Human papillomaviruses (HPVs) are small, nonenveloped icosahedral viruses with a double-stranded DNA genome of approximately 8 kilobases. HPVs display strict species and cell type specificity in nature, exclusively infecting human epithelial cells (44). The association between certain high-risk HPV types (16, 18, 31, 33, and 45) and human cancer is well established, especially for cervical cancer (58). Additional epithelial cancers are also linked to HPV infections, including other anogenital cancers, cancers of the head and neck, and possibly some types of nonmelanoma skin cancers (16, 20, 62).

The initial step in the life cycles of papillomaviruses is their interaction with cellular receptors that allow attachment and viral internalization. This presumably occurs in the basal layer of the epithelium in order to establish a persistent infection. Epidermal cells are not fully permissive for papillomaviruses at the onset of their differentiation process, but become permissive with increasing cellular differentiation (27, 33, 63). Productive in vivo HPV infections are thought to occur only in benign or lower grade genital lesions, and particles are made at very low levels (40). Although infectious particles can be obtained from growths caused by low-risk HPV types (46), high-risk HPV virions have never been purified directly from anogenital lesions (27). In the past decade, the organotypic (raft) tissues and performed experimental infections in a variety of cells. Successful infection following viral attachment, internalization, and nuclear transport was assayed by detecting newly synthesized, spliced HPV transcripts using reverse transcription (RT)-PCR or RT-quantitative PCR. Most human epithelial cells were infected with HPV31b at a multiplicity of infection as low as 1 to 10 viral genome equivalents per cell. HPV31b infection was detected in other cell lines, including COS-7 monkey kidney cells, but higher viral multiplicities of infection were required. Heparin preparations of various molecular weights or heparinase I treatment of viral receptors. However, whether VLPs and pseudovirions accurately reflect the infection process of HPV virions has not been verified. We generated infectious HPV31b virions from organotypic (raft) tissues using reverse transcription (RT)-PCR or RT-quantitative PCR. Most human epithelial cells were infected with HPV31b at a multiplicity of infection as low as 1 to 10 viral genome equivalents per cell. HPV31b infection was detected in other cell lines, including COS-7 monkey kidney cells, but higher viral multiplicities of infection were required. Heparin preparations of various molecular weights or heparinase I treatment of cells prevented HPV31b infection of COS-7 cells and C-33A human cervical cancer cells in reproducible and dose-dependent manners. However, these reagents were unable to block infection of human keratinocytes, including HaCaT and N/TERT-1 cells and low-passage human foreskin keratinocytes. These data suggest that HPV31b infection of human keratinocytes, the natural host cell for HPV infections in vivo, does not require a heparan-sulfonated receptor, whereas heparan sulfate is important for infection of some other cells.

Oncogenic human papillomaviruses (HPVs) are difficult to study experimentally as they replicate at low levels in vivo. This has precluded the purification of high-risk HPV virions from in vivo lesions. Virus-like particles (VLPs) and pseudovirions from low- and high-risk HPV types can emulate various aspects of HPV virion attachment and infections. These studies suggest that HPV infection is mediated by α6-integrin and/or heparan-sulfonated receptors. However, whether VLPs and pseudovirions accurately reflect the infection process of HPV virions has not been verified. We generated infectious HPV31b virions from organotypic (raft) tissues and performed experimental infections in a variety of cells. Successful infection following viral attachment, internalization, and nuclear transport was assayed by detecting newly synthesized, spliced HPV transcripts using reverse transcription (RT)-PCR or RT-quantitative PCR. Most human epithelial cells were infected with HPV31b at a multiplicity of infection as low as 1 to 10 viral genome equivalents per cell. HPV31b infection was detected in other cell lines, including COS-7 monkey kidney cells, but higher viral multiplicities of infection were required. Heparin preparations of various molecular weights or heparinase I treatment of cells prevented HPV31b infection of COS-7 cells and C-33A human cervical cancer cells in reproducible and dose-dependent manners. However, these reagents were unable to block infection of human keratinocytes, including HaCaT and N/TERT-1 cells and low-passage human foreskin keratinocytes. These data suggest that HPV31b infection of human keratinocytes, the natural host cell for HPV infections in vivo, does not require a heparan-sulfonated receptor, whereas heparan sulfate is important for infection of some other cells.
only a small percentage of exposed cells become detectably infected, suggesting experimental infections in vitro are inefficient (26, 29, 32, 34, 37, 38).

