Cryoelectron Microscopy Maps of Human Papillomavirus 16 Reveal L2 Densities and Heparin Binding Site

Highlights
- 4.3-Å resolution 3D reconstructions based on gold-standard FSC
- Classification of HPV overcomes heterogeneity that limits the resolution
- L1 protein was built and used to reveal L2 minor protein
- Heparin binding elicits conformational changes to L2

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In Brief
Guan et al. solved near-atomic resolution cryo-EM maps of HPV16 and HPV16-heparin, building the L1 protein structure including C-terminal arm. The structural analysis revealed the heterogeneity of HPV16, the location and features of L2 protein, the heparin binding site, and suggested L2 conformational changes during cell entry.

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Cryoelectron Microscopy Maps of Human Papillomavirus 16 Reveal L2 Densities and Heparin Binding Site

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SUMMARY

Human papillomavirus (HPV) is a significant health burden and leading cause of virus-induced cancers. The current commercial vaccines are genotype specific and provide little therapeutic benefit to patients with existing HPV infections. Host entry mechanisms represent an excellent target for alternative therapeutics, but HPV receptor use, the details of cell attachment, and host entry are inadequately understood. Here we present near-atomic resolution structures of the HPV16 capsid and HPV16 in complex with heparin, both determined from cryoelectron micrographs collected with direct electron detection technology. The structures clarify details of capsid architecture for the first time, including variation in L1 major capsid protein conformation and putative location of L2 minor protein. Heparin binds specifically around the capsid icosahedral vertices and may recapitulate the earliest stage of infection, providing a framework for continuing biochemical, genetic, and biophysical studies.

INTRODUCTION

Human papillomavirus 16 (HPV16) is the most prevalent oncogenic genotype in HPV-associated anogenital and oral cancers. Having co-evolved with the human host for more than a million years, HPV develops chronic infections by activating the cell cycle as epithelial cells differentiate in order to create a replication competent environment that allows viral genome expression cycle as epithelial cells differentiate in order to create a replica.

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remaining 60 capsomers are each surrounded by six other capsomers and referred to as hexavalent capsomers. An extension of the L1 C terminus, or C-terminal arm, links capsomers together and provides interactions leading to the formation of disulfide bonds between Cys175 and Cys428 from two neighboring L1 proteins (Buck et al., 2005a, 2005b; Cardone et al., 2014; Sapp et al., 1998). Although the invading arm structure of bovine papilloma structure is known (Wolf et al., 2010), the stabilizing C-terminal arm structure has not been solved yet for HPV.

Besides the L1 major capsid protein, there is an uncertain number of L2 minor structural proteins that co-assemble with L1 to form infectious virus capsids. The L2 minor protein has a number of functions including encapsidation of DNA, involvement in the conformational changes of the capsid during cell entry, disruption of the endosomal membrane, and subcellular trafficking of the incoming viral genome (Bromann et al., 2013; Buck et al., 2008; Raff et al., 2013). Specifically, the N-terminal residues of L2 (1–88) are exposed on the surface of the capsid although the anti-L2 antibody, RG-1 recognition of L2 (17–36) is dependent on maturation status or cell attachment (Day et al., 2008a, 2008b; Finnen et al., 2003; Lowe et al., 2008; Okun et al., 2001). An N-terminal L2 motif (RXXR, furin cleavage site) is conserved across HPV genotypes and thus has promise as a new target for vaccine development (Bromann et al., 2013; Conway et al., 2011; Roden et al., 2001). A previous structural study using pseudovirus maps (23 and 25 Å) interpreted small densities beneath each capsomer as belonging to L2 (Buck et al., 2008). Based on analysis of the late gene products, L2 protein has been predicted to bind within the center of the L1 capsomer with the antigenic region facing outward (Lowe et al., 2008). Stoichiometric studies have indicated that there are about 12–36 L2 molecules per HPV capsid (Okun et al., 2001); however, other works have suggested the amount of L2 per capsid is variable, and may include up to 72 L2 molecules per capsid, perhaps one L2 at each capsomer (Buck et al., 2008; Doorbar and Gallimore, 1987; Trus et al., 1997). Naturally produced virions average approximately 24–36 L2 molecules per capsid (Bromann et al., 2013; Buck et al., 2008; Campos and Ozbin, 2009). Despite these variable reports, there is general acceptance that between 12 and 72 L2 proteins per capsid are incorporated, each into a central internal cavity of the capsomer via L2 C-terminal interactions.

