Extensive subunit contacts underpin herpesvirus capsid stability and interior-to-exterior allostery

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The herpesvirus capsid is a complex protein assembly that includes hundreds of copies of four major subunits and lesser numbers of several minor proteins, all of which are essential for infectivity. Cryo-electron microscopy is uniquely suited for studying interactions that govern the assembly and function of such large functional complexes. Here we report two high-quality capsid structures, from human herpes simplex virus type 1 (HSV-1) and the animal pseudorabies virus (PRV), imaged inside intact virions at ~7-Å resolution. From these, we developed a complete model of subunit and domain organization and identified extensive networks of subunit contacts that underpin capsid stability and form a pathway that may signal the completion of DNA packaging from the capsid interior to outer surface, thereby initiating nuclear egress. Differences in the folding and orientation of subunit domains between herpesviruses capsids suggest that common elements have been modified for specific functions.

Herpesviruses are a leading cause of human viral diseases, including oral and genital blisters (herpes simplex viruses HSV-1 and HSV-2), chicken pox and shingles (varicella zoster virus), and cancers (Epstein–Barr virus and Kaposi sarcoma herpesviruses (KSHV)), among others. Chronic herpesvirus infections exhibit periods of latency interspersed with recurrent reactivations that can be treated with drugs only temporarily, if at all. Conversely, beneficial applications include herpesviruses modified to replicate specifically in tumor cells and direct tumor-specific cell lysis1 or stimulate antitumor immunity2. Understanding herpesvirus structure and function is essential for developing its use as a therapeutic target and agent.

The virion is enveloped and includes membrane-bound glycoproteins, a deep and dense internal layer of proteins called ‘ tegument’, and the icosahedral capsid, in which the double-stranded DNA (dsDNA) chromosome is packed. The capsid structure and biochemistry have been studied extensively, but many important architectural details remain uncertain or unknown. As with all herpesviruses, the 200-MDa HSV-1 capsid is icosahedrally symmetric, composed primarily of 955 copies of the 150-kDa major capsid protein, VP5, arranged as 11 pentamers (pentons) on the vertices and 150 hexamers (hexons) elsewhere1 (Fig. 1). Other critical components include the dodecameric UL6 portal complex, which occupies the 12th vertex4 and thus breaks the local and icosahedral symmetry; hundreds of copies of the VP19C–VP23 ‘triplex’ molecule located between hexon and penton capsomers5; the VP26 protein, which caps only hexons6; and a recently identified heterodimer of the pUL17 and pUL25 proteins, called the capsid-vertex-specific component (CVSC), which binds specifically to triplexes adjacent to pentons6. Understanding how this three-dimensional network functions in assembly, packaging of the viral genome, exiting the cell nucleus, and acquiring tegument proteins and the viral envelope is challenging because of its complexity as well as its resistance to atomic-resolution structural methods. Indeed, X-ray structures have been determined for parts of only two capsid proteins: (i) a 65-kDa upper-domain fragment of VP5 including residues 484–1045 (ref. 7) and (ii) the C-terminal 134–580 residues of CVSC subunit pUL25 (ref. 8).

Cryo-EM has provided medium-resolution structures of entire herpesvirus capsids purified from the nucleus (nucleocapsids), including immature procapsids, DNA-filled C-capsids, and the aborted empty A capsids and partially protein-fil lled B capsids. A striking conclusion of these studies is that herpesviruses share the canonical HK97 capsid protein fold with the ubiquitous dsDNA tailed bacteriophage family9. Indeed, the functions of some HSV-1 proteins have been suggested on the basis of their clear homology with the better-characterized phage counterparts, including the portal and terminase subunits. However, the herpesvirus capsid is considerably more complex, comprising more and larger subunits. Cryo-EM visualization of chemically depleted capsids or of bulk labels (such as GFP) attached to specific amino acids has furthered understanding of capsid topology, although at times imperfectly. Most importantly, details of subunit interfaces and folds that may be exploited for designing antiviral drugs remain obscure, as do some subunit locations and even stoichiometry.

Cryo-EM is making rapid advances in resolution10,11 because of direct-electron-detecting cameras coupled with automated data collection, which together allow the large herpesvirus capsids to be modeled at unprecedented resolution and in the full context of the intact virion. Here we set out to use this new technology to study herpesvirus capsids. We present a comprehensive and robust analysis of capsids from human HSV-1 and the animal pseudorabies virus (PRV), both imaged inside intact virions. HSV-1 and PRV are alphaherpesviruses that share...
important features of capsid architecture and allow limited cross-complementation of an essential minor capsid subunit, pUL25 (ref. 12), which has been a focus of our recent studies13–15. Together, these two implementations of an essential minor capsid subunit, pUL25 (ref. 12), provide corroborating and complementary information that substantially advances understanding of the enveloped capsid organization. We resolve previous confusion regarding the subunit location underpins the robustness of our analyses, thus adding confidence in our interpretation of regions for which no atomic models are available and providing an objective resolution standard for evaluating the density maps (Supplementary Fig. 2).

