Multiple Heparan Sulfate Binding Site Engagements Are Required for the Infectious Entry of Human Papillomavirus Type 16

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Human papillomavirus (HPV) entry is accompanied by multiple receptor-induced conformational changes (CCs) affecting both the major and minor capsid proteins, L1 and L2. Interaction of heparan sulfate (HS) with L1 is essential for successful HPV16 entry. Recently, cocrystallization of HPV16 with heparin revealed four distinct binding sites. Here we characterize mutant HPV16 to delineate the role of engagement with HS binding sites during infectious internalization. Site 1 (Lys278, Lys361), which mediates primary binding, is sufficient to trigger an L2 CC, exposing the amino terminus. Site 2 (Lys54, Lys356) and site 3 (Asn57, Lys59, Lys442, Lys443) are engaged following primary attachment and are required for infectious entry. Site 2 mutant particles are efficiently internalized but fail to undergo an L1 CC on the cell surface and subsequent uncoating in the endocytic compartment. After initial attachment to the cell, site 3 mutants undergo L1 and L2 CCs and then accumulate on the extracellular matrix (ECM). We conclude that the induction of CCs following site 1 and site 2 interactions results in reduced affinity for the primary HS binding site(s) on the cell surface, which allows engagement with site 3. Taken together, our findings suggest that HS binding site engagement induces CCs that prepare the virus for downstream events, such as the exposure of secondary binding sites, CCs, transfer to the uptake receptor, and uncoating.

Human papillomaviruses (HPVs) are small, nonenveloped epitheliotropic DNA viruses. HPV infection usually induces benign papillomas of the skin and mucosa. However, certain species, especially HPV16, are known as “high risk” due to their involvement in the progression to invasive carcinomas. Infection by HPV is considered necessary, though not sufficient, for the development of cervical cancer (1, 2). HPV infection is also associated with various anogenital and head and neck cancers (3). Despite the clear medical importance of preventing HPV-induced lesions, limited molecular detail regarding the attachment and entry of the virus is available. HPVs productively infect only epithelial cells in the skin and mucosa and depend on the differentiation of these cells for the completion of the viral life cycle (4). To bypass this obstacle, an *in vitro* surrogate system for viral propagation using a marker gene encapsidated into the viral capsid proteins was developed (5–7). This pseudovirus system overcomes the tropism and species specificity for viral propagation displayed by HPVs, allowing for study of the early events in the infection process.

The papillomavirus virion is composed of the major and minor capsid proteins, L1 and L2, respectively. L1 is present in 360 copies organized into 72 pentamers, referred to as capsomeres (8–10). The L2 protein is present in an undetermined number of copies and is initially hidden inside the L1 structure prior to attachment to the cell surface (11). The outer virion shell, formed via pentavalent and hexavalent capsomere interactions between L1 molecules, mediates viral attachment (9, 12, 13). Invading carboxy-terminal arms, from neighboring capsomeres, provide stability to the capsomere structure, strengthened by disulfide bonds between two highly conserved cysteine residues (10, 14, 15). L2 is not required for the formation of the L1 capsid structure; however, it is essential for infection, and its presence increases the level of DNA encapsidation (16, 17). The virion contains a chromatinized circular double-stranded DNA genome of approximately 8,000 bp.

Efficient infection with HPV16 requires the engagement of heparan sulfate proteoglycans (HSPG) on the extracellular matri-

ces (ECM) or surfaces of basal-layer keratinocytes (12, 13, 18–20). HSPG are ubiquitous molecules that are involved in a number of normal cellular processes, such as wound healing, blood coagulation, and embryonic development (21). HSPG molecules are large, complex structures composed of core proteins and covalently attached glycosaminoglycans capable of posttranslational sulfate and acetyl modifications (22). A dynamic model of the initial events during HPV infection includes primary attachment to heparan sulfate (HS), transfer to/recruitment of secondary HS molecules, and subsequent transfer to the uptake receptor (20, 23–25). Many candidates for the non-HSPG uptake receptor have been identified, including integrins, tetraspanins, growth factor receptors, and annexin A2 (26–34). Previous reports also indicate the involvement of a non-HSPG ECM receptor, possibly laminin 332 (LN332), whose binding can support successful HPV infection when virions are preincubated with heparin (20, 35). Primary HS attachment is believed to induce conformational changes in the capsid proteins, presumably allowing for interaction with secondary HS sites as well as these proposed uptake receptors (23, 24). Published evidence for this includes the fact that neutralizing antibodies against the L1 protein that do not prevent cell surface binding, such as H16.V5, sequester the virus onto the primary attachment site, probably by preventing these shifts from occurring (36). Along these lines, antibodies that sterically hinder interactions with secondary receptors, such as the L2-specific monoclonal antibody (MAb) RG-1, relocate the virus to the ECM, indicating that there must be a loss of affinity following

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primary attachment (24). A conformational shift in L2 is well defined, requiring host cell cyclophilin B (CyPB) and mediating the exposure of the amino terminus of the protein on the capsid surface, thus revealing the RG-1 epitope as well as a furin cleavage site required in later steps following internalization (24, 37, 38). Indirect evidence supports the idea of conformational changes in the L1 protein, including observations of antibodies capable of neutralizing only cell-bound particles and a switch from heparin-sensitive to heparin-insensitive virus following cell binding (20, 39, 40).