During host entry and endocytosis, HPV conformational changes are triggered by binding to a cell receptor (Abban and Meneses, 2010; Kines et al., 2009; Selinka et al., 2003). Cell surface heparan sulfates (HSs) serve as a primary attachment receptor (Buck et al., 2006; Rommel et al., 2005; Schowalter et al., 2011; Shafti-Keramat et al., 2003) and it is not the protein backbone but the glycan moieties, specifically the sulfation pattern, that are required for virus infection (Cerqueira et al., 2013). HS proteoglycans are expressed and secreted by nearly all mammalian cells, and are located on both the cell surface and extracellular matrix (Bernfield et al., 1992; Buck et al., 2006; Schowalter et al., 2011; Selinka et al., 2003). However, because HS polysaccharides are expensive and difficult to obtain, heparin has been used in lieu of HS in most biological and structure studies (Cerqueira et al., 2013; Selinka et al., 2007). Both heparin and HS binding have been shown to induce conformational changes of virus during entry (Florin et al., 2012; Knappe et al., 2007; Levy et al., 2009). Co-crystallization of a truncated L1 capsomer together with heparin oligosaccharides (8 and 10 monosaccharide units) suggested four distinct heparin binding sites that mapped to the L1 protein (Dasgupta et al., 2011). Three of these HS binding sites were verified by subsequent mutational analysis (Dasgupta et al., 2011; Richards et al., 2013). Nonetheless, the molecular mechanisms regarding the binding of heparin during entry remain fragmented and not completely understood.

With recent technological advances in cryo-EM image detection and the introduction of new software classification schemes, we can now investigate HPV at near-atomic resolution. Here, we present the near-atomic resolution structures of HPV16 alone and HPV16 interacting with heparin molecules at 4 Å resolutions. Three-dimensional (3D) classification of the data revealed that HPV capsids are heterogeneous in nature but contain sub-populations of capsids with consistent diameters. The classification of particles improved the high-resolution maps, which facilitated building de novo the L1 protein (residues 3–485), revealing the structure of the C-terminal arm. Solving the L1 asymmetric unit allowed us to calculate an L1-only capsid that was used to identify the location of L2 densities in our maps. Binding of heparin induced no conformational changes in the capsid that include local changes in L1, but there are alterations to putative L2 densities. Besides the identification of the heparin binding site in the context of the intact capsid, the changes induced by heparin binding provide the first glimpse of an HPV entry intermediate. We propose an entry mechanism model that may include isolation of specific 5-fold capsomers due to potential interaction between heparin and L2 protein.

**RESULTS**

Near-Atomic Resolution Maps Were Reconstructed by Selecting Homogeneous Sub-populations

HPV16 capsids and capsids incubated with heparin in excess were vitrified for cryo-EM data collection using new direct electron detector technology. The drift-corrected micrographs of HPV and HPV-heparin displayed bumpy spherical shapes with evident capsomer protrusions (Figures 1A and 1B). Capsids contained internal density corresponding to packaged DNA and appeared to have diameters of about 60 nm. However, 3D classification using RERegularized Likelihood OptimizatioN (RELION) showed that the populations of HPV and HPV-heparin capsids were heterogeneous in size (Figures S1 and S2). Both capsid types included a large group of approximately 50% of the total number of particles that had the same diameter of 569–570 Å. Final reconstructions using AUTO3DEM with the gold-standard approach used 50% of the HPV particles and 50% of the HPV-heparin complex particles to reconstruct a homogeneous population in each case to near-atomic resolution (Figures 1C and 1D). The 4.3Å resolution of the maps was estimated where the Fourier shell correlation (FSC) dropped below 0.143 (Figure 2) (Table 1). Central sections through the density maps illustrate the modest quality of the reconstructions (Figures 3E and 1F). At this resolution, we can see strand separation, the secondary structures of α helices and β strands (Figures 3AI, 3AII, 3BI, and 3BII), and side chains...
of some residues can be identified (Figures 3A1–3A3 and 3B1–3B3).

