Differential binding patterns to host cells associated with particles of several human alphapapillomavirus types

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INTRODUCTION

Human papillomavirus (HPV) is a non-enveloped, double-stranded DNA virus that infects the epithelial cells of the skin and mucosa. A subset of HPVs infect the anogenital tract and are agents of sexually transmitted disease. The papillomavirus life cycle is coupled to the terminal differentiation process of the stratified epithelium. Successful infection is thought to be dependent on proper targeting of virions to the mitotically active basal cells lining the basement membrane.

The viral receptor(s) utilized by papillomaviruses is currently unknown. Two main candidates have been identified: heparan sulfate (HS), found on heparan sulfate proteoglycans (HSPGs), and α-6 integrin (Evander et al., 1997; Giroglou et al., 2001; Patterson et al., 2005). HSPGs are expressed ubiquitously through all layers of the epithelium, whereas α-6 integrin is mainly restricted to basal keratinocytes (Andriessen et al., 1997; reviewed by Jones et al., 1998; Oksala et al., 1995). Previous research from our laboratory also established a direct binding interaction between HPV11 and laminin 5 (LN5), a key extracellular matrix (ECM) component involved in hemidesmosome formation. We hypothesized that HPV11 virions utilize LN5 as a transreceptor for proper targeting to basal keratinocytes migrating to the site of injury (Culp et al., 2006a).

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Five supplementary figures are available with the online version of this paper.

RESULTS

Differential binding of HPV VLPs to fixed HaCaT cells and ECM

We tested the binding capabilities of various HPV VLPs to fixed HaCaT cells and ECM via immunofluorescence (IF),
utilizing two different detection methods. Firstly, $1.8 \times 10^{11}$ HPV11, 16 and 18 L1 and HPV45 L1/L2 VLPs incubated with fixed HaCaT cells were detected by type-specific mAbs. Secondly, dye-coupled particles for the four HPV types were analysed by direct IF binding (Fig. 1a, b).

Following mAb detection, HPV16, 18 and 45 VLPs bound to the HaCaT cells, but HPV11 VLPs showed preferential binding to exposed ECM near the edges of the fixed HaCaT cells, as evidenced by co-staining with the ECM marker LN5 (co-staining data not shown). Incubation of VLPs to fixed HaCaT ECM demonstrated that papillomavirus particles bound to a HaCaT ECM component(s) (Fig. 1a). We also stained for LN5 because we previously showed a direct binding interaction between HPV11 virions, VLPs and LN5 (Culp et al., 2006b). We determined that these conformationally sensitive mAbs are useful tools for detecting VLPs bound to fixed cells and ECM.

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**Fig. 1.** Binding of VLPs to HaCaT cells and ECM. (a) Indicated VLPs were incubated with fixed HaCaT cells or ECM and detected with primary antibodies H11.B2, H16.V5, H18.J4 and H45.N5. Anti-LN5 pAb was included on ECM staining. Secondary antibodies were anti-mouse–Alexa Fluor 488 and anti-rabbit–Alexa Fluor 594; Hoechst stain was used to detect cellular DNA. Column (i) displays HPV11, 16, 18 or 45 VLPs binding to HaCaT cells; column (ii) displays VLP binding to HaCaT ECM; column (iii) shows staining of the ECM for LN5; column (iv) is the merge of VLPs and LN5. (b) Alexa Fluor 488-conjugated HPV16 and 18 VLPs were incubated with fixed HaCaT cells or ECM. Column (i) displays unconjugated VLPs binding to cells, detected as described above; column (ii) shows conjugated VLPs binding to cells; column (iii) shows conjugated VLPs binding to HaCaT ECM; column (iv) displays LN5 staining. The merge of column (iii) and (iv) staining is indicated. Bars, 50 μm.
Dye-coupled HPV particles were incubated with HaCaT cells and ECM (Fig. 1b). Alexa Fluor 488-coupled HPV16 VLPs did not show a difference in binding patterns from uncoupled VLPs, but Alexa Fluor 488-coupled HPV18 VLPs no longer bound to HaCaT cells or ECM. ELISA data [see Supplementary Fig. S1(a), available in JGV Online] show that the coupled VLPs are intact, as they are detected by conformationally sensitive mAbs. Dye coupling was also attempted for HPV18 VLPs with a different manufacturer’s kit with the same results. HPV16 coupled VLPs appear unaffected, but HPV18 coupled VLPs lost their binding affinity for HaCaT cells and ECM. Dye-coupled HPV11 particles showed binding similar to uncoupled controls, predominantly to ECM components, and dye-coupled HPV45 L1/L2 VLPs lost binding similarly to dye-coupled HPV18 VLPs (data not shown).