HS binding sites on L1 have been identified previously and confirmed recently from a cocrystallization of HPV with size-defined heparin, a highly sulfated form of HS (23, 41). These binding sites are composed of positively charged lysine residues capable of creating charge-charge interactions with the highly negative HS or polar residues capable of forming hydrogen bonds. The heparin binding sites are conformational and contain residues scattered on several surface loops of different L1 molecules (41). Four different binding sites have been identified for HPV16: two binding sites on the tip of the capsid surface and two more binding sites reaching along the capsid vertex into the intercapsomeric cleft. The combination of binding sites observed for HPV16 and HPV18 is unusual for viruses, most of which possess a sole binding pocket. These structural observations led us to hypothesize that HS is engaged multiple times and in a variety of ways during the attachment and entry of HPV16. Structure-guided mutagenesis indicated that the binding site composed of residues Lys278 and Lys361 is responsible for primary viral attachment, since the absence of these residues results in a virus unable to bind to the ECM or cell surface (41). Mutations in other binding sites encompassing the other residues identified (Lys54 and Lys356; Asn57, Lys59, Lys442, and Lys443) did not impact binding to the cell surface or ECM; however, the infectivity of these viruses was significantly impaired. These data indicated that secondary binding sites are not required for primary binding, which is accomplished via Lys278 and Lys361, but are important for downstream events that are required for infectious internalization. Secondary HS engagements have been shown to be important for infectious internalization; however, the molecular detail of these engagements is not completely understood (20, 39). Using a virus deficient in secondary HS binding sites, we sought to determine how the loss of specific HS engagements would impact the infectious entry process of HPV16 after primary attachment. Using conformational antibodies as molecular tools, we can track the capsid as it undergoes multiple receptor engagements that eventually allow the entry of a particle capable of uncoating and delivering viral DNA to the nucleus.

MATERIALS AND METHODS

Cell lines. HeLa and 293TT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, and antibiotics. HaCaT cells were grown in low-glucose DMEM containing 5% FBS and antibiotics.

Plasmids and pseudovirions (PsVs). Codon-optimized HPV16 L1 and L2 expression plasmids were a kind gift from Martin Müller, Heidelberg, Germany. Pseudoviruses harboring pEGFP (where EGFP is enhanced green fluorescent protein) were generated using the 293TT cell line as described previously (5). Particles were characterized by L1- and L2-specific Western blotting, and viral genome equivalents (vge) were determined by green fluorescent protein (GFP)-specific quantitative real-time PCR (qRT-PCR).

Inhibitors and reagents. Cyclosporine (CsA) was obtained from Toronto Research Chemicals (C988900). Bafilomycin A1 (BafA1) was purchased from Alexis Biochemicals (ALX 380030). Marimastat (444289), batimastat (196440), GM 6001 (364206), and γ -secretase inhibitor XXI (565790) were obtained from EMD Biochemicals. Genistein (G6649) was purchased from Sigma. Heparin and heparin oligomers were purchased from Iduron.

Antibodies. HPV16 L1-specific mouse monoclonal antibodies (MAbs) H16.56E, H16.V5, H16.E70, and 33L1-7, polyclonal antibody K75, and the L2-specific MAb RG-1 have been described previously (20, 42–45). H16.V5, H16.E70, and RG-1 were kind gifts from N. D. Christensen, Hershey Medical Center, and R. B. Roden, Johns Hopkins University. A rabbit polyclonal antibody (ab14509) against laminin 5 (also known as laminin 332 [LN332]) was purchased from Abcam. A fluorescein isothiocyanate (FITC)-conjugated antibody (Ab) against CD147 (MA1-19588) was purchased from Affinity Bioreagents. A Click-iT EdU (5-ethynyl-2'-deoxyuridine) imaging kit, Alexa Fluor-labeled secondary antibodies, and phalloidin were purchased from Invitrogen. Peroxidase-conjugated AffiniPure goat anti-mouse antibodies were purchased from Jackson ImmunoResearch.

Infection assay. HeLa or 293TT cells were seeded into 24-well plates, and inhibitors or oligomeric heparin diluted in complete DMEM was added 24 h later. Thirty minutes later, PsVs yielding wild-type (wt) virus infection rates of 10% to 20% were added. Mutant PsV levels were normalized to wt virus levels using cycle threshold (C_T) values for all infectivity experiments, so that equal numbers of genome equivalents were added in all cases. In order to avoid cytotoxicity associated with prolonged genistein incubation, cells were washed after a 15-h incubation with high-pH (pH 10.5) phosphate-buffered saline (PBS) to destroy particles not yet internalized, and infection was then allowed to proceed in the absence of inhibition (46). Infectivity was scored by counting GFP-expressing cells at 72 h postinfection (hpi) using flow cytometry.

Enzyme-linked immunosorbent assay (ELISA). Pseudovirions from two preparations were diluted in PBS, added to a 96-well plate (Nunc-Immuno module; Nunc) in replicates, and bound for 1 h at 37°C. In order to monitor the presence of the 33L1-7 epitope, particles were bound in a buffer containing a Click-iT reaction cocktail to allow for denaturation (47). Subsequently, the plate was washed with PBS–0.1% Tween 20 (PBST). Where indicated, PsVs were fixed to the plate using 4% paraformaldehyde (PFA) in PBS for 20 min. After washing with PBST, wells were blocked with 0.01% bovine serum albumin (BSA) in PBST for 1 h at 37°C. The primary Ab was added for 1 h at 37°C. Bound primary antibody was detected by the addition of a horseradish peroxidase-coupled secondary antibody for 30 min at 37°C. The assays were developed with trimethylbenzidine (Promega) and were stopped with 1 N HCl. Absorbance was measured at 450 nm using a FLUOstar-Omega plate reader (BMG Labtech).