Building L1 into the Cryo-EM Density Solved the Structure of the C-Terminal Invading Arm and Revealed the Location of L2

Strong continuous density facilitated the modeling of the L1 protein structure from residues 3 to 485, which included the C-terminal arm that links capsomers. The structure building was initiated with a combination of the previously solved pseudoatomic L1 model (PDB: 3J6R) and the crystal structure of the truncated L1 (PDB: 3OAE). During the iterative modeling and refinement, six full-length L1 molecules were built separately to make an asymmetric unit of the capsid (Figure 4A). After applying icosahedral symmetry to this asymmetric unit an L1-only density map was generated (see Experimental Procedures). A difference map was calculated by subtracting the L1-only density from the L1/L2-containing HPV16 experimental map. Difference density mapped to both pentavalent and hexavalent capsomers (Figures 4B and 4C). Putative L2 density began at the inside base of the capsomer (Figure 4C), rose along the outside of the capsomer to the shoulder, ending at the outside center of the capsomer (Figure 4B). Corresponding densities in the pentavalent capsomer were significantly smaller than the densities in hexavalent capsomers, both at the surface and the internal part at the base.

Distinct Structural Differences Were Observed between Pentavalent and Hexavalent Capsomers

Our near-atomic structure of the HPV16 capsid supplied more detailed information on the capsid surface topology than the maps we produced before (Guan et al., 2015a, 2015b; Lee et al., 2014). Besides the entire C-terminal arm, surface loops could be distinguished separately, especially the BC and EF
loops that were seen as merged densities in the previous lower-resolution maps (Figure 4A). To further visualize the differences between L1 conformations in the two different environments, the newly built structures of the pentavalent and hexavalent capsomers were superimposed (Figure 5A). The conformational differences were obvious on both surface loops and C-terminal arms, with a root-mean-square deviation (RMSD) value of 4.77 Å between the entire sets of L1 atoms of the two capsomers. Since the C-terminal arm conformations were noticeably different, the RMSD was recalculated with this part of the structure truncated, and found to be 1.49 Å, indicating significantly different loop structures throughout. Specifically the hexavalent C-terminal arm extended further away from the hexavalent capsomer, whereas the pentavalent arms were arranged closer in a curved arc at the site of interaction with the neighboring hexavalent capsomer. The individual BC, DE, FG, HI, and EF loops are in decidedly different conformations throughout; however, overall the hexavalent capsomers formed a more open crown-like structure compared with the pentavalent capsomers.

Differences between the pentavalent and hexavalent capsomers were also apparent in the local resolution map (Figures 5B and 5C). The pentavalent capsomer surface had lower local resolution compared with the hexavalent capsomer, especially near the center, suggesting the pentavalent capsomer loops had more flexibility. Overall the very center and the outer edges of the capsomers had lower resolution than the mid-regions of the capsomers. Specifically, the BC and EF loops, which were located at the tip of the star-shaped capsomer, had the lowest resolution (6.5 Å) in both pentavalent and hexavalent capsomers.

**The HPV-Heparin Complex Showed that Heparin Receptors Only Bound at the 5-fold Vertex of the HPV16 Virus**

Significant extra densities were found at each 5-fold vertex of the HPV-heparin complex map compared with the HPV map. This putative heparin density was comparable with the magnitude of the average capsid density, which suggested nearly full occupancy of bound heparin. The regions of strongest density were located between neighboring pairs of BC and EF loops; however, there was noisy discontinuous density stretching out and away from the bound heparin (Figure 1E). The mixture of polysaccharide chain lengths (10–30) in the heparin incubated with the virus in our study likely caused the lesser densities extending away from the bound heparin, which corresponded to longer polysaccharide chains of bound heparin that were averaged with shorter chains during the reconstruction process.