RESULTS

Data collection, reconstruction and validation

We collected 50,000 micrographs of HSV-1 virions in the frozen hydrated state (Online Methods); from these micrographs, we selected 25,637 images of capsids inside intact virions. Similarly, 32,956 micrographs of PRV virions yielded 13,242 virion capsid images for comparison. Central sections through the reconstructions revealed excellent localization of density and low background noise (Fig. 2 and Supplementary Movie 1). The representation of capsid elements such as pentons, hexons, and triplexes was strong and detailed, as was the density of the triplex-bound CVSC molecule, which has been underrepresented in reconstructions of nucleocapsids16. We also observed penton-capping density, which has been previously identified on capsids thermally extracted from virions as an ordered portion of the large pUL36 tegument protein17. The capsid interior was tightly packed with viral dsDNA that appeared in the symmetrized reconstruction as concentric shells of density spaced ~26 Å apart.

Fitting of the VP5 upper-domain crystal structure (PDB 1NO7 (ref. 7)) into the HSV-1 cryo-EM map was straightforward (Fig. 3a). Helices aligned well with the numerous tubes of density, and connectivity among the helical regions was excellent for both HSV-1 and PRV. This fit was also consistent with results18 from an antibody-labeling study (Supplementary Fig. 1). This validation of capsid density quality underpins the robustness of our analyses, thus adding confidence in our interpretation of regions for which no atomic models are available and providing an objective resolution standard for evaluating the density maps (Supplementary Fig. 2).

Figure 1 Architecture of the HSV-1 capsid. The locations of capsid elements are shown in representations of the HSV capsid structure determined from cryo-EM images (left half) and as a simplified schematic (right half) including the internal packaged DNA. The table lists the major capsid elements, as identified by color in the capsid images. Not included in the schematic are the portal, which occupies one vertex in place of a penton, and the accessory protein that binds the outer tips of hexons. Scale bar, 200 Å.

Figure 2 HSV-1 and PRV density maps. (a,b) HSV-1 (a) and PRV (b). Left, representative portions of cryo-micrographs showing intact virions from which capsid images were collected. Elements of the virion are marked in a. Scale bar, 1,000 Å. Surface renditions of the capsid reconstructions, colored by radius, are shown as viewed from the exterior (center) and interior (right) after computational removal of internal density. White dashed circles mark the locations highlighted in c–e, as indicated. Superimposed on each interior view is a gray-coded section (protein is dark) corresponding to the sectioning plane. H, hexon; P, penton; T, triplex; Teg, density attributed to tegument; CVSC, density attributed to the CVSC molecule. Scale bar, 200 Å. Inset, diagram of the icosahedral lattice geometry with triangulation number $T = 16$ indicating the locations of hexons (purple) and pentons (light blue). (c) Close-up view of a hexon (purple) with the domains of one VP5 subunit colored dark blue (upper domain), light blue (middle domain) and dark green (lower domain). The VP26 subunit bound to the VP5 tip is colored white. (d) Close-up view of a triplex molecule with the proposed subunit segmentation of magenta, green and blue. (e) Close-up view from beneath the HSV-1 penton, revealing pairs of tubes (magenta). Scale bars, 50 Å.
The phage HK97 fold in the herpesvirus major capsid protein

The 150-kDa major capsid protein of herpesviruses has three structural domains: a lower domain that forms the connected capsid density, a middle domain extending above it, and a top-most 60-kDa upper domain, for which a crystal structure from the HSV-1 VP5 protein is available. The lower domain has been proposed to adopt the canonical HK97 fold of dsDNA tailed phages. In our structure, in contrast to recently reported herpesvirus structures, we readily modeled all major features of the HK97 fold (PDB 2FT1 (ref. 21)) into the HSV-1 density map (Fig. 2b). Although the core A and P domains of the fold have been previously identified, we found that both the E loop and extended N-terminal arm were also well accommodated, thus suggesting that these extensions serve critical common functions. However, the spine helix was ~10 Å longer (approximately eight residues) in the herpesvirus maps than in any HK97-like phage capsid observed to date, and the consequent lengthening of the lower domain affected capsomer packing, as described below. Additionally, the central channel in the lower domain of the herpesvirus hexon had a larger diameter of ~35 Å compared with the HK97 capsomer, in which the backbone-to-backbone spacing is only 13 Å, and the center is filled with side chain density (Fig. 3d). The herpesvirus channel was wider than the ~24-Å diameter of dsDNA, thus suggesting that the VP5 lower domain alone is incapable of retaining the viral genome in the capsid. Instead, a constriction plugged the capsomer channel at the level of the middle domain (Fig. 3a).