IF. HaCaT cells were grown on coverslips to ~50% confluence and were incubated with HPV16 pseudovirus. Approximately 1×10^6 to 1×10^7 vge were used per coverslip. The PsV preparations used maintained similar vge-to-capsid ratios and were added in equal quantities as determined by ELISA and qRT-PCR. For experiments with inhibitors, cells were incubated with the drug for 30 min prior to infection, and then PsVs and the drug were added for the duration of the infection. The following inhibitors were used: BafA1 (100 nM), the BMG matrix metalloprotease (MMP) inhibitor cocktail, consisting of 20 μ M (each) batimastat, marimastat, and GM6001; and CsA (10 μ M). At the times postinfection indicated in the figure legends, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, washed, permeabilized (where indicated) with 0.2% Triton X-100 in PBS for 2 min, washed, and blocked with 5% goat serum in PBS for 30 min, followed by a 1-h incubation with primary antibodies at 37°C. After extensive washing, cells were incubated with Alexa Fluor-tagged secondary antibodies and fluorescently labeled phalloidin, where indicated. In experiments using FITC-conjugated anti-CD147, cells were washed after incubation with second-

FIG 1 Analysis of secondary HS binding sites. (A) Structural analysis of the HPV16 capsomere in complex with oligomeric heparin revealed multiple binding sites located at the top and side of the capsomere. Structure-guided mutational analysis revealed 3 important binding sites; site 1 (K278, K361), site 2 (K54, K356), and site 3 (N57, K59, K442, K443). (B) Results of Western blot analysis for capsid proteins and *Ct* (cycle threshold) values are shown with those for the wt control. (C) PsVs were analyzed for reactivity with various antibodies in an ELISA after binding for 1 h at 37°C. (D) HeLa cells were infected with wt and mutant PsVs. The infectivities of mutant PsVs were normalized to that of the wt. Raw percentages of infectivity were 14.67% for the wt, 0.93% for the site 2 mutant, 0.81% for the site 3 mutant, and 0.83% for the site

ary antibodies, and then FITC-CD147 was added for 20 min at 37°C, followed by washing with PBS. Cells were mounted in Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). All immunofluorescence (IF) images were captured by confocal microscopy with a 63× objective (Leica TCS SP5 spectral confocal microscope) and were processed with Adobe Photoshop software. Within individual experiments, the same microscope settings and exposure times were used. For ECM binding of PsVs, the ECM was generated by removal of HaCaT cells from coverslips after 48 h using EDTA (0.5 mM in Dulbecco's PBS [DPBS]). RG-1 staining was performed as described previously (24). In brief, infected HaCaT cells were shifted to 4°C and were incubated with RG-1 and K75 for 1 h in the presence of 2% normal goat serum. After extensive washing and incubation with fluorescently labeled secondary antisera, cells were fixed for 20 min in 2% paraformaldehyde. After washing, cells were incubated for 5 min with Alexa Fluor 647-conjugated phalloidin and were mounted. For 33L1-7 staining following denaturation, cells were incubated with the Click-iT reaction cocktail according to the manufacturer's instructions (without the addition of the EdU label to virions or subsequent Alexa Fluor staining) for 30 min as described previously (47). Quantification for Fig. 2 and 3 was performed by obtaining the pixel sums of approximately 20 regions of interest (ROIs) using LAS AF software to crop confocal maximum projections so that they contained either LN332 (ECM binding) or CD147 in the absence of any LN332 signal (cell surface). For internalization and uncoating, z-stack slices through the middles of cells were analyzed for the presence of the L1 signal. All values were compared and expressed relative to wt values. In Fig. 3B, only site 3 particles were analyzed, and 1-h cell surface binding was used as the reference. For Fig. 3C, the ECM was not stained, so quantification was based solely on the amount of virus on the cell surface that was detected by phalloidin, and only the uppermost slices of maximum projections were used in quantification. The H16.56E conversion percentage (see Fig. 6 and 7) was determined by comparison of the pixel number for each antibody in 20 ROIs. The results are expressed as an average percentage of H16.56E reactivity compared to K75 reactivity for virus bound to the ECM alone or in the presence of cells for 18 h.

ECM cell transfer assay. HaCaT cells were seeded onto coverslips for 24 to 48 h, after which cells were removed by EDTA (0.5 mM in PBS). The ECM was either left untreated, incubated with 20 µg/ml DSTP27, or fixed for 20 min using 4% paraformaldehyde in PBS. After washing with PBS, virions were bound to the ECM for 1 h at 37°C. 293TT cells were used as detector cells, and infectivity was scored at 72 h. Where indicated, heparin and DSTP27 were added at 10-µg/ml and 20-µg/ml concentrations, respectively.

RESULTS

Identification of primary and secondary HS attachment sites on the viral capsid. We have recently identified multiple types of HS binding sites on HPV16 capsomeres by using structural analysis (41) (Fig. 1A). Mutation of viral residues Lys54 and Lys356 (site 2) and Asn57, Lys59, Lys442, and Lys443 (site 3), in combinations suggested by the crystal structure, yielded PsVs that incorporated L2 protein and the pseudogenome at similar levels (Fig. 1B) and retained conformational epitopes, as measured by reactivity with conformation-dependent monoclonal antibodies (Fig. 1C), and their ability to bind to the ECM and the cell surface (41) (see Fig. 2) but displayed strongly impaired infectivity (Fig. 1D). Before we