**Heparin Binding Triggers Conformational Changes of L2 Protein without Altering the Capsid Diameter**

No significant difference in diameter between HPV16 and HPV16-heparin complex maps was seen in the radial density plots (Figure 6A), indicating that heparin binding did not trigger a change in the overall size of the capsid. The structure of L1 was built into the HPV-heparin complex map to make an asymmetric unit and an L1-only capsid map was calculated as described above for the capsid. The mass axis of L1 proteins in pentavalent and hexavalent capsomers in the HPV and the HPV-heparin maps were calculated (Figure 6B) to track both rotational and translational movements of L1 proteins. L1 proteins rotated no more than 0.4° and shifted less than 0.1 Å (Figure S3). Thus, no significant movement of L1 protein was found after binding with heparin.

To check the surface loop rearrangement of the L1 protein, the HPV and HPV-heparin asymmetric unit structures were superimposed. An RMSD of 0.76 Å suggested slight changes may have occurred after heparin binding. Superimposing the HPV hexavalent and pentavalent capsomers on

![Figure 3. Secondary Structure and Residue Side Chain of the L1 Protein Were Identified in the Maps](https://example.com/figure3)

**Table 1. Cryo-EM Image Reconstruction Data**

<table>
<thead>
<tr>
<th></th>
<th>HPV</th>
<th>HPV-Heparin</th>
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<tbody>
<tr>
<td>No. of micrographs</td>
<td>9,137</td>
<td>6,175</td>
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<tr>
<td>Defocus level range (μm)</td>
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<td>0.78-4.85</td>
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<tr>
<td>No. of particles selected from micrograph</td>
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<td>102,829</td>
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<tr>
<td>No. of particles selected for reconstruction</td>
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<td>51,422</td>
</tr>
<tr>
<td>Final resolution</td>
<td>4.3</td>
<td>4.3</td>
</tr>
</tbody>
</table>

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256 Structure 25, 253–263, February 7, 2017
the corresponding HPV-heparin capsomers resulted in RMSD values of 0.51 and 1.12 Å, respectively (Figure 6C). There was no significant loop rearrangement among all HI, EF, DE, FG, and BC loops (Figure 6C), and no significant change in the diameter of the capsid.

By subtracting the calculated HPV-heparin L1-only map from HPV-heparin experimental map, we found a difference density that had significant conformational changes compared with the difference densities from the HPV map (Figures 4B, 4C, 6D, 6E, and S4). After binding with heparin, difference densities in the HPV-heparin complex map included putative L2 density that mapped to both pentavalent and hexavalent capsomers. Compared to virus alone, there was redispersion within the HPV-heparin capsomer and more putative L2 densities on the top surface of the capsomer and inside the capsomers, especially for the pentavalent capsomers. These changes suggest that heparin binding initiates conformational changes that may result in additional L2 residues becoming exposed at the capsid surface and the side wall of capsomers, which is consistent with previous studies.

The HPV-heparin local resolution map was compared with the HPV map to show that areas of poorer local resolution mapped to the centers of the capsomers in the complex map (Figure 5C). However, resolution at the rim of the BC and EF loops on pentavalent capsomers improved slightly after binding with heparin, possibly due to a stabilizing effect (Figure 5C, indicated by arrow). Overall, both the pentavalent and hexavalent capsomers showed regions of poorer resolution after heparin binding, suggesting local flexible movements.

The Receptor Binding Site Is between Hexavalent and Pentavalent Capsomers

The structures of multiple disaccharide units of heparin were manually fitted one-by-one into the difference map calculated by subtracting HPV from the HPV16-heparin reconstruction. The fitting result for ten units was then refined in the HPV16-heparin complex map. The resultant heparin model and the L1 protein model were then used to describe the binding site (Figure 7A).

