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Entry and trafficking of human papillomavirus type 31 into human keratinocytes

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B.S. Molecular, Cellular, and Developmental Biology, UC Santa Cruz, 2001

DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

Human papillomaviruses (HPVs) are small, nonenveloped double stranded DNA viruses that display a strict species and cell type tropism for human epithelial cells. The association between high-risk HPV types and cervical cancer is well established. Additionally, HPVs have been implicated as cofactors in development of several other epithelial cancer types. The difficulty associated with producing infectious HPV stocks, coupled with the complexity of detecting infection using standard virological methods has hindered research into some basic aspects of HPV biology. Recent advances have allowed for the production of high yield stocks of HPV particles for use in infectivity studies and has facilitated research on HPV cellular entry. Using a combination of fluorescent tracking and infectivity studies, we have elucidated the infectious entry pathway and trafficking of HPV31 particles following internalization into human keratinocytes (HKs). Upon initial attachment, HPV31 associates with lipid rafts before

internalization *via* caveolae, a process that is dependent on dynamin-2. Virions are observed trafficking through caveosomes before transport to the endosomal pathway in a Rab5-dependent fashion. Progression through the endosomal pathway results in exposure to decreasing pH, which promotes conformational changes in the virion capsid. These changes are associated with escape of the viral genome and/or genome-L2 complex to the cytoplasm, an event necessary for trafficking to the nucleus and production of viral transcripts. This pathway represents a unique viral trafficking route. In addition to entry and trafficking events of HPV31, the research presented here elucidates signaling cascades induced during HPV31 attachment to cells and internalization, as well as cytoskeletal rearrangements observed during these events. Signaling through protein tyrosine phosphatases was found to regulate microtubule reorganization that is required for association of HPV31 particles with lipid rafts. Following lipid raft localization, several classes of cellular signaling are activated. Tyrosine kinase and phosphatidyl-inositol-3 kinase activation was found to be required for actin cytoskeletal rearrangements that result in filopodia formation at the cell surface. Retrograde transport of virions along filopodial projections suggests that these structures play a role in facilitating uptake of virions from the extracellular matrix of host cells and increase infection efficiency of HPV31.

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Chapter I

Introduction

Papillomavirus Background

Human papillomaviruses (HPVs) are small viruses with a nonenveloped icosahedral capsid that encloses a double stranded DNA genome of ~8 kilobases (80). HPVs are members of the *Papillomaviridae* family and are further classified by nucleotide sequence into genera and species. Although distribution of PV types into genera is based solely on nucleotide sequence, biological properties, such as transmission and cell type infected, are conserved within genera. HPV types that infect the genital mucosa are members of the *Alpha-papillomavirus* genus and include both low-risk (i.e., HPV6, 11) and high-risk (i.e., HPV16, 18, 31) types (41), classified as such for their association with cervical cancer.

Papillomaviruses display a strict cell type and species tropism, infecting only epithelial cells of their host species (80, 133). Whether this tropism is dictated by receptor usage or ability to replicate in infected cells, or a combination of both, still remains unclear. However, the requirement for differentiation for completion of the PV life cycle, which will be discussed in more detail later in this chapter, plays an important role. It is important to keep in mind the stringent tropism of PVs when researching them in the laboratory. The use of cell types that are irrelevant to HPV infection can confuse the field's understanding of HPV biology.

HPV and Disease

HPVs have been established as the causative agents of cervical cancer, as more than 99% of cervical cancer tissues are HPV DNA positive (9, 205). Genital HPV types are classified as high or low risk, depending on association with cervical cancer. HPV16

and HPV18 are generally considered to be the primary cancer-inducing types, accounting for ~70% of cervical cancer and CIN cases, while a handful of other types, including HPV31, account for the remaining 30% (8, 172). High-risk HPV infection is considered a necessary but insufficient cause of cervical cancer, and a number of cofactors including age, parity, immune function, other genital infections, and smoking can contribute to progression (19). In addition to the association with cervical cancer, HPVs have also been implicated as risk factors for other cancer types, including other anogenital cancers (135), nonmelanoma skin cancer (83, 147), and head and neck cancer (68).

The recent advances in HPV vaccine development have been promising. Two VLP-based vaccines have been developed: the first a quadrivalent HPV6/11/16/18 vaccine that was approved in 2006 (4); the second a bivalent HPV16/18 vaccine slated to be released this year (76). Clinical trials with both vaccines have demonstrated that both are safe and highly immunogenic, demonstrating virtually 100% efficacy against development of HPV-related neoplasia. Additionally, follow-up studies have indicated that protection against infection can last up to five years (204). However, some drawbacks of the vaccines do exist, most notably price and distribution difficulties. Although high-risk HPV infection is widespread in women, both in developed and underdeveloped countries, screening practices differ considerably, resulting in dramatic differences in cervical cancer mortality. In developed countries such as the United States, efficient screening for early signs of cervical cancer has been effective in controlling, for the most part, cervical cancer deaths. In stark contrast, Pap smear screening is very low in developing countries, and cervical cancer mortality is high. For example, in 2002, more than 80% of the ~300,000 worldwide cervical cancer deaths

occurred in developing countries (136). Thus, although the advent of the HPV vaccine shows great promise for controlling HPV infections where it can be efficiently administered, the high cost of this vaccine presents a major challenge for distribution to those women most in need of it (7). Moreover, although protection has been demonstrated for up to 5 years post-vaccination, in order for the vaccine to be effective in preventing cancer progression, protection must last for decades, a property of this vaccine that still remains to be demonstrated. Thus continued research on HPV biology is essential to understand these important human pathogens.

The HPV Life Cycle

A unique aspect of HPV biology is the strict requirement for infection of differentiating epithelia for life cycle completion (Figure 1.1). In the current understanding of HPV infection, virions are believed to require access to the mitotically active cells in the basal layer of a differentiated epithelium, which presumably can only occur through a break in the skin (50, 173). Following entry into these cells, an initial stage of low level genome replication occurs in this layer, and viral genomes are maintained episomally (47, 104). Episomal maintenance and segregation during mitosis is facilitated by the early proteins, E1 and E2 (151, 212). Cells of the basal layer maintain the viral genome, and transcription occurs solely from the early promoter (82). Only low-level genome replication occurs in this layer and production of late structural genes is undetectable. It is not until daughter cells derived from infected basal cells undergo differentiation that transcription is induced at the late promoter of structural gene products. As differentiating cells ascend in the epithelial layer, increased genome

replication occurs as well (5, 24, 82, 193). Production of late structural proteins, L1 and L2, that form the viral capsid, coupled with increased levels of viral genome promotes the formation of progeny virions that are released as the top layers of the epithelia are sloughed off.

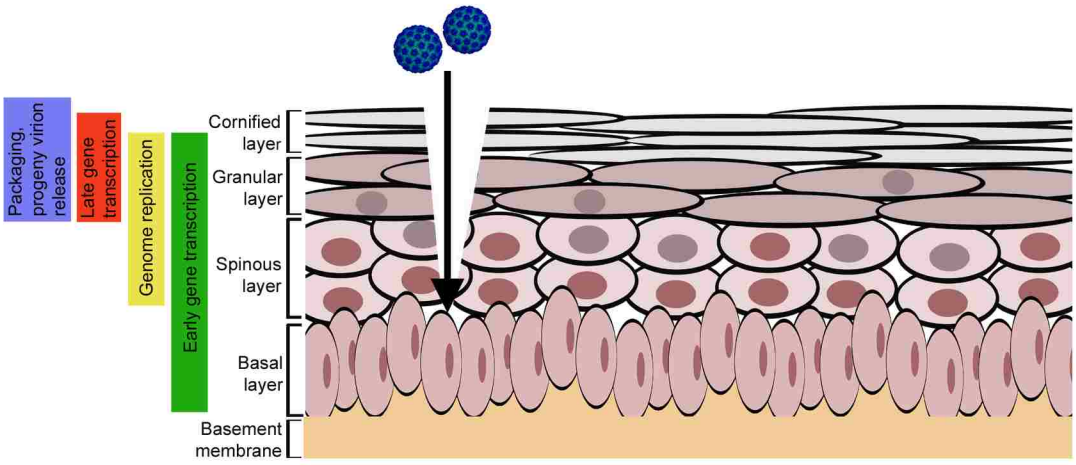

Figure 1.1 The differentiation dependent life cycle of papillomaviruses
Papillomaviruses infect the differentiated epithelium through a break in the layer, in order to access the basal layer. Infected basal cells exhibit low level genome replication and early gene expression. As infected daughter cells differentiate and rise in the epithelia, increased genome replication and early gene expression occur. Expression of late transcripts (capsid proteins) does not occur until infected cells reach the granular layer, and packaging of progeny virions can occur. As cells of the cornified layer are sloughed, progeny virions are released.

The functions of HPV early genes are still under investigation, but each gene product clearly has multiple functions. In addition to the roles E1 and E2 play in viral genome maintenance, they are important for replication of the viral genome as well as regulating expression of other early gene products (64, 117, 191). Functions of the E1^E4 and E5 products are less clear. It has been proposed that the E1^E4 protein, which is expressed at high levels late in infection, may be important for release of progeny virions from infected epithelium (45), although this function in natural infection

has yet to be demonstrated. HPV E5 is emerging as another possible tumorigenic protein, in addition to E6 and E7, because of its ability to affect cellular signaling, epidermal growth factor receptor expression, and cell cycle progression (57, 89, 109). The most well characterized high-risk HPV proteins are the oncoproteins, E6 and E7, both of which display potent transforming activity (123, 148). The most prominent function of HPV E6 is its ability to bind and degrade the tumor suppressor p53, promoting uncontrolled growth and cell cycle disregulation (169, 208). The ability of E7 to bind the cell cycle regulatory molecule pRb contributes significantly to the transforming capability of high-risk HPVs (119, 148). The levels of both of these proteins are relatively low in early infection, and it is not until integration of the viral genome, which typically occurs within the E1 or E2 region, that repression is lost, and E6 and E7 expression is increased, contributing to the oncogenic potential of high-risk HPVs (164).

HPV virions are composed of 360 L1 molecules, arranged as 72 capsomers, and 12-72 L2 molecules, which together form an icosahedral capsid, encapsidating a single copy of the circular viral genome organized around cellular histones (16, 56, 90, 146, 194). The expression of HPV late proteins is tightly linked to the differentiation of infected cells, ensuring that viral progeny are released only in the uppermost layers of the epithelium (24, 193). As the viral capsid proteins are most likely to be those targeted by the host immune system, it is probable that this gene expression strategy contributes significantly to immune evasion by HPVs (199). The ability of HPV infection to persist for decades and avoid immune clearance plays a vital role in the ability of high-risk HPVs to cause cancer.

Virus Entry

Viruses must enter host cells in order to access cellular machinery to replicate and produce progeny virions. Entry into host cells occurs primarily by one of two mechanisms: (1) direct penetration of the host membrane, a method utilized principally by enveloped viruses; or (2) exploitation of the host cell endocytic machinery [reviewed in (108, 180)]. Overall, it appears the most common route of viral entry is endocytosis, as this mode allows for some specific advantages, such as passage across the cortical actin network, transport through a densely packed cytoplasm, and delivery to sites of uncoating/replication. Research on viral use of endocytic pathways has expanded considerably in recent years, and the existence of many unique mechanisms has become apparent. Knowledge gained from this research has given great insight not only into virology but cell biology as well.

At present, there are four well-defined internalization pathways that can be used by viruses for host cell entry (Figure 1.2). The first identified and best characterized pathway, clathrin-dependent endocytosis, was first observed by electron microscopy, which revealed electron-dense 'coated pits' internalizing ligands at the plasma membrane (65, 165). The protein forming the cage surrounding these vesicles was later identified as clathrin (140), and a wide variety of ligands have since been demonstrated to enter cells within these structures. Clathrin-dependent entry is a constitutive process, able to occur in the absence of ligand stimulation, in contrast to other endocytic pathways. Additionally, ligands entering *via* clathrin-coated pits are internalized rapidly (within minutes), another distinguishing property of this pathway. In the classic view of

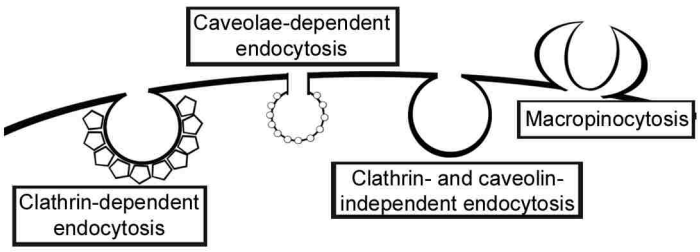

Figure 1.2 Endocytic routes of virus entry
At present, there are four characterized entry pathways utilized by viruses. *Clathrin-dependent endocytosis* is the most commonly used route for viruses and can be distinguished by a clathrin lattice surrounding invaginating vesicles. *Caveolae-dependent endocytosis* occurs in small, flask-shaped vesicles encircled by caveolin-1. *Clathrin- and caveolin-independent endocytosis* is at present not well understood but occurs in the absence of clathrin and caveolin function. *Macropinocytosis* is a nonspecific mechanism for uptake of extracellular material.

clathrin-dependent entry, ligands traffic to the early endosome, where sorting occurs to direct transport either through the endosomal pathway to the lysosome or back to the plasma membrane (30). More recently, intracellular trafficking following endocytosis has been revealed to be more complex and will be discussed in further detail later in this chapter. The first viruses demonstrated to use clathrin-dependent endocytosis for entry were Semliki Forest virus and influenza virus (107, 110), and subsequent research has revealed that most viruses whose entry pathways have been defined use this endocytic route.

The second most common mode of internalization is caveolae-dependent endocytosis. Caveolae were first described as small, flask-shaped, uncoated pits observed by electron microscopy forming at the plasma membrane and in the cytoplasm (134, 210). A key component of these structures was later identified as caveolin-1 or VIP-21 (93, 166), and other members of this family, caveolin-2 (171) and caveolin-3 (207), have since been identified. Caveolins are oligomeric proteins that interact with cholesterol and fatty acids directly (120, 201) and are present in low-density, detergent-insoluble membrane domains known as lipid rafts at the cell surface (60). Unlike clathrin, caveolins display cell-type specific expression, and caveolae are not detected in

every cell type. Internalization through caveolae is a ligand-stimulated process and occurs slowly as compared to clathrin-dependent entry (137). Since caveolae have been described, a wide variety of ligands have been demonstrated to internalize through these structures, including cholera toxin B, a classical marker of lipid rafts and caveolae, and GPI-linked receptor ligands (118). The first virus demonstrated to use caveolar entry was the polyomavirus, simian virus 40 (SV40), which was observed binding to cells, translocating to caveolin-rich lipid rafts, internalizing in caveolae, and trafficking to the endoplasmic reticulum (ER) (1, 190). Since this discovery, a number of other viruses have been shown to internalize within these structures, including several other polyomaviruses (PyV) (49, 159). Transport of viruses and ligands following entry *via* caveolae typically occurs to a compartment termed the caveosome, defined as a caveolin-1 positive, pH neutral organelle (125). Transport from the caveosome is not yet well understood but has been proposed to occur primarily to the ER (142). Although trafficking through caveolae to the caveosome and ER was originally believed to be distinct from clathrin entry to the endosomal pathway, new research suggests that these two pathways can converge in unique ways, depending on cell type and ligand (43, 142, 155).

Lipid rafts are detergent-insoluble low density domains that are rich in cholesterol, sphingolipids, and GPI-linked proteins present in the plasma membrane. A great deal of controversy exists as to how lipid rafts are defined and what assays are appropriate for identifying them and their associated ligands. Nevertheless, many researchers have identified regions of the plasma membrane that appear to be distinct patches in which cholesterol, GPI-linked receptors, signaling molecules, and often

caveolin are concentrated (182). These structures can act as signaling centers and areas where ligands concentrate before endocytosis *via* caveolae or other uncoated vesicles (14, 183). Although much research still needs to be done to understand lipid rafts, it is clear that they play important roles in virus entry, as internalization of numerous viruses—whether dependent or independent of caveolin--is sensitive to cholesterol depletion, a traditional method for dissolving lipid rafts and halting lipid raft-mediated entry (37, 67, 84, 150).

In addition to the two classic endocytic pathways discussed above, at least two other modes of entry are employed by viruses. Macropinocytosis is a nonspecific internalization pathway in which lamellipodia engulf extracellular material to form large intracellular organelles, a process that is independent of receptor engagement (180). Because of the inherent low specificity of such a process, macropinocytosis is rarely used for viral entry. However, there are a few examples, such as HIV-1 entry into macrophages (106), uptake of vaccinia virus intracellular mature virions (102), and internalization of some adenovirus types (112). A final, ill-defined mode of entry is simply defined as clathrin- and caveolin-independent endocytosis. This pathway is not well understood and likely represents several distinct pathways with as yet undefined markers. Examples of viruses that can enter by this pathway are echovirus-1, rotavirus, and in some cell types, SV40 (37, 150, 167).

What is becoming increasingly apparent as more research is performed is that defining endocytic pathways is not a simple task, as viruses often use previously undefined routes and/or use different pathways depending on cell type. Some prominent examples are SV40, the classic caveolae-dependent virus, which is able to enter caveolin-

deficient cells without defect (37) and herpes simplex virus, which has been shown to use three distinct entry pathways (115). Such divergent mechanisms demonstrate the complexity of viral entry and reveal interesting ways virology can inform cellular biology.

Intracellular Trafficking of Viruses

Following endocytosis, viruses must rely on cellular machinery to be transported to their site of replication. In the case of HPV, the virion must reach the nucleus to begin early gene transcription and genome replication. There are numerous mechanisms by which an entering ligand is trafficked to its appropriate cellular destination (Figure 1.3). Ligands entering cells through clathrin-dependent endocytosis are typically targeted to the early endosome, at which point recycling to the plasma membrane or trafficking through the endosomal pathway to the lysosome occurs (180, 184). Progression through the endosomal pathway is generally considered to be a degradative pathway, given that compartments become increasingly acidic as they approach the lysosome. Additionally the presence of proteases in endosomal and lysosomal compartments contribute to degradation of endosomal cargo (71). Sorting and trafficking in this pathway is tightly regulated by Rab GTPases. In the classic view, Rab5 GTPase is believed to mediate plasma membrane to early endosome trafficking, Rab7 GTPase controls late endosome to lysosome trafficking, and Rab11 GTPase directs recycling endosomal sorting to the plasma membrane (188). Recent evidence has implicated Rab5 in a novel trafficking pathway that occurs between early endosomes and caveosomes as well (144, 155).

Following caveolar entry into host cells, ligands are generally observed trafficking through an intracellular pH neutral compartment that is caveolin-1 positive, defined as the caveosome. Traditionally, the caveolar and the endosomal pathways were considered distinct from each other, with caveolar ligands trafficking exclusively to the

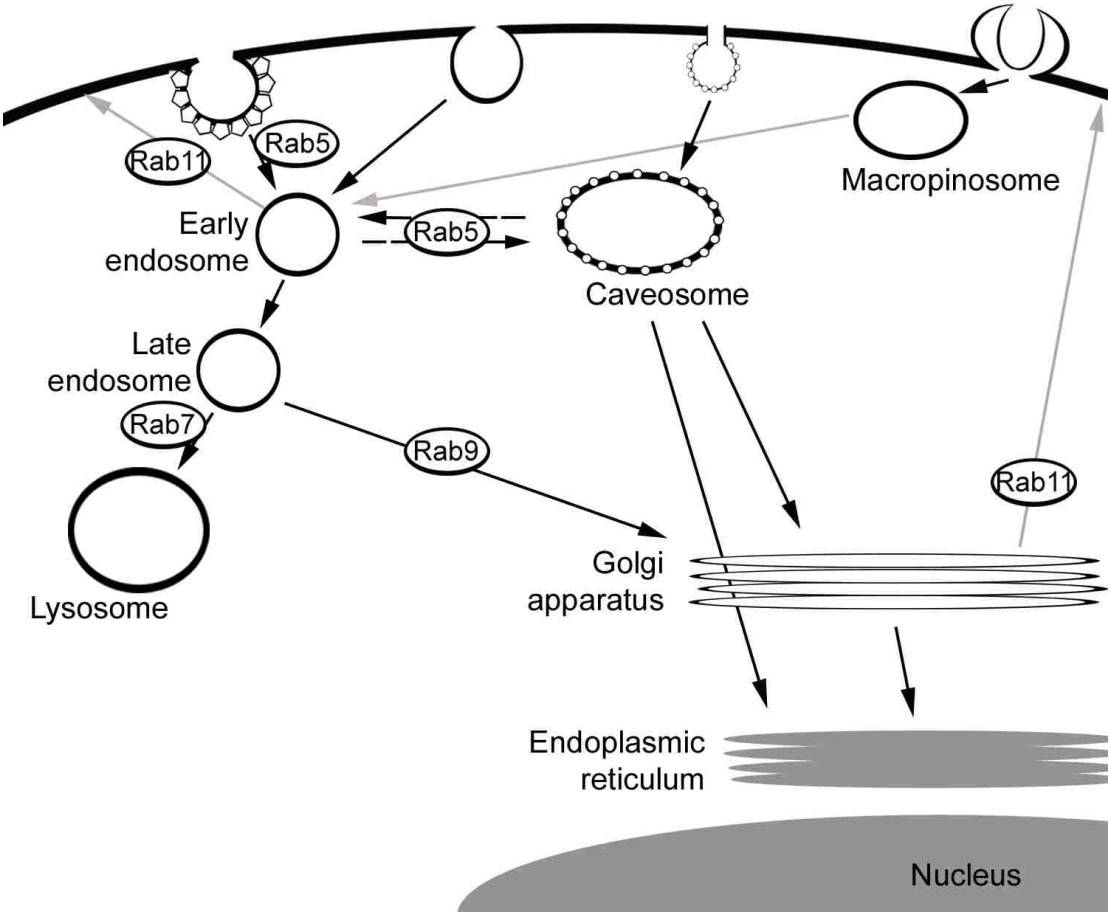

Figure 1.3 Trafficking routes following endocytic entry
Trafficking of ligands and viruses following endocytic entry into cells is complex and regulated by many factors. In the classical understanding of clathrin-dependent entry, ligands traffic to early endosomes and proceed through the endosomal pathway to the lysosome. Ligands entering *via* caveolae-mediated internalization traffic through the caveosome to the Golgi apparatus or ER. Continued research reveals many areas where crosstalk occurs, and these pathways intersect. Rab GTPases play critical roles in regulating the trafficking of endocytosed ligands.

ER or Golgi (180). However, as mentioned above, the existence of unique crosstalk mechanisms has emerged. The case of two classic caveolar ligands, SV40 and cholera toxin B (CtxB), is an interesting example. Due to the final cellular localization of these two ligands, it was originally believed that they both entered cells through caveolae and entered the caveosome, where SV40 was then targeted directly to the ER and CtxB to the Golgi (126-128). However, after careful observation of the trafficking of each of these ligands, it was found that both circulate between the caveosome and early endosome in a Rab5-dependent manner. When encountering the low pH environment of the endosome, CtxB dissociates from its receptor, while SV40 remains tightly bound and is transported back to the caveosome. CtxB then progresses through the endosomal pathway, where it is specifically transported from the intermediate endosome to the Golgi. SV40, having returned to the caveosome, is then transported to the ER (142, 144). A second example of viruses challenging the classic view of endocytic pathways is another polyomavirus, JCV, which enters cells through clathrin-dependent endocytosis, is transported to the caveosome, a process mediated by Rab5, before trafficking to the ER (155).