2/3 mutant. (E) Sensitivity of the infectivity of HS binding site mutant PsVs to known inhibitors of wt HPV16 infection used at the following concentrations: genistein, 200 µM; CsA, 10 µM; γ-secretase inhibitor XXI, 500 nM; BafA1, 100 nM. Raw control percentages of infectivity were 17.53% for the wt, 1.13% for the site 2 mutant, 0.94% for the site 3 mutant, and 0.87% for the site 2/3 mutant. Approximately 5×10^5 vge were added to each well. For panels D and E, infectivity was scored 72 h postinfection.

investigated the defect of these mutant PsVs in more detail, we tested whether the mutant viruses follow the same internalization pathway as wt HPV16. To test this, HeLa cells were infected with wt and mutant PsVs in the presence of well-established inhibitors of HPV16 infection targeting CyPs, γ -secretase, tyrosine kinases, and endosomal acidification and trafficking (38, 46, 48–53). All mutant PsVs tested displayed the same inhibition profile as wt HPV16 (Fig. 1E).

Internalization of mutant HPV16 pseudovirions. To explore the role of secondary binding sites in the infectious entry of HPV16, each mutant PsV was first tested for its ability to be internalized. We used a known inhibitor of uncoating, intracellular trafficking, and capsid protein degradation, BafA1, in order to facilitate the detection of internalized pseudovirions (47–49, 51). We reasoned that this treatment would prevent possible complications due to the different stabilities of mutant PsVs following internalization. PsVs were bound to HaCaT cells for 1 h at 4°C and were either directly processed for immunofluorescence (IF) or allowed to incubate overnight at 37°C in the presence of BafA1. Following fixation, internalized capsid proteins were denatured using the Click-iT reaction cocktail and were detected using MAb 33L1-7. MAb 33L1-7 is specific for a linear L1 epitope that is not accessible in intact particles but is exposed upon treatment with the Click-iT reaction cocktail (47). At 1 h after binding at 4°C, wt and mutant PsVs were detected at similar levels on the cell surface (Fig. 2A). As expected, the wt virus accumulated in perinuclear regions at 18 hpi, indicating internalization. The HS binding site 2 mutant was also internalized, like the wt (Fig. 2B). In contrast, the site 3 mutant virus was detected neither on the cell surface nor intracellularly at this time point, indicating that the defect of the site 3 mutations occurs prior to internalization. Instead of remaining bound to the cell surface, the site 3 mutant virus accumulated on the ECM at 18 hpi (Fig. 2C and D). PsVs harboring mutations in both site 2 and site 3 (the site 2/3 mutant) were not internalized or relocated to the ECM. Instead, they were present on the cell surface at 18 hpi to a higher degree than wt PsVs (Fig. 2C). However, this difference did not reach significance. Quantification of viral binding and internalization is shown in Fig. 2E. These data indicate that HS binding site 2 and 3 mutants, and the combination site 2/3 mutants, are defective for different steps of infectious entry.

Uncoating of site 2 mutant pseudovirions. Following internalization, HPV undergoes the process of uncoating, facilitating the release of L2/DNA from the L1 capsid. This event can be measured using monoclonal antibody 33L1-7, which is directed at a linear epitope exposed only following this process (30, 38, 53). Wt and site 2 mutant PsVs were incubated with HaCaT cells for 18 h and were subsequently analyzed for reactivity by use of MAb 33L1-7 without prior denaturation. Following internalization, the L1 protein of wt PsVs, but not that of site 2 mutant PsVs, became reactive with 33L1-7, suggesting that the site 2 mutant pseudovirus fails to be uncoated after internalization (Fig. 3A).

Loss of site 3 mutant pseudovirions from the cell surface. The loss of site 3 mutant pseudovirus and its accumulation on the ECM strongly suggested that it lost affinity for the primary HS binding site after initial attachment events. It has been suggested recently that the shedding of HSPG-bound pseudovirus triggered by cell surface-resident matrix metalloproteases (MMPs) or ADAM proteases contributes to HPV16 infections (31). To investigate whether shedding is responsible for the loss of site 3 mutant

PsVs from the cell surface, we bound and incubated site 3 mutant particles with HaCaT cells overnight in the presence of a mixture of broadly active inhibitors of major MMPs and ADAM proteases. As shown in Fig. 3B, this treatment did not prevent the loss of mutant particles from the cell surface, and at 18 hpi, site 3 mutant PsVs still accumulated on the ECM. The presence of these inhibitors did not affect the initial binding of the site 3 mutant to the cell surface. These data suggest that a mechanism other than shedding via MMP activity is responsible for the release of site 3 mutant PsVs from the cell surface.

This loss of affinity was further investigated using MAb H16.V5. Day et al. have demonstrated previously that preincubation of this antibody with HPV16 PsVs does not block cell binding but does prevent internalization by sequestering the capsids on the cell surface (36). They also proposed that H16.V5 binding prevents conformational changes of the capsid that normally participate in reducing affinity for the primary HS receptor (24, 36). To test whether these proposed postattachment conformational changes might be responsible for the change in affinity that results in the loss of site 3 mutant viral particles from the cell surface, we pretreated wt and site 3 mutant PsVs with H16.V5, bound them to HaCaT cells, and investigated the fate of viral particles at 18 hpi by IF. We found that wt and site 3 mutant particles remained attached to the cell surface in the presence of H16.V5 (Fig. 3C). Taken together, these observations indicated that the initial binding of wt and site 3 mutant PsVs to cell surface receptors occurs with similar affinities and that the loss of mutant particles is more likely due to a conformational change than to the release of a virus/HSPG complex from the cell surface.

