VP1-2, resulting in failure in its recruitment to the proper location or to recruit other tegument proteins, with a consequent defect in assembly in the cytosol. In this context, and as consequence of misfolding, VP1-2 could be targeted by cellular chaperones and/or the degradation machinery. A related but mechanistically distinct possibility for the ts defect could be with its single known enzymic activity, i.e. the function of its N-terminal ubiquitin-specific protease (USP) domain, rather than with its structural role per se. In this context, we wished to examine whether accumulated VP1-2 co-localized with accumulation of ubiquitin-conjugated proteins and whether we could observe any alteration in ubiquitinated species that might be attributed to a non-functional USP activity. We examined mock-infected cells and cells infected with tsB7 and HFEM, using the same schedule of infection and temperature shift as before, together with the FK2 antibody. This antibody specifically recognizes ubiquitin conjugated to other proteins but not free-monomer ubiquitin (Fujimuro et al., 1994) and has been used extensively to detect intracellular conjugated ubiquitin by immunofluorescence.

Uninfected cells showed a diffuse, predominantly nuclear localization of FK2 signal, with additional nuclear speckles. This distribution was roughly similar at both temperatures, although we noted increased nuclear aggregates at the elevated temperature in uninfected cells [Fig. 6a(i) and (ii)]. Infection at 33 °C resulted in a generalized loss of the FK2 signal, but some residual (or potentially induced) small nuclear foci were observed [Fig. 6a(iii–v), and higher-magnification images (vi–viii)]. There was little or no FK2 signal in the cytoplasm and what little signal was detected showed no co-localization or enrichment with cytoplasmic VP1-2 during infection at the permissive temperature [Fig. 6a(iii–viii)].

In striking contrast, tsB7 infection shifted to the non-permissive temperature resulted in a highly pronounced
accumulation of cytosolic FK2-positive signal [Fig. 6a(ix–xi), arrows]. The FK2 accumulations showed a strong but not completely overlapping relationship with the VP1-2 cytosolic accumulation. In each case, there was a single major aggregate, but the FK2 signal was frequently juxtaposed or wrapped around partially co-localizing VP1-2. This feature can be observed more clearly in the higher-magnification images [Fig. 6a(xii–xiv)]. In further experiments examining the localization of FK2 in relation to GFP–VP16, a very similar effect was observed, with FK2-reactive species accumulating in a single major aggregate that overlapped but did not completely co-localize with the accumulated GFP–VP16, an example of which is shown in Fig. 6(b). In control experiments, infection with the parental HSV-1 (HFEM) showed the same reduction in nuclear FK2 signal, independent of the temperature, with no discernible cytosolic accumulation of FK2 or VP1-2 (data not shown).

We next asked, with regard to the accumulation of polyubiquitinated species, whether a similar phenotype was also observed with the KAUL36 virus, which lacks intact VP1-2. This virus is now known to express a truncated N-terminal region, which would encompass the USP (Roberts et al., 2009). Consistent with the original characterization (Desai et al., 2001), we observed cytoplasmic foci containing the capsid components VP5 and VP26 [Fig. 7a(i–iii), arrows]. We noted a difference in this localization from that seen with tsB7, in that for KAUL36 the cytoplasmic foci were smaller and usually more numerous, whilst for tsB7, a single large cluster was mostly observed. Furthermore, when examining the localization of polyubiquitinated species, it was clear that, despite accumulated cytosolic components, there was no evidence for and no association with any polyubiquitinated foci [Fig. 7a(iv–vi)]. Lack of polyubiquitin accumulation was also observed after infection with this virus at the elevated temperature used for investigation of tsB7 (data not shown).

We asked a similar question for a virus mutant lacking UL37, which in this case expressed a full-length UL36, but was blocked in egress and assembly due to lack of the essential protein UL37 (Desai et al., 2001; Leege et al., 2009; Roberts et al., 2009). Again consistent with previous

Fig. 4. Localization of a trans-Golgi network marker in aberrant cytoplasmic clusters. Monolayers of cells were infected with tsB7.GD1 (i, iii, v, vi) or tsB7 (viii, ix) and incubated at 33 °C, and a subset was shifted to 39 °C at 10 h p.i. At 24 h p.i., cells were fixed and the distribution GFP–VP16 (ii, iii, vi) or VP1-2 (vii, ix) was compared with that of the cellular Golgi protein TGN46. In mock-infected cells, TGN46 showed no significant difference in localization (i, iv). In infected cells at both temperatures, TGN46 localization was disrupted with reduced abundance, making localization difficult. Nevertheless, TGN46 was observed to be present and incorporated in the cytoplasmic accumulation at 39 °C. Arrows indicate the cytoplasmic accumulation of GFP–VP16 (v, vi) or VP1-2 (viii, ix) co-localizing with TGN46 in cells after temperature shift.

http://vir.sgmjournals.org 2359
reports, we observed foci of the capsid component VP26 in cytosolic aggregates, although, as for the KΔUL36 comparison, these were smaller in size, rather than the singular large cluster seen with tsB7. Also, as with KΔUL36, infection with the ΔUL37 virus did not result in accumulation of polyubiquitinated species co-localizing with the capsid clusters [Fig. 7b(i–iii)]. These results are discussed below in relation to the phenotype of tsB7.