Much of our current understanding of HPV interactions with cells comes from the use of virus-like particles (VLPs) and pseudovirions. Overexpression of the L1 major capsid protein, or L1 plus the L2 minor structural protein results in efficient assembly of these virus-like structures (19, 23, 24, 41, 43, 55, 56). Although large numbers of VLPs can be purified and used for receptor studies, there is little way of determining whether the interactions might accurately reflect the function of true virions during the initiation of a productive infection. Pseudovirions are composed of the HPV capsid proteins packaging or attached to a reporter gene whose subsequent expression is used to identify and quantify pseudoinfected cells. Thus, the readout requires not only receptor engagement, but also internalization, transport, and uncoating of the particles. Additionally, the pseudovirus reporter gene is typically carried on a plasmid that must be amplified for detection in specific cell types. For example, simian virus 40-derived reporters require the use of cells expressing large T antigen such as COS-7 monkey kidney cells to amplify the input reporter DNA (3, 17, 55). However, COS-7 cells are not a natural host cell type for HPV infections.

The binding of VLPs to a variety of cells indicates that distribution of the papillomavirus receptor(s) is wide (36, 42), and the conservation of papillomavirus L1 sequences suggests the viruses may use the same cellular receptor(s). Treatment of HeLa cervical carcinoma cells with various compounds has demonstrated that a cell surface protein is involved in HPV11 and HPV33 VLP attachment (36, 57). The use of VLPs and pseudovirions to elucidate the cellular receptor for papillomavirus-infection of some nonhost cell and transformed human cell types, heparan sulfate does not appear to play an essential role in HPV31b infection of natural host human keratinocytes.

**MATERIALS AND METHODS**

**Cell and tissue culture.** The CIN-612 cell line was established from a cervical intraepithelial neoplasia (CIN) grade I biopsy (1). The CIN-612 clonal derivative 9E maintains the HPV31b genome episomally at an average of 50 copies per cell (21). CIN-612 9E cells were maintained in monolayer culture using E medium containing 5% fetal calf serum in the presence of mitomycin C-treated J2 3T3 fibroblast feeder cells as previously described (28, 31). Epithelial organotypic (raft) tissue cultures for in vitro differentiation were maintained as reported previously (28, 31, 33). Raft tissues were treated every other day with 10 μM 1,2-diocanoyl-sn-glycerol (C8:0; Sigma) in E medium containing 5% fetal calf serum to promote differentiation. Epithelial tissues were allowed to stratify and differentiate at the air-liquid interface for 14 days.

The HaCaT cell line (a kind gift of N. Fusenig, Deutsches Krebsforschungszentrum) is a spontaneously immortalized epithelial line derived from normal adult skin (2). The C-33A cell line was established from an HPV-negative cervical carcinoma (59). C-33A and HaCaT cells were maintained in Dulbecco’s modified Eagle’s medium/F12-Ham’s Nutrient Mixture containing 10% fetal calf serum. Unless otherwise stated, amino acids, 25 mM HEPES, 10 mM sodium pyruvate, 100 μM nonessential amino acids, 1 mg/ml streptomycin (Sigma). N/TERT-1 cells are human foreskin keratinocytes (HFKs) immortalized by the telomerase catalytic subunit and were generously provided by J. Rheinwald, Harvard University (11). Low-passage HKFs from a total of seven donors (strains 1 to 7) were a generous gift of C. Wheeler, University of New Mexico School of Medicine. N/TERT-1 cells and HKFs were each maintained in monolayer culture using E medium containing 10% fetal calf serum plus 10 ng epidermal growth factor per ml and were grown in the presence of mitomycin C-treated J2 feeder cells. COS-7 cells are a simian virus 40-transformed derivative of the CV-1 African green monkey kidney cell line (18). COS-7 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 25 μg/ml gentamicin.

**Virion purification and quantification.** CIN-612 9E raft tissues grown for 21 days at the air-liquid interface were extracted following a modified protocol of Favre et al. using a biologically contained beadbeater device (Biospec Products, Inc.) as previously reported (15, 38). Concentrated virion stocks were prepared from cleared raft tissue lysates by pelleting viral particles based on a sedimentation coefficient for DNA-containing particles (38). Viral DNA containing particles were quantified by DNA hybridization using a 32P-labeled probe in comparison to genome copy number controls and are henceforth referred to as viral genome equivalents (37, 38). Typical yields were ~107 viral genome equivalents per raft and resulted in virion stock concentrations of 107 to 108 viral genome equivalents per ml. Four batches of virion stocks grown from separate groups of raft tissues were used in this study.