It must be noted that HPV45 L1/L2 VLPs were used for all binding experiments because we currently do not have the capability to produce HPV45 L1-only VLPs. However, the data in Supplementary Fig. S1(b) show that the binding patterns of HPV11, 16, 18 and 45 PsVs show no major differences in binding patterns from L1-only VLPs, as also found in a previous study (Culp et al., 2006b).

Pretreatment of ECM with anti-LN5 antibody does not block binding of all VLP types tested

Previous blocking experiments showed that pretreatment of fixed HaCaT ECM with a high concentration (10 μg ml\(^{-1}\)) of polyclonal antibody (pAb) to LN5 blocked binding of authentic HPV11 virus and HPV11 L1 VLPs to HaCaT ECM (Culp et al., 2006b). This observation emphasizes the strong binding preference that HPV11 VLPs have for LN5. Similar anti-LN5 antibody-blocking experiments were conducted using HPV16, 18 and 45 VLPs. Antibody-mediated block in VLP binding is based on a qualitative comparison of the amount of bound VLPs on anti-LN5 pAb-treated versus untreated HaCaT ECM.

In contrast to the findings for HPV11 L1 VLPs, pretreatment of HaCaT ECM with anti-LN5 antibody did not block binding of HPV16, 18 or 45 VLPs to fixed HaCaT ECM (Fig. 2). These results indicate that HPV16, 18 and 45 VLPs do not share the same specificity as HPV11 for LN5, and must bind an additional receptor(s) in the ECM. Supplementary Fig. S2 (available in JGV Online) shows the control VLP binding to HaCaT ECM.

Heparin-mediated block of HPV VLP binding

HSPGs have previously been identified as candidate binding receptors for papillomaviruses (Shafti-Keramat et al., 2003). A series of heparin-mediated VLP-blocking experiments was conducted to characterize HPV capsid binding components further. Soluble heparin and HS are closely related in structure, both consisting of heavily sulfated disaccharides (Selinka et al., 2003). Therefore, it is believed that these two molecules bind to similar sites on the VLP capsid.

Three classes of heparin were used in these studies: H1027 (undefined high molecular mass), H8537 (4–6 kDa) and H3400 (3 kDa). First, we investigated whether the heparins interfered with the mAb detection of the VLPs. The four VLP types were incubated with 50 μg H1027 ml\(^{-1}\) and then bound to an ELISA plate and detected by H11.B2, H16.V5, H18.J4 or H45.N5 (Fig. 3). All mAbs were able to detect VLPs incubated with heparins at the same levels as unincubated VLPs. This experiment also indicates that the capsid structure remains intact with respect to the epitopes recognized by these mAbs.

IF studies (Fig. 4a) showed that HPV11 VLP binding to fixed HaCaT cells appeared mostly unaffected by the different heparin treatments. Similarly to previously published data (Day et al., 2007), treatment of HPV16 VLPs with 10 μg H1027 ml\(^{-1}\) appeared to sequester VLPs to the ECM near the edges of the cells. VLP binding was re-established when incubated with H8537 and H3400 heparins. HPV45 VLPs displayed a binding profile similar to that of HPV16 VLPs; however, there was a larger degree
of blocking, as binding was not re-established fully until incubation with H3400. In contrast, treatment of HPV18 VLPs with H1027 blocked binding to HaCaT cells completely and did not sequester the VLPs to the ECM. Similarly to HPV45 VLPs, binding was blocked substantially when VLPs were treated with H8537.