**DISCUSSION**

The earliest results indicating an essential role for VP1-2 in HSV replication came from examination of the phenotype of the ts mutant tsB7, which exhibited defects in a very early aspect of virus entry and a second later defect in replication (Batterson & Roizman, 1983; Batterson et al., 1983; Knipe et al., 1981). Both defects were reported by marker-rescue experiments to reside in the UL36 gene. The defect in the later aspects of virus replication was identified in temperature-shift experiments where infection was initiated at the permissive temperature, allowing entry and gene expression, followed by a shift to the non-permissive temperature. This regime resulted in reduced late gene expression, unless infection was allowed to proceed for at least 4 h at the permissive temperature prior to the shift, in which case late protein expression was relatively normal. However, the effects of the ts mutation on synthesis and localization of VP1-2 itself or on other candidate proteins have not been reported. Here, we showed, consistent with the original analysis, that a shift up to the non-permissive temperature after 10 h had relatively little effect on the accumulation of late proteins. However, the production of infectious virus rapidly declined. This decline is likely to be due to a combination of the thermosensitivity of pre-formed infectious virus combined with cessation of the production of new virus. We showed a pronounced feature of the ts defect in that VP1-2 and associated structural proteins coalesced in a singular large perinuclear cluster, which contained at least some Golgi components. These clusters were seen by electron microscopy to represent coalesced capsids that were not progressing to the envelopment stage.

This ts phenotype in VP1-2 resembles aspects of the phenotype resulting from partial deletion (Desai, 2000) or full deletion (Roberts et al., 2009) of the protein, although interestingly there are differences between the two. Thus, the HSV partial deletion mutant KΔUL36 (containing intact VP1-2 from the complementing line) infects cells,
expresses late proteins, including an N-terminal truncation product of VP1-2 itself (Roberts et al., 2009), and produces DNA-filled cytosolic capsids. However, the lack of intact VP1-2 results in failure of the mutant particles to become enveloped, possibly due to a failure in recruitment to a cytoplasmic maturation compartment. This was accompanied by aggregation of cytoplasmic capsids as seen by electron microscopy (Desai, 2000). However, in a subsequent study of a mutant HSV lacking the entire UL36 gene whilst the main defect in cytoplasmic envelopment was also observed, the capsid clustering phenotype was not observed (Roberts et al., 2009). It was speculated that, in the HSV partial deletion mutant, the presence of the N-terminal fragment of VP1-2, encompassing the USP domain, could be involved in capsid clustering, and indeed the fragment was observed to co-fractionate with capsids. The proposal for VP1-2 involvement in capsid aggregation in mutant or defective situations has also been suggested based on observations of capsid clustering in mutant viruses of HSV and PrV lacking UL37 or the UL37-binding site in VP1-2 (Desai et al., 2001; Fuchs et al., 2004; Klupp et al., 2001). The clustering we observed in tsB7 at the non-permissive temperature, taken together with the observed clustering in the VP1-2 deletion mutant retaining the N-terminal fragment versus the complete deletion, is consistent with the proposal of altered presentation or a role of the N-terminal USP-containing region due to the tsB7 defect.

Considering this, we examined the distribution of ubiquitin-conjugated species in infected cells at the permissive and non-permissive temperatures. During productive infection at 33 °C, we observed a generalized loss of ubiquitin-conjugated species, although with some retention of small punctate nuclear foci. This effect is similar to that reported previously (Burch & Weller, 2004), although these authors also observed some increase in diffuse cytoplasmic ubiquitin-conjugated species, an effect we have not observed. In fact, we observed very few ubiquitin-conjugated cytoplasmic species during the course of normal infection. In striking contrast, we observed a major accumulation of ubiquitin-conjugated species when infection was shifted to the non-permissive temperature, an effect not observed in control experiments in either mock-infected cells or cells infected with the parental virus.