Strong density in both herpesvirus maps corresponding to the HK97 E loop indicated that this loop is well stabilized by capsid contacts, although it does not form the covalent cross-links found in the mature HK97 capsid that confer robustness. In HSV, unlike HK97, the E loop did not reach as far as an adjacent capsomer but instead made multiple interactions with adjacent subunits, including the lower and middle domains of an intracapsomeric VP5 subunit, and a triplex molecule (Fig. 3c). In addition to overlaying the spine helix of an adjacent subunit, each E loop also contacted the N arm, similarly to the contacts in HK97 (Fig. 3d). Density corresponding to the extended N-arm motif was strongly represented in both the HSV and PRV maps, thus suggesting that its conformation and function are conserved and may be crucial for maintaining capsid structure.

In our new model of the HSV lower domain, the geometry of capsomer interactions at the three-fold sites differed from that in HK97 (Fig. 3e). In this region, herpesvirus VP5 subunits were too distant from each other for the E loops to contact or cross-link with adjacent P domains; instead, the triplex molecule mediated contacts, whereas the spacing between intracapsomeric E loops and P domains was relatively tight in HK97. Indeed, the P loop appears to be particularly important for stabilizing the HK97 procapsid as well as participating in the covalent cross-linking of the mature capsid and is likely to act as a hinge in the transition from procapsid to capsid. In contrast, the herpesvirus triplex molecule positioned on the local three-fold sites appears to carry out equivalent roles.

A new helix-pair motif under the pentons of HSV-1

A striking feature of our HSV-1 density map was a set of five pairs of tubes lining the inner capsid surface beneath each penton (Figs. 2e and 4a). Cross-sections through the map showed that this density was equal in strength to that of the capsid, thus demonstrating that the tubes were a robust feature of the reconstruction. To our knowledge, this motif has not been described before for any herpesvirus or HK97-like phage capsid, or for any virus capsid structure. We interpreted these pairs of tubes as helices, on the basis of their 10-Å center-to-center spacing and 40-Å lengths. The tubes had weak but distinct connections with the VP5 lower domain, thus suggesting that they were extensions of VP5. Alternative sources seemed unlikely because the tightly packed center-to-center spacing was ~25 Å, and not the 10 Å observed between the tubes.

We found that the most N-terminal ~50 residues of VP5 are predicted to adopt an extended α-helix (Supplementary Fig. 3), and this region was both sufficient to account for the new density and
well positioned to occupy it as an extension of the N-arm density. We propose that each tube pair represents an antiparallel arrangement of two VP5 N-terminal helices contributed by a penton subunit and an opposing hexon subunit. Although the formation of similar tube pairs might be expected among hexons, we found instead that the density followed a curved path of a length similar to that of the tubes (Fig. 4a). We believe that these curved features probably comprised the same region of VP5 that forms the helix in the penton-associated tubes, but this region was induced to form an alternative fold by unique factors associated with hexon–hexon packing. If the two conformations, elongated (penton–hexon) and curved (hexon–hexon), indeed arise from the same part of the same protein, we expect that this region would be more likely to occur at one end of the protein rather than in the middle. Furthermore, the PRV capsid map (Fig. 4b) lacked straight tube pairs, but the density in the hexon–hexon region appeared to resemble the curved tubes of HSV, although this region was shorter. This result was consistent with VP5 sequence alignments showing that the VP5 N-terminal domain has ten residues fewer in PRV than in HSV (Fig. 4c), thus supporting assignment of both the straight and curved tubes to the most N-terminal part of VP5.

The helix pairs are not visible in an HSV B-capsid map7,30. However, a C-capsid map at 16-Å resolution (data not shown) revealed tracks of density occupying the same location beneath pentons as the helix pairs. The difference suggests that the tubes stabilize pentons in DNA-filled capsids, perhaps serving to buttress the pentons against the internal pressure of packaged DNA.

**Organization of the major capsid protein VP5**

The middle and upper domains of the VP5 protein that form the protruding capsomer turrets appeared to be a large C-terminal extension of the HK97 fold. Although the resolution was insufficient to allow modeling of the VP5 middle-domain fold, combining information from the atomic model of the upper domain, the HK97 pseudoatomic model of the lower domain, and secondary-structure prediction allowed us to generate a reasonable model for the organization of VP5 domains (Supplementary Fig. 3). We readily detected elements of the HK97 fold in the N-terminal region of the VP5 sequence, which was anchored on the long helix predicted between residues 150 and 190, and which we assigned as the 50-Å spine helix. Directly upstream, we observed a 12-residue helix (residues 130–142) corresponding to helix 1 of the HK97 fold and preceded by a series of β-strands compatible with the E loop. Further upstream, we assigned the N arm, which was predicted to be α-helical (residues 50–78), as in phages such as T4 and T5, in which it binds scaffolding proteins on the capsid interior during assembly31. The ~50 most N-terminal residues extending beyond the fold of the mature HK97 capsid protein were associated with the tube motifs observed beneath capsomers (as described above).