The HPV-heparin interaction appears to be conferred by sulfated polysaccharide residues and six different side chains from two contributing L1 proteins. Specifically, in the model two BC loops, one EF loop, and one C-terminal arm from two neighboring L1 proteins participated in the binding. One hexavalent L1 protein contributed the 59.Lys of the BC loop and 176.Thr and 177.Asn of the EF loop for interaction. All the other binding residues in the model were from one pentavalent L1 that contributed a 57.Asn, 58.Asn, and 59.Lys from the BC loop, and C-terminal arm residues of 428.Cys, 429.Gln, 431.His, 432.Thr, and 433.Pro (Figure 7B).

DISCUSSION

Variations in the size of the HPV capsid have been attributed previously to progressive stages of maturation of the capsid through formation of disulfide linkages conferred by the C-terminal arm using the pseudovirus model. Similarly, our 3D classification of the quasi-HPV capsids used in our study (Experimental Procedures) showed different-diameter capsids existing with similar surface features (Figure S1). This size variation in capsids may relate to the CRPV genome or maturation status. Variation also suggests that heterogeneity is a natural attribute of HPV, which may be linked to heterogeneity of the host in a hostile environment and may represent an evolving adaptability. For instance, the range in diameter may be linked to stability, providing classes of more (or less) stable particles. Once assembled into capsids, the heterogeneous characteristic of HPV during and after the maturation process may provide an advantage ensuring the survival of an existing sub-population, within the mixed population of capsids.
Compared with the well-understood nature and structure of the L1 major protein, the location and number of L2 minor proteins per capsid have remained controversial, despite the essential requirement for genome encapsidation and subcellular trafficking. During the past 25 years, extensive biochemical and structural studies have attempted to lift the veil shrouding L2 protein incorporation. Using the L1-only calculated map, we identified non-L1 density in our experimental map and suggest that difference density can be attributed to L2. L2 protein was observed to intertwine with L1 protein at the base part of the capsomer and extend along the side wall of the capsomer in the canyon to the top surface until reaching the upper center of each capsomer (Buck et al., 2008). This observation was consistent with a previous model, L1 residues 261–269 map near the L2 density and, presumably, the most flexibility. (C) The resolution of the BC and EF loop densities (arrow) improves after binding with heparin.

The incorporated L2 protein may start from the middle center of the capsomer, extending to the surface along the side wall to reach the capsid floor with most of the molecule including the L2 C terminus intertwining with the L1 protein and interacting with the genome at the base of the capsomer. At the current resolution we cannot solve the molecular structure of L2 peptide within the density (Figure 4) or define the orientation. However, due to the magnitude of these difference densities they cannot be definitively apportioned or assigned to L2 molecules.

An anisotropic distribution of L2 protein in the capsid implies that a unique vertex may exist, which may function during host cell binding and infection. However, due to the magnitude of these difference densities they cannot be definitively apportioned or assigned to L2 molecules.

The L2 N terminus has been shown previously to be accessible at the surface of the capsomer (Conway et al., 2011; Lowe et al., 2008), and such a location is suggested in our map (Figure 3B). The incorporated L2 protein may start from the middle center of the capsomer, extending to the surface along the side wall to reach the capsid floor with most of the molecule including the L2 C terminus intertwining with the L1 protein and interacting with the genome at the base of the capsomer. At the current resolution we cannot solve the molecular structure of L2 peptide within the density (Figure 4) or define the orientation. However, the morphological features and conformational changes of the putative L2 protein densities might be used to guide future studies to contribute to understanding the mechanisms of entry and infection.

Variations in the micro-environment of the T = 7d icosahedral capsid (Guan et al., 2015a, 2015b; Lee et al., 2014; Xia et al., 2016; Zhao et al., 2014) underlie functional differences between pentavalent and hexavalent capsomers. Specifically, the U4 antibody binds preferentially at the icosahedral 5-fold (Guan et al., 2015a), which is consistent with the previous studies where U4 antibody prohibited the virus from binding cells (Day et al., 2007; Deschuyteneer et al., 2010). Considering the functional differences between pentavalent and hexavalent L1 environments, preferential heparin binding, and the established importance of L2 function during entry, we conclude that the 5-fold vertex can be considered the functional site for binding host cells during entry. This model suggests L2 incorporation must include the vertex pentamers.