Pretreatment of VLPs with heparin also resulted in variable blocking on HaCaT ECM. Fig. 4(b) shows the binding patterns upon incubation of VLPs with 50 μg H8537 and H3400 ml⁻¹ (dark grey and white bars, respectively) and subsequently bound to a 96-well plate. Untreated VLPs (black bars) were added as controls. VLPs were detected by H11.B2, H16.V5, H18.J4 or H45.N5 and a rabbit anti-mouse secondary conjugated to alkaline phosphatase. Values are means ± SEM (error bars).

Heparinase treatment of HaCaT cells and ECM

Although similar in structure, heparin and HS differ in size, degree of sulfation, site of synthesis and glycosamine linkages. To investigate the role of HS and to compare with the heparin blocking studies, HaCaT cells and ECM were treated with heparinase I (5 U per coverslip), which cleaves heparin blocking studies, HaCaT cells and ECM were treated with heparinase I (5 U per coverslip), which cleaves heparin and HS molecules at the linkages between the glycosamine and uronic acid residues in the heparin polymer (Joyce et al., 1999). Heparinase treatment greatly diminished the staining for HS on HaCaT cells (Fig. 5a).

No change in HPV11 VLP binding was observed on heparinase-treated and untreated HaCaT cells. Similar to what was seen with heparin treatment of VLPs in Fig. 4, pretreatment of HaCaT cells with heparinase sequestered HPV16 and 45 VLPs to the ECM (Fig. 5b). Comparable to results of the previous heparin treatments, binding of HPV18 VLPs to HaCaT cells was blocked almost completely.

On HaCaT ECM, HPV11 VLPs bound with equal intensity with or without heparinase treatment (see Supplementary Fig. S4, available in JGV Online). Conversely, heparinase treatment of HaCaT ECM blocked binding of HPV18 VLPs almost completely. Interestingly, heparinase treatment did not appear to diminish the binding of HPV16 and 45 VLPs greatly. Studies with heparinase III, which cleaves only HSPGs, were also conducted, but no difference was seen compared with heparinase I (data not shown).

Concurrent treatment of VLP binding with heparin and anti-LN5 pAb

Incomplete block of binding of HPV16 and 45 VLPs after heparinase treatment of ECM and heparin treatment of VLPs suggests the presence of multiple ECM-binding receptors for these VLP types. We next tested dual-treatment studies aimed at blocking both heparin and LN5 receptors on HaCaT ECM. VLPs were treated with 10 μg H1027 ml⁻¹ prior to incubation with fixed HaCaT ECM that was also treated with 10 μg anti-LN5 pAb ml⁻¹. A complete block in both HPV16 and 45 VLP binding was observed when both treatments were applied, whereas individual treatment of both types of VLP or HaCaT ECM still permitted VLP binding (Fig. 6; Supplementary Fig. S5 (available in JGV Online) shows the control individual treatments). These results suggest a possible dual-receptor binding to LN5 and HS for VLPs of these HPV types.

VLP binding to purified LN5 and heparin–BSA via ELISA

Given the heterogeneous nature of HaCaT ECM, we could not assess the binding affinity of VLPs to the individual ECM components directly. In order to overcome this limitation, ELISA studies were conducted using purified human LN5 and heparin–BSA. VLPs were either incubated with 10 μg H1027 ml⁻¹ or left untreated, and added to empty wells or wells containing either purified LN5 or heparin-conjugated BSA. Fig. 7 shows that, in multiple independent experiments, all VLPs displayed strong binding to both purified LN5 and heparin–BSA. When VLPs were treated with heparin prior to addition to coated ELISA wells, HPV11 VLPs were no longer able to bind to heparin–BSA, HPV16 VLP binding was largely unaffected and HPV45 VLP binding showed a 1.5-fold decrease.