Density downstream of the putative spine helix was not sufficiently well connected to allow conclusive assignments of sequence to structural domains. However, we propose that the lower HK97-like domain continues after the spine helix, accounting for residues 200–440, and is followed by the middle domain, which is broken into two parts encompassing residues 440–483 and 1046–1374, and brackets the upper-domain fragment 484–1045 (PDB 1NO7 (ref. 7)). This assignment includes insertions after the spine helix (residues 200–270) as well as after helix 4 (residues 350–390), which are consistent with smaller insertions observed in some HK97-fold family members32, such as HK97 itself (PDB 2FT1 (ref. 21)), epsilon 15 (PDB 3140 (ref. 33)), the CTF073 prophage protein (PDB 3BQW), and the Thermotoga maritima encapsulin (PDB 3DKT34).

**The triplex molecule**

The triplex composition is putatively a heterotrimer of VP19C (50 kDa; one copy) and VP23 (34 kDa; two copies)3,35, but gel-based stoichiometry varies, and indeed subpopulations of triplexes formed from different combinations of the two constituent proteins cannot be ruled out. The quality of our maps allowed us to make a definitive assessment that was aided in part by comparison with a third herpesvirus, KSHV (EMD-6038 (ref. 36)) (Fig. 5). In each of our capsid maps, the triplexes at the five local three-fold sites within the asymmetric unit were well resolved and identical in shape, thus indicating that they are specifically oriented at each site and share a common composition. The sixth triplex site on the icosahedral three-fold axis yielded regions of strong and weak density. We were able to reproduce this distribution of density by imposing three-fold symmetry on any of the other triplex regions, and we concluded that triplexes at all six distinct locations in the asymmetric unit were identical in composition.

Inspection of the triplex density revealed an organization consistent with a 1:2 heterotrimer composition, i.e., a nonsymmetric...
region associated with a quasidimeric motif (Fig. 5b). We observed a similar quasidimer motif in the KHSV map but a markedly smaller nonsymmetric region (Fig. 5c). The similar quasidimer densities suggested two copies of VP23, whereas the differently sized nonsymmetric regions are consistent with one copy each of VP19C in HSV-1 and its shorter homolog in KHSV. Interestingly, the VP23 subunits were related by different rotations according to height above the capsid floor, being 120° apart at the base, as if they adopt a more trimeric arrangement with the lone copy of VP19C, and ~140° at the top (Fig. 5d). In addition, the conformation of each copy of VP23 in a triplex differed by an ~40° bend between the top and bottom portions (Fig. 5e), thus further departing from dimeric symmetry and disguising the organization of the molecule.

We also observed density that penetrated the capsid floor and was contiguous with the triplex (Fig. 5a,b). This density did not obey local three-fold symmetry, thus ruling out contributions from the three adjacent copies of the VP5 major capsid protein. Further, the N-terminal 72 residues of VP19C have been shown to bind dsDNA nonspecifically. Consequently, we propose that the N-terminal region of the VP19C subunit is embedded in the capsid-floor layer and accesses the capsid interior.

**Figure 5** Triplex organization. (a) Views of the HSV-1 triplex density segmented into a dimeric region, corresponding to two copies of VP23 (red and blue), and an additional region corresponding to VP19C (green). Left, top view. Center, bottom view of asymmetric density on the capsid interior surface, assigned to VP19C. Right, bottom view with VP5 density replaced by the HK97-based model. (b) Top views of triplex density extracted from the HSV-1 map, and a side view including models of the VP5 lower domain. Right, the VP19C monomer (green) and VP23 dimer (red and blue). (c) Views from a KSHV reconstruction (EMD-6214 (ref. 49)) corresponding to a KSHV reconstruction (EMD-6214 (ref. 49)) corresponding to b. (d) Rotational displacements between VP23 subunits at different heights, as indicated by dashed lines in b. (e) Superposition of the two VP23 regions from one triplex.

Arrangement of the CVSC subunits pUL17 and pUL25

Covalent attachment of GFP to the pUL17 (ref. 14) and pUL25 (ref. 15) proteins has confirmed their proposed contribution to the CVSC molecule that surrounds pentons on the capsid exterior. Visualization of the tags by cryo-EM has also suggested that pUL17 is proximal to the penton, and pUL25 is distal, as originally proposed. However, we found that the pUL25 C-terminal model (PDB 2PSU, residues 134–580) did not fit into the distal region in our new maps. Consequently, we sought to test the arrangement of pUL17 and pUL25 by knocking out each protein in turn. Western blot analysis of HSV-1 nucleocapsids lacking pUL25 (~Δ25) confirmed the absence of pUL25 but detected pUL17 (Fig. 6a). In contrast, both proteins were absent from the western blot when pUL17 was deleted (~Δ17), thus suggesting that pUL25 requires pUL17 to bind capsids, but pUL17 does not require pUL25 for...
capacids binding, in agreement with Thurlow et al.38. We verified equal loading of the capacid samples by SDS–PAGE (Fig. 6a).

