The DNA cleavage and packaging protein encoded by the U_{L33} gene of herpes simplex virus 1 associates with capsids

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Abstract

The U_{L33} gene of herpes simplex virus 1 (HSV-1) encodes a protein (pU_{L33}) that is essential for the cleavage and packaging of concatameric herpesvirus DNA into preformed capsids. Previous data have suggested that the U_{L33} protein interacts with the cleavage and packaging proteins encoded by UL15 and UL28 that are known to associate with capsids. Examination of purified A capsids that lack DNA and are derived from aborted packaging events, B capsids that lack DNA, and C capsids that contain DNA revealed an association of the U_{L33} protein with all three capsid types. More U_{L33} protein was detected in A capsids than was present in B capsids. Capsid association was susceptible to guanidine–HCl treatment and independent of the presence of UL15 or UL28. Capsid association of pU_{L33} was also independent of UL6, which is believed to encode the portal into which DNA is inserted. These data suggest that pU_{L33} may act as part of the capsid-associated molecular machinery that translocates cleaved genomic DNA into the capsid interior.

Keywords: Cleavage; Packaging; U_{L33}; Herpes simplex virus 1; Capsids

Introduction

An essential step in the assembly of new herpesvirus nucleocapsids is the cleavage of viral DNA concatamers into individual genomes that are inserted into preformed capsids (reviewed by Baines, 2001; Homa and Brown, 1997). This step represents the merging of the DNA replication and capsid assembly pathways and contains features reiterated in a wide range of double-stranded DNA viruses.

The herpes simplex virus 1 (HSV-1) gene U_{L33} is essential for the cleavage and packaging of HSV DNA (Alkobaisi et al., 1991) and encodes 19000 apparent Mr proteins found in the cytoplasm and nuclear replication compartments of infected cells (Reynolds et al., 2000). In addition to pU_{L33}, five other genes are similarly dispensable for capsid formation but essential for HSV-1 DNA cleavage and packaging: U_{L6}, U_{L15}, U_{L17}, U_{L28}, and U_{L32} (Addison et al., 1984, 1990; Baines et al., 1997; Cunningham and Davison, 1993; Lamberti and Weller, 1996, 1998; Patel et al., 1996; Poon and Roizman, 1993; Salmon et al., 1998; Sherman and Bachenheimer, 1987; Taus et al., 1998; Tengelsen et al., 1993; Weller et al., 1987; Yu et al., 1997). In the absence of any of these six proteins, the viral DNA is not cleaved and empty capsids accumulate in the nuclei of infected cells.

Four types of capsids have been identified in cells infected with HSV-1. The procapsid is believed to be the progenitor of the other capsid types and has a spherical, unstable, and relatively porous shell (Newcomb et al., 1996; Trus et al., 1996). The shells of the other three capsid types, A, B and C, are icosahedral, containing 20 faces and 12 vertices of fivefold symmetry (Booy et al., 1991; Wildy et al., 1960; Zhou et al., 2000). Procapsids consist of an outer shell surrounding uncleaved scaffold protein (preVP22a), A capsids consist only of the outer capsid shell, B capsids contain cleaved scaffold protein within the capsid shell, and C capsids contain packaged DNA but lack most scaffold proteins (Gibson and Roizman, 1972; Newcomb et al., 1996). The cleavage of the scaffold protein in the procapsid is believed to trigger a rearrangement of the outer shell such that it forms the more stable icosahedron (Heymann et al., 2003; Trus et al., 1996). Data largely support the hypothesis that the entry of DNA is closely linked to this structural

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rearrangement during morphogenesis of C capsids. In the same reaction, A capsids are believed to arise from aborted packaging events in which the scaffold is lost but DNA is not retained, whereas B capsids are the consequence of premature or asynchronous scaffold protein cleavage such that the shell undergoes rearrangement before the scaffold is degraded or expelled (Newcomb et al., 1996; Sherman and Bachenheimer, 1987; Trus et al., 1996).