Fig. 3. Heparin does not alter the conformation of VLPs. VLPs were incubated with heparins [10 μg H1027 ml⁻¹ (light grey bars) or 50 μg H8537 or H3400 ml⁻¹ (dark grey and white bars, respectively)] and subsequently bound to a 96-well plate. Untreated VLPs (black bars) were added as controls. VLPs were detected by H11.B2, H16.V5, H18.J4 or H45.N5 and a rabbit anti-mouse secondary conjugated to alkaline phosphatase. Values are means ± SEM (error bars).
Fig. 4. Heparin-mediated block of VLP binding to HaCaT cells and ECM. (a) The first column, labelled UN, shows untreated VLPs; TR1 shows VLPs that were incubated with 10 μg H1027 ml⁻¹ prior to binding to cells; TR2 shows VLPs that were incubated with 100 μg H8537 ml⁻¹ prior to binding to cells; TR3 shows VLPs incubated with 100 μg H3400 ml⁻¹ prior to binding to cells. The insets show enlarged regions of interest (boxed). (b) The first column, labelled UN, shows untreated VLPs; TR1 shows VLPs treated with 50 μg H8537 ml⁻¹; TR2 shows VLPs treated with 50 μg H3400 ml⁻¹. The merge columns show the overlay of VLPs and LN5 for each treatment. Bars, 50 μm.
(P-value of 0.02 by Student’s t-test) in binding to LN5 compared with untreated VLPs. HPV18 VLP binding to heparin–BSA and plastic (i.e. empty wells) remained largely unaffected after treatment, whereas HPV18 VLP binding to purified LN5 was nearly eliminated (P-value of 0.0015).

**DISCUSSION**

Our current results show differential binding patterns to host-cell structures for several alphapapillomavirus VLPs. In these studies, which were focused on the primary binding events rather than uptake/infection, we found that

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**Fig. 5.** Binding of VLPs to heparinase-treated HaCaT cells. (a) HaCaT cells were treated with 5 U heparinase I in KCl isotonic buffer for 1 h at 37 °C. Cells not treated with heparinase I were incubated in buffer alone. The first column shows HPV11 VLPs (red) detected by a polyclonal rabbit antiserum and the second column shows HS detected by an anti-HS mAb (F58-10E4) (green). The top row shows untreated cells and the bottom row shows cells treated with heparinase I. The indicated VLPs were added to untreated (UN) or heparinase-treated (TR) cells. (b) HaCaT cells were treated as described for (a). Indicated VLPs (green) were added to untreated (UN) or treated (TR) cells and detected by mAbs as described in the legend to Fig. 1. Bars, 50 μm.

