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ABSTRACT

Infections by high-risk human papillomaviruses (HPV) are the main causative agents for the development of cervical cancer. HPVs are uniquely associated with and dependent on host epithelial stratification and differentiation for completion of the viral life cycle. As with other non-enveloped viruses, HPVs are taken up by the cell through endocytosis following primary attachment to the host cell. Through studies using recombinant pseudovirus particles (PsV) many host cellular proteins have been implicated in the process. Data reported in literature suggest there are structural and functional differences between recombinant and tissue-derived HPV particles. PsV particles are produced by over-expression of the viral capsid proteins in monolayer cells and thus bypass the need for stratified and differentiated epithelial tissue. Knowledge of attachment and entry by tissue-derived HPV particles is lacking due to the more efficient production and ease of use of PsV particles.

The objective of this thesis was to investigate some of the cell surface interactions reported to play a role in attachment and entry, using tissue-derived HPV particles. Further, we wanted to explore whether some of the most common cancer-causing HPV types shared the same requirements. Glycosaminoglycans (GAGs), especially heparan sulfate (HS), are the primary attachment receptor mediating infection by HPV PsV. Here we show that native HPV particles produced in a differentiated epithelium have developed different strategies to infect the host. In biochemical inhibition assays using purified GAG polysaccharides and GAG-negative cell, the four most common cancer-causing HPV types, HPV18, HPV31, and HPV45 were largely dependent on GAGs to
initiate infection. However, their specificities varied. In contrast, HPV16 could bind and enter through a GAG-independent mechanism. Infections of primary human keratinocytes, natural host cells for HPV infections, supported our conclusions.

The proprotein convertase furin cleaves the N-terminus of the minor capsid protein L2 post-attachment to host cells and is required for infectious entry by HPV PsV. In contrast, using biochemical inhibition by a furin inhibitor and furin-negative cells, we show that infecting with tissue-derived HPV16 native virus (NV) was independent of cellular furin. We show that HPV16 L2 may be cleaved during virion morphogenesis in differentiated tissue. In addition, HPV45 was also not dependent on cellular furin for infection, but infecting with two other alpha papillomaviruses, HPV18 and HPV31, were dependent on the activity of cellular furin.

We extended our analysis of GAG- and furin-dependence to HPV16 molecular variants. HPV16 variants of non-European origin are associated with an increased risk for the development of cervical cancer. Our data suggests that HPV16 variants, similarly to different HPV types, have different specificities for interactions with cellular molecules for infection of the host.

Importantly, current HPV vaccines target the major capsid protein L1 of HPV16 and HPV18, which together account for 70% of cervical cancer cases, protecting against virus infection and development of neoplasias. However, since current vaccines are type-specific, they do not offer protection against all cancer-causing HPV types. In addition, they are cost-prohibitive to most women around the world. Thus, there is a need for the development of less expensive alternatives, such as universal microbicides in addition to
the current vaccines. We tested carrageenan, a polysaccharide that has microbicidal activities against HPV PsV particles by competing with virus-GAG interactions. Similarly to the different GAG-specificities of the various tissue-derived HPV types, they displayed differential susceptibilities to carrageenan.

Our data demonstrate that ordered maturation of papillomavirus particles in a differentiating epithelium may alter the virus entry mechanism. This study should facilitate a better understanding of the attachment of and infection by the main oncogenic HPV types. Further, our work sets a framework for future research on attachment and entry by tissue-derived particles and for the development of inhibitors of HPV infection.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Asian American</td>
</tr>
<tr>
<td>Af</td>
<td>African</td>
</tr>
<tr>
<td>As</td>
<td>Asian</td>
</tr>
<tr>
<td>BPV</td>
<td>Bovine papillomavirus</td>
</tr>
<tr>
<td>C or Cys</td>
<td>Cystein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulfate</td>
</tr>
<tr>
<td>E</td>
<td>European</td>
</tr>
<tr>
<td>E1, E2, E5, E6, and E7</td>
<td>Early gene/protein</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GF</td>
<td>Growth factor</td>
</tr>
<tr>
<td>GFR</td>
<td>Growth factor receptor</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HCK</td>
<td>Human cervical keratinocyte</td>
</tr>
<tr>
<td>HFK</td>
<td>Human foreskin keratinocyte</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>K or Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>L</td>
<td>Late gene/protein</td>
</tr>
<tr>
<td>LCR</td>
<td>Long control region</td>
</tr>
<tr>
<td>NA</td>
<td>North American</td>
</tr>
<tr>
<td>NE</td>
<td>Non-European</td>
</tr>
<tr>
<td>NV</td>
<td>Native virions</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAVE</td>
<td>Papillomavirus Episteme</td>
</tr>
<tr>
<td>PC</td>
<td>Proprotein convertase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PsV</td>
<td>Pseudovirion</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>QV</td>
<td>Quasivirion</td>
</tr>
<tr>
<td>R or Arg</td>
<td>Arginine</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------</td>
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<tr>
<td>URR</td>
<td>Upstream regulatory region</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Micro</td>
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</table>
CHAPTER I  LITTERATURE REVIEW

INTRODUCTION - DIVERSITY OF HUMAN PAPILLOMAVIRUSES IN DISEASE

Human papillomaviruses (HPVs) compromise a large family of non-enveloped, double-stranded DNA viruses. To date more than 150 different types of HPVs have been discovered and fully sequenced. In addition, more than 60 different animal papillomaviruses have been found and sequenced (see Papillomavirus Episteme (PAVE); http://pave.niaid.nih.gov/#home and [1]). HPVs are separated into five genera (alpha, beta, gamma, delta, and epsilon) based on DNA sequence diversity. The different types are found in different mucosal and non-mucosal epithelial sites and differ in their life-cycle and disease association, ranging from asymptomatic infection to common warts and cancer. The mucosal HPV types are further classified as low-risk, intermediate risk, or high-risk according to the frequency with which they are associated with cancer lesions. The alpha papillomavirus group includes the high-risk cervical cancer-associated (squamous cell carcinoma and adenocarcinoma) virus types [1-5].

The main focus of this review will be on the high-risk HPVs, as they are the causative agents of cervical cancer and other anogenital and oral cancers. The four main cancer-causing HPV types are the targets of the experimental analyses in this thesis. Further, the development of recombinant HPV particles that may bypass epithelial
differentiation-dependence has rapidly evolved the field of HPV receptor and entry research. However, these discoveries need to be confirmed and extended in the context of the natural host, using human tissue-derived particles. Using tissue-derived particles the focus of this proposal is on the viral life cycle with special emphasis on the virus-host interactions that occur during viral attachment and entry of the natural target cell, human keratinocytes. Using tissue-derived virus particles is of great interest and importance as the understanding of entry events in the context of the natural host may identify potential antiviral targets for diverse or common strategies of treatment against the various cancer-causing HPV types.

**HPV TYPES IN CANCER DEVELOPMENT**

HPVs are the causative agents of cervical cancer and other anogenital cancers and oropharyngeal cancers [6, 7]. In 2008, cervical cancer was the third most prevalent cancer type in women universally. Furthermore, 80% of cervical cancer incidences occur in the developing world where cervical cancer is the most commonly diagnosed type of cancer in women in many countries [8]. The majority of the high-risk HPV types are alpha papillomaviruses and cluster in the alpha-5, -6, -7, and -9 groups (pre-dominantly in alpha-7 and alpha-9) (Table 1.1) [9-11]. Further, a pooled analysis of case-control studies from eight countries identified 15 HPV types that are associated with a more than 200-fold increased risk of cervical cancer. Together, HPV16 and HPV18, account for 70% of all cases of cervical cancer, with HPV16 being the most prevalent. HPV45 and HPV31 are the third and fourth most common types, respectively, accounting for less than 10%
of all cervical cancers [12]. Current papillomavirus vaccines target the most common cancer-causing types, HPV16 and HPV18, protecting against virus infection and development of neoplasias [2, 13]. However, since current vaccines are type-specific, they do not offer protection against all cancer-causing HPV types. In addition, they are cost-prohibitive to most women around the world [14, 15]. Infections by diverse papillomaviruses remain a significant disease burden as there are about half a million cases of cervical cancer diagnosed every year worldwide with almost half of the cases resulting in fatalities ([WHO/ICO Information Centre on Human Papilloma Virus (HPV) and Cervical Cancer; http://www.who.int/hpvcentre/en/]). Thus, there is a need for the development of less expensive alternatives, such as universal microbicides, in addition to the current vaccines.
Table 1.1 Phylogenetic and cancer-risk classifications of alpha papillomaviruses.


<table>
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<tr>
<th>HPV SPECIES (alpha)</th>
<th>HPV GENOTYPE</th>
<th>RISK</th>
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<td>High</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>HPV 77</td>
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Obtained from Munoz et al. 2006.
EPITHELIAL DIFFERENTIATION DEPENDENCE IN THE HPV LIFE CYCLE

HPV infections are found at mucosal or cutaneous sites of the human body. Completion of the viral life cycle and shedding of new viral particles is strictly dependent on epithelial cell differentiation with expression of the late, structural proteins, L1 and L2, and production of viral particles restricted to the suprabasal cells [16-20]. As the basal cells in the epithelium divide, the daughter cells that are forced up begin to stratify and differentiate forming five sub-layers: the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and the stratum corneum (figure 1.1) [21]. Terminal differentiation is driven by molecular signals that turn on a program in which the cells start to change shape, filling up with keratin filaments, and eventually exiting from the cell cycle before they undergo nuclear disintegration [21-23]. The resulting squames are sloughed off the outer layer of the epithelium. The cells are continuously replaced by a new generation of cells continuously renewing the epithelium about every two weeks [23, 24].

The differentiation status of a tissue can be monitored by the expression of specific keratins and other cellular markers in specific layers of the epithelium. The dividing cells in the basal layer express mainly keratins 5 and 14 [24, 25]. As the cells leave the basal layer, in the spinal layer differentiation-specific proteins such as involucrin and transglutaminase are expressed forming a scaffold for the deposition of the cornified envelope and catalyzing the formation of peptide bonds, respectively, forming a network with the keratins. Keratins 1 and 10 are now expressed [23, 25-28]. In the
normal tissue in the spinal layer, cellular DNA replication and the cell cycle are down-regulated by the increased activity of tumor suppressors and other inhibitors of the cell cycle [29-31]. In the granular layer loricrin and profilagrin are expressed, which will ultimately make up components of the cornified envelope forming a physical barrier to the environment [23, 27]. In pre-cancerous or cancerous tissues these expression patterns are disrupted. Specifically, in HPV-infected tissues, cells maintain their ability to stay active in the cell cycle due to the expression of the E6 and E7 viral oncoproteins [5].
**Figure 1.1 Differentiation-dependent HPV life-cycle.** HPV infections occur at localized sites where microabrasions allow access of the viral particles to the basement membrane and mitotically active basal cells. The viral genome is established and maintained in the lower levels of the epithelium. Expression of the viral non-structural, early proteins stimulates cellular proliferation. The epithelial cells divide and differentiation is delayed as they move up through the epithelium. Different events in the viral life cycle are triggered as the cells move up through the epithelium. In the mid layers the viral genome amplifies as viral protein levels needed for genome amplification are expressed at higher levels. In the upper layers at later stages of differentiation the viral capsid proteins are expressed. Viral particles begin to assemble and are released from the cornified layer. Modified from [32]: Frazer, 2004.
PRODUCTION OF TISSUE-DERIVED HPV PARTICLES

Epithelial differentiation can be induced in culture by methods such as cell suspension in methylcellulose, allowing the cells to grow to confluency, and high levels of serum [33-36]. Although all these methods induce variable levels of differentiation, none of them recapitulate differentiation of a stratified epithelium, which is required for the productive life cycle of PVs. The only way to fully recapitulate the development of a differentiated multilayered epithelium \textit{in vitro} is by organotypic raft culture. In organotypic raft culture isolated keratinocytes are grown on a dermal equivalent, including collagen and fibroblasts, at the air-liquid interface to mimic the physiological environment [16, 37]. When the undifferentiated keratinocyte monolayers are raised to the air-liquid interface, cells are induced to become polarized; they form tight junctions and begin to differentiate, losing their proliferative capacity. The morphology of organotypic raft cultures is comparable to biopsy-derived tissues [38]. A closer investigation of protein markers, however, suggests that organotypic tissue resemble a wound healing environment favoring hyper-proliferation rather than fully health tissue [39]. Organotypic raft culture allows for the completion of the HPV life cycle, including DNA replication, induction of the viral promoters with early and late gene transcription and protein expression, in a physiological host environment \textit{in vitro} (figure 3) [16, 40-42]. Using raft cultures, cell lines stably maintaining episomal copies of the viral genome have been created to successfully produce HPV16, HPV18, HPV31, HPV33, HPV39, HPV45, and HPV11 [40-43]. In addition, a variety of chimeric and mutant viruses have been created [44-48]. Thus, this method allows for the study of multiple steps of the viral life cycle as well as for the production of tissue-derived viral particles.
PRODUCTION OF HPV PARTICLES BYPASSING DIFFERENTIATION

Because the HPV life cycle is tightly linked to the host cell differentiation program a system to bypass epithelial differentiation was developed. Synthetic or recombinant particles are produced by expression of codon-modified viral capsid proteins, L1 and L2, in eukaryotic or prokaryotic monolayer cells such as 293T, BSC-1, E. coli, yeast, or Sf9 cells (figure 1.2), but do not go through the natural maturation process. Expression of L1 by itself, or L1 and L2 together results in the self-assembly into viral capsids, called virus-like particles (VLPs) [49-55], For the production of pseudovirions (PsV) in addition to the viral capsid proteins, a reporter plasmid such as GFP is incorporated in place of the histone-associated viral genome [56, 57]. Quasivirus (QV) particles incorporate the full-length 8kB HPV genome by co-expression with the viral capsid proteins in 293T or 293TT cells [56, 57].

Synthetic particles do indeed resemble authentic virions as they have a similar structure by cryoelectron microscopy and they also retain the majority of surface-exposed conformational-dependent epitopes [58-62]. Further, L1 only virus-like particles elicit a strong antibody response which is efficient against authentic virions and are the basis for the current vaccines [49]. Due to the ease of production, these methods allow for rapid molecular alterations and have been used for the study of PV structure and neutralization, as well as entry and infectivity pathways. However, it is unclear what structural differences may exist and how these may affect the biology and entry pathway of the virus. Due to the ease of monitoring internalization and delivery of a reporter genome to
the nucleus, PsV particles are the most widely used particles for HPV entry and infection studies. Thus, most studies reviewed in this chapter will refer to studies performed using PsV reporter plasmid gene transduction as the main model to simulate PV infection events. As has been the standard in most previous studies PsV transduction will be referred to as infection throughout this thesis.
• **Virus-like particles (VLPs)-**  
  L1 or L1+L2 expressed in yeast, *E. coli*, 293TT cells, etc. can self-assemble…

• **Pseudovirions (Psv)-**  
  Same VLP technology plus marker plasmid

• **Quasivirions (QV)-**  
  Same VLP technology plus 8 kb viral genome

• **Native virions (NV)**  
  Produced in terminally differentiated epithelia
  *in vitro* in the organotypic raft culture system

---

**Figure 1.2. In vitro systems for producing HPV particles.** VLPs are produced by expression of codon-modified viral capsid proteins, L1 by itself or L1 and L2 together, in eukaryotic or prokaryotic monolayer cells. PsV particles are produced by co-expression of L1 and L2 with a marker plasmid. QVs are produced by co-expressing L1 and L2 with the full-length viral genome. NVs are produced by stratification and differentiation of HPV-harboring keratinocytes in organotypic raft cultures.
HPV GENOME AND GENE FUNCTIONS

The genome organization of the high-risk alpha papillomaviruses is highly conserved (figure 1.3). The genome consists of an upper regulatory region (URR) and six non-structural and two structural genes that are expressed at and required for different stages of the virus life cycle in the host epithelium. Cellular transcription factors, including SP1, AP1, and Oct1, as well as the early viral E1 and E2 proteins bind to the LCR and control viral replication and gene expression. Two viral promoters, the early and the late viral promoters, regulate the expression of viral mRNAs during epithelial differentiation [5]. The E1 and E2 early viral replication proteins bind to sequence at the viral origin and recruit polymerases and other cellular factors to mediate replication. The E1 protein itself also acts as a helicase for the separation of the viral DNA strand in this process. In addition to playing a role in replication E2 also acts in the regulation of transcription from the early viral promoter [63]. E6 and E7 encoded by the high-risk HPV types are considered the main viral oncoproteins since they in addition to stimulating cell growth also can transform cells. The main functions of E6 and E7 have been well studied. Degradation of p53 by high-risk E6 and degradation of pRB by E7, results in increased cell replication. Many additional cellular binding partners regulated by E6 and E7 have been identified [5, 63, 64]. The E5 protein, while not expressed in tumors, is another viral oncoprotein that plays an important role early on in infection by stimulating the transforming activity of the epidermal growth factor receptor [65]. The E1^E4 spliced protein is thought to stimulate genome replication by inhibition of the G2- to M-transition in the cell cycle. It is also thought to interact directly with the epithelial keratin network,
causing it to collapse and thus facilitate the release of viral particles in the cornified layer [66-69]. Finally, the late L1 and L2 proteins serve as the major and minor capsid proteins, respectively, encapsidating the 8 kB circular, double-stranded genome [54, 61].
Figure 1.3. Genome organization of alpha papillomaviruses. The alpha papillomavirus genome is composed of an upper regulatory region (URR) and eight different genes, including the early (E) and late (L) ORFs. mRNA splicing results in a larger number of gene products. The URR contains binding sites for cellular transcription factors and the viral E1 and E2 and is the control center for viral replication and gene expression. Obtained from [11]: Munoz et al. 2006.
**BASICS OF HPV CAPSID STRUCTURE**

**Major Capsid Protein, L1**

The viral capsid consists of 72 pentamers from 360 monomers of the major capsid protein, L1, arranged in a T=7 icosahedral lattice. The L1 protein serves as the main structural protein as it alone can self-assemble to form viral particles [54]. The core of the capsomer consists of eight anti-parallel β-strands forming a β-sandwich structure. The β-strands are connected by small loops, the BC, DE, FG, and HI that face outwards from the capsid and contain the major neutralizing epitopes (figure 1.4) [54]. These loops are responsible for the greatest diversity between different HPV types and contribute to the differences in antigenicity and lack of cross-neutralization between HPV types [54, 70]. Individual capsomers interact tightly with neighboring capsomers as the C-terminus of each L1 molecule acts like an arm invading neighboring capsomers and receiving 5 arms in return [54, 71]. While not essential for particle formation, disulfide bonds between L1 molecules form a network of intra- and interpentameric interactions, making the particles more stable and resistant to environmental influences from nuclease and proteases [72, 73]. Specifically, the invading C-terminal arm brings Cys-428 close to Cys-175 allowing for disulfide-bond formation. The importance of these two residues is confirmed as they are conserved among all PV L1 proteins [71]. Disulfide-bond formation is thought to happen slowly during virion maturation in an oxidizing environment present in the uppermost layers of the epithelium [47, 73].
In addition to serving as the main structural protein, the C-terminus contains a conserved polybasic motif that mediates import into the nucleus where capsid assembly occurs [74]. The polybasic motif is also thought to play a role in encapsidation of the viral genome [75]. As the main protein exposed to the virion surface, the L1 protein is responsible for primary attachment and interaction with the cell surface [76-78].
Figure 1.4. HPV16 pentamer structure and surface loops. A top and sideview of the HPV16 pentamer with the variable surface loops in grey shades. Figure obtained from [79]: Ahmed et al. 2013.
Minor Capsid Protein, L2

Including L2 in synthetic particles increases the yield of particles, enhances the stability, DNA encapsidation and infectivity of individual particles, and has an impact on the overall structure of the resulting particles [50, 80-84]. The amount of L2 incorporated in the virion is controversial. Cryoelectronmicroscopy of native bovine papillomavirus type 1 (BPV1) suggests that there is only one L2 protein in the center of each of the 12 pentameric capsomeres [85]. In contrast, SDS-PAGE analysis from native HPV1 particles, co-immunoprecipitation of HPV11 L1 pentamers with L2, and cryoelectron microscopy images of HPV16 PsV, suggests that up to 72 copies of L2, associate with the L1 capsomeres [61, 85, 86]. In cryo-EM images L2 is observed as a density in the central cavity of the L1 pentamers (figure 1.5). Only part of the L2 protein can be observed and the rest of the chain is hidden [61]. Although not revealed by the cryo-EM images, an N-terminal region of L2, residues 60 to 120 in BPV1, are surface exposed, as antibodies to epitopes in this region bind to intact particles [81]. Antibody-binding studies for HPV16 yield non-overlapping results, implicating residues 32-81, 212-231, 272-291 and 347-381 in one study [87], 1-120 in others [88-90]. This difference may be due to conformational changes that occur during infection and increase the exposure of the L2 N-terminus after attachment of the virus to cells. It has been suggested that the most N-terminal portion of L2 somehow folds back into the capsid resulting in it being unavailable to antibody binding as well as protease cleavage [90, 91]. Although the N-terminus is hidden inside intact mature particles, the hidden N-terminal epitopes, including residues 17-36, become exposed during infection [92]. Of great interest for
future vaccine development are antibodies generated to peptides from L2 residues 17-36 (RG-1) and 56-75 (anti-P56/75), as they result in a cross-neutralizing antibody response among many HPV types [60, 81, 93, 94]. The L2 protein is depicted in the central hollow of the L1 pentamers, but the size of the cavity is too small to allow passage of polypeptide chains. Therefore, it is more likely that the N-terminus of the L2 polypeptide passes between neighboring capsomers [95]. [13, 92]. In summary, most of the L2 protein is hidden inside the viral capsid, but short regions of the L2 N-terminus appear to be exposed on intact particles in solution prior to infection, and additional residues may be exposed during early stages of infection.

Homotypic interactions between L2 molecules as well as heterotypic interaction with L1 on the inside of the capsid are thought to be responsible for L2’s role in capsid stability and overall structure. When L1 disulfide bonds were not allowed to form, L1-only capsomers did not assemble into capsids; in contrast, when L2 was included, VLPs were observed [84]. This data suggests L2 plays a role in capsid formation and stabilization. In support of the role of L2 in capsid formation and stabilization, L2 amino acid residues 396 to 439 in HPV11, 129 to 246 and 384 to 460 in BPV1 are thought to mediate hydrophobic interaction with the L1 pentamers [96]. Further, the C-terminal L1-binding region contains several proline residue motifs (PxxP) that participate in protein-protein interactions. Based on sequence-predictions of conserved residues in the L1 residues, it has been suggested that residues 113-130 and 247-269 in two of the L1 β-sheets (D and F) are involved in the reciprocal interaction with L2 [97].
Figure 1.5. L2 in the papillomavirus capsid. A 3D model of HPV L1-only capsid (top and middle) and L1 + L2 capsid (bottom). Central sections (middle and bottom) show the arrangement of L2 (red) as a density in the central cavity of the L1 pentamers. Obtained from [61]: Buck et al. 2008.
HPV LIFE CYCLE

Micro-abrasions in the epithelium are thought to be required to allow the viral particles to gain access to and infect the basal cells (figure 1.1). In fact, wound healing has been suggested to stimulate HPV infection [2]. Active cell division is required for the viral genome to enter the nucleus of the infected cells. Thus, initial infection of the mitotically active basal cells is thought to be required for establishing a lesion [98]. After initial infection the viral genome is amplified and maintained as an episome at a low copy number. The E1 and E2 viral proteins mediate initial genome amplification [5]. Based on the study of episomal epithelial cell lines isolated from cervical lesions the genome copy number in basal keratinocytes is approximately 200 copies per cell [98, 99]. In contrast, in benign lesions of the rabbit oral papillomavirus animal model, the basal copy number is around 50 to 100 copies [100]. In HPV-containing foreskin keratinocytes established by electroporation of the viral genome which support the production of infectious particles, the basal level of episomal viral genomes has been estimated from 10-50 copies for HPV45 [41] to up to 10,000 copies per cell for HPV16 [47]. The basal level of genomes does not appear to correlate with the number of particles produced and it is likely that there will be variations between distinct lesions and sites.

The E6 and E7 proteins of PVs stimulate increased entry into the cell cycle and enhanced proliferation in the basal cells, as well as the super-basal cells. This active cellular replication allows for the viral genome to become amplified in the upper layers of the epithelium. Activation of the differentiation-dependent promoter in the upper layers of the epithelium increases the amount of E1 and E2 expressed, which are needed for
genome amplification. Expression of E4 and E5 are also though to mediate viral genome amplification by modifying the cellular environment [5].

The late proteins L1 and L2 are expressed only in the uppermost layers of the epithelium as their expression is regulated not only by the activity of the late promoter, but also by a switch in splice site usage [2]. Genome amplification is replaced by genome packaging and particle formation in the upper layers. Encapsulation of the viral genome gets started as the minor capsid protein, L2, is recruited by E2 to regions of replication in the nucleus. As L1 is expressed its localization to the same nuclear domains has been suggested to be influenced by L2 [80, 101]. The E4 protein is very abundant in the upper layers and it assembles into amyloid fiber structures. These structures disrupt keratin structure thus facilitating the release of viral particles [66, 68]. Desquamation of cells from the cornified layers of the epithelium facilitates transmission of viral particles [102].

**VIRUS MATURATION**

After expression of the viral capsid proteins and initial assembly, there is tissue-dependent maturation of HPV16 NV [47]. After 10 days (10-day particles) in organotypic raft culture the viral DNA is amplified, L1 expression is observed in the suprabasal layers of the tissue, and encapsidated viral genomes from infectious HPV16 NV particles can be harvested. Over a period of another 10 days (20-day particles), the harvested particles exhibit a more mature phenotype than particles harvested after 10 days. The particles harvested after 20 days display increased stability manifesting enhanced resistance to the
physical stress imposed by fractionation through ultracentrifugation. In addition, infecting with 20-day particles produces twice as much E1^E4 spliced transcript as compared to 10-day particles, suggesting that the particles are twice as infectious. In addition, maturation of the particles increases their susceptibility to L1 and L2 antibody-mediated neutralization. This data suggests that there are slight structural changes causing neutralizing epitopes to become more available. Maturation of the HPV16 NV particles correlates with a natural redox gradient in the tissue, as the lower layers of the tissue reside in a more reducing environment and the upper layers in a more oxidizing environment. Immunohistochemical staining for the L1 protein suggests that the 10-day particles reside mainly in the nuclei of the suprabasal layers, whereas the 20-day particles are found mainly in the denucleated, cornified layers of the epithelium. The effect of oxidation on virus maturation was demonstrated by the addition of the oxidizing agent, GSSG, to the tissue during virus production. Addition of GSSG to the tissue during early time-points enhanced maturation of virus particles harvested earlier than 20 days. In addition, L1 cysteines form disulfide bonds in the oxidizing environment in a differentiation-dependent manner and stabilize HPV16 NV particles [48].