We then confirmed the subunit positions by cryo-EM reconstruction of the two knock-out mutants (Fig. 6b). The Δ25 map revealed a bridge of density across the triplexes that resembled CVSC density seen in central sections through native capsids (Fig. 6c), thus demonstrating that pUL17 forms this bridge, but pUL25 does not. The low occupancy of this pUL17 density is typical for nucleocapsid reconstructions, especially empty capsids16, compared with virion capsid reconstructions (Fig. 6c). The Δ17 map instead lacked all CVSC density, thus indicating that pUL25 does not bind capacids directly but depends on pUL17 for binding. However, with pUL17 now correctly assigned to the triplex-spanning bridge density, the remaining CVSC density in the HSV-1 map appeared to be insufficient and inconsistent with the large Δ25 C-terminal crystal structure.

We finally located the large pUL25 C-terminal domain by examining the CVSC density in our new PRV capacid map. Both maps revealed a striking group of 80-Å-long parallel tubes projecting from the triplex bridge toward the penton and consistent with a four-helix bundle (Fig. 7a–c). Density at the side of this bundle and adjacent to the penton was weak and fragmentary in the HSV-1 map and has not previously been identified with the CVSC. In contrast, this region was very well defined in our PRV map (Fig. 7c), where it contacted the CVSC helical bundle and was large enough to accommodate much of the pUL25 subunit. Indeed, the fit of the HSV-1 pUL25 C-terminal model (PDB 2F5U) into this region of the PRV density map was straightforward and convincing (Fig. 7c,d) and placed the model’s N-terminal residue 134 near the CVSC helical bundle. We then transferred the pUL25 fit into the corresponding but weaker density of the HSV-1 map, thus demonstrating that pUL25 has the same fold and location in both viruses. The conservation of the fold of pUL25 between HSV-1 and PRV is not surprising because the two proteins share 50% sequence identity. Furthermore, the pUL25 protein of HSV-1 can partially complement pUL25-deficient PRV capsids12, thus indicating conservation of structure and function.

Interactions among CVSC, penton and tegument

We observed the pUL25 C-terminal domain contacting two VP5 subunits of the adjacent penton as well as density sitting atop the penton (Fig. 7e). Fitting of pUL25 and the VP5 upper domain in the HSV-1 map revealed that the two pUL25 loops (A249–D254 and R335–G345) approaching the penton were negatively charged, whereas the opposing VP5 loops (R512–R519 and R798–A803) were positively charged, thus suggesting an electrostatic attraction (Fig. 7f). However, the fragmentary HSV-1 density suggested that the C-terminal domain was mobile and not stabilized by binding to VP5, or that it bound in several alternative locations, thus causing blurring in the density map by superposition. The complimentary charges across the interface may function more to align the pUL25 C-terminal domain relative to VP5 rather than to lock it in place.

The penton-capping density in HSV-1 has been ascribed to the pUL36 protein17, and indeed pUL36 is known to bind pUL25 (ref. 39). We observed that the pUL36 density was contacted by pUL25 loop T510–P514, which is essential for virus replication, as shown by a transient virus replication assay in which the loop was deleted40. In contrast, the same study has reported no effect of deleting the two VP5-contacting loop regions of pUL25 identified above, thus indicating that those contacts are not essential for HSV-1. A similar deletion study in PRV would address whether the additional stability observed for pUL25 affects viability.

Model of the CVSC

The results above indicated three distinct CVSC domains: the triplex bridge composed of pUL17; the vertex-binding region including pUL25 and putatively pUL36 as a member of the CVSC molecule;
The contacts that we observed between pUL25, VP5 and pUL36 identifies interfaces that may be critical to assembly and function. The triplex and CVSC molecules clarifies this structural linkage and density above pentons. Resolving the molecular organization of the triplex to the CVSC decoration molecule that interacts with the pUL36 tegument density; orange, CVSC density; black arrow, density connecting the pUL25 C-terminal and N-terminal domains. Scale bar, 100 Å. 

(c) Model of the CVSC subunit organization, viewed from above (left), including the previously reported locations of two GFP tags and an N-terminal tandem affinity purification tag, and from the side (right).