Conformational changes in the L2 protein. As mentioned, the HPV16 capsid proteins are believed to undergo conformational changes following attachment (20, 36–39). The conformational change in L2 releases an initially hidden amino terminus and is required for infectious entry. The conformational change can be detected using MAb RG-1, which is specific to a linear epitope close to the amino terminus (45). We next sought to determine if there is a link between specific HS engagements and the L2 conformational changes in the capsid proteins. We bound wt and HS binding site mutant PsVs to HaCaT cells at 4°C for 1 h and monitored reactivity with RG-1 following 3 h of incubation at 37°C. None of the PsVs showed significant reactivity with RG-1 when tested prior to the shift to 37°C (Fig. 4A). In contrast, the wild-type virus and all mutant viruses tested exposed the L2 amino terminus at 3 h after the temperature shift (Fig. 4B), suggesting that engagement with the primary binding site via lysine residues 278 and 361 is sufficient to trigger the exposure of the L2 amino terminus.

Conformational changes in the L1 protein. Evidence for an L1 conformational change during cell surface events has been indirect until now, and no tools to detect such conformational changes have been described (20, 39, 40). Testing of conformation-dependent neutralizing MAbs for their abilities to detect epitopes that are exposed only after the binding of HPV16 PsVs to cells identified H16.56E as one such antibody (42, 54). We found that H16.56E reacted only weakly with formaldehyde-fixed particles by ELISA, even though it readily bound to unfixed particles (Fig. 5A). Similarly, it did not recognize particles bound to the ECM or to HaCaT cells at 4°C following fixation (Fig. 5B). However, incubation for extended periods at 37°C following binding to the cell surface, but not to the ECM, rendered HPV16 PsVs reactive with H16.56E, indicating that the L1 protein undergoes a

FIG 2 Binding properties of site 1, site 2, site 3, and site 2/3 mutant pseudovirions. HaCaT cells were seeded onto coverslips for 48 h; then PsVs were bound to cells for the indicated times, fixed, and subsequently stained for virus with 33L1-7 after denaturation (green). Other markers are as follows: CD147 (blue) for the cell surface, LN332 (red) for the ECM, and DAPI (gray) for nuclei. (A) Initial binding of PsVs was determined after 1 h of incubation at 4°C. (B) Internalization was measured 18 h postinfection. Bafilomycin A1 (100 nM) was used to maintain similar levels of stability for all PsVs measured. (C and D) The locations of the mutant virions were determined 18 h postinfection using maximum projections of z-stack layers containing either CD147 in the absence of LN332 (C) or CD147 and LN332 (D). (E) Quantifications for all images are provided. All pixel sums are shown relative to those for wt virus. Approximately 1×10^7 vge/coverslip were added. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

conformational change following cellular attachment. The gain of reactivity with H16.56E could not be blocked by CyP inhibitors, suggesting that the L1 conformational change does not require CyP activity, in contrast to the L2 conformational change (38).

Using H16.56E, we tested the panel of HS binding site mutant viruses for the ability to undergo a conformational change in L1.

The epitope for H16.56E involves the amino-terminal portion of the FG loop encompassing residues 260 to 270 but has not been fully elucidated (40). To ensure that HS binding site mutations did not affect the binding site of the antibody, an ELISA was performed in the absence of fixation. All viruses were able to bind H16.56E efficiently (Fig. 1C). After 4 h of viral binding to the cell

FIG 3 Further characterization of site 2 and site 3 mutant PsVs at 18 h postinfection. (A) Uncoating of wt and site 2 mutant PsVs was detected using 33L1-7 without prior denaturation. Wt and site 2 mutant PsVs were added to HaCaT cells for 18 h. Infected cells were then fixed, permeabilized, and stained for virus with 33L1-7. (B) Eighteen hours after the infection of HaCaT cells with or without MMP inhibition (BMG), site 3 mutant PsVs were detected with 33L1-7 after denaturation (green). Staining with antibodies against CD147 (blue) and LN332 (red) for quantification of cell surface and ECM bound PsVs, respectively. The MMP inhibition cocktail (BMG) consisted of 20 μ M batimastat, 20 μ M marimastat, and 20 μ M GM6001. (C) Wt and site 3 mutant PsVs were bound to HaCaT cells for 18 h in the presence or absence of H16.V5 (1:100), fixed, and then stained with K75 (green) and labeled phalloidin (blue). The left-hand graph shows the level of binding of the wt virus to the cell surface after 18 h with or without H16.V5; the right-hand graph compares the levels of binding of the site 3 mutant to the cell surface after 18 h in the presence or absence of H16.V5 with those of the wt virus. Approximately 5×10^7 vge/coverslip were added. **, $P < 0.001$; ***, $P < 0.0001$.

surface at 37°C followed by fixation with PFA, site 2 mutant virus particles displayed dramatically lower reactivity with H16.56E than did wt and site 3 mutant particles, suggesting that engagement with site 2 is required to induce the L1 conformational change, which leads to reactivity with H16.56E (Fig. 6, top). The combination site 2/3 mutant showed a similar reduction in H16.56E reactivity, although this virus also had reduced reactivity with K75 (due to mutations affecting epitopes of the polyclonal K75 antisera) (Fig. 1C). Quantification of H16.56E signal strengths in relation to those for K75 (thus correcting for reduced K75 reactivity) further supports the notion that site 2 mutants have reduced reactivity with H16.56E (Fig. 6, bottom).