PsV maturation similarly involves an increase in stability, an increased formation of disulfide bonds, and more ordered particles as imaged by electron microscopy [73]. However, in contrast to HPV16 NV, HPV16 PsV maturation occurs within 24 hours. Maturation of PsV particles requires the presence of unknown cellular factors. Overnight incubation of PsV particles in the presence of a cellular lysate, but not particles clarified from cellular lysates, resulted in more mature particles, as defined by their more ordered structure as well as resistance to trypsin digestion. The identities of the cellular factors
required for this process are unknown. It is likely that these cellular factors regulate the slow maturation observed in tissue.

**HPV CELL SURFACE EVENTS**

Much attention and effort has been put into elucidating the mechanisms of PV binding to and entering host cells. Despite the identification of several candidate receptors and entry pathways, much of the data remains controversial. Some of the confusion is most likely attributed to the use of different cell types and model systems, but also to the use of different HPV types and attempts to attribute a single mechanism to all PV types. Recently, research on virus entry has evolved where a single receptor is no longer thought to be responsible for the entry of viruses but rather an intricate interplay between several virus-specific receptors, co-receptors, and other co-factors is thought to occur [103-105].

Our current understanding of the PV infection strategy proposes that microabrasions allow access for virus particles to the basal cells as well as the basal lamina. Further, using synthetic particles, most papillomaviruses have been observed to infect cells via L1 by first attaching to a form of glycosaminoglycan (GAG), heparan sulfate (HS), on the cell surface or extracellular matrix (ECM) [13, 106, 107]. Initial binding to laminin-332 (also named laminin 5) on the ECM has also been demonstrated [108, 109]. HS attachment induces a conformational change allowing for the L2 N-terminus to be cleaved by a proprotein convertase (PC), furin and/or PC5/6, inducing a conformational change [91, 92]. The conformational change allows for the virus to be
transferred to (a) secondary entry receptor/s [110, 111]. Alpha6-integrin, growth factor receptors, and annexin A2 have been implicated as potential candidate receptors; however their role in infection is still unclear [112-116].

**Glycosaminoglycans**

Proteoglycans are a component of all extracellular matrices. They play important structural roles, regulate activities of enzymes, and influence the availability of growth factors and chemokines etc. They may also act as cell surface receptors. Proteoglycans are made up of a core protein with covalently attached glycosaminoglycan chains (GAGs). GAGs are long, un-branched polysaccharides, consisting of repeating disaccharides (figure 1.6). The GAGs themselves may either be attached to a core protein or secreted. The different types of GAGs include heparan sulfate (HS) and the closely related heparin, chondroitin sulfate (CS), dermatan sulfate (CS), keratan sulfate (KS) and hyaluronan (HA). The number of GAGs is limited but epimerization and modifications such as N- or O-sulfation during their synthesis in the Golgi compartment add to their diversity. The exception is HA which is synthesized on the cell surface and does not get sulfated. Further, the GAGs may attach to a number of different core proteins (Table 1.2). Some core proteins are specific for a single type of GAG and others can be modified by several GAGs [117]. Depending on the incorporation of N- acetyl- glucosamine or N-acetyl- galactosamine, GAGs can be divided into two broad categories; the glucoaminoglycans, including heparan sulfate, and the galactosaminoglycans, including chondroitin sulfate. In addition, the glucosaminoglycans are linked by 1,4 disaccharide
bonds whereas the galactosaminoglycans are linked by alternating 1,3 and 1,4 disaccharide bonds [14].

The extracellular matrix (ECM) and cells of most tissues are likely to possess a combination of multiple proteoglycans and GAGs [117]. Several proteoglycans are found on the basement membrane. Three of them; perlecan, type XVIII collagen, and agrin, contain HS as their GAG. HS in the basement membrane plays an important role in sequestering growth factors, protecting from proteases and receptor recognition [118-120]. Another major GAG of the ECM is CS [121]. Many of the core proteins to which CS attaches are directly secreted into the matrix and include aggrecan, brevican, decorin, and biglycan [122]. CS proteoglycans may play a structural role in the ECM and bind to different components of the ECM and cross-link them; they also bind to and regulate growth factors [123].

With the exception of erythrocytes, all other cell types have one or more proteoglycans on the cell surface. The majority, including CD44 and syndecans, are transmembrane proteins, but several others, including the glypicans, are glycosylphosphatidylinositol-linked to the outer membrane. The syndecans and the glypicans have HS as the main GAG, but the syndecans may also have CS and DS [117,124]. The syndecans are transmembrane receptors that may signal either independently, or in combination with other receptors. For example, HS on syndecan may associate with growth factors such as FGF and EGF and signal through growth factor receptors. Another common receptor collaborating with syndecans are the integrins [125-128]. Syndecan-1 plays an important role in keratinocyte biology as it is the most plentiful HS-decorated proteoglycan in this cell type. In addition to cellular binding partners, syndecan-1 has
also been implicated as a cell surface receptor for many viruses including HPV, retroviruses, herpesviruses, and flaviviruses [129, 130]. The diversity in molecular and functional properties of syndecan-1 is due to the varying sulfation, polysaccharide length, charge, and attachment of either HS or CS [131].
**Figure 1.6. Glycosaminoglycan composition.** GAGs are long, un-branched polysaccharides, consisting of repeating disaccharides. The different types of GAGs include heparan sulfate (HS) and the closely related heparin, chondroitin sulfate (CS), dermatan sulfate (CS), keratan sulfate (KS) and hyaluronan (HA). Epimerization and modifications, N- or O-sulfation, are added during their synthesis. Obtained from [117]: Couchman and Pataki, 2012.
Table 1.2. Proteoglycans core proteins and characteristics. Obtained from [117]: Couchman and Pataki, 2012.

<table>
<thead>
<tr>
<th>Proteoglycan</th>
<th>Core Protein Size, kDa</th>
<th>Type of GAG Chains</th>
<th>Human Chromosome Localization</th>
<th>Tissue Location</th>
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</tr>
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<tr>
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<td>58</td>
<td>CS</td>
<td>Chromosome: 20 location: 20p11.2</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>CD44 (19 isoforms)</td>
<td>37–81</td>
<td>CS/DS</td>
<td>Chromosome: 11 location: 11p13</td>
<td>Transmembrane, extracellular, intracellular</td>
</tr>
<tr>
<td>NG2/CSPG4</td>
<td>251</td>
<td>CS</td>
<td>Chromosome: 15 location: 15q24.2</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Invariant chain</td>
<td>31</td>
<td>CS</td>
<td>Chromosome: 5 location: 5q33</td>
<td>Cell surface, intracellular</td>
</tr>
<tr>
<td>Neuroglycan-C</td>
<td>120–150</td>
<td>CS</td>
<td>Chromosome: 3 location: 3p21.3</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Type XVIII collagen</td>
<td>180–200</td>
<td>HS</td>
<td>Chromosome: 21 location: 21q22.3</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Perlecian</td>
<td>400–450</td>
<td>HS</td>
<td>Chromosome: 2 location: 1p36.1-p34</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Agrin</td>
<td>212</td>
<td>HS</td>
<td>Chromosome: 1 location: 1p33.3</td>
<td>Transmembrane, extracellular</td>
</tr>
<tr>
<td>Betaglycan</td>
<td>110</td>
<td>HS/CS</td>
<td>Chromosome: 1 location: 1p33-p32</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>SV2</td>
<td>20</td>
<td>KS</td>
<td>Chromosome: 1 location: 1q21.2</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Serglycin</td>
<td>10–19</td>
<td>HS/CS</td>
<td>Chromosome: 10 location: 10q22.1</td>
<td>Intracellular</td>
</tr>
<tr>
<td>Endocan</td>
<td>50</td>
<td>DS</td>
<td>Chromosome: 5 location: 5q11.2</td>
<td>Circulating extracellular</td>
</tr>
<tr>
<td>Neutroplin-1</td>
<td>130</td>
<td>HS/CS</td>
<td>Chromosome: 10 location: 10p11.2</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Type IX collagen</td>
<td>270</td>
<td>CS</td>
<td>Chromosome: 6 location: 6q12-q14</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Testican 1</td>
<td>48</td>
<td>HS/CS</td>
<td>Chromosome: 5 location: 5p31.2</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Testican 2</td>
<td>45</td>
<td>HS/CS</td>
<td>Chromosome: 10 location: 10pter-q25.3</td>
<td>Extracellular</td>
</tr>
</tbody>
</table>

CS, chondroitin sulfate; DS, dermanan sulfate; GAG, glycosaminoglycan; GPI, glycosylphosphatidylinositol; HS, heparan sulfate; KS, keratan sulfate; SLRP, small leucine-rich family of proteoglycans.
**GAGs and different synthetic particles.** The first demonstration of the involvement of GAGs as attachment receptors for HPV used HPV11 L1 VLPS in the spontaneously immortalized HaCaT keratinocyte cell line. [132]. Binding to HaCaT keratinocytes was blocked by heparin and chondroitin C but not chondroitin A, suggesting that L1-interactions with GAGs are specific and depend on the type of GAG. Enzymatic removal of HS further supported the conclusion of the importance of GAG-binding for initial attachment. Further, the importance of GAG sulfate modifications was demonstrated by showing decreased binding of VLPs to sodium chlorate-treated cells which has under-sulfated GAG chains, as well as GAG-deficient mutant CHO cells. Interestingly, this study also identified a role for the carboxy-terminus of L1 in mediating GAG interactions. Cell-surface engagement of HS was soon verified for HPV16 and HPV33 PsV [107]. In contrast to what was observed with HPV11 L1 VLPs, HPV16 and HPV33 PsV attachment to and infection of Cos-7 cells was inhibited by heparin but not chondroitin or dermatan sulfate, indicating that either the difference between VLPs and PsV or the HPV types was responsible. Again, a role for GAG-sulfation modifications was demonstrated. In contrast to carboxy-terminally deleted L1 VLPs, studies with PsV did not support a role for the L1 C-terminus in GAG interactions.

**Importance of proteoglycan core protein.** Wound healing facilitates HPV16 PsV infection of the vaginal canal in mice [133]. Following wounding, HSPGS are down-regulated throughout the subrabasal layers as well as in the basement membrane. In contrast, migrating keratinocytes arriving to repair the wound express high levels of HSPGs, including syndecan-1 and CD44 [134, 135]. A role for syndecan-1 as a candidate
for the main HS-carrying proteoglycan involved in HPV attachment is suggested not only because it is highly expressed in keratinocytes, but also because its expression is increased during wound healing [136]. However, an absolute requirement for syndecan-1 has not been demonstrated experimentally. Rather, several HSPGs may serve as binding receptors. In the K562 (human erythroleukemia) cell line, which lacks expression of cell surface syndecans and glypicans [137], exogenous expression of syndecan-1, syndecan-4, or glypican-1 enhances cell surface attachment by HPV16 L1 VLPs and infection by HPV11 NVs [138]. The results further suggested that the level of HSPG expression rather than the specific core protein is important for supporting attachment and infection by HPV16 PsVs and infection by HPV11 NVs [138]. The results further suggested that the level of HSPG expression rather than the specific core protein is important for supporting attachment and infection by HPV16 PsVs and infection by HPV11 NVs [138]. The results further suggested that the level of HSPG expression rather than the specific core protein is important for supporting attachment and infection by HPVs. In addition, using mice nulligenic for syndecan-1 does not significantly block HPV16 PsV infections of the female genital tract [139]. Thus, syndecan-1 expression may be sufficient to support attachment and infection by HPV16 PsV, but syndecan-1 may not be essential. Rather, several HSPGs may function in cellular attachment. Removal of GPI-linked glypicans with phosphoinositol-specific phospholipase C from the cell surface of Cos-7 cells does not impact binding of HPV33 L1L2 VLPs or HPV33 PsV infection. In contrast, cleavage and removal of general cell surface HSPGs by heparinase prevents both binding and infection [140]. Together, these data suggest that removal of one type of cell surface proteoglycan is not sufficient to prevent virus attachment, and so different types of proteoglycans may be used interchangeably.

**Cell surface and basement membrane.** Initial binding to host HSPGs may occur directly on the epithelial cell surface, or at the basement membrane, as demonstrated in the mouse vaginal challenge model for HPV16, HPV31, and HPV5 PsV [13, 141]. In this
model, heparin blocks attachment to the basement membrane and the cell surface, and infection by HPV16 and HPV31, but less so HPV5. Noticeably, chondroitin-6-sulfate does not inhibit infection by any of the viruses. Heparinase treatment to remove cell- and basement membrane-associated HS also prevents cell surface and basement membrane attachment, as well as infection.

**GAGs and tissue-derived virus.** Although primary attachment to HS has been suggested as a universal entry step for all papillomaviruses, tissue-derived HPV31 NV infection of human keratinocytes does not require HS [115]. Infection of HaCaT and N/TERT-1 immortalized but non-transformed keratinocyte cell is not blocked by exogenous heparin competition, nor by the enzymatic removal of cell surface HS. Similar results are obtained using primary foreskin keratinocytes. In contrast, infections of Cos-7 monkey kidney cells and transformed C-33A human cervical cancer cells are efficiently blocked by heparin or removal of cell surface HS. These data suggest that the virus uses more than one receptor/s and entry pathway/s depending on the cell type. It is interesting to note the contradictory findings for HPV31 PsV [141] and HPV31 NV [115] in HaCaT cells. The 50% inhibitory concentrations of heparin for HPV16 and HPV31 PsV infections were almost identical, but no dose-dependent inhibition was observed for HPV31 NV infections. Possible explanations include differences between PsV and NV in particle structure due to differences in maturation, and/or accessibility of furin cleavage sites.
**GAGs and L1 interactions.** For both HPV16 and HPV18 the L1 interaction with heparin molecules was mapped to specific residues on the pentamer surface by site-directed mutagenesis and co-crystallization of heparin molecules with purified L1 pentamers [142, 143]. Negatively charged heparin oligosaccharides interacted with multiple binding-sites on L1 by charge-charge interactions and hydrogen bonds (figure 1.7). The heparin molecules bind to two locations on the top outer rim and two more locations on the side wall of the pentamer. Despite the presence of negative charges in the central hole of the pentamers, no heparin molecules bind to this region. The heparin binding sites of L1 are conformational, made up of residues that cluster in the three dimensional structure, arising from more than one surface loop and more than one subunit. One of the two top rim binding patches of HPV16 is made up of residues from the FG and HI loops of two neighboring monomers (K278, T266, N285, K361 site 1). The other patch is also composed of residues from two monomers in the FG, HI, and BC loops (K356, T358, T266, K54, N56 site 2). On the side wall of the pentamer, the heparin molecules bind vertically, with both patches starting in the BC loop (N57, K59) at the top of the pentamer, reaching down to the α4-loop at the base of the pentamer (K443 site 3 and N450, K452 site 4) (figure 1.7). The negatively charged Lys 278 (FG loop) and Lys 361 (HI loop) site 1 at the top of the HPV16 pentamer are important for primary attachment, as mutations of these residues completely ablate ECM and cell surface-binding. Site 2 and 3 are dispensable for binding, but they are important for entry of HPV16 PsV. Site 4 has not been studied further, as mutations in these residues do not result in assembly-competent PsV. The binding of heparin to HPV18 involves the same surface loops, although the residues differ some [143].
Figure 1.7. Structure of L1 binding to heparin. (A, B, C) HPV16 and (D, E, F) HPV18 L1 pentamer complexed with short-chain heparin molecules (heparin depicted as stick model in A, B, D, E, and surface model in C and F). A and D are top views, B, C, E, and F are side views of the L1 pentamer. Red color indicates negative charges; blue, positive charges; and white, neutral charges of the capsid surface. Obtained from [143]: Dasgupta et al. 2010.
**GAGs and conformational changes.** Initial HS attachment by HPV PsV is thought to induce conformational changes in the L1 and L2 proteins of the viral capsid. The conformational changes have been suggested to allow interactions with secondary HS sites, transfer to an uptake receptor or receptor complex, as well as exposing of hidden epitopes that may be important for the interaction with secondary receptors or other cellular proteins in the entry process [92, 142]. A conformational change in L2 is supported by the finding that the N-terminal RG-1 epitope is exposed only post-attachment [92]. Evidence for conformational changes in L1 include 1) that a subset of antibodies neutralizes only cell-bound virus and 2) that the virus switches from a heparin-sensitive state to a heparin-insensitive state while bound to the cell surface [110, 144, 145], and 3) there are several HS binding sites on the capsid surface [142, 143]. A recent study investigated a role for the HS binding sites in conformational changes and downstream entry events [146] (summarized in Table 1.3). Based on the observation that mutants for site 2 and 3 already have the L2 N-terminus exposed, attachment to site 1 is thought to be sufficient to trigger the L2 conformational change. The site 2 mutant is attachment and internalization competent but fails to undergo a conformational change in L1 and does not uncoat in the endosomal compartment. Therefore the uncoating process may begin with an L1 conformational change that depends on HS interactions with site 2. The site 3 mutant undergoes conformational changes in both L1 and L2, but fails to internalize and instead accumulates on the ECM. So, interactions between HS and site 3 may be necessary for transfer to a functional uptake receptor. In summary, the data suggests that each of the HS binding sites is involved in inducing a series of conformational changes in the viral capsid, each which then is required for a subsequent
step of entry. These interactions may be sequential or occur by coincident multivalent interactions of a high molecular weight HSPG, as suggested by the finding that shorter chain heparin oligosaccharides do not block infection but longer chain molecules do [143].
Table 1.3. L1 HS-binding sites and their role in HPV infection.

<table>
<thead>
<tr>
<th>HS Binding Site</th>
<th>Main Function</th>
<th>Conformational changes WT</th>
<th>Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>Primary Attachment</td>
<td>L2 CC, exposing N-terminus</td>
<td>Fail to bind</td>
</tr>
<tr>
<td>Site 2</td>
<td>Infectious Entry</td>
<td>L1 CC, un-coating</td>
<td>Fails L1 CC and stays in endosome</td>
</tr>
<tr>
<td>Site 3</td>
<td>Infectious Entry</td>
<td>L2 CC, L1 CC</td>
<td>Fail to internalize, Accumulates on ECM</td>
</tr>
<tr>
<td>Site 4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
**Capsid association with GAGs in solution.** An interesting observation is that HPV16 PsV particles may associate with HSPGs on cells, but then be released from the cell surface during the continuous shedding of HSPGs. The virus may then associate with growth factors in solution via the HSPGs. It has been suggested that the virus-HSPG-growth factor complex then can bind directly to growth factor receptors on target cells and enter in an HS-independent manner \[114\]. This matter is of some dispute. First, virus can be released from cells without the continuous release of HSPGs \[146\]. Second, a virus-heparin growth factor complex is not able to bind to cells \[109\]. Thus, virus-heparan sulfate-growth factor association appears to play a role in attachment and conformational changes, but not in bridging virus particles to the cells for HS-mediated uptake.

**Laminin**

Laminin-332 is a major component of the basement membrane. In addition, migrating keratinocytes at sites of wound healing secrete high levels of laminin-332 within a few hours. This event occurs prior to the secretion of other basement membrane components including type VII collagen and HSPGs \[147, 148\]. In addition to binding to cellular GAGs, on the cell surface or ECM, HPVs may bind to laminin-332 on the ECM. This interaction was first shown using HPV11 produced in xenografts in immunocompromised mice \[108, 149\]. HPV11 particles colocalize with laminin-332 secreted by keratinocytes and binding to ECM is blocked by antibodies against laminin-332. In addition, particles bind to purified laminin-332. Binding to the ECM was not blocked by pre-incubation with heparin, but adsorption to the cell surface and infection of
HaCat cells was efficiently blocked by heparin [149]. These results suggest that laminin-332 is important for initial attachment to the ECM, but subsequent transfer to a HSPG-receptor on the cell is required for efficient infection by HPV11 NV. Interestingly, binding to the basement membrane of tissue sections from human cervical mucosa is not affected by enzymatic removal of GAGs, or interfering with laminin-332 binding, or the combination of the two treatments [108]. Although tissue sections of intact cervical mucosa may not mimic the environment of cervical microabrasions completely, these data emphasize an increased complexity of virus binding to cervical lesions.

An important consideration when studying attachment to host molecules are the potential implications derived from comparing binding to mouse versus human cells. HPV binding to the ECM of human cells occurs via laminin-332, or HSPGs [108-110, 150]. In contrast, binding to the mouse basal lamina as well as ECM of cultured mouse cells is dependent exclusively on HSPGs [109, 141]. A recent study using HPV16 PsV demonstrated that heparin-preincubated paricles bind efficiently to ECM secreted by HaCaT cells. Binding to the ECM is mediated via laminin-332 for heparin-coated as well as uncoated virus, since removal of HSPGs did not affect binding. Interestingly, when the heparin-coated, but not un-coated particles were allowed to bind laminin-332, they infected cells in a HS-independent manner [109]. The data suggest that initial attachment by HPV16 PsV is initiated by laminin-332 but that a GAG-mediated step is required for efficient infection.
Attachment by different HPV types

Due to structural differences among different HPV types, initial attachment and interactions with the ECM and cell surface may be type-dependent. Although the L1 protein displays an overall high sequence similarity and structural conservation, structural diversity and conformational differences in the loop structures on the pentamer surface have been demonstrated for diverse types HPV11, HPV16, HPV18, and HPV35 [70]. For instance the conformation of the HI loop, which includes parts of the site 1 HS-recognition site, of HPV18 L1 differs significantly from that of the HI loop of HPV16. This difference may be due to the two additional proline residues found in the HPV18 L1 HI loop. The consequence of such amino acid variations at this site, and other locations in L1, is not well understood. Minor structural differences may have an impact on engagement of receptors for primary attachment, as well as putative entry receptors. A study of VLP binding by four different HPV types to the ECM and cell surface demonstrated some differences [150]. HPV11 VLPs were dependent on laminin-5 for binding to the ECM, but removal of HS from the ECM or cells did not alter binding. In contrast, attachment by HPV18 VLPs to both ECM and cells depended solely on HS. HPV16 and HPV45 VLPs displayed dependence on HS moieties for binding to the cell surface but preventing binding to the ECM required treatment with both heparin as well and an antibody to laminin-332 preventing interactions with this molecule.

In summary, slight variations in structure of the different HPV types may favor binding to a specific type of GAG and/or GAG modification, laminin-332 or another receptor to facilitate attachment and trigger subsequent entry events. Different members of the closely related polyomavirus family also use different receptors for infection [95,
Thus, any one hypothesis for HPV attachment may not encompass all HPV types. Attachment to GAGs, using tissue-derived NV particles of the main cancer-causing HPV types will be addressed in Chapter III.

**Furin proprotein convertase**

Proprotein convertases (PCs) are part of a family of cellular calcium-dependent serine proteases whose main function is to activate proproteins. There are seven family members, among these, furin, PACE4, PC5/6 and PC7/8 are widely expressed, and PC2 and PC1/3 are restricted to neuro-endocrine and testicular tissues, respectively. Target recognition and cleavage occur at a protease-specific basic motif. Furin, which is considered the prototype PC, recognizes the R-X-K/R-R (R-Arg, K-Lys, X-any amino acid) consensus motif. Cellular substrates cleaved by PCs include neuropeptides, peptide hormones, growth and differentiation factors, receptors, enzymes, and adhesion molecules. Furin is a type 1 membrane protein localized primarily in the trans-Golgi network; it is also present in an active form both on the cell surface and within the endosomal compartment. Furin is also found in a cleaved, soluble form without the transmembrane domain [153]. Furin cleavage is required for escape from the endosome by several bacterial toxins, including anthrax toxin and pseudomonas exotoxin. Many viruses require the activity of furin-like proteases for their infectivity. Enveloped particles are cleaved during virion maturation while transiting through acidic compartments of the secretory pathways prior to release of the particles and attachment to uninfected host cells [154-157].
**Furin in HPV PsV infection.** During a screen for common protease inhibitors and their effect on infection by HPV16, a PC inhibitor (decanoyl-RVKR-cmk) was identified with an IC\textsubscript{50} of about 50 nM. The inhibitor had similar IC\textsubscript{50} for infection by HPV5, HPV6, HPV18, HPV31, BPV or CRPV PsV. A role for furin in the life cycle of other non-eveloped viruses has not been demonstrated. BK and JC viruses, which are structurally related polyomaviruses, contain a conserved furin cleavage site at the C-termini of the minor capsid proteins VP2 and VP3. Despite the presence of the furin cleavage site, infection by these viruses is not sensitive to furin inhibition [91].

The role of furin in HPV16 PsV infection was confirmed by the inability of the virus to infect furin-deficient CHO FD11 and LoVo (human colon carcinoma) cells. Reconstitution of furin-expression in the CHO FD11 cells rescued infection. These results suggest that other PCs expressed in these cells cannot compensate for the lack of furin expression. However, PC5/6 is not expressed in these cells. There is a conserved consensus site for furin cleavage N-terminus of all described PV L2 sequences (figure 1.8). Mutation of arginine 12 to serine (R12S) reduces the infectivity of HPV16 PsV to almost undetectable levels. In the presence of the furin inhibitor, wildtype virus and the virus carrying the R12S mutation are retained in a late endosomal/lysosomal compartment. However, furin cleavage is not required for uncoating as the viral genome can be detected. These results suggest that furin cleavage is required only after uncoating and shedding of L1, allowing L2 and the viral genome to escape the endosome, but before trafficking to the nucleus [91].
Figure 1.8. Conservation of the furin cleavage site in the L2 N-terminus.

Alignment of the L2 N-termini of HPV16, HPV18, HPV31, and HPV45. The conserved amino acids for furin recognition and cleavage are highlighted in bold.

