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Published in:
Journal of Virology

DOI:
10.1128/JVI.00123-17

Document Version
Final author's version (accepted by publisher, after peer review)

Publication date:
2017

The vertex specific proteins pUL17 and pUL25 mechanically reinforce Herpes Simplex Virus capsids

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Abstract word count: 120

Main text word count: 2462

Running title: The CVSC mechanically reinforces HSV-1.

Abstract

Using atomic force microscopy imaging and nanoindentation measurements, we investigated the effect of the minor capsid proteins pUL17 and pUL25 on the structural stability of the icosahedral Herpes Simplex Virus capsids. pUL17 and pUL25 that form the capsid vertex-specific component (CVSC) particularly contributed to the capsid resilience along the 5-fold and 2-fold, but not along the 3-fold icosahedral axes. Our detailed analyses, including quantitative mass spectrometry on the protein composition of the capsids, revealed that pUL17 and pUL25 are both required to stabilize the capsid shells at the vertices. This indicates that herpesviruses withstand the internal pressure that is generated during DNA genome packaging by locally reinforcing the mechanical sturdiness of the vertices, the most stressed part of the capsids.

Importance

In this study the structural, material properties of Herpes Simplex Virus type 1 were investigated. The capsid of Herpes Simplex Virus is built up of a variety of proteins and we scrutinized the influence of two of these proteins on the stability of the capsid. For this we used a scanning force microscope that makes detailed, topographic images of the particles and that is able to perform mechanical deformation measurements. Using this approach we revealed that both studied proteins play an essential role in viral stability. These new insights support us to form a complete view on viral structure and could furthermore possibly not only help to develop specific anti-virals, but also to build protein shells with improved stability for drug delivery purposes.

Main Text

Herpes Simplex Virus type 1 (HSV-1) is an important human pathogen that causes a variety of diseases ranging from common cold sores to life threating encephalitis(1-3). Herpesvirus particles are enveloped virions with $T = 16$ icosahedral capsids harboring the dsDNA genomes. After synthesis and nuclear import of the capsid proteins, they initially assemble into rather spherical immature procapsids (4, 5). Upon proteolytic cleavage of the internal scaffold, consisting mostly of the protein VP22a, these procapsids mature into three icosahedral capsid types (6-9). B-type capsids have failed to expel the protein scaffold, A-type capsids are considered to have aborted DNA packaging and lack both DNA and the internal scaffold, and C-type capsids, also called nucleocapsids, result from successfully replacing the internal protein scaffold with the 152 kb dsDNA genome of HSV-1. C capsids then leave the nucleus and undergo secondary envelopment in the cytoplasm to generate mature, infectious, enveloped virions (10, 11). Recent nanoindentation experiments using atomic force microscopy (AFM) have revealed remarkable insights on the mechanical basis of HSV1 genome packaging and capsid maturation (12-14).

In AFM-nanoindentation experiments, viral capsids are deposited on a glass surface, imaged by AFM, and subsequently indented to probe the mechanical resilience of the particle (15, 16). Such AFM studies have revealed how the structural stability of capsids depends on environmental conditions, packaged genome length, and the protein composition of the particle (17-23). Moreover, it has been shown that the mechanical resilience of viral capsids is directly related to (i) local conformational dynamics (Minute Virus of Mice) (24), (ii) the virus's infectivity (HIV-1) (25), and (iii) the particle's propensity for efficient uncoating (Adenovirus) (26, 27).

In the case of HSV-1 capsids, we have shown that scaffold expulsion and genome packaging result in molecular changes that strengthen the particles (12). This is reflected by an increase in the threshold for the breaking force F_{break} required for structural collapse. By treating HSV1 capsids with a moderate, partially denaturing concentration of guanidine hydrochloride (GuHCl), the penton-fraction of the major capsid protein VP5, the small capsid protein VP26 located on the tips of the VP5 hexons, the scaffold protein VP22a, the minor capsid proteins pUL17 and pUL25 as well as the DNA genomes are extracted (12, 28, 29). Using such penton-less B, A, and C capsids , we showed that their stiffness is reduced, indicating that the vertex proteins of HSV-1 capsids are especially important for the mechanical resilience of the capsids (12, 13). In addition, it has been recently reported that the protein pUL25 reinforces the capsid (30). The two minor capsid proteins pUL25 and pUL17 form heterodimers that are attached to the capsid vertices (c.f. Fig. 1a), and hence have been called capsid vertex-specific components (CVSC) (31-44).