Conformational changes of site 3 mutant particles residing on the ECM after 18 h. Having been able to link L1 and L2 conformational changes with specific HS binding sites, we wanted to further investigate the loss of site 3 mutant particles from the cell surface. We reasoned that if the loss of site 3 mutant particles from the cell surface and their accumulation on the ECM are due to a loss of affinity triggered by conformational changes, particles found on the ECM should have gained reactivity with RG-1 and H16.56E. Therefore, we tested the reactivities of ECM-resident site 3 mutant particles with RG-1 and H16.56E following 18 h of incubation at 37°C after direct binding to cell-free ECM or following incubation with HaCaT cells (Fig. 7). We found that the ma-

FIG 4 Abilities of HS binding site mutants to undergo the conformational change in L2. Wt and HS binding site mutant PsVs were bound to HaCaT cells for either 1 h at 4°C (A) or 3 h at 37°C (B). The conformational change in L2 was detected using RG-1 (green); viral binding was detected with K75 (red); and labeled phalloidin (blue) was used to delineate the cell surface. Approximately 1×10^7 vge/coverslip were added.

jority of site 3 mutant particles residing on the ECM after incubation with cells, but not after direct binding to the ECM, were reactive with RG-1. They also gained reactivity with H16.56E. However, mutant particles that bound directly to the ECM and were incubated for 18 h at 37°C also reacted with H16.56E, though not to the extent seen in the presence of HaCaT cells.

Multivalent interaction of HPV16 pseudovirions with HS. Pseudoinfection of 293TT and HeLa cells with wild-type HPV16 is sensitive only to long-chain heparin, not to oligomeric heparin, indicating the existence of a multivalent, extended HS binding site (41). Given the existence of multiple HS binding sites on the viral capsid, we wondered if these sites contribute to an extended HS binding site that would be more susceptible to inhibition by short-chain heparin. Therefore, we tested wt and mutant PsVs for sensitivity to oligomeric heparin. We found that all mutant viruses were highly sensitive to short-chain HS. A chain length of 8 saccharides was sufficient to suppress the residual infectivity of mutant viruses by 50%. In contrast, wt HPV16 was fully resistant to 16-mer oligomeric heparin (Fig. 8).

Enhanced infectivity of mutant PsVs in the absence of HS interactions. In addition to directly binding to cell surfaces, HPV16 has been shown to interact with the basement membrane or ECM and subsequently to transfer to the cell surface in an HS-dependent manner (20). HPV16 binds to the ECM secreted by HaCaT cells via HS and the non-HSPG ECM receptor LN332 (20,

FIG 5 Description of L1-specific mouse monoclonal antibody H16.56E. (A) HPV16 PsVs were bound to ELISA plates and were either fixed with paraformaldehyde or left unfixed prior to incubation with H16.56E. Epitope availability was measured by the detection of H16.56E binding via horseradish peroxidase-labeled secondary antibodies. (B) PsVs were bound to the ECM secreted by HaCaT cells or to the HaCaT cell surface for 1 h or 4 h, followed by fixation and then staining for H16.56E (green). The ECM is detected by use of an anti-laminin 5 Ab (red), and nuclei are stained with DAPI (blue). The bottom right panel shows reactivity with H16.56E in the presence of 10 μ M C5A, which inhibits the Cyp-dependent conformational change in L2. Approximately 1×10^6 vge/coverslip were added.

35). We have previously observed a higher rate of infection of HaCaT cells than of 293TT or HeLa cells with mutant PsVs (41). To test whether this may be due to the presence of LN332, we employed an ECM-to-cell transfer assay. HaCaT cells were seeded onto coverslips and were then removed by EDTA, leaving the ECM behind. Virus was then bound to the ECM for 1 h at 37°C; unbound virus was removed by washing; detector 293TT cells were added; and infection was scored at 72 h. The infection rate was increased for mutant viruses in this assay; however, if the ECM was fixed with PFA, infectivity returned to the levels seen in HeLa or 293TT cells alone (Fig. 9A). Under these conditions, LN332 is denatured. DSTP27 treatment was then used to remove interactions with HS on the ECM, the cell surface, or both, and the assay was repeated. DSTP27 binds to HS moieties, inhibiting interaction with the virus (20, 55, 56). For the wt virus, DSTP27 treatment of the ECM, the cell surface, or both resulted in decreased infectivity. However, mutant viruses benefited from the removal of HS, becoming as much as 3-fold more infectious than wt virus in the absence of HS on the ECM and the cell surface (Fig. 9B). Wt infectivity could be rescued from the lack of ECM or cell surface HS by the addition of exogenous heparin, suggesting that soluble HS can function as a ligand activator in the presence of a non-

FIG 6 Determining whether the HS binding site mutations affect the conformational change in L1 as detected by H16.56E. (Top) Wt and HS binding site mutant PsVs were bound to HaCaT cells for 4 h at 37°C. The cells were fixed and were subsequently stained with H16.56E (green), K75 (red), and labeled phalloidin (blue). Approximately 1×10^7 vge/coverslip were added. (Bottom) The signal strengths of H16.56E relative to those for K75 were quantified.

HSPG receptor, in line with a recent report (35). The addition of heparin caused site 3 and site 2/3 mutant PsVs to become noninfectious again. However, the site 2 mutant was not affected by the addition of heparin in the assay. The enhanced infectivity of all

FIG 7 Site 3 mutant PsVs released from the cell surface have undergone conformational changes. Site 3 mutant PsVs were bound to the ECM or the cell surface for 18 h. The cells and ECM were then fixed and stained for the L2 conformational change with RG-1 or for the L1 conformational change with H16.56E. Monoclonal antibody reactivity is shown in green; binding was also detected in all cases with K75 (red), and nuclei were stained with DAPI (blue). The percentage of bound particles undergoing the L1 conformational change was determined for 20 ROIs by comparing the quantity of PsVs bound to the ECM with the quantity of PsVs released from the cell surface. Approximately 1×10^7 vge/coverslip were added. ***, $P < 0.0001$.