L2 HPV16 MRHKRSAKRTKRASATQLYKTCKQAGTCPDPDIIPK 35
L2 HPV18 MVSHRAARR-KRASVTDLYKTCKQSGTCPDPDVVPK 34
L2 HPV31 MRSKRSTKRTKRASATQLYQTCKAAGTCPDVIPK 35
L2 HPV45 MVSHRAARR-KRASATDLYRTCKQSGTCPDVINK 34
**Furin cleavage and conformational changes in the capsid.** Exogenous furin enzyme cannot access the L2 N-terminus of mature PsV particles for cleavage, supporting the notion that the L2 N-terminus and the furin cleavage sites are initially hidden inside the viral capsid. On the other hand, immature particles, which are more loosely packed, can be pre-treated with exogenous furin, allowing them to bypass the requirement for cellular furin during infection. Thus it is likely that a conformational change is required during entry before cleavage by furin can occur [91]. Conformational changes at the cell surface allowing for exposure of the L2 N-terminus, including the furin cleavage site and the 17-36 epitope recognized by the RG-1 antibody, have been observed. The RG-1 epitope cannot bind and immunoprecipitate mature PsV particles. However, when virus binds to the cell surface the epitope becomes increasingly exposed over a period of several hours. Exposure of the epitope is blocked by furin inhibitors, suggesting that an initial conformational change induced by cell surface attachment is followed by a second conformational change post furin-cleavage exposing additional residues in the L2 N-terminus [92]. Cleavage of L2 may take place after attachment to heparan sulfate (HS) on the cell surface or on the extracellular matrix (ECM) [13, 91, 92].

**GAG interactions and furin-cleavage.** A link between HSPG-interactions and furin-cleavage was established by the use of HPV16 PsV particles that were cleaved *in vitro* by furin. In addition to bypassing the need for cellular furin during infection, these particles bind to and efficiently infect cells deficient in HSPGs expression [111]. Observations from the mouse vaginal challenge model contradict these data somewhat as the pre-cleaved virus bypassed a need for cellular furin but could not infect heparinase-
treated, HSPG-deficient epithelium [13]. The pre-cleaved virus binds to all layers of the epithelium, suggesting that in vivo cellular HSPGs target the virus to the basement membrane. These conclusions are compromised by the fact that whereas in mice PsV binding to basement membrane and ECM is mediated primarily by HSPGs, but in human cells basement membrane and ECM interactions are mediated primarily by laminin-332 [109].

**Exposure of the L2 N-terminus mediated by cyclophilin.** The conformational changes required for exposure of the L2 N-terminus and infectious entry of virus particles are mediated by cyclophilin B for some, but not all, HPV PsV types tested [158]. Cyclophilin B is a cellular chaperone that mediates cis/trans isomerization of peptidyl-prolyl bonds and associates with HSPGs on the cell surface as well as with membrane structures intracellularly. In the presence of cyclophilin inhibitors, wildtype HPV16 and HPV18 PsV do not expose the L2 N-terminus post-attachment and are internalized by a non-infectious pathway. Surprisingly, a mutation in the cyclophilin B recognition site (85-PXXXGPXXP-93) that exchanges the glycine and proline at positions 99 and 100 in the L2 N-terminus bypasses a requirement for cyclophilin B on the cell surface and efficiently exposes the L2 N-terminus. It was proposed that the amino acid residue exchanges increased the flexibility and exposure of the L2 N-terminus in mature PsV particles. The mutant remains sensitive to cyclophilin inhibition since intracellular cyclophilin also helps remove L1 from L2 and the genome by interacting with a second binding site in the L2 C-terminus [159]. Noticeably, HPV6, HPV45, and HPV58 exhibit
similar sensitivity to cyclophilin inhibition, whereas BPV1, HPV5, HPV31, and HPV52 are comparably resistant to cyclophilin inhibition [158]. The observed variations in cyclophilin sensitivity among HPV types may reflect differences in entry strategies [160].

**Functional implications from furin-cleavage of L2.** The reason for cleavage of the L2 N-terminus during PV entry has not yet been elucidated. One possibility is to expose new sites for cellular binding partners that guide the incoming viral particles through the endocytic pathway and ultimately to the nucleus. In this regard the virus loses affinity for the primary cell surface receptor after undergoing the initial conformational changes and instead becomes sequestered on the ECM. Noticeably, the interaction domain for syntaxin 18, an intracellular receptor protein suggested to play a role in HPV entry, is immediately downstream of the 17-36 epitope [161].

**Co-Receptors**

Several reports suggest that after engaging with cell surface HSPGs HPV16 PsV particles interact with one or more non-HSPGs receptors required for endocytosis [92, 110, 111]. First, initial colocalization of capsids with HSPGs on the cell surface is lost after the particles enter intracellular vesicles, suggesting that the particles are transferred to a non-HSPG receptor before endocytosis occurs [110]. Further, when blocked by antibodies, virus that has gone through post-attachment conformational changes loses affinity for the cell surface and relocates to the ECM rather than being taken up by the
cells [92]. In addition, furin pre-cleaved virus binds to and infects HSPG-deficient cells, providing strong evidence for additional post-attachment receptors [111].

The identity of the keratinocyte-specific entry receptor for PV is currently unknown. Therefore many proposed candidates and the subject is of much debate. The virus may use several receptors acting in concert for efficient infection and identification strategies that depend on deletion of only one receptor result in partial inhibition of infection. Another possibility is that the virus prefers one receptor but when that receptor is no longer available it uses another receptor/s. Another source of complexity is the use of model systems with different particles, PV types, and cell types.

**Integrin.** One of the first candidate receptors was α6-integrin, an epithelial cell adhesion protein [113]. The study compared attachment of HPV6b L1 VLPs to CV-1 (monkey kidney) and HaCaT epithelial cells with attachment to DG75 cells (human B lymphoma) that do not bind VLPs. α6-integrin from the attachment-permissive CV-1 and HaCaT cells immunoprecipitated with VLPs. No co-precipitation was observed in α6-integrin-negative DG75 cells. Further, an α6-integrin-specific monoclonal antibody reduced HPV6b L1 VLP binding to the permissive cells by 60%. In a study of 10 different cell lines HPV16 L1 VLP-binding correlated with α6-integrin expression levels. HaCaT cells had the highest α6-integrin expression-levels and the highest amount of HPV16 L1 VLP-binding [162]. Again, an α6-integrin-specific antibody reduced attachment by up to 60%. Complicating the issue, PsV attach to and infect cells that do not express α6-integrins. Primary bovine keratinocytes that express α6-integrin and primary bovine fibroblasts that do not, bind native BPV4 particles to similar levels,
suggesting that at least this virus does not depend on α6-integrin for attachment. Also, BPV4 binds to human BO-SV keratinocytes deficient for α6-integrin expression to the same extent as α6-integrin-expressing KH-SV human keratinocytes. Both cell lines support infection by BPV4, suggesting that α6-integrin is not essential for this process [163]. The α6-integrin-deficient BO-SV cells also bind to HPV16 L1 VLPs and are permissive for infection by HPV11 NV to the same extent as α6-integrin-expressing cells [138]. However, in another study with HPV11 NV α6-integrin-deficient cells were not infected as efficiently as α6-integrin-expressing cells when the virus was first bound to the ECM of HaCaT cells [108], suggesting that the route of infection may affect receptor usage. HPV16 PsV infection of conditional skin epithelium β4-integrin knockout mice, which also have undetectable levels of α6-integrin, results in modest but consistent decreases in infection levels [139]. These results support the notion that α6-integrin contributes to the efficiency of infection but is not essential. In addition to a role for integrin in attachment and entry, α6-integrin activates intracellular signaling pathways for Ras/MAPK and PI3K which may promote infection indirectly by creating a more proliferative cellular environment [164]. α6-integrin signaling through the focal adhesion kinase may activate endocytosis [165]. In summary, the data suggest that α6-integrin is a co-receptor for HPV infection. Interestingly, interactions between α6-integrin and other factors reported to be important for HPV infection, including HSPGs, cyclophilin B, and tetraspannins, have been observed, supporting the hypothesis that α6-integrin is part of a receptor/signaling complex rather than serving as a single entry receptor [160].
**Growth factor receptors.** A role for growth factor receptors (epidermal growth factor receptor, EGFR and keratinocyte growth factor receptor, KGFR) in PV entry and infection has been suggested [114]. HPV16 PsV activates GFRs although direct binding to GFRs by PsV has not yet been demonstrated. Blocking of the EGFR inhibits internalization and infection. However, the kinetics of signaling does not coincide with virus internalization leaving to uncertainty as to the actual role of the EGFR in PV infection, whether it be in entry events or triggering signaling events important for viral gene expression [114, 166].

**Annexin A2.** Annexin A2 is a calcium and phospholipid binding protein. It is expressed on the cell surface as part of a heterotramer (A2t) consisting of two Annexin A2 monomers associated with a S100A10 dimer [167]. The discovery of annexin A2 as a candidate HPV16 PsV receptor provided direct evidence for an L2 receptor interaction [112]. Initially, indirect evidence was provided by the demonstration that an HPV16 L2 peptide from amino acid 108 to 126 interfered with HPV16 PsV infection. Independently of L1 the peptide interacts with and binds to the cell surface of the human cervical cancer cell lines HeLa, SiHa, and CaSki [168]. This same L2 peptide was later shown to directly interact with the A2t subunit, S100A10, and annexin A2 co-immunoprecipitates with HPV16 L1L2 VLPs and PsV particles. A role in infection for A2t was supported by the observation of a significant decrease in HPV16 L1L2 VLP internalization and HPV16 PsV infection in HaCaT cells, after knockdown of expression of annexin A2 by short hairpin RNA (shRNA). Additionally, a mutation in the L2 108-126 region reduces binding to A2t as well as HPV16 PsV infection [112]. HPV16 PsV infects annexin A2-
deficient HepG2 cells (human hepatocellular carcinoma cells), suggesting that HPV16 PsV uses an alternative entry strategy in the absence of A2t [160]. However, suggesting a role for annexin A2 in HPV16 PsV internalization and infection, experiments with the HaCaT keratinocyte cell line may be more biologically relevant. Annexin A2 mediates cellular processes such as cell adhesion, membrane trafficking, exocytosis, and endocytosis and is implicated in the binding and uptake of other viruses including HIV-1, enterovirus 71, and cytomegalovirus [169-175]. Interestingly, the annexin A2 C-terminus may interact with heparin [167], as might be expected for A2t being a part of a potential PV entry and uptake complex. In addition, similarly to HPV16 PsV surface interactions including HSPGs, EGFR, and integrins are implicated for cytomegalovirus infection [176]. A study demonstrated a link between EGFR signaling and increased translocation of annexin A2 to the plasma membrane. In the same study, a possible direct or indirect association between HPV16 PsV and annexin A2 and S100A10 of A2t may or may not have been mediated by HSPGs. The association with annexin A2 was observed in endocytic vesicles, supporting a role for annexin A2 in entry [177]. Annexin A2 is involved in endocytic pathways, including clathrin- and caveolae-mediated endocytosis and macropinocytosis [178-180], all of which have all been implicated in cellular entry by HPV16 and other HPV types [160]. The involvement of annexin A2 in multiple endocytic pathways offers a possible explanation for why more than one entry route has been observed for PsV entry. Interactions between A2t and other PV types, has yet to be investigated.
Proposed model of HPV cell surface interactions

A comprehensive model attempting to incorporate the many implicated molecular interaction observed for HPV16 PsV infections of keratinocytes was proposed by Raff et al. (figure 1.9) [160]. In this model initial attachment is mediated through L1 interactions with HSPGs to the cell surface and the basement membrane as well as laminin-332 on the basement membrane. Interactions with GFRs through HSPGs and GFs initiate an early signaling cascade. Additional signaling cascades are mediated through interactions with α6-integrin. Additionally, initial attachment to cells occurs preferentially to extended filopodia rather than directly to the cell body. The virus moves along the extended filopodia towards the cell body before immobilizing prior to internalization [181]. Whether the virus is associated with HSPGs or EGFR, both, or another molecule during this movement is unknown. An interaction with EGFR is suggested by activated EGFR moving along filopodia, similarly to PV [182]. HSPG binding induces L1 and L2 conformational changes which are mediated by cyclophilin B, and furin cleavage, resulting in further exposure of the L2 N-terminus. HPV16 then binds to A2t after the initial signaling and conformational changes. However, conformational changes are not required as the A2t binding site in L2 is already exposed on mature virus particles [112], but signaling events trigger increased translocation of A2t to the cell surface [177]. Binding to A2t then triggers endocytosis of the virus particles through a macropinocytosis-like pathway [166] or alternatively through one of the other proposed endocytic pathways reviewed in the section on HPV entry pathways. Tetraspannin-enriched microdomains (TEMs) which are a form of receptor-enriched membrane domains, associate with HSPGs, integrins, and GFRs and play a role in HPV16 PsV
infection [183]. This study showed that tetraspannin CD151 integrin-interaction-domain is required to facilitate HPV16 PsV infection and knockdown of α6-integrin decreased infection. TEMs may thus act by bringing together into a receptor complex and stabilize the interactions between the aforementioned molecules to facilitate infection. However, no direct evidence exists for interactions between all of these molecules during HPV16 PsV infection. Alternatively, the implications of several receptor molecules may suggest they act in a sequential manner. Additionally, more than one pathway may be used by the virus. It is possible that when interactions with one receptor are blocked, the virus might be shuttled into an alternative pathway for infection.

A simplified model for the initial steps in HPV infection, focusing on interactions with the proposed attachment molecules, GAGs and laminin-332, and the subsequent cleavage by furin, is provided in figure 1.10. This model sets the framework for the hypothesis and questions addressed in this thesis proposal.
Figure 1.9. Model of HPV16 PsV cell surface interactions resulting in entry.

HPV16 first binds to HSPGs on the cell surface or basement membrane or via laminin-332 on the basement membrane. Growth factor receptors and integrins may initiate signaling pathways. Conformational changes result in the exposure of the L2 N-terminus and cleavage by a PC. Additional conformational changes results in engagement of an entry complex including A2t, and endocytosis is triggered. All of the mentioned molecules may be part of a big complex stabilized in tetraspannin-enriched microdomains. Obtained by [160]: Raff et al. 2013.
**Figure 1.10. Simplified model of initial steps by HPV16 PsV infection in tissue.** HPV16 PsV gains access to the basement membrane at sites of trauma. Initial attachment to HSPGs on the basement membrane is preferred over direct cell surface attachment. Binding to HSPGs results in conformational changes and exposure of the L2 N-terminus on the capsid surface. The L2 N-terminus is cleaved by furin. Furin-cleavage results in additional conformational changes and release from the HSPGs, facilitating transfer to an entry receptor/complex on the basal cells.
RECEPTOR USE BY THE RELATED POLYOMAVIRUSES

Lessons can be learned from the existing literature from other virus families. Similarly to the papillomaviruses, the related polyomaviruses consist of a family of structurally similar animal-associated as well as human-specific viral pathogens with different tissue tropisms. Revisiting studies performed on the polyomaviruses, various polyomaviruses preferentially associate with different receptors and evidence also exist for the sequential interaction with more than one receptor being required for entry. The murine polyomavirus, simian virus-40 (SV40), as well as human polyomavirus BKV use sialic acid linked to gangliosides, for entry into cells [95, 151]. Human polyomavirus, JCV, binds specifically to a sialylated pentasaccharide, LSTc, decorating either proteins or gangliosides expressed only on a limited number of cell types [152]. Merkel cell polyomavirus (MCV) in contrast, instead of sialylated host factors, relies on GAGs for primary attachment to host cells [184]. This initial interaction is followed by sequential interactions with sialylated host cell factors. The different carbohydrate specificities also send these viruses into different endocytic pathways. Murine polyoma, SV40, and BKV enter cells through cholesterol-dependent caveolar endocytosis, whereas JCV enters through clathrin-mediated endocytosis [95]. This is an example of where closely related viruses with a small number of amino acid changes in the receptor-binding site may determine receptor specificity as well as targeting of the virus to a specific entry pathway [152].

Depending on the serotype, another family of non-enveloped viruses, adeno-associated viruses (AAV), also uses a diverse set of receptors for attachment and
infection. For example, where AAV 4 and AAV 5 use protein-linked sialic acids [185], AAV 2 depends on HS to attach to target cells [186]. AAV6 on the other hand, uses either sialic acid residues or HS for infection, depending on the cell type [187, 188]. Thus, a general hypothesis for HPV attachment and entry may not encompass all HPV types.

**ENTRY PATHWAYS**

Once the virus initiates endocytosis and becomes internalized, the PVs are transported through the endosomes, the viral genome uncoats, and cellular sorting determines the fate of L1, and L2 along with the viral genome. L2 forms a complex with the viral genome and traffics to the nucleus, while the L1 protein is retained in the late endosome/lysosome for degradation [166, 189].

Entry by HPVs is very slow. A recent study on HPV16 PsV infection, reported an average half-time of 12 hours in HaCaT cells. Similar half-times were reported previously for both HPV16 PsV and HPV18 PsV [107, 110]. The average entry time for HPV31 NV as well as HPV31 QV is similarly slow at about 14 hours. In contrast, HPV16 QV entry is comparably rapid with an average half time of 4 hours in HaCaT cells [190], suggesting type-dependent as well as model system-dependent differences in HPV infections. Cell surface events such as interactions with several receptors and conformational changes are thought to be responsible for the slow entry kinetics [116, 191]. Cellular entry for HPV16 PsV is asynchronous with several waves of
internalization. Endocytosis of individual particles is very rapid, within 120 seconds after movement of the particle on the cell surface stops [166].

Early studies on endocytosis by different HPV types, reported a role for both clathrin- as well as caveolae-mediated pathways. Using biochemical inhibitors of endocytosis, internalization via clathrin-mediated endocytosis was observed for HPV16 PsV and HPV58 PsV in COS-7 cells (african green monkey fibroblasts), while HPV31 PsV was dependent on caveolae [192]. These studies are in conflict with a more recent study where HPV31 PsV, similar to HPV16 PsV, was reported to use a clathrin-mediated pathway in COS-7 and 293TT cells [193]. In contrast, caveoale-mediated entry was demonstrated for HPV31 QV, using transfection of dominant-negative inhibitors of endocytic pathways in HaCaT cells. Biochemical inhibitors confirmed caveolae-mediated entry by HPV31 QV. HPV16 QV entry was again suggested to be mediated by clathrin-coated pits [190]. In a recent study, HPV16 PsV entry into HeLa, 293TT, and HaCaT cells clathrin- and caveolae-independent entry was observed, using dominant negative inhibitors and siRNA knockdown experiments, [166, 191]. Recently, in a comparative study, HPV16 PsV, HPV18 PsV and HPV31 PsV entry was similarly clathrin- and caveolae-independent [194]. The clathring- and caveolae-independent entry pathway by HPV16 PsV, HPV18 PsV and HPV31 PsV is described as a “clathrin-, caveolin-, lipid raft-, flotillin-, cholesterol-, and dynamin-independent mechanism, distinct from macropinocytosis” [166, 194]. The pathway is defined by a requirement for actin polymerization and tetraspannin-enriched microdomains, and all particles traffic to the late endosomal/lysosomal compartment with similar kinetics [194].
In summary, multiple pathways have been implied to play a role in HPV infection depending on the HPV type, virus-system, cell type, and the experimental approach. Considering the diversity of HPV types, their various tropisms and HPV-associated diseases, side-by-side studies should be done before making broad conclusion regarding the mechanism used for entry. Despite non-specific effects by inhibitors on other cellular processes [166], the various responses by the different HPV types suggest that these viruses rely on host cellular factors differently from each other for infection. Detailed analyses of the various HPV types using PsV, QV, and NV, in relevant keratinocyte cell lines and primary keratinocytes, would likely show many similarities but also highlight differences among the types, reflecting the conflicting observations existing in current literature.

**Interactions with Host Factors during Entry**

Multiple steps in HPV entry and infection are attributed to functions of the minor capsid protein, L2 (figure 1.11). These steps range from L2 interactions with secondary receptors, facilitating endosomal escape of L2 along with the viral genome, interactions with the cellular sorting and trafficking machinery transport via the cytoskeleton to the nucleus, to the involvement in new capsid assembly [195]. Facilitated by cyclophilins, an L2/DNA complex separates from L1 in the endosomal compartment [159]. After disassembly, L2 accompanies the viral genome to the nucleus. Several cellular factors have been implicated to interact with L2 to facilitate transport to the nucleus, through yeast two hybrid, tandem affinity purification, and proteomic approaches. The identified
proteins include; membrane proteins, proteases, chaperones, trafficking molecules, nuclear import factors and nuclear proteins, and regulators of transcription [195].

A 23-amino acid peptide at the L2 C-terminus disrupts bacterial and eukaryotic membranes. Mutations in the L2 C-terminus compromises PsVs infectivity retaining the particles within the endosomal compartment, suggesting that the C-terminus of L2 is exposed in the endosome and facilitates endosomal escape [196]. Inhibitors against protein disulfide isomerases (PDIs) prevent the viral DNA from reaching the nucleus, suggesting that PDIs also act during endosomal escape of the L2/DNA complex [197]. However, the details of how this normally ER resident protein interact with the L2/DNA requires further investigation. The L2/DNA complex then traffics along the cytoskeleton guided by interactions with syntaxin 18 [161], sorting nexin 17 [189], and dynein [198, 199]. Interaction domains in L2 have been mapped for all of these proteins and mutations within the respective domains disrupts infection. Syntaxin 18 and Sorting nexin 17 are both involved in correctly trafficking and directing cargo proteins associated with intracellular vesicles. However, the details of how they play a role in the L2/DNA trafficking are not well understood. Interaction of L2 with dynein, a microtubule motor protein complex, directs the L2/DNA complex along microtubulues towards the nucleus [198, 199]. The L2/DNA complex then transits through the trans-Golgi network after leaving the late endosome [200]. How the L2/DNA complex reaches the nucleus in unknown. Interactions with the dynein subunit DYNLT1, which localizes to the Golgi apparatus and PMLs in mitotic cells, may play a role in the delivery of the L2/DNA complex [200-203]. Also, retention of L2 in the cytoplasm following depletion of the
hsc70 chaperone, suggests that hsc70 plays a role in release of the L2/genome complex from the microtubules before nuclear import [204].

There are several positively charged nuclear import- and export-sequences found in the C-terminus, N-terminus, and in the middle of L2, that are required for L2 homing to the nucleus [205, 206]. However, as early stages of mitosis are required for HPV infection [98] interactions with nuclear import factors may not be involved in the establishment of infection. Rather, these sequences may play a role in virion morphogenesis. During entry, the L2/DNA complex associates with subnuclear structures called PML nuclear bodies [101, 207]. L2 interacts with Daxx, a transcriptional co-repressor localizing to PMLs [205, 207]. L2 then recruits the major capsid protein, L1, and the non-structural protein, E2, as well as other cellular proteins to the PMLs [101, 204, 205, 208, 209], to mediate early viral transcription [210]. Most of the L2 interactions during early infection have been studied for HPV16 PsV and it remains to be determined whether the same cellular factors play a role in infection by other HPV types.
Figure 1.11. L2 minor capsid protein conserved domains and protein interactions. A linear representation of the L2 protein with conserved domains and known protein interactions labeled. Obtained from [211]: Wang and Roden, 2013.

<table>
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<th>Domains</th>
<th>References</th>
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<tr>
<td>Nuclear localization signals (aa 1-9) and (aa454-462)</td>
<td>Darshan et al., 2004</td>
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<td>DNA binding domains (aa1-12) and (aa456-461) [BPV1 L2]</td>
<td>Fay et al., 2004</td>
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<td>Furin Cleavage site (aa 9-12)</td>
<td>Richards et al., 2006</td>
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<td>Yang et al., 2003b</td>
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<td>Cell surface exposed sites (aa 13-31)</td>
<td>Yang et al., 2003a</td>
</tr>
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<td>Cell surface exposed and Annexin A2 binding site (aa108-120)</td>
<td>Woodham et al., 2012</td>
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<td>Sytinx 18 binding site (aa43-47) [BPV1 L2]</td>
<td>Bossis et al., 2004; Lanioz et al., 2007</td>
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<td>Transmembrane-like domain (45-67)</td>
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<td>SORTIN Nexin 17 binding site aa (245-257)</td>
<td>Bergant et al., 2012</td>
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<td>Dynein interacting domain (aa456-461)</td>
<td>Florin et al., 2006</td>
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<td>ND10 localization domain (aa390-420) [HPV33 L2]</td>
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<td>L1 binding domain (396-439) [HPV11 L2]</td>
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<td>Arginine rich nuclear retention signal (296-316)</td>
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<tr>
<td>Nuclear export signal (462-471)</td>
<td>Mamoor et al., 2012</td>
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DIFFERENCES IN NATIVE PARTICLES COMPARED TO SYNTHETIC PARTICLES

L2 N-terminus

Studies using chimeric L1 and L2 HPV18/HPV16 NV particles show that L2 has a major impact on L1 and overall structure [46]. In particles with the L2 protein chimeric for both HPV16 and HPV18, with wildtype HPV16 L1, the overall structure is altered to escape neutralization by the H16.V5 antibody which targets the major neutralizing epitope, as well as escaping neutralization by the H16.7E antibody. Using a variety of chimeras for L1 and L2, it was observed that changes in L2 impact L1 structure, and similarly that change in L1 impacts the display of L2 on the capsid surface.

The slow maturation of native virions in a differentiating epithelium plays an important role in regulation of capsid formation and conformational changes over time. A more detailed review of HPV16 NV and PsV maturation is found in the Virus Maturation section of this chapter. The cellular environment and cellular factors influence the final structure of tissue-derived particles [47, 48, 73, 212-214]. Antibody-mediated neutralization targeting L1 and L2 epitopes is enhanced over a 10-day period as the particles become more mature, suggesting that controlled conformational changes occur in context of the differentiating tissue. Cross-neutralization by an array of L2 specific antibodies targeting surface-exposed epitopes of L2 displayed a different pattern of neutralization for tissue-derived particles as compared to previously published data on PsV particles, suggesting that there are differences in NV as compared to PsV maturation.
Specific temporal changes in L2 exposure on the virion surface have been mapped on the HPV16 PsV capsid surface with the monoclonal antibody RG-1 (amino acids 17-36). The L2 N-terminus along with the furin cleavage site is masked in the mature PsV particles but is displayed only hours post-attachment to host cells [91, 92]. Neutralization of HPV16 NV particles by RG-1 is inefficient when immature 10-day virions are extracted, as compared to the efficient neutralization observed of mature 20-day virions [47]. These results suggest that conformational changes may occur during virion morphogenesis as well as during infection.