Next to HSV-1, similar CVSC complexes are present on purified capsids of the swine alphaherpesvirus pseudorabies virus with even higher occupancy levels (45-47). Furthermore, homologs of these minor capsid components exist in other alphaherpesviruses: the betaherpesviruses (e.g. pUL77 and pUL93 in human cytomegalovirus) (48), and the gammaherpesviruses, (e.g. ORF32 and ORF19 in Kaposi-sarcoma associated virus) (44), suggesting that functional stabilizing CVSCs are a feature of all herpesviruses (49).

In HSV-1 the CVSCs also mediate interactions with the inner tegument protein pUL36 and the outer tegument protein VP13/14 that link the capsids to envelope components during assembly (50-52). Previous studies have shown that pUL17 and pUL25 depend on each other for optimal capsid binding, since capsids derived from either UL17 or

98 UL25 deletion mutants lack most of the CVSC altogether (53). Furthermore, a recent
99 study using cryo-electron microscopy reconstructions clearly shows that the CVSCs
100 directly link the pentons to the adjacent triplexes (45). In the current study, we used
101 AFM to determine at the single particle level how the CVSC contributes to the
102 mechanical properties of HSV-1 capsids.
103
104 **Materials and Methods**
105 ***Capsid purification.*** Nuclear capsids were isolated from cells infected with HSV-1 wild-
106 type (WT HSV-1 strain F, ATCC VR-733), or with the mutants HSV1-ΔUL17 (derived
107 from HSV-1 strain F, see ref (38)) or HSV1-ΔUL25 (HSV-1 strain KUL25NS derived from
108 strain KOS, see ref (32)) after cell homogenization and purification on a linear 20 to 50
109 % (w/w) sucrose gradient in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA
110 supplemented with 10 mM dithiothreitol as described before (12, 54, 55). While during
111 WT infection B, A, and C capsids are assembled, B- and A-type capsids are formed in the
112 absence of pUL25 (32), and only B-type capsids in the absence of pUL17 (35, 38, 56).
113 ***AFM imaging and nanoindentation.*** The capsids were deposited onto silanized glass
114 substrates and analyzed at room temperature in 50 mM Tris buffer pH 7.5, 150 mM
115 sodium chloride, by AFM imaging and nanoindentation as described in detail elsewhere
116 (12, 57, 58). The experiments were performed with a Nanotec AFM (Tres Cantos, Spain),
117 using cantilevers with an approximate tip-radius of 15 nm and a spring constant of 0.05
118 N/m (Olympus OMCL-RC800PSA). Imaging was performed in jumping mode AFM, which
119 is a very gentle imaging mode where lateral forces are almost absent, and which is
120 therefore ideally suited to image proteinaceous assemblies such as viral caspids (59).
121 The probe velocity during nanoindentation was 60 nm/s. The data were analysed with
122 the WSxM software (Nanotec; Version 4) and a home written Labview programme (58).

123 Capsid absorption to the surface was expected to be random with respect to the
124 icosahedral orientation; in addition to absorption to the 2-, 3- or 5-fold symmetry axes,
125 we detected also intermediate positions. As the intermediate positions were difficult to
126 classify, we focused on particles that adhered to the 2-, 3- or 5-fold symmetry axes.
127 ***Protein extraction and LC-MS/MS and data analysis:*** HSV-1 capsids were resuspended
128 in 50 mM ammonium bicarbonate, 5% (w/v) sodium deoxycholate and heated at 90 °C
129 for 5 min. For each reaction, 100 µg of protein were reduced using dithiothreitol (DTT)
130 for 30 min at 56 °C and then alkylated by iodoacetamide for 30 min in the dark. After
131 dilution to a final concentration of 0.5% sodium deoxycholate, each sample was digested
132 overnight at 37°C with trypsin at an enzyme to protein ratio of 1:50. The sodium
133 deoxycholate was precipitated, and the reaction/digestion quenched by adding formic
134 acid to a final concentration of 2% (v/v). The samples were centrifuged for 20 minutes
135 at 20,000 x g, and the supernatants were analyzed on a mass spectrometer (Q-Exactive
136 Plus coupled to an Agilent 1290 Infinity UHPLC system). Briefly, the peptides were
137 loaded onto the trapping column (Dr Maisch Reprosil C18, 3 µm, 2 cm × 100 µm) with a
138 flow rate of 5 µl/min for 10 min with reversed-phase solvent A, whereas peptide
139 separation was performed at a column flow rate of ~300 nl/min (Agilent Poroshell 120
140 EC-C18, 2.7 µm, 50 cm × 75 µm). Nanospray was achieved with an in-house pulled and
141 gold-coated fused silica capillary (360 µm outer diameter, 20 µm inner diameter, 10 µm
142 tip inner diameter) and an applied voltage of 1.9 kV. Full-scan MS spectra (from m/z 350
143 to 1500) were acquired in the Orbitrap with a resolution of 35,000. HCD fragmentation
144 was performed with a data dependent mode, as previously described(60).
145 Peak lists were generated (Proteome Discoverer; version 1.4, Thermo Scientific,
146 Bremen, Germany) and searched against a database containing the Human Herpes Virus
147 1 strain 17 sequences (77 protein entries) using Mascot (version 2.4 Matrix Science,