Previous work has shown that HS binding triggered conformational changes to the capsid, which resulted in reduced affinity to the primary HS attachment receptor and more exposure of...
L2 protein for furin cleavage (Day et al., 2008a, 2008b; Selinka et al., 2003). The HPV-heparin complex captured in our study showed no significant conformational changes of the L1 protein, but suggested major L2 alteration. Post-attachment alterations to the HPV capsid have been reported previously (Guan et al., 2015a) and may correspond to the heparin-triggered conformational changes we report here that include small L1 protein movements at the apical surface of the capsomers. The surface loops on hexavalent capsomer had no significant changes after binding heparin (Figure 6C), which coincides with the retained ability of antibody HPV16.V5 to neutralize well after cell attachment (Guan et al., 2015a; Lee et al., 2014). Compared with HPV16.V5, the redisposition and more exposure of L2 densities on the surface may affect other antibody binding, for example, H33.J3, which displayed improved neutralizing efficiency to cell-bound virions (Selinka et al., 2003). The lack of global conformational changes of L1 protein after binding heparin and the presence of possible L2 density near the bound heparin molecule suggested that the L2 protein was likely participating in the binding with heparin (Figures 6D and 6E). If heparin binds to both L1 and L2 proteins, perhaps the L1 interactions facilitate the orientation and the L2 binding with heparin provides the contacts that triggered major conformational changes. The movement of L2 after heparin binding also includes conformational changes of L2 at the side wall of the capsomer near the canyon (Figures 6D, 6E, and S4). These changes may indicate that the binding sites for furin cleavage, cyclophilin B, or other factors of the L2 protein are located at the side wall of the capsomer, and the release of L2 and the genome may be from the canyon during the latter dis-assembly of the capsid; however, further work remains to reveal the L2 structure and subsequent changes.

Of the three heparin binding sites identified previously, the site occupied by heparin in our density map overlapped with two residues of site 3 (57.Asn, 59.Lys, 442.Lys, 443.Lys), whereas no traces of bound heparin were found at the top of the capsomers involving the FG and HI loops previously identified as sites 1 and 2 (Richards et al., 2013), and as the primary binding sites. However, considerable differences exist among these three experiments including the form of HPV capsid (L1 only pentamers, pseudovirus, or quasivirus) or the type of heparin (heparin oligosaccharide 8–10, heparin oligomers, or heparin 10–30) used. The seemingly disparate results suggest several possibilities. First, due to the variations in the reagents, the heparin used in these experiments was only capable of interacting at site 3 on our quasivirus capsids due to steric limitations of binding. Certainly, it seems likely from our structure that heparin binding to site 3 would interfere with binding at site 2. Second, a stepwise sequence of events is conceivable. For example, heparin bound HPV capsids at site 1 during the 1-hr incubation; the bound heparin initiated the conformational changes revealed in the maps; whereupon heparin dissociated from site 1 and then bound site 3 as revealed in the HPV-heparin complex structure.