Once a virus has entered a cell through endocytosis, it technically remains in the extracellular space and still faces the challenge of crossing a membrane barrier to access the cytoplasm. Viruses use a variety of mechanisms for crossing intracellular membranes. Viruses that traffic through the endosomal pathway typically use acidic pH as a cellular cue for promoting endosomal escape (72). Two enveloped viruses, HIV-1 and influenza, for example, use acidic pH as a trigger for glycoprotein conformational change from nonfusogenic to fusogenic forms, promoting fusion of the viral envelope with the endosomal membrane (18, 44). Poliovirus, reovirus, and adenovirus are all

examples of nonenveloped viruses that use acidic pH and/or proteases of the endosomal pathway as triggers to expose viral epitopes that either form pores in or disrupt the endosomal membrane. Additionally, low pH treatment of nonenveloped viruses often promotes partial or complete disassembly of the viral capsid and uncoating of the viral genome (20, 200, 209). A second strategy utilized by viruses to cross intracellular membranes is to access cellular chaperones present in the ER that facilitate uncoating and/or exposure of membrane destabilization epitopes, as is the case for murine PyV and SV40 (27, 105, 170).

Virus Entry and Cellular Signaling

Regulation of cellular signaling by viruses during attachment, endocytosis, and trafficking within the host cell is well documented. When considering signal induction by viruses during host cell invasion, there are essentially three categories of signals observed: (a) signaling required for recruitment of cellular factors involved in endocytosis and trafficking of the endocytosed viral particle; (b) signaling that promotes cytoskeletal rearrangement; and (c) host cell anti-viral response to infection (141).

Upon initial interaction with a host cell, virus-receptor interactions often promote signaling that recruits cellular factors involved in endocytosis. SV40, for example has been demonstrated to promote a tyrosine kinase signal that results in the recruitment of dynamin, a factor important in pinching off of budding vesicles, to cell surface caveolar domains. Inhibition of this signaling results in the trapping of SV40 particles at the mouths of caveolar pits, unable to bud from the surface (23, 145). Entry of JCV, another PyV, also exhibits sensitivity to inhibition of tyrosine kinases that results in trapping of

virions at the plasma membrane. Interestingly JCV uses clathrin-dependent endocytosis for entry and thus may represent an example of signaling to recruit the clathrin machinery to endocytic domains (154). Recruitment of signaling molecules important for activating endocytosis has also been demonstrated, such as in the case of human rhinovirus infection, which requires the localization of Syk kinase to clathrin pits for internalization (96).

Many viruses translocate along the cell surface following initial attachment in order to access internalization receptors or move to areas where endocytic machinery is located. This translocation is dependent on cytoskeletal factors. Additionally the cytoskeleton plays an important role in endocytosis, and following entry, viruses must traverse the cortical actin skeleton and frequently rely on cytoskeletal elements for transport to the cell interior (156, 187). Signaling induced at the plasma membrane by viruses often promotes cytoskeletal rearrangements that facilitate these events, and it is rare that a virus is able to enter and navigate through the cellular cytoplasm without the aid of microtubules, the actin network, or both. Specific roles of the cytoskeletal network in viral infection will be discussed in further detail later in this chapter.

Signaling upon viral attachment is a common innate immune response host cells use to adopt an anti-viral state. Downstream events of such signaling can include cytokine/chemokine release, apoptosis of infected cells, and recruitment of immune effector cells (73). The popularity of adenoviral vectors as gene therapy platforms has resulted in extensive research into how these viruses induce innate immune responses (101). For example, signaling activated through integrin engagement by adenoviruses at the cell surface results in both ERK1/2 and p38 MAPK signals that activate NFκB-

mediated transcription, resulting in transcription of inflammatory genes (15, 198). Numerous other examples of innate immune response activation during virus entry and attachment have been reported, including hepatitis, influenza, and herpesviruses to name a few (88, 121, 157).

Virus Entry and the Cytoskeleton

Utilization of the cellular cytoskeletal network is a common mechanism viruses employ to facilitate entry and movement within cells. When considering initial viral-induced cytoskeleton rearrangements, there are essentially three classes of interactions that occur: (a) movement of virions at the cell surface directed by cytoskeletal interactions with the cytoplasmic regions of viral receptors (termed "surfing"); (b) cytoskeletal factors affecting endocytosis of virions and passage through the cortical actin network; and (c) transport of virions from the periphery to the interior of the cell on cytoskeletal networks. The vast majority of research on viral-cytoskeletal interactions has focused on the actin microfilament and microtubule networks, and these two classes of cytoskeletal arrangements will be concentrated on here as well (152, 156, 185).

Upon initial interaction with the cell, many viruses bind to an attachment receptor, rather than an internalization receptor. Thus, virions must be transported to the receptor that it will utilize for endocytosis. The cytoskeletal network is frequently employed for this transport. In the case of coxsackievirus, attachment to decay accelerating factor (DAF) at the apical surface of an epithelial layer mediates an intracellular signal that induces actin rearrangements. Interaction of the cytoplasmic domain of DAF and the actin network promotes surfing of the virion along the surface to epithelial tight

junctions, where the virion is delivered to its internalization receptor, coxsackie and adenovirus receptor (CAR) (31). Surfing of virions along filopodia regulated by the actin cytoskeleton has been documented for poxviruses and vesicular stomatitis virus (VSV), and this movement appears to act as a means for virions to be delivered to lipid rafts, caveolae, or clathrin pits (97, 113). Diffusion of virions at the cell surface is also regulated by the actin cytoskeleton and is important for directing and restricting murine polyomavirus and SV40 within internalization locales (53, 145).

Normal cellular endocytosis is accompanied by changes in the cortical actin network to allow passage of the internalized vesicle (2). Therefore, disregulation of actin network dynamics usually has profound effects on entry of virions that enter cells by endocytosis, as is often observed in polyomavirus infection. Changes in the actin network induced by virus attachment, such as breakdown of the cortical actin network and actin stress fibers, further supports the assertion that cytoskeletal networks play vital roles in viral entry (48, 145, 178).

The most well characterized function of cytoskeletal elements in viral infection is that of transport from the cell periphery to the nucleus. This transport is typically regulated by the microtubule motor proteins, dynein and dynactin, and takes place on intact microtubule networks. Virions can be transported either in vesicles or after egress into the cytoplasm (152, 156). Herpesviruses, polyomaviruses, and adenoviruses are all examples of viruses that rely heavily on microtubule-mediated transport for delivery to their respective replication sites (122, 168, 196).

Human Papillomavirus Entry and Trafficking in Human Keratinocytes

A crucial aspect of virology that is often overlooked is the utilization of relevant cell types during *in vitro* experimentation. When investigating HPV biology, and especially early entry and infection events, the importance of using the correct cell type is critical, as HPVs display a very strict species and cell type tropism in nature. Difficulties associated with producing HPVs, as well as infection detection, have prevented researchers from always using cell types that are comparable to those infected in nature. Many of these studies have produced conflicting results, causing confusion in the field of HPV biology, especially within the discipline of HPV entry. All of the research presented herein was performed in human keratinocytes, cells analogous to those infected by HPV in nature. For many years, due to the differentiation-dependent life cycle of PVs, producing complete infectious HPV virions in tissue culture relied on reproduction of differentiated epithelial tissue, a technique that is quite difficult and expensive. As a result, the bulk of research on early HPV infection events has been performed with virus-like particles (VLPs) or pseudovirus (PsV). The use of VLPs, which are composed solely of the capsid proteins, L1 or L1/L2, should be carefully considered, as DNA-containing virions display a tighter conformation than empty particles (61, 175). Additionally, one can only monitor adsorption at the cell surface of VLPs, as there is no downstream measure of infection, and many of these binding events represent nonproductive interactions. Research with PsV, comprised of L1 or L1/L2 proteins encapsidating a reported plasmid, is more relevant to natural HPV infection, as these particles exhibit a more analogous structure to infectious HPV virions (61, 175). However, due to the

nature of detecting pseudoinfection by reporter plasmid expression, much of the research performed with PsV has been performed in cell types irrelevant to HPV infection.

HPV Receptors. Low specificity interaction with negatively charged glycosaminoglycans (GAGs), such as heparan sulfate proteoglycans (HSPGs), is a common mechanism viruses use for increasing efficiency of infection. These interactions typically occur for the purpose of concentrating virions at the cell surface so that specific internalization receptors can be easily accessed (189). HSPGs were first identified as receptors for HPV11, HPV16, and HPV33 using PsV and VLP binding assays (69, 86). Using both competition with free heparin molecules and removal of HSPGs from cells by heparinase treatment, HSPGs have been implicated in the attachment of many other HPV types (11, 29, 46, 176, 177). In many of these reports, adsorption of VLPs was used as a measure of HSPG dependence. Unfortunately, VLPs have been demonstrated to interact more promiscuously with HSPGs than DNA-containing particles (175), calling into question this mode of investigating HSPG requirements. Additionally, when the requirement for HSPGs for PsV infection was measured, COS-7 cells (a monkey kidney cell line) were often used, and the relevance of binding and entry into this cell type is uncertain. In contrast, we demonstrated that authentic HPV31 infection of human keratinocytes was independent of HSPGs, whereas infection of COS-7 cells did require HSPG interaction (138). Whether these seemingly contradictory results are a function of viral type or cell type used still remains unclear. However, these data do demonstrate that using different cell types for investigating HPV infection has profound effects on the results obtained.

Regardless of HSPG interaction, it appears that following cell surface attachment, HPV virions interact with a secondary internalization receptor (174). Changes in heparan sulfate requirements, antibody neutralization sensitivity, the unusually long internalization time of PVs, and the role of L2 in internalization, although dispensable for virion binding, all suggest that a conformational change in the viral particle occurs upon attachment that facilitates interaction with a secondary receptor (34, 69, 163, 175). The identity of this receptor still remains unknown, although α6 integrin has been proposed as a candidate receptor for HPV6 (52, 103). However, this molecule has since been demonstrated to be dispensable for entry of multiple papillomavirus types (69, 86, 133, 177, 179). Unfortunately, the same challenges exist in interpreting much of this research as a result of using different cell/viral types, and VLPs/PsV.

In recent years, the importance of HPV binding to the extracellular matrix (ECM) deposited by keratinocytes has become apparent. During tissue culture infection, a small percentage of virions are observed binding directly to the plasma membrane during immediate attachment; however the vast majority of HPV particles are detected bound at the ECM of cultured cells (32, 33). This phenomenon presumably has important implications in the context of natural HPV infection. As discussed above, the current model of natural HPV infection indicates that virions must access the mitotically active basal layer through a wound in the differentiated epithelium. One cellular response to wounding is the robust upregulation of secreted ECM factors, including laminin 5 (LN5), a molecule implicated in HPV-ECM binding (33, 124). Additionally, nonspecific binding to ECM factors present at the basement membrane could act as a means for concentrating virions close to target cells in the basal layer. Consistent with the

relevance of this binding to natural infection, it has been demonstrated that HPV binding to ECM deposited by keratinocytes is much stronger than to ECM deposited by less relevant non-keratinocyte cell types (32). Although it has been suggested that binding to LN5 of the ECM could direct virus interaction with a plasma membrane receptor (33), this claim has not been fully substantiated, and more research needs to be performed to understand how virions are able to access the plasma membrane from the ECM, and why such a seemingly inefficient process precedes HPV infection. This issue will be addressed in Chapter IV.

Following attachment to cells, PV particles enter cells by endocytosis. As discussed above, there are essentially four ways a virus can enter cells (Figure 1.2): clathrin-dependent endocytosis, caveolae-dependent endocytosis, clathrin- and caveolae-independent endocytosis, and macropinocytosis. The role of clathrin and caveolae in entry has been studied for several PV types, including HPV16, 58, 31, 33 and bovine papillomavirus type 1 (BPV1), in various cell lines. One of the first reports investigating PV entry examined the colocalization of BPV1 virions and HPV16 VLPs during entry into C127 cells (mouse fibroblasts)(38). Having established that HPV16 VLPs and BPV1 virions colocalize throughout entry into these cells, the researchers used immunofluorescence and biochemical inhibitors coupled with infectious entry assays to establish that BPV1 uses clathrin-dependent entry, traffics through the endosomal pathway, and localizes to the lysosome at late time points. Although the data presented in this report are quite solid, some difficulties arise when interpreting these results in the context of natural HPV infections. The authors certainly established a role for clathrin-dependent endocytosis in BPV1 entry into mouse fibroblasts. Whether this mode of entry

is applicable to entry of HPV types into human epithelial cells has not been determined. The significance of both viral type and cell type when performing these types of experiments has already been illustrated and should be kept in mind when considering these data. Another report demonstrated that HPV16 and HPV58 PsVs use clathrin-dependent endocytosis, while HPV31 PsVs use caveolae-dependent entry, to infect COS-7 (monkey kidney) cells (12). The researchers used electron microscopy, biochemical inhibitors, and dominant negative constructs to make these conclusions. Analysis of HPV33 PsV entry into COS-7 cells also revealed a role for clathrin-dependent endocytosis (176). Again, the relevance of these results to natural HPV infection of HKs is difficult to determine. Clathrin-dependent endocytosis has also been implicated in HPV31 entry into COS-7 and 293TT (human embryonic kidney) cells (79). Unfortunately, due to the variety of cell types and viral types used in these experiments, a clearly defined pathway for HPV infection still remains elusive. Research presented in the following chapters will demonstrate that caveolae play an important role in entry of authentic HPV31 virions into the natural host cell type, human keratinocytes (186).

Trafficking of PVs Following Entry. The fate of PV virions following entry into cells has been investigated using biochemical inhibitors and colocalization with specific organelle markers. These studies demonstrated that HPV16, HPV33, and BPV1 traffic through the endosomal pathway and require endosomal acidification for infection (38, 176). These reports are the same as those cited above, using cell types whose relevance to HPV infection is lacking. A recent conflicting report establishes that BPV1 enters 293TT cells *via* clathrin pits as previously established (38); however following clathrin entry, a role for caveolin-1 was found, suggesting that BPV1 traffics through the

caveosome during infection (95). To date, no research has been performed to understand the role that Rab GTPases play in directing HPV trafficking following entry. A thorough examination of trafficking events following HPV31 entry into human keratinocytes will be presented herein.

Signaling During HPV Entry. Although a wealth of research exists defining the signaling events associated with HPV early proteins, such as E5, E6, and E7 (111), the understanding of early signaling pathways induced by and required for HPV entry is surprisingly lacking. The studies that have been conducted fall into two categories: activation of signaling pathways in immune surveillance cells, such as Langerhans cells (LCs) and dendritic cells (DCs) (54, 55); and activation through engagement of α6β4 integrin, a putative receptor for HPV6 VLPs (63, 139). However, to date, no research has been conducted to understand the signaling pathways activated by authentic HPV virions upon attachment and entry into their natural host cell type.

Activation of phosphatidyl-inositol-3 kinase (PI3K) and mitogen activated protein kinase (MAPK) signaling during interaction between HPV VLPs and immune cells is important in the context of understanding immune evasion by the virus and induction of immunity by HPV VLP vaccines. In these studies (54, 55), the researchers established that HPV VLPs are able to activate MAPK and PI3K signaling and NFκB transcription in DCs, resulting in upregulation of cytokine release and antibody-mediated immunity. Interestingly, in LCs the response was quite different, with induction of PI3K signaling resulting in suppression of other signaling pathways and an overall unactivated state of the cell, a mechanism presumably related to evasion of the immune response. These studies, although unrelated to entry-associated signaling responses in host keratinocytes,

do illustrate that specific signaling responses to HPV exposure can be initiated, and that cell type does play a major role in the nature of that response.

Research on the signaling pathways induced by HPV VLPs in A431 (human genital epithelial) cells has introduced the idea that early signaling events are likely to be induced during HPV VLP attachment and entry into cells that are naturally infected (63, 139). The researchers demonstrated that HPV VLPs are capable of inducing Ras-MAPK and PI3K signaling, the former pathway resulting in increased cell proliferation. Although intriguing, the fact that these signaling events are induced through engagement of the $\alpha 6\beta 4$ integrin receptor raises some questions, as the relevance of this receptor to HPV infection is somewhat controversial (52, 69, 86, 177, 179). Additionally, the use of VLPs, which have a different conformation than DNA-encapsidating virions, may result in confusing results. Nevertheless, this work is an interesting starting point for understanding how this virus can manipulate a host cell to internalize virions, induce production of viral products, and aid in immune system evasion. Experimental data presented in this work will expand on these reports, characterizing the initial signaling events induced by complete HPV31 virions during attachment and entry into human keratinocytes.

HPV Infection and the Cytoskeleton. Utilization of the cellular cytoskeleton, primarily the actin and microtubule networks, is a common mechanism employed during viral infection for internalization and transport from the cell periphery to the site of viral replication (152, 156). Some experimentation into the requirements of cytoskeletal factors for HPV infection has been performed using biochemical inhibitors. These reports implicate both the microtubule and actin networks in BPV1, HPV16, and HPV33

entry, although the exact effects of these inhibitors on PV infection have yet to be elucidated (38, 176, 214). Additionally, several reports demonstrate a role for the minor capsid protein, L2, in transport and localization of PV particles following entry through interaction with β-actin and the microtubule motor protein, dynein (62, 211). Results presented herein will elucidate several interactions with and effects on the cytoskeleton induced by HPV31 during entry in human keratinocytes.

HPV31 Entry and Trafficking in Human Keratinocytes. The goals of the research presented in this dissertation are to (a) define the endocytic entry pathway utilized by complete HPV31 virions into its natural host cell type, human keratinocytes; (b) elucidate the trafficking of virions following entry; (c) determine the requirements for and downstream effects of cellular signaling events induced during HPV31 entry; and (d) ascertain the role of the cellular cytoskeleton in HPV31 entry and trafficking. Due to the abundance of research on HPV entry that has been performed using PsV, VLPs, and inappropriate cell types, the field of HPV entry has been obscured, and the exact requirements and mechanisms of endocytic entry still remain unclear. Therefore, in this research, I have attempted to clearly define the mechanism and requirements of complete HPV31 entry into the natural host cell type, human keratinocytes.

Chapter II

Human papillomavirus type 31 uses a caveolin 1- and dynamin 2-mediated entry pathway for infection of human keratinocytes

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Abstract

Papillomaviruses are species specific and epitheliotropic DNA viruses that cause tumors in their natural hosts. Certain infections with genital human papillomavirus (HPV) types are causally related to cervical cancer development. Most papillomaviruses are thought to infect cells *via* a clathrin-dependent pathway; yet no studies have determined the entry route in permissive host epithelial cells. Employing fluorescently labeled and native virions, we tested the effects of dominant negative and biochemical inhibitors of cellular endocytosis pathways. Infections of human keratinocytes, a natural host cell type for HPVs, were assessed visually and by infectious entry assays. We found that HPV type 31 (HPV31) entry and initiation of early infection events requires both caveolin 1 and dynamin 2 and occurs independently of clathrin-mediated endocytosis. Treatment with chlorpromazine and filipin had opposing effects on HPV31 and HPV16 infection. HPV31 entry was remarkably slow, with a half time of ≈14h, whereas the entry half-time of HPV16 was 4h. Consistent with a caveolae-mediated entry pathway for HPV31, the virions associated with detergent-resistant lipid rafts. During a 16-hour microscopic tracking of HPV31 and HPV16 virions, no colocalization of the two viral types was observed. These data suggest that HPV31 and HPV16 virions use distinct routes for host epithelial cell entry.

Introduction

Human papillomaviruses (HPVs) are small nonenveloped viruses that encapsidate a double-stranded circular DNA genome of ≈ 8kb. HPVs display strict species and cell type specificity, infecting human keratinocytes (HK) exclusively in nature. The association between high-risk HPVs (i.e., HPV16, 18, 31, 45) and cervical cancer is well-established, as >99% of cervical cancers are positive for HPV DNA (8). Additionally, these HPV types have been linked to ≥20% of head and neck cancers (36, 68).

A number of qualities that permit HPV persistence *in vivo* have impeded research leaving many basic aspects of HPV biology poorly understood. HPVs have an absolute requirement for differentiating epithelia for life cycle completion, and thus cannot be grown or studied to great extent in traditional cell culture. Low-level replication and protein expression during early infection hinders detection of HPV infection. This has prompted many researchers to study the interaction of HPV pseudovirions (PsV) or virus-like particles (VLP) with non-relevant cell types. However, this has resulted in confusion as to the natural infectious pathway used by high-risk HPVs. For example, Giroglou *et al*. found that interaction of HPV types 16 and 33 PsV with heparan sulfate proteoglycans (HSPG) was required for infection as measured by reporter gene expression in COS-7 cells, a monkey kidney cell line (69). To determine whether HSPG mediates HPV infection in host HKs, we used authentic HPV31 virions produced in the organotypic system. Infection was measured by quantification of a spliced predominant early viral RNA, E1^E4 (131, 132). Using the same experimental design as Giroglou *et al.,* we confirmed that HPV31 infection of COS-7 cells required HSPG. However, we also demonstrated that HPV31 virion interactions with cell surface HSPG was not required for

infection of HK (138). These findings underscore the importance of using relevant cell types and authentic virions to study viral early infection events.

Viruses generally enter cells by way of receptor-mediated endocytosis (184). The well-characterized clathrin-dependent pathway for ligand endocytosis has long been recognized as the major route used by many nonenveloped viruses to enter cells. Entry *via* this pathway proceeds through endosomes, and the decreasing pH of these vesicular compartments (66) often acts to stimulate endosomal escape (184). A clathrin-independent entry pathway involves caveolae and provides an advantage of access to signaling centers at the cell surface and direct delivery to caveosomes, which can mediate transport to several cellular organelles where uncoating may occur. There are examples of highly related viruses using different infection routes. For example, the polyomaviruses SV40 and BKV use a caveolae-mediated pathway for cellular entry (1, 149) whereas their closely related counterpart JCV initially employs a clathrin-dependent route (49).

The endocytic requirements for HPV entry have not been studied in HKs. HPV16 VLP were found to co-localize with BPV1 VLP and virions, and these particles enter mouse C127 cells *via* clathrin-dependent endocytosis with a relatively long internalization time (half-time of ≈4 h)(38). In COS-7 cells HPV16 and HPV58 PsV entry was clathrin-dependent, while HPV31 entry was caveolae-dependent (12). However, a recent publication showed clathrin-dependent and caveolae-independent entry of both HPV16 and HPV31 into COS-7 cells as well as 293TT cells, an SV40 large T antigen transformed human kidney cell line (79). Although some of these studies were performed in human cells, none of the cell types is relevant to natural HPV infection.