FIG 8 Oligomeric heparin has various effects on infectivity. The sensitivity of the infectivity of wt virus and HS binding site mutants to heparin was measured on 293TT cells. PsVs were incubated with heparin of increasing chain lengths (concentration, 50 μ g/ml). The viruses were then added to 293TT cells and were incubated for 72 h, after which infection was scored by flow cytometry. Raw percentages of infectivity were 9.8% for the wt, 0.72% for the site 2 mutant, 0.66% for the site 3 mutant, and 0.65% for the site 2/3 mutant.

mutant PsVs remained similarly affected by all inhibitors used in Fig. 1E (data not shown).

DISCUSSION

The results of this study, along with those of previously published studies, provide strong evidence of sequential cell surface engagement of HS requiring multiple binding sites on the viral capsid. Initial attachment to HS binding site 1 via Lys278 and Lys361 is required to induce conformational changes that allow interactions with secondary HS binding sites. HS interaction with site 1

FIG 9 Differences in infectivity with ECM present. (A) Virus was bound to fixed or unfixed (control) ECM, and infectious transfer was measured in 293TT cells 72 hpi. (B) Infection was scored either on HeLa cells alone or on 293TT cells after the virus had been bound to the ECM. The ECM or cells were treated with DSTP27 as indicated. Heparin (hep) was added at a 10- μ g/ml concentration. Infection was scored by flow cytometry.

residues also seems to be responsible for the L2 conformational change, with the aid of host cell CyPB (38). This conformational change exposes the amino terminus of the L2 protein, allowing for furin cleavage during downstream steps of infection (24, 37, 45). Other nonenveloped viruses have similar sequential attachment mechanisms. Sequential receptor engagement has recently been reported for a structurally similar virus, Merkel cell polyomavirus (MCV) (57). MCV uses glycosaminoglycans for initial attachment and proceeds to interact with sialylated host factors; both steps are vital to successful infection. While the uptake receptor for HPV16 has not been identified and may consist of a platform of multiple receptors, all of which are required for infectious entry (reviewed in reference 58), we see a similar requirement for multiple, sequential attachment receptor engagements, which we predict to be involved in the exposure of secondary, possibly conserved binding sites as well as in the beginning of the uncoating process.

Secondary HS binding sites on the capsid are not required for the virus to bind efficiently to the cell surface or ECM. Engagement of both site 2 and site 3 on the cell surface is, however, necessary for efficient internalization, uncoating, and infection. Further investigation into the defect in infectivity of site 2 and site 3 mutant virions identified two distinct cell surface events occurring after primary engagement of HS. While site 2 mutant particles were internalized in a manner similar to that for wt particles, they failed to be uncoated. This phenotype has been seen previously when the L2 conformational change was blocked by using CsA or after CyPB knockdown (38). Site 2 mutant PsVs were also unable to undergo an L1 conformational change that is detected with H16.56E. Taken together, these results suggest that interaction of cell surface HS with site 2 residues on the viral capsid allows for an L1 conformational change and that this shift is required for virus uncoating following internalization. However, the L2 conformational change triggered by engagement with site 1 residues is not essential for a gain of reactivity with H16.56E, suggesting that they are not interdependent. Furthermore, our findings indicate that the processes leading to capsid destabilization, necessary for uncoating, are already initiated on the cell surface. The exposure of limited linear epitopes on the cell surface was also reported recently by the Schelhaas group, supporting the notion that L1 conformational shifts occur, and uncoating is started, on the cell surface (35). These data provide evidence that interactions with HS on the cell surface serve as more than just viral sinks; they also initiate conformational changes that are required for downstream steps of infection. This has been shown previously for members of the picornavirus family, which utilize cell surface receptors for attachment and viral uncoating (58–60). Alternatively, the conformational changes induced by engagement with HS binding site 2 might act in a manner similar to those induced by engagement with site 1 and might be responsible for exposing other viral residues for downstream interactions. However, the similar internalization pathways of wt and site 2 mutant PsVs would appear to rule out this possibility.

Site 3 mutant PsVs are not internalized and are not found on the cell surface at 18 hpi; instead, the virus is relocated to the ECM. Relocation is not due to sheddase activity, since the presence of MMP and ADAM protease inhibitors does not cause the virus to be retained on the cell surface. However, in the presence of H16.V5, the virus is no longer accumulated on the ECM and instead remains bound to the cell surface at 18 hpi, like the wt virus. H16.V5 inhibits cell surface conformational changes, suggesting