Close to the N-terminal surface exposed loop, there are two highly conserved cysteine residues, C22 and C28, which form an intra-molecular disulfide bond creating a small loop in the L2 N-terminus. Mutation of either cysteine residue in HPV16 PsV and QV allows for normal capsid formation, but the resulting particles are non-infectious [216, 217]. Neither study observed any effect on attachment or early trafficking of the viral genome and L2 protein. Mutant PsV particles also displayed the 17-36 epitope during infection similar to wildtype, suggesting that these cysteine residues do not play a role in the exposure of the L2 N-terminus. The observed effect on infection may be due to the mutations preventing interactions of the region with a cellular factor. Or, the disulfide-bond holds the N-terminus of L2 in a particular conformation, thus acting as a molecular switch through a subtle conformational change, which is important for an unknown step during infection. A much lesser effect on infectivity was observed for BPV-1 PsV, suggesting type-dependent differences in the dependence of these residues during infection [217]. In contrast, studies using HPV16 NV found that not only do the
C22 and C28 cysteine mutants retain wildtype levels of infectivity but their infectivity increases [218]. However, the stability of the particles is compromised. Also, neutralization targeting the L2 N-terminus is prevented, suggesting that the N-terminus is not properly exposed in the mutant particles. Alternately, if exposed, the virions are still able to bind antibodies but are no longer susceptible to neutralization. The increase in infectivity may be due to an enhanced ability of unstable capsids to uncoat during infectious entry. Or, the mutated particles may display the N-terminus more efficiently for proteolytic cleavage or binding to a putative cellular entry factor. Overall, these data suggest there are structural and functional differences among different HPV types, genome-encapsidating versus not, and monolayer versus tissue-derived particles. Despite the uncertainty of the role of the L2 N-terminus in infection, due to the cross-reactive nature of antibodies targeted to the region, it remains an important target for the development of a cross-neutralizing HPV vaccine [219].

**Viral DNA**

The incorporation of DNA into particles alters the final structure [72]. When comparing DNA-free VLPs to DNA-containing PsV in cesium chloride density gradient centrifugation, the particles band in a light as well as heavy fractions. DNA-containing particles band exclusively in the heavy fractions. Further, the heavier fractions contain particles with more extensively cross-linked L1 molecules, suggesting they have a higher degree of disulfide formation. A higher degree of disulfide-bond formation is also observed in native HPV particles derived from hand warts [72]. Native BPV-1 capsids
are also more extensively cross-linked than HPV16 and HPV18 PsV [73]. Also, these particles are more resistant to trypsin-digestion, indicating a tighter association of capsomeres due to association with the 8-kb, histone-associated viral genome. In contrast, PsV incorporates a heterogeneous population of plasmid and cellular DNA of various sizes, with an upper limit of 8 kB [72]. It is not well understood what structural and functional differences are imposed by interactions with the 8-kb, histone-associated viral genome that is specifically incorporated in native virions.

**Other viral and cellular factors impacting assembly and structure**

Papilloma- and polyomaviruses share the ability to self-assemble *in vitro*. However, *in vivo* assembly is a highly regulated process that only occurs in the nucleus, and only in the suprabasal cells for papillomaviruses. Cellular chaperones, hsp70 and karyopherins, interact with the major capsid proteins and regulate assembly and disassembly in monolayer studies, for both virus families [213, 214, 220]. An increase in HPV31 NV particle production is observed when hsp70 expression is induced at higher levels, possibly due to interactions between L1 and hsp70 observed in raft cultures [212]. During the slow maturation of native HPV particles [47], disulfide bonds form between the capsid proteins that stabilize the viral particles [48]. Maturation of PsV particles takes place in the presence of the cellular lysate but not when capsids are cleared from the cellular lysate [73]. These data provide additional support that cellular factors are involved in the process. It is not well understood what other cellular proteins might be involved in forming the final mature and stable capsids.
**Binding and entry into cells**

Functional differences are observed in various binding and entry assays among various types of synthetic particles. These differences are most likely due to subtle variations in overall structure among PV types. In studies looking at the heparin sulfate side-chain modification preference, O-linked sulfation is sufficient for HPV33 L1L2 VLP binding to COS-7 monkey cells, whereas HPV33 PsV also show an additional requirement for N-linked heparan sulfates. These data suggest differences in particle structure imposed by the presence of L2, impacts L1 interactions with HS [144]. Also, HPV33 VLP internalization occurs with a half-time of 3.5 hours, whereas HPV33 PsV internalization is much slower with a half-time of about 7.5 hours [144]. Thus, suggesting that differences in surface interactions have an impact on downstream entry events.

**MICROBICIDES TARGETTING VIRAL ENTRY**

Two effective vaccines, targeting infections by high-risk HPV16 and HPV18, Cervarix and Gardasil, as well as low-risk HPV6 and HPV11 (Gardasil only), are currently available. They are not cross-protective against the full spectrum of high-risk and low-risk HPVs that are prevalent in human disease. Also, the relatively high cost limit their use in developing countries; and the cost effectiveness is questioned in countries with low rates of cervical cancer due to effective Pap smear programs. Thus, the development of less expensive topical microbicides that would also offer coverage against additional HPV types would complement the current vaccine programs.
Due to the observed importance of GAGs, mainly HS, in HPV attachment to and infection of host cells (reviewed in the “Glycosaminoglycans” section) by HPV16 PsV as well as other genital HPVs, agents targeting GAGs are of much interest. High molecular weight sulfated or sulfonated polysaccharides or polymers cellulose sulfate, dextran sulfate, and polystere sulfonate show microbicidal activity against BPV-1 in mouse cells and HPV11 and HPV40 in human A431 cell (human epithelial carcinoma cells) at a 50% inhibitory concentration of 1 to 10 µg/ml and 10 to 100 µg/ml, respectively. Importantly, these concentrations do not show toxicity in cell cultures [221]. Carrageenan, a highly sulfated polysaccharide extracted from red algae, was identified as a potential microbicide against PsVs of several genital HPVs, including HPV16, 18, 31 and HPV45 PsVs [14]. Inclusion of carrageenan in gel lubricants prevents HPV16 PsV infection in vitro and in vivo when applied to the mouse vaginal tract [14, 133]. Carrageenan is readily available commercially as a thickening agent in food and cosmetic products. As it is already approved and considered safe for food and topical application, it represents an ideal candidate as a microbicide. Carrageenan resembles HS structurally and blocks infection primarily by direct binding to the viral particles. However, carrageenan is much more efficient than the cell free HS, heparin, in inhibiting PsV infection, with 50% inhibition observed in the ng/ml range [14]. New compounds targeting GAG interactions are continuously being developed. Cebulagic acid and punicalagin isolated from the Terminalia chebula tree, are effective in preventing GAG interactions by human cytomegalovirus, hepatitis C virus, dengue virus, measles virus, and respiratory syncytial virus, demonstrating the potential of GAG targeting compounds as broad spectrum antivirals [222].
In addition to attachment, other steps of HPV entry can be targeted by antivirals. A high throughput screen for antivirals against HPV16 PsV infection of 293TT cells, identified 2 compounds from 40,000 that inhibit infection at post-entry steps, and that have good antiviral properties, including a low IC$_{50}$ and low cytotoxicity [223]. The cellular factors involved in HPV attachment and entry all represent promising targets for the development of novel, cost-effective antiviral drugs. Thus, elucidating the identity and function of these players for individual cancer-associated HPV types in order to identify type-specific as well as cross-reactive targets, is a key interest in HPV research. Some identified attachment and entry factors and the step/s in which they function during HPV16 PsV infection can be seen in figure 9.

**SPECIAL CASE OF HPV VARIANTS**

Genotypic variants of circulating HPV types have been identified for most oncogenic HPV types. Individual HPV types are identified by the DNA sequence of the major capsid protein, L1, differing by at least 10% from any other characterized HPV type [1, 224]. Speciation of different HPV types is a result of sequential genetic changes over millions of years as PVs replicate their genome using the host replication machinery with a very low mutation rate [225, 226]. A subtype or variant is characterized by the L1 open reading frame (ORF) differing by less than 10% [227]. Variants specifically, are defined as having less than a 2% nucleotide difference in the L1 ORF. In addition to the changes in L1, correlated changes and polymorphisms specific to a certain variant are
found throughout the genome [228, 229]. Interestingly, the evolution of viral variants of HPV16 and HPV18 can be traced back to the migration of the human population and the content of origin [230-232]. Variants have been identified for other HPV types as well, but human co-evolution and geographical association has not yet been determined for other types [233-238]. Despite their close phylogenetic relatedness variants display differences in their pathogenic abilities and the development of cervical cancer [239-248]. Based on phylogenetic analyses HPV16 variants were initially divided into four major variant lineages including; European (E), Asian (As), Asian-American (AA), African type 1 (Af-1), and African type 2 (Af-2) [229, 249, 250]. For HPV16, a German isolate belonging to the European family is considered to be the prototype or reference sequence [251]. Additional sequencing efforts later described an additional lineage, North American (NA) [250, 252, 253]. When a phylogenetic tree was created using sequence information from the E6 ORF and the LCR, using 953 samples gathered from across diverse geographical regions of the world, the variant lineages segregated into four major branches; the European-Asian (EAS), including the E and As sublineages, Af-1, including Af-1 a and b sublineages, Af-2, including Af-2 a and b, and North-American/Asian-American, including NA and AA1 and 2 sublineages (figure 1.12) [254].
Figure 1.12. Phylogenetic tree of HPV16 variants genomes. Segregation of the variant lineages into four major branches; the European-Asian (E-As), including the E and As sublineages, Af-1, including Af-1 a and b sublineages, Af-2, including Af-2 a and b, and North-American/ Asian-American, including NA and AA1 and 2 sublineages. Obtained from [79]: Ahmed et al. 2013.
Variants and cancer development

Not all HPV lesions result in the development of cancerous lesions. The development of cervical cancer may be influenced by genetic variability in the host itself, viral genome, and other environmental factors [255, 256]. Each stage of the viral life cycle, from initial exposure and infection, persistence of infection, development of a precancerous low-grade lesion, to the progression to invasive high-grade lesions [257], may be influenced by genetic variations in the viral genome and the other aforementioned factors. Dividing into biologically observable events, HPVs may vary in (1) infectivity (ability to infect the host), (2) persistence over time (differences in immune clearance, (3) progression to low-grade lesion given persistence (deregulation of host differentiation and other cellular changes), (4) progression to invasive cancer [227]. Epidemiological studies support the influence of HPV16 variants on viral persistence (2-fold effect) [246, 247], development of pre-cancerous lesions (2-4 fold effect) [246-248, 258-260], and progression to cancer (elevated risk) [239, 240, 260]. Most evidence point to the non-European (NE) lineages, African and Asian-American, as having a more pathogenic phenotype when compared to the European variants [227]. Less evidence is available for variants of other types, including HPV31 and HPV45, complicating the analysis for the association of one variant versus another with an increased risk for the development of cervical neoplasia [227].

Whole-genome analyses of HPV16 variants have demonstrated co-segregation of nucleotide changes in their genomes such that specific changes in one ORF correspond to specific sets of changes in the other ORFs [229, 231, 252]. This co-segregation supports
further analyses of biological differences as they relate to the whole genome of viral variants. Epidemiological studies of the natural history and carcinogenic potential of HPV16 variants are unlikely to be able to identify causal from non-causal lineage-specific variations [261]. In addition to lineage-specific amino acid changes, other variations in the genome do exist and need to be kept in mind as they could explain the biological behavior of a specific variant isolate [262, 263].

Several molecular studies have been performed focusing mostly on the effect of variations in the E6 and E7 viral oncogenes, the LCR, and E1 and E2 early proteins in isolation from the complete viral genome, and their effect on oncogenic properties [264-273], confirming the role of variant lineages in the development of cancer. In contrast, no studies on relative viral infectivity and establishment of infection have been done for any viral variants [227]. A few studies have analyzed the effects from variations of specific amino acid residues identified in the L1 ORF of viral variants on particle production and cross-neutralization [50, 274, 275].

Infections by HPVs are generally self-limiting and cleared within 8-12 months. Data has shown that HPV16 is unique in viral fitness compared to other types as HPV16 has a higher prevalence with a greater number of total infections that are able to persist longer, and a greater ability to progress to high-grade lesions [9, 10, 276]. The higher prevalence and [277] ability of variants to persist in certain populations is related to their carcinogenic potential within that particular population [243, 278, 279]. It is therefore reasonable to hypothesize that the infectivity of a given variant may be related to the infection rate (prevalence) and ability to establish a persistent infection. In chapter V we
are exploring the infectivity and infection pathways of a limited number of HPV16 molecular variants.

**CONCLUDING REMARKS**

Current literature on HPV entry is based largely on studies using PsV. Existing data on HPV capsid structure and virus maturation describe several discrepancies between synthetic and native particle morphogenesis and structure. Much of the evidence is indirect, as exemplified by antibody neutralization and stability studies. A detailed structural analysis has not been done comparing the high-risk HPV types derived from monolayer- versus tissue-dependent systems. However, detailed side-by-side comparisons are warranted to discover similarities as well as possible differentiation-dependent aspects of the HPV life cycle. In this thesis we will focus on the attachment and entry of tissue-derived particles.
CHAPTER II MATERIALS AND METHODS

Ethics Statement

The use of human cervical and foreskin keratinocyte tissues to develop cell lines as well as for infectivity assays for these studies was approved by the Institutional review Board at the Pennsylvania State University College of Medicine and by the Institutional review Board at Pinnacle Health Hospitals. Discarded, de-identified tissues were exempt from needing informed patient consent. Informed consent was waived by both Institutional Review Boards.

DNA reagents and mutagenesis

pBSHPV16(114B) and pBSHPV18 DNA were used as the wildtype template for mutations in HPV16 and HPV18, respectively, for site-directed mutagenesis using QuikChange II XL site-directed mutagenesis kit (Stratagene). The L1 lysine 278 to alanine (K278A) mutation was created using the following complementing oligonucleotides:

forward 5’CAGACGATTTATACATTGCAGGCTCTGGGTCTACTG3’
reverse 5’CAGTAGACCCAGAGCCTGCAATGTATAAATCGTCTG3’.

The L2 serine to alanine mutations were created using the following sets of primers:

forward 5’CAGACGATTTATACATTGCAGGCTCTGGGTCTACTG3’
reverse 5’CAGTAGACCCAGAGCCTGCAATGTATAAATCGTCTG3’. 
5’CAATGCGACACAAAAAGTTCTGCAAAACGC3’ and reverse
5’GCGTTTTTCAGAACTTTTGTCGCATTG3’ for HPV16 L2 R12S, forward
5’CAATGCGACACAAAAAGTTCTGCAAAACGC3’ and reverse
5’GCGTTTTGCAGAACTTTTGTCGCATTG3’ for HPV16 L2 R5S, and forward
5’GGTATCCACCGTGCCGCAAGTCGCAACCGGCTTTCGGTAAC3’ and reverse
5’GTACGGAAAGCCCCTTTCGTGCGACTTGGCGGCACCGGTAAC3’ for HPV18
L2 R11S3’, and forward
5’GGTATCCACCGTGCCGCAAGTCGCAACCGGCTTTCGGTAAC3’ and reverse
5’GTACGGAAAGCCCCTTTCGTGCGACTTGGCGGCACCGGTAAC3’ for HPV18
L2 R8S3’. An L2 expression plasmid was used as a template to create a plasmid with an
N-terminal deletion (amino acids 2-12) corresponding to the conserved furin cleavage
site [91]. The p16L2h plasmid [280] was utilized as templates for site-directed
mutagenesis The deletion was created using the follow complementing oligonucleotides:
forward 5’ GTTATTACTTAACTGGGATCGGTATCCACCGGCTTTCGGTAAC 3’ and reverse 5’
GTACGGAAAGCCCCTTTCGTGCGACTTGGCGGCACCGGTAAC 3’. The resulting plasmids were
sequenced to verify the presence of the correct changes.

Variant genomes EVA2716 and EVA3724 were a kind gift from Dr. Long Fu Xi
at Washington University from cervical brushings. Genomes were amplified by rolling
circle amplification and cloned into a pBS backbone by Horng-Shen Chen, a previous
student in the lab.
Keratinocyte cultures and electroporations

Primary human keratinocytes from newborn foreskin circumcision and cervical biopsies were isolated as previously described [41]. Primary keratinocytes were maintained in 154 medium supplemented with Human Keratinocyte Growth Supplement Kit (Cascade Biologics, Inc., Portland, OR). For electroporations, 30 µg of the mutated viral DNA was digested with EcoRI, linearizing the viral DNA and separating it from the vector sequence. HFKs were electroporated with the prepared DNA as described previously [16, 41]. Immortalized keratinocytes stably maintaining HPV episomes were cultured in E-medium with J2 3T3 feeder cells [16].

Production and isolation of NVs

To produce native HPV virions immortalized HPV-containing keratinocytes were grown in organotypic raft cultures as previously described [16]. Mature virus particles were harvested from tissues after 20 days [47]. Rafts were harvested and virus was isolated by homogenization in phosphate buffer (.005M Na-phosphate, pH 8, 2mM MgCl₂) as previously described [47]. All virus preps for titering and infectivity assays were treated with benzonase (375U) at 37°C for one hour to remove any un-encapsidated viral genomes. Samples were adjusted to 1M NaCl and centrifuged at 4°C for 10 minutes at 10,500rpm to remove cellular debris. Virus preps were stored at -20°C for short-term storage and -70°C for long-term storage.
**Virus Titers**

To release the viral genomes, 10µl of a virus prep was re-suspended in a 200µl HIRT DNA extraction buffer (400mM NaCl / 10mM Tris-HCl, pH 7.4 / 10mM EDTA, pH 8.0), with 2µl 20mg/ml Proteinase K, and 10µl 10% SDS for 2 hours at 37°C. The DNA was purified by phenol-chloroform extraction followed by ethanol precipitation and re-suspended in 20µl TE [47]. Titers were determined using a qPCR-based DNA encapsidation assay utilizing a Qiagen Quantitect SYBR Green PCR Kit. Amplification of the viral genome target was performed using previously described E2 primers against a standard curve of 10-fold serial dilutions from 10^8 to 10^4 copies per µl [47, 215].

A SYBR green PCR kit (Bio-Rad) and Bio-Rad CFX-96 Real-Time qPCR machine and software were utilized for PCR amplifications and subsequent data analysis.

**Cell cultures**

HaCaT cells were maintained in DMEM supplemented with 10% FBS, 0.025mg/ml Gentamicin, and 0.11mg/ml sodium pyruvate. CHO parental, CHO FD11 and FD11 plus furin cells were a gift from Stephen Leppla (National Institute of Allergy and Infectious Disease, NIH) [281]. CHO pgsA-745 cells were obtained from the ATCC. CHO cells were maintained in minimal essential α-medium supplemented with 10% FBS and antibiotics. 293TT cells were maintained in DMEM supplemented with 10% FBS, 1mM pyruvate and 0.4 mg/ml hygromycin.
Inhibitors and antibodies

The polysaccharides heparin (H4784, high molecular weight) and chondroitin sulfate A/C (C4384), ι-carrageenan (C4014), and sodium chlorate (403016), were purchased from Sigma-Aldrich. Furin inhibitor I (decanoyl-RVKR-chloromethylketone) was purchased from Calbiochem. The anti-L1 antibodies were a gift from Neil D Christensen. H16.V5, H18.J4, H31.A6, and H45.N5 were used to neutralize HPV16, HPV18, HPV31, and HPV45, respectively.

Infections and infectivity analysis by qPCR

HaCaT cells were seeded in 24-well plates, 50,000 cells per well 2 days prior to infection. CHO and 293TT cells were seeded 30,000 per well and healthy low-passage primary cells were seeded 70,000 per well, to account for growth rates. Compounds were mixed with virus and media in a total volume of 500 µl prior to addition to cells. Compounds were left on for the duration of the infections. For sodium chlorate, cells were grown in the presence of the compound 2 days prior to and during the infection. Virus was incubated with the cells for 48 hrs at 37°C. An MOI of 10 particles per cell was used unless otherwise noted. mRNA was harvested using a Qiagen RNAeasy Kit. Infections were analyzed using a previously described RT-qPCR-based infectivity assaying for E1^E4 transcript levels. Primers and probes to amplify the E1^E4 viral target and TATA-binding endogenous cellular control target were previously described [47, 215]. Amplifications were performed in duplicates for each sample 96-well qPCR
plates (Bio-Rad) using the Quantitect probe RT-PCR kit (Qiagen) and the CFX-96 instrument (Bio-Rad). Relative levels of viral transcripts were determined by using the REST software. Results are representative of means and standard deviations of at least three independent infections for each virus type. Students t-test was performed with statistical significance calculated with p < 0.05. Sensitivity of PsV to the furin inhibitor was tested by seeding cells and incubating with inhibitor the same way as native virus. Two days after the addition of PsV to the cells, PsV infection was assessed by immunofluorescent microscopy monitoring GFP-expression. Briefly, cells were washed extensively, fixed in 4% paraformaldehyde for 10 min at 4°C, washed and mounted with aqueous mounting media Aqua Poly/Mount (Polysciences). GFP expression was analyzed using a Nikon Eclipse 80i microscope. Images were captured using a Nikon Digital Sight SD-Fi1 camera using NIS-Elements 3.10 software.

Attachment Assays

Cells were seeded the same way as in the infectivity assays. The virus was incubated with the cells at an MOI of 25 for 2 hours at 4°C with or without compound, and then shifted after the indicated number of hours to 37°C. Cells were washed 3x with PBS before lysing on the plate for 2 hours at 37°C using a HIRT lysis buffer, followed by phenol-chloroform extraction and ethanol precipitation. The number of particles attached to the cells was determined as the number of viral genomes by amplifying a target sequence in the E2 gene, as in the DNA encapsidation assay. Results are representative of
means and standard deviations for at least three independent attachments assays from at least 2 different virus batches. Students t-test was performed with statistical significance calculated (p < 0.05).

**In vitro furin enzyme-treatment and fluorogenic furin assay**

Virus preparations were adjusted to 100 mM Hepes, and 1 mM CaCl₂, and 5U furin enzyme, followed by an 7 hour incubation at 37°C prior to infections [91]. As a control cleavage of the Boc-RVRR-AMC fluorogenic peptide (Alexis biochemicals) was assayed under the same conditions with the addition of 0.1M 2-mercaptoethanol and 5% Triton X-100 at Ex/Em 350/450.

**Histology and immunofluorescence staining**

Un-infected and infected foreskin and cervical tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (4 µm) were cut and either stained with hematoxylin and eosin (H&E) as previously described [16], or assessed for the expression of cellular furin. Briefly, tissue sections were deparaffinized in xylene 2 x 10 minutes. Slides were washed by 3 x 3 minute in 100% ethanol to rinse xylene, followed by rehydration in dH₂O for 5 minutes. Antigens were retrieved by boiling in Tris-EDTA buffer (pH 9.0) for 10 minutes. Slides were allowed to cool to room-temperature and rinsed in .05% TBS-Tween for 5 minutes. Samples were blocked with background sniper blocking agent (Biocare Medical) for 15 minutes at room temperature. Tissue sections
were incubated with the furin primary antibody, MON-139, diluted 1:200 in DaVinci green antibody diluent (Biocare Medical) at 4°C overnight. Slides were then rinsed in TBS-Tween 3 x 5 minutes before incubating with Alexa-Fluor 488 diluted 1:200 in DaVinci green antibody diluents for 1 hour at room temperature. Slides were then stained with 10 μg/ml Hoechst 33342 (Molecular Probes/Invitrogen) in TBS-Tween for 10 minutes at room temperature, followed by 2 x 5 minutes rinses in TBS-Tween. Slides were mounted with Aqua Poly/Mount. Images of tissue sections were captured using a Photometrics CoolSnap cf2 camera and Nikon Digital Sight SD-Fi1 camera using NIS-Elements 3.10 software on a Nikon Eclipse 80i microscope. Images of uninfected and infected tissues were adjusted identically for brightness and contrast.

**SDS-PAGE and L2 Western Blots**

Equal aliquots (50 μl) from homogenized HPV16 virus preps and non-infected tissues were re-suspended in 6% 2 mercaptoethanol loading buffer and boiled for 10 minutes. HPV16 PsV was used for the detection of full-length L2 [111]. An L2 expression plasmid with an N-terminal deletion was used as a control for N-terminally cleaved L2. Samples were loaded onto a 7.5% polyacrylamide gel followed by transfer onto a nitrocellulose membrane. Nitrocellulose membranes were blocked for 2 hours at room temperature using StartingBlock (Thermo Scientific) with .05% Tween. To detect HPV16 L2, membranes were incubated overnight with the RG-1 monoclonal antibody (a kind gift from Richard Roden, Johns Hopkins) at a 1:200 dilution in blocking buffer.
Membranes were then incubated with Biotin-Goat Anti-Mouse IgG (H+L) (Invitrogen) at a dilution of 1:10,000 for 1 hour, followed by Streptavidin Horseradish Peroxidase (HRP) Conjugate (Invitrogen) at a dilution of 1:5,000 for 20 minutes to amplify the signal. Membranes were washed with PBST after each incubation step. HRP was detected using an ECL kit (Perkin Elmer).

**PsV and QV production and expression of plasmid constructs in 293TT cells**

Purified HPV16 PsV produced in 293TT cells using the HPV16 L1 and L2 codon modified p16LIw plasmid, packaging a GFP-expression plasmid (pfwB), was a kind gift from Patricia Day (Center for Cancer Research, NIH) [282].