Figure 6. Conformational Changes of HPV Capsid Triggered by Heparin
(A) Radial density plots of HPV16 and HPV16-heparin.
(B) The mass axis of L1 protein in pentavalent and hexavalent capsomers for HPV (red) and HPV-HS (blue) maps.
(C) The conformational changes of L1 surface loops induced by heparin binding are indicated by superimposing the pentavalent and hexavalent capsomer models from HPV16 (red) and HPV16-heparin (blue). The movement of FG, HI, BC, DE, and EF loops were indicated. The RMSD value of pentavalent and hexavalent capsomers before and after heparin binding were measured as 1.12 and 0.51 Å, respectively.
(D) L2 densities (blue) calculated by subtracting the L1 only capsid model from HPV16-heparin map was shown from top, whereas the heparin densities were highlighted in black.
(E) The cross-section view L2 densities (blue) together with heparin (black) to show the densities inside the capsomer. Pentavalent and hexavalent capsomers were indicated by black pentagon and hexagon.
Cryo-EM
HPV16 was incubated with a 5-fold excess of heparin molecules (SigmaAldrich, catalog no. H1027) according to an average mass of 16 kDa per molecule and 360 binding sites predicted per capsid. HPV-heparin was incubated for 1 hr at 37°C and concentrated to 0.8 mg/mL in PBS buffer. An aliquot of 3 μL of virus-Fab complex was vitrified on QUANTIFOIL R2/1 holey carbon support grids (Quantifoil) that were vitrified by plunge-freezing into liquid ethane using a Cryoplunge 3 (Gatan). Low-dose micrographs were recorded using an FEI Polara G2 microscope operating at 300 kV. Images were collected under the software control of the EPU program using an FEI Falcon 2 direct electron detector with a nominal magnification of 93,000× yielding a calibrated pixel size at the sample of 1.15 Å. A similar procedure was followed to prepare and collect data for untreated HPV particles. Defocus ranges of 1.04–5.19 and 0.78–4.85 μm (Table 1) were measured respectively for micrographs of HPV16 and HPV16-heparin, respectively. RELION, AUTO3DEM, and EMAN2 program suites were used for image processing and 3D reconstructions (Scheres, 2012; Tang et al., 2007; Yan et al., 2007).

Image Processing
Whole-frame alignment was carried out using MotionCorr to account for stage drift (Li et al., 2013). The microscope contrast transfer function parameters were estimated for each micrograph using ctffind4 (Rohou and Grigorieff, 2015). Semiautomatic particle selection was performed using EMAN2 e2boxer.py to obtain the particle coordinates, followed by particle extraction, linearization, normalization, apodization, and 3D reconstruction of the images using AUTO3DEM 4.05 following the gold standard of initiating the process by splitting the dataset into two independently refined halves (Tang et al., 2007; Yan et al., 2007). The refinements for sharpening high-resolution information were performed using EMIFACTOR (Fernández et al., 2008; Rosenthal and Henderson, 2003). Reported resolutions are estimated according to the gold-standard FSC = 0.143 criterion (Scheres and Chen, 2012).

For heterogeneity investigation, 3D classifications and refinements were performed using RELION software (Scheres, 2012). A windowing operation was applied to the particles in Fourier space to downsize the particle images to yield a final box size of 260 pixels (corresponding to a pixel size of 2.3 Å). The final pixel size was chosen after optimization of the balance between the Nyquist frequency limit and the memory requirements for computations.

Model Building
The atomic model of the HPV16 L1 capsomer with the C-terminal arm truncated (PDB: 3OAE; residues 20–403, 438–474) was fitted as a rigid body into the cryo-EM map using UCSF Chimera (Darvuga et al., 2011; Pettersen et al., 2004). Placement of the pseudoatomic model of an asymmetric unit of HPV16 capsid (PDB: 3J6R; residues 9–486) was used as a guideline during building (Cardone et al., 2014). Model building proceeded de novo to place the missing C-terminal arm residues 404–437 and the N and C termini through COOT (Emsley et al., 2010). Specifically, 17 residues were built into density at the N-terminal end and 11 residues at the C-terminal end. Each L1 molecule in the asymmetric unit was built independently using COOT (Emsley et al., 2010). Icosahedral symmetry was applied to the refined asymmetric unit model to generate the whole capsid through SITUS (Wriggers, 2010; Wriggers et al., 1999), with the parameters of mass-weight of the atom, pixel size 1.147 Å, kernel width ~4.8 Å, Gaussian smoothing kernel, and scaling factor of 1. The capsid atomic model was then further refined iteratively using the Phenix Real Space Refinement program (Adorine et al., 2012). At each iteration, the best model was visually inspected in COOT and adjusted manually accordingly to the best fit into the density and guided by torsion restraints, planar