London, UK) and a mass tolerance of 50 ppm for precursor masses and ±0.05 Da for fragment ions. Enzyme specificity was set to trypsin with 2 missed cleavages allowed. Carbarmidomethylation of cysteines was set as fixed modification while oxidation of methionine, was used as variable modification. False discovery rate was set to <1%. To further filter for high quality data we used the following parameters: high confidence peptide spectrum matches, minimal Mascot score of 20, minimal peptide length of 6, and only unique rank 1 peptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005104(61).

Results

From AFM images taken immediately prior to the nanoindentation experiments, we determined the orientation of each capsid based on its capsomer morphology and the orientation of the triangular facets on the capsid surface. Figure 1b shows a projection of the facets on the AFM images. UL17- and UL25-null capsids that adhered to the surface in different orientations were compared to similarly oriented B-, A- and C- type capsids of the WT strain. There was a marked decrease in the spring constants *k* of the capsids from both deletion strains (Figure 2a).

We then stratified these data into B, A, and C capsids, and based on our AFM images further into measurements along the 2-fold, the 3-fold, or the 5-fold axes. The deposition onto the 2-, 3- or 5-fold axes occurred at a ratio of 61:63:47 (Fig. 2b). In an icosahedral particle, there are 30 2-fold axes, 20 3-fold axes and 12 5-fold axes. A similar ratio of deposition was determined previously using Hepatitis B Virus (HBV) capsids (62). In the current study, there was roughly the same number of particles deposited on the 2- or

the 3-fold axis. Thus, compared to the T=3 and T=4 HBV capsids of ~30 nm diameter, the larger T=16 HSV-1 capsids of 125 nm likely elicit additional surface interaction effects that slightly favor a stable deposition on a 3-fold axis over a 2-fold axis. The spring constant analysis revealed that the reduction in stiffness was particularly prominent for certain icosahedral orientations (Figure 2b). Capsids that had been deposited on a triangular facet of the icosahedral shell, and thus probed along the 3-fold icosahedral symmetry axis of the capsid, exhibited no significant loss of stiffness for the UL25-null or UL17-null mutants as compared to WT capsids. However, there was a significant decrease in the stiffness of UL25- or UL17-null capsids compared to WT capsids when the particle had been deposited on the edge between two facets (i.e. 2-fold icosahedral symmetry axis), or deposited on a vertex (5-fold icosahedral symmetry axis).

We then determined the protein composition of the different capsid type of the wildtype and the two deletion mutants by quantitative mass spectrometry using a label-free approach in which the number of peptide-spectrum matches (PSM's) serve as a proxy for the relative protein amounts (see Supplementary Table S1). We used the major capsid protein VP5 (pUL19) that forms the pentons and hexons in each capsid for normalization since it is considered to be present in constant amounts among different capsid types (54) (see Figure 3). Based on this normalization, we then determined the amount of the other capsid proteins in the different samples. As expected, the abundancy of two triplex proteins VP19c (pUL38) and VP23 (pUL18) were also similar in the different samples, indicating that the different preparations from the HSV-1 wild-type and the mutants indeed contained capsids with an identical backbone architecture In contrast neither of the CVSC proteins pUL17 and pUL25 could be detected in either of the deletion mutants. This indicates that none of the CVSC components was recruited or

maintained on the capsids if one of them had been missing. This analysis of the protein composition of all capsid types fits to our measurements of the capsid stability, since both deletion mutants displayed identical mechanics of their HSV capsids.