Two crucial components of the cleavage and packaging reaction of dsDNA viruses are the portal protein and terminase enzyme. The bacteriophage portal or connector structure is found at one vertex of the capsid and is the site of terminase and capsid interaction. The bacteriophage portal structure is typically a ring-shaped dodecamer of the portal protein, with the center of the ring forming the channel through which the DNA is translocated into the capsid (Bazinet and King, 1985; Lurz et al., 2001; Simpson et al., 2000; Valpuesta et al., 2000; Yeo and Feiss, 1995). The herpesvirus protein UL6 (pUL6) can assemble into ring-shaped dodecamers and is found at only 1 of 12 vertices of fivefold symmetry in herpesvirus B capsids (Newcomb et al., 2001), suggesting that it forms the portal of the herpesvirus capsid.

The terminase is a protein complex that binds and cleaves concatameric DNA at the junction of two adjacent genomes. Using the terminase complexes of bacteriophages as a model, it is believed that the HSV terminase remains bound to one free end of the cleaved DNA as it attaches to the capsid. It then participates in DNA translocation until exactly one genome is inserted into the capsid. The terminase then cleaves the DNA a second time, releasing the genome from the concatamer, and the terminase and remaining concatameric DNA disengage from the packaged capsid (Catalano, 2000). There is evidence that the HSV-1 UL15 and UL28 genes encode at least part of the terminase enzyme. The proteins have been shown to interact in cells infected with HSV-1 and when transiently expressed in the absence of other HSV proteins (Abbotts et al., 2000; Beard et al., 2002; Koslowski et al., 1997, 1999). In addition, the UL15 gene encodes a region with similarities to a Walker A motif present in a wide range of ATPases, including bacteriophage terminases (Davison, 1992; Mitchell et al., 2002), and the UL28 protein has been shown to bind sequences near the genomic terminus that ensure the accuracy of the second DNA cleavage event (Adelman et al., 2001; Hodge and Stow, 2001). Both pUL15 and pUL28 can be detected in B capsids but are less abundant in C capsids, suggesting that they are lost once DNA is inserted (Salmon and Baines, 1998; Taus and Baines, 1998; Yu and Weller, 1998).

Recently, pUL33 has been shown to associate with both UL15 and UL28, raising the possibility that pUL33 may represent a third component of the terminase (Beard et al., 2002). Preliminary results suggested that pUL33 was largely absent from B capsids (Reynolds et al., 2000); however, the discovery of the UL15–UL28–UL33 complex encouraged further studies to determine whether pUL33 was a component of capsids, as might be expected of a terminase subunit.

**Results**

The U133 protein associates with A, B, and C capsids in a continuous sucrose gradient

Capsids were purified from HSV-1(F)-infected Vero cells on a continuous 20–50% sucrose gradient as described in Materials and methods. The gradient was then fractionated and the proteins present in each fraction electrophoretically separated on 8% and 15% denaturing polyacrylamide gels. The proteins were transferred to PVDF membranes and probed with polyclonal rabbit antisera directed against the major capsid protein VP5 (Cohen et al., 1980) or pUL33 (Reynolds et al., 2000). Bound immunoglobulin was detected using the ECL+ system as recommended by the manufacturer (Amersham Biotech). A photograph of the immunoblot is shown in Fig. 1A. The UL33 protein is present in the regions of the gradient containing A, B, and C capsids, which are indicated by the presence of the VP5 protein. Identical capsid purification protocols were then carried out in the presence of either DNase or RNase, the gradient fractionated and the fractions examined for the presence of VP5 and pUL33 as described above. Neither DNase nor RNase treatment of the capsids affected the association of pUL33 with capsids (data not shown).