In the current study our goal was to determine the internalization pathway used by infectious HPV31 virions in their host cell type, HKs. We first examined the time course of HPV31 entry into several cell lines relevant to natural HPV infection and found it to be extremely slow, even compared to HPV16 internalization. Monitoring the effects of dominant negative inhibitors of clathrin- and caveolae-dependent endocytosis on HPV31 entry and infection, we found both caveolin-1 and dynamin-2 to be important mediators. Inhibition of clathrin-mediated endocytosis did not affect HPV31 entry or initiation of viral early gene transcription. Infections with HPV31 and HPV16 in the presence of the clathrin-mediated endocytosis inhibitor, chlorpromazine, or caveolae-mediated endocytic inhibitor, filipin, confirmed that HPV31 entry was caveolae-dependent, whereas HPV16 entered HKs through clathrin-dependent endocytosis. Consistent with caveolar-mediated entry, HPV31 virions associated with detergent resistant microdomains (DRM). Conversely, HPV16 displayed low DRM association. We conclude that HPV31 entry into host HKs is lipid-raft mediated and occurs by way of caveolae. These entry requirements differ from those reported for HPV16 (38) and our observations with HPV16 virions in HKs. These data suggest that HPV31 and HPV16 may use different pathways to enter cells. Furthermore, when observing fluorescently labeled HPV31 and HPV16 virions upon binding and entry into HK, at no time during entry did the two HPV types colocalize. These data support the hypothesis that HPV31 and HPV16 use distinct entry pathways to infect HKs.

Methods

Cell Culture and Transfections. HaCaT cells are a spontaneously immortalized epithelial line derived from normal adult skin (10). HEK-293T cells are derived from a human embryonic kidney cell line immortalized with SV40 large T antigen. Cells were maintained in DMEM/F12-Ham's Nutrient Mixture containing 10% fetal calf serum, 4X amino acids, 2mM L-glutamine, 100U/ml penicillin, and 1ug/ml streptocymin (Sigma). End1E6/E7 and Ect1E6/E7 cells were derived from normal endocervical and ectocervical tissues and immortalized with HPV16 E6 and E7 proteins (59). Low passage human foreskin keratinocytes were pooled from three donors and cultured as described (138). Plasmids encoding wild-type and dominant negative proteins tagged with GFP have been reported: caveolin-1 (144), Eps15 mutants (6), and dynamin-2 (129). HaCaT cells were transfected using the Nucleofector kit (Amaxa). Briefly, cells were tryspinized, pelleted, and resuspended in 100ul transfection solution V with 4ug of DNA per reaction. Following electroporation, cells were seeded onto coverslips in 6-well plates or in 12-well plates. Entry studies and infections were assessed at 48h post-transfection.

Virion Production and Purification. HPV31 virions were obtained from organotypic cultures of CIN-612 9E cells as previously described (131, 132). The transfection-based method for papillomavirus production was modified from that previously published (153). 293T cells were transfected by the calcium phosphate method with recircularized HPV31 genome and codon optimized HPV31-L1/L2 expressing plasmid. At 48h post-transfection, cells were tryspinized, pelleted, and resuspended at $1x10^8$ cells/ml in Dulbecco's PBS/9.5mM $MgCl_2$. Cells were lysed and subjected to 3X freeze-thaw

cycles; DNase treatment and virion maturation were as described (153). Supernatants were layered atop a 1.25g/ml-1.4g/ml step CsCl gradient. Following centrifugation at 20,000xg for 16-18h, the viral band was extracted by side puncture. Virions were dialyzed 2X at 4°C against HSB (25mM HEPES pH7.5, 500mM NaCl, 0.02% Brij58, 1mM $MgCl_2$ 100uM EDTA, 0.5% ethanol). Virions were concentrated using an Amicon Ultra-4 centrifugal filter 100K (Millipore) as needed. Virion stocks were quantified by dot blot based upon viral genome equivalents (vge) as previously described (131, 132). Virion purity was assessed by SDS-PAGE and Coomassie staining and by transmission electron microscopy (TEM). Virus stocks were visualized by TEM (Hitachi 7500) at 80 kV following binding to a carbon-coated electron microscopic grid and negative staining with 2% uranyl acetate.

Infections. Cells were exposed to virion stocks as previously described (131, 132). Briefly, stocks were sonicated at 0°C and diluted in media. Viral inocula were added to HaCaT cells and incubated at 4°C for 1h with rocking to permit viral attachment. Inocula were aspirated, cells were washed with media and fresh media were added. Cells were incubated at 37°C for various times before harvest. In virus neutralization experiments H31.A6 monoclonal IgG1 antibody to HPV31 VLP (26) diluted 1:1000 in media and added to cells at times indicated post-attachment. Infections in the presence of endocytic inhibitors were performed after pretreatment with each inhibitor for 1h at 37°C. Inhibitors were present in media during viral attachment and throughout 48h incubation at 37°C following attachment. Chlorpromazine (Calbiochem) concentrations used: 10uM, 20uM. Filipin III (Sigma) concentrations used: 0.8uM, 1.6uM.

RNA Isolation and RT-qPCR Analysis. Total RNAs were extracted from cells using TRIzol (Invitrogen) and nucleic acid concentrations were determined by spectrophotometer. Reverse transcription of total RNAs (2-3ug) and triplicate quantitative PCR (qPCR) reactions were performed using GeneAmp RNA PCR reagents and AmpliTaq Gold DNA polymerase (Applied Biosystems) using qPCR primers, probes, and conditions as previously described (131, 132, 138). Acceptable slope values were between -3.2 and -3.5 and correlation values were between -0.9800 and -0.9999. Results are shown as the average of 3 values falling between 0-4 standard deviations of the Ct value. Error bars represent standard error of the mean (SEM).

Virion Labeling with Fluorescent Dyes. Approximately 1ug of Alexa Fluor (AF) 594 or AF488 carboxylic acid, succinimidyl ester (Molecular Probes) was mixed with 10^8 virions (vge) and incubated for 1h at RT. Labeled stocks were washed 3X with 1X HSB and recovered using a Millipore Amicon Ultra-4 centrifugation filter to remove unincorporated dye.

Fluorescent Virion Entry Studies. Cells were seeded at 5000 cells/slip onto coverslips in 6-well plates one day prior to binding. AF594-HPV31 virions were diluted in media, added to pre-chilled cells, and allowed to attach for 1h on ice at 5000-10,000 vge/cell. Following binding, virus was removed, and cells were washed with media before addition of fresh media. For dominant negative inhibitor studies, virus was bound to cells at 48h post-transfection, and cells were incubated at 37°C, 24h. Fixation was with 2% paraformaldehyde/PBS for 15 min at RT. Coverslips were slide mounted using Vectashield+DAPI (Vector Labs). Microscopy photos were taken on a Zeiss META

confocal microscope with a 63X objective.

DRM Association Assay and Flotation Gradient Centrifugation. AF594-labeled TF (Molecular Probes), AF594-CT-B (Molecular Probes), AF594-HPV31, or AF594-HPV16 were bound to cells seeded onto coverslips for 1h at 4°C, before incubation at 37°C for indicated times in the presence or absence of 10mM methyl-ß cyclodextrin (MßCD; Sigma) as previously described (37, 155). Cells were then incubated with PBS or 1%TX-100 on ice for 10 min before paraformaldehyde fixation. Coverslips were slide mounted with Vectashield+DAPI (Vector Labs) and viewed on a Zeiss META confocal microscope. Alternatively, unlabeled virions were cell bound in the presence or absence of 10mM MßCD (Sigma) as above, flotation gradient centrifugation was performed as previously described (155). Cells were harvested in lysis buffer (20mM Tris-Cl pH 7.4, 150mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1mM EDTA, 1mM PMSF, 1X protease inhibitor, 1mM sodium orthovanadate), sonicated, dounce homogenized, and cellular debris was pelleted. Supernatants were layered under a discontinuous 40/30/5% Optiprep gradient and centrifuged at 40,000 rpm in a SW41 rotor. Fractions were collected from the top and analyzed by 10% SDS-PAGE and western blot for HPV31 L1 with polyclonal α-HPV31 L1 antisera.

Results

HPV31 Production and Infectivity. For years HPV virion production was only possible using systems that yield differentiated epithelial tissue, such as the organotypic culture system (114) or the mouse xenograft model (92). These systems are limited by technical difficulty and expense, and typically result in low virion yields. Recent advances permit

complete HPV virion production using a transfection-based, differentiation independent method (153). Papillomavirus virions produced in this system are indistinguishable from tissue-derived virions in infectivity kinetics and clinical outcome in an *in vivo* animal model (35). We used this method to produce infectious HPV31 virions that display normal morphology and infectivity properties (Figure 2.1). Virions were quantified by dot blot for viral genome equivalents (vge) and by Coomassie blue staining following SDS-PAGE with protein standards to estimate capsid equivalents. We reproducibly produced 1000-5000 capsids per vge. Similar to what we find with tissue-derived HPV31 (131, 132, 138), we detected a dose-

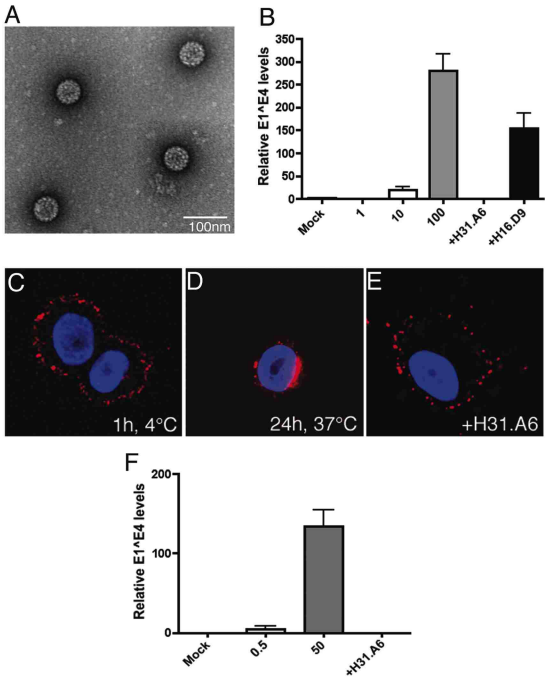

Figure 2.1 HPV31 virions produced by a transfection-based method display normal morphology and infectivity properties
(A) Transfection-derived HPV31 virions visualized by transmission electron microscopy. (B) HaCaT cells were exposed to increasing doses of HPV31 (1, 10, 100 vge/cell) as described in Methods. Monoclonal antibodies H31.A6 and H16.D9 were diluted in media and added to cells immediately following viral attachment. Total RNAs were subjected to RT-qPCR in triplicate to quantify spliced HPV31-E1^E4 transcripts indicative of infection (48h pi). Error bars represent SEM. (C) AF594-labeled HPV31 (red) was bound to HK at 10,000 vge/cell and visualized after 1h at 4°C, after incubation at 37°C for 24h (D), or after incubation at 37°C for 24h in the presence of H31.A6 neutralizing antibody (E). Coverslips were mounted in the presence of DAPI to visualize nuclei. (F) HaCaT cells were inoculated with AF594-labeled HPV31 at indicated doses; neutralizing antibody H31.A6 was used as in panels B & E. Infection was quantified as in panel B.

dependent increase in HPV31-E1^E4 transcript levels by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) following human keratinocyte infection with increasing HPV31 doses. Additionally, post-attachment treatment with H31.A6, an HPV31 neutralizing antibody completely blocked infection, while an HPV16-specific antibody did not significantly inhibit infection (Figure 2.1B). These data further confirm that virions produced by this transfection-based method display infectivity properties indistinguishable from those produced in tissue-based systems.

Alexa Fluor (AF) Labeled Virions as a Tool to Study Cellular Entry. Experiments were performed to determine the utility of AF594 fluorescent virion labeling and whether the label might affect early infection events (Figure 2.1C-F). We first tested entry of AF594-HPV31 and the effect of adding a neutralizing antibody post-attachment (Figure 2.1C-E). AF594-HPV31 binding at the surface of HK was readily visualized when cells were exposed to ≈10,000 vge per cell after 1h at 4°C (Figure 2.1C). As previously reported (32, 39) we observed a clear majority of fluorescent virions bound to the extracellular matrix secreted by HKs and remained bound throughout incubation. The focal point at which images were taken was in the central plane of the cell, and thus the extracellular matrix binding is not visible. Therefore despite the high dose of virions added to the HKs, the number of particles binding at the plasma membrane and entering cells appears to be a small fraction thereof. After incubation at 37°C for 24h, the virus was observed distributed perinuclearly within cells (Figure 2.1D). Post-attachment treatment with the neutralizing antibody H31.A6 completely inhibited AF594-HPV31 entry (Figure 2.1E), as AF594-HPV31 particles remained at the cell surface after 24h at 37°C. AF594-HPV31 retained full infectivity as measured by early viral gene expression

and infection was completely neutralized by antibody H31.A6 as expected (Figure 2.1F). These data indicate that AF-labeling of virions does not alter their biological properties.

HPV31 Entry into HK is Slow. The internalization half time ($t_{1/2}$) of BPV1 and HPV16 entry into mouse C127 cells was previously reported to be ≈4h, which is slow compared to other viruses (38). Post-attachment antibody-mediated neutralization experiments and confocal microscopy visualization of AF594-labeled virions were performed to determine the kinetics of HPV31 and HPV16 entry into HKs. Sensitivity to antibody neutralization at various times post-attachment was determined in three different cell types relevant to natural HPV infection (Figure 2.2A): a spontaneously immortalized epithelial line (HaCaT) (10), an ectocervical cell line (Ect1), and an endocervical cell line (End1) (59). Infections were performed by exposing cells to HPV31 for 1h at 4°C to promote attachment but not internalization. Cells were then washed, refed with regular growth media, and placed at 37°C to allow viral entry. Media were changed on replicate cultures at various times post infection (pi) where cells received no antibody, an HPV31 neutralizing monoclonal antibody (H31.A6), or an isotype control antibody. At 48h pi cells were harvested for total RNAs and RT-qPCR was used to detect and quantify HPV31 E1^E4 transcripts (Figure 2.2A). No significant HPV31 infection inhibition was observed at any of the assayed time points with media only (solid lines) or with the isotype control antibody (not shown). However, treatment of cells with antibody H31.A6 (dashed lines) in all three cell types considerably inhibited HPV31 infection from 0h up to 8h after internalization was initiated by shifting to 37°C. Post-attachment

neutralization by H31.A6 at 12h after temperature shift resulted in ≥50% inhibition of HPV31 infection, but the neutralizing antibody had no effect on infection by 16h after internalization began. Identical results were obtained with differentiation- and transfection-derived HPV31 virions. By interpolation the $t_{1/2}$ of HPV31 in HKs is ≈14h, where half the virions remain susceptible to neutralization following attachment, presumably as a result of retention at the cell surface signifying slow internalization.

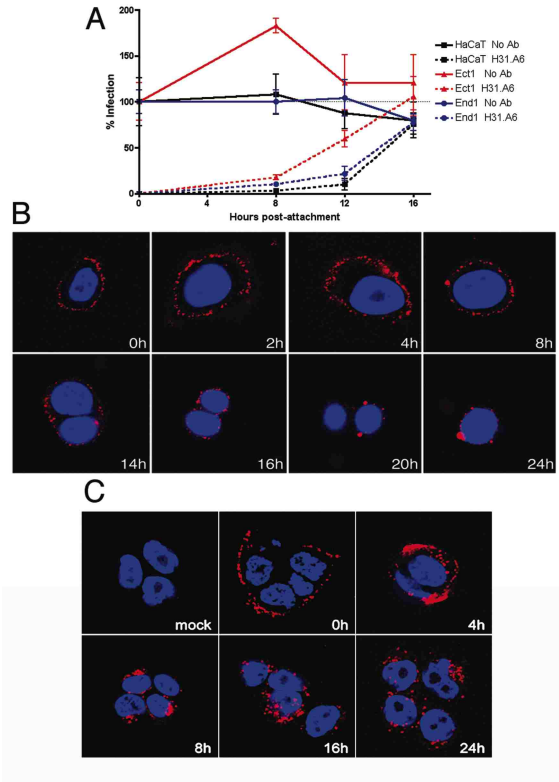

Figure 2.2 Time course of HPV31 internalization
(A) Organotypic culture-derived or transfection-derived HPV31 virions were bound to HaCaT (black boxes), Ect1 (red triangles), or End1 (blue circles) cells for 1h at 4°C at a dose of 50 vge/cell, cells were washed and refed with regular growth media. At the indicated times post-attachment, cells were treated with H31.A6 (dashed lines) or refed with new media (solid lines). The cells were harvested at 48h post-infection, and RNA was analyzed in triplicate by RT-qPCR for HPV31 E1^E4 levels; values were normalized to the average of the untreated controls at 0h. Error bars represent SEM. (B) AF594-labeled HPV31 was bound to HaCaT cells on glass coverslips at 10,000 vge/cell for 1h at 4°C. Cells were washed, refed with regular growth media, and shifted to 37°C. At indicated times post-attachment, cells were fixed and stained with DAPI to visualize nuclei. (C) AF594-labeled HPV16 was bound to HaCaT cells on glass coverslips at 10,000 vge/cell for 1h at 4°C. Cells were washed, refed with regular growth media, and shifted to 37°C. At indicated times post-attachment, cells were fixed and stained with DAPI to visualize nuclei.

It has been suggested that the long period of time following attachment during which some papillomaviruses are susceptible to antibody-mediated neutralization may result from neutralizing antibodies chasing the virus into vesicles and neutralizing infection post-entry (34). Although some evidence supports this method of neutralization by IgA, it does not appear to be a neutralization strategy used by IgG antibodies (158). To directly observe the entry time course of viral particles, we tracked AF594-labeled virion uptake into HK (Figure 2.2B, C). Following virion attachment the cells were washed and incubated at 37°C. At the indicated time points, cells were fixed and visualized by confocal microscopy. HPV31 was observed binding to the cell surface at 0h post-attachment, and remained surface-bound up to 8h following temperature shift to induce internalization. At 14h post temperature shift, virions had begun to enter cells, and by 24h post-shift, all virus particles appeared to have entered the cells where they localized perinuclearly. These data are in agreement with the results in Figure 2.2A that suggest a long $t_{1/2}$ of ≈14h for HPV31 in HKs. A more rapid internalization time for HPV16 into C127 cells has been reported (38). Our results using AF594-labeled HPV16 confirm a $t_{1/2}$ of ≈4h HPV16 in HKs, where by 8h post-internalization HPV16 particles are perinuclear (Figure 2.2C). The considerable difference in internalization time displayed by the two viral types in HKs suggests that HPV31 and HPV16 may be using different cellular entry pathways in these cells.

HPV31 Entry and Early Infection Events are Caveolin 1- and Dynamin 2-Dependent. To determine the endocytic route utilized by HPV31 to enter HKs, well-characterized dominant negative inhibitors of various endocytic pathways were used. HaCaT cells were transfected with various wild type and dominant negative expressing

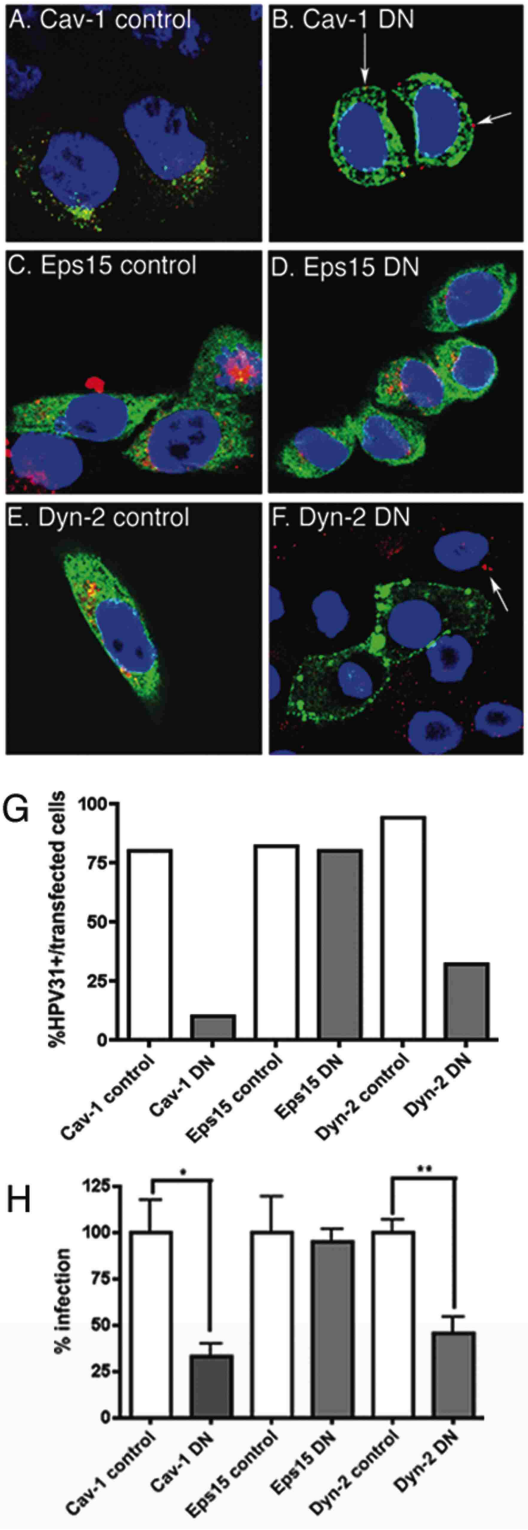

Figure 2.3 Effects of Dominant Negative Endocytosis Inhibitors on HPV31 Entry and Early Transcription.
(A-F) HaCaT cells transfected with GFP-tagged dominant negative inhibitors of endocytosis and their corresponding controls. At 48h post-transfection, AF594-HPV31 virions were bound to cells for 1h at 4°C, cells were washed, refed with regular growth media, and placed at 37°C. Cells were fixed and stained with DAPI 24h after 37°C shift to promote virus internalization. (A-B) wild type caveolin-1-GFP (Cav-1 control) and dominant negative caveolin-1 (Cav-1 DN); (C-D) wild type Esp15 (Eps15 control) and Eps15 dominant negative (Eps15 DN); (E-F) wild type dynamin-2 (Dyn-2 control) and dominant negative (Dyn-2 DN). Results are representative of 3 replicate transfection/infection experiments.
(G) Quantification of HPV31 particle entry into transfected cells was performed by blinded evaluation of internalization state of AF594-HPV31 in a total of 100 transfected cells (expressing GFP). (H) Triplicate cultures of transfected HaCaT cells were infected 48h post-transfection with HPV31 virions at 50 vge/cell. RNA isolated 48h pi was quantified in triplicate for HPV31 E1^E4 transcript levels by RT-qPCR. Error bars represent SEM and statistical significance was achieved with $p<0.05$ (*) and $p<0.01$(**).

constructs, and 48h later AF594-HPV31 virions were bound and allowed to enter cells for 24h at 37°C. Caveolin 1 tagged at the N-terminus with GFP (Cav-1 DN) was previously

shown to inhibit caveolar-mediated endocytosis as demonstrated by blockage of SV40 entry, whereas a chimeric protein with GFP placed at the C-terminus of caveolin 1 (Cav-1 control) does not display this inhibitory effect (144). In HaCaT cells inhibition of caveolae-mediated endocytosis by expression of Cav-1 DN reduced HPV31 entry, and virions were visible still bound at the cell surface following 24h at 37°C (Figure 2.3B, arrows). Conversely, Cav-1 control transfected cells showed no block in HPV31 entry, and AF594-HPV31 was observed at a perinuclear location (Figure 2.3A). When cells were transfected with a dominant negative inhibitor of Eps15 (Eps15 DN), a necessary adaptor for clathrin coat assembly, or its corresponding control (Eps15 control) which lacks the AP-2 binding site (6), no HPV31 entry inhibition was observed (Figure 2.3C-D). Dynamin 2, a motor protein that pinches off vesicles from the cell surface, has been observed to function in both clathrin- and caveolae-mediated pathways (78, 130, 197). A well-characterized mutant of dynamin 2 (K44A) has been previously shown to inhibit dynamin 2 activity (129). Expression of GFP-dynamin-2K44A (Dyn-2 DN) appeared to have an inhibitory effect on HPV31 entry, as many transfected cells were not positive for HPV31 entry, while HPV31 entry was not inhibited in adjacent untransfected cells (Figure 2.3F, arrow). Expression of wild type dynamin 2 (Dyn-2 control) caused no blockage of HPV31 entry (Figure 2.3E). HPV31 entry into transfected cells was quantified in a blinded assessment by counting the number of transfected cells positive for HPV31 entry after 24h at 37°C (Figure 2.3G). Similar results were obtained when primary human foreskin keratinocytes were transfected with each of the dominant negative mutants or their controls (data not shown). The microscopy results indicate that clathrin-mediated endocytosis plays a negligible role in HPV31 entry, while >90% of

HPV31 entry is caveolin-1 dependent. Additionally, dynamin 2 appears to mediate ~70% of HPV31 entry into HKs.