**Fig. 6.** Concurrent treatment of VLPs and ECM inhibits HPV16 and HPV45 VLP binding. HaCaT ECM was prepared and blocked with anti-LN5 pAb as described in the legend to Fig. 2. HPV16 and 45 VLPs were incubated with 10 μg H1027 ml⁻¹ for 1 h. Untreated VLPs merged with LN5 staining are shown in the first column, heparin-treated VLPs added to anti-LN5 pAb-blocked ECM are shown in the second column and the merged image with LN5 staining is also indicated. Bars, 50 μm.
and ECM for the four HPV types. These observations may
strated different patterns of binding interactions to cells
achieve complete blocking. The studies clearly demon-
combination of HS and anti-LN5 pretreatments in order to
of binding of HPV16 and 45 VLPs to ECM required a
mediated HPV18 VLP blockage to LN5. Finally, prevention
2007) and may provide an explanation for this HS-
awaits additional experimentation. We showed that the
presence of an HS-binding site on the
LN5 in ELISA was also blocked by heparin pretreatment.
Interestingly, HPV18 VLP binding to purified
was blocked strongly by heparin, but not by anti-LN5
b). In contrast, HPV18 L1 VLP binding to cells and ECM
lead to changes in the specificity and affinity of different
other HPV types tested, suggesting that fluorochrome labelling is
similarly to heparin-pretreated VLPs for two of the four
HPV types tested, suggesting that fluorochrome labelling is
not always a neutral event. The labelled HPV18 and 45
particles retained surface conformational epitopes as
determined by mAbs, indicating that, if the labelling
introduced any structural changes, then such changes were
subtle.
Recent studies on L1 pentamer structure have shown
significant differences in the positioning of the hypervari-
able loops of L1 sequences for several alphapapillomavi-
sus (Bishop et al., 2007). Prior to these studies, the
structural solution for L1 of only one HPV type (HPV16)
had been resolved, and this structure was used by
vestigators as a template for the capsids of other HPV
types. However, Bishop et al. (2007) showed that the five
major surface loops of HPV18 in particular show
substantial differences in organization from that observed
for the surface loops of HPV16 L1. For example, they
found that Asn58 and Gly57 in the BC loop of HPV18 L1
form hydrogen bonds with Arg178 and Cys185 in the EF
loop. HPV11, 16 and 45 L1 proteins do not share these
amino acids, suggesting that these latter types cannot form
such hydrogen bonding. The consequences of these amino
cid differences may impact on the differences in binding
of HPV18 VLPs to cells, HS and ECM components that
were observed in our studies presented here.
Local structural differences may alter binding affinities of
different HPV L1 proteins to HS moieties, which would
lead to changes in the specificity and affinity of different
HPV capsids to the list of binding structures and entry
receptors (HS, α-6 integrin, syndecan-1, LN5, cyclophilins)
that have been identified so far (Culp et al., 2006b; Da Silva
et al., 2007; Evander et al., 1997; Joyce et al., 1999; Sapp &
Day, 2009; Shafti-Keramat et al., 2003). These possibilities
are supported by recent observations indicating that there
are differences in surface charges of the L1 proteins for
different HPV types (Mistry et al., 2008).
HS-interaction sites on HPV L1 have been characterized by
several investigators (Giroglou et al., 2001; Joyce et al.,
1999; Knappe et al., 2007). The major sites include a
conserved region of the C terminus that contains clusters of
basic amino acids (Joyce et al., 1999), and a more recent
study located at least two different HS-interaction sites on
help to explain a number of apparently discordant
observations between different investigations that, collect-
ively, have used various HPV types, several cell lines and
different HPV capsid sources (VLPs, PsVs and native
virions).
The failure of fluorochrome-labelled HPV18 and 45 VLPs
to bind to cell surfaces and ECM components was an
unexpected observation, given that fluorochrome-labelled
HPV11 and 16 particles showed normal binding activity.
Other investigators have also shown no effects of
fluorochrome labelling on the binding and infectivity of
HPV16 PsVs (Day et al., 2007; Schelhaas et al., 2008). Our
data showed that fluorochrome-labelled VLPs behaved
similarly to heparin-pretreated VLPs for two of the four
HPV types tested, suggesting that fluorochrome labelling is
not always a neutral event. The labelled HPV18 and 45
particles retained surface conformational epitopes as
determined by mAbs, indicating that, if the labelling
introduced any structural changes, then such changes were
subtle.

VLPs and PsV particles showed similar binding patterns to
those found previously for HPV11 L1 VLPs, HPV11 PsVs
and native HPV11 virions (Culp et al., 2006a). The data
suggest that the four types of VLP capsid tested in this
study showed qualitative differences in binding to HS,
ECM and purified LN5, although direct quantification of
Kd values for binding to purified individual components
awaits additional experimentation. We showed that the
binding of HPV11 VLPs to ECM and to purified LN5 was
inhibited by anti-LN5 pAb, but not by heparin pretreat-
ment, confirming previous observations (Culp et al., 2006a,
b). In contrast, HPV18 L1 VLP binding to cells and ECM
was blocked strongly by heparin, but not by anti-LN5
antibody. Interestingly, HPV18 VLP binding to purified
LN5 in ELISA was also blocked by heparin pretreatment.
The presence of an HS-binding site on the γ2 chain of LN5
(which is involved in associations between LN5 and
 syndecan-1, a putative HPV receptor; Shafti-Keramat
et al., 2003) has been reported previously (Ogawa et al.,
2007) and may provide an explanation for this HS-
mediated HPV18 VLP blockage to LN5. Finally, prevention
of binding of HPV16 and 45 VLPs to ECM required a
combination of HS and anti-LN5 pretreatments in order to
achieve complete blocking. The studies clearly demonstra-
ted different patterns of binding interactions to cells
and ECM for the four HPV types. These observations may
the HPV16 L1 capsid surface, including lysine residues 278, 356 and 361 located within the FG and HI loops (Knappe et al., 2007). These findings, together with structural solutions of the capsomeres of several HPV capsids, could help to explain the differential interactions of HPV capsids with HS and ECM observed in our current study and by other investigators (Johnson et al., 2009; Mistry et al., 2008).