DISCUSSION

The quality of our density maps and the comparisons between them have led to crucial insights into herpesvirus capsid structure. Key among these insights is that triplex density links the capsid interior to the CVSC decoration molecule that interacts with the pUL36 tegument density above pentons. Resolving the molecular organization of the triplex and CVSC molecules clarifies this structural linkage and identifies interfaces that may be critical to assembly and function. The contacts that we observed between pUL25, VP5 and pUL36 generally confirm a recent report examining nuclear and cytoplasmic HSV-1 capsids at ~20-Å resolution. The results of that study suggest that pUL36 and the CVSC molecule have a costabilizing effect and that pUL36 forms an integral part of the CVSC molecule. In light of our assignment of pUL17 to the triplex bridging density, we reevaluated older HSV-1 and PRV data to confirm that pUL25 is present on capsids only when both pUL17 and the penton-capping pUL36 density are present. Further, nucleocapsids have been suggested to bind a truncated form of the pUL36 protein that is later replaced by full-length pUL36 after nuclear export. pUL36 is a large protein that extends well into the tegument layer, owing to its elongated central domain, and may act as the interface between capsid and tegument. We propose that pUL25 acts in concert with the exposed VP5 tips of pentons and forms a site that recruits tegument onto the capsid, most probably the C-terminal region of pUL36, which may be truncated (in the nucleus) or full length (postnucleus), and that the binding of pUL36 stabilizes pUL25 on the capsid.

Although triplexes are abundant on the capsid surface, the CVSC molecule binds only to a specific pair adjacent to vertices. As shown in Figure 9a, where the orientation of each triplex is marked by an arrow, triplexes immediately adjacent to the vertex and those in the next layer out ‘point’ toward each other (green pair). This arrangement is also evident between a pair of triplexes around the two-fold hexons (blue pair) but not elsewhere on the capsid surface. The correct triplex epitope for CVSC binding is thus limited at the vertices. Consequently, hexons at the empty site block the next layer out of adjacent capsids, whereas hexons adjacent to CVSC occupy both sites. This difference in packing density explains the morphology of the sectioning surface.

Figure 8 Organization of the CVSC molecule. (a) Transverse sections through this bundle revealed four spots arranged on a 10-Å-sided square (Fig. 8a), thus strongly supporting our interpretation that the bundle comprises four helices up to 80 Å in length. A fifth spot beneath the others suggested that an additional, shorter helix may also be present. Longitudinal sections captured parts of the helices (Fig. 8b) but not their full lengths as they twisted in and out of the sectioning surface.

With these new data from HSV-1 and PRV, we propose a more accurate model for the arrangement of the CVSC subunits around pentons that remains consistent with the results of our previous labeling experiments (Fig. 8c). Fitting of the crystal structure anchors the pUL25 C-terminal domain (A134–V580) in contact with the penton, and the pUL25 labels previously visualized place the N terminus and residues 50 and 51 in the penton distal region. Spanning the gap are the N-terminal residues A48–E110, which are predicted to fold as a helix (Supplementary Fig. 4) and are likely to contribute one 60-residue-long strand to the helical bundle. Beneath the bundle is the pUL17 density, which bridges triplexes but probably also contributes up to two helices to the four-helix bundle. This new organization of CVSC explains why pUL17 is required for pUL25 to bind capsids, but the converse is not true, and confirms the previous finding that pUL25 binds though the N-terminal 50 residues. Further, if pUL36 is indeed an integral part of the CVSC, and its C-terminal portion binds both pUL25 (refs. 39,41) and the penton, then the 40 most C-terminal residues of pUL36 are predicted to fold as a helix and could potentially form a shorter member of the helical-bundle domain of CVSC. Although this newest aspect of the CVSC remains to be confirmed, the structural framework that we developed here will serve to guide experiments addressing the precise composition and assembly order of the subunits.

Figure 9 Organization of the CVSC molecule. (a) Transverse sections through the CVSC helical bundle of PRV, shown at distances of 88 Å, 99 Å, 110 Å, and 121 Å from the vertex, from left to right, respectively. Purple, hexon density; green, triplex density; arrows, helical bundle, which is magnified three-fold in the insets. Scale bars, 100 Å (black) and 10 Å (white). (b) Radial sections in which helices are sliced longitudinally (arrowheads). The top section is at lower radius; the bottom section at higher radius. Purple, hexon density; blue, penton density; orange, CVSC density; black arrow, density connecting the pUL25 C-terminal and N-terminal domains. Scale bar, 100 Å. (c) Model of the CVSC subunit organization, viewed from above (left), including the previously reported locations of two GFP tags and an N-terminal tandem affinity purification tag, and from the side (right).
Figure 9 CVSC location is limited by triplex orientation and hexon crowding. (a) Surface view of the CVSC molecule bound to pairs of triplexes (green) adjacent to the penton (P). H, hexon. The triplex density is asymmetric but oriented consistently on the capsid, as indicated by the assignment of direction (arrows). Right, schematic of a capsid facet. (b) Surface view of a superposition between the green triplex pair that binds CVSC and the blue pair that does not, as shown in side view. (c) Comparison of the gap between hexons adjacent to the green triplex pair that binds CVSC (left) and those adjacent to the blue triplex pair (right). At right, the CVSC density binding the green site in the left panel is superimposed onto the blue triplexes and clashes with the hexon above it.

by both the orientations of triplexes and the differences in crowding by adjacent hexons.