**HPV31b infections and blocking assays.** Cells were seeded at 2.5 to 4.0 x 104 cells per well in the absence of fibroblast feeder cells in 4 cm2 wells and were allowed to attach overnight. The monolayers were 60 to 80% confluent at the time of infection. Plating efficiency of cell lines was scored visually just prior to infection. For low-passage HKFs, the plating of the pooled cells and individual strains was determined by counting cells of replicate plates prior to infection. Virion stocks were thawed from ~80°C to room temperature and were sonicated for 20 seconds at 0°C. For all cell lines, virus dilutions were added to each well in 0.25 ml of normal HaCaT medium (see above). The plates were rocked for 4 h; viral inocula were subsequently removed. The cells were then washed in an excess of their normal medium, and refed with their respective normal medium. Following infection, N/TERT-1 cells and HKFs were cocultured with mitomycin C-treated J2 fibroblast feeder cells. The cells were moved to 37°C, and medium was changed every other day until the cells reached confluence and were expanded or were harvested for nucleic acid purification.

Heparin preparations of three different molecular weights (H-1027, low molecular weight; H-3400, 3,000 Mr; and H-4784, 20,000 Mr; Sigma) were diluted to 10 mg/ml in sterile phosphate-buffered saline (PBS). The heparin preparations were further diluted in normal HaCaT medium (see above) to final concentrations of 3.0, 1.5, 0.5, or 0.0 mg/ml. Sonicated virion stocks diluted in HaCaT medium were combined with the heparin dilutions or with PBS and were incubated at 4°C for 1 h as described (17). The heparin or PBS plus virion suspensions were added to cells, which were rocked at 4°C to allow viral attachment as described above. Inocula were removed and the cells were washed in an excess of medium and then refed with their respective usual medium and handled as described above.

For heparinase I treatments, monolayer cells were washed once with heparinase buffer (20 mM Tris-Cl, 30 mM NaCl, 4 mM CaCl2, 0.01% bovine serum albumin) and then refed with fresh medium. Heparinase I treatments were performed for 1 h at 37°C, and the medium was replaced with fresh medium, and the cells were washed once with heparinase buffer and then refed with fresh medium.
albument, pH 7.5). Heparinase I (Sigma) was diluted in heparinase buffer added at 20, 10, 5.0, 1.0 and 0 units per well in 0.25 ml to cells as previously reported (17, 22). Cells were incubated for 1 h at 37°C, washed twice on ice with cold PBS, and then infected with HPV31b at various multiplicities of infection (MOI) as described above or processed for flow cytometry.

Nucleic acid purification and qPCR analysis. Total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies) and nucleic acid concentrations were determined by optical density measurement. RNA concentrations and quality were verified by electrophoresis through agarose gels containing ethidium bromide. RNA (1.5 to 4.0 μg) was reverse transcribed using random hexamer primers in a final volume of 20 μl. End-point and quantitative PCR were performed using GeneAmp PCR RNA PCR reagents and AmpliTaq Gold DNA polymerase (Applied Biosystems) under conditions previously reported (37, 38).

For quantitative PCR (qPCR) 5 μl of each cDNA reaction was analyzed in triplicate. HPV31b primer pairs E7.3A and E4.3B or E7.4A and E4B (37, 38) were used at 200 nM each with 2.5 mM MgCl2. For qPCR, the TaqMan probe spanning the E1 < E4 splice site (HPV31b nucleotides 3295 to 3325; 6FAM-CAG TGA CGA AAT ATC CTT TGC TGG GAT TGT T-TAMRA) was used at final concentration of 100 nM. Oligonucleotides were synthesized at Sigma Genosys. An ABI 5700 Quantitative PCR Machine was used for the PCR amplifications and data analysis. The qPCR thermocycling profile was as follows: 2 min at 50°C, 12 min at 95°C, 40 to 50 cycles at 95°C for 15 seconds and 65°C for 30 seconds.