The importance of HS moieties as the primary binding receptor for HPVs has received general acceptance in the literature (reviewed by Sapp & Day, 2009), although several studies describing HS-independent uptake and infection in vitro and in vivo have also been reported (Day et al., 2008; Patterson et al., 2005). The HS receptors have been identified as present on both the cell surface (Giroglou et al., 2001; Joyce et al., 1999) and the ECM (Selinka et al., 2007). The role of HS receptors in vivo has been examined recently in a mouse vaginal infection model using PsVs of several HPV types and, interestingly, preferential initial binding of HPV PsVs to basement membrane (BM) components was demonstrated (Johnson et al., 2009; Roberts et al., 2007). In addition, preferential binding of several HPV VLPs to the BM in human cervical tissue sections was reported previously (Culp et al., 2006a). These recent studies support an important role for BM/ECM components in the initial binding and uptake of HPV capsids in vivo, as a reservoir for uptake of virions via cellular filopodia (Schelhaas et al., 2008; Smith et al., 2008), and support a role for the importance of ECM-binding events that can be observed in vitro when keratinocyte cell lines representing the natural host cell are used. The selective targeting of HPV virions to the BM in vivo would provide the needed targeting of the virions to the appropriate cell type (keratinocytes) necessary for HPVs to initiate their life cycle. Our studies described here suggest a role for two or more ECM components (e.g. HS and LN5) that contribute to HPV binding and, if such ‘dual’ binding could lead to increased affinity/avidity of HPV capsid binding, then a mechanism describing the selective targeting of HPV capsids to ECM/BM in vivo can be accommodated.

Earlier studies on HPV binding and infection focused predominantly on one HPV type and one cell type to identify papillomavirus binding receptors for HS, α-6 integrin, syndecan-1 and LN5 (Culp et al., 2006a; Evander et al., 1997; Joyce et al., 1999; Patterson et al., 2005; Shafti-Keramat et al., 2003). These earlier studies led investigators to propose general mechanisms for HPV infection that should be applicable to all HPV types. More recent studies have demonstrated that HPV types show differences in capsid structure (Bishop et al., 2007), surface charge (Mistry et al., 2008), binding patterns to HS (Buck et al., 2006; Johnson et al., 2009) and to ECM (this study), and uptake pathways (Bousarghin et al., 2003; Johnson et al., 2009). In addition, in vivo studies using a mouse vaginal PsV infection model showed differences in binding properties between two alphapapillomaviruses and a betapapillomavirus type (Johnson et al., 2009; Roberts et al., 2007). We conclude from our current studies and from recent publications that HPV types do not show identical patterns of binding to host-cell components, and that unifying models of binding and infection should not be based on observations with a single HPV type or model system.

METHODS

Cells, virus particles and reagents. HaCaT cells (Culp et al., 2006a) were maintained in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Atlanta Biologicals). John Schiller (National Cancer Institute, NIH, Bethesda, MD, USA) provided 293TT cells and plasmids. 293TT cells were grown in DMEM with 10% FBS and supplemented with 400 µg hygromycin ml⁻¹ (InvivoGen). All VLPs were produced in 293TT cells transfected with codon-optimized L1 or, for HPV45, a bicistronic plasmid encoding L1 and L2, and isolated by methods described previously (Buck et al., 2005; Pastrana et al., 2004). VLPs were labelled with Alexa Fluor 488 carboxylic acid tetrafluorophenyl ester (A10235; Invitrogen) according to the manufacturer’s suggestions and following the protocol provided by Day et al. (2007). A plasmid secreting the alkaline phosphatase gene was transfected along with L1 and L2 to produce PsVs. VLPs and PsVs were analysed in a 96-well plate (Evergreen Scientific) by an ELISA for properly folded L1 and L2 (Christensen et al., 1996) using 1 mg p-nitrophenyl phosphate ml⁻¹ (Sigma), pH 9.5, followed by colorimetric analysis at 405 nm. The concentration of VLPs was determined by BCA assay (Pierce 23227). Multiple VLP preparations of each HPV type were utilized to perform the experiments described in this study.