To determine whether the effects of the dominant negative proteins on HPV31 entry observed by microscopy were a reflection of the actual infectious pathway, an infectious entry assay was performed to examine early viral transcription in transfected HaCaT cells. HK transfected with each construct in triplicate were exposed to HPV31 virions at 25 vge/cell on day 2 post-transfection. RNA was harvested at 48h pi and HPV31 E1^E4 transcripts were quantified by RT-qPCR. Although we were unable to obtain transfection efficiencies >75%, the data from these infections confirmed the requirements for caveolin-1 and dynamin-2 that were observed in the microscopic analysis (Figure 2.3H). Transfection with Cav-1 DN caused a 67% inhibition of HPV31 infection as compared to infection of Cav-1 control transfected cells, and inhibition was statistically significant. We believe that remaining infection observed in these samples is due to incomplete transfection, although some infection may result from entry through a minor pathway that is caveolae independent. Inhibition of clathrin-mediated endocytosis by transfection with Eps15 DN had no effect on HPV31 infection when compared with Eps15 control transfected cells, confirming that HPV31 infection is independent of clathrin-mediated endocytosis. HPV31 infection was inhibited 55% following transfection with Dyn-2 DN as compared with Dyn-2 control transfected cells; these results were highly significant.

Biochemical Inhibitors Confirm Clathrin Dependent HPV16 Entry and Caveolae Dependent HPV31 Entry. To verify the dependence on caveolae for HPV31 infection in HKs, as well as to confirm previous reports of clathrin-dependent HPV16 entry, infections with both viral types were performed in the presence of the well-characterized endocytic inhibitors, chlorpromazine and filipin. Chlorpromazine, an inhibitor of clathrin-mediated endocytosis (206), blocked HPV16 infection in a dose-dependent manner resulting in 72% inhibition at the highest concentration used (Figure 2.4A; triangles), confirming the clathrin dependence of HPV16 infection in HKs. Treatment with chlorpromazine had a negligible effect on HPV31 infection (Figure 2.4A; boxes). In contrast, treatment with filipin, a cholesterol binding drug that blocks caveolae-dependent endocytosis, inhibited HPV31 infection by 69% at the highest concentration used (Figure 2.4B; boxes), while HPV16 infection was unaffected by filipin treatment (Figure 2.4B;

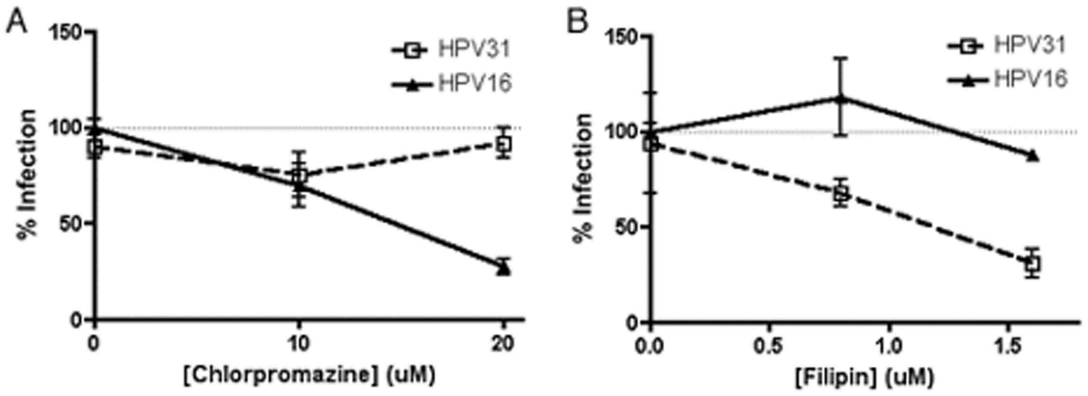

Figure 2.4 Effects of Biochemical Endocytic Inhibitors on HPV31 and HPV16 Infection of HKs

HaCaT cells were pretreated with increasing concentrations of chlorpromazine (A; 0, 10, 20 uM) or filipin (B; 0, 0.8, 1.6 uM) for 1h at 37°C. HPV31 (open boxes, dashed lines) or HPV16 (triangles, solid lines) virions at 50 vge/cell were bound for 1h at 4°C to promote viral attachment. Cells were then washed, refed with media/inhibitor, and placed at 37°C. RNA isolated at 48h pi was quantified for HPV31 E1^E4 transcript levels by RT-qPCR. Infections were performed in duplicate and RT-qPCR was performed in triplicate with error bars representing SEM.

triangles). These inhibitor studies confirm that HPV31 infection of HKs is caveolae dependent, while HPV16 infection is clathrin dependent.

HPV31 Associates with Detergent-Resistant Microdomains (DRM). Lipid rafts are low-density regions of the plasma membrane where high concentrations of cholesterol localize along with GPI-linked proteins and several other key factors, especially caveolins. These domains typically act as signaling centers that send messages from the plasma membrane into the cell, and caveolae-mediated endocytosis is thought to originate in these centers [reviewed in ref.(94)]. One characteristic of lipid rafts is their resistance to detergent solublization, thus they are denoted as detergent resistant microdomains (DRM). To investigate whether HPV31 associates with DRM as part of caveolar-mediated entry, we performed a Triton X-100 (TX-100) washout experiment (37). Cholera toxin B (CT-B) is known to use caveolar mediated entry and associate with DRM (74), whereas transferrin (TF) is a ligand that is internalized by clathrin-mediated endocytosis independent of DRM (75). AF594-labeled CT-B and TF proteins were used as controls. Labeled ligands were bound to HaCaT cells for 1h at 4°C, then placed at 37°C for 30 min in the presence or absence of methyl β-cyclodextrin (MßCD), a cholesterol and DRM disruptor (84). Cells were then washed with TX-100 on ice to remove detergent soluble membranes, fixed, and visualized by confocal microscopy. As expected CT-B displayed resistance to TX-100 treatment (Figure 2.5A, top row), and all CT-B signal was lost following cholesterol disruption by MßCD treatment. TF does not associate with DRM, and as expected TX-100 treatment removed all AF594-TF (Figure 2.5A, second row). AF594-HPV31 displayed strong DRM association as demonstrated by resistance to TX-100 washout (Figure 2.5A, third row), and MßCD treatment

confirmed cholesterol dependence for viral signal retention. AF594-labeled HPV16 (Figure 2.5A, bottom row) was also tested for its resistance to TX-100 washout at 30 minutes post- attachment. Although some signal was retained following the TX-100 wash, the majority was lost, suggesting that HPV16 binding to HKs does not occur at lipid rafts. HPV31-DRM association was also examined over time (Figure 2.5B). DRM association was low immediately following attachment (0h), increased over time (2-4h), then decreased at later time points (6h). HPV31 association with DRM is consistent with a caveolar-mediated pathway for HPV31 entry in HKs.

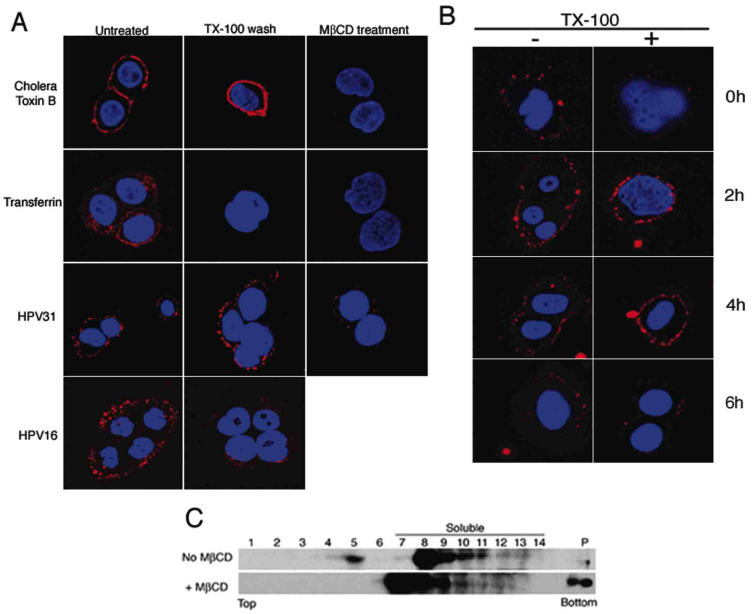

Figure 2.5 HPV31 Associates with Detergent-Resistant Lipid Rafts
(A) AF594-labeled ligands (CT-B, TF, HPV31, or HPV16) were bound to HaCaT cells for 1h at 4°C, then washed and refed with regular growth media before incubation at 37°C for 30 min. MßCD treatment for 30 min at 37°C was performed to disrupt cholesterol (right column). Cells were subjected to a control PBS wash (left column) or TX-100 wash (middle and right columns) on ice to remove detergent-soluble membranes. Cells were then fixed and stained with DAPI to visualize nuclei. (B) AF594-HPV31 was bound to cells for 1h at 4°C, then washed, refed with regular growth media, and placed at 37°C. At indicated times post-attachment, cells were subjected to a control PBS wash (left column) or TX-100 wash (right column) before fixation. (C) HPV31 was bound to HaCaT cells for 1h at 4°C, cells were washed and refed with normal growth media before incubating at 37°C for 30 min in the presence or absence of MßCD. Cells were then lysed, and the samples were subjected to centrifugation on a discontinuous density gradient. Fractions were collected from the top and analyzed for HPV31 L1 protein by western blot.

Another lipid raft characteristic due to their specific lipid composition is

a density lower than that of soluble plasma membrane components (182). We thus investigated the density of HPV31-membrane interactions as a second means of determining HPV31 association with lipid rafts. Virions were bound to cells for 1h at 4°C prior to incubation at 37°C for 30 min in the presence or absence of MßCD. Cell lysates were loaded under a discontinuous density gradient and subjected to high-speed centrifugation. Lipid rafts and their associated ligands typically float up in the gradient and are detected in lower density fractions, separate from soluble fractions. Indeed, a fraction of the HPV31 L1 signal was detected in lower density fractions (Figure 2.5C, top panel, fractions 4, 5), although a significant amount was still present in soluble fractions. Since a predominant proportion of HPV31 virions remain associated with the extracellular matrix at this time pi, we interpret the excess virions detected in the soluble fractions to be those that are extracellular matrix-associated. Importantly, MßCD treatment completely disrupted the virus signal present in the low-density fractions, confirming the cholesterol requirement for HPV31 association with low-density lipid rafts (Figure 2.5C, bottom panel). Together the data in Figure 2.5 confirm the association of HPV31 with lipid rafts at the plasma membrane of HKs prior to entry.

HPV31 and HPV16 do not Colocalize During Entry into HKs. The findings that HPV31 entry is slow and uses a caveolae-dependent pathway involving dynamin-2 and lipid rafts differ significantly from the clathrin-dependent entry pathway reported for HPV16 (12, 38) and from our observations of HPV16 in HKs. Further, the data imply HPV31 and HPV16 virions may use distinct cellular entry pathways in HKs. The nature of HPV virion binding to the HK extracellular matrix (32) and the vastly different internalization times for HPV16 and HPV31 on the plasma membrane have prevented us

from performing informative VLP-virion competition experiments for evaluating whether one virus type can block authentic infection of the other measured by virus gene expression in the HKs. However, we labeled HPV31 virions with AF488 (green) and HPV16 virions with AF594 (red) and monitored plasma membrane binding and entry into HK over time (Figure 2.6). No significant colocalization of the two viral types was observed at any time during the binding and entry process. In fact, at later time points (8h) and as expected from the observed differences in $t_{1/2}$ for each virus, HPV16 (red) was observed at a perinuclear location within cells, while the HPV31 signal (green) remained at the cell surface. Observation of AF594-labeled HPV31 and AF488-labeled HPV31 entry displayed high levels of colocalization, confirming that particles entering by the same pathway would be expected to display colocalization by microscopy (data not shown). These data suggest that the bulk of observable HPV31 and HPV16 virions enter HK *via* distinct pathways.

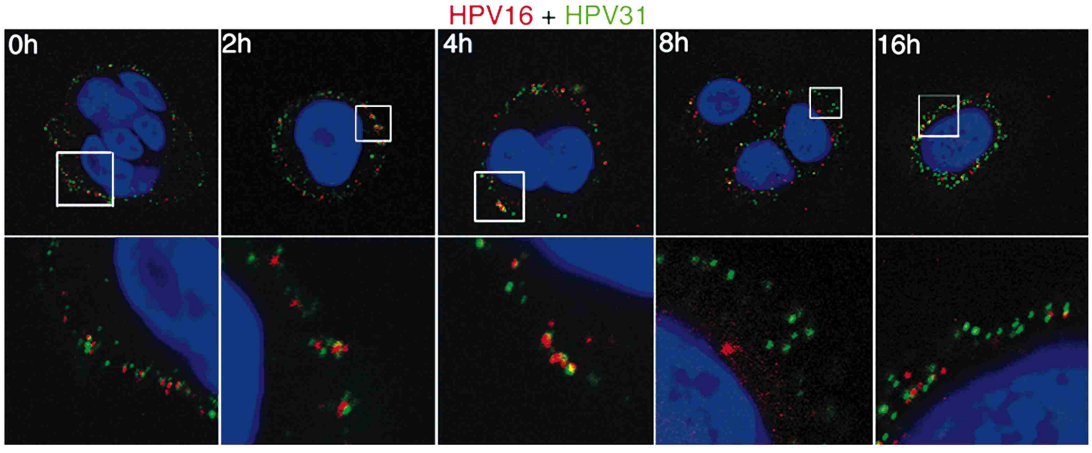

Figure 2.6 HPV31 and HPV16 Fail to Colocalize During HK Entry
AF594-HPV16 (red) and AF488-HPV31 (green) virions (each at 10,000 vge/cell) were added to HaCaT cells for 1h at 4°C, cells were washed, refed with regular growth media, and placed at 37°C. At the indicated time points, cells were fixed, DAPI stained, and visualized by confocal microscopy. Lower panels are higher magnifications of boxed areas in each upper panels.

Discussion

HPVs have co-existed with humans over millions of years *via* persistent infections and have therefore evolved means of immune evasion involving low gene expression and tightly regulated life cycles in their target tissues, the differentiating epithelium of HKs. These aspects have made it challenging to study early infection events because it is difficult to obtain high-titer virion stocks and to detect experimental infections in HKs. To avoid these obstacles, many researchers have used VLP and PsV with nonrelevant cell types for examining initial HPV-cell interactions. Such studies have generated conflicting data regarding attachment requirements (i.e., HSPG interactions), receptor usage (i.e., a6-integrin), endocytic entry (whether clathrin or caveolae mediated), and internalization kinetics. The range of cell types used may partially explain the disparate results. Here we have used authentic HPV31 virions to investigate the entry pathway into HKs, including human foreskin keratinocytes and human cervical keratinocytes, which are the naturally infected host cell type *in vivo*. Additionally, we utilized both visual and infectious entry assays to confirm our findings.

New methodology for HPV virion production (153) allowed us to obtain high-titers of authentic HPV31. We found that HPV31 entry in HK is very slow, requiring more than 16h for completion. This long internalization time for HPV31 is similar to that observed for HPV11, HPV40, and CRPV (25, 34), although it differs from that for HPV16 (Figure 2C and ref. (38)). This remarkably long internalization time suggests that multiple events may occur upon viral attachment before internalization. Transfer of virions from an initial attachment receptor to an internalization receptor, conformational changes in the viral particle and/or the viral receptor, lipid raft clustering, and signaling

events have been widely reported for the internalization of many viruses (reviewed in (108, 184)) and are possible events that may occur prior to HPV entry as well. Additionally, transfer of papillomavirus from an extracellular matrix component to a plasma membrane receptor may be required, an event that could also affect internalization kinetics (32, 39).

Here we show that HPV31 uses a caveolae-mediated pathway that is dynamin-2 dependent for HK entry. We demonstrate that HPV31 and HPV16 have differential sensitivities to chlorpromazine and filipin treatments, suggesting that they are using distinct entry pathways. Our results are consistent with the observations of Bousarghin *et al*. using PsV to transduce COS-7 cells (12), but differ from those recently reported by Hindmarsh and Laimins using PsVs in COS-7 and 293TT cells (79). In agreement with a caveolae-dependent entry pathway for HKs, we also find that HPV31 associates with lipid rafts as early as 30 minutes following attachment suggesting a role for cell surface signaling in entry. Indeed signaling upon VLP binding has been reported for HPVs (52, 63, 139).

Our data indicate that HPV31 may use a cellular entry pathway distinct from that used by HPV16 in their normal host cell, the human epithelial keratinocyte. Consistent with this possibility, we find the two viruses displayed markedly different internalization rates, are differentially susceptible to endocytic inhibitor treatment, differ in their association with DRM, and fail to colocalize in HK over 16h post attachment and internalization. Therefore, the two viral types appear to use independent entry pathways upon infection of HKs. A difference in internalization pathway, were it shown to influence the ability to establish infection might help explain the difference in prevalence

of infections with these two viruses in the population. This work emphasizes the importance of using cells relevant to *in vivo* infections, and demonstrates the diverse nature of the interaction of HPVs with their hosts.

Endocytic routes were previously thought of as strictly defined and distinct in that non-clathrin/non-caveolae mediated endocytosis and clathrin-mediated entry deliver particles to the early endosome, where they then reach late endosomes. Caveolar-mediated routes typically pass through the caveosome (bypassing endosomes) and then move to the Golgi and/or endoplasmic reticulum (181). However, exceptions occur, and each trafficking ligand may have distinct routes in a given cell (77). For example, JCV enters cells by clathrin-dependent endocytosis, is transported immediately to early endosomes, and is then sorted to a caveolin-1-positive endosomal compartment; this is the first example clathrin-early endosome to caveosome sorting (155). Alternatively, some caveolar vesicles join the early endosomes rather than caveosomes (77). Therefore, it will be important to determine which organelles are involved in the trafficking and uncoating of these two virus types, and we are pursuing experiments to this end. Furthermore, the entry receptor for these two oncogenic viruses has not been unequivocally confirmed and it will be important to determine whether HPV16 and HPV31 utilize different molecules to penetrate host cells. Such results might have implications for design and assessment of anti-virals and second-generation vaccines to target multiple HPV types.

Chapter III

Caveolin-1 dependent infectious entry of human papillomavirus type 31 in human keratinocytes proceeds to the endosomal pathway for pH-dependent uncoating

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Abstract

High-risk human papillomaviruses (HPVs) are small nonenveloped DNA viruses with a strict tropism for squamous epithelium that are associated with cervical cancer and some head and neck cancers. The differentiation-dependent life cycles of HPVs has made them difficult to study in simple cell culture. Thus, many aspects of early HPV infection remain mysterious. We recently showed the high-risk HPV type 31 (HPV31) enters its natural host cell type *via* caveolae-dependent endocytosis, a distinct mechanism from the closely related HPV16 (186). In this report, we have determined the downstream trafficking events following caveolar entry of HPV31 into human keratinocytes. After initial plasma membrane binding, HPV31 associates with caveolin-1 and transiently localizes to the caveosome before trafficking to the early endosome and proceeding through the endosomal pathway. Transport between the caveosome and early endosome was found to be Rab5 GTPase-dependent. Although HPV31 capsids were observed in the lysosome, Rab7 GTPase was found to be dispensable for HPV31 infection, suggesting that viral genomes escape from the endosomal pathway prior to Rab7-mediated transport. Consistent with this, the acidic pH encountered by HPV31 within the early endosomal pathway induces a conformational change in the capsid resulting in increased DNase susceptibility of the viral genome, which likely aids in uncoating and/or endosomal escape. The entry and trafficking route of HPV31 into human keratinocytes represents a unique viral pathway by which the virions use caveolar entry to eventually access a low-pH site that appears to facilitate endosomal escape of genomes.

Introduction

Human papillomaviruses (HPVs) are small, naked viruses that carry a circular doubled-stranded DNA genome and infect human squamous epithelial cells. The causal relationship between HPVs and cervical cancer is well established (8), and HPVs have been implicated in other epithelial cancers as well, including head and neck cancer (51, 68). The development of an extremely effective vaccine against four types of HPV is likely to be a very valuable tool for preventing new HPV infections (4). However, the cost of this vaccine series is likely to be prohibitive in the populations of women in developing countries who would benefit most (7). Additionally, the long-term protection offered by the vaccine, an essential property given the role of HPVs in cancers, has not been established. Therefore, as many aspects of basic HPV biology remain undefined, continued research of high-risk HPVs is essential.

Many viruses enter cells through endocytosis, hijacking the cellular machinery for entry and invasion of the host cell (180). The majority of viruses have been demonstrated to use clathrin-mediated endocytosis for entry, whereas a few have been shown to enter through caveolae. Classically, ligands employing clathrin-dependent endocytosis proceed through the endosomal pathway, while cargo entering via caveolae enter an intracellular caveolin-1 positive and pH neutral structure known as the caveosome before trafficking to the endoplasmic reticulum (143). These pathways, previously believed to be distinct, have recently been shown to display crosstalk, whereby cargo can move between them. For example, the polyomavirus JCV has been shown to enter glial cells through clathrin-mediated endocytosis. However, following entry the virions localize to caveolin-1 positive structures, presumably the caveosome, and inhibition of caveolin-1

activity blocks a downstream, non-entry event in JCV infection (155). Conversely, following entry *via* caveolae-mediated endocytosis, SV40 is observed to traffic between caveosomes and early endosomes. However, SV40 infection, as defined by nuclear delivery of active genomes, requires transport back to the caveosomes and on to the endoplasmic reticulum (142). In both cases, movement between the caveolar and endosomal pathways was found to be dependent on the small GTPase, Rab5.