It would be expected that if pUL33 were associated with capsids, it would remain associated with highly purified capsids. To test this possibility, the light scattering bands containing the A, B, and C capsids from the first gradient were collected, pooled, and separated on a second 20–50% sucrose gradient, and the gradient was fractionated. The proteins from each fraction were separated on 8% and 15% polyacrylamide gels and probed with antisera directed against VP5 or pUL33, respectively (Fig. 1B). Immunoreactivity specific for pUL33 was present in the region of the gradient corresponding to the A and B capsid bands, confirming the results obtained from the analysis of capsids purified on a single gradient. Because relatively few C capsids survived the additional purification steps, we could not derive conclusions concerning the presence of pUL33 in C capsids in this particular experiment.

As a further control experiment to ensure that pUL33 associated specifically with capsids and did not coincidentally migrate in capsid-containing fractions, an identical capsid purification protocol was carried out on Vero cells infected with the mutant virus K23Z. The genome of K23Z contains a LacZ expression construct replacing most of the U18 gene and is therefore unable to assemble capsids (Desai et al., 1993). Examination of the gradient containing lysates of K23Z-infected cells revealed no U133 protein-specific immunoreactivity (Fig. 1C), confirming that the
normal migration of pUL33 in the sucrose gradient was dependent on the presence of capsids.

The pUL33 protein is present in purified A capsids

To determine if the pUL33 protein was present in A capsids as well as B, nuclear lysates from twenty 175 cm² flasks (approximately 4 × 10⁸ cells) were purified as described above on two successive sucrose gradients. The A and B light scattering bands were collected separately from the initial sucrose gradient, the capsids within them pelleted, resuspended in minimal volume, and recentrifuged on separate sucrose gradients. The light scattering bands were then collected, the capsids within them pelleted, and solubilized in SDS and β-mercaptoethanol. The A and B capsid samples were then analyzed on immunoblots probed with antibody specific for pUL33. Fig. 2A shows that pUL33 was detected in both the A and B capsid samples. Aliquots of the A and B capsid samples were also examined on an 8% polyacrylamide gel stained with Coomassie blue. The major scaffold protein VP22a, which is absent from A capsids, was identified only in the B capsid sample, confirming the fact that the A capsid preparation was largely devoid of B capsids (Fig. 2B).

Quantification of the amount of pUL33 in A and B capsids

The relative amount of VP5 and pUL33 in highly purified A and B capsids was compared in seven separate experiments. On six occasions, as seen in Fig. 2A, there was comparatively more pUL33 present in the A capsid sample, even though, as the Coomassie-stained VP5 band in Fig. 2B reveals, there were less A capsids than B capsids present. In one of the seven experiments, there appeared to be more pUL33 in B than A capsids. As it was not possible to purify C capsids to the same standard as A and B capsids, they were not included in the quantification analysis. Extensive efforts were made to determine the absolute number of copies of pUL33 in A and B capsids, as has been described for pUL15 and pUL28 (Beard et al., 2004); however, the substantial variability of the results prevented any conclusions being drawn.
The effect of guanidine–HCl on the association of pUL33 with B capsids

To investigate whether pUL33 was tightly or loosely associated with capsids, B capsids were treated with varying concentrations of GuHCl (Fig. 3A). The U133 protein was found to be removed from the capsid when exposed to increasing concentrations of GuHCl, similar to the association of the capsid scaffold protein VP22a (Fig. 3B).

The proteins encoded by genes U1, U15, and U18 are not necessary for pUL33 capsid association

Vero cells were infected with previously described viruses individually lacking intact U1, U15, or U18 genes (Baines et al., 1997; Patel and Maclean, 1995; Tengelsen et al., 1993). The mutant B-like capsids from the respective nuclear lysates were purified on two sequential sucrose gradients as described above. The second gradient was fractionated, and the proteins present in each fraction separated on two denaturing 8% polyacrylamide gels, transferred to nitrocellulose membrane, and immunoblotted with antisera directed against VP5 or pUL28. Immunoreactivity to the pUL28 protein was detected only in fractions containing capsids (Fig. 5).