Heparin (H1027), 4–6 kDa heparin (H8357), 3 kDa heparin (H3400), heparinase I (H2519) and heparinase III (H8891) were purchased from Sigma-Aldrich. Heparin–BSA (H1027; Sigma-Aldrich) was conjugated as described previously (Wang et al., 2005). Human LN5, a gift from Peter Marinkovich (Stanford University School of Medicine, Stanford, CA, USA), was mAb affinity column-purified.

Immunocytochemistry/IF. For VLP–cell binding assays, HaCaT cells were seeded on sterile glass coverslips at 1.5 × 10⁵ cells per well or, for ECM, cells were seeded at 0.9 × 10⁵ cells per well. The next day, wells were rinsed with PBS and cells were fixed with cold methanol. For ECM binding, 10 mM EDTA was added to wells and cells were removed by gentle pipetting. Coverslips were rinsed again with PBS and the ECM was fixed with methanol as described above. All coverslips were blocked with 2% BSA in PBS with 0.05% Tween 20 (PBS/T), which was also used as a diluent. Coverslips were washed in PBS/T. All experiments were performed using approximately 1.8 × 10¹⁵ VLPs per well (John Schiller laboratory technical files; http://ccr.cancer.gov/staff/links.asp?profileid=5637). mAbs from our laboratory include H11.B2, which binds a conformationally sensitive epitope on HPV11 L1 as described previously (Christensen et al., 1990), and H18.J4, H16.V5 and H45.N5, which bind a conformationally sensitive epitope on HPV18, 16 and 45, respectively (Christensen et al., 1996). The purchased antibodies were purified human anti-LN5 (ab14509; Abcam) and anti-HS (F58-10E4 epitope; Seikagaku). Fluorophore-labelled secondary antibodies were anti-mouse–Alexa Fluor 488 IgG or IgM and anti-rabbit–Alexa Fluor 594 (Invitrogen). All coverslips were stained with Hoechst 33342 (Molecular Probes) to detect cellular DNA.

Heparinase I and III digestions were performed at 37 °C in an isotonic buffer containing KCl (10 mM KCl, 130 mM NaCl, 20 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg glucose ml⁻¹ and 0.5% BSA, pH 7.4) in order to avoid disruption of clathrin-mediated
endocytosis (Bayer et al., 2001; Culp et al., 2006a). Cells and ECM were rinsed with PBS and then fixed following digestion. HPV11 L1 was detected by a rabbit polyclonal antiserum raised against HPV11 L1. For heparin treatments of VLPs, VLPs were incubated with indicated dilutions of heparins in PBS for 1 h at room temperature.

Fluorescence microscopy for all IF studies was performed by using a Nikon Eclipse E600. Photographs were digitally prepared by using Adobe Photoshop. Within each figure, all images were photographed and digitally prepared in an identical manner.

ELISA. VLPs were treated with 50 μg heparin ml⁻¹ or left untreated and added to 96-well plates. Plates were rinsed with PBS/T and then blocked with 5% milk in PBS/T. Heparin-coated VLPs were detected by mAbs H11.B2, H116.V5, H18.J4 or H45.N5, followed by an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Pierce) and detected as described above.

VLP binding to heparin–BSA and purified LNS. Heparin–BSA or LNS (200 ng per well) was plated in 96-well plates in 50 μl sodium carbonate buffer (pH 9.6) overnight at 4 °C. Wells were washed with 200 μl PBS/T and then blocked with 5% milk PBS/T. After washing, untreated VLPs or VLPs incubated with 10 μg H1027 ml⁻¹ were added in 1% BSA/PBS/T, followed by the indicated primary and secondary antibodies. Detection was as described above.

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