HPV16 QV was generated in 293TT cells as previously described [283]. Briefly, 293TT cells were co-transfected by using Lipofectamine 2000 with 25 µg each of HPV16 wild-type genome and a bi-cistronic L1/L2 expression vector [284]. The HPV16 L2 N-terminal deletion expression plasmid was similarly expressed by lipofectamine transfection in 293TT cells. Cells were harvested 48 hours post-transfection. Cell pellets were harvested by dounce homogenization and resuspended in phosphate buffer similarly to the native virus harvest. Virus lysates were incubated overnight at 37°C for virion maturation prior to spinning down the cellular debris [73].
**Optiprep gradient purification**

Optiprep purification was performed as described previously [47, 285]. Briefly, a 27, 33, and 39% Optiprep step gradient was produced by under-layering. Gradients were allowed to diffuse at room temperature for at least one hour. Approximately 350 µl of virus was layered on top of the gradient. Tubes were centrifuged in a SW55 rotor (Beckman) at 234,000 X g for 3.5 hours at 16°C. After centrifugation, 11 500 µl fractions were collected starting from the top of the gradient.
CHAPTER III DIFFERENTIAL DEPENDENCE ON HOST CELL GLYCOSAMINOGLYCANS FOR INFECTION OF EPITHELIAL CELLS BY HIGH-RISK HPV TYPES

To further the understanding of HPV entry, we set out to investigate whether diverse HPV types produced under physiologically relevant conditions of differentiating host tissue are dependent on GAG-mediated binding for infection. Here, we examined the requirement of the high-risk HPV types HPV16, HPV18, HPV31, and HPV45 for GAG binding during infection. The data in this chapter was published prior to completion of this dissertation [286].

Infection and neutralization for analyses of high-risk HPVs

The production and neutralization of the most common high-risk papillomaviruses HPV16, HPV18, and HPV45 in foreskin-derived organotypic raft culture has been previously shown [41, 42, 47]. Native HPV31 was produced from a cervical intraepithelial neoplasia type 1 biopsy-derived cell line CIN-612 9E [16]. To verify the specific particle-mediated infectious entry as well as the specificity of the RT-qPCR assay for the analysis of HPV infection, we neutralized each virus with a type-
specific monoclonal antibody targeting a major epitope in the L1 major capsid protein (figure 3.1).
Figure 3.1. Particle-mediated infection. HPV16, HPV18, HPV31, and HPV45 were incubated with L1 type-specific antibodies H16.V5, H18.J4, H31.A6, and H45.N5, respectively, for 1 hour at 37°C prior to infection of HaCaT cells. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection.
Inhibitory effects of the HS mimetic heparin

Heparin, a highly sulfated form of HS produced from mast cells, is thought to bind to HPV particles and prevent binding to the cells [107]. HPV16 infection of HaCaT cells was not blocked by heparin (figure 3.2A). In contrast, infection by HPV18 was efficiently blocked by heparin in a dose-dependent manner (figure 3.2B). HPV31, which is evolutionarily related to and found in the same species (α9) as HPV16, showed the same resistance to heparin as HPV16 (figure 3.2C). This confirms data from a previous study on infection by HPV31, where the presence of heparin had no effect on infection of HaCaT cells [115]. Infection by HPV45, which is related to and found in the same species (α7) as HPV18, was also resistant to inhibition by heparin (figure 3.2D). Thus, functional studies rather than sequence conservation and relative relatedness between different HPV types are necessary for the prediction of responsiveness to a given blocking agent. At a multiplicity of infection (MOI) of 10 particles, HPV18 infection was unaffected by the presence of 1µg/ml heparin, with 90% inhibition observed at 10µg/ml heparin (figure 3.2B). When infections were done with an MOI of 1,000 particles the inhibitory ability of heparin decreased, with 50% inhibition observed at 10µg/ml heparin. This is in contrast to a PsV inhibition assay at a similar inoculum where a 10-fold lower concentration is needed for 50% inhibition of infection [14], suggesting that HPV18 NV particles are less sensitive to inhibition by heparin than PsV. The observed capsid dose-dependence of inhibition further supports the direct binding of heparin to the HPV18 NV particles.
Figure 3.2. Inhibitory effects of heparin against various HPV types. A) HPV16, B) HPV18, C) HPV31, and D) HPV45 were incubated with heparin at increasing concentrations (0 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml) for 30 min’s at 37°C prior to infection and during infection of HaCaT cells. All infections were done at an MOI of 10. For HPV18 additional infections at a MOI of 100 and 1000 were performed. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentrations with infection at 0 µg/ml of heparin set equal to one.
Heparin and virus attachment

The effect of heparin on total virus adsorption to HaCaT cells and the extracellular matrix was analyzed by measuring attachment at 4°C. No significant block was observed for attachment by HPV16 in the presence of heparin (figure 3.3A), suggesting HPV16 may attach to cells using a non-HS receptor. HPV18 attachment was completely blocked by heparin (figure 3.3B), supporting that the first step in the infectious pathway by HPV18 is to bind to HS.

Despite resistance to inhibition of HPV31 and HPV45 infection in the presence of heparin (figure 3.3C, D), a nearly complete block of attachment was observed for both virus types in the presence of heparin (figure 3.3C, D). After binding to cells, HPV virus particles have been shown to be surface bound for several hours [92, 166, 190, 287] and conformational changes of surface-bound papillomavirus particles are well documented [90, 92, 144]. It has been suggested that conformational changes are responsible for the slow entry kinetics of the virus particles [166]. Thus, heparin, despite not being able to block infection may still bind to the particles and prevent efficient conformational changes thus preventing initial attachment to cells. We tested this by chasing attachment for 2 hours at 4°C with increased times at 37°C. HPV16 and HPV18 attachment in the presence of heparin did not change over time (figure 3.3E, F). For HPV31, switching the cells to 37°C allowed for an increased number of particles to adhere to the cells in the presence of heparin over time (figure 3.3G), suggesting that coating of the particles by heparin at 4°C may present a block to conformational changes that need to take place. HPV45 attachment in the presence of heparin remained blocked, even up to 8 hrs at 37°C.
(figure 3.3H and data not shown). Initial HPV45 receptor-binding may be of very low affinity and/or HPV45 transfer to a higher affinity entry receptor may require more time.
Figure 3.3. Effect on absorption in the presence of heparin. A) HPV16, B) HPV18, C) HPV31, and D) HPV45 were mixed with heparin at increasing concentrations (0 µg/ml, 10 µg/ml, and 100 µg/ml) and added to HaCaT cells at an MOI of 25. The virus was allowed to attach to HaCaT cells for 2 hours at 4°C. E) HPV16, F) HPV18, G) HPV31, and H) HPV45 in the presence or absence of heparin (0 µg/ml and 100 µg/ml). After attachment at 4°C, the cells were shifted to 37°C for an additional 2 to 4 hours. Analysis of the number of particles was done by SYBG q-PCR with attachment in the absence of heparin set to 100%.
Effects of Chondroitin sulfate

Two main categories of GAGs, glucosaminoglycans, including HS, and galactosaminoglycans, including chondroitin sulfate (CS), exist. To examine the possibility of HPV interactions with different GAG types, we investigated the role of CS for infection by adding chondroitin A/C exogenously during infection (figure 3.4). Only HPV18 was sensitive to the addition of CS, whereas HPV16, HPV31, and HPV45 were resistant. The sensitivity of HPV18 to both heparin and CS suggests that the determinant for attachment is a combination of the type of GAG and/or GAG sulfate modifications.
Figure 3.4. Inhibitory Effects of Chondroitin A/C against various HPV types.

HPV16, HPV18, HPV31, and HPV45 were incubated with or without chondroitin A/C (0 µg/ml, and 100 µg/ml) for 30 min’s at 37°C prior to infection and during infection of HaCaT cells. All infections were done at an MOI of 10. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentration with infection at 0 µg/ml of chondroitin A/C set equal to one.
Infection in absence of sulfate modifications

The sulfation patterns of GAGs have been shown to play a role in the ability to inhibit HPV PsV infection [288]. To examine infection of NV particles in cells deficient in cell surface GAG sulfate modifications, we treated HaCaT cells with increasing concentrations of sodium chlorate. HPV16 efficiently infected sodium chlorate-treated cells (figure 3.5A), suggesting HPV16 does not depend on GAG sulfate modifications for infection. In contrast, HPV18 was efficiently blocked (figure 3.5B). HPV31 and HPV45 were also inhibited by the absence of GAG sulfation in a dose-dependent manner (figure 3.5C, D). Given that HPV31 and HPV45 infections are resistant to both heparin and chondroitin during infection, this supports a requirement for GAG sulfation by these virus types during infection that may display a different GAG specificity.
Figure 3.5. Infection of sodium chlorate-treated HaCaT cells. HaCaT cells were treated with increasing concentrations of sodium chlorate (0mM, 10mM and 25mM) 2 days prior to infection and during infection by A) HPV16, B) HPV18, C) HPV31, and D) HPV45 at an MOI of 10. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentration with infection at 0 mM sodium chlorate set equal to one.
Attachment and infection in the absence of GAGs

To further analyze the specificity of NV HPV types for GAG binding, we took advantage of CHO cells either expressing or deficient for the expression of GAGs. We found that GAG-deficient pgsA-745 cells were infected by HPV16 at similar levels compared to CHO parental cells (figure 3.6A). Further, HPV16 binding to pgsA-745 cells was equivalent to that of CHO parental cells (figure 3.6B), supporting the ability of HPV16 to infect cells in a GAG-independent manner. In contrast, HPV18 infection was compromised in pgsA-745 cells (figure 3.6A). A decrease in HPV18 binding added to the evidence for a requirement of GAGs for primary attachment and infection (figure 3.6B).

Infection of CHO cells by HPV31 and HPV45 was too low to reproducibly detect in RT-qPCR infectivity assays. However, binding by HPV31 and HPV45, was consistently lower in the pgsA-745 cells compared to parental cells (figure 3.6C), suggesting that cell surface binding by HPV31 and HPV45 is dependent on cellular GAGs.
Figure 3.6. Attachment and infection in CHO parental cells and GAG-negative pgsA-745 cells. CHO Par and pgsA-745 cells were plated 2 days prior to infection by A) HPV16 and HPV18 at an MOI of 10 or attachment by B) HPV16 and HPV18, and D) HPV31 and HPV45. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection normalizing to infection by the CHO Par cells. Analysis of the number of particles was done by SYBG q-PCR after incubating for 2 hours at 4°C, normalizing attachment to the CHO Par cells.
Differential susceptibilities to carrageenan in HaCaT cells

An *in vitro* screen of compounds that can effectively block infection by high-risk HPV PsV identified carrageenan, a highly sulfated polysaccharide derived from red algae, as a powerful inhibitor [14]. In contrast, for NV at an MOI of 10, carrageenan failed to inhibit infection by HPV16 at concentrations up to 100µg/ml (figure 3.7A). For HPV18, when an MOI of 10 and 100 were used, significant levels of inhibition were observed at 1µg/ml (figure 3.7B). When the titer was increased to an MOI of 1000, 50% inhibition increased to 10µg/ml (figure 3.7B). In contrast, a 1000-fold lower IC$_{50}$ in the ng/ml range was observed for various HPV PsV types [14], suggesting that the NV is more resistant to inhibition than PsV. Infection by HPV31 was also sensitive to inhibition by carrageenan (figure 3.7C), in contrast to the observed resistance to heparin and CS. This suggests a very selective requirement for a specific type of sulfated GAG for HPV31 infection. HPV45 did not show a dose-dependent decrease in infection in the presence of carrageenan (figure 3.7D), suggesting yet another preference for a different type of sulfated GAG.
Figure 3.7. Antimicrobial effects by iota-carrageenan on infection by native HPV particles. Infections of HaCaT cells were performed with A) HPV16, B) HPV18, C)HPV31, and D) HPV45 at an MOI of 10 for all virus types as well as an MOI of 100 and 1000 for HPV18. Virus was incubated with iota-carrageenan at increasing concentrations (0 µg/ml, 1µg/ml, 10 µg/ml, and 100 µg/ml) at 37°C for 30 min prior to and during infection. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentration with infection at 0 µg/ml of carrageenan set equal to one.
We then analyzed virion attachment in the presence of increasing concentrations of carrageenan. HPV16 was not affected at the level of attachment (figure 8A). HPV18 attachment was completely blocked by carrageenan (figure 3.8B). HPV31 attachment was not as sensitive (figure 3.8C) as to account for the level of inhibition observed during infection (figure 3.7C). This suggests that carrageenan might have an additional post-attachment inhibitory effect on HPV31. HPV45 attachment in the presence of carrageenan was also not significantly reduced (figure 3.8D).
Figure 3.8. Effect of i-carrageenan on virus adsorption. A) HPV16, B) HPV18, C) HPV31, and D) HPV45 were incubated in the presence or absence of carrageenan (0µg/ml and 100µg/ml) and added to HaCaT cells at an MOI of 25. The virus was allowed to attach to HaCaT cells for 2 hours at 4°C. Analysis of the number of particles was done by SYBG q-PCR with attachment in the absence of carrageenan set to 100%.
**Antiviral activities by carrageenan in primary cells**

We used primary cells in culture to verify key data generated using HaCaT keratinocytes. As PsV infection of primary cells in culture has been shown to be very inefficient [111], we first wanted to establish the ability to infect primary keratinocytes with NV. Infections by HPV16, HPV31, and HPV45 were comparable to, or better than, infection of HaCaT keratinocytes (figure 3.9A, C, D). In contrast, HPV18 infection is reproducibly weaker in primary keratinocytes (figure 3.9B).

We next investigated the efficiency of inhibition by carrageenan against infection by the different high-risk HPV types in primary keratinocytes. The lack of a dose-dependent inhibition of HPV16 infection of primary cells supports that HPV16 NV is insensitive to inhibition by carrageenan (figure 3.9E). An MOI of 100, instead of 10, was used for HPV18 infections, as this resulted in infection levels similar to that observed in HaCaT cells (data not shown). Infection by HPV18 was blocked by carrageenan, (figure 3.9F), supporting the need for GAGs as primary attachment receptors for HPV18. HPV31 was not significantly blocked in the presence of carrageenan at 10µg/ml, but was blocked only at the higher concentration of 100µg/ml (figure 3.9G). Similarly to that seen in HaCaT cells, HPV45 infection of primary keratinocytes was not significantly blocked by carrageenan (figure 3.9H).
Figure 3.9. Infection of primary cells and the inhibitory effects of carrageenan on the infection of primary cells. HaCaT and primary cells were infected at an MOI of 10 by A) HPV16, B) HPV18, C) HPV31, and D) HPV45. Infections of primary cells were performed in the presence ι-carrageenan at increasing concentrations (0 µg/ml, 10 µg/ml, and 100 µg/ml) for; E) HPV16, F) HPV18, G) HPV31, and H) HPV45. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentration with infection at 0 µg/ml of carrageenan set equal to one.
DISCUSSION

As summarized in Table 3.1, a complicated pattern of dependency on cell surface GAGs and/or GAG modifications, emerges from the comparison of infection by the four main cancer-causing HPV types, HPV16, HPV18, HPV31, and HPV45. We show here that raft-derived HPV18 requires HS binding for infection, similar to previously reported data using recombinant HPV particles. Noticeably, HPV18 infection is also sensitive to CS, whereas infections by HPV16 and HPV18 PsVs, are not substantially inhibited by the presence of CS [109, 141].

HPV31 and HPV45 display a different pattern of inhibition under our experimental conditions. The reduction in infectivity after sodium chlorate treatment of the cells, and the inability to attach to GAG-deficient cells, suggests a preference for a different type of sulfated GAG. Previous research has shown that the type and level of sulfation may play an important role for the interaction of PsV with polysaccharides [109, 288]. Interestingly, despite not blocking infection, heparin effectively blocked attachment by HPV31 and HPV45. It is possible that despite not being able to block infection, heparin still attaches to the particles. HPV5 PsV has been shown to bind carrageenan beads even though no block was observed at the level of infection [14]. In a recent study attachment to the cell surface but not the ECM was blocked by heparin pre-incubated HPV16 PsV [109]. Further, it was demonstrated that bound heparin conferred a conformational change to the particles [109]. Thus, it is possible that residual levels of heparin-coated virus binding to the ECM are able to transfer directly to a secondary entry receptor on the cell surface.
Unexpectedly, native HPV16 does not follow the same mode of dependence on GAGs for attachment. Binding to GAGs is not required for attachment or infection by HPV16, as shown by incubation with soluble heparin, the ability of HPV16 to infect GAG-deficient cells, and by preventing sulfate modifications of GAGs by sodium chlorate. In addition, PsV do not efficiently infect primary keratinocytes in culture [111], which was hypothesized to be due to altered structure of HS modifications during in vitro culturing of keratinocytes [289]. Interestingly, infection of primary keratinocytes by HPV18 NV is also inefficient, whereas primary keratinocytes are readily infected by HPV16, HPV31, and HPV45 NV. Together, these results suggest that different HPV types have distinct requirements for entry into their natural host cells. Further research is required to determine whether the GAG-dependent and independent HPV types post-attachment share the same receptor for internalization into their natural host cells. Interestingly, a recent publication demonstrated that HPV particles may be released from the cell surface in complex with HS, followed by re-attachment to the cell surface independent of cell surface GAGs, to initiate infection [114]. Additional studies are needed to determine the composition of native particles as they are released from the cells and whether that plays an important role in HPV NV infection.

The observed differences in GAG-dependency between different HPV types, as well as between PsV and NV particles, are unlikely to be explained simply due to the use of different cell lines and different requirements for in vivo and in vitro infections. Using the same HaCaT keratinocyte cell line, as used in previous PsV infection studies [14, 141], we found that the sensitivity to polysaccharide compounds is greatly reduced or completely lacking for native HPV particles. It has been shown that the need for initial
attachment to HS is tightly correlated with the need for cleavage by cellular furin during infection, where furin pre-cleaved PsV particles can bypass binding to HS for infection [111]. Bypassing HS-binding could allow for direct binding to a functional entry receptor. It is possible that HPV16, which does not need HS binding during infection, is pre-cleaved during virion morphogenesis. It is also conceivable that different HPV species utilize different molecules for initial attachment to the basement membrane and the host cell. Laminin-332 has been shown to be the preferential receptor for HPV11 and HPV16 PsV for initial attachment [108, 109]. Studies using PsV show that different types of HPV may utilize different entry pathways [106], suggesting that binding to different receptor molecules on the cell surface may direct these viruses into different endocytic pathways. Moreover, although not required, it is still possible that HPV16 interacts with GAGs through conserved motifs on the virus surface [143]. In vivo, this may serve to concentrate the virus to the site of infection.

Importantly, neutralization by carrageenan was shown to be effective against HPV18 and HPV31 infections. However, HPV16 and HPV45 could not be blocked by carrageenan, suggesting carrageenan may not protect against all HPV types. Inhibition of dengue virus infection by carrageenan also showed differential efficacy depending on the serotype tested [290]. Different carrageenan compounds need to be considered, as a preparation of more than one type of carrageenan might be able to protect against more HPV types [14, 290].

The efficient production of synthetic particles is invaluable for rapid analysis of many aspects of the papillomavirus life cycle. However, a complete comparison of the
structure of PsV and NV has not been published and experimental results using PsV particles as a surrogate need to be verified with NV [72, 73, 291]. As described here, genital HPVs may use different strategies to attach to and infect their host. As a result, they may naturally exhibit differential susceptibility to various agents, an important factor to consider when developing new agents to block HPV transmission.
Table 3.1. GAG Dependence during Infection. Summary of the sensitivity of the different HPV types to neutralization by monoclonal antibodies, various polysaccharide compounds, sodium chlorate treatment, and GAG-positive or negative cells. Nd=not done.

<table>
<thead>
<tr>
<th></th>
<th>Neutralized</th>
<th>Heparin</th>
<th>Chondroitin</th>
<th>Sod. Chl. (sulfation)</th>
<th>CHO +/− GAGs</th>
<th>Carrageenan (HaCat)</th>
<th>Carrageenan (HFKs)</th>
</tr>
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<tbody>
<tr>
<td>HPV16</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<tr>
<td>HPV18</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HPV31</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Nd</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>HPV45</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
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CHAPTER IV - CLEAVAGE OF THE HPV16 MINOR CAPSID PROTEIN L2 DURING VIRION MORPHOGENESIS ABLATES THE REQUIREMENT FOR CELLULAR FURIN DURING DE NOVO INFECTION

A close relationship between L1-mediated primary attachment to HS, followed by cleavage of the L2 N-terminus by furin, has been demonstrated for the infectious entry of HPV16 PsV. We thus wanted to investigate if the L2 protein of GAG-independent HPV16 NV may be cleaved during maturation in tissue, thereby allowing for furin-independent entry and infection by HPV16 NV.

HPV16 NV is independent of cellular furin and furin-related proprotein convertases for infection of HaCaT keratinocytes

To determine whether native HPV16 produced in differentiating epithelial tissue is dependent on cellular furin, we infected HaCaT cells, a human spontaneously immortalized keratinocyte cell line, in the presence or absence of increasing amounts of a furin inhibitor. HPV16 NV produced in differentiating foreskin tissue was previously characterized [47]. HPV16 NV infection was not inhibited but rather a dose-dependent small increase in infectivity was observed in response to increasing concentrations of the
furin inhibitor (figure 4.1A). To rule out the possibility that the virus was processed by residual PC activity in the homogenate during virus harvest (homogenization and benzonase-treatment) we added the furin inhibitor during harvest. Addition of the inhibitor had no effect as compared to when no inhibitor was added during harvest (figure 4.1A). To confirm previous data that infection of HaCaT keratinocytes by HPV16 is particle-mediated [47, 286], and to ensure that addition of the furin inhibitor to the cells does not cause uncharacterized virus uptake, infections were done in the presence of the L1-specific, conformation-dependent, monoclonal antibody H16.V5 [292, 293]. Indeed, infection in the presence of the furin inhibitor is particle-mediated supporting that in vitro infection by tissue-derived HPV16 is a furin-independent process (figure 4.1B). As this result is in stark contrast to previous data using HPV16 PsV we wanted to confirm previously shown data using our experimental conditions. We used 293TT cells due to the superior signal intensity by GFP-expressing PsV in this cell line [284]. Addition of the furin inhibitor during PsV infection of 293TT cells and HaCat cells nearly completely ablated the GFP signal (figure 4.1C and data not shown), confirming that in vitro infection by HPV16 PsV is a furin-dependent process. The ability to infect cells in the absence of active cellular furin was not cell-type dependent as infection of 293TT cells by HPV16 NV was not blocked by the furin inhibitor (figure 4.1D). As the furin inhibitor also blocks substrate-binding to other related PCs [294] this data also suggests that HPV16 NV during entry does not require proteolytic cleavage by any furin-related PC for efficient infection.
Figure 4.1. Furin-independent HPV16 NV infection. (A) Infection of HaCat cells with foreskin-derived HPV16 at increasing concentrations (0.25 µM, 2.5µM, 25 µM) of the furin peptide inhibitor. The infection at 25 µM inhibitor concentration was also repeated after harvesting the virus in the presence of 25 µM inhibitor. B) Virus preparations were incubated with 1:100 dilutions of H16.V5 antibody for 1 hour prior to infections in the absence or presence of 25 µM furin inhibitor. C) HPV16 PsV D) HPV16 NV infections of 293TT cells in the absence or presence of 25 µM furin inhibitor. HPV16 NV infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentration with infection at 0 µM furin inhibitor set equal to one. HPV16 PsV infection was assessed by immunofluorescent microscopy monitoring GFP-expression two days post-infection.
**HPV16 NV can infect furin-deficient cells**

To further characterize the furin-independence by HPV16 NV and avoid potential off-target effects by the furin inhibitor, we performed infections with furin-negative cells. CHO FD11 cells have a genetic mutation that eliminates expression of furin and they also do not express PC5/6 [281]. HPV16 NV infected CHO FD11 cells at similar levels to infection of a furin-expressing derivative CHO FD11 +furin cell line [281]. Addition of the furin inhibitor during infection with HPV16 did not change infection of the furin-negative cells, but rather an increase in infection was observed in the furin-positive cells when the inhibitor was present (figure 4.2).
Figure 4.2. Infection of furin-negative CHO FD11 cells and furin-positive CHO FD11 + furin cells. Infection of furin-negative and the furin-positive CHO FD11-derivative cell lines with HPV16 in the presence or absence of 25 μM furin inhibitor. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection normalizing to infection by the CHO Par cells.
**Exogenous furin has no impact on infection**

While insensitive to the addition of a furin inhibitor at the time of and during infections, it remained possible that furin may be able to impact the virus prior to infection. We first investigated this by incubating the virus with exogenous furin prior to infection. Incubation of the virus preps with exogenous furin prior to infection of HaCaT or CHO FD11 cells failed to enhance infection (figure 4.3A). This is in contrast to HPV16 PsV where pre-treatment with exogenous furin may enhance infection of furin-deficient as well as furin-expressing cells [111]. To verify that the furin enzyme and inhibitor were fully functional in our assay conditions, an *in vitro* cleavage assay using the Boc-RVRR-AMC fluorogenic peptide containing a furin cleavage site utilizing the same buffer conditions as in the viral preparations was performed. The fluorogenic peptide was efficiently cleaved by exogenous furin and was effectively blocked by the inhibitor already at the lowest concentration, suggesting the assay conditions are not responsible for the observed results (figure 4.3B).
**Figure 4.3.** A) Infection of HaCat cells and furin-negative CHO FD11 cells after incubating the virus for 7 hours at 37°C with 5U exogenous furin enzyme. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection normalizing to virus infections not treated with exogenous furin. B) Cleavage of the Boc-Arg-Val-Arg-Arg-AMC fluorogenic peptide at increasing concentration (0 µM, 0.25 µM, 2.5µM, 25 µM) of furin inhibitor in virus 0.06 M phosphate buffer. Fluorescence was recorded at Ex/Em 350/450.
Expression of furin in organotypic foreskin and cervical cultures

Due to the strict differentiation-dependent tissue tropism of PVs, we were interested in the furin expression in virus-infected organotypic epithelium. Expression of furin throughout the epithelium has been shown in sections of human epithelium [153]. Expression and localization of furin in the intact mouse genital tract indicates the presence of furin through all layers of the epithelium. Following wounding, the furin expression is intensified, particularly in the basal layer [13]. Organotypic cultures may resemble to some extent a wound-healing environment as the cultures are initiated as a single layer before proliferation, stratification, and differentiation takes place. Primary human foreskin (HFK) and cervical (HCK) keratinocytes are both physiologically relevant cells for the maintenance, spread, and pathogenesis of HPV. To determine the effect of virus infection and replication in foreskin and cervical tissue, we grew uninfected as well as HPV16-infected rafts from both tissues (figure 4.4A, C, E, G). The expression of furin in foreskin- and cervix-derived organotypic raft cultures was found diffuse throughout basal and lower nucleated suprabasal layers, but with more intense staining observed in the basal layer (figure 4.4B, F). In HPV16-infected foreskin- and cervix-derived tissues, furin-staining was more intense and was found primarily in the upper layers of the epithelium with decreased intensity observed in the basal cells (figure 4.4D, H).
Figure 4.4. Expression of furin in raft cultures. Hematoxylin and eosin (H&E) staining of A) primary foreskin tissue C) HPV16-infected foreskin tissue E) primary cervical tissue G) HPV16-infected cervical tissue. Immunofluorescent staining using the furin MON-139 antibody of B) primary foreskin tissue D) HPV16-infected foreskin tissue F) primary cervical tissue H) HPV16-infected cervical tissue. Nuclear stain was done using Hoechst 33342. Furin staining (green). Nuclear stain (blue). Sections were harvested from tissues grown for 20 days in organotypic raft cultures.
Cleavage of HPV16 during virion morphogenesis