Flow cytometry analysis. HaCaT or COS-7 cells were seeded in monolayer at 104 cells per 100-mm dish. The next day, cells were treated with heparinase I at 0, 1.0, or 10 units per dish for 1 h at 37°C as described above. Cells were washed one time with PBS and detached with 25 mM EDTA/PBS. Following detachment, the cells were pelleted and fixed in 3.7% formaldehyde/PBS for 10 min at room temperature. The cells were then pelleted, resuspended in 1:200 dilution of an anti-heparan sulfate antibody (F58 to 10E4; Seikagaku America) in PBS-1% bovine serum albumin, pH 7.5). Heparinase I (Sigma) was diluted in heparinase buffer added at 20, 10, 5.0, 1.0 and 0 units per well in 0.25 ml to cells as previously reported (17, 22). Cells were incubated for 1 h at 37°C, washed twice on ice with cold PBS, and then infected with HPV31b at various multiplicities of infection (MOI) as described above or processed for flow cytometry.

RESULTS

Reverse transcription and quantitative PCR assay for experimental HPV31b infections. In previous studies using RT and end-point PCR to detect the presence of newly synthesized, spliced viral RNAs, we found that HPV31b reproducibly and efficiently infected HaCaT cells (37). To explore the validity of using RT and quantitative TaqMan PCR (qPCR) as a means to compare the relative efficiencies of HPV31b infections under various conditions, as shown for HPV11 infection in cultured cells (9), HaCaT cells were infected with authentic HPV31b virions at various multiplicities of infection. At 4 days postinfection, the cells were harvested for total RNA, and the RNA was subjected to RT.

In earlier work we found that targeting the spliced HPV31b E1 < E4 RNAs by RT-PCR was the most sensitive means of detecting HPV31b infection (37, 38). In Fig. 1 we compared the method of RT and end-point PCR to that of RT and qPCR for detecting HPV31b E1 < E4 transcripts following infection of HaCaT cells. Subtle differences may be seen among the samples infected with HPV31b MOI from 1.0 to 100 viral genome equivalents per cell using RT and end-point PCR (Fig. 1A). However, the qPCR data clearly demonstrate a 10-fold variation in E1 < E4 levels corresponding to the 10-fold serial dilutions of viral genome equivalents supplied to the cells, indicating the accuracy and validity of using RT-qPCR to investigate the relative levels of HPV31b infection.

Experimental HaCaT cell infection and quantification of HPV31b E1 < E4 cDNA via qPCR was repeated a number of times with remarkable reproducibility. Additionally, β-actin transcripts were targeted in RT plus end-point PCR to control for the presence and integrity of RNA in samples, for RT, and for PCR amplification (Fig. 1A). β-Actin RNAs were also targeted in separate qPCR reactions to attempt to control for the amount of input cDNA; however, we were unable to perform internal multiplex qPCR analysis of the two genes, and in separate reactions we found this to be an unreliable control for normalization. Instead, we chose to rely on the reproducible detection of HPV31b E1 < E4 cDNAs as analyzed in triplicate from each of multiple separate experimental infections.

Quantitative comparison of experimental HPV31b infections in cultured cells from various sources. Studies of HPV receptors and entry pathways have employed the binding of virus-like particles (VLPs) or the binding and internalization of pseudovirions to a number of nonhost cell lines including COS-7, 293T, C127, and CHO, as well as cell lines originating from each of multiple separate experimental infections.
from human epithelium such as C-33A, HeLa, HaCaT and simian virus 40-transformed human keratinocytes (10, 12, 14, 17, 22, 30, 46, 57, 60). Recent studies have used HPV11 virions in HaCaT keratinocytes and HPV11 and HPV40 virions in A431 vulvar carcinoma cells (4, 8, 46). Yet there have been no such investigations using bona fide high-risk HPV virions in human keratinocytes, the natural host cells targeted by HPV infections in vivo.

We wished to evaluate the properties of HPV31b infection among a few of the aforementioned cell lines. However, as papillomaviruses have a narrow host range and strict tissue tropism, which is thought to be dictated at the transcriptional level (52), we were unsure as to whether our transcription-based endpoint assay for measuring infection would permit such comparisons. We employed RT-qPCR to directly compare the relative efficiency and reproducibility of detecting HPV31b infection, as measured by E1^E4 transcript levels, among various cell lines targeted by HPV infections in vivo.