Pathogens that proceed through the endosomal pathway during trafficking in the host cell typically take advantage of the decreased pH of the endosomal compartments (72, 202). Acidic pH acts as a trigger for many viruses to undergo conformational changes leading to any number of events that facilitate endosomal escape of virion proteins and/or viral genomes. Such events may include modification of the viral-receptor interaction, exposure of protease digestion motifs, viral envelope-endosomal membrane fusion or partial to complete uncoating of the viral genome (44, 192). Indeed, a C-terminal region of BPV L2 has been identified that displays pH-dependent membrane destabilizing activity (87), although the exact mechanism by which this structure may assist in endosomal escape remains unclear.

Previous work suggests that HPV16 utilizes a clathrin-dependent entry mechanism (38). In contrast, we recently demonstrated that HPV31 infection of human keratinocytes, the natural host cell type for HPVs, occurs through caveolae-mediated endocytosis and the initial entry route is distinct from that of HPV16 (186). In this report, we investigated the trafficking of HPV31 virions following caveolar entry into human keratinocytes. Colocalization studies with various markers of cellular organelles reveal that after entry, HPV31 traffics through the caveosome to the endosomal pathway,

and this trafficking is dependent on the small GTPase Rab5. Consistent with passage through the endosomal pathway, HPV31 infection is dependent on the acidification of endosomes, and low pH treatment of virions induces a conformational change in the HPV31 capsid that appears to promote genome uncoating.

Materials and Methods

Cell Culture, Virion Production, and Infections. HaCaT cells are a spontaneously immortalized epithelial line derived from normal adult skin (10). 293T cells are derived from the human embryonic kidney cell line HEK293 and express SV40 large T antigen. Cells were maintained in DMEM/F12-Ham's Nutrient Mixture containing 10% fetal calf serum, 4X amino acids, 2 mM glutamine, 100U/mL penicillin, and 1 ug/ml streptomycin. HPV31 virions were produced and purified by density gradient centrifugation as previously described (153, 186); stocks were quantified by blot hybridization to determine viral genome equivalents (vge) per unit volume as reported (131, 132). Transmission electron microscopy (TEM; Hitachi 7500) was performed to visualize virus stocks at 80 kV following binding to a carbon-coated electron microscopic grid and negative staining with 2% uranyl acetate. Infections were performed as previously described (132). Briefly, virion stocks were diluted in media and exposed to HaCaT cells for 1 hour at 4°C with agitation to allow viral attachment. Inocula were aspirated, cells were washed, and refed with fresh media. Cells were incubated for various amounts of time at 37°C before analyzing.

RNA Isolation and RT-qPCR Analysis. Total RNA was extracted at 48 hours post infection using Tri Reagent (Sigma). Nucleic acid concentrations were determined by spectrophotometry. Reverse transcription of 2 ug total RNA and triplicate quantitative PCR (RT-qPCR) was performed using GeneAmp RNA PCR reagents and AmpliTaq Gold DNA Polymerase (Applied Biosystems) using primers, probes, and conditions as previously described (131). Error bars represent standard error of the mean (SEM).

Virion Labeling with Fluorescent Dyes. Approximately 1 ug of Alexa Fluor (AF) 594 carboxylic acid, succinimidyl ester (Molecular Probes) was mixed with 10^8 virions and incubated for 1 hour at room temperature. Labeled stocks were washed 3X with 1X HSB (25mM HEPES pH 7.5, 500 mM NaCl, 0.02% Brij58, 1 mM $MgCl_2$, 100uM EDTA, 0.5% ethanol) and recovered using a Millipore Amicon Ultra-4 centrifugation filter to remove unincorporated dye.

Colocalization Studies. The following antibodies were used for immunofluorescence at the indicated dilutions: α-caveolin-1 (1:500; BD Biosciences clone 2297), α-protein disulfide isomerase (PDI; 1:500; Stressgen clone 1D3), α-golgin 97 (1:500; Molecular Probes clone CDF4), a-early endosome antigen 1 (EEA1; 1:1000; Abcam ab15846), a-lysosomal-associated membrane protein 1 (LAMP1; 1:500; Abcam clone H4A3), AF488-goat a-mouse IgG (1:500; Molecular Probes), AF680-goat α-mouse IgG (1:500; Molecular Probes). Cells were seeded at 5000-10,000 cells per glass coverslip in 60 mm dishes one day prior to virus exposure. AF594-HPV31 virions were diluted in media to 5000-10,000 vge/cell and exposed to cells for 1 hour at 4°C. Cells were washed, refed

with fresh media, and incubated at 37°C for various times. For caveolin-1 staining, cells were fixed with 3.7% paraformaldehyde and permeabilized with cold methanol. For PDI and golgin-97 staining, cells were fixed with 3.7% paraformaldehyde. For EEA1 and LAMP1 staining, cells were fixed and permeabilized with cold acetone. Following fixation, slides were blocked in 4% BSA/PBS and incubated with the appropriate primary antibody diluted 1:100 in 4%BSA/PBS for 1 hour at room temperature. Cells were washed extensively with PBS, then incubated with AF488- or AF680-anti mouse IgG diluted to 5 ug/mL in PBS. Cells were washed and mounted with VectaShield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Labs). Photomicroscopy was performed using a Zeiss META confocal microscope with a 63X objective and the appropriate filters.

Rab GTPase Transfections. Wild type (wt) and dominant negative (dn) Rab5, Rab7 and Rab11 proteins were fused with green fluorescent protein (GFP) (GFP-Rab5wt, GFP-Rab5 S34N, GFP-Rab7 wt, GFP-Rab7 T22N, GFP-Rab11wt, GFP-Rab11 S25N) as previously reported (21, 22, 58, 160, 161). HaCaT cells were transfected with each construct using the Amaxa Nucleofector V protocol as previously described (186). At 48 hours post-transfection, cells were exposed to virions in triplicate (as described above); colocalization studies were performed at various times post-attachment. Following identification of transfected cells by GFP expression, AF680-stained caveolin-1, EEA1, and LAMP1 were pseudocolored green, and ≈100 AF594-labeled virion signals were assessed for colocalization with each respective immunostained organelle.

DNase Susceptibility Assay. Approximately $1x10^8$ virions were treated with 10 mM DTT overnight at room temperature or at the indicated pH for 2 hours at 37°C. Each sample was then washed over a Millipore Amicon Ultra 4K centrifugation filter and exchanged into DNase I buffer (40 mM Tris-Cl pH7.9, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$). Samples were equally divided and either mock treated or treated with 5U DNase I for 1 hour at 37°C. Samples were then blotted onto a GeneScreen membrane and probed for HPV31 genome as previously described (132). Genome copy number was quantified by densitometry, and the percent of DNase-resistant genome was calculated by normalization of DNase treated to mock treated samples.

Results

HPV31 traffics through caveosomes to the endosomal pathway. Recently, we showed that HPV31 enters human keratinocytes through a caveolae-mediated and dynamin 2-dependent pathway in which uptake is quite asynchronous and occurs with a very slow entry half-time ($t_{1/2}$) of 14 h (186). To further investigate the trafficking route of HPV31 particles during and following entry into human keratinocytes, non-saturating numbers of AF594-labeled virions were allowed to bind the plasma membrane of HaCaT cells and enter for various amounts of

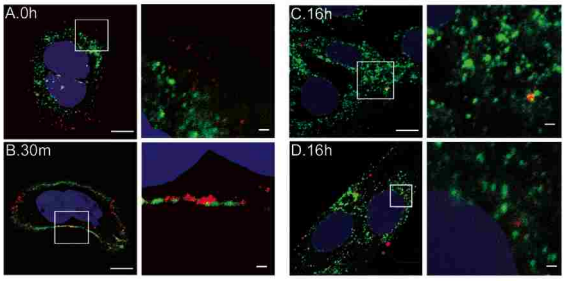


Figure 3.1 HPV31 transiently localizes to caveolin-1 structures at the plasma membrane and intracellularly
HaCaT cells were exposed to AF594 labeled HPV31virions at 10,000 vge/cell for 1h at 4°C. Cells were washed and incubated at 37°C for (A) 0h, (B) 30m, or (C,D) 16h. Cells were fixed, stained for caveolin-1 (AF488; green) and nuclei were visualized with DAPI. The microscopy is representative and the focal plane is mid-cell body/nucleus. Right panels are enlarged from boxed areas in left panels.

time before counterstaining for several cellular organelles. No colocalization occurred between HPV31 particles and caveolin-1 immediately following attachment (Figure 3.1A); however, as early as 30 minutes after attachment, HPV31 and caveolin-1 were observed in adjacent locales with some colocalization (Figure 3.1B). This suggests that incubation at 37°C is required for HPV31 -caveolae association, and is similar to what we observe for the organization of HPV31 into detergent resistant microdomains/lipid rafts (186). At later time points internalized HPV31 was only found associated with caveolin-1 positive structures near the cell periphery (Figure 3.1C), whereas virions located in perinuclear regions were no longer localized with caveolin-1 (Figure 3.1D). Thus, HPV31 appears to transiently associate with caveolin-1 at the plasma membrane and at a peripheral caveolin-1 positive structure consistent with the caveosome before trafficking to a caveolin-1 free intracellular compartment.

Cargo that traffic through the caveosome are typically transported to the Golgi apparatus and/or endoplasmic reticulum (143). To determine whether HPV31 traffics to either of these

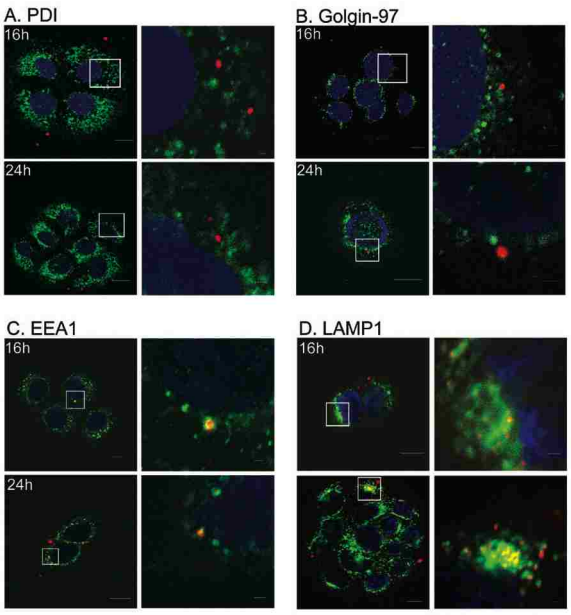


Figure 3.2 HPV31 localizes to early endosomal pathway at late times post-entry HaCaT cells were exposed to AF594 labeled HPV31virions at 10,000 vge/cell for 1h at 4°C. Unbound virions were removed and cells were incubated at 37°C for 16h or 24h. Cells were fixed, and immunostained (AF488; green) for PDI (A), Golgin-97 (B), EEA1(C), or LAMP1 (D) as described in the Materials and Methods section; nuclei were visualized with DAPI. The microscopy is representative and the focal plane is mid-cell body/nucleus. Right panels are enlarged from boxed areas in left panels.

structures, cells infected with AF594-HPV31 were stained at 16 and 24 hours post-attachment for protein disulfide isomerase (PDI), a marker of the endoplasmic reticulum (Figure 3.2A), or golgin-97, a marker of the Golgi apparatus (Figure. 3.2B). Surprisingly, at no time during infection was colocalization observed between HPV31 and either of these markers, indicating that HPV31 does not traffic to the Golgi or endoplasmic reticulum. However, high levels of HPV31 capsids were found consistently colocalized with markers of the endosomal pathway at later times in infection (Figure 3.2C, D). This included markers of the early endosome, EEA1 (Figure 3.2C), or the lysosome, LAMP1 (Figure 3.2D) at 16 and 24 hours post-attachment. Additionally, colocalization between HPV31 capsids and LAMP1 increased over time, indicating that particles accumulate in this structure. These data demonstrate that HPV31 virions enter cells *via* caveolae and transiently localize to the caveosome before trafficking to the endosomal pathway.

The role of pH in HPV31 trafficking and uncoating. Many ligands and viruses that traffic through the endosomal pathway require the acidic pH of these compartments for proper transport or to otherwise facilitate function or infection (72, 202). We investigated the importance of acidic pH during HPV31 infection of human keratinocytes by treating cells with three inhibitors of endosomal acidification, including the lysosomotropic agents ammonium chloride and chloroquine, as well as bafilomycin A, which inhibits the vacuolar H^+ ATPase. At 48 hours post-infection, we assessed infection levels by RT-qPCR quantification of the spliced viral transcript E1^E4 as previously reported (131, 138). All three inhibitors of endosomal acidification blocked HPV31

infection (Figure 3.3A), indicating that the acidic pH of endosomal vesicles is required during this process.

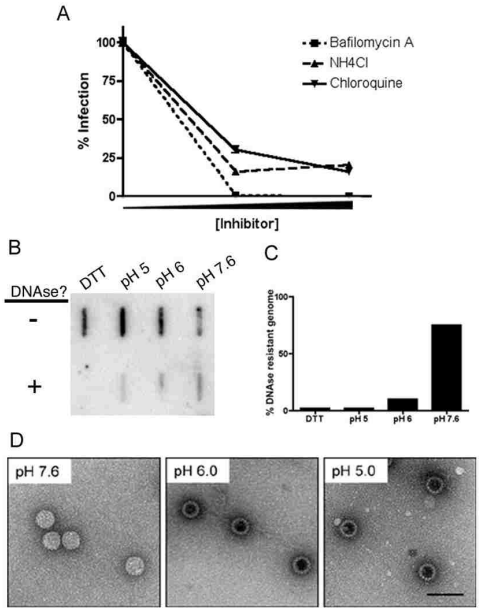


Figure 3.3 HPV31 infection requires endosomal acidification and low pH induces conformational changes in the HPV31 virion
(A) HaCaT cells were pretreated for 30 minutes with bafilomycin A (75 nM, 100 nM; dotted line), NH4Cl (15 mM, 20 mM; dashed line), or chloroquine (5 μm, 10 μM; solid line) then infected with HPV31 at 100 vge/cell in the presence of inhibitors for 48 hours; cells were monitored for viability. Total RNA was analyzed for HPV31 E1^E4 by RT-qPCR. Values are expressed as percentage infection normalized to mock treated cells. (B) HPV31 virions (~108 vge) were treated in vitro with 10 mM DTT at room temperature overnight or at the indicated pH for 2h at 37°C. Samples were equally divided and mock or DNase I treated for 1h at 37°C, then blotted onto GeneScreen membrane and probed for intact HPV31 genomes. (C) Quantification of (B) where values are expressed as % DNase resistant genome normalized to the corresponding mock-treated sample. (D) ~10^8 HPV31 virions were treated in vitro at indicated pH for 2 h at 37°C. Samples

The acidic milieu of the endosomal compartment is a common trigger for several viruses, often causing conformational changes in the capsid that promote genome uncoating and/or endosomal escape (20, 44, 209). To investigate how acidic pH affects HPV31 capsid structure, we exposed virions to lower pH *in vitro* and measured the effects visually and by access of the viral genome to DNase I. The tight conformation of HPV capsids formed during virion morphogenesis cause viral genomes encapsidated in mature virions to be DNase resistant (17, 153). Treatment with dithiothreitol (DTT), which reduces intercapsomeric bonds in papillomavirus particles, causes near complete

susceptibility of the viral genome to DNase (99) (Figure 3.3B). Whereas neutral pH had no significant effect on viral genome DNase sensitivity, lower pH had substantial effects. Exposure of virions to pH 6 (equivalent to endosomes) or pH 5 (equivalent to lysosomes) rendered the virion-associated genomes increasingly more susceptible to DNase (Figure 3.3B). Quantification after each treatment (Figure 3.3C) revealed that more than 98% of the viral genomes were susceptible to DNase digestion following pH 5 treatment, similar to the susceptibility of DTT-treated virions. Incubation at pH 6 resulted in 90% loss of viral genome after DNase treatment, while >75% of viral genome remained DNase resistant following treatment at neutral pH (Figure 3B). To observe changes in virion morphology upon lower pH exposure, virions were stained with uranyl acetate and visualized by transmission electron microscopy (Figure 3.3D). Virions treated at neutral pH displayed normal morphology. However, after treatment at pH 5 or pH 6, particles exhibited increased uptake of uranyl acetate dye, as evidenced by the darker staining in the center of virions. This change in staining suggests that acidic pH-treated particles have a looser conformation, consistent with the increased DNase susceptibility observed for these samples (Figure 3.3C). These data demonstrate that incubation at pH levels equivalent to those encountered by HPV31 virions during trafficking through the early endosomal pathway results in a conformational change in the virion, which likely contributes to uncoating and endosomal escape of the viral genome.

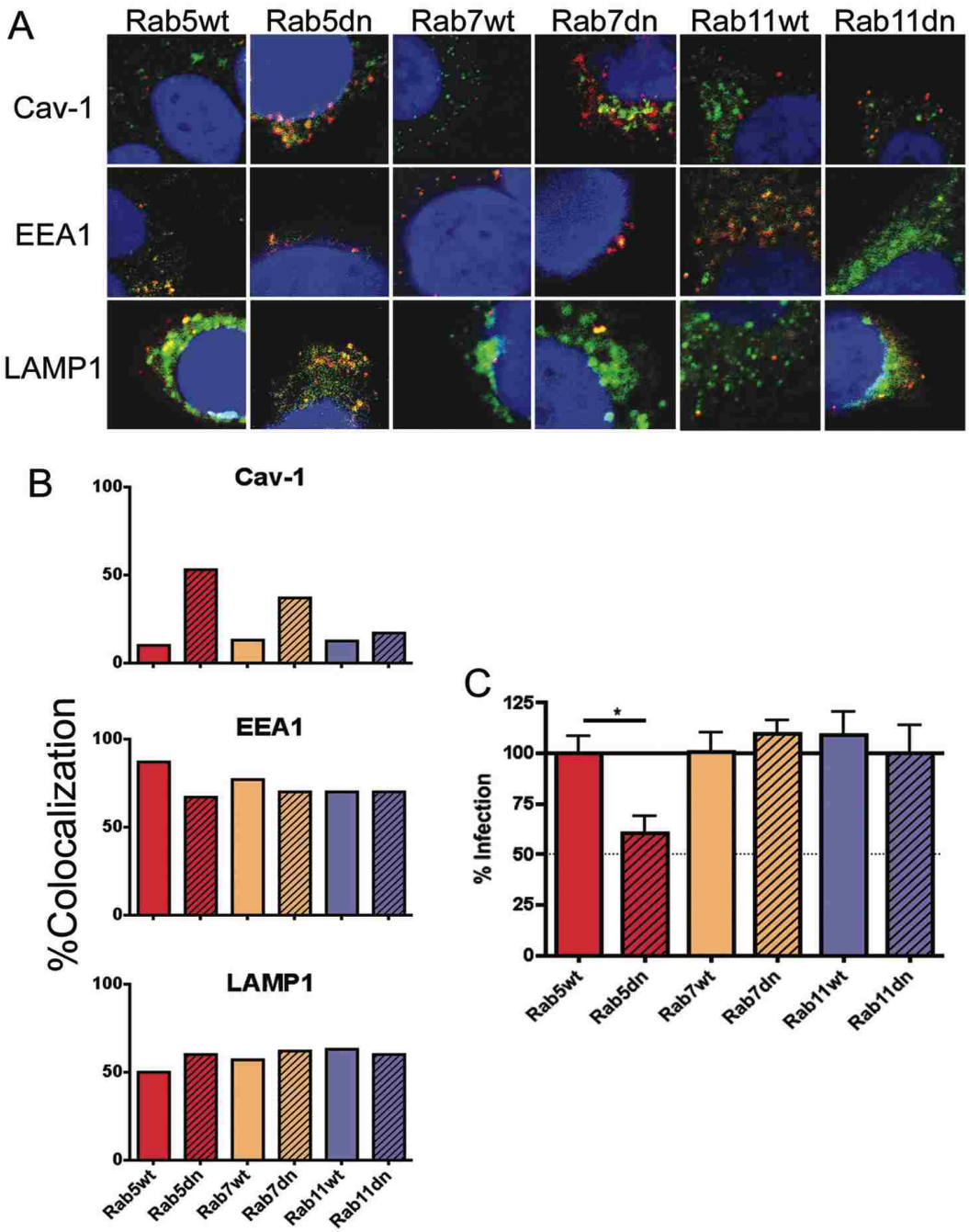
HPV31 trafficking between the caveosome and early endosomes is Rab5-dependent. Rab GTPases regulate many steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion. Rab5 is one of the more intensely studied members of the diverse family of Rab GTPases (28).

Functional studies demonstrate Rab5 to be a crucial regulator of early endocytosis, where it is involved in clathrin-coated vesicle formation, fusion between early endosomes, endosomal cargo recruitment, and endosomal movement (203, 213). Rab7 GTPases mediate cargo transport from the early to late endosome, and Rab11 GTPases control recycling endosome transport between the *trans* Golgi network and the plasma membrane (188). To investigate the importance of each of these Rab GTPases in HPV31 trafficking in human keratinocytes, cells were transfected with fluorescent-tagged wild type and dominant negative forms of Rab5, Rab7, and Rab11. The cells were then infected with AF594-labeled HPV31 virions for 24 hours, and immunostained for markers for the caveosome (caveolin-1; cav-1), early endosome (EEA1), and lysosome (LAMP1; Figure 3.4A). Rab-transfected cells were identified by fluorescent protein expression, and colocalization between HPV31 (AF594; red) and each of the organelle markers (AF680; pseudocolored green) was blindly quantified (Figure 3.4B). Expression of the dominant negative Rab5 S34N had the most profound effect on HPV31 localization. A 43% increase of HPV31 colocalization in caveosomes resulted from expression of the kinetically delayed dominant negative Rab5 GTPase, as compared to the more transient HPV31 localization with caveolin-1 in wild type Rab5 transfected cells. There was little obvious corresponding decrease in colocalization with endosomal or lysosomal markers where labeled HPV31 capsids accumulate in the lysosome. This shows that limited transport between the caveosome and endosomal pathway continues in the presence of the kinetically delayed Rab5 mutant as expected. This demonstrates HPV31 particle transport between the caveosome and the endosomal pathway is regulated by Rab5 GTPase, a route similar to that shown for SV40 and cholera toxin B (142).

Rab7 regulates transport between early and late endosomes, and cells expressing a kinetically delayed Rab7 T22N GTPase mutant demonstrated a similar, but less dramatic increase in HPV31 localization in caveosomes (Figure 3.4A, B). This result also supports a model whereby HPV31 circulation between caveosomes and early endosomes occurs regulated by Rab5: as virion traffic backs up into the early endosome due to Rab7 inhibition, Rab5-mediated circulation sustains particle cycling resulting in a net accumulation in the caveosome. As predicted, dominant negative Rab11 GTPase had no effect on HPV31 localization confirming that Golgi to endoplasmic reticulum sorting is not involved in virus entry.