Since furin and PC 5/6 are abundantly expressed in the keratinocyte epithelium, the natural target for HPV infection, we were interested if native virus might be processed by furin during assembly before cell attachment and entry. Expression of the L1 and L2 capsid proteins only in the suprabasal layers has been previously shown for wild-type HPV16 organotypic cultures \[47, 295\]. Because of the coincident intensification of furin expression in the same layers as the HPV capsid proteins are expressed, we hypothesized that the HPV16 L2 minor capsid protein may be cleaved during virion maturation. To examine the state of the L2 protein in tissue-derived HPV16 virus particles, we probed the harvested virus preparations separated on an SDS-PAGE gel with the RG-1 L2 monoclonal antibody. HPV16 PsV L2 migrated at about 70 kDa as previously shown despite its calculated molecular size of about 55kDa \[86, 296-298\]. A faster-migrating L2 species was observed in HPV16 NV viral preparations derived from organotypic raft cultures but not in the PsV lane (figure 4.5). The size of the faster-migrating L2 species was verified by an N-terminally modified L2 expression-plasmid lacking amino acids 2-12, corresponding to the consensus furin cleavage site. A mixture of full-length and N-terminally cleaved L2 species was observed on some of the western blots (figure 4.5). The cleaved species of L2 was found in foreskin- as well as cervix-derived particles from cultures immortalized by the 114/B and the 114/K HPV16 European isolates (figure 4.5 and data not shown). Non-HPV containing raft cultures were used as negative controls (figure 4.5). We also analyzed virus particles derived from the previously characterized HPV16 cell line carrying a cysteine 428 to serine mutation.
in L1. The cysteine 428 residue plays a differentiation-dependent stabilizing role in mature HPV16 capsids [47, 48]. These particles also had N-terminally cleaved L2 (data not shown). This data suggests that the HPV16 L2 N-terminus is cleaved during virus assembly and maturation in tissue. The size of the cleaved L2 corresponds to the expected size from cleavage at the furin cleavage consensus site.
Figure 4.5. Cleavage of the L2 capsid protein from native virus. Western blot analysis of HPV16 L2. Lane 1: HPV16 PsV, lane 2: HPV16 L2 expression plasmid with N-terminal deletion, lane 3: HPV16 NV, lane 4: non-infected primary raft. The arrow and asterisk indicate the two forms of L2 species observed in tissue-derived NV particles.
Infection of primary foreskin and cervical keratinocytes by

HPV16 NV derived from either tissue

To address the possibility that virus produced in various tissues may mature differently and thus be exposed and processed differently by cellular PCs, we harvested HPV16 NV from stratified HPV16-immortalized cervical tissue (figure 4.4G). Infections of HaCaT cells with HPV16 produced in HCKs, were not blocked by the furin inhibitor (figure 4.6A), similarly to the foreskin-derived virus (figure 4.1A). This supports the observation that HPV16 NV is independent of cellular furin and furin-related PCs for de novo infection and that it is not a cell line or cell-type specific effect. Next, we wanted to determine whether infection by HPV16 NV of primary keratinocytes is dependent on the activity of cellular furin. Infection of low-passage primary HCKs and HFKs by HPV16 NV was not blocked in the presence of the furin inhibitor (figure 4.6B and data not shown), further confirming the observation that infection by HPV16 produced under physiologically relevant condition of stratifying and differentiating tissue is independent of cleavage by cellular furin for de novo infection. Taken together, these results demonstrate that HPV16 NV does not require cellular furin or furin-related PCs during infection of its host cells.
Figure 4.6. Furin-independent infection cervical cell-derived HPV16 A) Infection of HaCat cells with cervical cell-derived HPV16 in the presence or absence of 25 µM furin inhibitor. B) Infection of primary cervical keratinocytes with foreskin and cervical cell-derived HPV16 in the presence or absence of 25 µM furin inhibitor. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection normalizing to infections done in the absence of furin inhibitor.
Conservation of the furin cleavage site in L2

The consensus site for cleavage by cellular furin in the L2 N-terminus is highly conserved between the most common cervical cancer-causing HPVs; HPV16, HPV18, HPV31, and HPV45 (figure 4.7A). We next sought to determine whether these high-risk HPV types necessitate cleavage by furin or a related PC for de novo infection of keratinocytes. The production of HPV18 and HPV45 virus from foreskin keratinocytes has been previously characterized [41, 42]. Native HPV31 was produced from a cervical intraepithelial neoplasia type 1 biopsy-derived cell line CIN-612 9E [16]. Infection of HaCaT keratinocytes showed that HPV45 displayed a similar independence of cleavage by cellular PC as HPV16 (figure 4.7D). In contrast, infections by HPV31 and HPV18 were clearly blocked in the presence of the furin inhibitor (figure 4.7B, C).
A.
L2 HPV16 MRHKRSAAKRKTKRASATQLYKTCKQA6TCPDIIIPK 35
L2 HPV18 MVSHRAARR-KRASVTDLYKTCKQSGTCPDVPVK 34
L2 HPV31 MRSKRSTKRTKRASATQLYQTCAAGTCPDVIPK 35
L2 HPV45 MVSHRAARR-KRASATDLYRTCKQSGTCPDVINK 34

B.

C.

D.
Figure 4.7. Furin inhibitory activities of various high-risk human papillomaviruses. A) Alignment of the L2 N-terminal sequences of HPV16, HPV18, HPV31, and HPV45. A furin consensus site in each type is indicated in bold type. Infection of HaCat cells with B) HPV31 NV C) HPV18 NV and D) HPV45 NV in the presence or absence of 25 µM furin inhibitor. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection normalizing to infections done in the absence of furin inhibitor.
The effect of furin inhibition on HPV16 QV

HPV16 QVs encapsidate the full-length viral genome similarly to HPV16 NVs. To test what effect the incorporation of the viral genome has on HPV16 sensitivity to furin inhibition, HPV16 QV particles were tested for their sensitivity to furin inhibition during infection. Similarly to HPV16 NV, HPV16 QV infection was not blocked in the presence of the furin inhibitor (figure 4.8A). This suggests HPV16 QV may resemble HPV16 NV more closely than HPV16 PsV. To test whether HPV16 QV particles are sensitive to furin inhibition during particle production, we added furin inhibitor during HPV16 capsid assembly and genome packaging in 293TT cells. HPV16 QV produced in the presence of the furin inhibitor was still not sensitive to the addition of the furin inhibitor during infection (figure 4.8B), suggesting HPV16 QV does not need the activity of furin during virus production or infection. To analyze whether HPV16 QV L2 is N-terminally cleaved during virus production we performed an L2 immunoblot assay. L2 from HPV16 QV migrated at the same size as L2 from HPV16 PsV (figure 4.9A). We did not observe any HPV16 QV L2 migrating at the same size as N-terminally cleaved L2 (figure 4.9B). This data further supports that HPV16 QV is furin-independent.
Figure 4.8. Effects of furin inhibition during infections by HPV16 QV.

HPV16 QV particles were incubated with the furin inhibitor at 0µM or 25 µM during A) infection of HaCaT cells or B) during production of QV in 293TT cells. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentrations with infection at 0 µM of furin inhibitor set equal to one.
Figure 4.9. The status of HPV16 QV L2 produced in 293TT cells. Western blot analysis of HPV16 L2. A) Lane 1: HPV16 PsV, lane 2: HPV16 L2 expression plasmid with N-terminal deletion. B) Lane 1: HPV16 PsV, lane 2: HPV16 QV.
Mutations in the L2 N-terminal furin cleavage site

In addition to the canonical furin recognition sequence R-X-K/R-R, furin may also cleave at alternative R-X-X-K/R-R and minimal R-X-X-R recognition sequences [299]. Sequence analysis of the L2 N-terminus of HPV16 reveals two possible furin cleavage site at arginine 5 (R5) and arginine 12 (R12, conserved site). Two sites, arginine 8 (R8) and arginine 11 (R11, conserved site), are found in HPV18 L2. Serine mutations at the conserved sites, HPV16 L2 R12S and HPV18 L2 R11S, did not result in a loss of infectivity (figure 4.10A and 4.11A). Rather, the mutant viruses were more infectious than wildtype virus. These data suggest that NVs do not rely on the conserved furin cleavage sites for infection. Additional mutations in the alternative furin cleavage sites, HPV16 L2 R5S and HPV18 L2 R8S, resulted in a complete loss of infectivity of the mutant particles (figure 4.10B and 4.11B), suggesting these sites may be more important for furin cleavage of NV particles. Further, these data suggest that furin cleavage is needed during HPV16 as well as HPV18 life cycles.
Infections of HaCat cells were done at an MOI of 10 for wildtype and A) L2 R12S and B) L2 R5S mutant particles. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection with wildtype set to one.
Figure 4.11. Analysis of the infectivity of HPV18 L2 mutant particles.

Infections of HaCat cells were done at an MOI of 10 for wildtype and A) L2 R11S and B) L2 R8S mutant particles. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection with wildtype set to one.
DISCUSSION

In this report we demonstrate that tissue-derived genital high-risk HPV16 NV does not require the activity of cellular furin or related PCs during de novo infections. Importantly, a furin peptide inhibitor did not block infection by HPV16 and the virus could infect cells not expressing furin as efficient as cells that do express furin. Our finding was confirmed using virus particles harvested from foreskin- and cervix-derived organotypic cultures and by infections of cells harvested from tissues of both origins. This is important as foreskin and cervix are both relevant tissues for the life cycle and transmission of HPV infections between males and females.

Furin is expressed at increased levels in the suprabasal cells of virus-infected tissues, compared to expression of furin mainly in the basal layers of normal un-infected human foreskin and cervical tissues. This raises the possibility that the L2 protein of the virus may be cleaved by furin not just during de novo infections, but also during virion morphogenesis in the tissue. Furin, in its transmembrane form, is found trafficking in the trans-golgi network and a cleaved smaller version may be secreted. The accessibility of the furin catalytic domain may change as the cells are transiting through the epithelial cell layers and has been shown to be more readily detected by antibodies is the granular layer where cells are progressing through terminal differentiation [153]. Here, the cells are going through remodeling and dissolution of membranous organelles and the golgi apparatus [300-302]. Thus, reorganization of the normal inhabitants in the organelles would allow access to a different subset of substrates in the cells, including cytoplasmic substrates [153]. Virions are observed in the nuclei of differentiated cells as well as in the
cytoplasm of denucleated cells [102], making it possible for assembled particle to be exposed to furin following nuclear degeneration. Indeed, the L2 N-terminus of immature HPV16 PsV can be cleaved prior to cell attachment in vitro. In contrast, the mature particles cannot be cleaved prior to cell attachment [91]. This suggests that the virions may go through a stage during maturation where the L2 N-terminus is exposed to cleavage by furin in vivo. During tissue-differentiation HPV16 NV goes through an extended period of increased maturation over a period of 10 days [47], during which the particles may be influenced by and interact directly with cellular factors [212].

Cleavage of the L2 N-terminus is incomplete. In vitro furin cleaved PsV particles retain full-length in addition to N-terminally cleaved L2 [111]. We also demonstrated incomplete cleavage of the L2 N-terminus in tissue-derived HPV16 particles. It is unclear whether the same particles contain the two types of L2 or if two distinct populations of particles exist. The physiological relevance of two forms of L2 being produced in differentiating tissue is supported by the demonstration of an L2 doublet by western blot from HPV11 particles derived from the athymic mouse xenograft system but not when the HPV11 L2 open-reading-frame was expressed alone. This was suggested to be a result of proteolytic cleavage of the full-length protein [298]. The increase in infection by HPV16 NV that is observed in the presence of the furin inhibitor suggests that there may a threshold for the stoichiometric level of full-length versus cleaved L2 protein for efficient infection, and that additional cleavage during entry may have a negative impact on infection. Cleavage of the L2 N-terminus is required for endosomal escape of L2 and the viral genome [91]. However, the N-terminus of L2 is highly conserved between different HPV types and may serve additional functions during entry. The L2 protein
directs the retrograde trafficking of the viral genome to the nucleus [211, 303], supporting a role for the cleaved as well as the un-cleaved form of L2 during entry.

Cleavage of the L2 N-terminus occurs post-attachment to HS moieties on the host and in vitro furin cleaved HPV16 PsV bypass the requirement of HS-binding to infect cells [111]. We reported previously the GAG-independent infection by tissue-derived HPV16 [286]. This suggests a model where proteolytic cleavage of the HPV16 L2 protein during tissue differentiation allows the particles to bypass GAG-binding for primary attachment. This may permit the virus to bind directly to a functional entry receptor. However, it is also possible that various HPV types utilize diverse molecules for initial attachment to the host cell [108, 109]. In addition, a requirement for GAGs during primary attachment is not predictive of furin-dependence for de novo infections. Tissue-derived HPV45 NV is dependent on the presence of cellular GAGs for primary attachment [286], but can infect cells de novo in a furin-independent manner.

We show here that HPV types 18 and 31 virions produced in differentiating tissue require the activity of cellular furin or a related PC during de novo infection, similarly to recombinant HPV PsV particles. Unexpectedly, native HPV16 and HPV45 could infect cells independently of active cellular furin. These results warrant further studies focusing on the entry pathways of the individual virus types. The evolutionary related but genotypically and serologically distinct alpha HPVs may share many features of the virus life cycle but differ in some. It is interesting to note that different types of HPV PsV have been reported to use distinct entry pathways under some experimental conditions but significant overlap in others [58, 106, 166, 192-194, 304]. Given the diversity of HPV
types and their associated diseases [5] perhaps we should be more careful when simplifying and taking a broad view in regards to the general biology and infection path for distinct HPV types. The finding that HPV16 NV L2 N-terminus is proteolytically cleaved during virion assembly and maturation in a differentiation-dependent manner, supports the possibility that inhibitors of furin or related PCs may be effective to prevent the spread of genital HPV infections, and is of great interest for future investigations.
CHAPTER V  NATURAL HPV16 MOLECULAR VARIANTS DIFFER IN THEIR REQUIREMENTS FOR HOST CELLULAR FACTORS DURING ENTRY AND INFECTION

The prevalence and persistence of HPV infection is related to the risk and development of cervical cancer. In this chapter we investigate whether the infectivity of a given variant may be related to the infection rate (prevalence) and ability to establish a persistent infection. Also, we explore the infectivity and infection pathways of a limited number of HPV16 molecular variants.

Characterization of HPV16 variant-infected cell lines

To develop cell lines stably infected with, and able to synthesize native HPV16 variant particles, HPV genomes obtained from cervical brushings were sequenced in the E6 ORF to determine the type and variant lineage. Sequencing of the complete viral genome was subsequently performed to identify nucleotide variations across the viral genome as compared to the reference genome and other known variant sequences to verify the identity of the viral genomes (figure 5.1).
Figure 5.1. HPV16 molecular variants sequence variations. Whole-genome sequencing was done for each of the molecular variants in this study along with other variant genomes. Sequences were compared to the E prototype sequence and nucleotide variations were identified and aligned with the corresponding nucleotide number in the prototype sequence. Sequencing was done by Robert Burk, Albert Einstein University.
A complete genome functional analysis has never been done for variants of any type. For this study we identified and used two European variants, E 114/B and E 114/K, two non-European variants, the North American NA EVA3724, and African type 2 Af-2 EVA2716. A phylogenetic tree was constructed along with other full-length HPV16 variant genomes of the different lineages to demonstrate their relatedness to variants of the same groups and the divergence from each other (figure 5.2).
Figure 5.2. Phylogenetic tree from variant nucleotide sequences. A phylogenetic tree was constructed along with other full-length HPV16 variant genomes of the different lineages to demonstrate their relatedness to variants of the same groups and the divergence from each other.
Genomes were amplified by rolling circle amplification and cloned into a PBS plasmid backbone. Primary keratinocytes of foreskin and cervical origin were electroporated with linear HPV16 wild-type and variant genomes. Upon immortalization re-circularization and stable maintenance of the viral episome was confirmed by Southern blot analysis before cell lines were allowed to stratify and differentiate in organotypic culture. Cultures were allowed to grow for 20 days to ensure complete maturation of the viral particles [47]. Representative variant tissues were H&E stained to confirm stratification and differentiation. All tissues stratified and displayed a disrupted differentiation pattern with nuclei appearing in the suprabasal compartments, indicative of an HPV infection (figure 5.3). The 114/B foreskin-derived cell line has been previously characterized [47].
Figure 5.3. Virus-producing HPV variant tissues. Hematoxylin and eosin (H&E) staining of representative virus-producing variant foreskin (HFK) and cervical (HCK) tissues. Sections were harvested from tissues grown for 20 days in organotypic raft cultures.
Productive infection in variant cell lines

To determine whether the variant cell lines were capable of supporting the complete viral life cycle and produce viral particles we harvested the tissues after 20 days in culture and dounce homogenized to release the viral particles into the supernatant. The virus preps were treated with benzonase nuclease to degrade unprotected viral and cellular DNA and to ensure analysis of only encapsidated viral DNA [47]. Titer of the different tissues varied between $10^5$ to $10^6$ viral particles/µl for a total of $10^8$ to $10^9$ particles for three rafts (figure 5.4). Viral titers did not appear to correlate and depend on the specific viral variants or type of tissue but rather seem to be cell line-specific. However, a larger number of cell lines and tissues would be required to definitively determine whether tissue-titers correlate with the viral variant and or tissue-type.
Figure 5.4. Virus titers from HPV variant tissues. Virus preps from three combined raft cultures were tittered by qPCR targeting an amplicon in the E2 ORF against a standard curve with known concentrations of viral genomes. Titers are reported as the number of genomes per µl of virus prep.
Virus infectivity assays were then performed at an equal MOI of 10 to determine the relative infectivity of the different virus types in the immortalized HaCaT keratinocyte cell line. We did not recognize a significant difference in the relative infectivity of the different variants nor variants derived from different tissue-types (figure 5.5). To confirm particle-mediated infection of the HaCaT cells by the variants we performed infections in the presence of the L1 neutralizing antibody H16.V5. Most of the variant particles were efficiently neutralized by this antibody (figure 5.5A, B). While the 114/B HCK-derived particles may be neutralized efficiently (figure 5.5B), they are more often than not neutralized by the H16.V5 antibody (data not shown). We have been unable to neutralize the 114/K-derived particles so far (figure 5.5B).
**Figure 5.5. Particle-mediated infection.** A and B. HPV16 variants were incubated with the L1 type-specific antibody H16.V5 for 1 hour at 37°C prior to infection of HaCaT cells. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection.
It is at this point unknown why the 114/B and 114/K HCK-derived particles are poorly neutralized as the H16.V5 antibody targets one of the major neutralizing antibodies. It is possible that the maturation status of these particles is such that the H16.V5 epitope is not properly exposed or that the antibody may still bind but is unable to neutralize these particles. Neutralization versus non-neutralization appears to be specific for certain virus preps from an entire set of tissues grown at the same date as batches the 114/B HFK-derived particles are sometimes not neutralized (data not shown). To investigate whether other epitopes are exposed in the H16.V5 non-neutralizable virus preps we attempted to neutralize the virus with human polyclonal serum from vaccinated individuals. The 114/B-derived particles that are not neutralized by H15.V5 may be neutralized by polyclonal serum (figure 5.6), suggesting improper display of the H16.V5-targetted epitope.
**Figure 5.6. Neutralization by patient serum.** HPV16 E HFK 114B variant was incubated with patient-derived polyclonal serum for 1 hour at 37°C prior to infection of HaCaT cells. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection.
Analysis of heparin susceptibility of HPV16 variant particles

Using biochemical inhibition assays and heparin sulfate negative cell lines we showed that foreskin-derived E HPV16 114/B variant binds and infects stable cell lines as well as primary keratinocytes in a GAG-independent manner (see Chapter III [286]). In contrast, HPV16 PsV is dependent on primary attachment to GAGS and more specifically HS for infection [13, 106, 107]. We were interested in whether this property is specific only to the E 114/B variant and also to determine whether it is a cell line-specific or general property of this variant. We did an assessment of the HS-dependence by incubating the virus particles with heparin prior to infection. The foreskin- as well as cervix-derived E 114/B and E 114/K variants infected HaCat cells in the presence of heparin as efficiently as without (figure 5.7A, B), suggesting that heparin-resistance may be a general phenotype of variants of the European lineage. Further, the data supports it being a variant- rather than cell line-specific effect. The NA EVA3724 variant was also resistant to inhibition in the presence of heparin (figure 5.7A, B). In contrast, the Af-2 EVA2716 variant derived from either foreskin or cervical tissue was sensitive to inhibition by heparin (figure 5.7A, B). This suggests that the different variant lineages have evolved differently in regards to the entry pathways used. Further, this suggests that small variations in the variant genomes are responsible for the observed phenotypic difference.
Figure 5.7. Inhibitory effects of heparin against various HPV16 variants. A) Foreskin-derived HPV16 variants and B) cervix-derived HPV16 variant particles were incubated with heparin at 100 µg/ml for 30 min's at 37°C prior to infection and during infection of HaCaT cells. All infections were done at an MOI of 10. Infections were analyzed by RT-qPCR measuring the relative amount of E1\(^{\text{E4}}\) transcript two days post-infection. The data is plotted as relative infection at the different concentrations with infection at 0 µg/ml of heparin set equal to one.
Sensitivity of HPV16 variants to furin inhibition

close relationship between L1-mediated primary attachment to HS followed by cleavage of the L2 N-terminus by furin has been demonstrated for the infectious entry of HPV16 PsV [13, 111] and GAG-independent infection of foreskin-derived E HPV16 114/B (see chapter III [286]) could also infect cells in a furin-independent manner (see chapter IV). We thus determined to analyze this property for the variant particles by performing infections in the presence of a furin inhibitor. Infection by the foreskin- as well as cervix-derived E 114/B and E 114/K variants infected cells as efficiently in the presence of the furin-inhibitor as without (figure 5.8A, B). Interestingly, both the NA EVA3724 and the Af-2 EVA2716 variants were susceptible to furin inhibition (figure 5.8A, B). This suggests the possibility the dependence on cellular furin for infection is a general feature that can be bypassed by European variants but is maintained by non-European HPV16 variants.
Figure 5.8. Effects of furin inhibition during infections by various HPV16 variants. A) Foreskin-derived HPV16 variants and B) cervix-derived HPV16 variant particles were incubated with the furin inhibitor at 25 µM during infection of HaCaT cells. All infections were done at an MOI of 10. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentrations with infection at 0 µM of furin inhibitor set equal to one.
**Effect of carrageenan on HPV16 variant infections**

Next, we were interested in extending our studies on the red algae-derived polysaccharide carrageenan as a potential microbicide (see chapter III [286]). We extended our observation that the E 114/B variant is resistant to inhibition by carrageenan to the E 114/K variant, which also displayed inhibition to carrageenan (figure 5.9A, B). The NA EVA3724 variant showed some sensitivity and the EVA2716 variant is clearly sensitive to the presence of carrageenan during infection (figure 5.9A, B). This data suggests a general GAG-dependence for non-European HPV16 variant lineages but that European variants may bypass the need for GAGs for infection.
Figure 5.9. Antimicrobial effects by ι-carrageenan on infection by HPV16 variants. Infections of HaCaT cells were performed with A) foreskin-derived HPV16 variants and B) cervix-derived HPV16 variant particles. Virus was incubated with ι-carrageenan at 100 µg/ml at 37°C for 30 min prior to and during infection. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentration with infection at 0 µg/ml of carrageenan set equal to one.
Genetic analysis of the capsid proteins

Each of the HPV16 variants has gene sequences that are specific just for that variant and others that are common for that entire variant lineage. In addition, some changes are shared between the non-European lineages, compared to the European lineage (figure 5.2). Using genetic analysis we aim to identify potential lineage-specific nucleotide changes that are responsible for the observed biological differences. We expect that the nucleotide differences in the viral genome that are responsible for the observed phenotypes of GAG- and furin-dependence or independence, to segregate with the viral capsid proteins. Studies using NV have shown that the L1 and L2 capsid proteins mutually impact capsid structure [46]. This suggests that changes in either capsid protein may ultimately impact timing of the exposure and/or usage of the furin cleavage site and GAG-preference for primary attachment.

The L1 protein has been shown to play a direct role in primary attachment of the virus particles to HS [132, 143, 305], and changes in L1 imparts changes in L2 [46]. Table 5.1 summarizes the major amino acid sequence variations in L1 between the HPV16 E reference sequence and the NE variants [229, 253]. More variations and lineage-specific changes have been identified in the L2 reading frame (Table 5.II) [229, 253]. The conserved heparin sulfate binding sites [143], and furin cleavage site [91], are retained in all the viral variants used in this study as confirmed by sequencing of the viral capsid proteins (figure 5.1). Our sequence analysis confirmed the presence of the lineage-specific changes in L1 and L2 of the variants included in this study. Variations that co-segregate with the non-European lineages are highlighted in bold. Currently known
locations and interactions in structurally important domains are noted (Tables 5.I and 5.II).
Table 5.1. HPV16 variant lineage-specific amino acids in L1 and their location in the L1 structure. The two sets of numbers represent two different numbering systems.