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Investigation of a role for heparan-sulfonated molecules in HPV31b infections of various cells. In the past few years, much attention has been given to a potential role for heparan sulfonated molecules and glycosaminoglycans in papillomavirus infections (6, 12, 17, 22, 46). Yet most studies on virus attachment and infection pathways have simply investigated VLP binding to cells of various types or the use of pseudovirion infection of nonhost, nonkeratinocyte cells. Using reagents and methods previously reported to block HPV11 VLP binding to HaCaT cells as well as HPV33 and HPV16 pseudovirion infection of COS-7 cells (17, 22), we investigated the role of heparan-sulfonated molecules in HPV31b infection of cultured cells of human keratinocyte origins and COS-7 cells (Fig. 3).

HPV31b virions were incubated with various molecular weights of heparin and used to initiate infections as described in the Materials and Methods section and as reported by Giroglou et al. (17). For all experiments replicate PBS-treated virion controls were included, and E1^E4 levels were expressed in percentages relative to those in the controls for each cell line. Consistent with previous reports using HPV33 and HPV16 pseudovirions (17, 45), heparin pretreatments vastly reduced the infectivity of HPV31b virions in COS-7 cells in a clear and dose-dependent manner (Fig. 3A and B). HPV31b infection of COS-7 cells was reduced 70% to 100% when virions were pretreated with heparin compared with infections using control PBS-treated virions. However, no reproducible heparin dose-dependent reduction in HPV31b infectivity was observed in HaCaT human keratinocytes under the same conditions (Fig. 3D and E). The results in COS-7 cells are representative of four separate infections each analyzed 2 to 3 times in triplicate; experiments with HaCaT cells were repeated four times to verify the results. The data in Fig. 3D showing nearly 50% inhibition of HaCaT cell infection was the only analysis indicating possible heparin blocking in these cells.

Removal of cell surface heparan sulfate also was performed to assess the requirement for interaction of HPV31b virions with heparan sulfate (Fig. 3C and F and Fig. 4). Heparinase I was used to remove heparan sulfate from COS-7 and HaCaT cells and removal was monitored by fluorescence-activated cell sorting (FACS) analysis. Treatment of COS-7 cells with 40 units per ml (10 U) of heparinase I reduced the cell surface expression of heparan sulfate by 55% compared to untreated cells (Fig. 4A), and this correlated with a >75% reduction of HPV31b infection of COS-7 cells (Fig. 3C). Although heparinase I treatment of HaCaT cells reduced cell surface expression of heparan sulfate by 79% compared to untreated cells,
negligible, if any effects were observed on HPV31b infection (Fig. 3F). The effect of heparinase I treatment of HPV31b infection of COS-7 cells was repeated three times with the same results; heparinase I effect on HaCaT cell infectivity was verified five times. These results are in full agreement with the heparin blocking experiments and indicate that although interaction with heparan sulfate is important for HPV31b infection of the nonhost COS-7 cells, it is not essential for HPV31b infection of HaCaT human keratinocytes.

To further determine whether the interaction of HPV31b virions with heparan sulfate is important for infection of other biologically relevant human keratinocyte cell lines or low-passage HFKs, we performed experiments using heparin treatment of virions and heparinase I treatment of cells in the C-33A cervical carcinoma cell line, the N/TERT-1 immortalized HFK cell line, and in pools of low-passage HFKs from a number of donors (Fig. 5). HPV31b infection in C-33A cervical cancer cells was reproducibly blocked in a dose-dependent manner when virions were incubated with heparin of various molecular weights (Fig. 5A and B). These results were similar in three separate infections. Likewise, heparinase I treatment of C-33A cells resulted in a substantial reduction in HPV31b infection (Fig. 5C) that was verified in four individual infections.

From the data in Fig. 2B, we expected N/TERT-1 cells and low-passage HFKs to show more variable detection of HPV31b infections, and this held true in the heparin experiments as well. In many of these experiments we observed random infection fluctuations that were not heparin dose-dependent (e.g., see Fig. 5D and G). We attribute this to the more variable infectivity in these HFK-derived cells, likely due to the more heterogeneous nature of the cell populations (see Discussion). On more than one occasion both N/TERT-1 cells and low-passage HFKs exposed to virions treated with the low molecular weight heparin (H-1027) had greater than 50% infection reduction in a dose-dependent manner (e.g., Fig. 5E). Yet, considering the five separate experiments each testing heparin blocking in N/TERT-1 cells and HFKs, HPV31b infections of neither HFK-derived cell population were reproducibly blocked in a dose-dependent manner. Furthermore, heparinase I treatment of N/TERT-1 cells and three different pools of HFKs from seven donors (strains 1 to 7) failed to reduce HPV31b infection in these cells (Fig. 5F, H, I), and the triplicate RT-qPCR analyses were repeated to confirm the results. Taken together, our data suggest that heparan sulfonated molecules play a role in HPV31b infection of COS-7 monkey kidney cells and the C-33A cervical carcinoma cell line, but that interaction with heparan sulfate is not essential for HPV31b infection of human keratinocytes, the host cell type for HPVs.