Discussion

The association of the U133 protein in A, B, and C capsids purified from HSV-1(F)-infected cells is consistent
with previous reports showing that the UL33 gene is required for DNA cleavage and packaging (Alkobaisi et al., 1991), and that the UL33 protein localizes in the nuclear replication compartments of infected cells (Reynolds et al., 2000) where at least some of the DNA cleavage and packaging events likely occur (Lamberti and Weller, 1998; Taus et al., 1998; Ward et al., 1996). In addition, pUL33 forms a complex with pUL15 and pUL28, two other capsid-associated proteins required for cleavage and packaging (Beard et al., 2002). This work describes the capsid association of pUL33 to be similar to that of pUL15 and pUL28 (Salmon and Baines, 1998; Taus and Baines, 1998; Yu and Weller, 1998), consistent with the hypothesis that pUL33 interacts with, or forms a third component of, the terminase enzyme.

Because A and B capsids migrate closely together on a 20–50% sucrose gradient, it is often difficult to separate them on a single sucrose gradient. In addition, relatively few A capsids are produced in HSV-1(F)-infected cells, making purification difficult in routine analyses. To overcome these difficulties, A capsids from multiple gradients were pooled.
(original material was derived from a total of approximately \(4 \times 10^8\) cells) and purified through two successive sucrose gradients. The absence of the scaffold protein VP22a from the electrophoretic profile of the A capsids (Fig. 2B) confirmed that this sample was not extensively contaminated with B capsids. A capsids are believed to represent aborted packaging events during which DNA has begun to be inserted into capsids, but for some reason is not retained (Sherman and Bachenheimer, 1987). The observation that pUL33 can be detected in A capsids suggests that the protein remains attached to the capsid after DNA translocation has been initiated.

It has been reported recently that there is significantly more pUL15 in A than B capsids, but similar amounts of pUL28 in both capsid types (Beard et al., 2004). In six out of seven separate experiments comparing the amount of pUL33 in A and B capsids, there was comparatively more pUL33 detected in A than B capsids, a situation similar to that of pUL15.

The resistance of the U33 protein in B capsids to guanidine–HCl extraction is similar to that of the scaffold protein VP22a and other cleavage and packaging proteins U15 and U28 (Salmon and Baines, 1998; Taus and Weller, 1998), suggesting that they are all associated with the capsid less avidly than the major capsomere protein (VP5) and triplex proteins VP19c and VP23.

The U33 protein was found to associate with capsids lacking the U6, U15, or U28 proteins. This was not unexpected because previously reported immunofluorescence data showed that the distribution of pUL33 in infected cells was not altered by the absence of pUL15, pUL28, or pUL6 (Reynolds et al., 2000), and the lack of any one of these three proteins does not preclude the interaction of the remaining two in vitro (Beard et al., 2002). It is therefore apparent that the association of pUL33 with B-like capsids is not dependent on pUL15, pUL28, or the putative portal protein U6. The converse is also true: the U6, U15, and U28 proteins all associate with capsids lacking pUL33 [(Patel et al., 1996; Salmon et al., 1999), this paper].

Although the association with intermediate capsids and other putative terminase components provide indirect support for the hypothesis that pUL33 plays a role as part of the terminase holoenzyme, more direct proof will require further studies, including documentation of terminase-like activities using purified UL33 protein.

**Materials and methods**

**Viruses, cells, and antibodies**

All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) newborn calf serum, penicillin, and streptomycin. The wild-type HSV-1(F) strain has been previously described (Ejercito and Roizman, 1968) and was propagated and titrated on Vero cell monolayers. The mutant viruses lacking U16 (Patel and Maclean, 1995), U15 (Baines et al., 1997), U18 (Desai et al., 1993), U28 (Tengelsen et al., 1993), and U33 (Cunningham and Davison, 1993) were propagated as described, except for the U33 mutant that was grown on D4 cells (Salmon et al., 1999). The U33- and U28-specific rabbit polyclonal antibodies have been previously described (Beard et al., 2002; Reynolds et al., 2000); the VP5 antibody (NC1-1) was a generous gift of Drs. R. Eisenburg and G. Cohen (Cohen et al., 1980).