If Rab proteins are necessary in the HPV31 infection process prior to genome uncoating and nuclear transport, the delayed mutants of involved Rabs would be expected to cause a reduction in our infectious entry assay which measures HPV31 early transcription following virion entry. Therefore, to firmly establish a role for Rab GTPases in HPV31 early infection, we quantified the abilities of wild type and mutant Rab5, Rab7, and Rab11 proteins to block HPV31 early transcription as a measure of nuclear viral genome delivery (Figure 3.4B). At 48 hours post-transfection with the Rab constructs, cells were exposed to HPV31 and incubated at 37°C for an additional 48 hours to allow viral entry and initiation of early transcription. Infection was assessed by RT-qPCR to measure levels of spliced viral E1^E4 RNAs (Figure 3.4C). As expected, blocking Golgi to endoplasmic reticulum trafficking by dominant negative Rab11 did not alter HPV31 infection. Surprisingly, mutant Rab5 GTPase was the only protein to cause significant inhibition of HPV31 infection. Despite the change in localization of HPV31 observed in mutant Rab7 transfected cells (Figure 3.4A, B), no infection inhibition was

detected in these cells. This finding along with the fact that viral genomes are susceptible to DNase I at an early endosomal pH is consistent with a model in which the HPV31 genome exits the endosomal pathway before Rab7-mediated trafficking occurs. Although AF594-labeled particles are observed in the lysosome (Figure 3.2D, 3.4A), this signal is likely to represent empty capsids.


A
Rab5wt
Rab5dn
Rab7wt
Rab7dn
Rab11wt
Rab11dn
Cav-1
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LAMP1
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LAMP1
%Colocalization
100
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Rab5wt
Rab5dn
Rab7wt
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Rab11wt
Rab11dn
C
% Infection
125
100
75
50
25
0
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Figure 3.4. Trafficking of HPV31 between caveosome and endosomal pathway is Rab5 GTPase dependent and essential for infection of human keratinocytes
A. HaCaT cells were transfected with fluorescent-tagged Rab5 wild type (wt), Rab5 S34N dominant negative mutant (dn), Rab7 wt, Rab7 dn T22N, Rab11 wt, or Rab11 S25N dn constructs. At 48 hours, AF594-HPV31 was exposed to cells at 10,000 vge/cell for 1 hour at 4°C, washed, refed and incubated at 37°C for 24 hours. Cells were fixed and immunostained for caveolin-1 (cav-1; top row), EEA1 (middle row), or LAMP1 (bottom row) (AF680; pseudocolored green), and nuclei were visualized with DAPI. Images were captured on a Zeiss META confocal microscope with a 63X objective, and are representative; the focal plane was not limited to mid-cell body, but was variable to assess colocalization. B, Blinded quantification of HPV31 virions (red; AF594) colocalizing with caveolin-1, EEA1, or LAMP1 positive structures (pseudocolored green). Values are expressed as percent of HPV31 virions per cell colocalized with indicated marker compared to total virions. C, Triplicate transfected HaCaT cell cultures were infected with HPV31 at 100 vge/cell. Total RNA was harvested at 48 hours post-infection and quantified in triplicate by RT-qPCR for spliced viral E1^E4 transcripts. Values are expressed as percent infection.

Discussion

Human papillomaviruses are important pathogens, causing substantial morbidity and many deaths each year worldwide (205). Despite the recent advent of a highly effective vaccine against four HPV types, political and financial barriers will likely hinder distribution of this important technology to developing countries, where cervical cancer remains a widespread cause of death. Therefore, defining the basic aspects of HPV biology is essential to further understanding of this significant pathogen.

Trafficking of cargo following caveolar entry for many years was believed to be a parallel but separate pathway from transport following entry by clathrin dependent endocytosis into the endosomal pathway. Typically, ligands and viruses that enter *via* caveolae move through the caveosome to the Golgi apparatus or endoplasmic reticulum

avoiding the endocytic route (144). Yet recently more complex interactions and crosstalk between these two pathways has become evident. For example, the polyomavirus SV40 has been shown to traffic between the caveosome and the early endosome in a Rab5-dependent manner. However, the SV40 remains tightly associated with its receptor and is not released into the endosomal pathway. Instead, the virus is transported to the endoplasmic reticulum, where resident proteins direct genome uncoating (142, 144, 170). In this regard, caveolae to endosome transport is not required for SV40 infection. In contrast, polyomavirus JCV enters host cells *via* clathrin-mediated endocytosis, but then

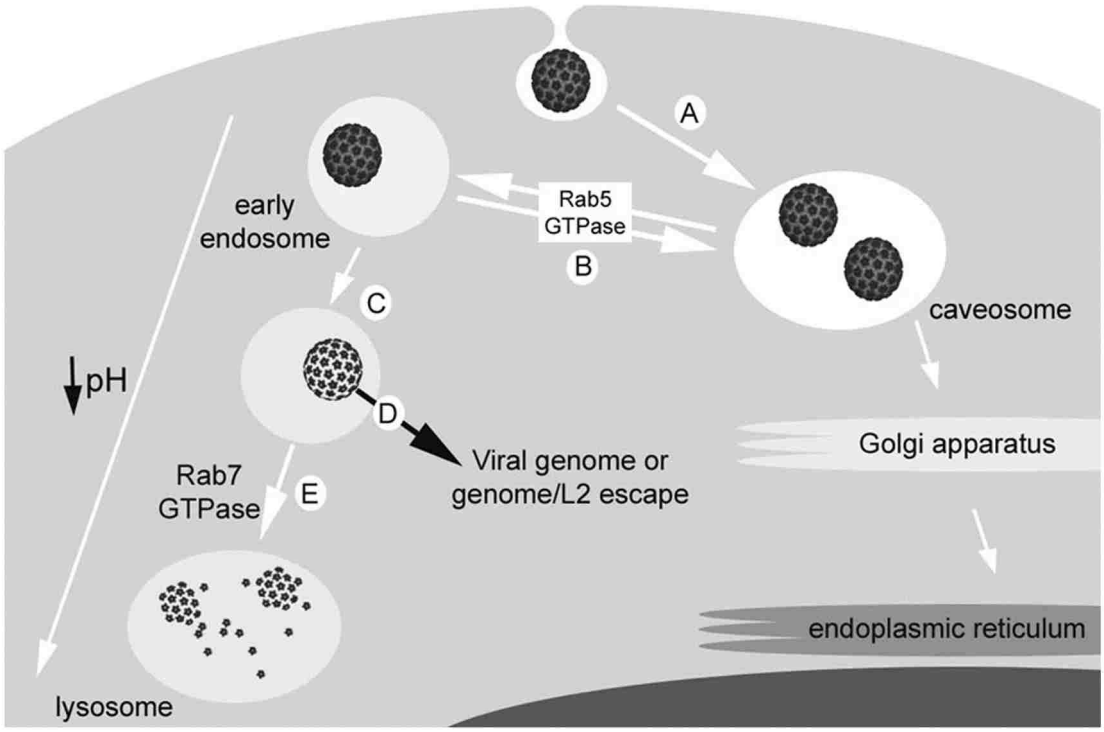

Figure 3.5 Model of HPV31 infectious entry in human keratinocytes
HPV31 enters via caveolae- and dynamin 2- mediated endocytosis and traffics to caveosomes (A). Thereafter, virions are transported from the caveosome to the early endosome dependent on Rab5 GTPase (B). As HPV31 proceeds through the endosomal pathway (C), the decreasing pH of the endosomal compartments causes a conformational change in the viral capsid, which results in DNase I sensitivity of genomes. We propose that this conformational change leads to endosomal escape of the viral genome or genome/L2 complex (D) before Rab7 GTPase-mediated capsids are transported to the lysosome (E). Empty/disassembled capsids can be visualized in the lysosome.

virions travel to the caveosome (155). Finally, cholera toxin B circulates between the caveosome and early endosome and uses the acidic pH of the early endosome as a trigger for disassociation from its receptor to allow continued trafficking within the endosomal pathway (142).

In this report, we have delineated the trafficking route of HPV31 virions following entry into the natural host cell type, human keratinocytes (Figure 3.5). The unusually slow and asynchronous uptake of HPV31 into host cells makes this type of work challenging, as colocalization studies required investigation at many and extensive times post infection. However, our infectious entry assays using unlabeled virions, which depend upon nuclear delivery of and transcription from viral genomes post infection, corroborate the each of the informative localization results using labeled virions. HPV31, which we previously demonstrated to enter HKs through caveolar dependent endocytosis, first traffics to the caveosome as expected (Figure 3.5A). HPV31 then exits the caveosome to join the endosomal pathway (Figure 3.5B). Consistent with previous reports that implicate the small GTPase Rab5 as an important factor involved in circulation between caveosomes and early endosomes, trafficking of HPV31 between these structures is also Rab5 dependent (Figure 3.5B). Unlike for SV40, this transport is necessary for HPV31 infection. Whereas, JCV traffics through endosomes to caveosomes for infectivity (155), HPV31's entry is to our knowledge, the first example of a virus that requires the caveosome to endosomal pathway for infectivity.

An interesting role for the Rab7 GTPase was also identified, as the kinetically delayed Rab7 mutant altered localization of HPV31 particles by increasing localization in the caveosome, supporting a model in which particles backed up into the early endosome

by Rab7 inhibition are trafficked back to the caveosome. However, inhibition of Rab7 function had no effect on the delivery of viral genomes to the nucleus for transcription. These results suggest that viral genome uncoating and escape occurs prior to Rab7 transport (Figure 3.5D), and that lysosomal localization of fluorescent signal at late times post infection reflects empty viral capsids. Consistent with this idea, significant changes in capsid structure and DNase susceptibility of viral genomes were observed following virion exposure to an early endosomal pH of 6.0. This suggests that the environment of endosomal pathway can trigger capsid conformational changes (Figure 5C), promoting uncoating and endosome escape (Figure 3.5D).

We recently demonstrated that the closely related types HPV16 and HPV31 enter human keratinocytes through distinct pathways (186). Our present findings that downstream trafficking of HPV31 requires the acidic compartments of the endosomal pathway is, however, similar to what is observed for HPV16 (38)(Campos and Ozbun, unpublished results). Thus, it appears that the trafficking routes of the two types, despite utilizing distinct entry mechanisms at the plasma membrane, converge in the endosomal pathway, sharing a requirement for low pH during the infectious process.

The internalization receptor(s) for HPV16 and HPV31 still remain unknown. Whereas HPV16 binding to and infection of human keratinocytes requires heparan sulfonated proteoglycans, HPV31 infections do not (138). Furthermore, HPV16 entry $t_{1/2}$ is nearly twice the rate as that of HPV31 (186). The different binding requirements and entry mechanisms of these two HPV types may therefore reflect different receptor usage. One might imagine a scenario where using one of the two receptors and/or entry mechanisms might give that particular type an evolutionary advantage in specific host

cells, whether cervical epithelium, oral epithelium, or some other cell reservoir. For example, the high levels of signaling induced within lipid rafts during caveolar entry (183), which we find for HPV31 (Smith and Ozbun, in preparation) could contribute to differential innate antiviral responses for HPV31 explaining in part why HPV31 infections are far less prevalent and pathogenic compared to HPV16 infections (40). Regardless of initial entry mechanisms, convergence of the two HPV types in the endosomal pathway strongly suggests these viruses take advantage of the existing properties of this pathway (i.e., acidic pH), as has been demonstrated for many other viruses (72, 202). The unique nature of HPV31 trafficking following caveolar entry into human keratinocytes described here represents yet another cellular pathway exploited by viruses to facilitate entry into and infection of host cells.

Chapter IV

Signaling Events Induced by HPV31 During Entry into Human Keratinocytes Promote Actin and Microtubule Cytoskeletal Rearrangements, Resulting in Localization to Lipid Rafts and Increased Viral Uptake

Jessica L. Smith, Diane S. Lidke, and Michelle A. Ozbun

Manuscript in preparation

Abstract

High-risk human papillomaviruses (HPVs) are widely accepted as the causative agents of cervical cancer, and an association with other epithelial cancers is becoming apparent as well. Research from our laboratory has revealed that, despite being closely related, HPV16 and HPV31 use divergent mechanisms to enter their natural host cell, human keratinocytes (HKs). During caveolar entry into HKs, HPV31 associates with lipid rafts, regions of the plasma membrane where high levels of cellular signaling can be initiated. Therefore we have investigated the signaling events induced by and required for HPV31 entry into HKs, as well as the roles that the cytoskeletal elements play in internalization. Induction of phosphatase activity was observed, and this signaling was found to regulate the microtubule cytoskeleton, promoting HPV31 association with lipid rafts, an event that is required for internalization. Additionally, HPV31 activation of tyrosine kinases and phosphatidyl inositol-3 kinases precedes induction of filopodia formation in HKs. Transport of HPV31 virions on filopodia structures, coupled with changes in extracellular matrix-bound virus patterns, lends evidence to a model in which HPV31 induction of filopodia contributes to uptake of virions bound to the extracellular matrix.

Introduction

Human papillomaviruses (HPV) are small, nonenveloped DNA viruses that are associated with cervical cancer, as well as other epithelial cancers (9, 68, 83). The differentiation dependent life cycle of HPVs have hindered research into the basic cellular entry requirements of these important pathogens. Although entry of HPV16 has been reported to occur through clathrin coated pits and proceed through the endosomal pathway (38), results from our laboratory have demonstrated that HPV31 enters its natural host cell, the human keratinocyte, *via* caveolae-mediated endocytosis (186). Following internalization, trafficking occurs through the caveosome to the endosomal pathway, where the acidic environment promotes uncoating of the viral genome and/or endosomal escape (Smith et al. submitted for review). This entry and trafficking mechanism represents a unique route for virus infection. During plasma membrane attachment to human keratinocytes, HPV31 associates with lipid rafts, cholesterol-rich low density regions of the plasma membrane, an event consistent with caveolae-mediated endocytosis (186). Due to the nature of lipid rafts as signaling centers at the cellular surface (183), it is probable that this association leads to signaling events prior to and during HPV31 entry.

Activation of signal cascades upon viral attachment and during entry are common mechanisms employed by viruses to prime a cell for infection. Endocytosis is a highly regulated process, and cellular signaling often initiates the assembly of endocytic machinery required for internalization of ligands. Several members of the polyomavirus (PyV) family have been demonstrated to initiate cellular signaling upon attachment that

leads to virus enclosure within endocytic vesicles (23, 154). These types of signaling events are typically initiated through tyrosine phosphorylation, and the downstream effects result in recruitment of proteins that make up the clathrin coat or caveolar pits. Further signaling may occur once endocytic domains are formed to promote the pinching off of internalizing vesicles from the plasma membrane.

In addition to promoting internalization, viruses often induce cellular signaling that promotes changes in cytoskeletal structure coupled to internalization. At the level of initial cellular attachment, cytoskeletal rearrangements can contribute to confinement of virions at the cell surface within endocytic centers (53, 144), delivery from an attachment to an internalization receptor (31), or transport towards the cell body, such as in the case of transport of virions along filopodia (97, 113). Recent evidence suggests that viruses are capable of inducing the formation of filopodia, in addition to moving along previously formed structures, and this mechanism can act as a means of increasing viral uptake (85, 113, 178). Once a virion has accessed its internalization receptor, endocytosis occurs, and vesicles must cross the thick cortical actin cytoskeleton. Pre-existing pathways involved in endocytosis usually promote passage of endocytic vesicles through this barrier (2); however rearrangement of cortical actin filaments has been observed in virus infected cells, an event that contributes infection efficiency (98, 145). Once internalized, numerous viruses have been demonstrated to utilize the microtubule cytoskeleton and the motor protein machinery for transport from the cell periphery towards the nucleus (122, 168, 195, 196).

In contrast to virus-activated signaling induced for priming of the target cell, another class of responses often occurs in response to virion attachment and

internalization with antiviral results. These signaling events are induced following recognition of certain viral characteristics, such as nucleic acid structure or the repetitiveness of the viral capsid, and can occur at initial attachment, during endocytosis, or following delivery of the virion to the cytoplasm (70). The downstream events associated with this class of signaling are inflammatory in nature, resulting in the production of cytokines/chemokines, recruitment of immune effector cells, or apoptosis of the infected cell (101). Although the factors involved in these pathways can be quite diverse, certain classes of signaling molecules are often associated with these types of responses, including toll-like receptors (TLRs), interferon regulatory factors (IRFs), p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor kappa B (NFκB) (116).

Although some research has been conducted on the signaling events induced by and required for HPV infection, the vast majority is focused on late infection events such as those regulated by early HPV proteins, such as E5, E6, and E7 (111). Several reports have investigated early signaling induced in immune cells, such as dendritic cells (DCs) and Langerhans cells (LCs)(54, 55). Although these studies are intriguing in the context of immune system regulation by HPVs, the importance in infection of the natural host cell type has not been confirmed. Signaling induced upon engagement of the α6 integrin complex during HPV6 VLP binding to epithelial cells has been documented (63, 139). However, due to the controversy surrounding the identification of α6 integrins as receptors for HPVs (52, 69, 86, 177, 179), it remains unclear whether such signaling is induced by other HPV types and what relevance it may have in the context of natural HPV infection of epithelial cells.

Studies with biochemical effectors of the actin and microtubule cytoskeletons implicate both networks in HPV entry and infection (38, 176, 214), although the precise roles each plays in HPV infection has yet to be elucidated. Additionally, identification of a region of the HPV minor capsid protein, L2 that interacts with the microtubule motor protein, dynein, and directs intracellular transport, identifies a role for microtubule trafficking in HPV infection (62, 211).

In this report, we examine early signaling events induced by and required for complete HPV31 infection of human keratinocytes (HKs). Signaling through tyrosine kinases, phosphatidyl inositol-3 kinases (PI3K), and protein tyrosine phosphatases (PTPases) are demonstrated to be required for HPV31 entry, while protein kinase C (PKC) and mitogen activated protein kinase (MAPK) signaling are dispensable. Induction of signaling through PI3K, p38 MAP kinases, and PTPases was also observed. We have begun to elucidate the downstream events associated with these signaling pathways and establish a link between PTPase signaling and microtubule reorganization, which appears to regulate association of HPV31 and lipid rafts at the cell surface. Signaling through PI3K and tyrosine kinases is demonstrated to regulate actin cytoskeleton rearrangement and filopodia induction by HPV31. Finally, HPV31 retrograde transport along filopodia is demonstrated, and induction of and transport along these structures increases uptake of virions from the extracellular matrix into the cell.

Materials and Methods

Cell Culture, Virion Production, and Infections. HaCaT cells are a spontaneously immortalized epithelial line derived from normal adult skin (10). 293T cells are derived

from the human embryonic kidney cell line HEK293 and express SV40 large T antigen. Cells were maintained in DMEM/F12-Ham's Nutrient Mixture containing 10% fetal calf serum, 4X amino acids, 2 mM glutamine, 100U/mL penicillin, and 1 ug/ml streptomycin. HPV31 virions were produced and purified by density gradient centrifugation as previously described (153, 186); HPV31 pseudovirus encapsidating pClNeo-GFP plasmid was produced as previously described (186). Stocks were quantified by blot hybridization to determine viral genome equivalents (vge) per unit volume as reported (131, 132). Transmission electron microscopy (TEM; Hitachi 7500) was performed to visualize virus stocks at 80 kV following binding to a carbon-coated electron microscopic grid and negative staining with 2% uranyl acetate. Infections were performed as previously described (132). Briefly, virion stocks were diluted in media and exposed to HaCaT cells for 1 hour at 4°C with agitation to allow viral attachment. Inocula were aspirated, cells were washed, and refed with fresh media. For inhibitor studies, cells were preincubated with biochemical inhibitors [genistein (Calbiochem; 100uM, 150uM), wortmannin (Calbiochem; 300nM, 450nM) calphostin C (Calbiochem; 500nM, 750nM), PD90859 (Calbiochem; 20uM, 30uM), sodium orthovanadate (Sigma; 100uM, 150uM), cytochalasin D (Calbiochem; 10uM, 5uM), colchicine (Calbiochem; 100nM, 50nM)] for 30 minutes prior to viral attachment. Following attachment, infections were allowed to proceed for 16 hours in the presence of inhibitors, at which point media/inhibitor was removed and replaced with media containing an HPV31-neutralizing antibody (H31.A6)(26) diluted 1:1000. Cells were harvested at 48 hours for total RNA extraction.

RNA Isolation and RT-qPCR Analysis. Total RNA was extracted at 48 hours post infection using Tri Reagent (Sigma). Nucleic acid concentrations were determined by spectrophotometry. Reverse transcription of 2 ug total RNA and triplicate quantitative PCR (RT-qPCR) was performed using GeneAmp RNA PCR reagents and AmpliTaq Gold DNA Polymerase (Applied Biosystems) using primers, probes, and conditions as previously described (131). Error bars represent standard error of the mean (SEM).

Virion Labeling with Fluorescent Dyes. Approximately 1 ug of Alexa Fluor (AF) 594 or AF647 carboxylic acid, succinimidyl ester (Molecular Probes) was mixed with 10^8 virions/pseudovirions and incubated for 1 hour at room temperature. Labeled stocks were washed 3X with 1X HSB (25mM HEPES pH 7.5, 500 mM NaCl, 0.02% Brij58, 1 mM $MgCl_2$, 100uM EDTA, 0.5% ethanol) and recovered using a Millipore Amicon Ultra-4 centrifugation filter to remove unincorporated dye.

Phosphoprotein and Protein Tyrosine Phosphatase Analysis. HaCaT cells were seeded at $1x10^5$ cells/well in 6-well dishes. Cells were fed with serum-free media (SFM) for 48 hours with one media change at 24 hours. HPV31 was diluted to a multiplicity of infection of 100vge/cell in SFM and added to cells in 0.5ml. Equal volumes of 1X HSB diluted in SFM were used for mock-treated controls. Cells were incubated for indicated amounts of time at 37°C with agitation, harvested in SDS-PAGE running buffer, and nucleic acids were removed by passage through QiaShredder columns (Qiagen). Approximately 1/5 of total cell lysate was analyzed by SDS-PAGE and western blot with α-phosphotyrosine (Upstate Biotechnology), α-phospho-Akt Thr308 (Cell Signaling

Technology), α-phospho-p38 MAPK (Cell Signaling Technology), α- Akt (Cell Signaling Technology), α-actin (Sigma). PTPase assays were performed with SensoLyte Protein Phosphatase Assay Kit (AnaSpec) according to manufacturer instructions. Briefly cells were treated as for phosphoprotein analysis and harvested in 1X lysis buffer. Samples were centrifuged to remove cellular debris, supernatants were mixed with pNPP solution, and analyzed by plate reader for absorbance at 405nm.

DRM Association Assay. Association with detergent-resistant microdomains (DRMs) was performed as previously described (186). Briefly, cells were treated with biochemical inhibitors (100uM genistein, 450nM wortmannin, 10uM cytochalasin D, LY294002 (Cell Signaling Technology), 20uM U0126 (Cell Signaling Technology), 150uM sodium orthovanadate, 10uM nocodazole (Calbiochem), 25mM colchicine, 5mM methyl-β-cyclodextrin (Sigma), 1uM latrunculin B (Calbiochem), 750nM calphostin C (Calbiochem), 20uM PD90859 (Calbiochem) for 30 minutes prior to virus attachment. AF594-labeled HPV31 was diluted to 5000 vge/cell in media and bound to cells for 1 hour at 4°C. Virus/media were removed and cells were incubated for 30 minutes at 37°C in the presence or absence of inhibitors. Detergent-soluble domains were washed out and cells were fixed and mounted as described (186).