**DISCUSSION**

VLPs and pseudovirions have been used to study the requirement for heparan-sulfonated receptors in the binding to and internalization into a number of immortalized, transformed, and nonhost cell types for HPVs (6, 12, 17, 22, 46). However, to date there have been no reports investigating high-risk HPV infections in their natural host cells, nonimmortalized human keratinocytes. The purpose of our study was to assess the requirement of heparan-sulfonated molecules as potential HPV31b attachment moieties resulting in authentic infection in cell types most relevant to infection in vivo. To this end we have purified infectious HPV31b virions from CIN-612 9E raft tissues and have obtained virion stocks containing ~10^9 viral genome equivalents per ml.

Our previous work using RT and end-point PCR to detect spliced viral RNAs as a measure of HPV31b infection in a number of cell lines demonstrated HaCaT cell infection was most reproducibly and robustly detected (37, 38). These findings also held true in this study where we have modified our assay to include RT-qPCR to compare the relative levels of HPV31b infection among cells and cell lines as a function of viral E1^E4 transcript quantities. Using this assay to measure levels of spliced E1^E4 transcripts, HPV31b infections can be quantified and relative levels of infection compared, albeit not the absolute number of cells infected. Because the absolute levels of E1^E4 transcripts are different following infection of various cells (Fig. 2), we have not compared differences in transcription among cell lines as a basis for infection inhibition. Rather, we have compared the ability of potential blocking agents to inhibit HPV31b infection within a given cell line as normalized to untreated cells.

We analyzed COS-7 monkey kidney cells and C-33A cervical carcinoma cells, two transformed cell lines, as controls for relation to other studies (6, 17, 42). We also investigated cells that more closely represent the target cell for HPV infection in vivo: HaCaT and N/TERT-1 cells, which are immortalized human keratinocytes that retain the ability to differentiate in...
vitro (2, 11), and low-passage HFK pools. Using the methods of Giroglou and coworkers and Joyce et al. (17, 22), we found that interactions with heparan sulfate are required for HPV31b infection of COS-7 monkey kidney cells. Heparin treatment of virions or heparinase I treatment of COS-7 cells caused a 70 to 100% inhibition of HPV31b infection, even at the lowest chemical concentrations, and our findings using authentic HPV31b virion infection of COS-7 cells are in agreement with the previous studies exposing COS-7 cells to pseudovirions from HPV types 16, 18, 31, 33, 39, 45, 58, 59, and 68 (6, 17, 45).

We found a similar requirement for heparan-sulfonated molecules with HPV31b infection of C-33A cervical carcinoma cells. Heparin treatment of virions or heparinase I treatment of COS-7 cells caused a 70 to 100% inhibition of HPV31b infection, even at the lowest chemical concentrations, and our findings using authentic HPV31b virion infection of COS-7 cells are in agreement with the previous studies exposing COS-7 cells to pseudovirions from HPV types 16, 18, 31, 33, 39, 45, 58, 59, and 68 (6, 17, 45).

We can envision at least three explanations as to why HPV31b infection of COS-7 and C-33A cells is heparan sulfate
dependent, whereas infection of human keratinocytes does not require heparan sulfate interactions. These possibilities also address why our results differ from other studies indicating HPV31b infections do not require heparan sulfate for cellular binding and/or infection. First, our study investigated HPV infection of human keratinocytes, rather than VLP binding to or pseudojunction formation of transformed cells or cells relevant to in vivo HPV infection. HPV31b infections, like other viruses, may use distinct receptors to infect different cells. The heparan sulfate-dependent phenotype could result from the fact that COS-7 monkey kidney cells are not the correct species or cell type for HPV infection, have been transformed by simian virus 40 large T antigen, and/or have undergone an indeterminate number of passages in culture.