that it prevents the release of site 3 mutant particles by blocking conformational changes that result in reduced affinity of binding site 1 for HS (24). This notion is supported by our finding that the site 3 mutant particles that are relocated to the ECM after initial cell surface binding have undergone conformational changes and are reactive with MAbs H16.56E and RG-1. The phenotype of site 3 mutants is similar to the effects of MAbs RG-1 and H16.U4, as well as heparin, on wt HPV16 pseudovirions, which also induce the relocation of viral particles to the ECM (24, 25, 36). Interestingly, the MAbs bind to an area of the capsid in close proximity to site 3 within the intercapsomeric cleft, either to L2 (RG-1) or to L1 (H16.U4) (45, 61). These observations are in line with a model in which initial primary binding is required to induce conformational changes that allow for secondary HS engagements and reduce affinity for the initial binding site (Fig. 10). After these shifts, the virus must be able to engage secondary receptor binding sites, or the virus will be lost from the cell surface and will accumulate on the ECM. In the alternative model, which proposes a required “shedding” of HSPG/virus complexes, it is suggested that structural changes in L1 and L2 are not capable of releasing the capsid from primary binding. However, our data indicate that the HSPG-mediated conformational changes in L1 and L2 are indeed capable of releasing the virus from primary binding and allowing secondary receptor engagements in the absence of MMP activity. This would suggest that the virus does not need to be released from the cell surface following attachment and that the HSPG-virus interaction is not responsible for bridging receptors but instead induces capsid protein rearrangements that allow for infectious entry. The site 3 residues could also be directly involved in mediating an interaction with non-HSPG receptors on the cell surface. However, the sensitivity of site 3 mutant PsVs, but not wt PsVs, to oligomeric heparin points to an involvement of HS interactions and suggests that sites 2 and 3 may be engaged simultaneously, thus constituting an extended HS binding site. Whether or not this interaction is required for further conformational changes or for facilitating interaction with non-HSPG receptor complexes on the cell surface can only be elucidated after unequivocal identification of the uptake receptor. However, our finding that mutant particles that are bound to the non-HSPG ECM-resident receptor LN332 are fully infectious in the absence of HS suggests that engagement with the uptake receptor is not impaired. This finding also excludes the possibility that mutant particles are defective due to pleiotropic effects of mutations on downstream events.

The structure-function analysis is based on a structure obtained using L1 pentamers in complex with oligomeric heparin, leaving open the possibility of unidentified HS interactions in the intercapsomeric cleft. However, the recent cryoelectron microscopy (cryo-EM)-derived structure of bovine papillomavirus 1 (BPV1) suggested that the C-terminal arm of L1 is reinserted into the pentamer of origin after forming a cross-bridge with the neighboring pentamer (62). The L1 pentamer used for X-ray structure analysis has the invading helix 4 residues removed, but the remaining portion of the C-terminal arm assumes a conformation similar to that seen by cryo-EM, making the existence of additional HS binding sites unlikely.

While the sequence of events discussed so far describes the fate of viral particles directly bound to the cell surface via HSPG, the process seems to be different for particles bound to non-HSPG receptors present in ECM depositions. Evidence is accumulating that HPV16 utilizes ECM-resident LN332 in addition to HS to

FIG 10 Model of multiple receptor engagements during HPV16 entry. HS binding sites are shown on the surface of the L1 pentamer (red, site 1; blue, site 2; yellow, site 3). Structural changes in the capsid are indicated by various patterns around the cartoon capsid. Steps in viral entry are shown for the wt virus and for site 2 and site 3 mutants by using different lines, indicating what occurs during attachment in the presence and absence of various engagements. Circled numbers 1, 2, and 3 represent binding sites. (Site 1) HPV binds the ECM or cell surface via HS binding site 1. Prior to binding, the capsid is in the metastable conformation, which allows it to persist in the environment. Following primary binding, initial conformational changes occur in both the L1 and L2 proteins. This includes the L2 conformational change, which is facilitated by CyPB and results in the exposure of the RG-1 epitope (N terminus of L2) on the capsid surface. (Site 2) Secondary engagement of site 2 is required for producing conformational changes in the L1 protein, as detected by the H16.56E antibody. These shifts are required for downstream entry events, and their absence results in an internalized virus with a capsid that is not destabilized enough for uncoating. (Site 3) Site 3 residues are not required for primary binding or for the conformational changes recognized by RG-1 or H16.56E. However, these residues are necessary for post-conformational-change attachment to the cell surface, and in the absence of these residues, viral particles are released from the cell surface, where they most likely bind to ECM receptor LN332 and are unable to interact with the uptake receptor complex. The wt virus, which is able to engage all three HS binding sites, proceeds to interact with the uptake complex and to become internalized. The wt virus has undergone all necessary conformational changes; it is therefore prepared for the process of uncoating and can deliver its genome to the nucleus, completing the process of infectious entry.

bind to the basement membrane (20, 35). The data presented here and in previous publications indicate that in the presence of LN332, soluble HS can function as a ligand activator. Mutant PsVs bound to ECM-resident HS (ECM deposited by HeLa cells or HaCaT cell ECM pretreated with PFA to inactivate LN332) display the same noninfectious phenotype as particles directly bound to cell surfaces. However, in ECM cell transfer experiments, mutant virus becomes fully infectious—even more infectious than wt PsVs—when redirected, by pretreatment of ECM depositions and cell surface HS with DSTP27, so as to preferentially bind to LN332. These data suggest that interactions of viral capsids with LN332 trigger outcomes different from those with HS, even though both interactions require HS binding site 1 (41). Since exogenous addition of soluble HS is detrimental to infectious transfer under these conditions, it seems likely that HS-induced conformational changes prevent/interfere with the putative changes induced by LN332 binding. However, the combination of binding to LN332 and the presence of mutations in site 2 and site 3 leads to an outcome similar to that for the cleavage of immature PsV with furin convertase, such that infectious entry becomes independent of HS (25).

In summary, we have shown that HPV16 requires the engagement of multiple HS binding sites for infectious entry. Our data further indicate that the interaction with HS in the form of ligand activation may be necessary only while the virus is bound to the ECM. This suggests a model in which HPV attachment to the ECM occurs in an HS-independent manner, but subsequent HS interaction is required for triggering conformational changes that allow the virus to undergo crucial steps in the entry process, such as exposure of secondary HS binding sites on the capsid surface, interaction with the uptake receptor(s), and uncoating.

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