<table>
<thead>
<tr>
<th>L1 Amino Acid #</th>
<th>European</th>
<th>Asian</th>
<th>African-1</th>
<th>African-2</th>
<th>Asian American</th>
<th>North American</th>
<th>Structural Element</th>
</tr>
</thead>
<tbody>
<tr>
<td>-24/2</td>
<td>Q (Gln)$^{UP}$</td>
<td>E (Glu)$^N$</td>
<td>E (Glu)$^N$</td>
<td>E (Glu)$^N$</td>
<td>E (Glu)$^H$</td>
<td>E (Glu)$^H$</td>
<td></td>
</tr>
<tr>
<td>76/102</td>
<td>H (His)$^P$</td>
<td>Y (Tyr)$^H$</td>
<td>Y (Tyr)$^H$</td>
<td>Y (Tyr)$^H$</td>
<td>Y (Tyr)$^H$</td>
<td>$\beta$-C sheet</td>
<td></td>
</tr>
<tr>
<td>176/202</td>
<td>T (Thr)$^{UP}$</td>
<td>N (AsN)$^{UP}$</td>
<td>N (AsN)$^{UP}$</td>
<td>N (AsN)$^{UP}$</td>
<td>N (AsN)$^{UP}$</td>
<td>EF-loop</td>
<td></td>
</tr>
<tr>
<td>181/207</td>
<td>N (AsN)$^{UP}$</td>
<td>T (Thr)$^{UP}$</td>
<td>T (Thr)$^{UP}$</td>
<td>T (Thr)$^{UP}$</td>
<td>T (Thr)$^{UP}$</td>
<td>EF-loop</td>
<td></td>
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<tr>
<td>282/308</td>
<td>S (Ser)$^{UP}$</td>
<td>P (Pro)$^H$</td>
<td>P (Pro)$^H$</td>
<td>P (Pro)$^H$</td>
<td>P (Pro)$^H$</td>
<td>FG-loop</td>
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</tr>
<tr>
<td>353/379</td>
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<td>P (Pro)$^H$</td>
<td>P (Pro)$^H$</td>
<td>P (Pro)$^H$</td>
<td>P (Pro)$^H$</td>
<td>HI-loop</td>
<td></td>
</tr>
<tr>
<td>389/415</td>
<td>T (Thr)$^{UP}$</td>
<td>S (Ser)$^{UP}$</td>
<td>S (Ser)$^{UP}$</td>
<td>S (Ser)$^{UP}$</td>
<td>S (Ser)$^{UP}$</td>
<td>$\alpha$-2 loop</td>
<td></td>
</tr>
<tr>
<td>474/500</td>
<td>L (Leu)$^H$</td>
<td>E/F</td>
<td>F (Phe)$^H$</td>
<td>F (Phe)$^H$</td>
<td>F (Phe)$^H$</td>
<td>$\alpha$-5 loop</td>
<td></td>
</tr>
</tbody>
</table>

P – Positively charged
N – Negatively charged
UP- Uncharged polar
H – Hydrophobic
Table 5.2. HPV16 variant lineage-specific amino acids in L2.

<table>
<thead>
<tr>
<th>L2 Amino Acid #</th>
<th>European</th>
<th>Asian</th>
<th>African-1</th>
<th>African-2</th>
<th>Asian American</th>
<th>North American</th>
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<tr>
<td>108</td>
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<td>F (Phe)↓</td>
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<td></td>
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</tr>
<tr>
<td>122</td>
<td>S (Ser)↑</td>
<td></td>
<td>P (Pro)↑</td>
<td>P (Pro)↑</td>
<td>P (Pro)↑</td>
<td>P (Pro)↑</td>
</tr>
<tr>
<td>245</td>
<td>T (Thr)↑</td>
<td></td>
<td></td>
<td>A (Ala)↑</td>
<td>V (Val)↑</td>
<td></td>
</tr>
<tr>
<td>266</td>
<td>L (Leu)↑</td>
<td></td>
<td>F (Phe)↑</td>
<td>F (Phe)↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>269</td>
<td>S/P</td>
<td>P (Pro)↓</td>
<td>P (Pro)↑</td>
<td>P/A</td>
<td>P (Pro)↑</td>
<td>P (Pro)↑</td>
</tr>
<tr>
<td>270</td>
<td>S (Ser)↑</td>
<td></td>
<td>N (AsN)↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>325</td>
<td>H (His)↑</td>
<td></td>
<td>R (Arg)↑</td>
<td></td>
<td></td>
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<td>D (Asp)↑</td>
<td></td>
<td>N (AsN)↑</td>
<td>N (AsN)↑</td>
<td>N/P</td>
<td>N (AsN)↑</td>
</tr>
<tr>
<td>352</td>
<td>T (Thr)↑</td>
<td></td>
<td>A (Ala)↓</td>
<td>P (Pro)↓</td>
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<td>F (Phe)↑</td>
<td>F (Phe)↑</td>
<td>V (Val)↑</td>
<td>V (Val)↑</td>
</tr>
<tr>
<td>384</td>
<td>S (Ser)↑</td>
<td></td>
<td>F (Phe)↑</td>
<td></td>
<td>A (Ala)↑</td>
<td>S/A</td>
</tr>
<tr>
<td>385</td>
<td>V (Val)↑</td>
<td></td>
<td>I (Ile)↑</td>
<td></td>
<td>I (Ile)↑</td>
<td>I (Ile)↑</td>
</tr>
<tr>
<td>420</td>
<td>I (Ile)↑</td>
<td></td>
<td>T/I</td>
<td></td>
<td>T/I</td>
<td>T/I</td>
</tr>
<tr>
<td>424</td>
<td>A (Ala)↑</td>
<td></td>
<td>T (Thr)↑</td>
<td>T (Thr)↑</td>
<td>T (Thr)↑</td>
<td>T (Thr)↑</td>
</tr>
<tr>
<td>428</td>
<td>I (Ile)↑</td>
<td></td>
<td>L (Leu)↑</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

In this first ever functional analysis of complete-genome, infectious HPV16 variant particles of European and non-European lineages, we show that variant lineage may play a role in the attachment and entry process.

Importantly, developing infectious HPV16 variant particles allowed us to confirm and extend previous studies, which investigated the role of changes in the major capsid protein, L1, in antibody neutralization studies as this is relevant for the efficacy of the current vaccine. In a neutralization study, using human serum from individuals vaccinated with HPV16 L1 VLPs from the E114K variant which is used for the current vaccine, Semliki Forest-derived pseudovirions from each of the major HPV16 variant lineages were efficiently neutralized. This suggests that from a vaccine perspective that the induced polyclonal antibody response from the E lineage is sufficient to neutralize all variants [275]. This is also confirmed by reports of the current vaccine efficacy from clinical trials performed at various geographical locations in the Americas, Europe, and Asia [306, 307]. Neutralization of tissue-derived HPV16 variants by the type-specific monoclonal H16.V5 antibody supports these observations. H16.V5 targets an epitope in the FG loop which represent a major proportion of antibodies elicited [308, 309], and is used to monitor HPV16-induced antibodies through competition studies [310-312].

A larger number of cell lines and tissues would be required to definitively determine whether virus titers and infectivity correlate with the viral variant and/or tissue-type. Viral persistence and pathogenesis correlates with the ethnical background of the host [243, 278, 279]. Therefore, persistence and/or pathogenesis may relate to the
susceptibility of a tissue of a specific ethnicity for initial incident infection. Also, persistence may relate to the ability of a specific tissue to produce a larger number of particles to maintain a persistent infection. The current study did not allow for this analysis as the genetic and ethnic background of the host donors for the different cell lines were unknown.

The data in this study extends our previous observations that GAG- and furin-dependence, or alternatively independence, generally are correlated [286]. Also, GAG-specificity is specific not only to the HPV type, but also to the intratype variant. In general, the E variants 114/B and 114/K do not require the presence of cell surface GAGs, or the activity of cellular furin for infection. In contrast, the non-E variants, NA EVA3724 and Af-2 EVA2716, both depend on cell surface GAGs for infection. Although, their specificities are different as the NA EVA3724 is still infectious in the presence of heparin, but the Af-2 EVA2716 variant is efficiently blocked. A more extensive analysis of various GAGs and GAG-deficient or depleted cells will allow for the characterization of the GAG-dependence of the variant lineages.

The crystal structures and main interacting amino acids of HPV16 and HPV18 particles bound to oligomeric heparin have been determined. Positively charged lysine residues or other polar residues in the FG and HI loops, as well as additional residues within the BC and alpha-4 loops, mediate interactions of the virus capsid with negatively charged heparin molecules. Mutations of lysine 278 (FG loop), and lysine 361 (HI loop), ablate binding to the ECM and cell surface [143]. While no amino acid substitutions at
these specific amino acids are found in the variants (figure 5.1) [79], the implications of variations in other close by residues in the FG and HI loops are unknown (Table 5.1).

Both non-E variants require the activity of cellular furin for infection, suggesting that this interaction may be a general feature of non-E variants. The furin cleavage site is found in the N-terminus of the L2 capsid protein, and exposure of the L2 N-terminus has been shown to be required for efficient cleavage. In HPV16 PsV particles the L2 N-terminus is initially buried in the particle and becomes exposed first as particles attach to target cell, or alternatively to the ECM [13, 91]. Antibody neutralization studies using HPV16 NV also suggest temporal changes in exposure of N-terminal L2 epitopes [215]. A study looking at a larger number of HPV16 variant genomes did not identify any variant genomes with amino acid changes in the L2 N-terminus [313]. This data suggests that the overall structure of the virion, rather than the L2 N-terminal sequence, is important in determining furin-dependence versus independence by a given HPV type or variant.

The differences in GAG- and furin-dependence by HPV16 variants of different geographical origins suggest that additional steps in the entry/infectious pathway may also differ. We propose that the amino acid variations in the capsid proteins result in biologically relevant structural changes which may impact function, including the requirement for cellular interaction partners such as GAGs and furin. This is supported by reports describing an overall structural change in the virus particles due to variations affecting a neutralizing epitope in the FG loop of in the L1 protein. These variations include the A266T mutation, which is present in 11% of analyzed HPV16 sequences.
This mutation affects binding and neutralization by the H16.E70 monoclonal antibody [79, 292, 309, 314]. This study sets the groundwork for identifying specific amino acids or amino acid combinations that are responsible for observed phenotypes of different variant isolates.

It is possible that variations in biological behavior may be due to a specific variant isolate and not related to a variant lineage. To establish a correlation with variant lineages, a larger study including more variant isolates is needed and is a current effort in our lab. Our current data strongly supports the need for analyzing more than one variant isolate when investigating the viral life cycle and possible targets for antiviral interventions. Using genetic analysis we aim to identify potential lineage-specific nucleotide changes that are responsible for the observed biological differences. This information may then be used to design strategies for mutational analyses to identify which variant sequences are responsible for certain biological behaviors. This approach may also be applied to other regions of the genome. In conclusion, this study highlights the importance of studying individual variant lineages in addition to types, to be able to determine the requirement for cellular interaction partners and identify potential targets for the intervention of infections.
CHAPTER VI DISCUSSION – DIVERSITY IN HOST-CELL FACTORS INVOLVED IN PAPILLOMAVIRUS ATTACHMENT AND ENTRY

The object of this thesis was to elucidate and compare the requirements for cell surface attachment and early entry, of tissue-derived, high-risk HPV types. We based our rationale on existing structural and functional data which suggest there are differences between monolayer culture-derived versus tissue-derived particles. We also examined the requirements of natural, molecular HPV16 variants. The results allowed us to develop models of HPV NV attachment and surface events during virus entry. These models will serve as a framework for future studies on the molecular interactions during HPV attachment and entry. In addition, we show that tissue-derived particles may be more accurate to use than PsV particles when screening for potential microbicides to target HPV infection. In the following discussion, the most important results will be reviewed, along with limitations and alternative interpretations, as well as our current working models. The implications and significance of the data will be discussed along with what we may learn in the future.
GLYCOSAMINOGLYCANS INVOLVED IN THE ATTACHMENT BY VARIOUS HIGH-RISK HPV TYPES

Specificities for different glycosaminoglycans by high-risk HPVs

Our studies in Chapter III show that when comparing diverse HPV types, a complicated pattern of dependency on cell surface GAGs and/or GAG modifications emerges. Data obtained with HPV18 NV show similar results to studies with diverse HPV PsV types, where HS-modified proteoglycans appear to be the main type of GAG used for attachment (reviewed in Chapter I). Also, soluble chondroitin is very efficient in preventing infection by HPV18 NV. Our data suggests that binding to either of these GAGs may mediate infection by HPV18 NV. However, our data cannot clearly define the actual GAG used for infection. It is possible that binding by either heparin or chondroitin to the viral capsid sterically hinders the interaction with any other GAG on the cell surface. Also, the GAG-deficient cell line we used is deficient in both HS and CS. To more clearly define the GAG-specificity of HPV18 NV, heparinase and chondroitinase enzymes, which are specific for cleaving HS versus CS respectively, could be used. In addition, additional cell lines deficient for individual GAGs are available for analysis.

HPV31 NV or HPV45 NV infections are not prevented by either heparin or chondroitin. Nonetheless, neither virus could bind to GAG-deficient cells. Also, HPV31 infection was inhibited by carrageenan, suggesting that both virus types are dependent on cellular GAGs for attachment and infection. HPV18, HPV31, and HPV45 were all shown to be sensitive to the sulfation-status of cellular GAGs. Rather than being specific for a
type of GAG, the viruses may bind to certain sulfation patterns. Alternatively, the type of GAG in combination with the sulfation status determines the efficiency to which a GAG may interact with the viral capsid. A limitation to the study is the number of different GAGs that were included in the analysis. Additional types of GAGs include dermatan sulfate (CS), and keratan sulfate (KS). Also, each type of GAG may differ in chain length, epimerization, and level of sulfation. Soluble heparin for instance, is structurally very similar to HS, but is more heavily sulfated. HS chains in contrast, consist of heavily sulfated iduronic acid-rich, heparin-like domains, as well as less sulfated glucuronic acid-rich domains [315]. Inclusion of additional GAG molecules and polysaccharides with varying degrees of sulfation as well as a combination of N- or O-sulfation [288], would further address the GAG-specificity and the specificity of these virus types for sulfate modifications. In addition, specific lyases for the removal of specific cell surface GAGs, and CHO cell lines deficient in various enzymatic steps for the production of GAGs and GAG sulfate-modifications can be used. That would allow a closer analysis not only of what compounds are able to bind to the viral capsids and block infection, but also what the preferences are for mediating infection.

Bypassing a requirement for glycosaminoglycans during infection

In contrast, binding to GAGs is not required for attachment to or infection of HaCaT cells, primary cells, or CHO cells, by HPV16. This was shown by 1) incubation with soluble heparin, chondroitin and carrageenan, and 2) the ability of HPV16 to infect GAG-deficient cells, and 3) by preventing sulfate modifications of GAGs by sodium
chlorate. While we showed that none of the compounds prevented infection by HPV16, we cannot exclude that they still bind to the viral capsids.

Furin pre-cleaved HPV16 PsV particle bypass HS-interactions to infect monolayer cells [111], suggesting that they are in a primed conformational stage which allows them to bind directly to the proposed entry receptor on the epithelial cells. In contrast, upon enzymatic removal of HS-modifications in the mouse vaginal tract, they are deficient for infection [13], suggesting that binding to GAG-modified proteoglycans, specifically HSPGs, is important for targeting the viral particles to the basement membrane \textit{in vivo}. Whereas non-cleaved, wildtype PsV particles show a binding pattern primarily localized to the basement membrane and basal cells at early time points after PsV inoculation, the furin pre-cleaved capsids bind throughout the epithelium [13]. These data suggest that HSPG play a role \textit{in vivo} by targeting the viral capsids to the basement membrane and mitotically active basal cells. However, an important consideration complicating the use of the mouse model is that binding to human ECM has been shown not to be equivalent to mouse ECM. Binding to the ECM of human cells by HPV16 PsV, is dependent on laminin-332 rather than HSPGS [108, 109], suggesting that binding by HPV16 NV to basement membrane of human tissue is independent on the presence of GAGs, and that laminin-332 may be sufficient. Also, HPV16 NV does not infect 293TT cells as well as HaCat cells. 293TT cells do not secrete laminin-332 in their ECM [149], supporting the preference of HPV16 NV for laminin-5 rather than HSPGs for initial attachment to the ECM.

Changing lysine 278 to alanine (K278A) in the L1 site 1 HS binding-site of HPV16 PsV, prevents attachment to and infection of sub-confluent HaCaT cells [143].
As attachment to the HaCaT ECM by HPV16 PsV depends on the presence of laminin-332 rather than HS, this data suggests that a mutation in this site interrupts the ability of the virus to interact with laminin-332 in addition to HSPGs. Initial experiments inserting the K278A mutation in site 1 of L1 in HPV16 NV ablated the infectivity of this virus (see Appendix A), suggesting that laminin-5 and or HS-binding is important for HPV16 NV infection, similarly to HPV16 PsV. Additional experiments addressing the attachment to the ECM and cell surface of HaCat cells by wildtype and the K278A mutant would address whether binding to the ECM is dispensable for infection by HPV16 NV, and whether laminin-332 or GAGs play a major role in the attachment of this virus to the host epithelium. Attachment to the ECM versus the cell surface can be done with the qPCR-based method analyzing the number of particles attached by viral genome equivalents, as used in chapter III of this thesis. To distinguish between ECM and cell surface binding, EDTA treatment will remove the cells but leave the ECM behind attached to the tissue culture plates, allowing binding to the ECM to be studied directly by adding virus to the remaining ECM on the plates. Binding to the cell surface can be analyzed by allowing virus particles to attach to cells in solution. In addition, the use of confocal fluorescence microscopy would be able to distinguish between binding to the ECM and cell surface, without having to separate the cells from the ECM.
Future studies on attachment by native HPV

Future studies should be aimed at learning more about the details of the specificity of the different virus types for various GAGs during infection. Those studies can be facilitated by the several experiments listed in the above sections. In addition, GAG oligosaccharide microarrays [109] would show the affinity of binding by the different HPV types to a larger array of specific GAGs with different chain lengths and modifications.

Antibodies specific for laminin-332 can be used for blocking studies to investigate its role in infection by tissue-derived HPVs, in particular HPV16 NV which does not seem to require GAGs for initial attachment to cells. It is also possible that HPV16 NV does not depend on either GAGs or laminin-332 for infection. To investigate a role for other basement membrane components, co-localization studies in combination with antibody blocking studies, would aid in further elucidating initial attachment by HPV16 NV.

PsV infection of primary cells is inefficient as compared to established cell lines. The lower infection levels correspond to a lower level of attachment to primary cells than HaCaT cells. Since furin pre-cleaved HPV16 PsV which can infect monolayer cultures in an HS-independent manner also infect primary cells more efficiently, it was proposed that an altered sulfation pattern of primary cells in culture than in the tissue is responsible for the low infection levels [111]. Also, HPV18 NV infection of primary cells compared to HaCaT cells is less efficient, as reported in chapter III. Investigations into the GAG-composition and sulfation pattern in different epidermal tissues and cell lines may be able
to shed light on the why the efficiency of infection by HPV particles in different cell types differs. Alternatively, genome replication and transcription may vary in different cell types.

Infections of cells in monolayer may not faithfully represent *in vivo* infections. The ECM secreted in monolayer cultures is not structurally the same as the basement membrane [316]. Also, as just discussed, since binding to mouse epithelial cells is not representative of human epithelial cells, there is a need for a human tissue model system. Further, the virus is faced with the challenge of finding the correct target cells, the basal cells, in the tissue. A limitation in our current ability to use the organotypic raft culture system for the study of the HPV life cycle is that continuous HPV-harboring cell lines bypass the initial steps of attachment, entry, and establishment of infection. The establishment of a tissue model for *de novo* productive HPV infection has not yet been established and is hampered by the naturally low productivity of high-risk HPV lesions. Several attempts in our lab have been aimed at developing a tissue-infection model. The basics of the model include; 1) allowing for the establishment of a layered epithelium using primary foreskin or cervical cells over a time-period of several days, and 2) wounding of the tissue using a scalpel prior to the addition of virus. These attempts have yielded low-level expression of the E1^E4 protein, and limited cells positive for the expression of L1 capsid protein after scanning multiple tissue slides. This low level of expression makes it very difficult to localize areas of productive infection in the tissue for qualitative analysis of infection. Further, E1^E4 transcript expression is too low to accurately and reproducibly detect differences in expression when adding perturbations in experiments for entry and life cycle analyses, following infection. It is possible that
additional efforts changing the timing of infection and/or adding higher titers of virus would be able to improve the system. Addition of progesterone to the cultures during the days leading up to infection and using a Cytobrush cell collector for mechanical disruption, may further aid in potentiating infection [133].

In our system for producing native tissue-derived HPV particles, we layer HPV-harboring epithelial cells directly on to a fibroblast/collagen matrix, which represents the dermal equivalent. Following lifting to the air-liquid interface, the epithelial cells are stimulated to stratify and differentiate, allowing for production of new HPV particles. In an analysis of the factors contributing to proper basement membrane assembly and epithelial growth in organotypic raft cultures, this method did not result in the formation of a basement membrane [316]. Rather, formation of a basement membrane requires the presence of pre-existing membrane components. A summary of the morphology, basement membrane formation and components, growth and differentiation of organotypic epithelium in the presence or absence of pre-existing membrane components and foreskin fibroblasts is found in Table 6.1. Noticeably, in the absence of pre-existing basement membrane components, decreased and aberrant expression of laminin-332 (laminin 5) and α6-integrin, is observed. Considering the role of laminin-332 in attachment to the basement membrane [108], and α6-integrin in entry and signaling [113], our current preparation of organotypic raft cultures may not be very permissive for infection by HPVs. Incorporating pre-existing membrane components on top of the collagen matrix, prior to the addition of epithelial cells, may improve the ability of the HPV particles to attach to and infect in the tissue culture model. Also, our current efforts have focused on productive infection as a read-out. Looking at attachment as a read-out
by confocal microscopy would allow for initial steps of attachment and entry to be elucidated.

<table>
<thead>
<tr>
<th>Tissue stratification</th>
<th>+ BM + HFF 9d</th>
<th>+ BM + HFF 14d</th>
<th>+ BM - HFF 9d</th>
<th>+ BM - HFF 14d</th>
<th>- BM + HFF 9d</th>
<th>- BM + HFF 14d</th>
<th>- BM - HFF 9d</th>
<th>- BM - HFF 14d</th>
</tr>
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<tbody>
<tr>
<td>Tissue architecture</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>aberrant, loss of polarity</td>
<td>aberrant</td>
<td>aberrant, flat basal cell layer</td>
<td>aberrant, flat basal cell layer</td>
</tr>
<tr>
<td>Basement membrane assembly</td>
<td>+ HD + HD</td>
<td>+ HD + HD</td>
<td>+ LD* + LD*</td>
<td>+ LD* + LD*</td>
<td>- HD ND</td>
<td>- LD ND</td>
<td>- BM - HFF</td>
<td></td>
</tr>
<tr>
<td>Laminin 5</td>
<td>linear</td>
<td>linear</td>
<td>patchy</td>
<td>patchy</td>
<td>patchy</td>
<td>patchy</td>
<td>patchy</td>
<td>patchy</td>
</tr>
<tr>
<td>α6 integrin</td>
<td>linear</td>
<td>linear</td>
<td>linear</td>
<td>linear</td>
<td>linear</td>
<td>linear</td>
<td>linear</td>
<td>linear</td>
</tr>
<tr>
<td>Filaggrin</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>B6u (LI)</td>
<td>43%</td>
<td>18%</td>
<td>18%</td>
<td>0%</td>
<td>36%</td>
<td>0%</td>
<td>24%</td>
<td>0%</td>
</tr>
</tbody>
</table>

BM – basement membrane, HFF – human foreskin fibroblasts.  
++++ – full stratification, +++ – moderate stratification, ++ – little stratification.  
HD – hemidesmosomes, LD – Lamina densa. *linear, **local.  
pc – pericellular staining only, sb – suprabasal staining.  
ND – not determined.  
+++ – strong staining, ++ – moderate staining.
A better understanding of the molecular interactions during attachment will allow for the development of more specific inhibitors targeting HPV this step of the HPV life cycle. The goal is to identify a compound that is cross-reactive, with high specificity, and a low IC$_{50}$, against several of the cancer-causing HPV types in infection studies. A mixture of more than one compound may be needed to efficiently block attachment by several HPV types. Larger screens that include a range of oligosaccharide compounds would facilitate such an investigation. Due to technical restraints, including a larger number of compounds, studies would initially have to focus on infection of monolayer cultures. Ideally, tissue-infections studies could then be used to verify the efficacy of the compounds against HPV infections.

**CLEAVAGE BY FURIN PROPRoteIN CONVERTase**

**DURING VIRION MORPHOGENESIS AND/ OR ENTRY**

**Furin cleavage of native HPV particles**

Our studies in Chapter IV show that infections by HPV18 NV and HPV31 NV are dependent on the activity of cellular furin. In contrast, neither HPV16 NV nor HPV45 NV infections are prevented in the presence of a furin inhibitor during infection. As the activity of furin results in N-terminal cleavage of the L2 protein [91], we investigated whether furin is never required during any step in the HPV16 NV life cycle, or if HPV16 NV is cleaved during virion morphogenesis in the tissue, prior to infection.
Enhanced expression of furin in HPV16-producing tissues, suggested that HPV16 NV may be cleaved during virion production and maturation in the tissue, rather than post-attachment to un-infected host tissue. Western blot analyses of tissue-derived HPV16 support the presence of an N-terminally cleaved form L2, in addition to full-length L2. Initial studies (see Appendix B) using HPV16 QV particles for infection suggest that similar to HPV16 NV, HPV16 QV infection cannot be blocked by the furin inhibitor. Furin-independence by HPV16 QV, suggest that the presence of the full-length viral genome may change the structure of the viral capsid, and thus the interactions with cellular factors during entry and infection. In contrast to HPV16 NV however, WB data only revealed the presence of full-length L2 in 293TT-derived HPV16 QV. Also, the addition of the furin inhibitor during virus production had no effect on the infectivity of the virus, whether in the presence or absence of the furin inhibitor. It is possible that the ratio of un-cleaved to cleaved L2 is below our detection level as it is unknown how many cleaved molecules of L2 are needed to facilitate infection. On the other hand, HPV16 QV infection may differ yet from HPV16 NV and PsV, and not require a cleaved version of L2.