The restricted pattern of CVSC molecules on the capsid surface depends in part on the specific orientations of the underlying triplexes, thus prompting us to ask what governs triplex organization. A recent cryo-EM study on HSV-1 procapsids has proposed that the accretion of capsomers by the nascent structure is sequential and specific, guided both by an internal protein scaffold and by the triplexes acting as an external scaffold. This inclusion of three-fold-located capsid proteins during assembly is different from that of the gpD protein of phage \( \lambda \) and the small outer capsid protein of T4, which bind during capsid expansion. Further, the triplexes have direct access to the interior of the mature capsid, where the N-terminal domain of the VP19C triplex subunit may interact with the viral DNA. We propose that, in addition to guiding capsomers into the correct \( T = 16 \) geometry and holding them together as if stapled, the triplexes may form a signaling pathway from the internal DNA to the CVSC molecule poised above. In addition to retaining encapsidated DNA, the CVSC subunit pUL25 has also been implicated in nuclear egress of the capsid and transport of the genome into a newly infected nucleus. We speculate that the triplex–CVSC complex may trigger capsid export in response to the pressure of DNA at the completion of packaging, or conversely may signal to the capsid interior when the incoming capsid is docked at a nuclear pore, or may do both.

Two herpesvirus capsid structures derived from cryo-EM data have recently been reported: the human cytomegalovirus (EMD-5695) and KSHV (EMD-6038). These have been claimed to be at 6 Å resolution, but the first shows overfitting artifacts and blurring that is incompatible with this estimate, whereas the second uses the low 'gold standard' correlation coefficient cutoff of 0.143 to estimate resolution. Nonetheless, the KSHV capsid density is comparable in quality to our maps and aided our understanding of the triplex structure. The authors also state that the KSHV penton is rotated axially by \( \pi \) in comparison to HSV and PRV; however, fitting of the HSV-1 VPS upper-domain fragment (Supplementary Fig. 5) indicates a rotation of at most \( 5^\circ \). We found instead that it is the C-terminal domain of the pUL25 analog, pORF19, that is displaced by \( \sim 30^\circ \) around the penton from the corresponding location in the HSV and PRV density maps, owing to a shorter and differently oriented helical bundle in the CVSC. Consequently, pORF19 contacts only one penton subunit, not two subunits as does pUL25 in PRV. More remarkable is that the pUL25 crystal structure, when fit into that putative pORF19 C-terminal region, is rotated by \( \sim 180^\circ \) about its connection to the remainder of the CVSC density, relative to the orientation of pUL25 in our PRV fit. If this fitting of pUL25 in KSHV is correct, then the fold elements of pORF19 that interface with binding partners such as the adjacent capsomers and tegument proteins are completely different from those of pUL25 in HSV and PRV.

In conclusion, our herpesvirus capsid maps reveal new details of capsid structure and function, thus allowing resolution of conflicting reports on subunit organization and providing a sound basis on which to design future studies. Further refinements in data collection and analysis are likely to bridge the gap to atomic resolution for large structures such as herpesvirus capsids, in particular by accounting for a focus gradient through the capsid density. The symmetry-breaking portal vertex is also an important target because of its role in capsid nucleation and in packaging and releasing the viral genome, but it awaits a successful labeling strategy to identify its location. This work demonstrates not only the continuing improvement in cryo-EM structure determination, which is particularly valuable for studying entire complexes in situ, but also the benefit of combining and contrasting such data from structurally related sources.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Capsid maps have been deposited in the Electron Microscopy Data Bank under accession codes EMD-6386 (HSV-1) and EMD-6387 (PRV).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.F.C. and F.L.H. developed the concepts and experiments; J.B.H. and F.L.H. prepared samples and performed biochemistry; A.M.M. prepared grids; M.V. and J.F.C. developed microscopy procedures and collected data; A.H. and J.F.C.
performed data analyses and interpretation; J.C.C., F.L.H. and A.H. prepared the manuscript, which was edited by A.M.M. and M.V.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Virion purification. Virions of HSV-1 (strain KOS) and PRV (strain Becker) were isolated from the medium of infected Vero cells (CCL-81 from ATCC) as described previously. Vero cells (1.5 x 10^6) were infected overnight (18 h at 37°C) with HSV-1 or PRV at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU) per cell. Infected cells were scraped into the cell medium, and 5 M NaCl was added to a final concentration of 0.5 M NaCl. Cells were pelleted, and the medium was transferred to SW28 rotor tubes; virions were pelleted out of the medium by centrifugation at 20,000 r.p.m. for 35 min. The resulting pellet was resuspended in TNE buffer (10 mM Tris, 150 mM NaCl, and 1 mM EDTA, pH 7.5) plus protease inhibitors, and the sample was layered on top of a 20–50% sucrose (in TNE) gradient (SW41 rotor at 24,000 r.p.m. for 1 h). The gradient was fractionated with a Beckman Fraction Recovery system, the virion fractions were pooled and resuspended in TNE buffer, and the virions were pelleted (SW41 rotor at 24,000 r.p.m. for 1 h). The virions were resuspended in TNE buffer for cryo-EM studies.