Discussion

Our results on the B and A capsids of the UL25-null mutant corroborate and extend the recent finding by Sae-Ueng *et al.* (30) who also reported a reduced stability of HSV-1 capsids upon deletion of pUL25. However, they did not detect any changes in the mechanical resilience of the B capsids upon deletion of UL17. In contrast, we measured a significant decrease in the stiffness for the B capsids of the UL17-null mutant (dark blue columns in Fig. 2b). Furthermore, we have been able for the first time for herpesviruses to separately analyze the spring constants along the different icosahedral axes. As our data show that the spring constants k along the 3-fold axis remain largely unaffected by deletion of either UL17 or UL25, it is possible that Sae-Ueng *et al*. (30) predominantly measured the spring constants of the UL17-null mutant upon probing the triangular sides, but not capsids with their 2-fold or 5-fold axes oriented towards the AFM tip. Moreover, using quantitative mass spectrometry analysis we have corroborated earlier findings that the capsid levels of pUL17 and pUL25 largely depend on each other for stable capsid association (42, 53). In contrast, the immunoblot of Sae-Ueng *et al.* (30) and Huet *et al.* (45) revealed residual amounts of pUL17 on the capsids of the UL25-null mutant. The reasons for this difference are unclear; it may be due to the presence of dithiothreitol in our purification buffers to generate a similar reducing environment as in the nucleoplasm or the cytoplasm.

Our new data and Sae-Ueng *et al.* (30) support the notion that the CVSCs provide substantial mechanical resilience to HSV-1 capsids, and here we also show that pUL17

and pUL25 are both required to increase vertex resilience. Our finding that deletion of either pUL17 or UL25 result in a reduced strength of capsids corroborates the recent report of the structure of the CVSC that clearly shows how both proteins are intimately linked to each other in the CVSC (45). As the CVSC is located at the 5-fold vertices and oriented along the 2-fold symmetry axis, it is very likely to impact the capsid resilience along these symmetry axes, which is exactly what we find. The three-fold axis on the other hand, does not appear to be affected by the presence or absence of the CVSC (45). This also correlates with our findings, explaining the differences in observed impact of CVSC removal for the different icosahedral orientations. The vertices are removed from the capsid first when the particles are stressed, e.g. nanoindentation or partial denaturation with urea or GuHCl (12, 28). Moreover, in the absence of the capsid stabilizing CVSCs, e.g. in mutants lacking UL25, the capsids cannot maintain the viral genomes in their lumena, presumably because the capsids are not stably sealed (32). Actually, herpesviruses depend on the DNA terminase complex consisting of pUL15, pUL28 and pUL33 and ATP hydrolysis to package their genomes into capsids, and to work against the repulsive force of the highly confined, negatively charged DNA (63-65). Thus, one major function of the CVSCs could be to reinforce the vertices of the nucleocapsids to ensure retention of the genome inside the particle. Recent experimental and theoretical studies of virus capsid nanoindentation have demonstrated that the mechanical response of a capsid is basically a local property of the capsid structure (24, 66). The local reinforcement of the capsid vertices by the CVSC is therefore an example of a virus specifically adapting to mechanical limitations imposed by packaging large genomes to near liquid crystalline density.

Acknowledgements. We are grateful to Fred Homa (University of Pittsburgh School of Medicine, USA) and to Valerie Preston (MRC-University of Glasgow Centre for Virus Research, United Kingdom) for providing the UL25 deletion mutant KUL25NS and the complementing Vero 8-1 cell line. This study was supported by FOM Projectruimte grants to GJLW and WHR, a NWO-VICI grant to GJLW, by the DFG (SFB 900, project C2) to BS, by a VIDI grant of the NWO to WHR, by National Institutes of Health award GM 507401 to JDB, and by the EraNet NanoSci-E+ Initiative to GJLW, WHR and BS (DFG, So303/4).

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Figure Legends

Figure 1. Atomic force microscopy imaging of HSV-1 capsids. A) Schematic of the HSV-1 capsid vertex region; modified from ref. (41, 44). The UL25 part of the CVSC is proposed to be closest to the vertex and likely touching it (45). B) AFM images of HSV-1 capsids. Based on the facet orientation and capsomer morphology, particles deposited on the 2-, 3- and 5-fold icosahedral symmetry axis can be distinguished. Scale bar is 50 nm.

Figure 2. Both CVSC components pUL17 and pUL25 contribute to the mechanical vertex stabilization of HSV-1 capsids. A) Frequency distributions of particle spring constants (*k)* from particle with or without the CVSC, showing the shift to lower *k* values

for the latter particles. B) The average spring constant (*k*) for each orientation is shown for all three capsid types, comparing capsids from UL17- or UL25-null backgrounds to WT capsids. Error bars represent standard error of the mean (SEM), the numbers of particles per type/orientation are indicated in white on each bar.

Figure 3. Protein copy numbers on capsids. Quantitative Mass Spectrometry results on the abundancy of pUL38, pUL18, pUL25 and pUL17 on the different capsids. On the y-axis the relative number of peptide-spectrum matches (PSM's) (67) is indicated.

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