Alternatively, the QV data suggests the cleaved version of L2 may not play an important role in HPV16 NV infection either. Also, despite expression of furin in un-infected foreskin tissue, HPV18 NV does not appear to be cleaved in the tissue like HPV16 NV, as HPV1 does not bypass the requirement for cellular furin during infection. Despite our efforts to amplify the signal during WB analysis we have not yet been able to visualize HPV18 NV L2. The amount of L2 which is incorporated in the viral particles in unknown and it is possible that it varies for different types. Improving the harvest
procedure of native particles to maximize the concentration particles in our viral preps should facilitate the analyses. A limitation using the organotypic raft culture is the number of particles produced in a raft, which makes biochemical analyses more difficult to perform. Ongoing efforts in the lab are aimed towards determining the status of L2 from HPV18 NV, as well as HPV16 variants. Knowing the status of L2 from several different viruses will shed light on the significance of the cleaved version of L2 in infections by HPV NVs. In addition, analyses of furin expression in the HPV-infected tissues from other types will begin to elucidate whether furin up-regulation is a mechanism induced by HPV16 NV for the purpose of inducing cleavage of its L2 N-terminus during infection. Also, tissue-sections from HPV-infected individuals would be of great interest. If furin is up-regulated in all HPV-infected tissues that may suggest that furin up-regulation is induced to facilitate differentiation and a cellular environment conductive for particle production. Alternatively, it may be an indirect response on the tissue due to the effect of virus infection.

**Future studies on furin dependence by native HPV particles**

Future investigations are aimed at elucidating the relevance of furin pre-cleaved particle in infections by native HPV particles. Serine replacement mutations at the conserved furin cleavage-sites in L2 (Chapter IV), HPV16 L2 R12S and HPV18 L2 R11S, did not result in a loss of infectivity as previously observed with PsV particles. Rather, the mutant viruses were more infectious than wildtype virus. This data suggests that NVs do not rely on the conserved furin cleavage sites for infection. Alternatively,
these mutations may have rendered the L2 N-terminus more flexible such that it is exposed on the virion surface on mature particles prior to attachment to cells. Exposure of the L2 N-terminus on the capsid surface of mature particles has been observed for mutants PsV particles (data not published, reviewed in [317]). The increase in infectivity may be due to the enhanced exposure of the L2 N-terminus, thus allowing for more efficient interactions with cellular factors. Additional mutations of NV particles, HPV16 L2 R5S and HPV18 L2 R8S which are also potential furin cleavage sites, resulted in a complete loss of infectivity of the mutant particles. Thus, suggesting that these sites may be more important for furin cleavage of NV particles. Alternatively, the L2 N-terminus is highly conserved and other functions other than furin cleavage may be altered in the mutant particles. Analysis of the size of L2 in mutant and wildtype particles to determine the cleavage-status would provide definitive evidence for the role of these sites in infection. It is also possible that exposure of the L2 N-terminus, with or without cleavage, is sufficient to mediate infection. Confocal microscopy for trafficking analyses during entry of wildtype and mutant viruses, in the presence and absence of the furin inhibitor, would aid in unraveling the role of the L2 N-terminus and furin cleavage in infection.

Inhibiting furin during virion morphogenesis should shed light on the importance of this enzyme during particle production. Initial efforts suggest the addition of lower concentrations of the furin inhibitor (concentrations that effectively block activity in monolayer cultures) does not block the activity of the enzyme, suggesting that addition of the inhibitor to the culture medium might not be the best way of delivery. On the other hand, addition of higher concentrations throughout virion morphogenesis is toxic to the tissue. It’s possible that extensive time-course and concentration titer experiments will be
able to identify a window suitable for inhibition. The use of a retroviral vector for delivery of shRNA for knockdown of furin expression may be a viable alternative approach to specifically target furin expression. An inducible promoter would allow for the growth and establishment of the tissue prior to knockdown.

**MATURATION STAGE AND ASSOCIATION WITH VIRAL AND HOST-CELL FACTORS BY NATIVE HPV PARTICLES**

**Implications on HPV entry from maturation stage**

The phenotype of HPV16 NV is reminiscent to that of furin pre-cleaved PsV and HS site 2 and 3 mutant PsV, which may all bypass a need for HS to infect host cells [111, 146]. The data on HPV16 NV suggest that the particles have reached a structural stage where they have already undergone the initial structural changes that are induced by HS-binding and furin-cleavage for HPV16 PsV. The more advanced structural stage of the HPV16 NV particles may be due to the extended period of maturation in the tissue environment, and/or cleavage of the L2 N-terminus by cellular furin during virus maturation.

HS-binding induces a structural change of HPV16 PsV, which exposes the L2 N-terminus to cleavage by furin. Furin cleavage induces another conformational shift, which allows for transfer of the particles to the putative secondary entry receptor/s. Furin
pre-cleaved PsV particles bypass the HS-induced conformational changes and directly bind to the cell surface. A structural feature of the furin pre-cleaved PsV particles is the exposure of the L2 N-terminus in solution prior to attachment [111]. Structural analysis of HPV16 NV and HPV18 NV wildtype particles as well as L2 N-terminal furin cleavage mutants, using the RG-1 antibody to target the L2 N-terminus in an ELISA assay, will add valuable structural information about the native particles. Also, these experiments may aid the understanding of why HPV16 is pre-cleaved in the tissue and not HPV18.

Alternatively, bypassing a requirement for GAG-interactions on the cell surface may not require pre-existing exposure of the L2 N-terminus. HPV16 PsV particles with mutations in site 2 and 3 HS-binding sites in L1, do not expose the L2 N-terminus in solution, but rather post-attachment to the host ECM or cell surface, similarly to wildtype particles. However, these particles may still infect cells in a HS-independent manner, as their infectivity is enhanced in the absence of HS-interactions during infection. Noticeably, the mutant particles require interactions with laminin-332 on HaCaT ECM to infect the cells in an HS-independent manner. Also, the infectivity of the mutants is greatly impaired in laminin-332-deficient 293TT and HeLa cells [146]. The data suggests that particle interactions with laminin-332 on the ECM, in the absence of HS-interactions, are sufficient to induce the L1 and L2 conformational changes that are required for entry and infection. Further, the data suggests that it is un-likely for HPV16 NV to bypass a requirement for binding to an attachment receptor prior to secondary interactions with an uptake receptor or complex. Future experiments investigating the interactions of HPV16 NV with laminin-332 on the HaCaT ECM and the basement membrane in a tissue-culture
model are warranted to investigate any such interactions. Mutations of the site 2 and 3 HS-binding sites in L1 of native virus particles would also be of great interest.

**Implications on HPV entry from associations with viral and cellular factors**

Infections in all our studies with NV were performed with non-purified virus preparations. HPV particles are released with the de-nucleated cornified layer as the outermost cells are continuously shed into the surroundings [5, 102]. Infections may take place with cell-associated virus or mechanically ruptured cells [102], which is likely to be the source of transmission to the genital epithelium during sexual activity. Thus, infections *in vivo* are unlikely to occur in isolation from cellular factors. As a result, it is possible that HPV16 NV is interacting with cellular molecules that are released during harvest. PsV may avoid interacting with some of these cellular molecules due to the inclusion of a detergent during harvest of the particles. Also, PsV particles are always gradient-purified before experimental analysis.

Recent publications suggest that HPV16 PsV virus particles upon infection associate with cellular factors, including heparan sulfate proteoglycans and growth factors, to facilitate infection [109, 114]. HPV16 PsV associating with heparin in solution undergoes a slight conformational change, indicated by the enhanced recognition by an L1-specific conformational antibody [109]. The heparin-associated virus is more infectious, independently of cell surface GAGs. Rather, binding to laminin-332 in the
ECM, mediates infection by heparin-associated HPV16 PsV. Interestingly, this was only seen for heparin, and not for other purified GAGs, including HS. Further studies of purified NV particles would determine what cellular factors have a strong association with the virus particles and how that impacts the viral life cycle. It is possible that HPV16 NV associates with cellular GAGs more efficiently than the other types. The conformational change induced by heparin-association by HPV16 PsV [109], was shown by the enhanced binding to the H16.B20 antibody, indicating induced exposure of amino acids 396-415 [293]. Experiments investigating the exposure of this epitope in HPV16 NV will result in additional structural knowledge on HPV16 NV particles.

Experiments looking at gradient-purified HPV16 NV particles using particles from fraction 7 and 8, representing the highest infectivity fractions [47], have been initiated (see Appendix B). The sensitivity to inhibition by soluble heparin was a lot more variable as compared to non-purified particles, especially at the higher 100 µg/ml concentration where inhibition ranged from 0% up to 70%. Additional experiments and the inclusion of lower levels of heparin are needed to address the significance of the results. An increased sensitivity to the furin inhibitor was observed for gradient-purified HPV16 NV. Interestingly, inhibition was not dose-dependent as maximum inhibition was obtained at the lowest amount of inhibitor added, and never reached above 60%. In comparison, furin inhibition during HPV18 NV infection is dose-dependent and reaches nearly 100% at the highest concentration. The data supports the presence of a furin-dependent as well as a furin-independent population of HPV16 NV particles. Alternatively, factors available in the cellular lysate rather than an L2 N-terminally cleaved population may be responsible for the observed resistance to inhibition by
soluble heparin and the furin inhibitor. Analysis of heparin- and furin inhibitor-sensitivity of additional fractions would be able to address whether particles in other fractions contain a mixture of virus populations as well. Re-adding cellular factors from an un-infected tissue lysate to the purified fractions will address whether the particles are likely to associate with cellular factors and regain resistance to inhibition. Another important consideration is that gradient-purification of virus particles may bias the analysis of a particular population of virus particles that does not represent the behavior of the population as a whole [47]. Further studies of different fractions from gradient-purified NV particles are warranted to analyze the biology of different subsets of virus particles. Laser-capture-microdissection techniques may allow for the harvest of immature particles from suprabasal nuclei in separation from mature particles found in the cornified layers of 20-day organotypic raft cultures.

DIFFERENCES IN HOST FACTORS INTERACTIONS BY MOLECULAR VARIANTS

HPV variants distribution and the vaccine

Almost all current molecular and functional correlation studies are performed using a single genome for each HPV type. Geographical distribution analysis of sub-lineages for the different HPV types shows whether these genomes are representative of the majority of the population for each type. For HPV16, a European (E) isolate is used as the model, and considered “wildtype”. In a big survey of HPV16 variants, HPV16
variant genomes of the E lineage are well represented among all the geographical regions surveyed. The Asian (A) was the most common in Eastern Asia, African (Af) lineages in Africa. The Asian American (AA) lineages were commonly observed in South-Central America, although, AA1 was more frequently found in Asia. The North American (NA) lineage was most commonly observed in genomes surveyed from North Africa (figure 6.1) [254]. Thus, the HPV16 E lineage is a good representative as a wildtype genome.
Figure 6.1. Global distribution of HPV16 variant lineages. Variant genome samples from different regions were sequenced and identified according to variant lineage. Obtained from [254]: Cornet at al. 2012.
While the current HPV vaccine targets the most common HPV types responsible for the development of cervical cancer, HPV16 and HPV18, the interest in developing a multivalent vaccine, that includes protection against other HPV types considered high-risk for the development of cervical cancer, is great [318]. The vaccine would include high-risk types, such as HPV31, HPV33, HPV45, HPV52, and HPV58. A study of over 1000 full-length L1 sequences from America, Africa, Asia, and Europe, of several HPV types of interest for a future vaccine, analyzed the degree of intra-type variations. The number of variations for HPV6, HPV11, and HPV52 is very low and found mainly in non-surface-exposed residues, suggesting that there is a negligible effect on vaccine-induced antibodies. In contrast, for HPV31, HPV33, and HPV58 intra-type changes are frequent and mapped to surface-exposed capsid domains, including recognized epitopes. Enough sequences were not available for HPV18 and HPV45, for a complete analysis. However, analysis of HPV45 revealed some changes in surface epitopes. Of great interest, and a possible cause of concern, the reference sequence for most types does not represent the major variant, but rather a minority variant. Also, the capsid protein constructs used to produce PsV for HPV18, HPV31, HPV45, and HPV58, do not represent the consensus L1 sequence for these types. For example, the reference sequence for HPV31 is only representative of 12% of analyzed sequences, and the HPV31 PsV L1 protein used, is identical to the reference sequence [79]. Importantly, T267A and T247N variations in the FG loop of HPV31 L1 may have an impact on neutralization, as several HPV31 neutralizing antibodies have been reported to target the FG loop [79, 319, 320]. The reference sequence for HPV45 only matched 1 out of 13 sequences, while the reference sequence for HPV18 matched 39% of analyzed sequences. Of interest, the
HPV18 VLP used in the Cervarix vaccine contain some, but not all variations that are part of the consensus sequence [321]. This suggests the existence of variant residues that may have a major influence on the efficacy of vaccine-induced antibodies in future vaccines as well as lifecycle studies of these HPV types [79].

For the more studied and characterized HPV16, a significant amount of variations was observed, but none which were not previously identified [79]. Semliki Forest-derived PsV, from each of the major HPV16 variant lineages, are efficiently neutralized by human serum that is derived from individuals vaccinated with HPV16 L1 VLPs from the E 114K variant which is used for the current vaccine. This data suggests that from a vaccine perspective, that the induced polyclonal antibody response from the E lineage is sufficient to neutralize all variants [275]. The efficacy of the vaccine is also confirmed by reports from clinical trials performed at various geographical locations in the Americas, Europe, and Asia [306, 307]. Neutralization studies of tissue-derived HPV16 variants, using the type-specific monoclonal H16.V5 antibody, further supports these observations (see Chapter V). H16.V5 targets an epitope in the FG loop, which represents a major proportion of antibodies induced [308, 309].

**Variants, GAG and furin interactions**

Our data in Chapter V suggests that HS- and furin-independence during *de novo* infections may be a general feature of HPV16 European isolates. Additional experiments, including various GAG molecules and infection of sulfate-modification- and GAG-
depleted cells, are needed to explore any GAG preferences. Preliminary data suggests that HPV16 isolates of different geographical origins do not display the same HS- and furin-dependency or independence during infection. Thus, molecular variants of a given HPV type may interact with a different set of host factors during infection. Our analysis thus far is a proof-of-principle study, demonstrating that variants may display different entry properties. Additional genomes for each variant, and the inclusion of more variant lineages, are needed for a comprehensive analysis.

**Implications from amino acid changes**

We did not detect any amino acid difference is the L2 N-terminus, including the conserved furin cleavage site, or in the HS-binding sites in L1. The lack of such amino acid changes suggests that the overall structure of the particles is the major determinant for the observed phenotypes. We reasoned that if HS-dependence and furin-dependence segregate together between our two non-E variant genomes compared to the E genomes, sequences variations common for non-E variants compared to E variants may be responsible for the observed phenotypes. To identify candidate amino acid differences, we compared the L1 and L2 sequences of the E variant genomes to the non-E variant sequences, and identified amino acid variations that are common for the non-E variant genomes but not found in the E variants (Table 5.I and 5.II). Residues highlighted in bold are common for the non-E variants compared to the non-E variants. To determine whether the observed differences in phenotypes segregate with variations in the L1 or L2 protein, HPV16 variant chimeras can be created by switching the L1 and the L2 ORFs of
the “wildtype” E ORFs, individually. The chimeric E/non-E viruses can then be tested for HS- and furin-dependence. To find the specific amino acid differences that are essential for a given phenotype, the lineage-specific sequence differences may be targeted by site-directed mutagenesis, using the 114B E genome as the “wildtype” backbone. Single mutations, or mutations in combination, may be required to change the phenotype of the wildtype genome. While the furin-cleavage and HS-binding sites are found on L2 and L1, respectively, these two functions may segregate together or separately. Thus, one mutant may display both phenotypes, or various mutants may display different phenotypes.

We expect that the amino acid changes responsible for the observed phenotypes, lie within a structural motif that may have an impact on the overall structure, or localized structure, thereby impacting the exposure of the L2 N-terminus and L1 HS-binding sites. Some known structural features that are associated with the amino acid changes found in the variant genomes are pointed out in Tables 6.I and 6.II. For example, in L1, within the 3-dimensional structure, amino acid number 176, which is a threonine (T) in the E variants and asparagine (N) in the non-E variants, is in close proximity to cysteine (C) 175, which forms an interpentameric disulfide bond with C428. Amino acid number 102 is found within a β-hairpin, histidine (H) in E and tyrosine (Y) in non-E variants, and amino acid number 500, lysine L in E and phenylalanine (F) in non-E variants, is found in an α-helix in the invading C terminal arm [48, 54, 70, 71]. Also, lysine (K) 361 in the L1 site HS-binding site, is in close proximity to, and in the same loop as amino acid 353, T in E and P in non-E variants [143]. In L2, two residues, 122 and 269, serine (S) in E and proline (P) in non-E variants, are buried inside the particle. Amino acid 424, the non-polar alanine (A) in E and polar T in non-E variants, mediates hydrophobic interactions
with L1. Amino acid 424 T is also in proximity to recognition sites for cellular cyclophilin and Daxx proteins [211, 303].

**MODELS OF CELL SURFACE INTERACTIONS BY NATIVE HPV PARTICLES**

The majority of structural, cellular receptor, and entry studies, have been performed using a variety of synthetic particles. These systems provide different strengths, and have provided much insight into the structure and entry pathways of papillomaviruses. Using tissue-derived NV particles, much of what has been determined using PsV, also holds true for specific types and variants. With the exception of the European HPV16 variants, the use of GAGs for primary attachment is universal for the types and variants we have studied so far. Our data added to the complexity, as different types and variants display different specificities for GAG binding, which needs to be considered, when developing potential microbicides. A requirement for active cellular furin during entry also is more common than not, as only E HPV16 and HPV45 NVs, are resistant to inhibition by a furin inhibitor. As summarized in Table 6.2., a complex pattern of GAG specificities and furin dependence exists for the different HPV types. Based on our data and current literature we also propose that a basement membrane attachment receptor exists for each HPV type. In Table 6.2 we propose the possible nature of the attachment receptor.
Table 6.2. GAG, Furin, and Attachment Receptor Dependence during Infection. Summary of the sensitivity of the different HPV types to neutralization by monoclonal antibodies, various polysaccharide compounds, sodium chlorate treatment, infectivity of GAG-positive or negative cells, furin dependence, and the proposed nature of the attachment receptor. Nd=not done.

<table>
<thead>
<tr>
<th></th>
<th>Neutralized</th>
<th>Heparin</th>
<th>Chondroitin</th>
<th>Sod. Chl.</th>
<th>CHO +/- GAGs</th>
<th>Carrageenan (HaCat)</th>
<th>Carrageenan (HFKs)</th>
<th>Furin</th>
<th>Proposed Attachment Receptor</th>
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<tr>
<td>HPV16</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<td>No</td>
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<tr>
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<td>Yes</td>
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</tr>
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<td>No</td>
<td>Yes</td>
<td>Nd</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>GAG</td>
</tr>
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<td>No</td>
<td>GAG</td>
</tr>
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</table>
Based on our results using HPV NVs, combined with existing data obtained using PsV particles, we have developed two working models for the role of GAGs and furin, for interactions with the basement membrane and cell surface, in HPV infections. In the GAG- and furin-dependent model (figure 6.2), the incoming virus, with full-length L2, first binds to GAGs that are exposed on the basement membrane, or on the basal cells, on sites of tissue trauma. We cannot exclude that initial binding to the basement membrane doesn’t also include laminin-332. Binding to GAGs induces a series of L1 and L2 conformational changes. Exposure of the L2 N-terminus allows for cleavage by furin. Additional conformational changes decrease the affinity between the GAGs and the virus. The virus then transfers to the putative entry receptor/complex on the mitotically active basal cells, which are migrating in to fill the wound. Binding to the entry receptor/complex ultimately triggers endocytosis.

In the GAG- and furin-independent model (figure 6.3), virus released from the tissue may already have its L2 N-terminus cleaved by furin. Particles with their L2 N-terminus cleaved by furin, may be in a conformational state which allows them to bind directly to the entry receptor/complex on the surface of basal cell. Alternatively, the furin pre-cleaved particles may use laminin-332 on the basement membrane for initial attachment and conformational changes, prior to transfer to the entry receptor/complex. It is also conceivable that tissue-derived particles, whether cleaved or not, interact with cellular factors, such as GAGs, as they are being released from the epithelium during the continuous sloughing off, of the top keratinized layers. Pre-cleaved particles, which associate with cellular GAGs, may be in a conformational state that allows them to directly bind to the entry receptor/complex. GAG-associated particles may also bind to
laminin-332 on the basement membrane, for additional conformational changes and furin-cleavage, before transfer to the entry receptor/complex. Based on current literature, using furin pre-cleaved and heparin-associated PsV particles, interactions with laminin-332 are likely needed to target the incoming viral particles to the basement membrane, for transfer and infection of basal cells.
1. Progressing conformational stages of HPV virions
2. 
3. 

GAGS
Entry receptor
L2 N-terminus
Virus particle
Laminin 5
**Figure 6.2. Tissue-model of the initial steps in infection by GAG- and furin-dependent HPV types.** The incoming virus, with un-cleaved, full-length L2, first binds to GAGs on the basal cells or exposed on the basement membrane on sites of tissue trauma or laminin-332 on the basement membrane. Binding to GAGs induces a series of L1 and L2 conformational changes. Exposure of the L2 N-terminus allows for cleavage by furin convertase. Additional conformational changes decrease the affinity between the GAGs and the virus. The virus is then transferred to the putative entry receptor/complex on the mitotically active basal cells migrating in to field the wound. Binding to the entry receptor/complex ultimately triggers endocytosis.
200

A

Stratified epithelium
Basement Membrane
Dermis

1. Progressing conformational stages of HPV virions
2.
3.

B

Proteoglycans
Entry receptor
Laminin-332
L2 N-terminus
GAG-molecules
Figure 6.3. **Tissue-model of the initial steps in infection by GAG- and furin-independent HPV types.**

A) Virus released from the tissue may already have its L2 N-terminus cleaved by furin in the tissue. Tissue-derived particles may also interact with cellular factors such as GAGs as they are released from the tissue.

B) Furin pre-cleaved particles and/or particles associated with cellular GAGs that are pre-cleaved may be in a conformational state allowing them to directly bind to the entry receptor/complex. Alternatively, the furin pre-cleaved particles and GAG-associated particles may use laminin-332 on the basement membrane for initial attachment and additional conformational changes.
BEYOND GAGS AND FURIN IN THE HPV LIFE CYCLE

This thesis dissertation has focused on the interactions with cellular GAGs and furin during HPV attachment and entry. Several groups have reported HPV16 PSV interactions with a number of different cell surface molecules and cellular entry pathways. It is of great interest to use HPV16 NV to confirm and/or elucidate the identities of additional cell surface molecules that interact with the virus. Infection of HS- and/ or sulfation-depleted cells may facilitate identification of additional receptor molecules as the virus would not bind GAGs. Immunoprecipitation and virus overlay-binding assays, using only proteins associated with the cell membrane fraction, would facilitate such investigations. Also, the use of diverse molecules for cell surface interactions, suggest that various HPV NV types may be shuttled into different entry pathways. Thus, thorough entry studies for the different HPV NV types are warranted. Mutational analysis of suggested interaction domains in L2 for cellular factors, along with inhibitor studies of the implicated cellular proteins, will be able to establish whether these proteins are involved in infections by HPV NVs.
REFERENCES


APPENDICES

In the appendices we will present data on complementary studies we have initiated to address some of the questions raised in the data sections and discussion of this thesis. The results obtained in the appendices and their implications are discussed in the relevant contexts in the Chapter VI Discussion.

Appendix A  K278A mutation in the L1 HS-binding site

A lysine to alanine mutation was created in the L1 site 1 HS-binding site of HPV16 (HPV16 L1 K278A). This same mutation in HPV16 PsV renders the particles attachment-deficient and non-infectious [142]. Preliminary experiments with tissue-derived HPV16 L1 K278A mutant particles show an almost complete loss in infectivity compared to wildtype HPV16 particles (figure A.1). The data suggests that this residue is important for HPV16 NV infectivity, similarly to HPV16 PsV.
Infections of HaCat cells were done at an MOI of 10 for wildtype and L1 K278A mutant particles. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection with wildtype set to one.
APPENDIX B  INFECTIONS WITH OPTIPREP

GRADIENT-PURIFIED HPV16 NV

To begin to address if the cellular lysate and different populations of HPV16 NV particles has an impact on the functional phenotypes of HPV16 NV we gradient-purified HPV16 NV. We used fractions 7 and 8 as those fractions have previously been shown to contain the most stable and infectious particles [47]. Cellular components in the non-purified virus prep that are not tightly associated with the stable virus particles are expected to migrate in the less dense upper fractions of the gradient. Any tightly virus-associated molecules would still co-migrate with the stable virus particles. This can be seen in western blots where fractions 1 through 5 have very high background staining. Similarly to non-purified HPV16 NV, purified virus was efficiently neutralized by the L1-specific H16.V5 antibody (figure B.1). Purified virus was resistant to inhibition by heparin at 10µg/ml. The sensitivity to inhibition by soluble heparin at the higher 100µg/ml concentration heparin was a lot more variable as compared to non-purified particles, with inhibition up to 70% inhibition observed (figure B.2). Purified particles were consistently more sensitive to the furin inhibitor during infection (figure B.3A), although this was not dose-dependent as the max amount of inhibition was observed already at lower concentrations of inhibitor (data not shown). In contrast, furin inhibiton of HPV18 NV infection is dose-dependent and may reach 100% (figure B.3B).
Figure B.1. Particle-mediated infection of gradient-purified HPV16 NV.

Fractions 7 and 8 from Optiprep gradient-purified HPV16 particles were incubated with the L1 type-specific antibody H16.V5 for 1 hour at 37°C prior to infection of HaCaT cells. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection.
Figure B.2. Inhibitory effects of heparin against gradient-purified HPV16

Fractions 7 and 8 from Optiprep gradient-purified HPV16 particles were incubated with heparin at increasing concentrations (0 µg/ml, 10 µg/ml, and 100 µg/ml) for 30 min’s at 37°C prior to infection and during infection of HaCaT cells. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentrations with infection at 0 µg/ml of heparin set equal to one.
Figure B.3. Effects of furin inhibition against gradient-purified HPV16 NV.

A) Fractions 7 and 8 from Optiprep gradient-purified HPV16 particles were incubated with the furin inhibitor at 0 and 25μM during infection of HaCaT cells. B) HPV18 NV were incubated with the furin inhibitor at increasing concentrations during infection of HaCaT cells. Infections were analyzed by RT-qPCR measuring the relative amount of E1^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentrations with infection at 0 μg/ml of heparin set equal to one.
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