Visualization of the Actin Cytoskeleton. For phalloidin staining, HaCaT cells were seeded onto coverslips and allowed to attach overnight. HPV31 was diluted to 100 vge/cell in media and added to cells in 0.1ml for indicated amounts of time (mock samples were treated with equal volume of 1X HSB diluted in media for 30 minutes).

Cells were then washed and fixed with 3.7% paraformaldehyde, permeablized with 0.1% TX-100, and stained with AF680 phalloidin (Molecular Probes). Coverslips were mounted with Vectashield+DAPI (Vector Labs). Alternatively, HaCaT cells were transfected with pGFP-actin (Invitrogen) and treated 2 days post-transfection with HPV31 for 30 minutes as in phalloidin staining. Cells were pretreated with inhibitors (100uM genistein, 450nM wortmannin, 150uM sodium orthovanadate) for 30 minutes and inhibitors were present throughout virus treatment. Cells were fixed with 3.7% paraformaldehyde and mounted with Vectashield+DAPI. Photomicroscopy was performed on a Zeiss LSM510 META confocal microscope with a 63X objective. Number of filopodia/microspikes per cell were quantified in a blinded fashion on 20 cells per slide.

Microscopy Time Series. Confocal laser scanning microscopy was performed with a Zeiss LSM510 META system using 63x 1.4 NA oil objective. GFP and AF647 were simultaneously excited using the 488 nm argon line and the 633nm diode laser. GFP emission was collected through 505-530 bandpass filter and the AF647 with a 650 longpass filter. Images were acquired using 2x line averaging at intervals of one image every 4s. Time series were Gaussian filtered with $\sigma=1$ in the x and y dimension for the AF647 channel and a $\sigma=2$ in the x, y, and z dimensions for the GFP channel. PsV particles undergoing transport were tracked using routines written in Matlab (The Mathworks, Inc) and fit to the equation describing active transport $MSD = 2D\Delta t + v^2\Delta t$, where MSD is the mean square displacement, D is the diffusion coefficient, v is velocity and Δt is the time interval.

Fluorescence Recovery After Photobleaching. Confocal laser scanning microscopy was performed with a Zeiss LSM510 META system using 40x 1.3 NA oil objective. AF647 was excited using the 633nm diode laser and emission collected with a 650 longpass filter. Images were acquired using 2x line averaging at intervals of one image every 2s.

Results

HPV31 Entry into HKs Requires Tyrosine Kinase, PI3 Kinase, and Phosphatase Signaling. Previously we demonstrated that HPV31 associates with detergent insoluble, low density lipid rafts prior to caveolar entry into HKs (186). In addition to concentrating GPI-linked proteins, cholesterol, and other molecules such as caveolin into specific regions of the plasma membrane, lipid rafts have also been shown to contain high numbers of signaling molecules (182, 183). Therefore, we hypothesized that HPV31 infection might induce and require cellular signaling for infection. To assess the role that signaling plays in internalization, the effects of several well-characterized inhibitors of common signaling pathways on HPV31 infection of HKs were investigated (Figure 4.1A). After virus exposure in the presence these kinase inhibitors for 16 hours, the chemicals were removed, a neutralizing antibody to HPV31 was added, and infection was allowed to proceed for an additional 32 hours. This experimental design was used to minimize cytotoxic effects of the chemicals. Additionally, as the HPV31-neutralizing antibody functions by sequestering virions at the plasma membrane and cannot access intracellular particles (186), any associated block in infection under these conditions

would result from chemical inhibition of virion internalization. Signal inhibition of PKC by calphostin C (green inverted triangles) and ERK1/2 by PD90859 (blue diamonds) had no effect on HPV31 entry into HKs (Figure 4.1). Conversely, treatment with genistein to block tyrosine kinases (black boxes), wortmannin to prevent PI3 kinase activity (red triangles), and Na_2VO_3 to inhibit PTPases (yellow circles) all resulted in dose-dependent inhibition of HPV31 internalization.

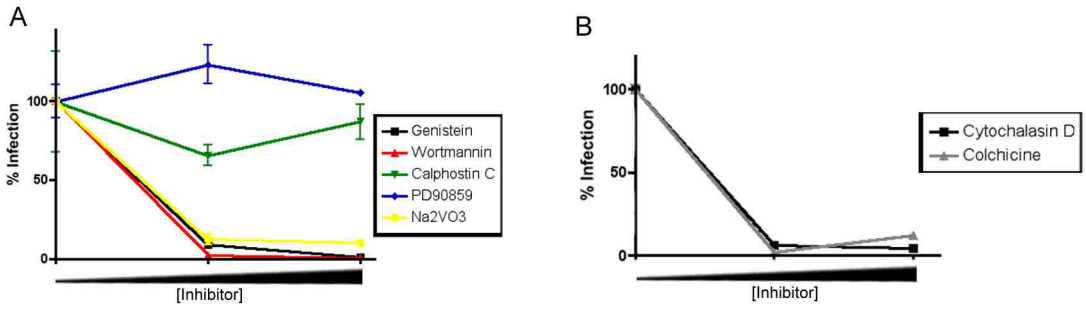

Figure 4.1 HPV31 Infection of HKs Requires Cellular Signaling and Cytoskeletal Reorganization

(A) HaCaT cells were pretreated and infected with HPV31 in the presence of 100uM, 150uM genistein (black boxes), 300nM, 450nM wortmannin (red triangles), 500nM, 750nM calphostin C (green inverted triangles), 20uM, 30uM PD90859 (blue diamonds), or 100uM, 150uM Na2VO3 (yellow circles). Infections were allowed to proceed for 16 hours in the presence of inhibitor, then inhibitors were removed, and cells were refed with media + neutralizing antibody (H31.A6; 1:1000). Infections were then allowed to proceed for 48 hours before RNA isolation to ensure effects of inhibitors reflected changes in virus internalization. (B) HaCaT cells were pretreated and infected with HPV31 in the presence of 5uM, 10uM cytochalasin D (black boxes) and 50nM, 100nM colchicine (grey triangles), and infections were conducted as in (A). Total RNAs were subjected to RT-qPCR in triplicate to quantify spliced HPV31-E1^E4 transcripts indicative of infection (48h pi).

HPV31 Internalization Requires Rearrangement of the Actin and Microtubule Cytoskeletal Networks. Rearrangements of the actin and microtubule cytoskeletal networks are frequently involved in endocytosis of ligands and viruses (2, 152, 156). Given that many viruses, including the closely related polyomaviruses (48, 145), have been demonstrated to require rearrangement of the actin and microtubule cytoskeletons

for internalization and infection, we investigated the role of these networks in HPV31 infection of HKs (Figure 4.1B). Treatment with agents that prevent actin polymerization (cytochalasin D; black boxes) or microtubule polymerization (colchicine; grey triangles) using the same experimental design described in the previous section revealed that rearrangements of both networks play important roles in HPV31 internalization.

HPV31 Binding to HKs Induces Early Tyrosine Kinase, PI3K, p38 MAPK, and PTPase Signaling. To determine the signaling events induced during HPV31 interaction with HKs, cells were exposed to HPV31, and protein phosphorylation levels were analyzed for a variety of common signaling molecules. Given reports of tyrosine kinase activation upon HPV6 VLP exposure to epithelial cells (139), as well as the demonstrated importance of this class of signaling in many other viral

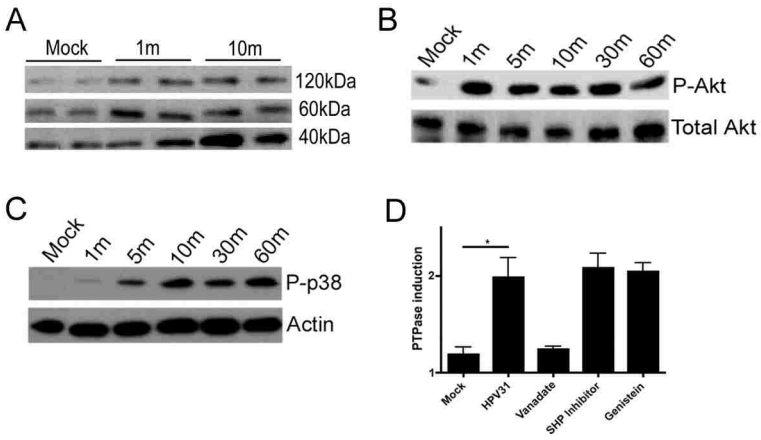

Figure 4.2 HPV31 Exposure to HKs Induces Phosphotyrosine, p38 MAPK, Phospho-Akt, and PTPase Signaling
(A-C) HaCaT cells were serum starved for 48 hours before treatment with HPV31 in SFM at a dose of 100 vge/cell for indicated amounts of time. Mock samples were treated with equal volume of 1XHSB diluted in SFM. Lysates were harvested in SDS-PAGE loading buffer and analyzed by SDS-PAGE and western blot for (A) Phosphotyrosine positive proteins (B) Phospho-Akt Thr308 (top) and total Akt (bottom), or (C) Phospho-p38 (top) and actin (bottom). (D) HaCaT cells were serum starved for 48 hours before exposure to HPV31 in SFM at a dose of 100 vge/cell in triplicate for 30 minutes at 37°C. Mock samples were treated with equal volumes of 1XHSB in SFM. For inhibitor treatments, samples were pretreated for 30 minutes at 37°C and HPV31 treatment was conducted in the presence of inhibitors (vanadate 150uM, SHP1/2 10uM, genistein 100uM). PTPase activity was measured using the Sensolyte Protein Phosphatase Assay Kit. Statistical significance was achieved with $p<0.01$(**).

infections (70), we sought to investigate whether tyrosine kinase signaling was activated upon HPV31 interaction with HKs (Figure 4.2A). After treatment with HPV31 for 1 and 10 minutes at 37°C, cells were lysed and analyzed by α-phosphotyrosine western blot. As compared to mock-treated cells, increased phosphotyrosine signaling was observed on at least three proteins at these time points at with molecular weights of approximately 40, 60, and 120kDa. This induction is consistent with the requirement for tyrosine kinase activity previously observed (Figure 4.1A). Although the exact nature of each of these proteins has yet to be defined, several classes of common signaling molecules exist as potential candidates, including growth factor receptors (120kDa), Src family kinases (60kDa), and MAP kinases (40kDa), and continued research is needed to identify these factors. Western blot analysis of lysates from HPV31-exposed cells also revealed strong induction of phospho-Akt and phospho-p38 MAPK signaling as early as 1 minute, and activation was prolonged, lasting as long as 1 hour (Figure 4.2B, C). The induction of P-Akt (a downstream effector of the PI3K pathway) is consistent with the requirement for PI3K activity we observed during the HPV31 entry/infectivity assay (Figure 4.1A). No changes in p44/p42 MAPK and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling were observed following HPV31 treatment at early time points (data not shown). Consistent with the requirement for PTPase signaling during HPV31 internalization (Figure 4.1A), induction of PTPase activity was observed following HPV31 treatment for 30 minutes (Figure 4.2D). As expected, HPV31 treatment in the presence of vanadate, an inhibitor of PTPase activity, abrogated this activation. Neither tyrosine kinase nor SHP1/2 activity were necessary for the PTPase induction observed, as treatment with inhibitors of these pathways had no effects.

HPV31-Lipid Raft Association is Dependent on PTPase activity and the Microtubule Network. Lipid rafts are detergent insoluble, low density domains present within the cellular plasma membrane. These regions are considered signaling centers, as many classes of signaling molecules can be observed concentrating within them, effecting activation and signal transmission within the cell. Additionally, caveolae-dependent endocytosis is initiated from within these domains (14, 182, 183). We previously demonstrated that upon initial interaction with the plasma membrane, HPV31 does not associate with lipid rafts. However, as early as 30 minutes following incubation at 37°C, the bulk of plasma membrane bound HPV31 particles migrate to DRMs and are resistant to Triton X-100 (TX-100) washout (186). Thus this association appears to require an active event, and is likely dependent on signaling events and/or cytoskeletal rearrangements within the cell. To investigate this possibility, the TX100 washout assay was performed in the presence of a variety of biochemical inhibitors to assess their effects on the localization of fluorescent HPV31 to DRMs (Figure 4.3). Consistent with previous observations at 30 minutes post-attachment (186), AF594-HPV31 signal was retained following TX-100 washout (Figure 4.3A). This retention was dependent on cholesterol, as treatment with MβCD, a compound that sequesters cholesterol from the plasma membrane, resulted in the loss of signal (Figure 4.3B). Treatment with disruptors of the microtubule network (nocodazole, colchicine; Figure 4.3C, D) resulted in loss of the fluorescent signal as well, indicating that rearrangements of the microtubule cytoskeleton contribute to HPV31 association with lipid rafts. However, disruption of the actin network (cytochalasin D, latrunculin B; Figure 4.3E, F) had no effect on HPV31-DRM association. Similarly, inhibition of a variety of signaling pathways did not affect

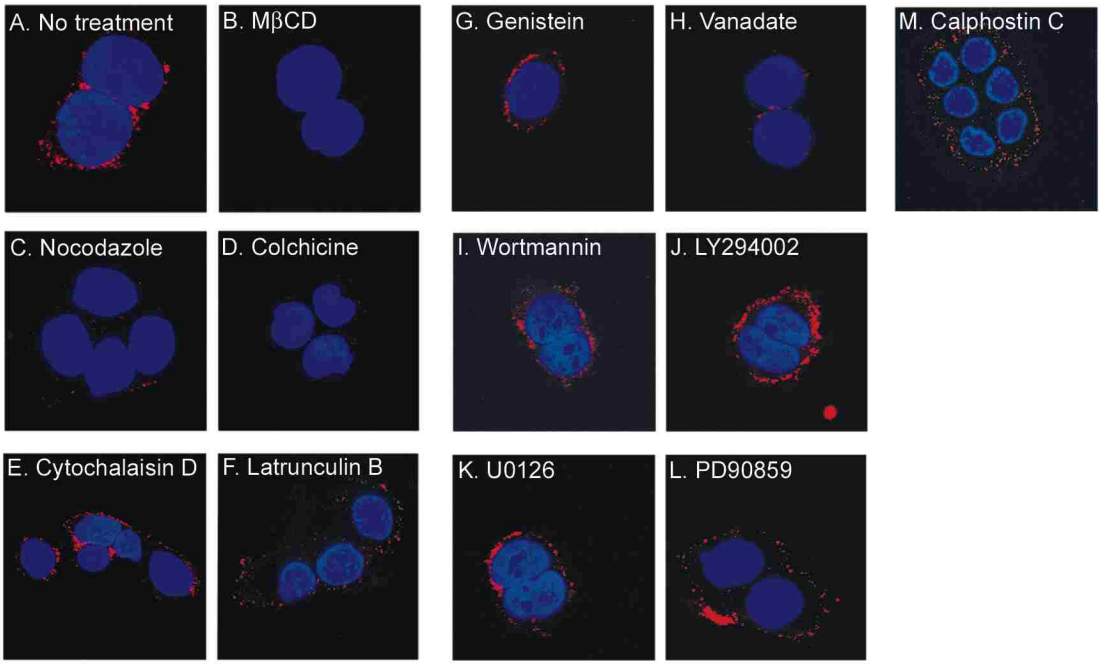

Figure 4.3 HPV31-DRM Association Requires PTPase Activity and Reorganization of the Microtubule Cytoskeleton

HaCaT cells were pretreated with (B) methyl-b-cyclodextrin [5mM] (C) nocodazole [10uM], (D) colchicine [25mM] (E) cytochalasin D [10uM], (F) Latrunculin B [1um] , (G) genistein [100uM], (H) sodium orthovanadate [150uM], (I) wortmannin [450nM], (J) LY294002 [50uM], (K) U0126 [20uM], (L) PD90859 [20um], and (M) calphostin C [750um] for 30 minutes at 37°C. AF594-HPV31 was bound to cells for 30 minutes at 37°C in the presence of inhibitors. Cells were then incubated in cold 1% TX100 for 10 minutes on ice before fixation. Coverslips were mounted with Vectashield+DAPI on slides and photos were taken on a Zeiss META confocal microscope with a 63X objective.

HPV31 localization to DRMs, including tyrosine kinase (genistein; Figure 4.3G), PI3K (LY294002, wortmannin; Figure 4.3I, J), MAPK (U0126, PD90859; Figure 4.3K, L), and PKC (calphostin C; Figure 4.3M). In contrast, inhibition of PTPase activity (vanadate; Figure 4.3H) had a profound effect on retention of AF594-HPV31 signal following TX-100 washout, suggesting that this signaling pathway directs DRM association, possibly through regulation of the microtubule cytoskeleton. Together these results demonstrate that the tyrosine kinase, MAPK, PI3K, and PKC signaling pathways, as well as actin

rearrangements, are not required for HPV31-DRM association. However, PTPase signaling and reorganization of the microtubule cytoskeleton do play important roles in localization of HPV31 to detergent-insoluble regions of the plasma membrane, consistent with the requirements for these events during infectious entry of HPV31 (Figure 4.1A, B).

Induction of Filopodia in HKs by HPV31 is Tyrosine Kinase and PI3K Dependent. Given that HPV31 infection is dependent on reorganization of the actin cytoskeleton (Figure 4.1B), it is probable that changes in the actin cytoskeleton would be observed following interaction of HPV31 and HKs. Therefore, HKs exposed to HPV31 at 100 vge/cell for indicated times were stained with AF680-phalloidin to visualize the actin network (Figure 4.4A). Untreated cells revealed a strong cortical actin network, stress fibers, and very few filopodia (Figure 4.4A; mock). After HPV31 incubation with HKs for short periods of time (Figure 4.4A; 1m, 5m), the cortical actin network and stress fibers appeared to break down, and at later time points (10m, 30m), filopodia structures were clearly observed. To understand the signaling pathways regulating filopodia induction by HPV31, GFP-actin transfected cells were visualized by microscopy following HPV31 exposure for 30 minutes in the presence of various kinase inhibitors (Figure 4.4B). Both genistein and wortmannin prevented filopodia activation by HPV31 exposure, suggesting that tyrosine kinase and PI3K activities regulate the actin cytoskeleton rearrangements leading to formation of filopodial structures. The PTPase inhibitor, vanadate, had no effect on filopodia activation. Quantitation of the number of filopodia/cell in blinded specimens confirmed the requirement for tyrosine kinases and PI3K during HPV31 filopodia induction (Figure 4.3C).

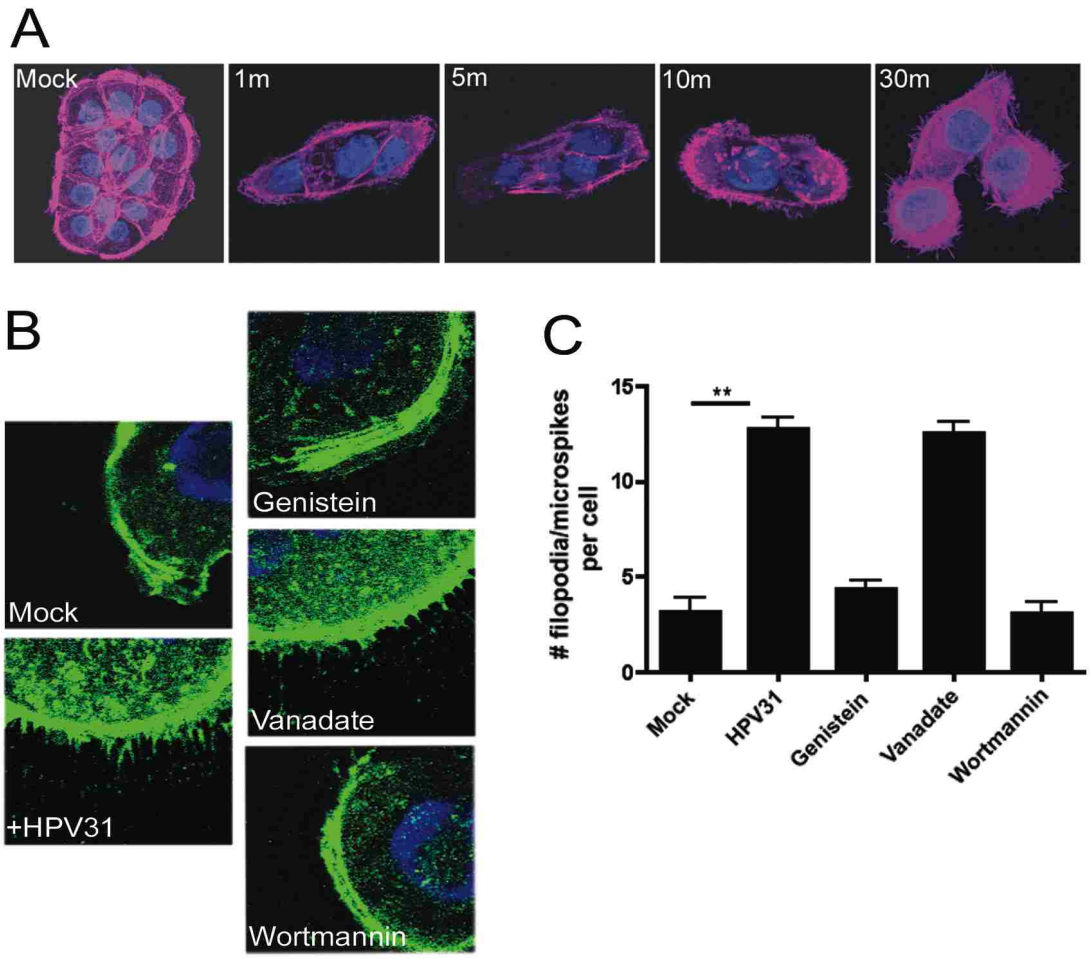

Figure 4.4 HPV31 Induced Filopodia Formation Requires PI3K and Tyrosine Kinase Activity

(A) HPV31 was added to HaCaT cells seeded onto coverslips for indicated amounts of time at 37°C. Cells were fixed, permeablized, and stained with AF680-phalloidin to visualize the actin cytoskeleton. Slides were mounted with Vectashield+DAPI and conocal microscopy photos were taken on a Zeiss META confocal microscope with a 63X objective. (B) GFP-actin transfected HaCaT cells were treatedwith HPV31 in the presence or absence of inhibitors of tyrosine kinase (genistein; 100uM), PTPases (vanadate; 150uM), or PI3K (wortmannin; 450nM) for 30 minutes at 37°C. Slides were mounted with Vectashield+DAPI and conocal microscopy photos were taken on a Zeiss META confocal microscope with a 63X objective. (C) Blinded quantitation of number of filopodia per cell

Retrograde Transport of HPV31 on Filopodia Facilitates Virion Uptake From the Extracellular Matrix. Upon exposure of cells to HPV31 virions, the large majority of virus is observed attached to extracellular matrix (ECM) deposited around the cell (32, 33)(Figure 4.5A, B). In the context of natural HPV infection, virion binding to ECM is relevant to the wounding environment as high levels of ECM are deposited during the wound healing process (124). Laminin 5 (LN5), a structure implicated in HPV attachment to the ECM, is one such component (33). Furthermore, attachment to ECM factors may represent a mechanism of concentrating virions near target cells, the basal cells of the epithelia. Nonetheless, this event would appear inefficient at first glance, especially if virions are unable to access the cellular plasma membrane following ECM attachment. This activity would be of little advantage to the virus unless there was an efficient means of transfer of virions from the ECM to the cellular membrane. Such transfer could be achieved in at least ways: (a) motility of virions on the ECM; or (B) cellular movement over the ECM. To investigate the mobility of ECM-bound virions, fluorescence recovery after photobleaching (FRAP) was performed on ECM-bound AF647-HPV31 pseudovirions (Figure 4.5A). A bleached region of ECM-bound PsV failed to recover after photobleaching, indicating that following attachment, virions remain immobile and are unable to move from the ECM to access the plasma membrane.