Analysis of capsid proteins. Intranuclear capsids were purified from Vero cells (1.5 x 10^6) infected overnight (18 h at 37°C) at an MOI of 5 PFU/cell by sucrose density gradient fractionation as previously described. Gradient fractions containing A, B, or C capsids were run on 4 to 12% SDS–PAGE, and the gels were either stained with Imperial blue (Pierce) to visualize capsid proteins or analyzed by immunoblotting with an anti-pUL25 mouse monoclonal antibody, 25E10 (ref. 15) provided by J. Brown (University of Virginia) or an anti-pUL17 chicken polyclonal antibody provided by J. Baines (Cornell University), diluted at 1:5,000 and 1:25,000, respectively. The diluted antibodies were reacted with the blocked nitrocellulose in 2 h at room temperature, washed five times in Tris-buffered saline with 0.5% Tween 20, and incubated with IRdye800-conjugated secondary antibodies diluted 1:15,000 in Rockland near-infrared blocking buffer (Rockland Immunochemicals) with 0.1% Tween 20 and goat anti-mouse (UL25) or donkey anti-chicken (UL17) obtained from LiCor. The blots were washed and scanned with an Odyssey system (LiCor).

Cryo-electron microscopy. 3 µl of sample was deposited on a Quantifoil R2/1 copper grid (Quantifoil Micro Tools) that had been glow-discharged for 13 s. Grids were blotted and plunge-frozen into a liquid-nitrogen-cooled mix of 2:1 ethane and propane with an FEI Vitrobot Mk IV. Grids were mounted in an FEI Krios microscope operating at 300 kV and imaged under conditions of parallel illumination at a nominal magnification of 75,000 x with the FEI EPU automated data collection software, version 1.2. Images were collected on an FEI Falcon 2 direct-electron-detecting camera with a postcolumn magnification of 1.6 x and pixel dimensions of 14 µm, thus yielding a calibrated pixel size at the specimen of 1.08 Å. Exposures lasted 2 s. For HSV-1, 21 grid squares from two grids yielded 50,000 micrographs, from which 49,660 capsid images were selected; 25,637 of these were inside intact virions, and the rest were DNA-filled capsids that were partially or completely emerging from broken virions. One grid of the PRV virion sample was imaged, and 32,956 micrographs were collected from 19 grid squares, thus yielding 28,374 capsid images, of which 13,242 were from intact virions. Although data collection extended over as many as 7 d per grid, no appreciable change in contrast due to ice contamination was observed.

Image reconstruction. Capsids were frequently obscured by the tegument and membrane layers, and often the distinctive DNA ‘fingerprint’ was the only indication of capsid location within the virion; consequently, particles were picked by hand. Defocus estimates were made automatically for each micrograph with CTFIND3 (ref. 55) and then adjusted by a local smoothed defocus function according to the location of each image within a grid square. Capsid images were reduced two-fold in size for an effective pixel size of 2.16 Å and were analyzed with AUTO3DEM software running on a Beowulf cluster with 192 processing elements, imposing icosahedral symmetry. Minor image-processing steps were performed with BSOFT. Resolution of the HSV-1 map was assessed at 6.8 Å with the Fourier Shell Correlation (FSC) at a limit of 0.3 (Supplementary Fig. 2). Comparison of the density map with the major capsid protein VP5 upper-domain–fragment cryo-crystal structure (PDB 1NO7 (ref. 7)) rendered at different resolutions was consistent with this estimate (Supplementary Fig. 2). Inspection of the density map revealed good localization of density as tubes where the atomic models indicated helices, but no convincing chirality was observed. Density maps were inspected and rendered in UCSF Chimera. Atomic models were fit as solid bodies and then used to segment density. The model of the VP5 floor domain was derived from the HK97 gp5 structure of the expanded capsid (PDB 2FT1 (ref. 21)). In the absence of successful sequence alignment or side chain features in the cryo-EM maps, we modified an initial solid-body fit by removing incompatible regions of the HK97 model—the loop after the spine helix and the tip of the A domain—and we extended the spine helix with the ‘build structure’ tool in Chimera and the long α-helix sequence predicted in PSIPRED (Supplementary Fig. 3) and adjusted the resulting structure to the density map.

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