Although we were surprised that C-33A cervical cells behaved differently from HFKs, it is to be expected that these cells derived from a malignant carcinoma have lost a number of their epithelial characteristics. This is likely true also for other transformed cells like HeLa, A431, and KH-SV (simian virus 40-transformed human keratinocytes), which have been reported to display heparin-dependent phenotypes. Thus, the cells may not retain expression of a keratinocyte-specific HPV receptor(s). Furthermore, there may be a difference in the ratios of surface expression levels of nonspecific receptors (e.g., heparan sulfate) versus specific (α6 integrin or as yet unidentified) receptors present on these different cell types. Our data and data from other labs are consistent with a model in which HPV may use heparan-sulfonated molecules as initial, nonspecific attachment receptors in COS-7 and C-33A3 cells. The virus may then be transferred to a second specific, heparinase-resistant molecule that mediates entry into and productive infection of cells as previously proposed (17, 45). In contrast, human keratinocytes may express the specific receptor(s) at much higher levels, minimizing the requirement for an initial heparan sulfate interaction.

Second, we have used significantly fewer virus particles for our infectivity assays (MOIs from 5 to 50 viral genome equivalents per cell) compared to most VLP binding or pseudojunction formation experiments in which thousands to tens of thousands of particles per cell were used (6, 12, 17, 22, 45, 46). A disadvantage of using very high numbers of particles, especially in simple binding assays, is that the data could reflect bulk interactions resulting in nonproductive events, while masking the actual infectious pathway used by only a few virions as is expected in vivo. This has been observed with mouse polyoma pseudovirions where mass interactions can occur via a receptor-independent pathway that is distinct from the receptor-specific pathways required to successfully deliver DNA to the nucleus (25). A study using HPV16 VLPs also exemplifies this concern where VLP binding was completely blocked in HaCaT cells using 10 μg per ml of heparin, whereas the more specific and sensitive measure of VLP internalization required at least 3 mg per ml of heparin to achieve no more than 70% blocking (12). Interestingly, this work also points to a heparin-independent uptake pathway in HaCaT cells consistent with our data as discussed above.

A third possibility is that HPV31 may use a receptor(s) different from other genital HPV types that have been tested. Data in support of this come from Bousarghin et al. who found that HPV31 pseudovirions use a caveolar-mediated entry pathway in COS-7 or 293T cells, whereas HPV16 and HPV58 pseudovirions enter via a clathrin-dependent pathway (3). As viral receptors generally dictate the entry pathway (49), this suggests that different genital high-risk HPV's could use distinct receptors. Nevertheless, the entry pathway of HPV31b could be distinct for large numbers of pseudovirions in non-relevant cells, and we are in the process of verifying the entry pathway in human keratinocytes.

Our data suggest that HPV31b can use separate receptors for infection of COS-7 cells and C-33A cells, both high passage transformed cell lines, versus the lower passage untransformed human keratinocytes (i.e., HaCaT cells, N/TERT-1 cells, and HFKs). Several viruses use heparan sulfate as an initial receptor, with specific secondary or tertiary receptors for viral uptake (5, 7, 35, 45, 54). Our work supports other data (17, 45) indicating that a heparin-independent receptor is used for HPV uptake, regardless of whether an initial heparan sulfate-dependent primary interaction is required.

An issue that remains to be addressed is whether COS-7 or C-33A cells versus normal keratinocytes use the same secondary (specific) heparin-independent receptor. Another question is whether COS-7 and C-33A cells require heparan-sulfonated receptors because the expression of this putative secondary receptor is extremely low in these cells compared to keratinocytes. Likewise, if virions employ different uptake pathways in these cells, the receptors may be different. This is consistent with a recent study where HPV11 virion entry and E1+E4 expression kinetics were different in COS-7 cells compared to more relevant cell lines (8).

It is of great interest to test the role of heparan sulfate in other HPV infections. Although we have purified infectious HPV16 and HPV18 virions from raft tissues (26, 34), virions from these tissues are made at lower levels and early transcripts are more difficult to detect following experimental infections with HPV types 16 and 18. Thus, we are still in the process of optimizing a robust RT-qPCR assay capable of reproducibly distinguishing 2- to 10-fold decreases in infection levels, which is essential to our ability to obtain interpretable data from infection blocking experiments.

The continued study of infectious HPV virions in cell types relevant to in vivo infections will be imperative for uncovering the answers to many of the questions related to early infection events. For true biological relevance, infections will require the investigation of basal cells in the context of a differentiating epithelium.

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