EXPERIMENTAL PROCEDURES
Preparation of HPV16 Quasiviruses
HPV16 quasivirus containing L1 and L2 proteins and encapsidating a CRPV genome having the SV40 origin of replication was prepared as described previously (Brendle et al., 2010; Mejia et al., 2006; Pyeon et al., 2005). In brief, HPV16 sheL plasmid (kindly provided by John Schiller, NIH) was transfected together with linear CRPV/SV40ori DNA into 293TT cells and prepared as described previously (Buck et al., 2005a, 2005b; Pastrana et al., 2004). HPV16 was allowed to mature overnight and then pelleted by centrifugation. The centrifuged pellet was resuspended in 1 M NaCl and 0.2 M Tris (pH 7.4). After CsCl gradient purification, the lower band was collected, concentrated, and dialyzed against PBS, as described previously (Guan et al., 2015a). The concentrated HPV16 particles were applied to Formavar-coated copper grids, stained with 2% phosphotungstic acid, and analyzed for integrity and concentration on a JEOL JEM-1400 electron microscope.

Cryo-EM
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Model Building
The atomic model of the HPV16 L1 capsomer with the C-terminal arm truncated (PDB: 3OAE; residues 20–403, 438–474) was fitted as a rigid body into the cryo-EM map using UCSF Chimera (Dasgupta et al., 2011; Pettersen et al., 2004). Placement of the pseudoatomic model of an asymmetric unit of HPV16 capsid (PDB: 3J6R; residues 9–486) was used as a guideline during building (Cardone et al., 2014). Model building proceeded de novo to place the missing C-terminal arm residues 404–437 and the N and C termini through COOT (Emsley et al., 2010). Specifically, 17 residues were built into density at the N-terminal end and 11 residues at the C-terminal end. Each L1 molecule in the asymmetric unit was built independently using COOT (Emsley et al., 2010). Icosahedral symmetry was applied to the refined asymmetric unit model to generate the whole capsid through SITUS (Wriggers, 2010; Wriggers et al., 1999), with the parameters of mass-weight of the atom, pixel size 1.147 Å, kernel width ~4.8 Å, Gaussian smoothing kernel, and scaling factor of 1. The capsid atomic model was then further refined iteratively using the Phenix Real Space Refinement program (Adorine et al., 2012). At each iteration, the best model was visually inspected in COOT and adjusted manually accordingly to the best fit into the density and guided by torsion restraints, planar
peptide restraints, and Ramachandran restraints. The quality of the final model was evaluated by MolProbity (http://molprobity.biochem.duke.edu/) (Chen et al., 2010) (Table S1). The same building and refinement procedures were performed for the HPV-heparin complex map. Difference maps were calculated using SITUS (Wrighers, 2010; Wrighgers et al., 1999).

Coordinates for the disaccharide unit of heparin isolated from (PDB: 3OAE) were used as the basis for the model building (Dasgupta et al., 2011). The heparin polysaccharide model was built manually using disaccharide units fitted one-by-one into the difference map made by subtracting HPV16 from the HPV16-heparin complex map. The result of initial fitting was refined using the HPV16-heparin complex map. The final result included ten disaccharide units of heparin built in the density with a correlation coefficient of 83% according to Chimera (Pettersen et al., 2004). Contacts between the fitted heparin and L1 protein structures were identified using Chimera with the criteria for van der Waals overlap distances set at −0.4 and 0.0 Å. Clashes between atoms were defined by any overlap of 0.6 Å or more.

ACCESSION NUMBERS

Cryo-EM maps for the HPV16 and HPV16-heparin complexes have been deposited in the EM database (www.emdatabank.org) with accession numbers EMD: 6620 and 6619. Coordinates for the atomic model of the asymmetric unit of HPV16 L1 proteins and the complex with the heparin molecule have been deposited in the PDB under accession codes PDB: 5KEP and 5KEQ.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.str.2016.12.001.

AUTHOR CONTRIBUTIONS

J.G., S.M.B., and S.A.B. performed the research; R.E.A. performed microscopy and collected the cryo-EM data; A.M.M. and J.F.C. collected the cryo-EM data; J.G. processed and analyzed the cryo-EM data; J.G., N.D.C., J.F.C., and S.H. wrote the paper.

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