Given that exposure of HKs to HPV31 results in the induction of filopodia (Figure 4.4), we investigated whether movement of these structures over ECM-bound virions could represent a mechanism for increasing viral uptake from the ECM. First, we observed the pattern of ECM-bound AF594-HPV31 over time by microscopy, hypothesizing that changes in this pattern would occur if virions were being taken up

through filopodia structures. At early time points, a continuous pattern of ECM-bound virions was observed immediately adjacent to the plasma membrane (Figure 4.5B; 30m, 2h). However, by 4 hours post attachment, breaks in the ECM pattern began to appear (Figure 4.5B, 4h). As virions bound to the ECM are immobile (Figure 4.5A), this loss of ECM-bound virions support a model in which filopodial projections induced by a subset of virions activating at the plasma membrane facilitate uptake of virions bound to the ECM.

Transport of viruses along filopodia prior to entry has been reported for several viruses, including murine leukemia virus (MLV), African swine fever virus (ASFV), and vaccinia virus (VV)(85, 97, 113). Given that HPV31 causes strong induction of filopodia (Figure 4.4) and that this event is a possible mechanism for increasing viral uptake from the ECM, the possibility that transport along filopodia of HPV31 was investigated using live video confocal microscopy. In these experiments, HPV31 pseudovirus (PsV) was used to prevent contamination of microscopy equipment with infectious virus. A431 cells stably expressing eGFP-ErbB1, which facilitates visualization of the plasma membrane and filopodial projections(13), were exposed to AF647-labeled HPV31 PsV and monitored over time. High levels of transport along filopodial projections were observed of AF647-HPV31 PsV towards the cell body (Figure 4.5C). Calculation of virion velocity along filopodia revealed a speed of 40nm/s $\pm$ 15nm/s, a speed higher than that reported for retrograde actin flow transport of other ligands (i.e., epidermal growth factor receptor)(100), suggesting that recruitment of a motor protein and/or increased actin flow could be occurring. Taken together, the induction of filopodia, disappearance of ECM-bound virus over time, and active retrograde transport on filopodial projections indicate

that movement of HPV31 virions on filopodia could represent a mechanism by which ECM-bound virions are able to access the plasma membrane of target cells.

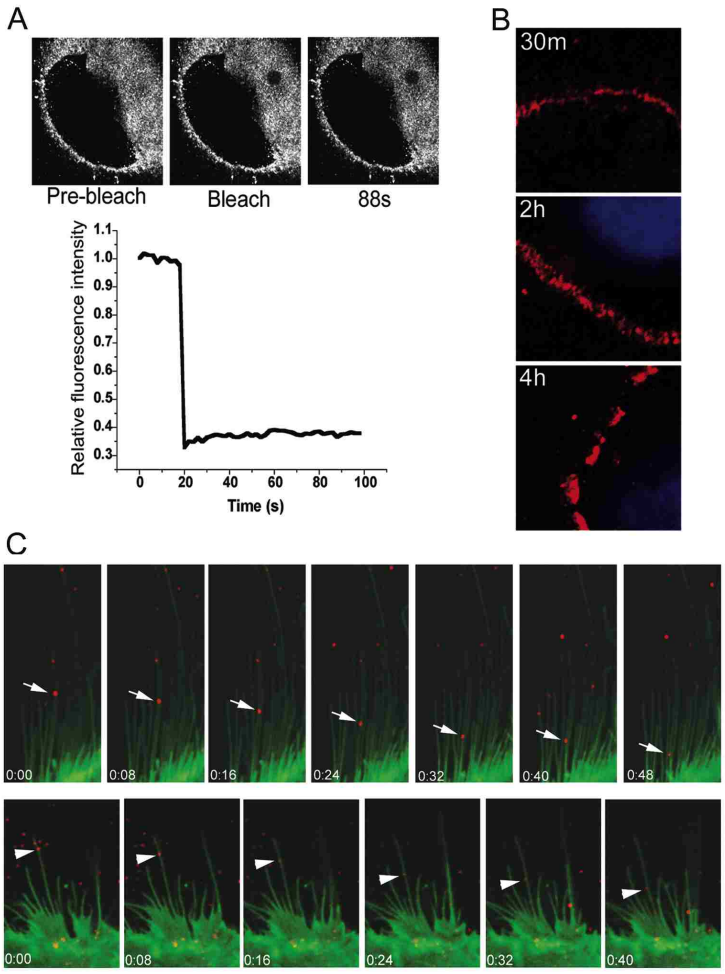

Figure 4.5 Transport of HPV31 on Filopodia Increases Uptake from the Extracellular Matrix

(A) Fluoresence Recovery After Photobleaching (FRAP) studies were conducted using AF647-HPV31 pseudovirus bound to the ECM of A431 cells. Photobleaching was performed by turning laser to full power. Following photobleaching, the affectedarea was monitored for fluorescence recovery. Quantitation is represented as fluorescence within bleached area versus time and normalized to pre-bleach intensity. (B) AF594-HPV31 virions (red) were bound to HaCaT cells and incubated at 37°C for indicated times. Cells were fixed and mounted with Vectashield+DAPI. Photos were taken with a 63X objective below the plane of the cell to visualize only ECM-bound virus. (C) AF647-HPV31 PsV (red) was bound to eGFP-ErbB1-A431 cells and monitored over time. Microscopy visualization was performed on a Zeiss LSM 510 META microsope.

Discussion

In this report, we have investigated the induction of and requirement for signaling events and cytoskeletal rearrangements during initial HPV31 attachment and entry into human keratinocytes. Activation of PI3K, tyrosine kinase, and PTPase signaling pathways was found to be required for HPV31 internalization, as well as reorganization of both the actin and microtubule cytoskeletons. Additionally, the activation states of several common signaling pathways were investigated, and induction of tyrosine kinase, PI3K, p38 MAPK, and PTPases was observed. We have begun to elucidate the downstream events associated with activation of these pathways. It was determined that activation of PTPases, as well as rearrangement of the microtubule cytoskeletal network, both contribute significantly to the localization of HPV31 virions to lipid raft domains. Given that localization within lipid rafts is necessary for caveolar entry, the function of PTPases and microtubules in regulating virus-lipid raft association is consistent with the requirement for these events for HPV31 internalization that were observed (Figure 4.1A, B).

Virus utilization of filopodia and other membrane associated cytoskeletal projections for targeting and infecting cells has become a popular field of study (85, 97, 113). In this report, we have demonstrated that HPV31 induces filopodia formation in human keratinocytes shortly after attachment, and that this induction is dependent on PI3K and tyrosine kinase activity. The binding of PVs to deposited extracellular matrix appears at first to be an inefficient method for virions to target host cells, especially considering that ECM-bound virus is immobile and appears unable to access the cellular membrane. However, given that HPV31 not only activates filopodia formation but also

is able to traffic along these projections towards the cell body, this transport emerges as a mechanism for increasing viral uptake from the ECM. Finally, the changes in the pattern of ECM-bound virus observed further support a model in which transport on filopodia represents a major route for viral uptake.

The results presented herein demonstrate an important role for cellular signaling and cytoskeletal reorganization in infectious entry of HPV31. Figure 4.6 illustrates the signaling pathways elucidated in this report and the established downstream effects as well as putative events that have yet to be examined. Our data support a model in which, upon immediate attachment to HKs, HPV31 induces PTPase signaling that promotes rearrangement of the cellular microtubule network (Figure 4.6A). These events are required for directing virion-detergent resistant microdomain association (Figure 4.6B). Further research must be performed to define the specific factors being activated and regulating microtubule rearrangements.

Following localization of virions to lipid rafts, several cellular signaling pathways are activated, including the p38 MAPK cascade (Figure 4.6C). The downstream events associated with this signaling are still under investigation. However, given the role that this signaling pathway commonly plays in cellular antiviral responses (157, 198), it is likely that this event results in expression of apoptotic and/or inflammatory factors, perhaps through NFκB activation. As the understanding of cellular signaling in response to HPV infection of relevant host cell types remains largely undefined, these data represent an exciting starting point for initiating this area of research.

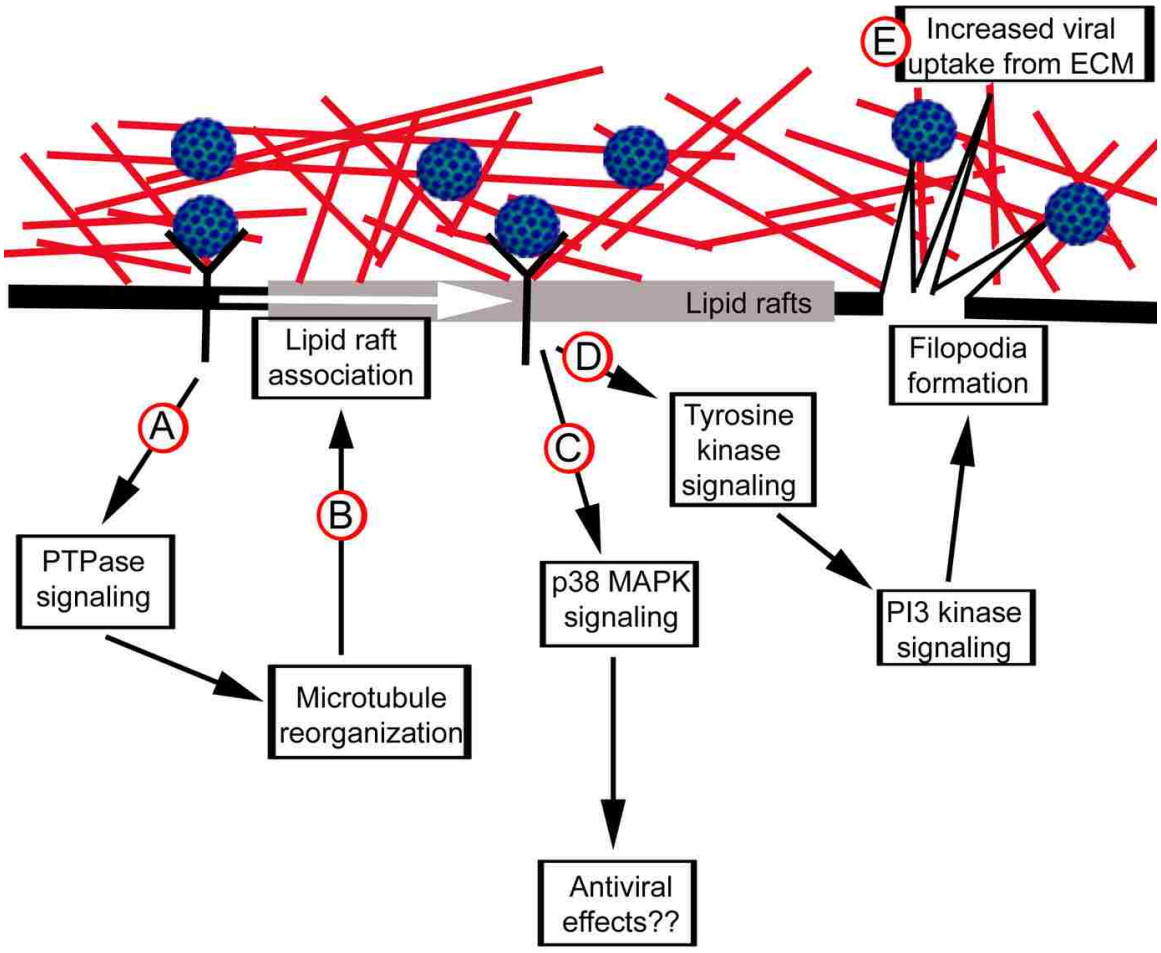

Figure 4.6 Model for HPV31-induced cellular signaling events and downstream events
(A) Upon initial attachment to the plasma membrane of HKs, HPV31 induces PTPase signaling that results in microtubule reorganization. (B) This reorganization of the microtubule network promotes localization of HPV31 virions to detergent-resistant lipid rafts. HPV31 virions present in lipid rafts induce several cellular signaling events, including p38 MAPK activation (C). The downstream effects of this cascade are currently under investigation; however induction of antiviral effects is suspected. Activation of tyrosine kinase and PI3 kinase signaling is also observed (D). These events are required for actin cytoskeletal reorganization that results in the formation of filopodial projections at the cell surface. (D) These actin structures function by increasing uptake of HPV31 virions bound to the extracellular matrix

In addition to p38 MAPK signaling, both tyrosine kinase and PI3K pathways are upregulated following virion localization to lipid rafts. Initiation of tyrosine kinase

signaling, likely through a membrane-associated receptor tyrosine kinase (RTK), appears to be acting upstream of PI3K signaling, which in turn regulates a massive reorganization of the actin cytoskeleton (Figure 4.6D). Although changes in the actin network are widespread throughout the cell, the formation of filopodial projections is the most profound change that was observed. Further elucidation of the role that these projections play in viral infection revealed that retrograde transport of virions along filopodia may represent an important mechanism for increasing uptake of virions bound to cell-deposited ECM (Figure 4.6E). These data are especially interesting when considered in the context of natural HPV infection. Several reports have recently demonstrated that PVs commonly associate with ECM factors prior to entry into host cells (32, 33, 39), and it is interesting to speculate on the how this association facilitates concentration of virions near target cells, upregulation of infection of wounded epithelium, and infection of cells that can support productive infection. Data presented in this report supports a model in which the induction of filopodia by HPV31 and subsequent transport along these structures plays a vital role in transfer of virus from the ECM to the plasma membrane for cellular entry and infection.

Chapter V

Discussion and

Future Directions

Human papillomaviruses significant infectious agents, due to their strong association with cervical cancer, as well as the role these viruses play in various other epithelial cancers (8, 68, 83). Cervical cancer is the second most common cancer killer of women worldwide, and causes approximately 300,000 deaths per year (136). This sobering figure reflects the importance of continuing the pursuit of knowledge within the field of cervical cancer research. Although progression to cervical cancer involves the contribution of numerous cofactors, infection with high-risk HPV of the cervical tissue is considered to be a necessary cause of cervical carcinoma (19). Therefore, a comprehensive understanding of HPV life cycle, infectivity requirements, and transmission characteristics is of crucial importance for combating the effects of this important human pathogen.

The development of two VLP-based vaccines targeting several types of high-risk HPVs have shown great promise as effective weapons against HPV infections and associated disease. High immunogenicity, safety, and long-term protection against development of cervical neoplasia are all characteristics of these vaccines that have been reported. Clinical trials have demonstrated nearly 100% effectiveness against formation of HPV-related precancereous lesions, an efficacy rarely reported for vaccines (4, 76, 91, 204). Unfortunately, some obstacles do exist for effective distribution of these vaccines. Due to the high prevalence of high-risk HPV infection in the sexually active population, administration to pre-adolescents is crucial for relaying protection before infection occurs, as no therapeutic consequences of the vaccines have been demonstrated. The politics surrounding vaccination of young girls against a sexually transmitted disease are complex. Additionally, for HPV vaccines to be really effective in decreasing the

population of infected individuals, vaccination of the male population must be performed as well to decrease transmission levels. Male cancers associated with HPV infection are rare, and vaccinating against a disease that is essentially asymptomatic in one group of the population also poses political difficulties. By far, the most problematic aspect of the HPV vaccine is cost. With the series totaling over $300, the possibility of combining the treatment in with other childhood vaccines or requiring vaccination for school enrollment becomes complicated. In addition to the complexity of effective HPV vaccination that is faced by the first world, distribution of the HPV vaccine to populations in the third world faces even more difficulties. Minimal screening practices in developing countries for early signs of cervical disease have resulted in the growth of cervical cancer to incredible proportions, and cervical cancer deaths in developing countries account for >80% of cervical cancer deaths worldwide. Unfortunately, the development of the new HPV vaccines may end up being of little consequence to women of these countries, due to the high cost (3, 7, 42, 162). Therefore, continued research on HPVs must be performed to continue expanding our knowledge of these viruses and contribute to the development of more affordable and accessible prophylactics and antivirals.

The differentiation-dependent life cycle of HPVs is an interesting, yet frustrating, aspect of HPV biology. For decades, production of complete HPV virions in standard cell culture as well as detection of infection were hindered by this characteristic of HPVs, and the understanding of early events of HPV infection has suffered. Because of the difficulty associated with studying HPVs, many researchers have relied on experimentation with VLPs and PsVs, which often require the use of cell types of questionable relevance to HPV biology. Thus confusion surrounding early HPV

infectious events exists. Fortunately, a relatively new technique for producing infectious HPV virions in culture has been established (17, 153). This method allows for production of significantly higher yield and greater purity preparations of infectious HPV than has ever been possible. Moreover, all research conducted to characterize the infectious qualities of these virions have concluded that virus produced in this system is indistinguishable from authentic virus produced by differentiation-based methods (35, 81). The body of work presented here details the modification of this system to produce infectious HPV31 particles that display similar infectivity properties. Using these authentic virus preparations, all experiments have been conducted in cell types relevant to natural HPV infection in order to present a clearer understanding of early events during HPV31 infection that can enhance our knowledge of natural HPV infection.

The field of virus entry and trafficking within host cells has blossomed in recent years, increasing our appreciation of how viruses manipulate host cells considerably. As more work is conducted, the incredible complexity of this area of research becomes apparent. For example, where one mode of entry, clathrin-dependent endocytosis, was once believed to be the primary route of virus internalization, we now know of numerous mechanisms, and many are yet to be defined. Similarly, the definition of cellular trafficking pathways has changed considerably, and the complexity of these routes only becomes more apparent as we understand more about how viruses utilize them. In this report, caveolar-dependent entry was demonstrated for HPV31 internalization into HKs, a surprising finding given that HPV16 appears to use a distinct route to enter the same cells. The implications of two such closely related viral types using separate internalization pathways are interesting, and more research must be conducted to

understand the aspects of early viral attachment, whether they be receptor usage or some other early event, dictate this disparity. Nonetheless, the differences emphasize that studying one viral type and assuming its properties apply to all other HPV types can be reckless. Monitoring of HPV31 virions during entry into HKs has revealed a unique infectious trafficking route, not previously reported for any other virus. After internalization, HPV31 moves to the caveosome before Rab5-dependent transport to the endosomal pathway. Although SV40 circulates between the caveosome and early endosome, this does not appear to be its infectious route, and it traffics back to the caveosome before transport to the ER (142). Similarly, JC virus uses Rab5-dependent circulation between the early endosome and caveosome for infection. This transport originates in clathrin pits, and the virus eventually traffics to the ER through caveosomes (155). Thus the internalization *via* caveolae, followed by localization to caveosomes, Rab5-dependent transport to early endosomes, and procession through the endosomal pathway by HPV31 represents a novel route and yet another example of viruses manipulating existing host cell trafficking pathways for infection. Interestingly, despite the distinct internalization routes taken by HPV31 and HPV16 into HKs, the two types both traffic through the endosomal pathway (38). Research presented herein demonstrates that HPV31 requires the decreasing pH of the endosomal pathway for proper infection of HKs, and that this low pH contributes to changes in the viral capsid that likely lead to viral uncoating and/or endosomal escape. The parallel trafficking through endosomes and requirement for endosomal acidification observed for HPV16 (Campos and Ozbun, unpublished observations)(38) suggests pH-induced changes in the viral capsid could be a universal requirement for HPVs.

During caveolar entry into HKs, HPV31 associates with lipid rafts, an event that results in several classes of signal induction. Signaling by HPV31 at the plasma membrane, in some cases, appears to facilitate entry of virions, whether by regulation of the microtubule cytoskeleton to promote localization to internalization compartments or by inducing changes in the actin cytoskeletal network that facilitate uptake of virus from the extracellular matrix. Although we have not defined the downstream effects of signaling through p38 MAPK that were observed, this class of signaling is generally associated with the antiviral response of infected cells (116, 198). High levels of cellular signaling through HPV31-lipid raft association could provide some insight into the consequences of HPV31 using this particular internalization pathway. Although many aspects of HPV biology likely contribute to the differences in distribution of HPV types within the human population, the differences we have observed between HPV31 and HPV16 in terms of internalization route and lipid raft association may contribute to the fact that HPV16 is so much more prevalent (40). The signaling induced by HPV31 attachment and entry, both through p38 MAPK and the other pathways we have identified, could contribute to more robust immune detection and more efficient clearance of this type.

One of the most exciting components of the research presented within this report is the signal-dependent induction of actin cytoskeletal structures in HKs by HPV31. Upon exposure to HKs, HPV31 transmits a signal through tyrosine kinases and PI3 kinases that induces filopodia formation at the cell surface. Given that the vast majority of HPV31 particles adhere to the ECM deposited by the cell rather than to the plasma membrane, it was intriguing to find that (a) virions bound to the ECM are immobile, and

(b) the pattern of ECM-bound virus changes over time. Together, the data suggest that the induction of filopodia upon HPV31 exposure at the plasma membrane is a mechanism used by the virus to increase uptake of virions from the ECM that would otherwise be unable to access the plasma membrane. Finally the finding that virion transport occurs towards the cell body along filopodial projections further supports a model in which virus actively promotes the formation of and utilizes these structures to increase infection efficiency. Although virus transport along filopodia is currently a popular topic, only a few reports have demonstrated extracellular virus utilization of the actin cytoskeleton for active transport (85, 97, 113), although it is likely that such a mechanism may be used by a wide variety of viruses.

Due to the previously mentioned difficulties associated with studying HPVs, the field of HPV internalization and trafficking within host cells remains largely open for exciting new research. With the advent of new technology to produce and study HPVs, combined with many interesting new techniques being applied to virological study, much is yet to be learned about HPVs, how they contribute to disease, and what can be done to combat infection. The work presented here focuses almost exclusively on HPV31 but has introduced some fascinating questions concerning the different mechanisms used by distinct viral types during early infection. Expansion of the research presented here into HPV16 and other high-risk types to discover whether these types induce cellular signaling similar to or divergent from HPV31, cause the formation of filopodia or other cytoskeletal structures, and use a parallel mechanism for viral uncoating and endosomal escape will inform our understanding of how related types of HPV have evolved and how

these differences and similarities relate to the divergent roles played in HPV-induced disease.

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