There are several non-mutually exclusive explanations for the accumulation of ubiquitin-conjugated species during tsB7 infection at the non-permissive temperature. One explanation could be, as outlined above, that altered folding of VP1-2 at the non-permissive temperature results in its being recognized as a misfolded protein by the cellular machinery and ubiquitinated in preparation for degradation. This could occur whether or not VP1-2 is a protein involved in the ubiquitination pathway. Therefore, a variant of this explanation could be that VP1-2 is itself more specifically modified by ubiquitination, that it is subject to autocatalytic de-ubiquitination and that at the non-permissive temperature this activity is eliminated with a resultant increase in its own ubiquitin content. However, it seems unlikely that the increase in ubiquitin-conjugated species seen by immunofluorescence represents solely an increase in ubiquitination of VP1-2. (We are currently attempting to examine temperature-dependent VP1-2 ubiquitination biochemically but this has proved technically difficult due to the size of VP1-2, with any modification likely to result in an undetectable change in migration, coupled with the appearance of VP1-2 as multiple species even under normal conditions.) More importantly, it was clear that the accumulated ubiquitin-conjugated species did not simply co-localize with VP1-2, but rather formed a distinct and tightly associated cluster around the accumulated VP1-2 and other tegument proteins. This could therefore represent accumulation of other viral proteins, but also potentially accumulation of cellular components of a distinct compartment, mechanistically or physically linked to the accumulated VP1-2 and tegument proteins.

![Fig. 7. Lack of accumulation of polyubiquitinated proteins during infection with ΔUL36 or ΔUL37 viruses. Monolayers of HEp-2 cells were infected with KAUL36 (a) or FRΔUL37 (b) at 37 °C. At 24 h p.i., cells were fixed and the distribution of capsid components VP5 and VP26 or ubiquitin-conjugated proteins (antibody FK2) was examined by immunofluorescence. (a) KAUL36-infected cells were examined for cytoplasmic clustering of capsid components showing co-localization of VP2 and VP26 (i–iii). There was no evidence for accumulation of polyubiquitinated species, and VP26-positive foci showed no co-localization with polyubiquitinated species (iv–vi). (b) FRΔUL37-infected cells showed accumulation of VP26 foci in the cytosol, but no associated accumulation of polyubiquitinated species. Arrows indicate the large cytosolic accumulation of VP26 or VP5.](http://vir.sgmjournals.org)
Although further work is necessary, it is tempting to speculate that the accumulations of tegument proteins, capsids and polyubiquitinated species could be related to malfunction of the VP1-2 USP. In this regard, we examined whether a virus lacking intact UL36, or a virus with intact UL36 but blocked in assembly due to lack of its partner UL37, would exhibit a similar phenotype in accumulation of polyubiquitinated species. The lack of such accumulation in either virus indicates that the expression per se of the USP in isolation in KAUL36-infected cells does not account for the accumulation of polyubiquitinated species seen in the intact VP1-2 from tsB7. As this is a small N-terminal product very likely to exhibit different properties, perhaps this is not surprising. However, the result with ΔUL37 indicated that, even when intact VP1-2 is produced in combination with a block in assembly, polyubiquitination accumulation was not seen. One implication is that the effect is specific to the nature of the USP function in the context of tsB7. For example, it could be that, in tsB7, the USP in the otherwise intact VP1-2 was defective in removing ubiquitin from target substrates that were relevant to late assembly, such as in the ESCRT pathway recently implicated in HSV maturation (Calistri et al., 2007; Crump et al., 2007). Interestingly, in recent work where the active site of the USP domain in the PrV VP1-2 was mutated, the results showed a significant reduction in virus yield together with large accumulations and clusters of non-enveloped cytosolic capsids similar to those observed here (Bottcher et al., 2008). An additional line of evidence suggesting a possible link between VP1-2, the USP in isolation in KΔUL36, would exhibit a similar phenotype in accumulation of polyubiquitinated species similar to those reported that VP1-2 is cleaved early after infection and that cleavage products of tsB7 VP1-2 at late times in infection, where the active site of the USP domain in the PrV VP1-2 was mutated, the results showed a significant reduction in virus yield together with large accumulations and clusters of non-enveloped cytosolic capsids similar to those observed here (Bottcher et al., 2008). An additional line of evidence suggesting a possible link between VP1-2, the USP in isolation in KΔUL36, would exhibit a similar phenotype in accumulation of polyubiquitinated species similar to those reported that VP1-2 is cleaved early after infection and that cleavage products of tsB7 VP1-2 at late times in infection, where the active site of the USP domain in the PrV VP1-2 was mutated, the results showed a significant reduction in virus yield together with large accumulations and clusters of non-enveloped cytosolic capsids similar to those observed here (Bottcher et al., 2008). An additional line of evidence suggesting a possible link between VP1-2, the USP in isolation in KΔUL36, would exhibit a similar phenotype in accumulation of polyubiquitinated species similar to those observed here (Bottcher et al., 2008). An additional line of evidence suggesting a possible link between VP1-2, the USP in isolation in KΔUL36, would exhibit a similar phenotype in accumulation of polyubiquitinated species similar to those.
Characterization of a temperature-sensitive mutant defective in the proteins.