**Capsid preparation and immunoblotting**

Capsids were purified as described previously with some modifications (Perdue et al., 1974). Unless otherwise stated, ten 175 cm² flasks of Vero cells were infected at an MOI of 5 and incubated for 16 h at 37 °C. Nuclear lysates were prepared by disruption in 1% triton-X and ultrasonification for 40 s at moderate power. After clarification in a Sorvall Legend RT centrifuge, Heraeus rotor #3334, 15 min at 8000 RPM (7227 × G) at 4 °C, capsids in the supernatant were pelleted through a 6 ml 35% (w/v) sucrose cushion in a SW28.1 rotor for 1.5 h at 20000 RPM. The resultant pellet was sonicated briefly, loaded on a 20–50% (w/v) continuous sucrose gradient, and centrifuged for 1 h at 23000 rpm in a SW41 rotor. Separate light scattering bands were collected with Pasteur pipettes and capsids were pelleted at 30000

**Fig. 5. Immunoblot of purified capsids from U33(-) virus. Capsids lacking pUL33 were purified through two sequential sucrose gradients and the second gradient fractionated from the top (fraction one). The protein in each fraction was separated on an 8% denaturing polyacrylamide gel, transferred to nitrocellulose membrane, and probed with antisera directed against VP5 (top panel) or pUL28 (bottom panel). Bound antibody was visualized by the addition of alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin followed by the addition of chromogenic substrate as described in Materials and methods.**
RPMS for 2 h in a SW41 rotor. Where mentioned in the text, the capsid-containing bands were then purified further on a second identical sucrose gradient, the bands collected, capsids pelleted, resuspended in 50 µl water (DNase-, RNase-, and protease-free, [Acros]), and stored at 4 °C. Examination of the material by electron microscopy showed minimal background material (not shown). Where mentioned, fractions of the sucrose gradients were collected using a fractioning device (Haake Buchler) beginning at the top of the gradient.

Immunoblotting was carried out essentially as previously described (Baines and Roizman, 1993). Unlike gradient. Immunoblotting was carried out essentially as described (Haake Buchler) beginning at the top of the gradient. Immunoblotting was carried out essentially as previously described (Baines and Roizman, 1993). Unlike previous studies (Reynolds et al., 2000), the antibody was not extensively preadsorbed against cells infected with the U133 deletion virus. This improved sensitivity and did not increase the number of nonspecific bands visualized in immunoblots of capsid-associated proteins (not shown).

Bound antibody was detected using either horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin followed by the ECL+ development reagent (Amersham Pharmacia Biotech) according to the manufacturer’s instructions and visualized using a Molecular Dynamics Storm Imager and associated Imagequant software, or with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin followed by the chromogenic substrates NBT and BCIP (Bio-Rad).

Guanidine–HCl treatment

B capsids were treated with varying concentrations of guanidine–HCl as described previously (Newcomb and Brown, 1991). A 4 M concentration of guanidine–HCl in TNE was slowly added with extensive mixing to purified B capsids such that the final concentrations were 0, 0.1, 0.25, 0.5, or 1 M GuHCl in a total volume of 1 ml. The samples were then incubated on ice for 45 min with intermittent agitation followed by centrifugation through a 25% sucrose cushion for 1 h in a SW50.1 rotor at 23 000 rpm. The pellet was resuspended and equal amounts analyzed on an 8% and a 15% denaturing polyacrylamide gel. The amount of pU133 present was detected using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin followed by the ECL+ development reagent (Amersham Pharmacia Biotech) according to the manufacturer’s instructions, visualized using a Molecular Dynamics Storm Imager and quantified using the associated Imagequant software. The major capsid bands were visualized on the 8% gel using Coomassie Brilliant Blue and quantified using a flatbed digital scanner and Scion Image densitometry for Windows.

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